Isolation and Structural Elucidation of Bioactive Compounds from Bangladeshi Medicinal Plants with a Focus on Novel Anticancer Compounds

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Abstract

The present study describes the bioactivity guided isolation and structural elucidation of novel anticancer compounds from the Bangladeshi medicinal plant *Blumera lacera*. At the outset nineteen Bangladeshi medicinal plants were selected and collected from different parts of Bangladesh. Plants underwent preliminary cytotoxicity screening based on their traditional medicinal uses, limited previous research on them, and their availability. The selected plants were extracted with methanol and screened for their cytotoxic potential using the MTT assay against two healthy cell lines (mouse fibroblast (NIH3T3), a healthy monkey kidney (VERO)) and four cancer cell lines namely, gastric (AGS), colon (HT-29), two breast (estrogen-dependent: MCF-7 and estrogen non-dependent: MDA-MB-231). Preliminary cytotoxicity assessment led to the identification of seven plants with significant cytotoxic potential, having $IC_{50} < 1.0$ mg/mL against a minimum of one cancer cell line. The identified plants were: *Avicennia alba*, *Caesalpinia pulcherrima*, *Diospyros peregrina*, *Ecbolium viride*, *Jasminum sambac*, *Clitoria terantea*, and *Saraca asoca.* The bioactivity detected correlated with their traditional uses as anticancer agents. Comparing cytotoxicity effects of the selected plants with that of *Blumea lacera* which was previously screened for cytotoxic potential in our research lab, *Blumea lacera* was found to be more cytotoxic and thus selected for bioassay-guided isolation of constituents.

Blumea lacera crude methanolic leaf extract was subjected to solid phase extraction (SPE, C-18) fractionation using differing gradients of H_2O/MeOH to separate the crude extract into different groups of constituents. Six SPE fractions were generated with varying polarity namely, SPE1 (100% H_2O), SPE2 (80%), SPE3 (65% H_2O), SPE4 (40% H_2O), SPE5 (20% H_2O) and SPE 6 (0% H_2O). The SPE fractions were tested for their cytotoxic activity against NIH3T3, AGS, HT-29, and MDA-MB-231 using the MTT assay. Of the six SPE fractions, SPE4 (IC_{50}: 2.4-39 µg/mL), SPE5 (IC_{50}: 0.2-2.7 µg/mL), and SPE6 (IC_{50}: 2.7-6.4 µg/mL) were identified with significant cytotoxicity against the cell lines tested. SPE4 and SPE5 were selected for further investigation based on their mass.

Reversed-phase C-18 HPLC (gradients of MeOH/H_2O or ACN/H_2O) was employed to isolate and purify constituents from SPE4 and SPE5 affording six compounds namely, **BL1**, **BL2**, **BL3**, **BL4**, **BL5** and **BL6**. Using NMR, UV, IR spectroscopy, mass spectrometry and optical rotation the isolated compounds were characterized as **BL1**: kaempferol-3-*O*-(2"6"-di-*O*-α-L-rhamnopyranosyl)-β-D-galactopyranoside; **BL2**: kaempferol-3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside; **BL3**: (25*R*)-3β-{*O*-β-D-glucopyranosyl-(1→4)-*O*-α-L-rhamnopyranosyl-(1→4)-[*O*-α-L-rhamnopyranosyl-(1→2)]-α-L-rhamnopyranosyl}-22αN-spirosol-5-ene; **BL4**: 3-*O*-β-D-glucopyranosyl-17-hydroxy-6*E*, 10*E*, 14*Z*-(3*S*)-geranyllinalool-17-*O*-β-D-glucopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→6)]-β-D-glucopyranoside;

BL5: 6*E*, 10*E*, 14*Z*-(3*S*)-17-hydroxygeranyllinalool-17-*O*-β-D-glucopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→6)]-β-D-glucopyranoside; and **BL6**: 2*E*, 6*E*, 10*Z*-12-hydroxyfarnesol-12-*O*-β-D-glucopyranosyl-(1→6)-α-L-rhamnopyranoside. Of the six isolated compounds, **BL1** and **BL2** were known flavonoid glycosides but isolated here for the first time from *Blumea lacera*, while **BL3** was characterized as a novel steroidal glycoalkaloid. **BL4** was characterized as a known terpenoid glycoside, but isolated here for the first time from *Blumea lacera*, while **BL5** and **BL6** were characterized as novel terpenoid glycosides, although **BL6** still requires absolute structural confirmation.

The cytotoxicity of **BL1-BL6** was assessed using the MTT assay against two healthy cell lines: a mouse fibroblast (NIH3T3), a monkey kidney (VERO) and four cancer cell lines: gastric (AGS), colon (HT-29), estrogen-dependent breast (MCF-7) and estrogen non-dependent breast (MDA-MB-231). Of the six compounds tested, the novel compound **BL3** exhibited the most potent cytotoxic effects. High activity was detected against estrogen-dependent breast cancer cells, MCF-7 with IC_{50} of 2.66 µg/mL, but also against HT-29, AGS and MDA-MB-231 with IC_{50} values of 10.99, 11.36 and 13.92 µg/mL, respectively. The novel compound **BL5** also showed significant cytotoxic potential against estrogen-dependent breast cancer cells, MCF-7 cells with IC_{50} of 6.66 µg/mL and estrogen non-dependent breast cancer cells, MDA-MB-231 with IC_{50} of 13.95 µg/mL. Interestingly, **BL5** and **BL6** were comparatively less toxic to both healthy cell lines, NIH3T3 and VERO with IC_{50} of > 22.66 µg/mL.

To investigate the cytotoxicity SAR relating to **BL3**, cytotoxicity assays were carried out on **BL3** along with the selected steroidal glycoalkaloid (SGA) analogues of **BL3**, namely β-solamargine, β-solamarine, α-solasonine, α-solanine, solasodine, khasianine and tomatidine HCl. Importantly, **BL3** showed the highest cytotoxic potential with an IC_{50} of 2.66 µg/mL. This indicates that the number and nature of the sugar moieties along with the sugar linkages to the aglycone are linked to cytotoxic activity.

To identify the mechanism by which **BL3** and **BL5** exert their cytotoxicity against MCF-7 cancer cells, flow cytometry studies looking at apoptotic and cell cycle effects using annexin V (AV) and propidium iodide (PI) staining were carried out. **BL3** induced apoptosis (24.35% AV+/PI-) to a slightly lesser degree than the known anticancer drug paclitaxel (35.05% AV+/PI-), whereas **BL5** induced apoptosis in MCF-7 cells to a slightly higher degree (45.5% AV+/PI-) than paclitaxel (39.9% AV+/PI-). Cell cycle analysis revealed that **BL3** arrested MCF-7 cells in the G1 phase, whereas **BL5** showed no considerable effects on any of the three phases of the cell cycle of MCF-7 cancer cells.

Apoptotic studies with the **BL3** analogues β-solamargine, β-solamarine, α-solasonine and α-solanine revealed that **BL3** had the highest apoptotic effects; **BL3** (24.35% AV$^+$/PI$^-$) > β-solamargine (13.35% AV$^+$/PI$^-$) > α-solanine (4.62% AV$^+$/PI$^-$).

Cell cycle analysis with these compounds revealed that **BL3**, β-solamargine, β-solamarine and α-solasonine, arrested MCF-7 cells in the G1 phase to similar degrees, with **BL3** having the largest effect; **BL3** (15.67%) > β-solamarine (12.27%) > β-solamargine (10.30%) > α-solasonine (11.68%). Given the closeness in effects, but the chemical differences of these compounds with regards to sugar moiety number and linkages, as well as aglycone structure it is difficult to assign a specific SAR that depends on these features.

Thus, the most significant cytotoxic activity of **BL3** was attributed to its apoptotic effects and G1 phase arresting potential, whereas the significant cytotoxic activity of **BL5** was attributed to its apoptosis induction as **BL5** showed very weak cell cycle arresting potential. However, further studies are required to confirm exact molecular mechanism/s of cytotoxic activity of **BL3** and **BL5**.

Antifungal activity of **BL1**, **BL2**, **BL3**, **BL4**, and **BL5** and some selected SGAs were investigated with none of the compounds showed antifungal effects.

Declaration

This thesis is the result of the author's original research and has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

Name:...

Signature:...

Date:..

LIST OF ABBREVIATIONS

Advanced DMEM	Advanced Dulbecco's modified Eagle's medium
AChE	Acetylcholinesterase
7AAD	7-Amino-actinomycin D
ACN	Acetonotrile
AIDS	Acquired Immune Deficiency Syndrome
AIHW	Australian Institute of Health and Welfare
AML	Acute myeloidleukemia
amu	atomic mass unit
AP	Aerial parts
app *t*	apparent triplet
APCI	Atmospheric pressure chemical ionization
APCI-MS	Atmospheric pressure chemical ionization-Mass spectrometry
ATCC	American type tissue collection
B	Bark
BV	Bed volume
BL1	*Blumea lacera* compound 1
BL2	*Blumea lacera* compound 2
BL3	*Blumea lacera* compound 3
BL4	*Blumea lacera* compound 4
BL5	*Blumea lacera* compound 5
BL6	*Blumea lacera* compound 6
CD$_3$OD	Deuterated methanol
CE-MS	Capillary electrophoresis-Mass spectrometry
CHCl$_3$	Chloroform
CE-MS	Capillary electrophoresis-Mass spectrometry
CHF	Congestive heart failure
CM E	Crude methanolic extract
CML	Chronic myeloid leukemia

LIST OF ABBREVIATIONS

CNS	Central nervous system
COSY	Correlation spectroscopy
COX	Cyclooxygenase
DNA	Deoxyribonucleic acid
DAD	Diode array detector
DAPI	4',6-Diamidine-2-phenylindole
DEPT	Distortionless enhancement by polarization transfer
DMSO	Dimethyl sulfoxide
EDTA	Ethylene diamine tetra-acetic acid
eIF	Eukaryotic initiation factor
ESI-MS	Electrospray ionization-Mass spectrometry
FITC	Fluorescin isothiocyanate
F	Flowers
Fr	Fruits
5-FU	5-Fluorouracil
GC-MS	Gas chromatography-Mass spectrometry
GM-CSF	Granulocyte macrophage colony-stimulating factor
^{1}HNMR	Proton NMR
HPLC	High Performance Liquid Chromatography
HR-ESI-MS	High resolution- Electrospray ionization-Mass spectrometry
HMBC	Heteronuclear multiple bond coherence
HSQC	Heteronuclear single quantum coherence
Hz	Hertz
IC_{50}	Inhibition concentration 50%
INF-γ	Interferon gamma
IR	Infrared
J	Coupling constant

LIST OF ABBREVIATIONS

Jmod	J-modulated spin-echo
L	Leaves
LC-MS	Liquid chromatography-Mass spectrometry
Log ε	Molar extinction co-efficient
LR-MS	Low resolution-Mass spectrometry
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
MeOH	Methanol
MS	Mass spectrometry
m/z	mass by charge ratio
NA	Not available
NF	Not found
NBCS	New born calf serum
NCI	National Cancer Institute
NF*k*-B	Nuclear factor kappa binding
NMR	Nuclear magnetic resonance
OD	Optical density
PBS	Phosphate Buffered Saline
PDA	Photodiode array
PI	Propidium iodide
PS	Phosphatidylserine
ppm	Parts per million
R_T	Retention time
RB	Root bark
RP	Reverse-phase
R	Root
ROS	Reactive oxygen species
S	Seeds

LIST OF ABBREVIATIONS

SB	Stem bark
SAR	Structure activity relationship
SDA	Sabouraud Dextrose agar
SDB	Sabouraud Dextrose broth
SGA	Steroidal glycoalkaloid
SPE	Solid phase extraction
SPE1	Solid phase extraction fraction 1
SPE2	Solid phase extraction fraction 2
SPE3	Solid phase extraction fraction 3
SPE4	Solid phase extraction fraction 4
SPE5	Solid phase extraction fraction 5
SPE6	Solid phase extraction fraction 6
T	Tomentum
TB	Tuberculosis
TFA	Trifluoroacetic acid
TNF-α	Tumor necrosis factor-alpha
UV	Ultraviolet
W	Wood
WHO	World Health Organization
WP	Whole Plant
XTT	[2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide]
δ	Chemical shift

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CHAPTER ONE

INTRODUCTION

1.1 Significance and approaches to drug discovery from natural products

Natural products have been recognized as a source for medicines since ancient times across the world for treating and preventing human diseases (Cragg, Newman et al. 1997). They have been derived from various sources including plants, microorganisms, marine organisms, vertebrates and invertebrates (Newman, Cragg et al. 2000). Despite major scientific and technological progress in combinatorial chemistry, drugs derived from natural products still make an enormous contribution to drug discovery today. Natural sources possess chemical diversity and therefore serve as an important reservoir of bioactive leads in the development of new drugs by providing novel templates for new drugs, as well as patterns for structural modifications to produce more potent and safer drugs (Gordon 2005).

Natural products and their derivatives represent over 60% of all drugs clinically used worldwide where natural products from medicinal plants alone contribute to 25% of total drugs (Gurib-Fakim 2011). Natural products and related drugs are reportedly used for example as antibacterial, anticancer, anticoagulant, antiparasitic and immunosuppressant agents to treat 87% of all categorized human diseases (Newman, Cragg et al. 2003). More than 28% of new chemical entities introduced into the market are derived from natural products (Xiong, Wang et al. 2013).

More than 100 compounds particularly, anticancer and anti-infectives, which are derived from natural products, are undergoing clinical trials at present and at least 100 natural products-derived compounds (primarily plant or microbial sources) are in preclinical development presently (Harvey Alan 2008).

The value of natural products in this regard can be assessed using 3 criteria: (i) the rate of introduction of new chemical entities of wide structural diversity, including serving as templates for semi-synthetic and total synthetic modification (Newman, Cragg et al. 2003), (ii) the number of diseases treated or prevented by these substances (Koehn 2005), and (iii) their frequency of use in the treatment of disease.

Natural products also serve as pharmacological tools, for example digitoxin from foxglove helped to elucidate the role of the sodium-potassium-ATPase pump in the human body; or morphine isolated from poppy seeds was used to explore the way endogenous opoids affect their receptors. In addition, muscarine, nicotine and tubocurarine helped to identify the different types of acetylcholine receptors (Harvey Alan 2008).

Table 1.1 shows some examples of drugs derived from natural sources that are currently used in clinical practice (Farnsworth 1990; Balandrin 1993; Cragg 1999). Apart from plant sources, drugs are also derived from animals, microorganisms or marine organisms.

Table 1.1 Some drugs developed from natural products

Drug	Medical use	Source	Mechanism of action
Aspirin	Analgesic, anti-inflammatory, antipyretic	Plant	Inhibition of COX
Digoxin	For atrial fibrillation and CHF	Plant	Inhibition of the Na^+/K^+ ATPase membrane pump
Quinine	Malaria prophylaxis	Plant	Protein synthesis inhibition
Insulin	Antidiabetic	Animal	Binding α-subunit of tyrosine kinase
Penicillin	Antibiotic	Microorganism	Inhibition of peptidoglycan synthesis
Tetracycline	Antibiotic	Microorganism	Protein synthesis inhibition
Aurantosides	Antifungal	Marine organism	Inhibition of tubulin polymerisation
Manoalide	Analgesic and anti-inflammatory	Marine organism	Phospholipase A2 inhibition

COX = Cyclooxygenase; CHF = Congestive heart failure

The table 1.1 highlights that drugs derived from natural sources have different modes of action and have been employed to treat different types of disease. Success in natural product research from plant sources demands careful plant selection, based on several approaches such as, a) chemotaxonomic data; b) information from traditional medicine which is based on i) plants used in an organized traditional medicine system, ii) herbalism, folklore, and shamanism, iii) use of

databases; c) field observations; d) random selection by chemical screening and; e) follow-up of biological activity reports.

One principal approach in the isolation of new lead compounds is bioactivity-guided isolation, in which pharmacological or biological assays are used to target the isolation of bioactive constituents (Hostettmann and Terreaux 2000; Fabricant and Farnsworth 2001). Another effective approach to isolate the bioactive constituents from extracts is metabolic profiling which involves the detailed analysis of an extract chemical composition by chromatographic-spectroscopic techniques and a subsequent activity evaluation (Halket 2005).

Bioactivity-guided isolation

Bioassay-guided isolation (Figure 1.1) is a multidisciplinary approach to drug discovery, which involves the evaluation of the biological activity of a crude extract from the natural source followed by fractionation. The individual fractions are then tested for their biological activity. Based on the biological activity, the active fractions undergo further fractionation, until pure compounds are obtained. Different technologies are used in this fractionation process, such as column chromatography, flash chromatography, vacuum liquid chromatography, thin layer chromatography, semi-preparative and preparative HPLC (Prathop 2005). Bioactivity-guided fractionation has resulted in most of the biologically active natural products currently in use (Pezzuto 1997).
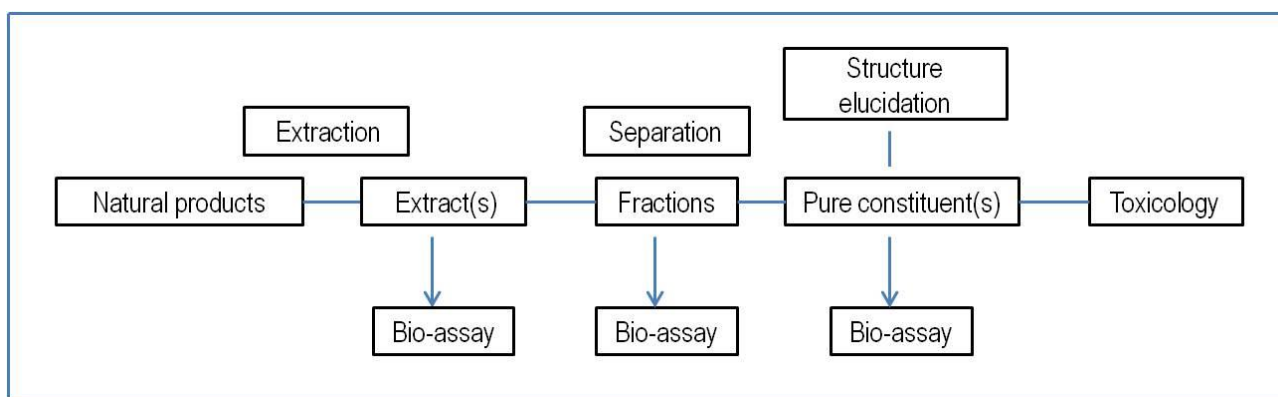
Figure 1.1 Overview on bioactivity-guided isolation

Metabolic profiling

Metabolic profiling provides information on absolute or relative quantities of metabolites present in natural source extracts, thus allowing for their detection and isolation (Torras-Claveria 2009). Furthermore, it provides information on the chemical composition of extracts, with known and unknown spectra, which can lead to the isolation and spectroscopic identification of new bioactive compounds (Berkov 2008). A metabolic profile of a plant extract can be obtained using techniques such as, liquid chromatography-mass spectrometry (LC-MS), gas chromatography-mass spectrometry (GC-MS) and capillary electrophoresis-mass spectrometry (CE-MS) (Halket 2005). In our study, we have employed bioactivity-guided isolation of extracts, to identify activity and isolate bioactive pure compounds.

1.2 Significance of medicinal plants in drug discovery

Plants have been utilized as medicines for centuries. Still, plants remain as the principal source of new drugs, new lead compounds and new chemical entities. Plant-based medicinal systems play a significant role in the health care systems of many countries worldwide. According to the World Health Organization (WHO), about 65% of the world's population and 80% of developing countries' population depend primarily on about 85% of plant-derived traditional medicines (Cragg and Newman 2013). In addition, about 3.5 to 4 billion people in the world rely on plants as sources of drugs (Farnsworth N.R. 1988). Approximately, 80% of 122 pure compounds on the market derived from 94 plant species were originally used for the same or related ethnomedical purposes (Cragg and Newman 2013). Plant studies indicate that there are about 250,000 to 350,000 plant species identified so far, and of them about 35,000 have been used for medicinal purpose across the world (Kong, Goh et al. 2003).

Plants serve as a source of bioactive compounds for direct use as drugs, for example sennosides A & B from *Cassia aculifolia* and *Cassia angustifolia*, vincristine and vinblastine from *Catharanthus roseus*, digitoxin from *Digitalis purpurea* and ephedrine from *Ephedra sinensis* (Fabricant and Farnsworth 2001; Lahlou 2013). Medicinal plants also led to the isolation of novel bioactive

compounds which in turn serve as lead compounds for semisynthesis of patentable entities with enhanced activity and/or reduced toxicity (Lahlou 2013). Table 1.2 provides some examples of lead compounds other than anticancer agents isolated from medicinal plants.

Table 1.2 Lead compounds other than anticancer agents isolated from medicinal plants

Lead compound	Plant source	Developed drug	Pharmacological use
Khellin	*Ammi visnaga*	Chromolyn	Bronchodilator
Galegine	*Galega officinalis*	Metformin	Anti-diabetic
Codeine	*Papaver somniferu*	Meperidine, pentazocine, and propoxyphene	Analgesics
Salicin	*Salix alba*	Aspirin	Analgesic and anti-coagulant
Papaverine	*Papaver somniferum*	Verapamil	Anti-hypertensive
Ephedrine	*Ephedra sinica*	Salbutamol and salmetrol	Anti-asthmatic
Quinine	*Cinchona officinalis*	Chloroquine and mefloquine	Anti-malarial
Artemisinin	*Artemisia annua*	OZ277 and the artemisinin dimeric analogue	Anti-malarial

These drugs are employed for the treatment of a broad array of diseases. In addition, bioactive compounds obtained from plants also serve as pharmacological tools, e.g. lysergic acid diethylamide, mescaline, and yohimbine (Fabricant and Farnsworth 2001). Some examples of the plant derived lead compounds are discussed below.

Bronchodialator: Khellin, isolated from *Ammi visnaga* L., led to the development of chromolyn, a bronchodilator (Cragg and Newman 2013).

Anti-diabetic: Galegine, isolated from *Galega officinalis* L., served as the model substrate for the synthesis of metformin and other bisguanidines used in the treatment of diabetes (Cragg and Newman 2013).

Analgesic and anticoagulant: Codeine was isolated from *Papaver somniferum* and served as the model substrate for the development of the analgesics, meperidine, pentazocine, and propoxyphene. In addition, salicin is a lead compound isolated from the bark of *Salix alba*, led to the development of a potent pain killer and anticoagulant drug, aspirin (Shaikh 2010).

Anti-hypertensive: Papaverine obtained from *Papaver somniferum,* formed the basis for the semi-synthetic anti-hypertensive drug, verapamil (Fabricant and Farnsworth 2001).

Anti-malarial: The anti-malarial drug, quinine was isolated from the bark of *Cinchona officinalis*, formed the basis for the synthesis of the anti-malarial drugs, chloroquine and mefloquine. Due to resistance to chloroquine and mefloquine in many tropical regions, Chinese scientists discovered an exciting new anti-malarial lead compound, artemisinin from *Artemisia annua* which had long been used in the treatment of fevers in Traditional Chinese Medicine (Wongsrichanalai, Pickard et al. 2002; Cragg and Newman 2013). Based on artemisinin, two promising analogues, OZ277 and an artemisinin dimeric analogue, with better efficacy and utility were synthesized which are now used for the treatment of malaria in many countries (Cragg and Newman 2013).

Anti-asthmatic: The anti-asthmatic drug, ephedrine, was isolated from *Ephedra sinica* and has formed the basis for the synthesis of anti-asthmatic beta agonists salbutamol and salmetrol (Cragg and Newman 2013).

These examples show that medicinal plants represent an important source of bioactive lead compounds, possessing chemical diversity and as such providing core scaffolds for the discovery and development of future novel drugs.

1.3 Cancer and anticancer drugs from natural sources

1.3.1 Cancer

1.3.1.1 Prevalence of cancer

Cancer is one of the leading causes of death worldwide. It is a disease characterized by unregulated proliferation of cells. Two factors primarily cause cancer: external factors, such as tobacco, infectious organisms, chemicals and radiation, and internal factors such as inherited mutations, hormones, immune conditions, and mutations that occur from metabolism. These factors either act together or in sequence to initiate and develop cancers.

The international agency for cancer research reported the estimated incidence, mortality and prevalence of 27 different types of cancers worldwide (Mondal, Bandyopadhyay et al. 2012). According to WHO 2010 statistics, "each year over 12 million people are diagnosed with cancer and it kills more people than AIDS, malaria, and tuberculosis (TB) combined". It is also estimated that nearly 84 million people will die due to cancer without intervention between 2005 and 2015. By 2030, there will be more than 13 million deaths from cancer around the world and nearly 21 million diagnosed cases annually, according to WHO (Berkov, Codina et al. 2008). In the year 2050, it is estimated that 17 million new cases of cancer will be diagnosed in less developed countries and 7 million new cases of cancer will occur in more developed countries (Cragg, Newman et al. 1997; Schwartsmann, Ratain et al. 2002).

Cancer is also identified as a major cause of illness in Australia. Cancer is the second most common cause of death after cardiovascular disease and accounts for the death of 3 in 10 Australians (AIHW 2012). According to the Australian Institute of Health and Welfare (AIHW), the most frequently reported cancers in males are prostate, bowel, breast, skin and lung cancer (AIHW 2012). The most commonly diagnosed cancers in females are breast, bowel, skin, lung and uterine cancer. More than 42,800 Australians died from cancer in 2010. According to the Cancer Council, Australia, 114,000 new cases of cancer were diagnosed in Australia in 2010. By the age of 85, 1 in 2 Australians will be diagnosed with cancer (Cancer Council Australia 2011; AIHW 2012).

Bangladesh is a developing country and is one of the most densely populated countries of the world. According to Global Cancer (GLOBOCAN), in Bangladesh 291,200 patients were diagnosed with cancer in a 5 year period (2003-2007). In Bangladesh, the top five malignancies in men and women occur in lung, breast, cervical, lip and oral cavity, and oesophagus. Lung cancer is estimated as being the most prevalent in men, whereas breast cancer is most prevalent in women. The top five paediatric cancers identified in Bangladesh are retinoblastoma, Ewing's sarcoma (a rare bone cancer worldwide), osteosarcoma, rhabdomyosarcoma, and nephroblastoma (Uddin, Khan et al. 2013)".

In our study, gastric, colon and breast cancer cells have been selected for screening the anticancer potential of selected Bangladeshi medicinal plants, hence the focus on these cancers in the following paragraphs.

Gastric cancer, commonly referred to as stomach cancer, is a common cancer worldwide. According to WHO, stomach cancer causes about 800,000 deaths worldwide each year (WHO 2010) Stomach cancer is also a common cancer in Australia. According to the Cancer Council of Australia, more than 2,000 new cases of stomach cancer are being diagnosed each year nationally. Stomach cancer affects nearly twice as many men than women. The risk of being diagnosed by the age of 85 is 1 in 61 for men and 1 in 137 for women (Cancer Council Australia 2011). Gastric cancer is the second most common cancer in Asia (56%), with more than half of the world's gastric cancer arising in Eastern Asia. The risk of gastric cancer varies among the countries and populations in the Asia-Pacific region. High-risk areas include East Asian countries such as China, Japan and Korea, (Sasako 2010). In Bangladesh, stomach cancer is the fifth most common cancer in men and 5.1% of men are diagnosed with stomach cancer each year (A. F. M. Kamal Uddin 2013).

Colon cancer is a major health burden worldwide. According to WHO, approximately 639,000 deaths occur from colon cancer per year worldwide (WHO 2010). Worldwide approximately 663,000 cases of colorectal cancer, 10% of the total in men, and 571,000 cases, 9.4% of the total in women, are diagnosed each year. In 2008, approximately 1.2 million cases of bowel cancer were

recorded; accounting for around 10% of all new cancer cases and the number of cases will increase to 2.2 million by 2030. More than half of all colorectal cancers occur in developed regions, such as Europe, Northern America and Australasia; about a third occur in developing countries, such as Asia (http://www.wcrf.org/cancer_facts/bowel_cancer_rates.php 2011). Colorectal cancer is the second most common cancer followed by prostate cancer in Australia (AIHW 2012). Approximately 15,840 new cases were diagnosed in 2012 in Australia (AIHW 2012). The risk of being diagnosed by the age of 85 is 1 in 10 for men and 1 in 15 for women (Cancer Council Australia 2011). In contrast, in Bangladesh colon cancer is not as common as breast and lung cancer.

Breast cancer is the most frequently diagnosed life-threatening cancer in women and the leading cause of cancer related death among women across the world. It is the second most common cancer and accounts for 1.38 million new cases every year across the world (Mondal, Bandyopadhyay et al. 2012). Based on WHO statistics in 2010, about 519,000 deaths occur from breast cancer per year worldwide (WHO 2010). Breast cancer is the second most common cancer in woman following lung cancer in Australia. More than 13,700 new cases are diagnosed each year in Australia and the risk of being diagnosed by the age of 85 is 1 in 8 for women (Cancer Council Australia 2011). In Bangladesh, breast cancer is the most common cancer in women with over 25.6% of women being diagnosed with breast cancer each year (A. F. M. Kamal Uddin 2013).

1.3.1.2 Pathophysiology of cancer

Cancer is characterized by uncontrolled growth and spread of cells. Abnormalities in the regulation of the cell cycle cause disturbances in the physiological cell death process that prevents or delays normal cell turnover, and can lead to pathogenesis of cancer (John C. 1996). Figure 1.2 illustrates the development of cancer stem cells from the normal cells and progenitor cells. Genetic and epigenetic alterations such as hormonal stimulation, DNA damage cause normal stem cell activation and this activated stem cell is further altered by genetic and epigenetic alterations, finally these alterations cause mutation of the stem cell. These genetically altered (mutated) cells undergo

proliferation and produce cancer stem cells. Similarly progenitor/transit-amplifying cells undergo mutations and these mutated cells produce cancer stem cells by mitosis.
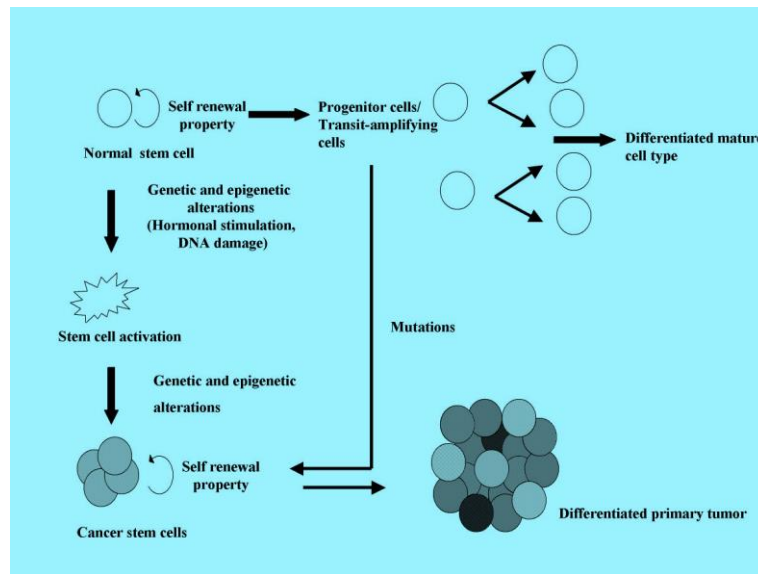
Figure 1.2 Development of cancer stem cells from normal stem cells and progenitor cells (Vaish 2007)

Carcinogenesis is considered as a multistep process which consists of initiation, promotion and progression. In the initiation phase, exposure of healthy cells to a mutagen induces mutation, deletions, substitutions, gene amplification or chromosomal translocation. Structural and functional alterations in the genetic apparatus are induced by the cellular oncogenes, recessive cancer genes and other regulatory sequences. If genetically altered, the cell undergoes mitosis, and at this stage normal DNA repair mechanisms cannot correct the genetic alteration of the initiated cell, or the cell is about to die and the alteration becomes permanent. In the initiated cells, cancer promoting agents act epigenetically altering genetic expression and causing proliferation in initiated tissue as these cells are more susceptible to cancer promoters. In the progression phase, these proliferated tissues undergo fast growth, autonomy, invasion and metastasis and are turned into malignant neoplasms (Hodges Gisele M. 1993).

1.3.2 Anticancer drugs from plants

Over the centuries, plants have been used in the treatment of cancer. Out of an estimated 250,000 plant species worldwide more than 3,000 have reported with significant anticancer potential (Mans 2000; Rajandeep Kaur 2011). Plant-based drug discovery has contributed and is contributing to the development of anticancer drugs as well as new anticancer lead compounds in clinical trials (Unnati, Ripal et al. 2013). For example, an extract of *Camptotheca acuminata* showed anticancer activity which led to the isolation of an anticancer drug, camptothecin (Rajandeep Kaur 2011). Over 60% of cancer patients use vitamins or herbs in their cancer therapy (Rajandeep Kaur 2011). Plants with good immunomodulatory and antioxidant effects have potential anticancer activity (Rajandeep Kaur 2011). Naturally occurring anti-inflammatory or immunomodulatory plant metabolites provide anticancer activity through induction or suppression of specific cellular inflammatory activites. In addition, they also act as anticancer agents by scavenging reactive oxygen species (ROS). For instance, shikonin (a naphthoquinone from *Lithospermum erythrorhizon*) provides anticancer activity through inhibition of TNF-α, granulocyte macrophage colony-stimulating factor (GM‑CSF), whereas resveratrol from grapes induces anticarcinogenesis through antioxidant activity (Aravindaram and Yang 2010).

However, there are numerous plants identified with anticancer potential that have not to date been investigated for the isolation and development of new anticancer drugs. There is much basis for such studies, with plant derived anticancer compounds (Table 1.3, Figure 1.3) such as vinblastine, vincristine, etoposide, teniposide, paclitaxel, docetaxel, topotecan, irinotecan, homoharringtonine, flavopiridol, 4-ipomeanol and silvestrol coming from such studies (Mans 2000; Ashis 2001; Mann 2002; Simmons T. 2005; Kinghorn, Carcache de Blanco et al. 2009; M. Joyce Nirmala 2011; Rajandeep Kaur 2011; Cragg and Newman 2013; Unnati, Ripal et al. 2013; Cragg and Newman 2009).

Table 1.3 Some representative plant-derived anticancer compounds in clinical use or drug development

Compound	Drug class	Plant source	Mechanism of action
Vinblastine, vincristine	Vinca alkaloids	*Catharanthus roseus*	Inhibition of tubulin polymerisation
Etoposide, teniposide	Lignans	*Podophyllum* species	Inhibition of topoisomerase II
Paclitaxel, docetaxel	Taxanes	*Taxus* species	Stabilisation of microtubules
Topotecan, irinotecan	Camptothecins	*Camptotheca acuminata*	Inhibition of topoisomerase I
Homoharringtonine	Cephalotaxanes	*Cephalotaxus harringtonia II*	Inhibition of protein synthesis
Flavopiridol	Flavones	*Dysoxylum binectariferum*	Inhibition of cell cycle progression at G1or G2 phase
4-Ipomeanol	Furanoterpene	*Ipomoea batatas*	Cytochrome P-450-mediated conversion into DNA binding metabolites
Silvestrol	Rocaglate derivative	*Aglaia foveolata*	Triggers apoptosome/mitochondrial pathway
Berbamine	Bis-benzylisoquinoline alkaloid	*Berberis amarensis*	Caspase-3 depenedent apoptosis
Beta lepachone	Orthonapthoquinone	*Tabebuia abellanedae*	Inhibition of topoisomerase I and II
Maytansine	Ansamycin macrolide	*Maytenus serrata*	Inhibition of microtube assembly

Vinblastine and vincristine: Vinka alkaloids such vinblastine and vincristine belong to an important group of anticancer drugs. They are naturally occurring active compounds isolated from the Madagascar periwinkle plant, *Catharanthus roseus*. These compounds inhibit tubulin polymerization and are primarily used in combination with other anticancer drugs for the treatment of a variety of cancers (M. Joyce Nirmala 2011; Cragg and Newman 2013).

Etoposide and teniposide: These are semi-synthetic derivatives of the natural product epipodophyllotoxin, an isomer of podophyllotoxin, which is an active antitumor agent, being isolated from the roots of various species of the genus *Podophyllum*. These antitumor agents act

through inhibition of topoisomerase II, an important enzyme involved in the replication of DNA. Etoposide and teniposide are used for the treatment of small cell lung cancer, glioma, bladder cancer, lymphomas, bronchial and testicular cancers (Australian medicines Handbook 2007; M. Joyce Nirmala 2011; Cragg and Newman 2013).

Paclitaxel and docetaxel: Paclitaxel was isolated from *Taxus brevifolia* and belongs to the taxanes whereas docetaxel is a semi-synthetic derivative of paclitaxel. Docetaxel was found to be more effective than paclitaxel due to it increased water solubility. Both docetaxel and paclitaxel are used as first- and second line treatments of metastatic cancer, breast cancer and ovarian cancer. They are also effective in the treatment of lung cancer, prostate cancer and lymphoid malignancies. They bind to the polymerized microtubules and prevent mitosis occurring, thus, stabilization of the microtubules (Cragg and Newman 2005; M. Joyce Nirmala 2011; Cragg and Newman 2013).

Topotecan and irinotecan: Topotecan and irinotecan are semi-synthetics, derived from camptothecin, which was isolated from *Camptotheca acuminata*. The compounds inhibit the enzyme DNA topoisomerase I (Cragg and Newman 2013; Cragg and Newman. 2009). Topotecan is used in patients with epithelial ovarian cancer and small cell lung cancer as a second-line treatment, whereas irinotecan was found to be effective as a first and second line defence in the treatment of metastatic colorectal cancer (M. Joyce Nirmala 2011).

Homoharringtonine: It is an alkaloid ester of cephalotaxine which was originally used as a traditional Chinese medicine to cure cancer. Cephalotaxine was isolated from coniferous shrubs of the *Cephalotaxus* species. Homoharringtonine is found to be effective against various leukaemic cell lines, and works by inhibiting protein synthesis and causing inhibition of chain elongation during translation. It is reported that a mixture of harringtonine and homoharringtonine can be used in treating both acute myeloidleukemia (AML) and chronic myeloid leukemia (CML) (Cragg and Newman 2005; M. Joyce Nirmala 2011).

Flavopiridol: A semi-synthetic flavone derived from the plant alkaloid rohitukine, which was isolated from *Amoora rohituka* and *Dysoxylum binectariferum*. Flavopiridol is reported to act

through arresting cell cycle progression at G1 or G2 phase by interfering with the phosphorylation activity of cyclin dependent kinases. Flavopiridol is currently undergoing phase I trials for treating solid tumors and is also undergoing phase II clinical trials for the treatment of a wide range of cancers such as colorectal, non-small cell lung, renal cell carcinoma, non-Hodgkin's lymphoma and chronic lymphocytic leukemia (Mans 2000).

4-ipomeanol: Is a pneumotoxic furan derivative that was isolated from *Ipomoea bataatas* and provides antitumor activity via cytochrome P-450 mediated conversion into DNA-binding metabolites. This cytotoxic agent showed promising results for lung-specific cancer in pre-clinical studies with animal models (M. Joyce Nirmala 2011).

Silvestrol: Was isolated from the fruits and twigs of *Aglaia foveolata*. It is found to be effective against prostate, breast and lung cancers. This compound exerts anticancer activity through impairment of the ribosomal recruitment step of translation initiation by affecting the composition of the eukaryotic initiation factor (eIF) 4F complex (Cencic, Carrier et al. 2009; Kinghorn, Carcache de Blanco et al. 2009).

Berbamine: Was isolated from *Berberis amarensis* and belongs to bisbenzyl-isoquinoline alkaloids. Berbamine provides anticancer effects through inducing caspase-3 dependent apoptosis. It was also reported to inhibit the bcr/abl tyrosine kinase and is used in the treatment of chronic myeloid leukemia (M. Joyce Nirmala 2011).

Beta-lapachone: Is an orthonapthoquinone compound, which was isolated from *Tabebuia avellanedae*. This compound has a broad spectrum of antineoplastic activity and was found to be effective against breast, prostate, lung and pancreatic cancer. It acts by inhibiting topoisomerase I and II (M. Joyce Nirmala 2011).

Maytansine: Very recently, maytansine isolated from the Ethiopian tree *Maytenus serrata* is in clinical trials for cancer treatment (Cragg and Newman 2013).
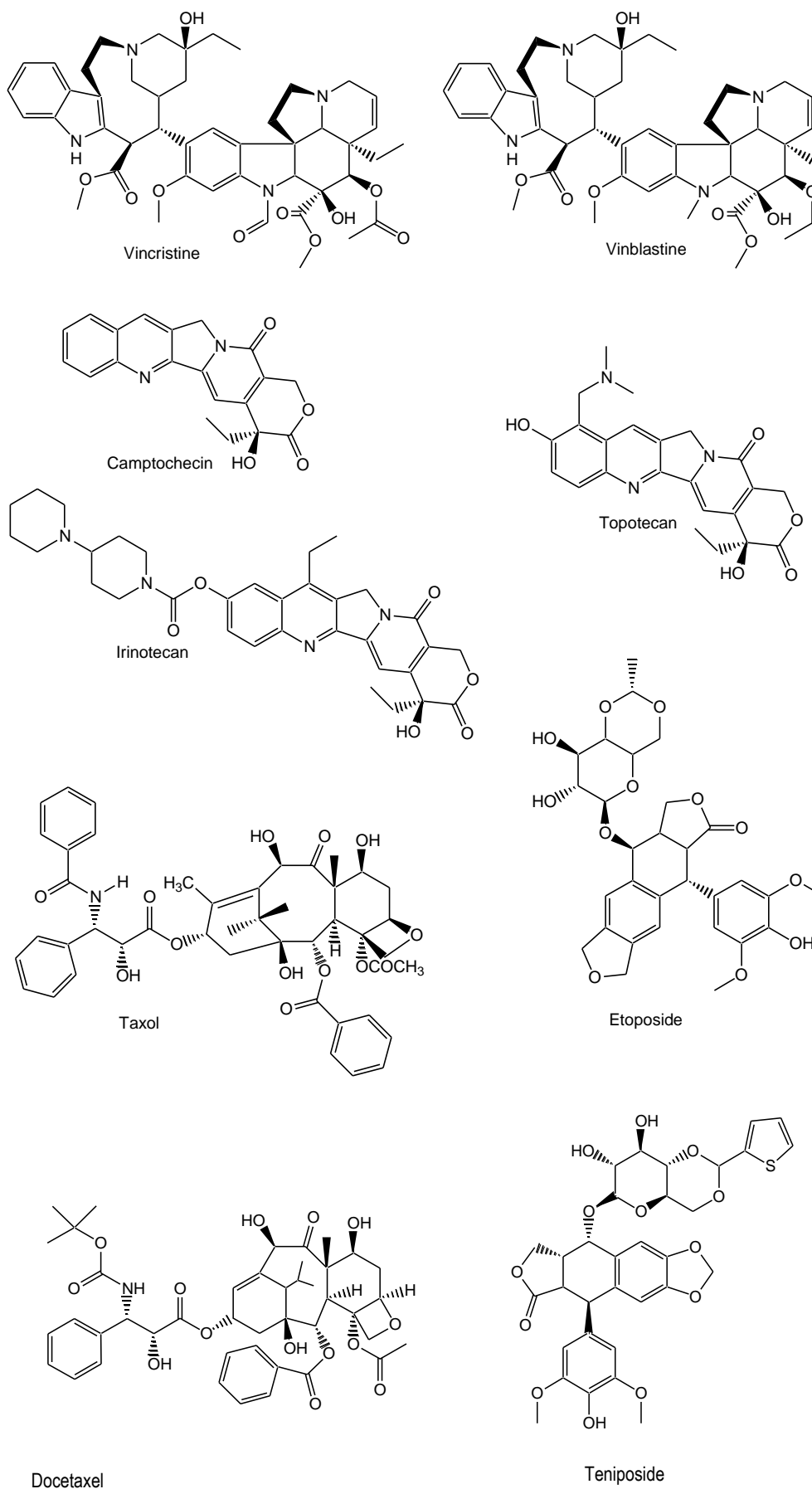
Figure 1.3 Some representative plant-derived anticancer drugs in clinical use or development
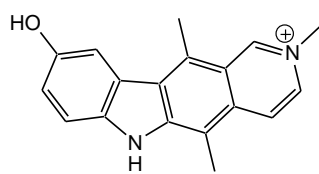
Elliptinium
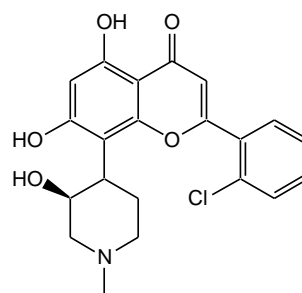
Falvopiridol
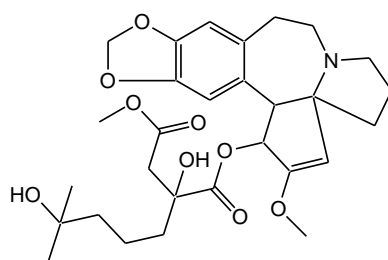
Homoharringtonine
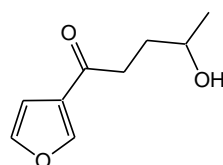
4-Ipomeanol
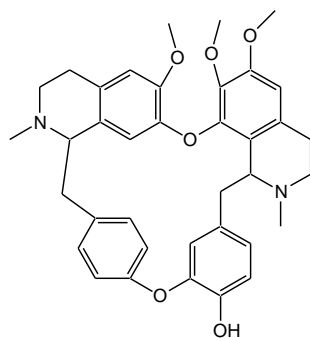
Berbamine
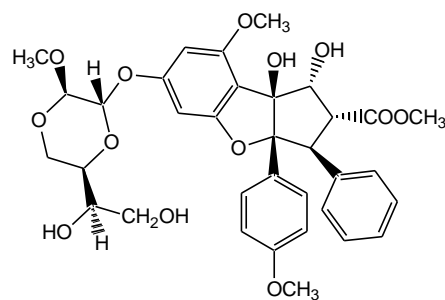
Silvestrol
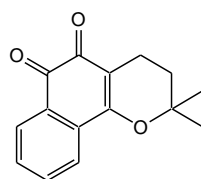
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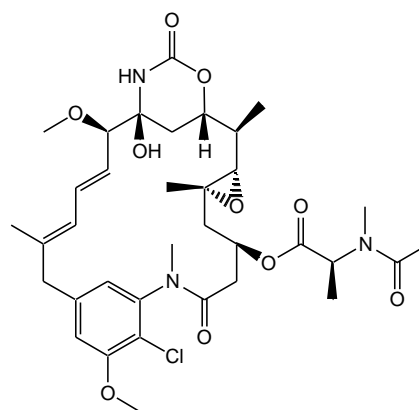
Maytansine

Figure 1.3 Some representative plant-derived anticancer drugs in clinical use or development (contd.)

1.3.3 Anticancer activity screening

Chemotherapy is the use of cytotoxic drugs to damage or kill malignant cells (Sloane 2002) and cytotoxic activity studies have been used to evaluate the potential of anticancer compounds (Wing-Yan Li, Shum-Wan Chan et al. 2007). Rapid screening of new cytotoxic agents for the treatment of cancer and identification of their mechanisms of action requires the use of effective methods. Over the last 50 years cell-based screening methods have been used as a major approach in detecting potential anticancer agents (Houghton 2007). In the process of identifying potential anticancer agents, new agents are screened in regard to their cytotoxic activity (Amit 2001; Ashis 2001; Wing-Yan 2007; Uddin 2010).

1.3.3.1 Cytotoxic activity

Though there are other modes of actions, most cytotoxic drugs cause DNA damage (e.g. melphalan, cyclophosphamide and cisplatin), block normal nucleic acid synthesis (e.g. methotrexate and 5-fluorouracil) or interfere with DNA replication (e.g. doxorubicin and irinotecan) (Houghton, Fang et al. 2007; Felth 2011). A number of experiments can be used to assay this cytotoxic activity and the basic principle is to compare the rate of proliferation of a cancer cell line in the presence and absence of the test substance, usually after a certain period of time. Ideally several different cancer cell lines and healthy cell lines can be used to determine the selectivity to an individual cancer cell line as well as selectivity between cancer cell lines and healthy cell lines. This approach provides an indication of potential usefulness in a clinical setting, for which a selectivity of at least two orders of magnitude in favour of the cancer cell line being the more susceptible is required (Houghton, Fang et al. 2007). Cytotoxicity tests are based on cell viability as well as on cell survival. Colonization of cells, their net change in population size, mass or gross metabolic activity is used to detect cell viability, whereas reproductive capacity of the cells determines cell survival (Jenkins). The colorimetric assay using MTT is one of the most common methods used to test for cytotoxic activity, whereas apoptosis or necrosis and cell cycle analysis can be used to identify the mechanism of the cytotoxicity observed.

1.3.3.1.1 MTT assay

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl-tetrazolium bromide] assay provides a quantitative, convenient approach for evaluating a cell population's response to external variables, whether it be an increase in cell growth, no effect, or a decrease in growth due to necrosis or apoptosis. The reduction of tetrazolium salts is now recognized as a safe, accurate alternative to radiometric testing and is widely used to quantify cell proliferation and cytotoxicity based on the cellular ability to maintain metabolic function and growth (Florian M. Freimoser. 1999). This method is applicable for the detection of drug sensitivity, cytotoxicity, response to growth factors, and cell activation. Figure 1.4 demonstrates the MTT assay procedure. The method is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT and produce dark blue formazan crystals which are largely impermeable to cell membranes, resulting in their accumulation within healthy cells. The number of viable cells is directly proportional to the level of formazan product being produced and can be readily measured colorimetrically (Sieuwerts 1995).
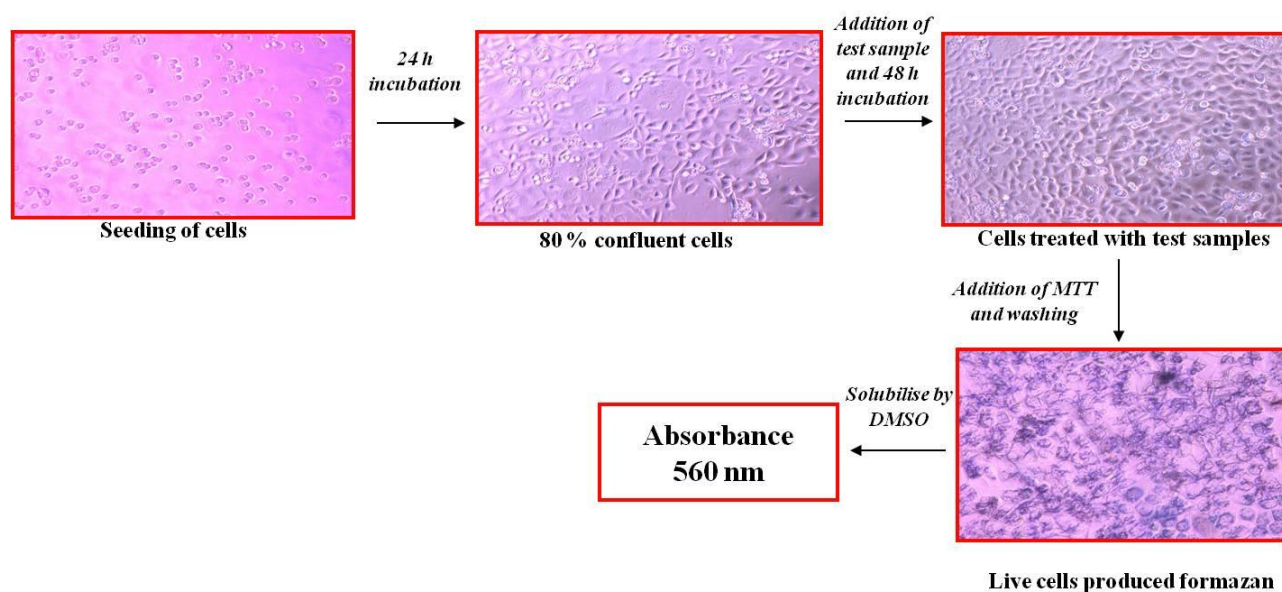
Figure 1.4 MTT assay procedure

1.3.3.1.2 Apoptosis and necrosis assays

The apoptosis and necrosis assay is used to detect apoptosis or necrosis inducing anticancer activity in multi-cellular organisms. Apoptosis is the prevalent form of programmed cell death, which plays a central role in the development and homeostasis in multi-cellular organisms. Disregulation of the apoptotic process can cause many debilitating diseases in humans including cancer (Shi 2005). Figure 1.5 illustrates the apoptosis and necrosis processes. Outstanding morphological features of apoptosis are condensation of nuclear heterochromatin, cell shrinkage, loss of positional organization of organelles in the cytoplasm and formation of apoptotic bodies (Amit 2001; Mooney 2002). Necrosis is a mechanism of cell death which involves cell swelling, chromatin digestion, disruption of plasma and organelle membranes (Mooney 2002; Elmore 2007).
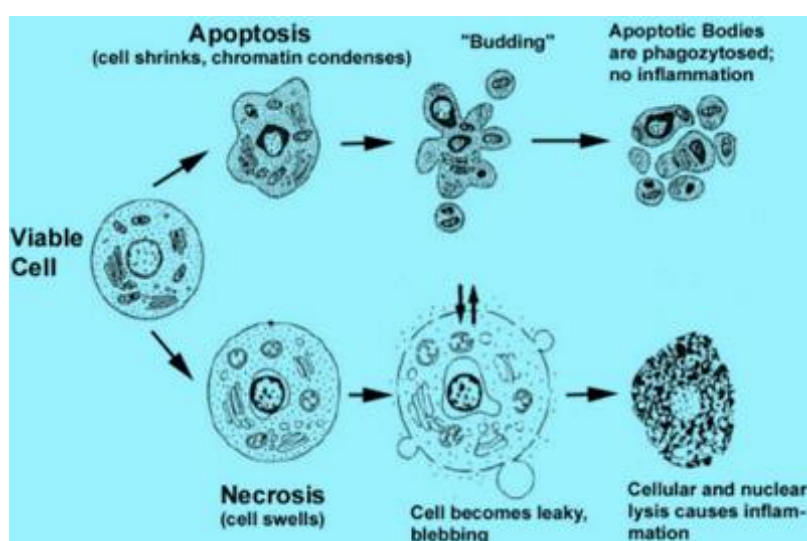
Figure 1.5 Apoptosis and necrosis (adapted from http://www.celldeath.de/encyclo/aporev/aporev.htm)

Flow-cytometry is used to detect apoptosis or necrosis. Flow cytometric identification and quantitation of apoptotic or necrotic cell death is based on the detection of endonucleolytic DNA degradation which results in excretion of low molecular weight DNA from the cell; such cells are then recognized by their fractional DNA content (Darzynkiewicz 1997; Gorczyca 1997; Herr 1997). Double staining fluorescein isothiocyanate (FITC)-labelled annexin V (AV) and propidium iodide

(PI) are used in this assay. In healthy cells the cell membrane integrity is intact which means that neither annexin V nor propidium iodide can enter the cells; this is presented as annexin V negative/propidium iodide negative AV^-/PI^-. In apoptotic cells phospolipid phosphatidylserine (PS) of the cell membrane is translocated from the inner leaflet to the outer leaflet of the plasma membrane. This causes annexin V to be present inside the cells due to alteration of cell membrane asymmetry in early apoptotic cells and is presented as annexin V positive/propidium iodide negaive (AV^+/PI^-). At the late apoptotic phase or at necrotic phase, translocation of phospolipid phosphatidylserine (PS) also occurs; cells lose their membrane integrity and can permeate PI to enter the inside of the cell, which presents as annexin V positive/propidium iodide positive (AV^+/PI^+) (Uddin 2012).

1.3.3.1.3 Cell cycle analysis

Cell cycle plays a critical role in the regulatory process of cell proliferation, growth as well as cell division after DNA damage. The cell cycle regulates the transition from quiescence (G0) to cell proliferation, which is comprised of four different phases: S phase (the periods associated with DNA synthesis), M phase (mitosis) are separated by gaps of varying length called G1 and G2 phases (Schwartz and Shah 2005) (Figure 1.6). Abnormalities in the regulation of the cell cycle causes an imbalance between cell proliferation and apoptotic process and thus, lead to cancer (John C. 1996).
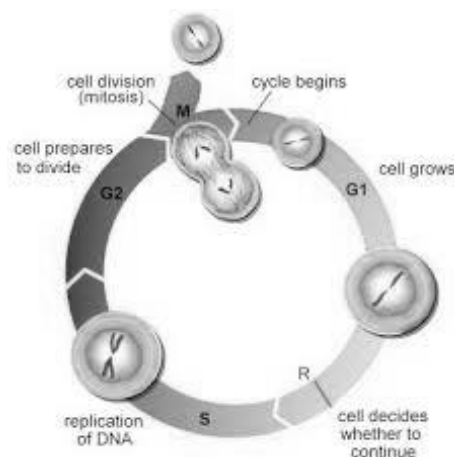
Figure 1.6 A cell cycle (adapted from http://teachline.ls.huji.ac.il/72373/substance_x/q2a.html)

Cell cycle analysis using flow cytometry is an important experimental technique in cell biology to detect anticancer activity of chemotherapeutic agents. Simple cell cycle analysis consists of DNA content measurements, as the DNA content of the cell can provide a great deal of information about the cell cycle, and consequently the effect on the cell cycle of added stimuli, such as transfected genes or drug treatment (Pozarowski ; James W. 2011). A number of dyes such as propidium iodide (PI), 7-amino-actinomycin D (7AAD) and 4',6-diamidine-2-phenylindole (DAPI) can be used to quantify the DNA content of the cells, as the viable cells actively excrete the dye, whereas dead cells passively take up the dye and fluoresce rightly (http://science.cancerresearchuk.org/sci/facs/facs_major_apps/cell_cycle_analysis/?version=1 2008). This analysis shows distribution of cells in three major phases of the cycle (G1 *vs*. S *vs*. G2/M) and can detect apoptotic cells with fractional DNA content as the fluorescence intensity of the stained cells at certain wavelengths correlate with the amount of DNA in the cells (Pozarowski). In our study, propidium iodide staining flow cytometry was employed to perform cell cycle analysis.

1.4 Antifungal drugs from plants

Over 600 plants with antifungal properties have been reported to date (Tasleem, Bhosale et al. 2009). Plants contain a wide variety of bioactive secondary metabolites such as terpenoids, flavonoids, saponins, alkaloids, and tannins which possess antifungal activities (Tasleem, Bhosale et al. 2009). Not surprising therefore, plant extracts have been used as alternative medicines for treating various diseases including fungal infections (Supattra, Kitisin et al. 2014). Table 1.4 shows some examples of herb/plant derived antifungal compounds such as caffeic acid, crassinervic acid, galangin, alizarin, CAY-1 and encelin.

Table 1.4 Some examples of herb/plant-derived antifungal compounds

Compound	Drug class	Herb/Plant source	Mechanism of action
Caffeic acid	Phenylpropane derivative	*Artemisia dracunculus* and *Thymus vulgaris*	Enzyme inhibition through reaction with sulfhydryl groups or interaction with proteins
Crassinervic acid	Phenolic acid	*Piper crassinervium*	Enzyme inhibition through Reaction with sulfhydryl groups or interaction with proteins
Galangin	Flavonoid	*Helichrysum aureonitens*	Complexation with extracellular protein and with fungal cell walls
Alizarin	Anthraquinone	*Rubia tinctorum*	Complex formation with fungal proteins
CAY-1	Triterpene saponin	*Capsicum frutescens*	Disruption of membrane integrity of fungal cells
Encelin	Sesquiterpene lactone	*Montanoa speciosa*	Membrane disruption by the lipophilic nature

Caffeic acid and crassinervic acid: Caffeic acid is a phenylpropane derivative which has been isolated from *Artemisia dracunculus* and *Thymus vulgaris*. It has been reported to be effective against fungi. Crassinervic acid, a phenolic acid derivative isolated from *Piper crassinervium,* exerts fungitoxic effects. Both of them provide antifungal activity through enzyme inhibition, possibly through the reaction with sulfhydryl groups or through interactions with proteins (Tasleem, Bhosale et al. 2009).

Galangin: Galangin belongs to a chemical class of flavonoids and was isolated from the perennial herb *Helichrysum aureonitens*. This compound has been reported to exert antifungal effects against a wide range of fungi through complexation with extracellular or soluble protein and with the fungal cell wall (Tasleem, Bhosale et al. 2009).

Alizarin: Is an anthraquinone aglycone, which was isolated from *Rubia tinctorum*. It provides antifungal activity through complex formation with surface-exposed adhesins, cell wall

polypeptides, membrane-bound enzymes and thus, inactivates the enzymes (Tasleem, Bhosale et al. 2009).

CAY-1: Is a triterpene saponin isolated from *Capsicum frutescens* and has been reported to be active against sixteen different fungal strains. The antifungal activity appears to be due to the disruption of membrane integrity of fungal cells (Tasleem, Bhosale et al. 2009).

Encelin: Encelin is a sesquiterpene lactone isolated from the Mexican species, *Montanoa speciosa*. It has significant fungicidal effects due to membrane disruption by its lipophilic nature (Tasleem, Bhosale et al. 2009).

Bangladesh is a rich repository of medicinal plants which have been traditionally used for treating various diseases including fungal infections. For example, three of our selected Bangladeshi medicinal plants namely, *Caesalpinia pulcherrima* (leaves), *Clerodendrum viscosum* (roots) and *Dillenia indica* (seeds) have been traditionally used to treat fungal infections (Ghani 2003).

A wide number of reports on the antifungal activity studies of Bangladeshi medicinal plants against different fungi strains have been found. Leaves of *Clerodendrum viscosum*, *Enhydra fluctuans*, *Andrographis peniculata* and *Plumbago indica*, and stem bark of *Clausena heptaphylla* have been reported for their fungitoxic potential against *Aspergillus niger* (*A. niger*), *A. fumigatus*, *Candida albicans* (*C. albicans*), *Trichophyton spp.* and other fungi spp. (Amin, Mondol et al. 2012; Saha and Paul 2012; Fakruddin, Mannan et al. 2012). Moreover, antifungal activity studies on another 17 different Bangladeshi plants have been reported against *A. niger*, *C. albicans* and *Saccharomyces cerevisiae* (Rahman, Rahman et al. 2008). In addition, alkaloidal compounds such as xylopine, liriodenine, lanuginosine, 16-oxocleroda-3, 13-*E*-dien-15-oic-acid, kolavenic acid, 16β-hydroxycleroda-3, and 13-dien-15,16-olide, isolated from different Bangladeshi medicinal plants, have been reported for their fungitoxic activity against *A. niger*, *A. fumigatus*, *C. albicans* and other fungi strains (Kaiser, Rahman et al. 2011).

1.4.1 Importance of new antifungals

The increased incidence of invasive fungal infections has been reported due to increasing patients with HIV infection (AIDS), cancer and other individuals receiving immunosuppressive treatment (Badiee, Kordbacheh et al. 2009). Invasive fungal infections remain a major cause of morbidity and mortality in immunocompromised patients (Zai, Khan et al. 1997). A study reported that cancer patients are immunocompromised due to underlying malignancies or cancer therapy. Specific malignancies may be linked to immune deficits that predisposes to infections with particular pathogens such as fungi. Cancer treated with cytotoxic drugs or corticosteroids and with localised or widespread irradiation further deteriorates cancer patients' defense mechanism. Thus, cancer patients remain vulnerable to invasive fungal infections.

Candidiasis is a fungal infection often observed in immunocompromised patients. Colonization of mucosal surfaces by endogenous *Candida* species is often followed by invasion of the vascular space, which carries a high risk of disseminated candidiasis in immunocompromised patients. For example, candida colonization was observed in approximately 57% of 310 immunocompromised patients (Badiee, Kordbacheh et al. 2009).

Widespread and indiscriminate use of antifungal agents also resulted in the development of resistance to current standard antifungal drugs (Tasleem, Bhosale et al. 2009). For example, due to selective pressure and increased use of antifungal drugs; fungi are developing resistance against amphotericin B and fluconazole. Therefore, this potentiates the necessity for searching for novel, safer and more effective antifungal agents to cure fungal infections (Tasleem, Bhosale et al. 2009). As plants provide a useful source of lead compounds with novel structures, a wide range of investigation for new antifungal agents from plants is a necessity.

1.4.2 Antifungal activity screening

An antifungal susceptibility test (AST) is usually used to evaluate the antifungal potential of new compounds. Once fungicidal efficacy has been determined, the AST is also used to compare these

activities to existing agents (UCAST 2008). Use of appropriate and effective screening methods is a requirement to determine the fungicidal ability of antifungal agents, as well as for identification of their mode of action.

Usually, the minimum inhibitory concentration (MIC, in mg/L or µg/mL) is regarded as the most basic measurement of the activity of an antifungal agent and is defined as the lowest concentration that completely inhibits the growth of a fungus (Rodriguez-Tudela, Barchiesi et al. 2003). It categorises fungi as 'susceptible' (S), 'intermediate' (I) or 'resistant' (R) to a drug. It is only valid under the given test conditions and can be used to make decisions about the treatment of patients after taking other factors into account such as pharmacokinetics or pharmacodynamics (UCAST, 2008).

Over the years, based on the principle, different testing methods have been used to detect antifungal activity such as the disc diffusion method (Nweze, Mukherjee et al. 2010), the broth dilution assay (Rodriguez-Tudela, Barchiesi et al. 2003), the gel assay (Troskie, Vlok et al. 2012) and the flow cytometry assay (Green, Petersen et al. 1994). The different methods were evaluated by our research group (Brechbühler 2013) and the broth dilution assay (Sub-section 2.7.2.11; Chapter 2) was chosen to conduct the antifungal tests, the results of which are included in this thesis in (Sub-section 5.5.6, Chapter 5 for steroidal glycoalkaloids; Sub-section 6.4.2.4, Chapter 6 for terpenoid glycosides; Sub-section 7.5.3.1, Chapter 7 for flavonoid glycosides).

1.5 Bangladeshi medicinal plants

1.5.1 Overview of Bangladeshi medicinal plants

Bangladesh is blessed with a rich flora of medicinal plants. Moreover, Bangladesh has the largest block of Sundarban mangrove forest in the world and many of these mangrove plants have been used traditionally as medicines. A long tradition of indigenous herbal medicines, based on the rich local plant diversity, is considered a very important component of the primary health care system. The majority of rural poor households still rely on the traditional system (known as Kabirajee system) for their primary health care needs. Over 500 plant species have been identified as

medicinal plants because of their therapeutic properties contributing to the treatment of various diseases such as cancer, inflammation, diabetes, hypertension, asthma, stomach problems, and cardiac problems (Ghani 2003). More than 250 of those medicinal plants are now commonly used for the preparation of traditional medicines in Bangladesh being grown naturally or under cultivation (Ghani 2003). A total of 400 herbal factories have been established for producing Ayurvedic and Unani medicines based on these medicinal plants. These plants also serve as important raw materials of many modern medicinal preparations and are extensively used in both raw and semi-processed forms as medicines in various pharmaceuticals.The wholesale price of the medicinal plant market in Bangladesh was estimated at US $ 14 million annually in 2005 (Akand 2005).

Although many medicinal plants suitable for commercial development as therapeutics have already been recognized (Akand 2005), they still remain unexplored scientifically (Ghani 2003). Proper scientific evaluation of these medicinal plants pharmacologically and phytochemically, is required to hopefully produce a great variety of new drugs (Ghani 2003). Thus well designed, systematic and objective research in this area will benefit the Bangladeshi people, as well as the international community.

1.5.2 Selection of Bangladeshi medicinal plants for the present study

Of the 500 medicinal plants that have been identified to exist in Bangladesh, only 60 have been studied in any regard for their pharmacological potential, including cytotoxic/anticancer activities (Rahman, Hasnat et al. 2001; Costa-Lotufo Leticia, Khan Mahmud Tareq et al. 2005; George, Bhalerao Siddharth et al. 2010; Uddin Shaikh, Grice et al. 2011). Thus, proper scientific evaluation of the remaining medicinal plants to explore their pharmacological and chemical potential holds future promise for the discovery and development of new therapeutics.

A total of 19 plant species were selected for cytotoxic activity screening in this study. Different plant parts were collected for 4 of the 19 plants selected for this study. Figure 1.7 represents photographs of some of the selected plants.

| A. corniculatum | C. pulcherrima | C. viscosum | C. terantea | D. indica |
| D. | E. viride | J. sambac | M. nagi | S. asoca |
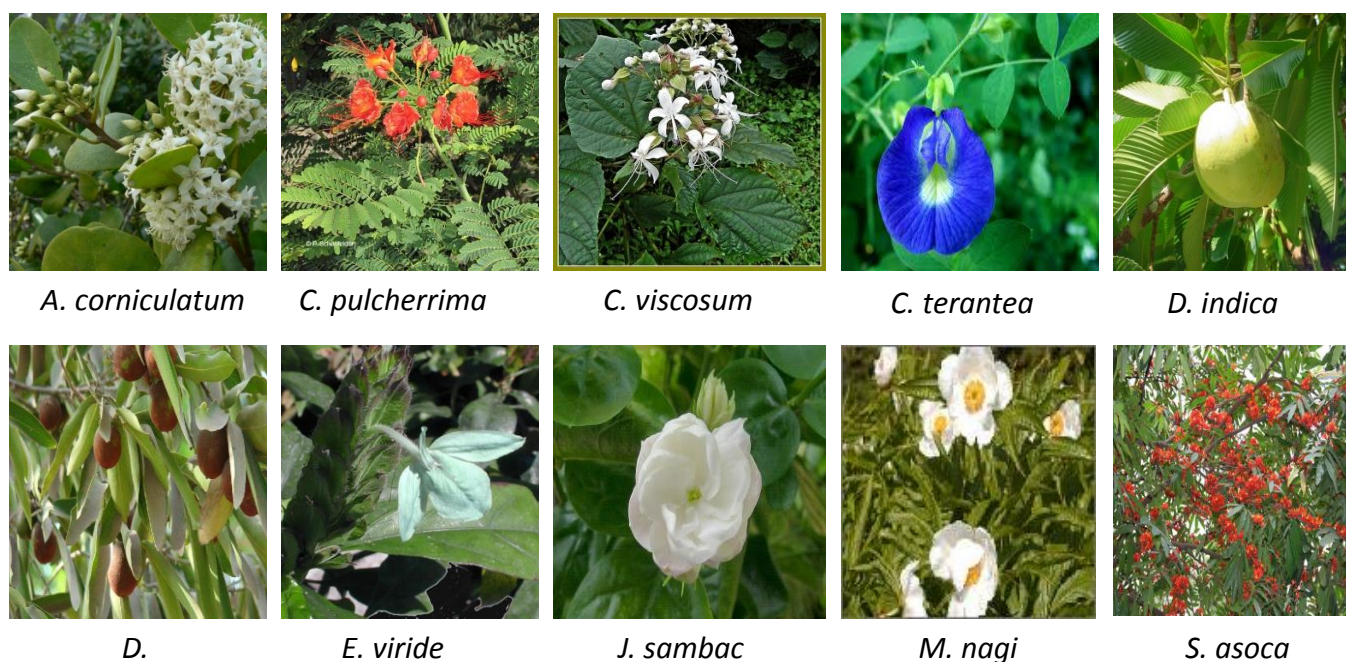
Figure 1.7 Photographs of some selected Bangladeshi medicinal plants

All 19 plants have been used traditionally as anticancer, anti-inflammatory, anti-infectous, antidiabetic, or antidiarrheal agents (Table 1.4). The decision to study the selected species was based on availability of an adequate quantity of plant material and the wide traditional use as anticancer, and anti-inflammatory agent. For example, different parts of 10 of the 19 plant species such as *Aegiceras corniculatum* (*A. corniculatum*), *Avicennia alba* (*A. alba*), *Ceasalpinia pulcherrima* (*C. pulcherrima*), *Clitoria terantea* (*C. terantea*), *Diospyros peregrina* (*D. peregrina*), *Hymenodictyon excelsum* (*H. excelsum*), *Ecbolium viride* (*E. viride*), *Jasminum sambac* (*J. sambac*), *Myrica nagi* (*M. nagi*), and *Saraca asoca* (*S. asoca*) have all been used traditionally as antitumor/anticancer agents. Inflammation and infections are involved in the pathophysiology cancer and it is noteworthy that 7 of our investigated plant species; namely *A. corniculatum*, *Clerodendrum viscosum* (*C. viscosum*), *Dillenia indica* (*D. indica*), *D. peregrina*, *Glycosmis pentaphylla* (*G. pentaphylla*), *H. excelsum*, and *Lannea coromandelica* (*L. coromandelica*) have been used traditionally in the treatment of inflammation and infectious diseases.

Table 1.5 List of the selected Bangladeshi medicinal plants with their traditional uses

Plant species	Family	Local name	Voucher no.	Traditional uses
Aegiceras corniculatum	Myrsinaceae	Kholisha	DACB 31584	**B, L** and **S:** fish poison, asthma, diabetes, *inflammation* and rheumatism (Bandaranayake 1998; Bandaranayake 2002)
Argyreia nervosa	Convolunaceae	Bichtarak	DACB 37954	**S:** hypotensive, hallucinogenic; **AP:** stomach trouble, small pox, syphilis, diarrhoea, dysentery; **L:** wound healing, local stimulant, skin diseases; **R:** rhematic affections, diseases of nervous system, diuretic, gonorrhoea, urinary diseases and *chronic ulcers* (Bandaranayake 1998; Ghani 2003)
Avicennia alba	Avicenniaceae	Morcha baen	DACB 40556	Treatment of anti-fertility, skin diseases, *tumors, ulcers,* (Resin) (Bandaranayake 2002)
Caesalpinia pulcherrima	Caesalpiniaceae	Krishnachura	DACB 32020	**L:** purgative, abortifacient, *anticancer*, fungi toxic, liver disorders; **F:** Cough, asthma, bronchitis, malaria fever; **R:** Cholera, infantile convulsions; **W:** diarrhoea, dysentery, skin diseases (Bandaranayake 1998; Ghani 2003)
Clerodendrum viscosum	Verbenaceae	Bhant	DACB 37953	**WP:** hypotensive, **L:** anthelmentic, emetic, antiperiodic in malaria, cough, asthma, *tumor*, skin disease, snakebite, **R:** antifungal (Ghani 2003)
Clitoria terantea	Papilionaceae	Aparajita	DACB 32021	**R:** demulcent, aperients, laxative, diuretic; **S:** cathartic, ascites, sore throat, *tumors*, dropsy and skin diseases; **AP:** colic, gonorrhoea and skin diseases (Bandaranayake 1998; Ghani 2003)
Dillenia indica	Dilleniaceae	Chalta	DACB 32019	**Fr:** expectorant, laxative, tonic, abdominal pain; **L:** astringent; **B:** astringent; **S:** antifungal and *antibacterial* (Bandaranayake 1998)
Diospyros peregrina	Ebenaceae	Gab	DACB 30323	**B:** astringent, dysentery, biliousness; **Fr:** astringent, sore throat, wounds, *ulcers*, cough, dyspnoea; **S:** diarrhoea, dysentery; **SB:** antiprotozoal, antiviral, diuretic and *anticancer* (Ghani 2003)
Diptreocarpus turbinatus	Dipterocarpaceae	Garjan	DACB 32026	Gonorrhoea, gleets, rheumatism, *ulcer*, ringworm and skin diseases (Bandaranayake 1998)
Ecbolium viride	Acanthaceae	Nilkanta	DACB 32018	**L:** *antitumor*; **R:** *tumor*, jaundice, rheumatism; **WP:** gout, dysuria, cardiovascular diseases (Bandaranayake 1998)

AP: aerial parts; **B:** bark; **F:** flowers; **Fr:** fruits; **L:** leaves; **R:** roots; **RB:** root bark; **S:** seeds; **SB:** stem bark; **T:** tomentum; **W:** wood; **WP:** whole plant

Table 1.5 List of the selected Bangladeshi medicinal plants with their traditional uses (contd.)

Plant species	Family	Local name	Voucher no.	Traditional uses
Glinus oppositifolius	Molluginaceae	Gima	DACB 32014	**WP:** CNS depressant, diuretic, stomachic, aperients, antiseptic and skin diseases (Bandaranayake 1998)
Glycosmis pentaphylla	Rutaceae	Daton	DACB 37931	**L:** fever, liver complaints, coughs, rheumatisms, anaemia, jaundice, eczema and other skin infections; **R:** fever; **Fr:** dysentery (Bandaranayake 1998)
Gnaphalium luteoalbum	Compositae	Boro karma	DACB 37955	**L:** astringent, diuretic, haemostatic, **T:** counter-irritant (Ghani 2003)
Hymenodictyon excelsum	Rubiaceae	Bhui kadam	DACB 32013	**B:** astringent, febrifuge, antiperiodic, hypotensive, *antimicrobial*, diarrhoea; **W:** herpes (Bandaranayake 1998)
Jasminum sambac	Oleaceae	Beli phul	DACB 31262	**AP:** CNS depressant, hypotensive; **L:** *indolent ulcer, breast tumors;* **R:** emmenagogue (Ghani 2003)
Lannea coromandelica	Anacardiaceae	Jeol/Jiga	DACB 35242	**B:** astringent, leprous and *obstinate ulcer,* mouth sores; **L:** local swellings and pain of the body (Bandaranayake 1998)
Mussaenda glabrata	Rubiaceae	Patralekha	DACB 32023	**R:** white leprosy; **L:** jaundice; **F:** diuretic; asthma, fever, dropsy, chest pain and *ulcer* (Bandaranayake 1998)
Myrica nagi	Myricaceae	Kaiphal	DACB 32029	**B:** astringent, carminative, antiseptic, fever, cough asthma, cholera, chronic dysentery, anaemia, piles, chronic bronchitis, *ulcer* and *tumors* (Ghani 2003)
Saraca asoca	Caesalpinaceae	Ashok	DACB 32007	**B:** *antitumor,* menorrhagia, bleeding haemorrhoids, haemorrhagic dysentery, colic, *ulcers;* **L:** blood purification, **F:** haemorrhagic dysentery, biliousness, syphilis, uterine tonic (Bandaranayake 1998; Ghani 2003)

AP: aerial parts; **B:** bark; **F:** flowers; **Fr:** fruits; **L:** leaves; **R:** roots; **RB**: root bark; **S:** seeds; **SB:** stem bark; **T:** tomentum; **W:** wood; **WP:** whole plant

1.5.3 Previous pharmacological and phytochemical evaluation of the selected Bangladeshi medicinal plants

A literature search on the selected plants for this study revealed specific information on pharmacological and phytochemical activities (Table 1.6).

Pharmacological activities:

Pharmacological activities such as cytotoxicity, anticancer, anti-inflammatory, anti-ulcer, analgesic, antipyretic, antidiabetic, hepatoprotective, antibacterial, antifungal, antiviral, antiprotozoal, antituberculosis and CNS activities have been previously reported for the selected plants in this research work.

Cytotoxic/anticancer activities: Of the 19 plant species, cytotoxic/anticancer activities of 9 plants, namely *C. pulcherrima*, *C. viscosum*, *D. indica*, *D. peregrina*, *G. pentaphylla*, *H. excelsum*, *J. sambac*, *M. nagi*, and *S. asoca* have previously been reported (see Table 1.6). Importantly, reported cytotoxic/anticancer activity of *C. pulcherrima*, *C. viscosum, D. peregrina*, *J. sambac*, *M. nagi*, and *S. asoca* correlates to their traditional uses as anticancer agents (see Table 1.5). In addition, *D. indica*, *G. pentaphylla*, and *H. excelsum* have been traditionally used for the treatment of infectious diseases and the reported anticancer activity may be attributed to their anti-infective properties (Table 1.5). The cytotoxic potential of *A. corniculatum* bark has been reported, but there are no reports on the cytotoxic activity of the fruits (Uddin 2011).

Anti-inflammatory potential: The relationship between inflammation and cancer is now well established. Proliferation of cells itself does not cause cancer, however cancer can be potentiated by sustained cell proliferation in an environment rich in inflammatory cells, growth factors, activated stroma, and agents that promote DNA-damage (Lisa 2002). The anti-inflammatory properties of different parts of 11 plants, namely *A. corniculatum, C. pulcherrima, C. terantea, E. viride, G. oppositifolius, G. pentaphylla, J. sambac, L. coromandelica, M. glabrata, M. nagi* and *S. asoca* have been reported (see Table 1.6). It is worth mentioning that *A. corniculatum,* and *L.*

coromandelica among these plants have been used traditionally for the treatment of inflammation (see Table 1.5).

Antioxidant/free radical scavenging activity: Some anticancer compounds act by scavenging free radicals, which otherwise would cause DNA/protein damage (Wing-Yan 2007). Antioxidant acitivities have been reported for 14 plant species, namely *A. corniculatum, A. alba, C. pulcherrima, C. viscosum, D. peregrina, D. indica, E. viride, G. oppositifolius, G. luteoalbum, J. sambac, L. coromandelica, M. glabrata, M. nagi* and *S. asoca* (Table 1.6). Among these plants, reported antioxidant activity of *A. alba*, *C. pulcherrima*, *C. viscosum*, *D. peregrina*, *E. viride*, *J. sambac*, *M. nagi* and *S. asoca* correlates to their traditional uses as anticancer agents (see Table 1.5).

Antimicrobial, antibacterial, antiviral, and antifungal activities: Antimicrobial activity of 11 species, namely *A. alba*, *C. pulcherrima*, *C. terantea*, *D. indica*, *G. pentaphylla*, *H. excelsum*, *J. sambac*, *L. coromandelica*, *M. glabrata*, *M. nagi*, and *S. asoca* have been reported (Table 1.6). Reports exist on the antibacterial activity of *A. nervosa*, *A. alba*, *C. pulcherrima*, *C. viscosum*, *J. sambac*, *M. Nagi*, and *S. asoca* (Table 1.6). Antiviral activities have been reported on *C. pulcherrima*, and *C. terantea*. Reports also exist on antifungal potential of *A. nervosa*, *C. pulcherrima*, *C. viscosum*, *C. terantea*, *G. oppositifolius*, and *J. sambac* (Table 1.6). All these plants (*A. nervosa*, *A. alba*, *C. pulcherrima*, *C. viscosum*, *C. terantea*, *D. indica*, *G. oppositifolius*, *G. pentaphylla*, *H. excelsum*, *L. coromandelica*, *M. glabrata*, *M. nagi*, and *S. asoca*) that have reported antimicrobial, antibacterial, antiviral and antifungal activities have traditionally been used to treat various infectious diseases (Table 1.5).

Other pharmacological activities: In addtiton to the activities mentioned above, antipyretic, antidiabetic, hepatoprotective, antiprotozoal, antituberculosis and CNS activities have also been reported for some of the selected medicinal plants (Table 1.6). No pharmacological studies have been reported for *D. turbinatus*.

Phytochemical investigation:

Phytoconstituents such as polyphenols, flavonoids and catechins have been associated with anticancer activities (Wenying 2003; Park 2004). Polyphenols are secondary metabolites present in higher plants, that can act as anticancer, antioxidant, anti-inflammatory, antihypertensive, anti-allergic, and antimicrobial agents (Ke, Wang et al. 2012).

Flavonoids have been reported to act as potent anticarcinogens for the treatment of different types of cancers. These compounds are present in plants, fruits and vegetables and are capable of inhibiting cancers in several ways, such as antioxidation, antiestrogenesis and antiproliferation (Liu, Gao et al. 2011). Modern scientific research on catechins, which are widely present in plants, have also shown potential to cure many diseases including cancer (Liu and Huo 2011).

Previous phytochemical investigations (Table 1.6) on the plants selected for this study revealed the isolation of sterols, flavonoids, glycosides, terpenoids, catechins, tannins, polysaccharides, organic acids, oils, vitamins and other polyphenolic compounds. These compounds have been isolated from different parts of the selected plants, including leaves, flowers, fruits, stem, bark, root, wood and whole plants.

No compounds have been isolated from *D. turbinataus* and *E. viride* to date, and compared to other plants only a few compounds have been isolated from *C. terantea* and *M. glabrata* (Table 1.6).

Quercetin is a widely distributed flavoind that has been isolated previously from a number of plants namely, *C. terantea*, *C. viscosum*, *G. luteoalbum*, *J. sambac*, *M. glabrata*, and *S. asoca*. In addition, betulinic acid is a commonly occurring triterpenoid that has been isolated from *A. alba*, *D. peregrina*, *D. indica*, and *J. sambac* (Table 1.6).

Bangladeshi medicinal plants offer great promise for the identification of novel bioactive compounds. Thus, the present study aims to explore the cytotoxicity of the selected medicinal plants and carry out the isolation of new bioactive anticancer compounds.

Table 1.6 Pharmacological and phytochemical activities previously studied on the selected Bangladeshi medicinal plants

Plant species	Pharmacological activity	Previously isolated compound (s)
Aegiceras corniculatum	■ Antioxidative properties of condensed tannins (Wei 2012) ■ Cytotoxic activity (Uddin Shaikh, Grice et al. 2011) ■ Antinociceptive effect (Roome 2011) ■ Antioxidant potential (Vadlapudi 2009) ■ Anti-inflammatory (Roome 2008) ■ Antioxidant, anti-inflammatory and hepatoprotectve actions (Roome, Dar et al. 2008) ■ Antioxidant polyphenols (Agoramoorthy, Chen et al. 2008) ■ Antioxidant activity and total phenolics (Banerjee, Chakrabarti et al. 2008) ■ Antidiabetic effect (Gurudeeban 2012)	■ 2-hydroxy-5-methoxy-3-nonyl-1,4-benzoquinone (I), 5-*O*-methyl-embelin (II), 5-*O*-methyl-rapanone (III), 5-*O*-ethyl-embelin (IV), 3,7-dihydroxy-2,5-diundecylnaphthoquinone (V) (Li 2010) ■ Four new isomeric macrolides of combretastatin D-2 congeners named isocorniculatolide A (I), 11-*O*-methylisocorniculatolide A, 11-*O*-methylcorniculatolide A, and 12-hydroxy-11-*O*-methylcorniculatolide A, and the known corniculatolide A (II), arjunolic acid, and maslinic acid (Ponnapalli 2012) ■ α-spinasterol (I), stigmasterol (II), oleanolic acid (III), protoprimulagenin A (IV), gallic acid Me ester (V), stigmasterol 3-*O*-beta-D-glucopyranoside (VI), α-spinasterol-3-*O*-beta-D-glucopyranoside (VIb), n-tetratriacontanol (VII), n-dotriacontyl alc. (VIII), and gallic acid (IX) (Xu and Long 2009) ■ 16α,28-dihydroxy-3-oxo-12-oleanene (I), 5-*O*-methylembelin (II), stigasterol (III), α -spinasterol (IV), fucosterol (V), palmitic acid (VI) (Zhang, Zhang et al. 2007) ■ 2-methoxy-3-nonylresorcinol, 5-*O*-ethylembelin, 2-*O*-acetyl-5-*O*-methylembelin, 3,7-dihydroxy-2,5-diundecylnaphthoquinone, 2,7-dihydroxy-8-methoxy-3,6-diundecyldibenzofuran-1,4-dione, 2,8-dihydroxy-7-methoxy-3,9-diundecyldibenzofuran-1,4-dione, and 10-hydroxy-4-*O*-methyl-2,11-diundecylgomphilactone (Xu, Deng et al. 2004) ■ Falcarindiol (1), p-hydroxyphenethyl anisate (2), dioctyl phthalate (3), 1-hentriacontanol (4), resveratrol (5), 1,5-dihydroxy-3-methoxy-7-methylanthraquinone (6), 1,3,5-trihydroxy-7-methylanthraquinone (7), quercetin (8) and lupeol (9) (Wang, Dong et al. 2006) ■ Pentacyclic triterpenes : protoprimulagenin (1), embelinone (2), and aegicerin (3) (Zhang, Zhang et al. 2005)

Table 1.6 Pharmacological and phytochemical activities previously studied on the selected Bangladeshi medicinal plants (contd.)

Plant species	Pharmacological activity	Previously isolated compound (s)
Aegiceras corniculatum (contd.)		■ A new oleanane triterpene, 16α-hydroxy-13,28-epoxyoleanan-3-one, protoprimulagenin, aegicerin, embelinone, syringic acid, gallic acid, isorhamnetin and isorhamnetin 3-*O*-α-L-rhamnofuranosyl-(1→6)-β-D-glucopyranoside (Zhang, Wu et al. 2005)
		■ Tannins, triterpenoids, benzoquinones, sterols (Xu and Long 2006)
		■ Toxicants from mangrove plants. V. Isolation of the piscicide, 2-hydroxy-5-methoxy-3-undecyl-1,4-benzoquinone (5-*O*-methylembelin) (Gomez, De la Cruz-Giron et al. 1989)
		■ Free amino acids, total methylated onium compounds and total nitrogen. Low molecular weight carbohydrates (Popp, Larher et al. 1984)
		■ Low molecular weight carbohydrates (Popp 1984)
		■ Inorganic ions and organic acids (Popp 1984)
		■ Tannins (Everett 1981)
		■ α-spinasterol, stigmasterol, syringic acid, and a new triterpene, aegicerin (Rao and Bose 1961)
		■ A new triterpenoid sapogenin (I), named aegiceradienol (Rao 1959)
		■ Genin-A and isorhamnetin (Rao and Bose 1959)
		■ Saponins (Weifs 1906)

Table 1.6 Pharmacological and phytochemical activities previously studied on the selected Bangladeshi medicinal plants (contd.)

Plant species	Pharmacological activity	Previously isolated compound (s)
Argyreia nervosa	■ Antibacterial (Jain 2011) ■ Antifungal activity (Mahule, Rai et al. 2012)	■ Ergoline, lysergic acid amide, isolysergic acid amide, steroidal glycoside: ergost-5-en-11-oxo-3β-ol-α-D-glucopyranoside, designated as argyroside (I), loline, N-methylloline and N-propionylnorloline (Crawford 1970; Miller 1970; Chao and DerMarderosian 1973; Tofern, Kaloga et al. 1999; Rahman, Ali et al. 2003)
Avicennia alba	■ Antioxidant, antibacterial and antimicrobial (Banerjee, Chakrabarti et al. 2008; Vadlapudi and Naidu 2009; Nagababu and Umamaheswara Rao 2012)	■ Taraxerol, β-amyrin, taraxerone, betulin, betulinic acid, and triacontanal (Majumdar and Patra 1979); Three new naphthoquinones and their analogs, named avicequinone-A (I), -B (II), -C (III), And avicenol-A , -B ,-C (Ito, Katsuno et al. 2000)
Caesalpinia pulcherrima	■ Anti-ulcer activity (Ali, Mujahid et al. 2013)■ Immunomodulatory activity (Madagundi, Pawadshetter et al. 2012) ■ Antidiabetic activity (Balasubramanian, Seetaram et al. 2012) ■ Anti-inflammatory activity and cytotoxicity (Shaikh, Kruger et al. 2012) ■ Cytotoxicity and anti-microbial activity (Chanda and Baravalia 2011) ■ Antioxidant activity (Hsu, Huang et al. 2012) ■ Anti-inflammatory and analgesic activity (Ramana, Raju et al. 2010) ■ Cytotoxic activity (Pawar, Mutha et al. 2009)	■ Thirteen polyphenolics: gallic acid (1), Me gallate (2), 6-*O*-galloyl-D-glucoside (3), Me 6-*O*-galloyl-β-D-glucoside (4), Me 3,6-di-*O*-galloyl-α-D-glucopyranoside (5), gentisic acid 5-*O*-α-D-(6'-*O*-galloyl)glucopyranoside (6), guaiacylglycerol 4-*O*-β-D-(6'-*O*-galloyl)glucopyranoside (7), 3-methoxy-4-hydroxyphenol 1-*O*-β-D-(6'-*O*-galloyl)glucopyranoside (8), (+)-gallocatechin (9), (+)-catechin (10), (+)-gallocatechin 3-*O*-gallate (11), myricetin 3-rhamnoside (12) and ampelopsin (13) (Hsu, Huang et al. 2012) ■ Flavonoid (F-1) (Madagundi, Pawadshetter et al. 2012) ■ 15 new cassane-type diterpenes, named pulcherrins D-R (1-15) together with eight known compds (Hsu, Huang et al. 2012) ■ A new diterpenoid, 12-demethyl neocaesalpin F (III) (Das, Srinivas et al. 2010) ■ A cassane furanoditerpene; (4aR,6aS,7R,11aS,11bR)-4,4,7,11b-tetramethyl-1,2,3,4,4a,5,6,6a,7,11,11a,11b-dodecahydrophenanthro[3,2-b]furan-4a-ol} (Fun, Yodsaoue et al. 2010)

Table 1.6 Pharmacological and phytochemical activities previously studied on the selected Bangladeshi medicinal plants (contd.)

Plant species	Pharmacological activity	Previously isolated compound (s)
Caesalpinia pulcherrima (contd.)	■ Antibacterial and antifungal activities of the isolated homoisoflavonoids (Das, Thirupathi et al. 2009)	■ One new homoisoflavonoid, (3*E*)-2,3-dihydro-6,7-dimethoxy-3[(3-hydroxy-4-methoxyphenyl) methylene]-4H-1-benzopyran-4-one and four naturally new analogs, (3*E*)-3-(1,3-benzodioxol-5-ylmethylene)-2,3-dihydro-7-hydroxy-4H-1-benzopyran-4-one, (3*E*)-3-(1,3-benzodioxol-5-ylmethylene)-2,3-dihydro-7-methoxy-4H-1-benzopyran-4-one, (3*E*)-2,3-dihydro-7-hydroxy-3-[(3-hydroxy-4-methoxyphenyl) methylene]-4H-1-benzopyran-4-one and (3*E*)-2,3-dihydro-3-[(3,4-dimethoxyphenyl) methylene]-7-methoxy-4H-1-benzopyran-4-one, along with four known homoisoflavonoids, bonducellin, sappanone A, 2'-methoxybonducellin and 7-*O*-methylbonducellin (Das, Thirupathi et al. 2009)
	■ Antioxidant and cytotoxic activities (Pawar, Mutha et al. 2009; Asadujjaman, Hossain et al. 2013)	
	■ Antibacterial activity of methanolic extract of (Prakash, Sharmistha et al. 2009)	
	■ Antioxidant activity (Padma, Sumathi et al. 2001; Chakraborthy 2009)	■ Cassane diterpenoids: pulcherrin A (I), pulcherrin B (II), pulcherrin C (III), neocaesalpin P (IV), neocaesalpin Q (V) and neocaesalpin R (VI), together with eight known compds. isovouacapenol C, 6β -cinnamoyl-7β -hydroxy-vouacapen-5α -ol, pulcherrimin E, pulcherrimin C, α-cadinol, 7-hydroxycadalene, teucladiol and bonducellin (Pranithanchai, Karalai et al. 2009)
	■ Antioxidant activity (Liu, Hu et al. 2007)	■ Three new cassane diterpenes (1-3) (Cheng, Roach et al. 2008)
	■ Antimicrobial activity (Sudhakar, Krishnaiah et al. 2005)	■ Two new homoisoflavonoids, (*E*)-7-methoxy-3-(4'-methoxybenzylidene) chroman-4-one (1) and (*E*)-7-hydroxy-3-(3',4',5'-trimethoxybenzylidene) chroman-4-one (5), along with three known homoisoflavonoids (*Z*)-7-hydroxy-3-(4'-methoxybenzylidene) chroman-4-one (isobon ducellin) (2), (*E*)-7-hydroxy-3-(4'-methoxybenzylidene) chroman-4-one (bonducellin) (3) and (*E*)-7-hydroxy-3-(2',4'-dimethoxybenzylidene) chroman-4-one (4) (Maheswara, Siddaiah et al. 2006)
	■ Anti-inflammatory and analgesic activity (Puratchikody and Nagalakshmi 2005)	
	■ The anti-inflammatory activities of five flavonoids isolated from this plant (Rao, Fang et al. 2005)	■ Four homoisoflavonids (4-*O*-methylsappanol, protosappanin A, brazilin, and caesalpin J) and Et 2,4,6-trihydroxy benzoate (Sudhakar, Krishnaiah et al. 2005)

Table 1.6 Pharmacological and phytochemical activities previously studied on the selected Bangladeshi medicinal plants (contd.)

Plant species	Pharmacological activity	Previously isolated compound (s)
Caesalpinia pulcherrima (contd.)	■ Analgesic, anti-inflammatory, and antipyretic properties (Kumar, Muthusamy et al. 2003) ■ Antitubercular activity (Promsawan, Kittakoop et al. 2003) ■ *In vitro* antiviral activities (Chiang, Chiang et al. 2003) ■ Antimycotic (antifungal) activity (Mohamed, Saka et al. 1996)	■ Five flavonoids, namely 5,7-dimethoxyflavanone (1), 5,7-dimethoxy-3',4'-methylenedioxyflavanone (2), isobonducellin (3), 2'-hydroxy-2,3,4',6'-tetramethoxychalcone (4) and bonducellin (5) (Rao, Fang et al. 2005) ■ A new cassane-type diterpene isovouacapenol E (1) together with the known compds. caesaldekarin A (3), spathulenol (4), caryophyllene oxide (5), phytol, and sitosterol (Ragasa Consolacion, Ganzon et al. 2003). ■ Antitubercular cassane furanoditerpenoids 6β -benzoyl-7β -hydroxyvouacapen-5α -ol (1) and 6β-cinnamoyl-7β -hydroxyvouacapen-5α-ol (2) (Promsawan, Kittakoop et al. 2003) ■ Cassane diterpenoids (Roach Joy, McLean et al. 2003) ■ Two new flavanoids, 5,7-dimethoxy-3',4'-methylenedioxyflavanone and isobonducellin along with 2'-hydroxy-2,3,4',6'-tetramethoxychalcone, 5,7-dimethoxyflavone and bonducellin (Srinivas, Koteswara Rao et al. 2003) ■ Anthocyanins (Banerjee and De 2001) ■ Pulcherrimins A-D, novel diterpene dibenzoates (Patil, Freyer et al. 1997) ■ 8-methoxybonducellin (Parmar, Singh et al. 1987) ■ A new diterpene ester, pulcherralpin (I, R1 = H, R2 = COCH:CHPh) (Che, McPherson et al. 1986) ■ Three new furanoditerpenoids of the caesalpin, vouacapen-5α-ol (I), 6β-cinnamoyl-7β-hydroxyvouacapen-5α-ol (II), and 8,9,11,14-didehydrovouacapen-5α-ol (III) (McPherson, Che et al. 1986)

Table 1.6 Pharmacological and phytochemical activities previously studied on the selected Bangladeshi medicinal plants (contd.)

Plant species	Pharmacological activity	Previously isolated compound (s)
Clitoria terantea	■ Anti-inflammatory and analgesic (Shyamkumar and Bhat 2012) ■ Antioxidant activity (Rao, Rao et al. 2011) ■ Hepatoprotective potential (Nithianantham, Shyamala et al. 2011) ■ Anti-asthmatic activity (Taur Dnyaneshwar and Patil Ravindra 2011) ■ Neuropharmacological effect (Anuradha, Pragyandip et al. 2010) ■ Antimicrobial activity (Kamilla, Mansor et al. 2009) ■ Larvicidal activity (Mathew, Anitha et al. 2009)] ■ Anti-inflammatory, analgesic and antipyretic activity (Devi, Boominathan et al. 2003) ■ Antifungal activity (Osborn, De Samblanx et al. 1995) ■ CNS activity (Jain Neeti, Ohal et al. 2003) ■ Diuretic activity (Piala, Madissoo et al. 1962)	■ Quercetin (Zafar and Humayun 2012) ■β-D-galactosides-specific lectin (Naeem, Haque et al. 2007) ■ Protein (Osborn, De Samblanx et al. 1995)

Table 1.6 Pharmacological and phytochemical activities previously studied on the selected Bangladeshi medicinal plants (contd.)

Plant species	Pharmacological activity	Previously isolated compound (s)
Clerodendrum viscosum/Cleroden drum infortunatum	■ Anticancer properties (Das, Haldar et al. 2010; Sannigrahi, Mazumder et al. 2012; Sun 2013) ■ Wound healing activity (Kumar and Tripathi 2013) ■ Antifungal activity (Kharkwal, Joshi et al. 2012) ■ Antineoplastic potential of the flavonoid component of this plant (Ha, Hoang et al. 2000) ■ Free radical scavenging, antioxidant enzymes and wound healing activities (Gouthamchandra, Mahmood et al. 2010; Dey, Chaudhuri et al. 2012) ■ Analgesic and anticonvulsant effects of saponin isolated from this plant (Pal, Sannigrahi et al. 2009) ■ Anthelmintic activity (Pal, Sannigrahi et al. 2007) ■ *In vitro* antibacterial potential of flavonoids in (leaves) (Hoang, Ha et al. 1999) ■ Antifungal activity of the flavonoids (Roy, Pandey et al. 1996)	■ Terpenoids: oleanolic acid and clerodinin A (Sannigrahi, Mazumder et al. 2012) ■ A neo-clerodane diterpenoid (Sultana, Akanda et al. 2005) ■ Anthocyanins (Banerjee and De 2001) ■ Flavonoids; medicarpin and demethylmedicarpin ; cabruvin and quercetrin (Roy, Pathak et al. 1994). ■ Sterols (Akihisa, Matsubara et al. 1989) ■ Acteoside: 2-(3',4'-dihydroxyphenyl)ethanol-1-O-α -L-rhamnopyranosyl-(1→3)-β -D-(4-O-caffeyl) glucopyranoside (acteoside*)* (Sinha, Pandey et al. 1983) ■ Flavonoids: apigenin, acacetin, and a new flavone glycoside, characterized as the Me ester of acacetin-7-O-glucuronide (Sinha, Seth et al. 1981) ■ Fumaric acid, the Et and Me esters of caffeic acid, β-sitosterol, and β-sitosterol glucoside (Sinha, Pandey et al. 1980) ■ Clerodin, hentriacontane, and (24S)-ethylcholesta-5,22,25-trien-3β–ol (Joshi, Prakash et al. 1977) ■ Scutellarein and hispidulin-7-O-glucuronide from the (leaves) (Subramanian and Nair 1973) ■ Steroidal glucosides, glucose and the aglycons β-sitosterol and stigmasta-5,25-dien-3β–ol (root) (Barua, Sanyal et al. 1967) ■ Clerodolone, clerodone, clerodol, and clerosterol (Manzoori-Khuda and Sarela 1965)

Table 1.6 Pharmacological and phytochemical activities previously studied on the selected Bangladeshi medicinal plants (contd.)

Plant species	Pharmacological activity	Previously isolated compound (s)
Diospyros peregrina	■ Antioxidant assay (Sahu 2012)	■ Two bioactive flavonoid glycosides, namely luteoline-4′-methyl-ether-7-*O*-glucoside and quercetin-3-*O*-(glucosyl)-glucoside (Sahu 2012)
	■ Antitumor activity (Venu Gopal 2011)	
		■ Fatty oils: oleic, stearic and linoleic acids found to be major fatty acids (Dayal, Sharma et al. 2009)
	■ Antidiabetic activity of the hydroalcoholic extract of *Diospyros peregrina* (Dewanjee 2011)	■ Betulin, betulinic acid and a naphthoquinone; 2,6'-Bis-7-methyljuglone (Pareek, Vidyapati et al. 2008)
	■ Antidiabetic and antioxidant activity of the methanol extract of on Type I diabetic rats (Dewanjee, Sahu et al. 2009)	■ Seven fixed oils (Sampurna and Rao 2003)
		■ Glyceride of myristic, palmitic, stearic, oleic, linoleic and palmitoleic acids; glycoside (β -sitosterol-D-glucoside); sugars (fructose, galactose, glucose, lactose, mannose and sucrose); pectin; protein (Sampurna and Rao 2004)
	■ Antidiabetic activity; hyperglycemia, hyperlipidemia and antioxidative activities (Dewanjee, Das et al. 2009)	■ Furano-(2'',3'',7,8)-3',5'-dimethoxy-5-hydroxyflavone: a new furanoflavone (Jain and Yadava 1997)
	■ Antiplasmodial properties (Kantamreddi and Wright Colin 2008)	■ A novel chromenoflavone: 3,6-dimethoxy-2-(3',5'-dimethoxy-4'-hydroxyphenyl)-8,8-dimethyl-4H,8H-benzo [1,2-b: 3,4-b'] dipyran-4-one (I) (Jain and Yadava 1996)
		■ Peregrinol, a lupane type triterpene (Jain and Yadava 1994)
		■ Peregrinol: a new lupene type triterpene (Jain, Alam et al. 1992)
		■ Flavanone glycoside ; 5,7,3',4'-tetrahydroxyflavanone-3-*O*-β-D-glucopyranosyl(1 -> 4)-α-L-rhamnopyranoside (Chauhan, Saraswat et al. 1982)
		■ Triterpenes; olean-9(11),12-dien-3-one and ursan-9(11),12-dien-3-one (marsformosanone) (Bhaumik, Dey et al. 1981)

Table 1.6 Pharmacological and phytochemical activities previously studied on the selected Bangladeshi medicinal plants (contd.)

Plant species	Pharmacological activity	Previously isolated compound (s)
Diospyros peregrina (contd.)		■ Nonadecan-7-ol-2-one, an aliphatic ketol (Chauhan and Kumari 1980)
		■ A new dihydroflavonol glycoside (Chauhan, Saraswat et al. 1979)
		■ A new leucoanthocyanin f (Chauhan and Kumari 1978)
		■ Lupeol (Sundararamaiah, Ramraj et al. 1976)
		■ Alkanes, β-sitosterol, β-sitosterol glucoside, betulin, gallic acid, and a new compd., monohydroxy triterpene ketone; betulinic acid (Misra, Misra et al. 1971)
		■ Myricyl alc., betulin, β-sitosterol, betulinic acid, oleanolic acid, and a saponin (Gupta and Tiwari 1964)
Dillenia indica	■ Antioxidant activity (Deepa and Jena 2011; Das, Sarma et al. 2012; Singh, Singh et al. 2012; Banerjee and De 2013)	■ Three triterpenoids, namely betulin, betulinic acid and betulinaldehyde, and the dicarboxylic acid 3-deoxytartaric acid (2-hydroxybutane-1,4-dioic acid) (Chowdhury, Halder et al. 1998)
	■ Cytotoxic and anti-microbial activities. (Apu 2010)	■ Pigments: 3',5-dihydroxy-4',3-dimethoxy flavone-7-*O*-β-D-glucopyranoside; 4,5,7,3',4'-pentahydroxy flavan-3-*O*-β-D-glucopyranoside; 1,8-dihydroxy-2-Me anthraquinone-3-*O*-β-D-glucopyranoside; and 5,7-dihydroxy-4'-methoxyflavone-3-*O*-β-D-glucopyranoside (Tiwari and Srivastava 1979)
	■ Anti-leukemic activity in human leukemic cell lines U937, HL60 and K562 (Kumar 2010)	■ Betulin and β-sitosterol (Sundararamaiah, Ramraj et al. 1976)
	■ Hepatoprotective activity (Padhya, Choudhary et al. 2008)	■ Betulinaldehyde, betulin, lupeol, sitosterol, betulinic acid, and myricetin (Banerji, Majumder et al. 1975)
	■ Antidiabetic, hypolipidemic and histopathological activity (Kumar, Kumar et al. 2011)	■ Flavone: naringenin (Pavanasasivan and Sultanbawa 1975)
		■ Flavonoids: (±)-dihydroisorhamnetin (I) and dillenetin (II) (Pavanasasivam and Sultanbawa 1975)

Table 1.6 Pharmacological and phytochemical activities previously studied on the selected Bangladeshi medicinal plants (contd.)

Plant species	Pharmacological activity	Previously isolated compound (s)
Dipterocarpus turbinatus	■ No reports found	■ No reports found
Ecbolium viride	■ Free radical scavenging (Ashoka, Arunachalam et al. 2011) ■ Anti-inflammatory (Lalitha and Sethuraman 2010)	■ No reports found
Glinus oppositifolius	■ Anthelmintic and free radical scavenging activity (Dutta, Ghosal et al. 2012) ■ Hypoglycemic activity (Sahu, Das et al. 2012) ■ Analgesic and anti-inflammatory activity (Hoque, Habib et al. 2011) ■ Antioxidant, antihyperlipidemic and antihyperglycemic activity (Behera, Satish Kumar et al. 2010) ■ Free radical and anti-oxidant activity (AsokKumar, UmaMaheswari et al. 2009) ■ Antiprotozoal activity (Traore, Faure et al. 2000) ■ Antifungal, larvicidal, molluscicidal, antioxidant and radical scavenging activities (Diallo, Marston et al. 2001)	■ Triterpenes: oppositifolone (1), spinasterol (2), squalene (3) and lutein (4) (Ragasa, Espineli et al. 2012) ■ A new triterpenoid saponin, glinoside C (Kumar, Shah et al. 2013) ■ An amino acid deriv., -(-)-(N-trans-cinnamoyl)-arginine (I) along with kaempferol 3-*O*-galactopyranoside, isorhamnetin 3-*O*-β-D-xylopyranosyl-(1→2)-β-D-galactopyranoside, vitexin, vicenin-2, adenosine and L-phenylalanine (Sahakitpichan, Disadee et al. 2010) ■ Two new triterpenoid saponins, glinosides A (I, R = OH) and B (I, R = H) (Traore, Faure et al. 2000) ■ Apigenin-8-*C*-glucoside (I), pelargonidin-3-sophoroside-7-glucoside (II), and naringenin-7-rhamnoglucoside (Singh, Singh et al. 1982)

Table 1.6 Pharmacological and phytochemical activities previously studied on the selected Bangladeshi medicinal plants (contd.)

Plant species	Pharmacological activity	Previously isolated compound (s)
Gnaphalium luteoalbum/affine	■ Antioxidant activity (Li and Wang 2012; Zeng, Zhang et al. 2013) ■ Prevent the complications of diabetes III. (Tachibana, Okada et al. 1995) ■ Insect antifeedant activity (Morimoto, Kumeda et al. 2000) ■ Radical-scavenging activity (Urabe, Sakai et al. 2008)	■ Two new phenolic glycosides, named gnaphaffine A and B together with six known compounds: caffeic acid (3), everlastoside (4), isorhamnetin-7-*O*-β--glucopyranoside (5), quercetin-3-*O*-β-glucopyranoside (6), scutellarein-7-*O*-β-glucoside (7) and api-genin-7-*O*-β-glucopyranoside (8) (Li, Huang et al. 2013) ■ Three new acylated flavonol glycosides, apigenin 4'-*O*-β-D-(6''-E-caffeoyl)-glucopyranoside, luteolin 4'-*O*-β-D-(6''-*E*-caffeoyl)-glucopyranoside, and quercetin 4'-*O*-β-D-(6''-*E*-caffeoyl)-glucopyranoside, together with 24 known compds (Xi, Chen et al. 2012) ■ Extraction and GC-MS analysis of constituents of essential oil: fatty acid: hexadecanoic acid 38.30%, linoleic acid 16.70%, Et linoleate 7.44%, myristic acid 6.33%, oleic acid 4.86%, stearic acid 2.28%, 6,10,14-trimethyl-2-pentadecanone 2.83%, pentadecanoic acid (Pan, Deng et al. 2009) ■ The essential oil composition (Demirci, Baser et al. 2009) ■ GnafC, a polysaccharide constituent (Aoshima, Hasegawa et al. 2003) ■ Insect antifeedant flavonoids: 5-hydroxy-3,6,7,8,4'-pentamethoxyflavone, 5-hydroxy-3,6,7,8-tetramethoxyflavone, 5,6-dihydroxy-3,7-dimethoxyflavone, and 4,4',6'-trihydroxy-2'-methoxychalcone (Morimoto, Kumeda et al. 2000) ■ Scopoletin (1), 4,2',4'-trihydroxy-6'-methoxychalcone-4'-*O*-β-glucoside (2), quercetin (3) and luteolin (4) ■ Four flavonoids: apigenin 4'-β-D-glucoside (I), apigenin (II), quercetin 4'-β-D-glucoside (III), and quercetin (IV) (Itakura, Imoto et al. 1975) ■ Flavonoids: gnaphaliin, jaceosidin, apigenin, luteolin, apigenin and luteolin 7-glucosides, and luteolin 4'-glucoside (Mericli 1980)

Table 1.6 Pharmacological and phytochemical activities previously studied on the selected Bangladeshi medicinal plants (contd.)

Plant species	Pharmacological activity	Previously isolated compound (s)
Glycosmis pentaphylla	■ Anticancer and apoptosis inducing activity (Sreejith, Mascarenhas et al. 2012) ■ Hepatoprotective effect (Nayak Siva, Jain et al. 2011) ■ Anti-inflammatory effect (Rao and Raju 2009) ■ Antitumor activity (Quader, Nutan et al. 1999) ■ DNA binding and antimicrobial activities (Chen, Yang et al. 2012)	■ Two new flavanols, glycoflavanones A (1) and B (2) together with 5 known compds. 4'-*O*-methylgallocatechine (3), β-sitosterol (4), alphitol (5), 3,4-dimethoxy-5-hydroxy-*trans*-cinnamyl alc. (6), and oxyresveratrol (7) (Wu, Hu et al. 2012) ■ A new simple carbazole alkaloid, 4-(7-hydroxy-3-methoxy-6-methyl-9H-carbazol-4-yl)but-3-en-2-one, and 2 new dimeric carbazole alkaloids, bisglybomine B and biscarbalexine A, together with 7 known alkaloids (Yang, Wu et al. 2012) ■ A new hydroperoxyquinolone alkaloid, glycopentaphyllone (1), along with nine known compds (2-10) (Sripisut, Ritthiwigrom et al. 2012) ■ Glycoborinine (1), glybomine B (2), carbalexin A (3) and N-*p*-coumaroyltyramine (4) (Chen, Yang et al. 2012) ■ Six new apiosyl-(1→6)-glucosyl isoflavones: 3',7-dihydroxy-4',5,6-trimethoxyisoflavone 7-*O*-(5-*O*-trans-*p*-coumaroyl)-β-D-apiofuranosyl-(1→6)-β-D-glucopyranoside (1), 2',7-dihydroxy-4',5',5,6-tetramethoxyisoflavone 7-*O*-(5-*O*-trans-p-coumaroyl)-β-D-apiofuranosyl-(1→6)-β-D-glucopyranoside (2), 2',7-dihydroxy-4',5',5,6-tetramethoxyisoflavone 7-*O*-β-D-apiofuranosyl-(1→6)-β-D-glucopyranoside (3), 7-hydroxy-4',8-dimethoxyisoflavone 7-*O*-β-D-apiofuranosyl-(1→6)-β-D-glucopyranoside (4), 7-hydroxy-4',6-dimethoxyisoflavone 7-*O*-β-D-apiofuranosyl-(1→6)-β-D-glucopyranoside (5), and 4',5-dihydroxy-3',7-dimethoxyisoflavone 4'-*O*-β-D-apiofuranosyl-(1→6)-β-D-glucopyranoside (6) (Wang, Yang et al. 2006) ■ Four hydroquinone diglycoside acyl esters, glypentosides A-C (I-III) and seguinoside F (Wang, Di et al. 2006) ■ A novel naphthoquinone and a new acridone alkaloid called glycoquinone (I) and glycocitrine III (II) (Ito, Kondo et al. 1999)

Table 1.6 Pharmacological and phytochemical activities previously studied on the selected Bangladeshi medicinal plants (contd.)

Plant species	Pharmacological activity	Previously isolated compound (s)
Glycosmis pentaphylla (contd.)		■ Three carbazole alkaloids, glycozolicine, 3-formylcarbazole and glycosinine (Jash, Biswas et al. 1992)
		■ A carbazole alkaloid: mupamine (Kamaruzzman, Roy et al. 1989)
		■ A new quinolone alkaloid named glycolone (Sinha and Kumar 1988)
		■ Carbazole and 3-methylcarbazole (Chowdhury, Mustapha et al. 1987)
		■ A new quinolone alkaloid homoglycosolone (Kumar, Das et al. 1986)
		■ A new carbazole alkaloid, glycozolidal (Bhattacharyya and Chowdhury 1985)
Hymenodictyon excelsum	■ *In vitro* cytotoxic activity (Khay, Toeng et al. 2012) ■ Anti-microbial activity (Chea, Jonville et al. 2007)	■ Two new acetylenic fatty acids [henicosa-6-yn-20-en-1-oic Acid (1); nonadeca-4-yn-18-en-1-oic acid (2)], a new triglyceride [1',2',3'-*O*-trinonadeca-4-yn-18-en-1-oyl-glycerol (3)](3), as well as, 11 known compounds, including 3b-hydroxy-11-oxours-12-en-28-oic acid (5) (isolated as its acetate derivative), 3b hydroxy-27-p-(*Z*)-coumaroyloxyolean-12-en-28-oic acid, 3-oxo-11a,12a-epoxyurs-13b,28-olide (4), 3b-hydroxy-11a,12a-epoxyurs-13b,28-olide (5), 3b-hydroxyurs-11-en-13(28)-lactone (6), oleanolic acid (7), β-sitosterol, uncarinic acid E (3b-hydroxy-27-(*E*)-*p*-coumaroyloxyolean-12-en-28-oic acid, 8), ursolic acid (9), ursonic acid (10), and 3b-(formyloxy)-urs-12-en-28-oic acid (11) (Leaves) (Nareeboon, Komkhunthot et al. 2009)
		■ Lipids (β-sitosterol and stigmasterol) (Joshi and Baxi 1993)
		■ Free amino acid and sugar (Joshi and Baxi 1990)
		■ Rubiadin (anthraquinone) and its 1-Me ether, lucidin, nordamnacanthal, damnacanthal, 2-benzylxanthopurpurin, anthragallol, 6-methylalizarin, soranjidiol, and morindone (Thomson and Brew 1971)

Table 1.6 Pharmacological and phytochemical activities previously studied on the selected Bangladeshi medicinal plants (contd.)

Plant species	Pharmacological activity	Previously isolated compound (s)
Jasminum sambac	■ Antibacterial and antifungal activities (Sheikh, Safiuddin et al. 2013)	■ Four new compds. (+)-jasminoids A, B, C, and D, together with seven known cpds (Zeng, Hu et al. 2012)
	■ Antioxidant activity (Wang and Wang 2012; Wen, He et al. 2012)	■ Hexacosanol, β-sitosterol, β-daucosterol, oleanolic acid, quercetin and tricin (Liu, Wei et al. 2009)
	■ Antiproliferative activity (Talib and Mahasneh 2010)	■ Five compds. were isolated and identified as (+)-cycloolivil (I), (+)-cycloolivil-4'-*O*-β-D-glucoside (II), iridane triol (III), iridane tetraol (IV), and β-daucosterol (V) (Zhang and Zhao 2006)
	■ Antimicrobial and anti-inflammatory activities (Tsai, Tsai et al. 2010)	■Compds. isolated: benzyl-*O*-β-D-glucopyranoside, benzyl-*O*-β-D-xylopyranoxyl (1->6)-β-D-glucopyranoside, tetraol, molihuaoside D, sambacoside A, sambacoside E, Rutin, kaempferol-3-*O*-(2, 6-di-*O*-α-L-rhamnopyranosyl)-β-D-galactopyranoside, and quercetin-3-*O*-(2,6-di-*O*-α-L-rhamnopyranosyl)-β-D-galactopyranoside (Liu, Ni et al. 2004)
	■ Antimicrobial activity (Tsai, Tsai et al. 2008)	
	■ Free radical scavenging activity (Huang, Luo et al. 2008; Tan, Yan et al. 2012)	■ Dotriacontanoic acid, dotriacontanol, oleanolic acid, daucosterol, and hesperidin (Zhang, Bian et al. 2004)
	■Antioxidant activity (Wetwitayaklung, Phaechamud et al. 2008)	■ Secondary metabolites: caryophyllene oxide (1), a mixt. of benzyl benzoate (2) and farnesyl acetate (3), Me isoeugenol (4), squalene (5), and sitosterol (6) (Ragasa, Tamboong et al. 2003)
	■ Antimicrobial and antioxidant activities (Tsai, Tsai et al. 2008)	■ Linalyl β-D-pyranoglucosidase (LGA) (Tu, Tong et al. 2001)
		■ Secoiridoid glycosides: syringalactones A and B, oleoside 7,11-dimethyl ester, jasminoside, jaslanceoside B, sambacoside A and molihuaside D (Shen, Chen et al. 2000)
	■ Inhibition of human leukocyte elastase (Baylac and Racine 2004)	■ Iridoidal glycosides: trimeric iridoidal glycoside, sambacoside A, five new oligomeric iridoidal glycosides, molihuasides A-E (Zhang, Liu et al. 1995)
	■ Free radical scavenging activities (Zeng, Xu et al. 2004)	■ Linalyl β-D-glucopyranoside and its 6'-*O*-malonate as aroma precursors (Moon, Watanabe et al. 1994)

Table 1.6 Pharmacological and phytochemical activities previously studied on the selected Bangladeshi medicinal plants (contd.)

Plant species	Pharmacological activity	Previously isolated compound (s)
Jasminum sambac (contd.)		■ Three tetrameric iridoid glucosides, sambacosides A (I), E (II), and F (III) (Tanahashi, Nagakura et al. 1988)
		■ New esters of (*Z*)-3,4-epoxyhexan-1-ol as well as the tetrahydrofuranyl derivatives (Kaiser 1988)
		■ A new secoiridoid glucoside, sambacolignoside (I), oleoside 11-Me ester, was elucidated as 7-*O*-[(+)-1-hydroxypinoresinol-β-D-glucoside-(7-6' '')]-oleoside 11-Me ester (Tanahashi, Nagakura et al. 1987)
		■ 9'-deoxyjasminigenin, jasminin, 8,9-dihydrojasminin (a new secoiridoid glucoside) (Ross and Abdel-Hafiz 1986)
		■ Triterpenoids: friedelin, lupeol, betulin, betulinic acid, α-amyrin, ursolic acid, oleanolic acid, and β–sitosterol (Dan and Dan 1985)
Lannea coromandelica	■ Antioxidant, antimicrobial and thrombolytic effects (Manik, Wahid et al. 2013)	■ Quercetin-3-*O*-rutinoside (1), stigmast-4-en-6β-ol-3-one (2), taraxerone (3), taraxerol (4), taraxeryl acetate (5), 5α-stigmastane-3, 6-dione (6), β-sitosterol (7), daucosterol (8), and 4-hydroxy-3-methoxybenzaldehyde (9) (Yun, Shu et al. 2012)
	■ Anti-inflammatory effect (Saravanan, Dhasarathan et al. 2010)	■ Dihydroflavonols, (2*R*,3*S*)-(+)-3',5-dihydroxy-4',7-dimethoxydihydroflavonol (I) and (2*R*,3*R*)-(+)-4',5,7-trimethoxydihydroflavonol (II) along with the known (2*R*,3*R*)-(+)-4',7-di-*O*-methyldihydroquercetin, (2*R*,3*R*)-(+)-4',7-Di-*O*-methyldihydrokaempferol and (2*R*,3*R*)-(+)-4'-*O*-methyldihydroquercetin (Tofazzal Islam and Tahara 2000)
		■ Quercetin 3-arabinoside and ellagic acid (Subramanian and Nair 1971)

Table 1.6 Pharmacological and phytochemical activities previously studied on the selected Bangladeshi medicinal plants (contd.)

Plant species	Pharmacological activity	Previously isolated compound (s)
Mussaenda glabrata/frondosa	■ Anti-oxidant and anti-inflammatory (Menon and Sasikumar 2011) ■ DNA cleavage activity (Gopalakrishnan and Vadivel 2011) ■ Antimicrobial activity (Jayasinghe, Jayasooriya et al. 2002)	■ Quercetin, rutin, hyperin, ferulic acid, sinapic acid, and β-sitosterol-glucoside (Lakshmi, Girija et al. 1985)
Myrica nagi/esculenta	■ Antioxidant and anti-proliferative activity (Saini, Garg et al. 2013) ■ Antimicrobial and anti-inflammatory (Agnihotri, Wakode et al. 2012) ■ Antimicrobial activity (Saklani 2012) ■ Anti inflammatory activity (Patel 2011) ■ Anti-allergic activity of (stem Bark) of *Myrica esculenta* (Patel 2010) ■ Antioxidant properties (Rawat 2011) ■ Antioxidative and radical-scavenging activities (Chen, Wang et al. 2007; Xu 2011) ■ Angiotensin I-converting enzyme (ACE) inhibition (Nguyen 2010)	■ Myricitrin (1), myricanol (2), myricanone (3), gallic acid (4), Et β-D-glucopyranoside (5), 3-hydroxybenzaldehyde (6), isovanillin (7), 4-methoxybenzoic acid (8), 4-(hydroxymethyl) phenol (9), β-rosasterol (10), β-sitosterol (11), daucosterol (12) (Yang 2011). ■ Taraxerol (A), barbaloin (B), myricanol (C) and myricetin (D) (Chen 2010) ■ One new monoterpenoid glycoside, myresculoside (I) (Nguyen 2010) ■ Flavonoid glycosides: flavone 4'-hydroxy-3',5,5'-trimethoxy-7-*O*-β-D-glucopyranosyl (1→4)-α-L-rhamnopyranoside (I) and flavone 3',4'-dihydroxy-6-methoxy-7-*O*-α-L-rhamnopyranoside (II) with three known compds. β-sitosterol, β-sitosterol-β-D-glucopyranoside and quercetin (Bamola, Semwal et al. 2009) ■ Amino acids, sugars, vitamins (Rawat and Rawat 1996) ■ Flavonols: myricetin 3-*O*-(3"-*O*-galloyl)-α-L-rhamnopyranoside and myricetin 3-*O*-(2"-*O*-galloyl)-β-D-galactopyranoside (Sun, Zhao et al. 1991)

Table 1.6 Pharmacological and phytochemical activities previously studied on the selected Bangladeshi medicinal plants (contd.)

Plant species	Pharmacological activity	Previously isolated compound (s)
Myrica nagi/esculenta (contd.)	■ Anti-neoplastic activity (Rana 2004) ■ Attenuates cumene hydroperoxide-induced cutaneous oxidative stress and toxicity (Alam, Iqbal et al. 2000)	■ Tannin: from *M. esculenta* was identified as partially 3-*O*-gallated prodelphinidin (bark) (Zhao, Luo et al. 1987) ■ New meta-bridged biphenyls, myricanol (I) and myricanone (II) (Campbell, Crombie et al. 1970) ■ A new proanthocyanidin (tannin) (Krishnamoorthy and Seshadri 1966) ■ Triterpenes: taraxerol, myricadiol (Agarwal, Roy et al. 1963) ■ Myriconol: a new phenolic rotenoid (Krishnamoorthy, Krishnaswamy et al. 1963) ■ A new glucoside: myricetine (Perkin 1902)
Saraca asoca	■ Anti-inflammatory activity (Gupta, Sasmal et al. 2013) ■ Antioxidant, antimutagenic, and antigenotoxic properties (Nag, Ghosh et al. 2013) ■ Antipyretic activity (Sasmal, Majumdar et al. 2012) ■ Chemo-preventive property of flavonoids from Saraca asoca (Cibin, Devi et al. 2012) ■ Molluscicidal activity (Singh and Singh 2009; Singh, Kumar et al. 2010) ■ Antibacterial activity (Shahid, Shahzad et al. 2007)	■ Five lignan glycosides, lyoniside, nudiposide, 5-methoxy-9-β-xylopyranosyl-(-)-isolariciresinol, icariside E3, and schizandriside, and three flavonoids, (-)-epicatechin, epiafzelechin-(4β→8)-epicatechin and procyanidin B2, together with β-sitosterol glucoside (Sadhu, Khatun et al. 2007) ■ (+)-catechin (I), (-)-epicatechin (II), and leucocyanidin (III), and quercetin (Indrani and Balasubramanian 1984) ■ (-)-epicatechin, procyanidin B2, and a new compd. named 11'-deoxyprocyanidin B (Middelkoop and Labadie 1985) ■ (+)-catechol, (-)-epicatechol, leucocyanidin, quercetin, leucocyanidin and gallic acid (Indrani and Balasubramanian 1985) ■ Leucopelargonidin 3-*O*-β-D-glucoside, leucopelargonidin, leucocyanidin and β-sitosterol (Duggal and Misra 1980)

1.5.4 *Blumea lacera*, a Bangladeshi medicinal plant

Based on the detected cytotoxic activity of *Blumea lacera* (Burn. f.) DC, screened by another PhD student in our lab (Uddin 2011) and compared to our selected 19 plant speices, *Blumea lacera* (Burn. f.) DC. was one of the most promising candidates for further study. In addition, a literature review indicated that only a few compounds had previously been isolated from this plant (Table 1.7; Figure 1.9). Therefore, *B. lacera* presented as the most attractive for constituent isolation in the present study.

ature*Blumea lacera* **(Burn. f.) DC.**

Species name: *Blumea lacera* (Burn. f.) DC.

Genous: *Blumea*

Family: Compositae/Asteraceae

1.5.4.1 Habitat and distribution of *Blumea lacera*

Blumea lacera (*B. lacera*) is commonly known as Kukursunga or Shialmutra (local names) in Bangladesh. It is an erect, weak herbaceous weed with very leafy stems, toothed alternate leaves and yellow, densly panicled flower heads. This plant is found throughout Bangladesh (Ghani 2003), although it also grows in Australia, India, Ceylon, China, Malaya, and tropical Africa (Agarwal, Singh et al. 1995). It is perennial herb up to 1 m in height, with leaves 2.0-19.0 cm x 0.6- 6.0 cm in area, lower obovate, short petiolated, upper elliptic-ovate to oblanceolate, cordate clasping; capitula axillary or terminal, with numerous yellow florets, rarely bisexual, marginal female, achenes brown, shining, sparsely pilos with white pappus (Singh and Parthasarathy 2012).

Figure 1.8 Photograph of *Blumea lacera*

1.5.4.2 Traditional medicinal uses of *Blumea lacera*

Blumea is a genus of shrubs and small trees which comprises about 80 species distributed in Asia, Africa, and Oceania [8]. This genus includes some important medicinal plants widely used in traditional medicine. For example, the essential oil from *Blumea mollis* and *Blumea perrottetiana* has shown notable insecticidal activities, whereas the essential oil from *Blumea membranacea* produces a significant and long-lasting fall in the blood pressure of anaesthetized dogs, exerts a direct depressant action on frog hearts, and a spasmolytic effect on rabbit ilea. *Blumea membranacea* also showed significant antifungal activity (Pednekar, Vakil et al. 2013). ***B. lacera*** is reported to cure bronchitis, blood diseases and fevers, and to alleviate burning sensations (Agarwal, Singh et al. 1995). The leaves of the plant are anthelmentic, and astringent, diuretic, antiscorbutic and are useful in catarrhal affections (Ragasa, Wong et al. 2007; Singh and Parthasarathy 2012). It is also effective in the treatment of sores and wound healing. The leaves are used as antidysentric, antimicrobial, and anti-inflammatory remedies. Juice of this herb is used as a 'carminative'. The root of this plant is also used in treating cholera (Singh and Parthasarathy 2012).

1.5.4.3 Previous pharmacological and phytochemical study on *Blumea lacera*

B. lacera has been reported to have cytotoxic, anti-leukemic, anti-inflammatory, antioxidant, antibacterial, antifungal, antiviral, antidiarrheal, and antidiabetic activities (Table 1.7). *B. lacera* methanolic leaf extracts were studied in our research group previously for its cytotoxic activity against various cancer cell lines (gastric: AGS, colon: HT-29, and breast: MCF-7 and MDA-MB-435). The plant showed the highest cytotoxic potential (IC$_{50}$: 0.01-0.08 mg/mL) of all plant tested against all cell lines tested (Uddin Shaikh, Grice et al. 2011). In other research the hot water extract of *B. lacera* revealed its broad anti-leukemic activity against anti-K562, L1210, P3HR1, Raji and U937 leukemia cells at magnitudes ranging from moderate to mild (Chiang, Cheng et al. 2004). *B. lacera* hot water extract also demonstrated antiviral activity, suppressing the replication of herpes simplex virus types 1 and 2 (HSV1/HSV-2) with IC$_{50}$ values below 100 µg/mL (Chiang, Cheng et al. 2004). Potent antibacterial and antioxidant properties of n-hexane, chloroform and methanol extracts of *B. lacera* aerial parts have also been reported (Shahwar, Ullah et al. 2010) along with anti-inflammatory activity through NF-κB inhibition (Nam and Jae 2009). Antibacterial and antifungal activities of monoterpene glycoside and flavonoids isolated from *B. lacera* leaf have also been examined (Ragasa, Wong et al. 2007). However, so far no reports have been found on the cytotoxic/anticancer activity of the isolated compounds from *B. lacera*.

Table 1.7 Previous pharmacological activity studies on *B. lacera*

Pharmacological activity	Type of extracts	Plant part	References
Cytotoxic	Methanol and water	Leaves	(Uddin Shaikh, Grice et al. 2011)
Antileukemic and antiviral	Water	Unknown	(Chiang, Cheng et al. 2004)
Antidiarrheal	Ethanol	Root	(Singh and Parthasarathy 2012)
Anti-diabetic	Methanol	Whole plant	(Shahwar, Ullah et al. 2011)
Antibacterial and antioxidant	n-hexane, chloroform and methanol	Aerial parts	(Shahwar, Ullah et al. 2010)
Anti-inflammatory	Methanol	Herbs	(Nam and Jae 2009)
Antifungal and antibacterial	Dicholomethane	Leaves	(Ragasa, Wong et al. 2007)

Previous studies have reported the isolation of a total of 17 compounds from *B. lacera* to date, belonging to natural product classes such as flavonoids, terpene glycosides, phenol glycoside, sterols, essential oils, coniferyl alcohol derivatives and terpenoid ketones (Table 1.8). These compounds have been isolated from the whole plant, aerial parts and leaves of this plant. However, there are no reports on the isolation of alkaloids, catechin or tannins.

Table 1.8 Compounds isolated previously from *B. lacera*
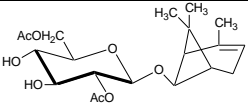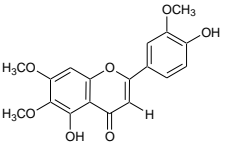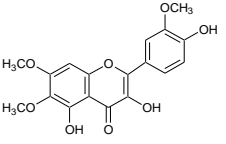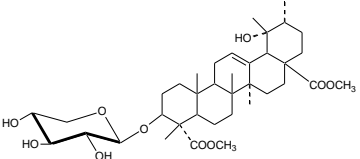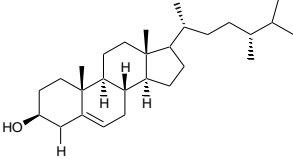
Compounds		Chemical class	Type of extract	Plant part	References
α-pinene-7β-*O*-β-D-2,6-diacetylglucopyranoside		Monoterpene glycoside	Dicholomethane	Leaves	(Ragasa, Wong et al. 2007)
5,4'-dihydroxy-6,7,3'-trimethoxyflavone		Flavonoid	Dicholomethane	Leaves	(Ragasa, Wong et al. 2007)
3,5,4'-trihydroxy-6,7,3'-trimethoxyflavone		Flavonoid	Dicholomethane	Leaves	(Ragasa, Wong et al. 2007)
19α-hydroxyurs-12-ene-24,28-dioate 3-*O*-β-D-xylopyranoside		Terpenoid glycoside and phenol glycoside	n-hexane	Whole plant	(Agarwal, Singh et al. 1995)
Compesterol		Sterol	Petrolium+CHCl$_3$	Aerial parts	(Pal, Moitra et al. 1972)

Table 1.8 Compounds isolated previously from *B. lacera* (contd.)
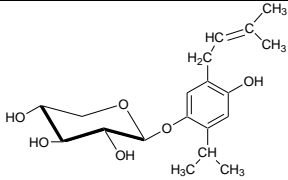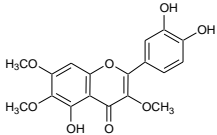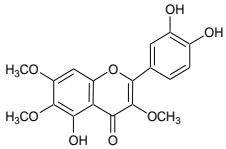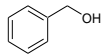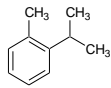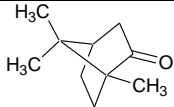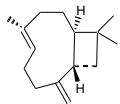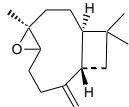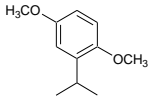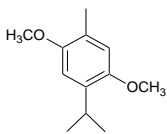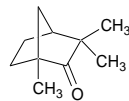
Compounds		Chemical class	Type of extracts	Plant part	References
2-isoprenyl-5-isopropylphenol 4-*O*-β-D-xylopyranoside		Phenol glycoside	n-hexane	Whole plant	(Agarwal, Singh et al. 1995)
5-hydroxy-3,6,7,3',4' pentamethoxyflavone		Flavonoid	n-hexane	Leaves	(Rao, Rao et al. 1977)
5,3',4'-trihydroxy-3,6,7 trimethoxyflavone		Flavonoid	n-hexane	Leaves	(Rao, Rao et al. 1977)
Benzenemethanol		Essential oil	Unknown	Unknown	(Iyer, Sane et al. 2004)
1-methyl-2-(1-methylethyl) benzene)		Essential oil	Unknown	Unknown	(Iyer, Sane et al. 2004)
Coniferyl alcohol derivatives	Not found	Coniferyl alcohol	Unknown	Unknown	(Bohlmann and Zdero 1969)

Table 1.8 Compounds isolated previously from *B. lacera* (contd.)

Compounds		Chemical class	Type of extracts	Plant part	References
1,7,7-trimethylbicyclo[2,2,1] heptan-2-one		Essential oil	Unknown	Unknown	(Iyer, Sane et al. 2004)
Caryophyllene		Essential oil	Water	Aerial parts	(Le, Tran et al. 2003)
Caryophyllene oxide		Essential oil	Water	Aerial parts	(Le, Tran et al. 2003)
Thymol hydroquinone di-me ether		Essential oil	Water	Aerial parts	(Le, Tran et al. 2003)
Thymoquinol di-me ether		Essential oil	Unknown	Leaves	(Laakso, Seppanen-Laakso et al. 1989)
Fenchone		Terpenoid ketone	Chloroform	Leaves	(Baslas and Deshapande 1950)

1.6 Rationale of the project

Cancer is considered one of the most life threatening problems worldwide and is identified as the second leading cause of death after cardiovascular diseases (Rajandeep Kaur 2011). Despite several therapeutic options for cancer treatment such as a combination of surgery, radiation therapy, and chemotherapy, cancer still remains associated with high mortality. Natural and some synthetic compounds can prevent, suppress, or reverse the progression of cancer (Huang, Lu et al. 2012).

Plants have been used for treating various diseases including cancer in humans and animals from past to present. Either directly or indirectly more than 60% of the current anticancer drugs have been obtained from natural sources (Rajandeep Kaur 2011). For example, vincristine, irinotecan, etoposide, and paclitaxel are widely applied anticancer drugs, derived from natural products which are playing a dominant role in chemotherapy (Huang, Lu et al. 2012). Cancer research is expanding and continually exploring new lead anticancer compounds from natural sources (Gordaliza 2007). Most of the conventional anticancer drugs available are non-selective, compromising both healthy and cancerous cells. Side effects such as vascular toxicity, cardiotoxicity, pulmonary toxicity, ocular toxicity, neurotoxicity, bone marrow suppression, hair loss and more are associated with current chemotherapeutic agents. Furthermore, cancer cells can undergo mutation and become resistant to anticancer drugs (Komarova 2005). New and innovative drugs with selective anticancer potential are in great demand. Although new strategies in cancer diagnosis, prevention, and treatment, such as gene therapy and cancer vaccines are emerging, innovation of more potent, selective and less toxic drug entities with the promise of improved efficacy and minimal side effects in cancer chemotherapy are being sought with urgency.

Based on the ethnomedical information, chemical diversity, and current literature, Bangladeshi medicinal plants are a great source of new bioactive compounds. Thus, the present study was undertaken to evaluate the cytotoxicity of the selected medicinal plants and to isolate new cytotoxic compounds from the potent plant extract of *Blumea lacera*.

1.7 Aims and objectives of the project

The overall aim of this PhD project was the "Isolation and structural elucidation of bioactive compounds from Bangladeshi medicinal plants with a focus on novel anticancer compounds".

Specific objectives are:

1. Selection of Bangladeshi medicinal plants according to ethnopharmacological records.

2. Extraction of plant materials and screening of cytotoxic activity of crude extracts using the MTT assay.

3. Fractionation and isolation of pure compounds from an active plant species using preparative chromatographic methods (SPE, HPLC).

4. Structural elucidation of pure compounds by spectroscopic (NMR, IR, UV) and spectrometric (MS) methods.

5. Evaluation of the cytotoxic activity of pure compounds using the MTT assay.

6. Identification of probable mechanisms` of cytotoxic activity using an apoptosis assay and cell cycle analysis.

7. Evaluation of antifungal activity of isolated compounds.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Collection and extraction of Bangladeshi plant materials

2.1.1 Plant collection and identification

The plants were collected from the tidal forest in the coastal Sundarban as well as from other parts of Bangladesh during March 2006 and May 2007. The plant materials were subjected to shade drying and identified by the Bangladesh National Herbarium, Mirpur, Dhaka, where a voucher specimen for each plant species was deposited for future reference (Table 2.1).

Table 2.1 Name and voucher numbers of the collected Bangladeshi medicinal plants

Plant species	Family	Local name	Plant part (s)	Voucher no.
Aegiceras corniculatum	Myrsinaceae	Kholisha	Fruit	DACB 31584
Argyreia nervosa	Convolunaceae	Bichtarak	Leaf	DACB 37954
Avicennia alba	Avicenniaceae	Morcha baen	Leaf	DACB 40556
Caesalpinia pulcherrima	Caesalpiniaceae	Krishnachura	Leaf	DACB 32020
Clerodendrum viscosum	Verbenaceae	Bhant	Leaf	DACB 37953
Clitoria terantea	Papilionaceae	Aparajita	Flower and Leaf	DACB 32021
Dillenia indica	Dilleniaceae	Chalta	Leaf	DACB 32019
Diospyros peregrina	Ebenaceae	Gab	Leaf	DACB 30323
Dipterocarpus turbinatus	Dipterocarpaceae	Garjan	Leaf and Bark	DACB 32026
Ecbolium viride	Acanthaceae	Nilkanta	Leaf	DACB 32018
Glinus oppositifolius	Molluginaceae	Gima	Whole plant	DACB 32014
Glycosmis pentaphylla	Rutaceae	Daton	Leaf	DACB 37931
Gnaphalium luteoalbum	Compositae	Boro karma	Leaf	DACB 37955
Hymenodictyon excelsum	Rubiaceae	Bhui kadam	Bark and Wood	DACB 32013
Jasminum sambac	Oleaceae	Beli phul	Leaf	DACB 31262
Lannea coromandelica	Anacardiaceae	Jeol/Jiga	Bark and Leaf	DACB 35242
Mussaenda glabrata	Rubiaceae	Patralekha	Leaf	DACB 32023
Myrica nagi	Myricaceae	Kaiphal	Leaf	DACB 32029
Saraca asoca	Caesalpinaceae	Ashok	Leaf	DACB 32007

2.1.2 Extraction of plant materials

The dried plant materials were ground to a coarse powder with a mechanical grinder (grinding mill) in Bangladesh and then imported to Australia. The extraction and any following processes were undertaken in Australia. For extraction, approximately 10 g of each plant material was soaked in 100 mL of methanol for 24 h. The extracts were then filtered and the solvent was evaporated using a rotary evaporator followed by freeze drying.

The % yield was calculated for each dry extract using the following equation:

$$\% \text{ of dry extract yield} = \frac{\text{Mass of extract (g)}}{\text{Mass of plant materials (g)}} \times 100\%$$

2.2 Chemicals, reagents, solvents and solutions

2.2.1 Chemicals and reagents

Names and sources of chemicals and reagents used for this project are listed in Table 2.2.

Table 2.2 Name and source of chemicals and reagents used for this project

Name of chemicals and reagents	Source
Advanced Dulbecco's modified eagle medium (DMEM)	Gibco
Dimethyl sulfoxide (DMSO) 99.9%	Sigma
Ethylene diamine tetraacetic acid (EDTA)	Sigma
Ethanol 99%	Merck
L-glutamine 200 mM	Gibco
New born calf serum (NBCS)	Gibco
Potassium chloride (KCl)	ChemSupply
Potassium di-hydrogen phosphate (KH_2PO_4)	Merck
Sodium chloride	Sigma
Di-sodium mono-hydrogen phosphate (Na_2HPO_4)	Sigma
Thiazolyl blue tetrazolium bromide (MTT)	Sigma
Trypan blue solution	Fluka
Trypsin-EDTA (trypsin 0.25% containing 0.02% EDTA)	Gibco

2.2.2 Solvents and solutions

2.2.2.1 Solvents for extractions, analytical and chromatographic purposes

Analytical grade solvents were used for all purposes except for HPLC, where HPLC grade solvents were used. Analytical grade solvents were fractionally distilled before use to remove any trace impurities including plasticizers. All solvents were stored in ventilated storage areas at 20°C and transferred into 500 mL bottles for routine use. Halogenated and non-halogenated solvents were stored separately. MilliQ water was used for all purposes. Analytical grade methanol (2.5 L, purity: 99.5%), ethanol (2.5 L, 99.5%) and HPLC grade methanol (4.0 L, purity: 99.8%) and acetonitrile (4.0 L, purity: 99.8%) were purchased from Merck Pty Ltd., Germany. Analytical grade trifluoroacetic acid was purchased from Sigma-Aldrich, Germany.

2.2.2.2 Solvent for NMR analysis

For NMR spectroscopic analysis, deuterated methanol (CD_3OD; 99.9% purity) was purchased from Sigma-Aldrich, Germany. The ^{1}H shift (multiplicity) and ^{13}C shift (multiplicity) for methanol were 3.30 (5) and 49.0 (5) ppm, respectively and the peak for trace water was 4.78 ppm.

2.2.2.3 Solutions for cell culture, MTT assay and flow cytometry

Phosphate Buffered Saline (PBS), pH 7.4

For mammalian cell culture and for the MTT assay, PBS was prepared by dissolving the chemicals below (Table 2.3) in 1 L MilliQ water. The pH (7.4) of the buffer was checked with a pH meter and the buffer was sterilized by autoclaving for 20 min at 121°C.

Table 2.3 Chemicals used to prepare PBS (pH 7.4) for cell culture

Chemicals	Molecular formula	Molar mass (g/mol)	Concentration (mM)	Weight (g/L)
Sodium chloride	NaCl	58.44	137	8.01
Potassium chloride	KCl	74.55	2.70	0.20
Di-sodium mono-hydrogen phosphate	Na_2HPO_4	141.96	7.90	1.12
Potassium di-hydrogen phosphate	KH_2PO_4	136.09	1.50	0.20

▪ EDTA solution

250 mL of 0.5 M EDTA solution was prepared by dissolving 36.53 g of EDTA in MilliQ water.

▪ PBS-EDTA solution

To prepare a 1 L PBS-EDTA solution, 10 mL of sterile 0.5 M EDTA solution was added to 990 mL of sterile PBS (pH 7.4). The solution was mixed thoroughly and then stored at 4°C.

▪ New born calf serum (NBCS)

NBCS 500 mL was purchased from Gibco Invitrogen (Australia) of which 50 mL aliquots were stored in -20°C.

▪ L-Glutamine

100 mL, 200 mM glutamine was purchased from Gibco Invitrogen (Japan) of which 5 mL aliquots were stored at -20°C.

▪ Trypsin-EDTA solution

Trypsin 0.25% containing 0.02% EDTA was purchased from SAFC Bioscience, USA and stored as 5 mL aliquots at -20°C.

▪ Dimethyl sulfoxide (DMSO) solution

DMSO 16% solution was prepared by adding 7 mL DMSO to 43 mL of MilliQ water. DMSO was purchased from Sigma Aldrich, Germany.

▪ MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl-tetrazolium bromide] solution

The 1 mg/mL MTT solution was prepared by dissolving 50 mg of MTT in 50 mL of PBS. A further 0.5 mg/mL solution of MTT was made by a 1:2 dilution of the 1 mg/mL MTT solution.

▪ Annexin V-fluorescein isothiocyanate (Annexin V-FITC)

Annexin V-FITC (500 µL, 20 µg/mL in Tris-NaCl) was purchased from BD Biosciences, NJ, USA, and stored at 4°C. Annexin V solution (5 µL, 0.1 µg) was used in each test of the annexin V-FITC staining assay.

▪ **Propidium iodide (PI)**

Propidium iodide (2 mL, 50 µg/mL in binding buffer) was purchased from BD Biosciences, NJ, USA, and stored at 4°C. PI solution (5 µL, 0.1 µg) was used in each test of the Annexin V-FITC staining assay and 10 µL of PI solution was used in each test of cell cycle analysis.

▪ **Annexin V binding buffer**

Annexin V binding buffer (10x, 0.1 M Heps/NaOH, 1.4 M NaCl, 25 mM $CaCl_2$) was purchased from BD Biosciences, NJ, USA, and stored at 4°C. For a 1x working solution, 1 part of 10x binding buffer was diluted with 9 parts of distilled water. Binding buffer solution (500 µL) was used in each test of the Annexin V-FITC staining assay.

2.3 Mammalian cell lines

Two normal and four cancer cell lines (Figure 2.1 and Table 2.4) were selected for cytotoxicity screening of Bangladeshi medicinal plants. All cell lines were purchased from the ATCC (American Type Culture Collection) USA, and are shown below:

(A) Mouse fibroblast, NIH 3T3   (B) Monkey kidney cells, VERO   (C) Colon adenocarcinoma, HT-29

(D) Gastric adenocarcinoma, AGS   (E) Estrogen-dependent breast carcinoma, MCF-7   (F) Estrogen-non-dependent breast carcinoma, MDA-MB-231
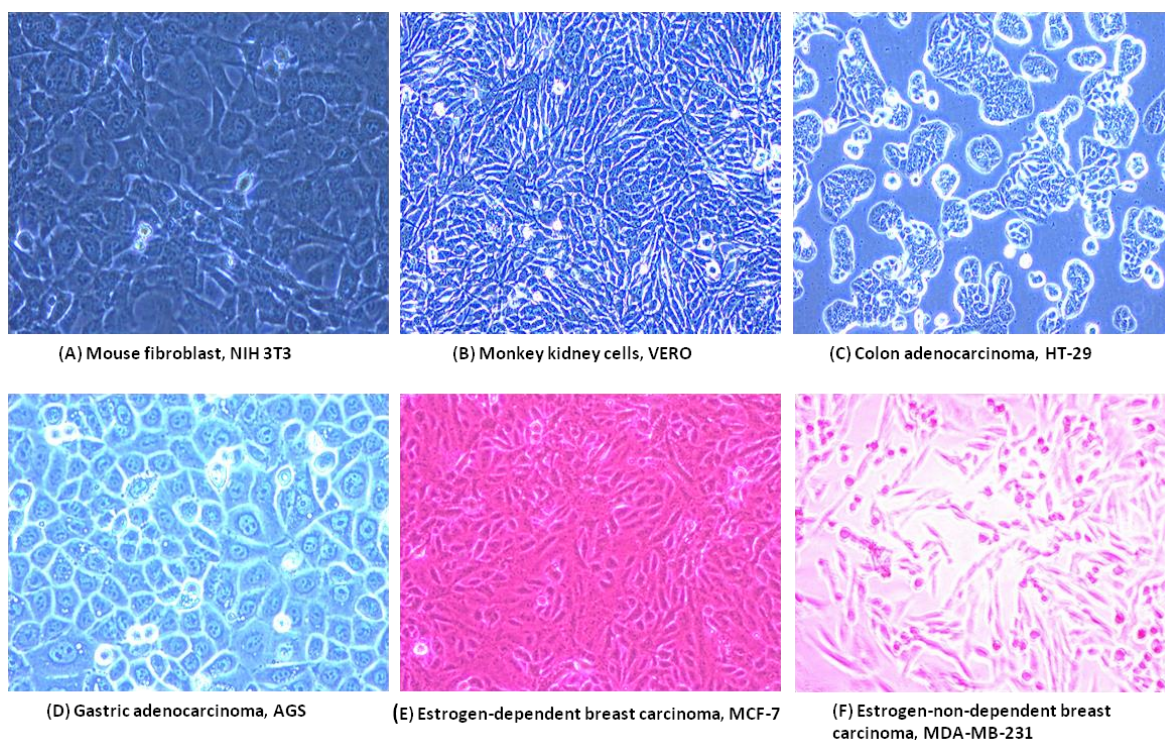
Figure 2.1 Cell lines (10 x magnification) used for cytotoxic screening

Table 2.4 Cell lines used for cytotoxicity screening

Common name	Generic name	ATCC Number
Mouse fibroblast cells	NIH3T3	CRL-1658
Monkey kidney cells	VERO	CCL-81
Human gastric adenocarcinoma cells	AGS	CRL-1739
Colorectal adenocarcinoma cells	HT-29	HTB-38
Estrogen-dependent breast carcinoma cells	MCF-7	HTB-22
Estrogen non-dependent breast carcinoma cells	MDA-MB-231	HTB-26

All cell lines were cultured in tissue culture flasks with supplemented advanced DMEM and incubated at 37°C with 5% CO_2.

2.4 Mammalian cell culture

2.4.1 Resuscitation of cells

To resuscitate the cell lines, frozen cells (stored at -80°C or in liquid nitrogen) were thawed and transferred into a falcon tube containing 5 mL pre-warmed supplemented Advanced DMEM medium. The cell suspension was centrifuged at 3000 rpm (Eppendorf-5810 R, Germany) at 37°C for 3 minutes. The supernatant was removed and cells were resuspended in 5 mL fresh supplemented advanced DMEM medium by knocking gently. Then the cell suspension was transferred into a 25 cm^2 tissue flask and incubated at 37°C with 5% CO2 to let the cells attach to the flask bottom. The medium was changed every 2 to 3 days.

2.4.2 Sub-culturing of cells

Cells were sub-cultured when the cells reached a minimum of 80% confluence. To detach the cells from the flask bottom, medium was removed and the cell layer was washed with 5 mL of PBS-EDTA solution to clear the cell layer from medium, dead cells and cell metabolites. After removing the PBS-EDTA, 1 mL of 0.25% Trypsin-EDTA solution was added to a 25 cm^2 flask for detaching the cells. The tissue flask was incubated at 37°C with 5% CO_2 for a maximum of 5 minutes. After complete detachment of the cells, approximately 330 µL of cell suspension was transferred into

each 75 cm^2 flasks already containing 10 mL fresh supplemented advanced DMEM medium. The flasks were returned to the incubator for further growth. The medium was changed when necessary.

2.4.3 Storage of cells

Cells were harvested in a similar way as described briefly in section 2.4.2. Cells were suspended in 1.5 mL supplemented advanced DMEM medium containing 5% (v/v) DMSO. The cell solution was then transferred into cryovials and stored in the -80°C freezer. For a longer preservation, the cryovials were transferred to liquid nitrogen.

2.4.4 Counting of cells

Cells were cultured as described earlier in section 2.4.2 and a uniform cell suspension was prepared. Cells were counted using an Improved Neubauer Haemocytometer (Figure 2.2).

Figure 2.2 Improved Neubauer Hemacytometer used for cell counting

Cell suspension (20 µL) was diluted (1:4) by adding 60 µL of trypan blue. About 10 µL of coloured cell suspension was placed onto the counting chamber. Non-viable cells absorb the blue dye whereas healthy cells remain uncoloured. The cells of each of the four corners and the central square (in total 5 squares) were counted. The cells per mL were calculated using the formula below;

$$\text{Cells/mL} = \frac{\text{Total number of cells counted in 5 squares x 4}}{5} \text{ x } 10^4 \text{ cells/mL}$$
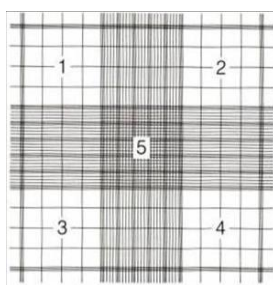
Figure 2.3 Grid of the Neubauer chamber

2.5 MTT assay

2.5.1 Procedure

The MTT colorimetric assay was performed according to the method previously described (Mosmann 1983) and further modified (Popiolkiewicz 2005) and (Uddin 2011). Briefly, the cells were seeded in 96 well plates (1.0×10^4 to 2.0×10^4 cells/well) in 150 µL of medium and incubated at 37°C with 5% CO_2 for 24 h. This seeding cell number would guarantee 80% confluence after 3 days incubation if untreated. Following attachment of the cells after 24 h, the cells were treated with different concentrations (2.5, 0.25, 0.025 and 0.0025 mg/mL) of Bangladeshi plant extracts and then the plates were incubated for further 48 h at the same condition. Following 72 h incubation, the supernatant was removed and the attached cells were washed once with 200 µL of PBS. A mixture of 150 µL of advanced DMEM and 50 µL of MTT in PBS (0.5 mg/mL MTT for cancer cell lines such as AGS, HT-29, MDA-MB-231 and 1 mg/mL for the healthy cell line NIH3T3) was added to each well. Following incubation for 2 h at the same condition, a purple formazan product was produced. DMSO (100 µL for cancer cell lines and 125 µL for healthy cell lines) was then added. The plates were incubated for a further 45 minutes at room temperature in a dark chamber to dissolve the formazan product. The absorbance was measured with a spectrophotometric microplate reader (Wallac 1420 Multilabel counter, PerkinElmer) at a wavelength of 560 nm. The assays were performed in triplicate; 2% DMSO served as the negative control while 20% DMSO served as the positive control.

The % of viable cells is directly proportional to the level of formazan produced. Therefore the higher the measured absorbance, the more viable the cells are present.

The cytotoxic activity was calculated with the measured absorbance values using the following formula:

$$\% \text{ of cytotoxic activity} = 100 - \frac{\text{Absorbance of test sample}}{\text{Absorbance of negative control}} \times 100\%$$

2.5.2 Sample preparation

2.5.2.1 Plant extract solutions for cytotoxic activity screening

For screening the cytotoxic activity of Bangladeshi medicinal plant extracts, four different concentrations (2.5, 0.25, 0.025 and 0.0025 mg/mL) were used. The total volume of each well (200 µL) consisted of 150 µL cell suspension and 25 µL test sample and 25 µL PBS.

To prepare 2.5 mg/mL concentration of a test sample in one well (200 µL) the following calculation was used:

$$= \text{Concentration of sample x volume of well}$$
$$= \frac{2.5 \text{ mg x } 200 \,\mu\text{L/well}}{1000 \,\mu\text{L}}$$
$$= 0.5 \text{ mg of extract/well}$$

To achieve a 2.5 mg/mL assay concentration of methanol plant extracts, 20 mg/mL of extract solution was prepared by dissolving 100 mg of dried extract in 5 mL of solvent (2% DMSO in MilliQ water). Then 25 µL of this extract solution was added in each well giving a concentration of 0.5 mg/25 µL. Other concentrations were prepared by serial dilution of the 20 mg/mL stock solutions. As a positive control, 20% DMSO solution and as a negative control, 2% DMSO solution were used in this cytotoxicity evaluation test.

2.5.2.2 Solid phase extraction (SPE) fraction solutions for cytotoxic activity screening

Cytotoxic activity of the SPE fractions obtained from the crude methanol extract (CME) of *Blumea lacera* was performed at four different concentrations (250, 50, 10 and 2 µg/mL) of the fractions in a 96 well plate. Each individual well had a total volume of 200 µL. It was filled with 150 µL of cell suspension, 25 µL of sample solution and 25 µL of PBS. A concentration of 250 µg/mL was obtained by preparing 2 mg/mL of each SPE fraction solution. To obtain a 2 mg/mL fraction solution, 5.0 mg of each fraction was dissolved in 2.5 mL of 2% DMSO solution in MilliQ water. Twenty five microliter (25 µL) of this fraction solution (50 µg in 25 µL) was then added to each well which ultimately represents 250 µg/mL concentration of the SPE fraction. Other concentrations were prepared by serial dilution of the 2.0 mg/mL stock solutions. As a positive

control, 20% DMSO solution and as a negative control, 2% DMSO solution were used in this cytotoxicity evaluation test.

2.5.2.3 Pure compound solutions for cytotoxic activity screening

For cytotoxic activity screening of the compounds isolated from *Blumea lacera* and of the purchased compounds, five different concentrations (100, 10, 1.0, 0.1 and 0.01 µg/mL) of the test compounds were added into a 96-well plate. The total volume of each well was 200 µL, which was comprised of 150 µL cell suspension, 25 µL of test sample solution and 25 µL of PBS. To obtain 100 µg/mL assay concentration, 0.8 mg/mL of pure compound solution was prepared by dissolving 4.0 mg of pure compound in 5 mL of solvent (16% DMSO in MilliQ water). Twenty five microliters of each compound solution (20 µg/25 µL) was then added to each well, which represents 100 µg/mL concentration of pure compound containing 2% DMSO. Other concentrations were prepared by serial dilution of the 0.8 mg/mL stock solutions. Cycloheximide, a known cytotoxic drug was used as a positive control and tested at the same concentrations used for the test compounds, whereas 2% DMSO served as a negative control.

2.5.3 Cell preparation

To prepare the cells for the MTT assay, based on cell type and their proliferation rate, 1.0×10^4 to 2.0×10^4 cells/well were suspended in 150 µL of advanced DMEM and seeded into a 96 well plate.

2.5.4 Cell susceptibility to DMSO

DMSO (2% v/v) solution was used to dissolve the methanol extracts. As DMSO itself is a cytotoxic agent, it was necessary to identify the sensitivity of DMSO to the cells and to identify the concentration which is acceptable in the MTT assay for the screening of cytotoxic activity of the extracts. To serve this purpose, different concentrations of DMSO; 0.5, 1.0, 2.0, 2.5, 5.0, 10 and 20% per well were used to test their cytotoxic effects against HT-29 and NIH3T3 cells. The MTT assay was performed as described in section 2.5.1, except that different concentrations of DMSO solution were used instead of plant extracts.

2.5.5 Calculation of IC$_{50}$

IC$_{50}$ (50% inhibition of cell growth) was calculated by using Probit analysis software (LdP Line software, USA) (Bakr, 2010).

2.6 Flow cytometry

2.6.1 Annexin V-FITC apoptosis measurement

Annexin V-FITC apoptosis assay by (Uddin, Grice et al. 2012) was used to measure the apoptosis of the isolated cytotoxic compounds from *Blumea lacera* as well as the purchased cytotoxic compounds against estrogen-dependent breast carcinoma cells (MCF-7). Briefly, cells were seeded in a 6-well plate at a density of 50 x 10^4 cells/well in 1.5 mL of advanced DMEM medium and incubated at 37°C with 5% CO$_2$ for 24 h. Following attachment of the cells after 24 h, the cells were treated with different cytotoxic compounds at IC$_{50}$ concentrations for 24 and 48 h (Section 2.6.1.2). Following the treatment incubation period, the supernatant was removed and collected in a 15 mL falcon tube in order to retain non-adherent and dead cells. Cells were washed with 2 mL of ice-cold PBS and the PBS wash was collected into the 15 mL falcon tube already containing the supernatant collected. The attached cells in the culture plate were trypsinised with 400 μL of trypsin-EDTA very rapidly in order to reduce false annexin V$^+$ staining. The trypsinised cells were then added to the falcon tube already containing the supernatant and PBS wash. The cell suspension was centrifuged for 10 minutes at 2000 rpm to pellet the cells. The supernatant was removed and 4 mL of cold PBS was added to each tube. The cells were briefly vortexed to resuspend the cells and centrifuged again for 10 minutes at 2000 rpm to pellet the cells. The supernatant was removed and the cells were resuspended in 100 μL of 1 x binding buffer. The cell suspension was then transferred to a 5 mL falcon polystyrene U bottom tube and 5 μL of annexin V-FITC and 5 μL of propidium iodide were added, followed by incubation for 15 minutes in the dark at room temperature. The cell suspension was diluted by adding 400 μL of 1 x binding buffer to each tube and apoptosis was measured within 1 hr using a CyAn^{TM} ADP flow cytometer (Beckman Coulter, USA) and the data was recorded by using HyperCyt (R) software. The assay was carried out as two

separate experiments and each experiment performed in triplicate. Cells with no treatment served as the negative control and paclitaxel (20 ng/mL) served as the positive control. The solvent effect was subtracted (2% DMSO) from the test sample.

2.6.1.1 Cell preparation

For annexin V-FITC apoptosis analysis, 50×10^4 MCF-7 breast cancer cells were suspended in 1.5 mL of advanced DMEM medium and seeded into 6-well plates.

2.6.1.2 Sample preparation

Identified cytotoxic compounds were further investigated by flow cytometric analysis using annexin V-FITC/PI staining to reveal their mechanism of cytotoxicity, whether they are causing apoptosis or necrosis to kill the breast cancer cells, MCF-7. The assay was performed in 6-well plates at the IC_{50} of each compound. The total volume of each well was 2 mL, which consisted of 1.5 mL cell suspension, 250 µL of test compound solution and 250 µL of PBS.

Compound **BL3** was tested at 2.66 µg/mL and was prepared by dissolving 16 µg of the compound in 750 µL of solvent (16% DMSO in MilliQ water), then 250 µL of this solution was added to each well (total volume was 2 mL), producing a final concentration of 2.66 µg/mL in the assay.

Compound **BL5** was tested at 6.66 µg/mL, β-solamargine at a 4.35 µg/mL, α-solasonine at a 4.5 µg/mL, β-solamarine at a 3.56 µg/mL, and α-solanine at a 24 µg/mL concentration. The samples were prepared as described above.

2.6.2 Cell cycle analysis

Propidium iodide staining cell cycle analysis (Rouf, Stephens Alexandre et al. 2013) was used to measure the cell distribution in 3 different phases of the cell cycle of the isolated cytotoxic compounds from *Blumea lacera* as well as the purchased cytotoxic compounds against estrogen-dependent breast carcinoma cells (MCF-7). Briefly, for cell cycle analysis using flow cytometry, 15 $\times 10^4$ MCF-7 cells/well in 1 mL of Advanced DMEM were seeded in 12-well plates and incubated at 37°C with 5% CO_2 for 24 h. Following attachment of the cells after 24 h, the cells were treated with different cytotoxic compounds at their IC_{50} for 24 h. Following 24 h incubation, the

supernatant was removed and washed with PBS-EDTA. Then the cells were trypsinized with 200 μL trypsin-EDTA/well and 800 μL Advanced DMEM was added to trypsinized cells and collected in a 15 mL falcon tube and vortexed shortly. The cell suspension was centrifuged for 2 minutes at 100xg to pellet the cells. The supernatant was removed and 500 μL of PBS was added to each falcon tube. The cells were vortexed briefly to resuspend the cells and centrifuged again for 2 minutes at 100xg to pellet the cells. The supernatant was removed and the cells were resuspended in 400 μL of PBS. Ice cold absolute ethanol (1 mL) (final concentration is approximately 70%) was added to each falcon tube for fixation with gentle shaking to avoid cluster formation and vortexed briefly to resuspend the cells. The cells were incubated on ice. After 15 minutes incubation, cells were centrifuged again for 2 minutes at 100xg to pellet the cells, ethanol was removed and 400 μL of PBS was added to resuspend the cells. Cells were centrifuged again for 2 minutes at 100xg to pellet the cells and supernatant was removed. Cell pellets were resuspended in 250 μL of RNase A solution (0.2 mg/mL RNase and 10% Triton X-100) in PBS and incubated for 40 minutes at 37°C. The cell suspension was then transferred to a 5 mL falcon polystyrene U bottom tube and 10 μL of propidium iodide was added and was analysed using CyAn^{TM} ADP flow cytometer (Beckman Coulter, USA) and the data was recorded by using HyperCyt (R) software. Cells with no treatment served as the negative control and paclitaxel (20 ng/mL) served as the positive control.

2.6.2.1 Cell preparation

For propidium iodide stained cell cycle analysis using flow cytometer, 15 x 10^4 MCF-7 breast cancer cells were suspended in 1 mL of advanced DMEM medium and seeded into 12-well plates.

2.6.2.2 Sample preparation

Identified cytotoxic compounds were further investigated by cell cycle analysis using propidium iodide staining to reveal the arrested phase of cell cycle causing death of the breast cancer cells, MCF-7. The assay was performed in 12-well plates at the IC_{50} of each compound. The total volume of each well was 1 mL. For sample application 250 μL of Advanced DMEM was removed and 250 μL of sample solution consisting of the IC_{50} of each compound was added to each well.

Compound, **BL3** was tested at a 2.66 µg/mL ($IC_{50} = 2.66$) and was prepared by dissolving 8 µg of the compound in 750 µL of solvent (8% DMSO in MilliQ water), then 250 µL of this solution was added to each well (total volume was 1 mL), producing a final concentration of 2.66 µg/mL in the assay.

Compound **BL5** was tested at 6.66 µg/mL, β-solamargine at a 4.35 µg/mL, α-solasonine at a 4.5 µg/mL, β-solamarine at a 3.56 µg/mL, and α-solanine at a 24 µg/mL concentration. The samples were prepared as described above.

2.7 Antifungal activity assay

2.7.1 Fungal strains used for antifungal activity assay

The *Candida albicans* fungal strain ATCC 10231 and the *Trichophyton mentagrophytes* fungal strain ATCC 18748 were purchased from ATCC. The fungal strains of *Aspergillus fumigatus* (ATCC 13073) and *Aspergillus niger* (ATCC 1004) were provided by Dr Joe Tiralongo, Institute for Glycomics, Griffith University, Gold Coast campus, Australia (Table 2.5).

Table 2.5 Fungal strains used for antifungal activity study

Fungal strain	ATCC number
Candida albicans	ATCC 10231
Trichophyton mentagrophytes	ATCC 18748
Aspergillus fumigatus	ATCC 13073
Aspergillus niger	ATCC 1004

2.7.2 Culture media

2.7.2.1 Sabouraud Dextrose broth and agar

The Sabouraud Dextrose broth contained the following ingredients:

Enzymatic digest of casein   10 g/L

Dextrose   20 g/L

Sabouraud Dextrose broth (SDB) powder (15 g) was dissolved in a final volume of 500 mL Milli-Q water and mixed well. The broth was adjusted to pH 7 by adding NaOH and sterilized by autoclaving it at 121°C for 20 min and then left to cool. The broth was stored at 4°C.

For the Sabouraud Dextrose agar (SDA) plates, in addition to the broth powder, 7.5 g of agar powder was dissolved in a final volume of 500 mL Milli-Q water to ensure a stable structure required for inoculation with a swab. The agar was adjusted to pH 7 by adding NaOH and sterilized by autoclaving it at 121°C for 20 min. After cooling to about 50°C, the agar was poured into petri dishes and dried until solid with a slightly open lid under aseptic conditions. The finished plates were stored upside down at 4°C.

2.7.2.2 Yeast Mould agar

Table 2.6 Ingredients of the Yeast Mould agar

Ingrdients	Concentration
Yeast Extract	3 g/L
Malt Extract	3 g/L
Peptone	5 g/L
Dextrose	10 g/L

Yeast Mould media powder (10.5 g) and agar powder (5 g) were dissolved in a final volume of 500 mL of Milli-Q water and mixed well. The agar was sterilized by autoclaving it at 121°C for 20 min. After cooling to about 50°C, the agar was poured into petri dishes or tissue culture (TC) flasks and

dried until solid with a slightly open lid under aseptic conditions. The finished plates were stored upside down at 4°C.

2.7.2.3 Pre-culturing of fungi on agar plates

Pre-cultures of the fungi were grown on YM agar for *C. albicans, A. niger* and *A. fumigatus* and on SDA for *T. mentagrophytes*, respectively.

A few drops of thawed cultures or standard inoculum were inoculated on agar plates and incubated at 25°C for 48 h (*C. albicans*) and 5-10 days (*T. mentagrophytes*), respectively.

Pre-culturing of *A. fumigatus* and *A. niger* was performed in tissue culture flasks by scratching some fungi with a loop from a frozen culture and dipping the loop onto the agar on five different spots. These were incubated at 25°C for 5-6 days until sporulation.

2.7.2.4 Harvesting

Candida albicans was harvested by picking up 4-5 fresh single colonies from an agar plate with a loop and suspending them in 5 mL of PBS. The suspension was centrifuged for 5-10 min at 2000 rpm. The supernatant was removed and the spores resuspended in PBS. This washing procedure was repeated twice. Following the final removal of the supernatant, the cells were resuspended in 5 mL PBS.

Harvesting of *Trichophyton mentagrophytes* was achieved by sweeping over a pre-culture (agar plate) with a wetted swab. This swab was swivelled in 10 mL of PBS and the procedure repeated, until the whole plate was harvested. The suspension was washed twice as described above and resuspended in 5 mL of PBS.

Harvesting of *Aspergillus fumigatus* and *Aspergillus niger* was performed by adding 10-20 glass beads and 8-10 mL of PBS into the TC flask where the fungi were grown. Then, the flask was shaken rigorously to dislodge the spores. The received suspension was filtered through glass wool and centrifuged for 10 min at 3000 rpm. The supernatant was removed and the spores resuspended

in PBS. This washing procedure was repeated twice. Finally, the spores were resuspended in 3 mL of PBS.

2.7.2.5 Storage of fungi

For storage, *C. albicans* was harvested by picking up 4-5 fresh single colonies with a loop and suspending them in 10 mL of SDB. The suspension was incubated at $25°C$ on an orbital shaker (200 revolutions per minute, rpm) for 2 days. After these 2 days, the suspension was centrifuged for 5-10 min at 2000 rpm. The supernatant was washed three times as described above (Subsection 2.7.2.4). After a final resuspending in 5 mL of SDB, 1 mL of this suspension was added to 240 µL of glycerol in a cryovial and stored at $-80°C$ and in liquid nitrogen.

For storage of *T. mentagrophytes* the fungi was harvested as described above (Subsection 2.7.2.4). A volume of 1 mL of final suspension was then added to 240 µL of glycerol in a cryovial and stored at $-80°C$ and in liquid nitrogen.

A. fumigatus and *A. niger* were harvested as described above (Subsection 2.7.2.4). A volume of 1 mL of the final suspension was added to 240 µLof glycerol in a cryovial and stored at $-80°C$ and in liquid nitrogen.

2.7.2.6 Inoculum standardisation

McFarland turbidity standard

McFarland turbidity standards are used to visually approximate the concentration of cells in a suspension. For this assay, according to the standard reference method (EUCAST 2008), only McFarland standard No. 0.5 was used, corresponding to a fungal suspension of $1\text{-}5 \times 10^6$ CFU/mL. The standard was prepared by mixing 0.05 mL of barium chloride 1.175% (w/v) and 9.95 mL of Sulphuric acid 1.00% (v/v), thoroughly. The optical density (OD) was measured with a UV spectrophotometer at a wavelength of 530 nm. The OD for the McFarland standard No. 0.5 was 0.158. The OD of the fungal suspension was adjusted to this value to ensure a density of $1\text{-}5 \times 10^6$ CFU/mL and is referred to in the following as standard inoculum. This was directly used in the agar

plate assay. The standard inoculum was diluted with SDB to a density of 2 x 10^4 CFU/mL to be used in the broth microdilution assay.

2.7.2.7 Cell counting using an Improved Neubauer Haemocytometer

To count cells, 10 µL of conidia or cell suspension in an appropriate dilution were added to the counting chamber. Using a microscope, all cells of the four corner squares plus central square were counted. The cells per mL were then calculated using the formula below:

$$\text{Cells/mL} = \frac{\text{Total number of cells counted in 5 squares x 4}}{5} \text{ x } 10^4 \text{ cells/mL}$$

Dilutions of the inoculum were made using SDB.

2.7.2.8 XTT and menadione solution

A saturated solution of XTT [2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] was prepared by dissolving 50 mg in 100 mL of PBS, leading to a concentration of 0.5 mg/mL. The solution was first heated to 37°C to dissolve the XTT as well as possible and then filter sterilized by filtering it through a 0.22 µm pore filter. The solution was divided into aliquots of 5 mL and the falcon tubes were wrapped in aluminium foil to protect the solution from the light. The tubes were stored in the -80°C freezer.

From menadione, a 10 mM solution was prepared by dissolving 17.218 mg in 10 mL of acetone. This solution was stored in the fridge.

The XTT and the menadione need to be stored separately until used. For usage, one aliquot of XTT was thawed and 62.5 µL menadione were added to a final concentration of 0.5 mg/mL XTT and 0.125 mM menadione.

Phosphate-buffered saline was prepared by dissolving one PBS tablet (Sigma Aldrich) in a final amount of 200 mL of Milli-Q water. The pH was measured and, if necessary, adjusted to 7.4 by adding NaOH or HCl.

Before usage, PBS was autoclaved at 121°C for 20 min.

2.7.2.9 Antifungal drug solutions

The antifungal drugs used in this study were amphotericin B, fluconazole and nystatin and were obtained from Sigma Aldrich. Nystatin possesses a potency of 6000 USP units per mg as declared by Sigma Aldrich.

Initial validation tests were performed to determine the MICs of these antifungals against the strains used under our assay conditions.

Solvents and concentrations of stock solutions are stated below (Table 2.8). Nystatin was not dissolved but suspended as it is active as a suspension.

Further dilutions, for example for the broth microdilution assay, were made with SDB. Amphotericin B and fluconazole solutions were stored in the fridge. Nystatin suspension was stored in the -80°C freezer. Amphotericin B was diluted to a concentration of 20 µg/mL with SDB to be used in the broth microdilution assay.

Table 2.7 Antifungal solutions

Antifungal drug	Solvent	Concentration of stock solution
Amphotericin B	100% DMSO	1.33 mg/mL
Nystatin	30% DMSO	1.33 mg/mL
Fluconazole	Milli-Q water	3.33 mg/mL

DMSO: Dimethyl sulfoxide

2.7.2.10 Sample preparation for testing

For an initial screening, all compounds (Table 6) were dissolved in DMSO at a concentration of 1 mg/mL. This solution was diluted 1:10 with PBS to get a compound testing solution of 100 µg/mL, with a DMSO concentration of 10%, which would lead to a final compound concentration in the wells of 20 µg/mL compound and 2% DMSO.

2.7.2.11 Antifungal assay

Broth microdilution assay

The assay was performed in 96 flat bottomed well plates which were either cultivated for 2 days (*A. fumigatus, A. niger* and *C. albicans*) or 5-7 days (*T. mentagrophytes*) in an incubator at 25°C. The total volume in one well was 200 µL. For testing Bangladeshi plant compounds, these 200 µL consisted of 100 µL fungal inoculum, 60 µL SDB and either 40 µL of amphotericin B solution (Subsection 2.7.2.8) as positive control, solvent of the test compound (10% DMSO in PBS) for the negative control or test compound solution for the actual test. Each experiment was conducted in triplicates and performed twice on two different days.

In order to manage the colour influence of the isolated compounds, wells containing 160 µL medium and 40 µL of either compound solution, solvent or antifungal solution were used as blank controls for the later calculation of the OD.

After the full incubation period, 50 µL of XTT and menadione (Subsection 2.7.2.8) were added to the wells and the plates were wrapped in aluminium foil and incubated for 2 more h at 25°C. After these 2 h, the absorbance of the plates was measured at 450 nm with the VICTOR[3] Multilabel counter 1420. Each compound was first tested at only one concentration which was 20 µg/mL, with the intention of testing with further concentrations if initial concentration showed antifungal activity.

As stated above, the blank values were first subtracted from the measured ODs to eliminate the colour influence of the well content.

$OD_{AF} = OD_{AF, measured} - OD_{AF, blank}$

$OD_{Solvent} = OD_{Solvent, measured} - OD_{Solvent, blank}$

$OD_{Test} = OD_{Test, measured} - OD_{Test, blank}$

The determination of the inhibition caused by the compounds was calculated as follows:

$OD_{100\% \text{ Inhibition}} = OD_{AF} - OD_{Solvent}$

$OD_{0\% \text{ Inhibition}} = OD_{Solvent}$

$$\Delta OD_{1\% \text{ Inhibition}} = OD_{100\% \text{ Inhibition}} / 100$$

$$X\% \text{ Inhibition} = 100 - (OD_{\text{Test}} / \Delta OD_{1\% \text{ Inhibition}})$$

OD_{AF} was the measured OD of a well consisting of 100 µL fungal inoculum, 40 µL amphotericin B (20 µg/mL in 10% DMSO) and 60 µL SDB

OD_{Solvent} was the measured OD of a well consisting of 100 µL fungal inoculum, 40 µL 10% DMSO and 60 µL SDB

OD_{Test} was the measured OD of a well consisting of 100 µL fungal inoculum, 40 µL compound solution in specified concentration and 60 µL SDB

Antifungal assay was developed and used by (Brechbühler 2013)

2.8 Chromatographic methods

2.8.1 Solid phase extraction (SPE)

Sample preparation

Fifty mg of extract were dissolved in 1mL (1 bed volume) of water and loaded onto a 300 mg SPE column. For a 10 g SPE column 500 mg of extract were dissolved in 20 mL (1 bed volume) of water and then loaded onto the SPE column.

Procedure

SPE fractionation of the crude methanolic extract of *Blumea lacera* was carried out for the crude extract using Alltech, 60 mL, 10 g High capacity C-18 SPE columns. The SPE columns were first preconditioned with 60 mL of methanol (3 bed volumes) and then equilibrated with 60 mL of water (3 bed volumes). Based on the separation of compounds with different gradients of mobile phase used at small scale, the sample was then eluted following loading of extract onto the column with a water/methanol mobile phase (60 mL) of 100% water, 20%, 35%, 60%, 80% and 100% methanol. The solvent was then evaporated by rotary evaporation from each fraction, followed by freeze drying (water fractions) or using a high vacuum system (methanol fraction).

The % of yield recovery was calculated as:

$$\% \text{ of yield recovery} = \frac{\text{Total mass of fractions}}{\text{Initial loading mass of the sample}} \times 100\%$$

2.8.2 High performance liquid chromatography (HPLC)

2.8.2.1 Analytical HPLC

Procedure

Analytical HPLC was employed for method development for small scale separation and purity assessment of isolated compounds. It was conducted using a Varian ProStar HPLC system (Varian Inc., Walnut Creek, CA, USA) comprising a 210 Binary pump, 410 Auto Sampler and 335 DAD (Diode Array Detector) monitoring 190-400 nm. A Luna C18 reverse phased column (5µm, 250 x 4.6 mm) served as the stationary phase. The mobile phase comprised of methanol and water containing 0.05% TFA (trifluoroacetic acid). Column temperature was set at 30°C and the solvent flow rate was maintained at 1 mL/min. The Star Work Station Sofware υ 6.41 was used to control the auto-sampler, gradient settings, DAD and data acquisition. The purity of each fraction was determined based on the sharpness of the peak and the detected UV profile (PDA) using Poly View 2000-Diode Array Spectral Processing software. A 10 mg/mL solution was prepared by dissolving 10 mg of each crude methanolic extract in 1 mL of methanol (HPLC grade). Vortexing for 5 min and sonication for further 5 min were applied to dissolve the extracts.

2.8.2.2 Semi-preparative HPLC

Procedure

Semi-preparative HPLC was used to isolate and purify the compounds from the crude extract of *Blumea lacera.* It was performed using a Luna C-18 column (5 µm) reversed-phase (150 x 21.2 mm) as the stationary phase and a gradient of 10% methanol in water with 0.05% TFA to 90% methanol in water with 0.05% TFA) as the mobile phase. The absorbance was monitored at 210 and 280 nm. The analytical method was converted to semi-preparative HPLC method by adjusting the flow rate and sample load using the following formula: $F_2 = F_1 \times (L_2/L_1) \times (r_2/r_1)$ where F_2 = semi-preparative HPLC solvent flow rate, F_1 = analytical HPLC solvent flow rate, r_2 = diameter of the

semi-preparative column, r_1 = diameter of the analytical column, L_2 = semi-preparative column length and L_1 = analytical column length. Purity of the isolated compounds from semi-preparative HPLC runs was confirmed by analytical HPLC analysis.

2.9 Spectroscopic methods

For structural characterization of the isolated compounds, principally NMR and mass spectromtery were performed. UV and IR spectroscopy along with melting point determination, and optical rotation analysis were also carried out when required for physical characterization and structural elucidation of isolated compounds.

2.9.1 UV-visible spectroscopy

UV spectroscopy was used for the preliminary identification of compounds, particularly for the identification of the presence of phenolic groups or conjugated double bonds. Compounds initial UV profiles were obtained on the ananlytical HPLC using PDA detection and these profiles were confirmed with a Shimadzu BioSpec-mini (A115247) UV-visible spectrophotometer (Shimadzu Corporation, Japan). Molar extinction co-efficients for each compound were calculated using the below formula:

$$\varepsilon = A/(c\ x\ l)$$

Where, ε = molar extinction co-efficient, A = absorbance at the particular wavelength, c = molar concentration, l = path length (1 cm).

2.9.2 IR spectroscopy

IR spectroscopy was further used to confirm any functional groups present in the isolated compounds. IR spectra were recorded with a Bruker Optics ALPHA QuickSnap™ (A220/D-01) FT-IR spectrophotometer with OPUS spectroscopy software. A solid sample of each compound was cast onto the diamond ATR-crystal plate and was scanned from 4000 to 375 cm^{-1} with 64-100 scans for analysis. Resulting peaks were compared to published data of functional groups.

2.9.3 NMR spectroscopy

The structures of the isolated compounds were elucidated primarily by 1D (homonuclear) and 2D (heteronuclear) NMR spectroscopy. 1D NMR experiments including ^{1}H and ^{13}C NMR were used to locate atom positions and fragment units. Further, 2D NMR experiments including COSY, HSQC, and HMBC were carried out on more complex molecules for accurate assignments of proton and carbon chemical shifts. NMR spectra were recorded on a Bruker Avance 300 MHz (300 and 75 MHz respectively) or a Bruker Avance 600 MHz (600 and 150 MHz, respectively) spectrometer was coupled with Topspin 2.1 acquisition software. Samples were dissolved in 500-600 μL of suitable deuterated solvent (Subsection 2.2.2.2). NMR experiments on samples with very little mass were carried out using Shigemi NMR tubes (100-200 μL sample) (Sigma-Aldrich Ltd.). Signals were recorded in chemical shifts (δ) and expressed in parts per million (ppm), with coupling constants (*J*) calculated in Hertz (Hz).

2.9.3.1 ^{1}H NMR

In the present study, ^{1}H NMR data was recorded for all isolated compounds and was used as a primary source of structural information. The chemical shifts and integration indicated the number and type of protons present in each molecule, whereas, the multiplicity and coupling constants indicated the adjacent protons and their spatial arrangements. The purity of the compounds was also determined from ^{1}H NMR spectra.

2.9.3.2 ^{13}C NMR

[13]C (Jmod) spectral data was recorded for all isolated compounds, to determine the number and types of carbons present in each molecule. In the spectra, quartenary and methylene carbon signals appeared in the positive phase, while methyl and methane signals were evident in the negative phase of the spectra.

Distortionless Enhancement by Polarization Transfer (DEPT) was performed on selected compounds. The DEPT pulse sequence experiment performs CH signal multiplicity and spin-spin coupling information into a phase relationship. In the DEPT spectrum, CH_3 and CH signals are

directed towards the positive phase of the spectrum and CH_2 signals to the negative phase of the spectrum. The number of quaternary carbons present in the molecule was determined by comparison of the ^{13}C (Jmod) with the relevant DEPT spectrum

2.9.3.3 Correlation spectroscopy (COSY)

COSY spectral data was recorded for all compounds. Cross-peaks were generated from the ^{1}H-^{1}H nuclei that share a mutual scalar coupling and normally evidenced for a germinal (^{2}J) and vicinal (^{3}J) couplings connectivity. This information provided the ^{1}H-^{1}H connectivity for the analysed compound.

2.9.3.4 Heteronuclear single quantum coherence (HSQC)

2D HSQC experiments were performed on all compounds. These experiments were used to identify one-bond (^{1}J) ^{1}H - ^{13}C connectivities. Cross peaks were generated from proton-carbon nuclei that are directly connected to each other through a single bond.

2.9.3.5 Heteronuclear multiple bond coherence (HMBC)

2D HMBC spectral data was recorded for isolated compounds, to detect long range 2 to 3 bond ^{1}H - ^{13}C couplings. Certain 4-bond cross-peaks were also observed in the HMBC experiments as a weaker intensity signal than that for the 2 and 3 bond cross-peaks. This experiment provided important structural information which helped to elucidate selected complex structures. It also provided information regarding connectivity between structural fragments.

2.10 Mass spectrometry

Positive- and negative-ion electrospray mass spectra (ESI-MS) and atomic pressure chemical ionization mass spectra (APCI-MS) were obtained on a Bruker Daltonics esquire Series 3000 mass spectrometer (LR-MS) (Bruker Daltonic GmbH, Bremen, Germany) with esquire Control software using a cone voltage of 4000 V with the source maintained at $250^{\circ}C$. For ESI-MS and APCI-MS spectra, samples were dissolved (0.4 mg/mL) in methanol:water (1:1) and injected at a flow rate of 0.04 mL/min. High-resolution electrospray ionization mass spectrometry (HR-ESI-MS) was

performed on a Bruker micrOTOF-Q 70 mass spectrometer, fitted with an Bruker Compass Data Analysis, at the University of Queensland.

2.11 Optical rotation

Optical rotation

Compounds were dissolved in methanol and the optical rotation of each compound measured at $25°C$ in a JASCO automatic polarimeter P-1010 series (JASCO Corporation, Tokyo, Japan) at the sodium wavelength, 589 nm, using Spectra Manager software. The solvent without sample was used as a blank and the sample cell (50 mm) was subsequently dried prior to taking the sample reading. The specific rotation [α] of a compound in degree at a specific temperature (T) and a wavelength (λ) is determined by the following formula:

$$[\boldsymbol{\alpha}]_{\boldsymbol{\lambda}}^{\mathbf{T}} = \left(\frac{\boldsymbol{\alpha}}{\mathbf{c}} \mathbf{x\, l}\right) \mathbf{x\, 10000}$$

Where, **α =** measured optical rotation, **c** = concentration (g/100 mL), **l** = length of the sample cell (mm).

CHAPTER THREE

CYTOTOXICITY SCREENING OF BANGALADESHI MEDICINAL PLANTS

3.1 Background

Based on ethnomedical information, the present study reports on the evaluation of cytotoxic activity of selected Bangladeshi medicinal plant extracts. A total of 19 medicinal plant species namely, *Aegiceras corniculatum* (*A. corniculatum*), *Argyreia nervosa* (*A. nervosa*), *Avicennia alba* (*A. alba*), *Caesalpinia pulcherrima* (*C. pulcherrima*), *Clerodendrum viscosum* (*C. viscosum*), *Clitoria terantea* (*C. terantea*), *Dillenia indica* (*D. indica*), *Dipterocarpus turbinatus* (*D. turbinatus*), *Diospyros peregrina* (*D. peregrina*), *Ecbolium viride* (*E. viride*), *Glinus oppositifolius* (*G. oppositifolius*), *Glycosmis pentaphylla* (*G. pentaphylla*), *Gnaphalium luteoalbum* (*G. luteoalbum*), *Hymenodictyon excelsum* (*H. excelsum*), *Jasminum sambac* (*J. samabac*), *Lannea coromandelica* (*L. coromandelica*), *Mussaenda glabrata* (*M. glabrata*), *Myrica nagi* (*M. nagi*) and *Saraca asoca* (*S. asoca*) were evaluated for their cytotoxic activity. Most of these plants grow all over Bangladesh, two plants (*A. corniculatum, A. alba*), which are mangrove plants, grow specifically in the coastal region, such as the Sundarban, and some (*D. turbinatus, M. glabrara* and *M. nagi*) grow specifically in the hilly area of Sylhet and Chittagong. Traditionally, these plants have been used as antitumor/anticancer, anti-infective and anti-inflammatory agents as well as for the treatment of other diseases (Chapter one; Table 1.6). Different plant parts of 9 plants namely *A. alba*, *C. pulcherrima*, *C. terantea*, *C. viscosum*, *D. peregrina*, *E. viride*, *J. sambac*, *M. nagi* and *S. asoca* have a reputation of being used traditionally as anticancer medications (Bandaranayake 1998; Ghani 2003; FAO 2004).

Cytotoxic activity screening of 19 Bangladeshi medicinal plants against healthy mouse fibroblast (NIH3T3), healthy monkey kidney (VERO), gastric cancer (AGS), colon cancer (HT-29), breast cancer (estrogen dependent: MCF-7 and estrogen non-dependent: MDA-MB-231) cells are reported here for the first time to confirm the traditional use of these selected plants as anticancer agents and to additionally identify new plants with significant anticancer potential.

3.2 Results and Discussion

3.2.1 Extraction yields from Bangladeshi medicinal plants

Extraction is the fundamental step in the discovery of drugs from medicinal plants and is the process of separation of medicinally active constituents from their sources (Handa 2008). For extraction purposes, classical techniques like maceration, decoction, percolation, soxhlet, microwave-assisted extraction, ultrasound extraction (sonication), supercritical fluid extraction have been used (Ghani 2003; Handa 2008; Biesaga 2011). Different solvents such as methanol, ethanol, acetone alone or in combination with water, ethyl acetate have usually been used for classical extractions (Biesaga 2011). The use of an appropriate extraction method, plant material, and solvent ensures a good quality extract (Handa 2008).

The amount of plant material available was taken into consideration when selecting plants, along with their traditional claims and unexplored cytotoxic/anticancer potential. Approximately 10 g of plant powder was extracted by maceration with 100 mL methanol and then dried using the rotary evaporator (Chapter 2, Section 2.1.2). Methanol was used for the extraction as it can separate both polar and non-polar compounds present in the material. A total of 23 extracts from 19 selected Bangladeshi plants were generated. Table 3.1 shows the yield of the dry extracts which ranged from 1.7% to 19.8% (w/w). The highest yield of dry extract (19.8% w/w) was obtained from *Myrica nagi* leaves whereas the lowest yield of dry extract (1.7% w/w) was afforded from *Dipterocarpus turbinatus* bark. Moreover, the dry extract yield for the leaves of *Dipterocarpus turbinatus* (7.1% w/w) was significantly higher than the yields obtained from the bark (1.7% w/w) indicating the presence of more methanol soluble constituents in the leaves of *Dipterocarpus turbinatus* than the bark. Previously the bark of *Aegiceras corniculatum* was extracted (Uddin 2011) and the yield of the dry extract was 8.0% w/w, whereas the yield for the dry extract from the fruits of *Aegiceras corniculatum* in this study was 5.7% w/w.

Table 3.1 Percent (% w/w) yield of dry extracts from the selected Bangladeshi medicinal plants

Plant name	Plant part #	Yield (% W/W)
Aegiceras corniculatum	Fruit	5.7
Argyreia nervosa	Leaf	5.0
Avicennia alba	Leaf	4.7
Caesalpina pulcherrima	Leaf	8.3
Clerodendrum viscosum	Leaf	4.3
Clitoria terantea	Leaf	11.3
Clitoria terantea	Flower	12.4
Dillenia indica	Leaf	8.9
Diospyros peregrina	Leaf	5.9
Dipterocarpus turbinatus	Leaf	7.1
Dipterocarpus turbinatus	Bark	1.7
Ecbolium viride	Leaf	13.3
Glinus oppositifolius	Whole plant	9.1
Glycosmis pentaphylla	Leaf	5.9
Gnaphalium luteoalbum	Leaf	1.9
Hymenodictyon excelsum	Bark	3.1
Hymenodictyon excelsum	Wood	4.5
Jasminum sambac	Leaf	2.3
Lannea coromandelica	Leaf	8.1
Lannea coromandelica	Bark	9.1
Mussaenda glabrata	Leaf	6.4
Myrica nagi	Leaf	19.8
Saraca asoca	Leaf	4.7

 # 10.0-10.1 g of each plant was extracted, with exception of *Clitoria terantea*-7.0 g

3.2.2 MTT assay validation

The MTT assay was used to investigate the cytotoxic activity of the selected Bangladeshi medicinal plant extracts against two different healthy cell lines and four different cancer cell lines in the present study. The method requires validation to obtain accurate and acceptable results. To serve this purpose, the MTT assay was validated previously in this lab (Uddin Shaikh, Grice et al. 2011) in regard to the cell concentration necessary for seeding of cell lines, time required for the cells to become attached, concentration of MTT solution, duration of cell incubation with MTT and volume of DMSO needed to ensure reproducible results. However, to confirm results regarding the influence of DMSO on cell viability, a healthy cell line: mouse fibroblast cells (NIH3T3) and a cancer cell line: colon cancer cells (HT-29) were tested.

3.2.2.1 Cell susceptibility to DMSO

Dimethyl sulphoxide (DMSO), an amphipathic solvent soluble in both water and organic substances, is usually used to dissolve the methanol extracts, as these do not dissolve fully in water. DMSO is a cytotoxic agent as it causes cell lysis through interaction with the membrane of cells (Da Violantem 2002). Furthermore, DMSO results in severe cell damage due to interaction with metabolism and cell membranes (Georges DA 2002). Although DMSO is an effective cryoprotective agent, it shows high cytotoxicity and reportedly affects the differentiation of neuron-like cells, cardiac myocytes and granulocytes (Kazuaki 2009). DMSO cytotoxicity is caused by inhibition of acetyl-cholinesterase activity by altering protein configuration (Michael 2006).

The MTT assay was performed with different concentrations of DMSO (0.5 to 20%) (Section 2.5.4). The results indicate that 2% DMSO has a low effect (<20% cell growth inhibition) on cell viability against NIH3T3 and HT-29 cell lines. Growth inhibition increased significantly when $\geq$ 5% of DMSO was used. At 20% DMSO concentration > 80% of cell growth inhibition was observed for both cell lines. Figure 3.1 summarizes the influence of DMSO on the growth of NIH3T3 and HT-29.
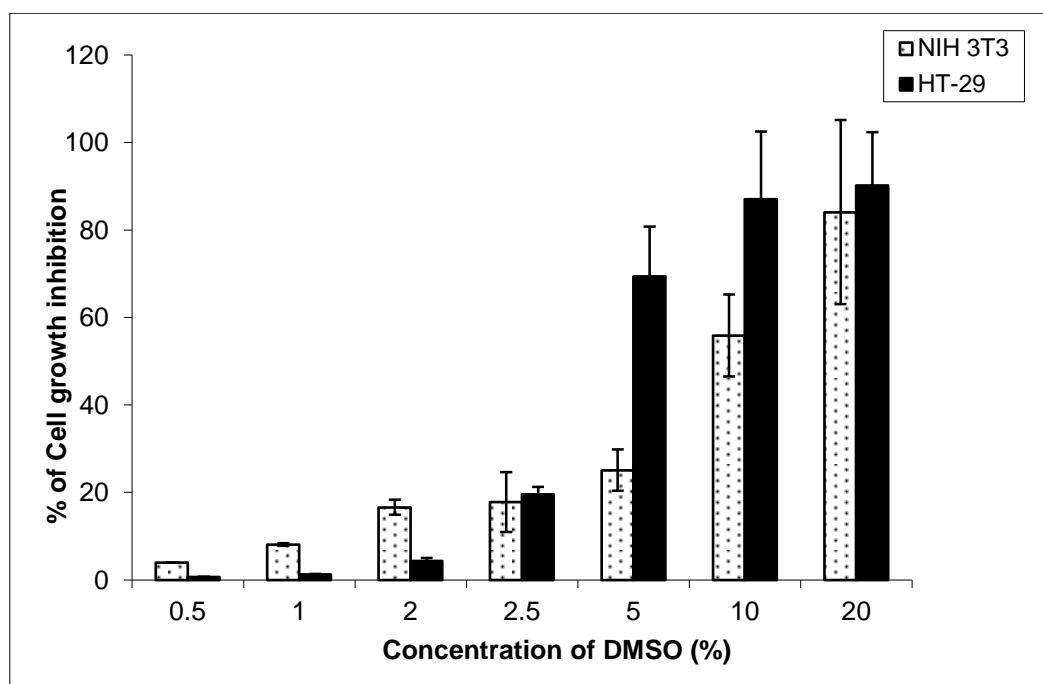
Figure 3.1 Effect of DMSO concentration on growth of HT-29 and NIH3T3 cell lines

A report has demonstrated that 5% DMSO does not affect the membrane integrity of isolated rat intestinal tissue (Georges DA 2002). But other reports state low inhibitory effects for 0.1 to 4% DMSO on different cell lines (Walker 1988; Adeniyi 2003). Our cell susceptibility study to DMSO confirmed that a DMSO concentration of 2% produced less than 20% of cell growth inhibition, while 20% DMSO concentration caused more than 80% cell growth inhibition against the healthy and cancer cell lines tested. Therefore, 2% DMSO was chosen as the vehicle to dissolve the dry methanol extracts and as the negative control, while 20% DMSO was selected as the positive control for the present study.

3.2.3 Cytotoxic activity of the tested Bangladeshi medicinal plants

Many Bangladeshi medicinal plants are traditionally known to have cytotoxic and antitumor properties, with some having a folkloric reputation of being used in the treatment of different types of cancer (Ghani 2003; Uddin Shaikh, Grice et al. 2011). This study reports on the investigation into the cytotoxic activity of 19 Bangladeshi medicinal plant species, comprised of 23 extracts which have not been investigated for such activity previously. Cytotoxic activities of the tested extracts are summarized in Table 3.2.

Table 3.2 Cytotoxic activity (IC_{50}) of selected Bangladeshi medicinal plant extracts

Plant species	Plant (part)	Cytotoxic activity (IC_{50})* (mg/mL)					
		VERO	**NIH3T3**	**AGS**	**HT-29**	**MCF-7**	**MDA-MB-231**
Aegiceras corniculatum	Fruit	**0.150**	**0.097**	**0.0005**	**0.998**	**0.091**	**0.461**
Argyreia nervosa	Leaf	>2.5	>2.5	2.20	>2.5	>2.5	>2.5
Avicennia alba	Leaf	>2.5	>2.5	>2.5	>2.5	1.17	1.34
Caesalpinia pulcherrima	Leaf	>2.5	>2.5	>2.5	>2.5	2.40	1.15
Clerodendrum viscosum	Leaf	>2.5	2.03	2.23	**0.88**	**0.05**	1.69
Clitoria terantea	Flower	>2.5	>2.5	>2.5	>2.5	1.14	**0.11**
Clitoria terantea	Leaf	>2.5	>2.5	>2.5	>2.5	1.70	**0.49**
Dillenia indica	Leaf	>2.5	>2.5	1.18	>2.5	**0.34**	**0.54**
Diospyros peregrine	Leaf	>2.5	>2.5	1.58	>2.5	**0.007**	**0.33**
Dipterocarpus turbinatus	Bark	>2.5	>2.5	1.81	>2.5	1.68	**0.27**
Dipterocarpus turbinatus	Leaf	>2.5	>2.5	1.50	1.50	>2.5	**0.008**
Ecbolium viride	Leaf	>2.5	>2.5	>2.5	>2.5	**0.06**	1.40
Glinus oppositifolius	Whole Plant	>2.5	>2.5	>2.5	>2.5	**0.15**	1.30
Glycosmis pentaphylla	Leaf	>2.5	>2.5	1.48	>2.5	>2.5	1.10
Gnaphalium luteoalbum	Leaf	>2.5	>2.5	**0.98**	>2.5	**0.34**	>2.5
Hymenodictyon excelsum	Bark	**0.23**	**0.07**	**0.09**	**0.16**	**0.08**	**0.44**
Hymenodictyon excelsum	Wood	>2.5	**0.18**	**0.58**	1.12	**0.72**	1.94
Jasminum sambac	Leaf	>2.5	>2.5	1.25	>2.5	**0.007**	2.5
Lannea coromendelica	Bark	>2.5	>2.5	**0.09**	>2.5	**0.27**	**0.16**
Lannea coromendelica	Leaf	>2.5	>2.5	**0.67**	**0.52**	1.61	**0.71**
Mussaenda glabrata	Leaf	>2.5	>2.5	1.15	>2.5	1.33	0.15
Myrica nagi	Leaf	1.77	>2.5	**0.02**	>2.5	1.72	>2.5

Significant activity is indicated by boldened data

3.2.3.1 High non-selective cytotoxicity

Among the 23 Bangladeshi medicinal plant extracts tested, *A. corniculatum* (fruit) and *H. excelsum* (bark) showed high cytotoxicity across all cell lines tested with IC_{50} values ranging from 0.0005 to 0.9980 and 0.08 to 0.44 mg/mL, respectively. Notably, high cytotoxicity (IC_{50} = 0.5 µg/mL) against AGS was shown by *A. corniculatum* fruit which is 2 times higher than the cytotoxicity (IC_{50} = 1.0 µg/mL) exhibited by the positive control, cycloheximide. Previously our research group reported on the cytotoxicity of *A. corniculatum* bark extract against NIH3T3, HT29, AGS and MDA-MB-435S cell lines (Uddin Shaikh, Grice et al. 2011), but no previous reports were found on the cytotoxicity evaluation of the fruits of this plant. Cytotoxic activity study of *A. corniculatum* bark (Uddin Shaikh, Grice et al. 2011) showed potent cytotoxicity against NIH3T3, HT-29, MDA-MB-435S (IC_{50} values of 0.02, 0.33, 0.66 mg/mL, respectively) but no cytotoxicity was exhibited against AGS cancer cells. In contrast, in our study, highly significant cytotoxicity was shown by *A. corniculatum* methanolic fruit extract against AGS cancer cells (IC_{50} = 0.5 µg/mL) and this extract also showed significant cytotoxic potential against MCF-7, MDA-MB-231, NIH3T3, VERO, and HT-29 cells (IC_{50} values of 0.091, 0.461, 0.097, 0.150, and 0.998 mg/mL, respectively).

Pentacyclic triterpenes such as maslinic acid, oleanolic acid and lupeol have been isolated from *A. corniculatum* bark previously (Wang, Dong et al. 2006; Xu and Long 2009; Ponnapalli 2012). Maslinic acid and lupeol are known to exert anticancer activity without effecting non-neoplastic cell lines through inhibition of NF-$_K$B activity which plays an anti-apoptotic role in the resistance to chemotherapy (Lee, Poon et al. 2007; Li, Yang et al. 2010). Another report reports on the apoptosis inducing effect of oleanolic acid on colon cancer cells (HT-29) (Juan, Planas et al. 2008). In addition, resveratrol, a natural phytoalexin which has been isolated from this plant

(Wang, Dong et al. 2006) as well as being found in grapes affects the cell cycle of cancer cells through inhibition of protein kinase C and D activities (Androutsopoulos, Ruparelia et al. 2011). It also induces apoptosis and decreases the activity of the transcription factors NF-$_{\kappa}$B and AP-1 (Androutsopoulos, Ruparelia et al. 2011). Some of these compounds could be responsible for the significant cytotoxic activity displayed by *A. corniculatum* fruit extract, however it is still unknown if and to what extent they are also present in the fruits of this plant.

Coumarin and its glycosides, such as scopoletin, aesculin, hymexelsin have previously been identified and isolated from the bark of *H. excelsum* (Gibson and Simonsen 1918; Rao, Asheervadam et al. 1988). A variety of coumarins have profound anticancer and antiproliferative potential, inhibiting the proliferation of some human malignant cell lines *in vitro* and also affecting the activity of several tumor cells lines such as human oral cancer cells, human squamous cell carcinoma and human colon cancer cells *in vivo* (Kawase, Sakagami et al. 2005; Yang, Protiva et al. 2005; Ishihara, Yokote et al. 2006; Ruiz-Marcial, Reyes Chilpa et al. 2007). A clinical study also proved their significant anticancer activity against prostate, malignant melanoma and metastatic renal cell carcinoma (Ruiz-Marcial, Reyes Chilpa et al. 2007). Thus, the high cytotoxicity of methanolic bark extract of *H. excelsum* could be due to the presence of coumarins. Furthermore, *H. excelsum* wood extract displayed high non-selective anti-proliferative activity (IC$_{50}$ <1.0 mg/mL) against NIH3T3, AGS, and MCF-7 cancer cells in our study. At this stage, however, it is not clear which constituents are present in the wood and responsible for the detected activity.

3.2.3.2 High selective cytotoxicity

High selective cytotoxicity with an $IC_{50} < 1.0$ mg/mL against at least one of the cancer cell lines tested was demonstrated by 11 plants, comprising 14 methanol extracts namely, *C. terantea* (flower and leaf extracts), *D. indica* (leaf), *D. peregrina* (leaf), *D. turbinatus* (bark and leaf), *E. viride* (leaf), *G. luteoalbum* (leaf), *G. oppositifolius* (whole plant), *J. sambac* (leaf), *L. coromandelica* (bark and leaf), *M. glabrata* (leaf) and *S. asoca* (leaf) in this study. It is worth mentioning that seven of these plants with high selective cytotoxicity namely, *A. alba*, *C. pulcherrima*, *D. peregrina*, *E. viride*, *J. sambac*, *C. ternatea* and *S. asoca* have traditionally been used to treat cancer (Bandaranayake 2002; Ghani 2003; Devmurari, Shivanand et al. 2009).

High selective-cytotoxicity against breast cancer cells

Interestingly, selective-cytotoxicity only against both of the breast cancer cells (MCF-7 and MDA-MB-231) was displayed with *A. alba* (leaf), *C. terantea* (flower and leaf), *C. pulcherrima* (leaf), *E. viride* (leaf) and *G. oppositifolius* (whole plant). *C. terantea* flower and leaf extracts exhibited high cytotoxic activity with an $IC_{50} < 1.0$ mg/mL against MDA-MB-231, whereas *E. viride* leaf and *G. oppositifolius* whole plant extracts showed high activity against MCF-7 cancer cells with an IC_{50} values of 0.06 and 0.15 mg/mL, respectively. It is also notable that the cytotoxicity of *E. viride* leaf extract was similar to that of the positive control, cycloheximide against the MCF-7 cancer cells (IC_{50} values of 0.060 and 0.061 mg/mL, respectively). To date, no reports exist on cytotoxicity studies for *A. alba*, *C. terantea*, *E. viride* and *G. oppositifolius* extracts against MCF-7 and MDA-MB-231 cell lines. Although interestingly the cytotoxic activity of aerial parts (methanolic) and wood (methanolic and water) extracts of *C. pulcherrima* (Pawar, Mutha et al. 2009; Chanda and Baravalia 2011) as well as anticancer activity of isolated

compounds from this plant (diterpenoid, 12-demethyl neocaesalpin F) against two cancer lines, HL-60 and HeLa have been reported (Das, Srinivas et al. 2010).

It is also worth mentioning that *G. luteoalbum* and *J. sambac* methanolic leaf extracts showed selectivity only to estrogen-dependent breast cancer cells, MCF-7 with IC_{50} values of 0.340 and 0.007 mg/mL, respectively. On the other hand, *D. turbinatus* and *S. asoca* methanolic leaf extracts exhibited potent cytotoxicity only against estrogen non-dependent breast cancer cells, MDA-MB-231 with IC_{50} values of 0.008 and 0.400 mg/mL, respectively. Noticeably, the cytotoxicity exhibited by *D. peregrina* and *J. sambac* leaf extracts (IC_{50} values of 0.007 mg/mL for both) against MCF-7 cells was about 9 times higher than the cytotoxicity displayed by cycloheximide (IC_{50} = 0.061 mg/mL). Interestingly, it is reported that *J. sambac* leaves have been traditionally used to treat breast cancer (Ghani 2003), while the flowers have reported anti-leukemic activity against K562, P3HR1, Raji, and U937 leukemia cells (Chiang, Cheng et al. 2004).

Reports also state *in vitro* antiproliferative activity of *S. asoca* crude extract towards human tumor cell lines, including human erythromyeloid K562, B-lymphoid Raji, T-lymphoid Jurkat, and erythroleukemic HEL leukemia. *In vivo* chemopreventive properties of *S. asoca* flower flavonoids on second-stage skin carcinogenesis are also reported (Dabur, Gupta et al. 2007; Cibin, Devi et al. 2012). Neither cytotoxic, nor anticancer activity studies of *D. turbinatus* and *G. luteoalbum* have been reported to date.

Cytoxicity against gastric cancer cells

Different plant parts of *M. nagi*, *M. glabrata*, *J. sambac*, *D. peregrina*, *A. nervosa*, *S. asoca*, *L. coromendelica*, and *D. turbinatus* have been used traditionally as ulcer healing agents (Bandaranayake 1998; Ghani 2003). Interestingly, we found high cytotoxic activity against

gastric cancer cells (AGS) for *M. nagi* methanolic leaf extract (IC_{50} = 0.020 mg/mL) and for *L. coromandelica* bark and leaf methanolic extracts (IC_{50} values of 0.090 and 0.067 mg/mL, respectively). Moreover, cytotoxicity at a lower extent against AGS was found with *M. glabrata* leaf, *J. sambac* leaf, *D. peregrina* leaf, *D. turbinatus* bark and leaf, *A. nervosa* leaf, and *S. asoca* leaf with IC_{50} values ranging from 1.15 to 2.22 mg/mL, respectively. Importantly, cytotoxic activity screening against gastric cancer cells of these plants had not been reported previously.

Cytotoxicity and selectivity to different parts of the same plant species

Significant differences in the cytotoxicity as well as in selectivity against MCF-7, VERO, and HT-29 cancer cells were exhibited with the bark and leaf extracts of *D. turbinatus,* the bark and wood extracts of *H. excelsum*, and the bark and leaf extracts of *L. coromandelica*. On the other hand, no significant difference in cytotoxic activity and selectivity was observed with fruits and leaf extracts of *C. terantea.* Furthermore, *H. excelsum* wood exhibited no cytotoxicity ($IC_{50} > 2.5$ mg/mL) to VERO, but exhibited high cytotoxicity with an IC_{50} of 0.18 mg/mL against NIH3T3 healthy cell lines. Thus, different parts of the same plant species contribute to differing magnitudes of selective cytotoxicity to the different cancer and healthy cell lines. This is no doubt due to the presence of different constituents and or concentrations of these.

3.2.3.3 Very low cytotoxicity

Of note, *A. nervosa* leaf extract showed very low cytotoxicity ($IC_{50} > 2.0$ mg/mL) against all cell lines tested in this study.

Phytoconstituents such as polyphenols, flavonoids and catechins have long been recognised as having potential anticancer, anti-inflammatory, antioxidant, and antimicrobial properties (Ren, Qiao et al. 2003; Park, Lee et al. 2004; Gonzalez-Mejia, Voss et al. 2010). Previous research reports exist on the isolation of flavonoids from different parts of *A. nervosa*, *A. corniculatum*, *C.*

pulcherrima, *C. viscosum*, *D. indica*, *D. peregrina*, *G. luteoalbum*, *G. oppositifolius*, *J. sambac*, *L. coromendalica*, and *M. nagi* (Pavanasasivan and Sultanbawa 1975; Singh, Singh et al. 1982; Roy, Pathak et al. 1994; Morimoto, Kumeda et al. 2000; Srinivas, Koteswara Rao et al. 2003; Liu, Ni et al. 2004; Wang, Dong et al. 2006; Das, Thirupathi et al. 2009; Prakash, Gautam et al. 2011; Yang, Tang et al. 2011; Sahu, Dewanjee et al. 2012; Yun, Shu et al. 2012). It is likely that these constituents are also associated with the anticancer activity observed in this study (Pawar, Mutha et al. 2009; Uddin Shaikh, Grice et al. 2011).

The 9 plant species showing significant cytotoxic activity in this study have all been used traditionally as antitumor/anticancer agents. Seven of these plants (*A. alba*, *C. pulcherrima*, *D. peregrina*, *E. viride*, *J. sambac*, *C. terantea* and *S. asoca*) showed high selective-cytotoxic properties against at least one of the tested cancer cells, but not against the two healthy cell lines. The remaining 2 species (*A. corniculatum* fruit and *H. excelsum* bark) showed the highest, but non-selective cytotoxicity overall. These results lend support for the traditional use of the *'active'* plants as anticancer agents.

However, the Bangladeshi plant *Blumea lacera,* which is also used in traditional medicine and was previously screened in our lab (Uddin Shaikh, Grice et al. 2011), showed higher cytotoxic activity, thus, it was given priority and was selected for bioactive compound isolation and characterisation.

CHAPTER FOUR

ISOLATION AND STRUCTURAL ELUCIDATION OF CYTOTOXIC COMPOUNDS FROM BANGLADESHI MEDICINAL PLANTS

4.1 Isolation of compounds from *Blumea lacera*

4.1.1 Background

Blumea lacera was selected for compound isolation as this plant showed significantly higher cytotoxic potential ((Uddin Shaikh, Grice et al. 2011) than our other screened Bangladeshi plants. The basic steps for separation of phytoconstituents involve authentication and extraction of plant materials, pharmacological screening of extracts, isolation and purification of the constituents of interest, characterization of the isolated compounds, biological, toxicological and clinical evaluation of the pure compounds (Sasidharan, Chen et al. 2011; Ankit Gupta 2012).

Extraction of plant material is the crucial first step in the pathway of preliminary separation and purification of constituents, with the nature of constituents extracted depending upon the choice of solvents used in the extractions. For example, polar solvents will extract out polar constituents, whereas non-polar solvents will extract out non-polar constituents (Sasidharan, Chen et al. 2011; Ankit Gupta 2012).

Crude extract contains a number of compounds as a mixture and thus, demands further separation into different groups of compounds. Solid phase extraction (SPE) is one of the techniques to serve this purpose.

SPE is a widely used sample purification technique that provides advantages over liquid-liquid separation which is labour intensive, difficult to automate, prone to problem such as emulsion formation, as well as requiring relatively large volumes of often expensive solvents. In addition, SPE is cost effective, time efficient with low solvent consumption, providing simpler processing procedures and ensures good separation of phytoconstituents present in crude extracts (Poole 2003). SPE is used to fractionate the crude extract into groups of compounds by eluting the extract with different solvents for chromatographic separations (Shaikh 2010).

As the crude SPE fractions usually occur as a combination of compounds or phytoconstituents with differing polarities, they still require further separation and purification by chromatographic methods such as TLC, column chromatography or HPLC (Shaikh 2010).

HPLC is considered a versatile, powerful, robust and widely used technique for analysis, isolation, purification and quantification of bioactive constituents from natural sources. Largely, this is due to the resolving power of HPLC which is ideally suited to separating minimal quantitities of compounds present in the extracts. HPLC coupled with a diode array detector (DAD) is often used for the acquisition of UV spectra of eluting peaks which aids in the identification plus characterisation of constituents (Sasidharan, Chen et al. 2011). The extent or degree of separation of constituents is mostly determined by the choice of stationary phase and mobile phase (Sasidharan, Chen et al. 2011). Basically, the stationary phase facilitates separation of compounds through phenomena such as adsorption, partition, ion-exchange, or size exclusion (Shaikh 2010). Polarity difference between the stationary and mobile phase facilitates the separation of compounds from the extracts/SPE fractions according to their polarity differences.

4.1.2 Results and discussion

In the present study, a methanolic extract of *Blumea lacera* was selected for the isolation of novel cytotoxic/anticancer constituents based on the cytotoxic activity detected in a previous screening of Bangladeshi medicinal plant extracts (Uddin Shaikh, Grice et al. 2011). Initially, a phytochemical profile of the crude extract was obtained using analytical RP-HPLC and the extract was screened for cytotoxic activity.

The crude extract was further fractionated using C-18 SPE cartridges utilizing differing gradients of water/methanol. The SPE sub-fractions were tested for their cytotoxic potential and among the 6 SPE sub-fractions; SPE4 and SPE5 exhibited the highest cytotoxic activity and thus were

selected for isolation and purification for bioactive constituents using reversed-phase HPLC (Figure 4.1). The % of yield was taken into consideration for compound isolation and purification.
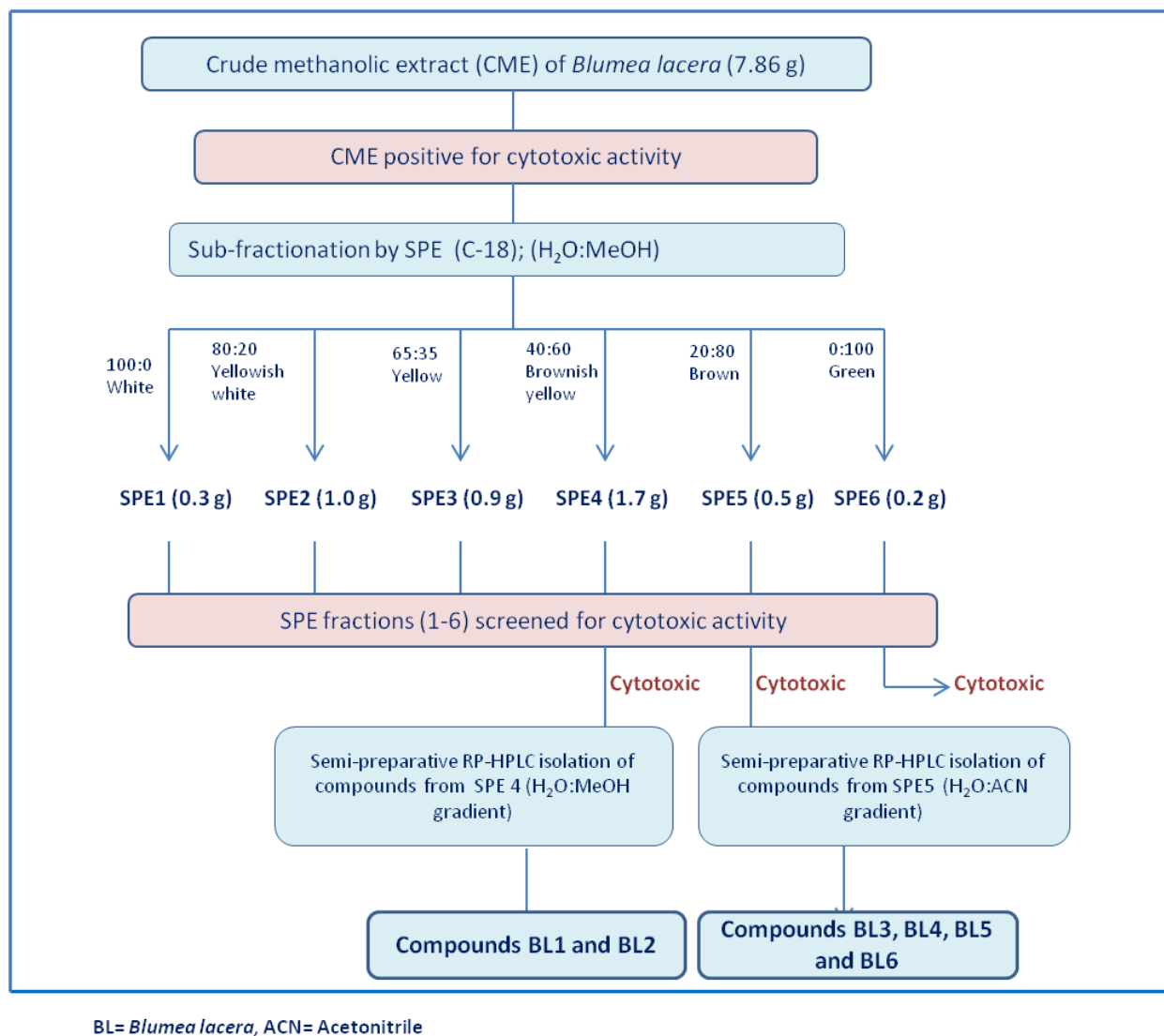
Figure 4.1 Overview on isolation of compounds from the crude methanolic extract of *Blumea lacera*

4.1.2.1. *Blumera lacera* crude methanolic extract (CME) profile analysis

A crude methanol extract (CME) of *Blumea lacera* (7.86 g afforded from 51.0 g of powdered plant materials of leaf) was obtained from a previous PhD student of our laboratory (Uddin

Shaikh, Grice et al. 2011). It was analyzed by reversed-phase analytical HPLC. The HPLC profile of CME (Figure 4.2) also indicated that a large number of compounds were of higher polarity (eluted at <3 min with 14% methanol), whereas another group of compounds were more lipophilic in nature (eluted at 55 min with approx. 87% methanol). However, compounds eluting between 3 min to 22 min needed further separation. Thus, the extract was subjected to fractionation using SPE column.
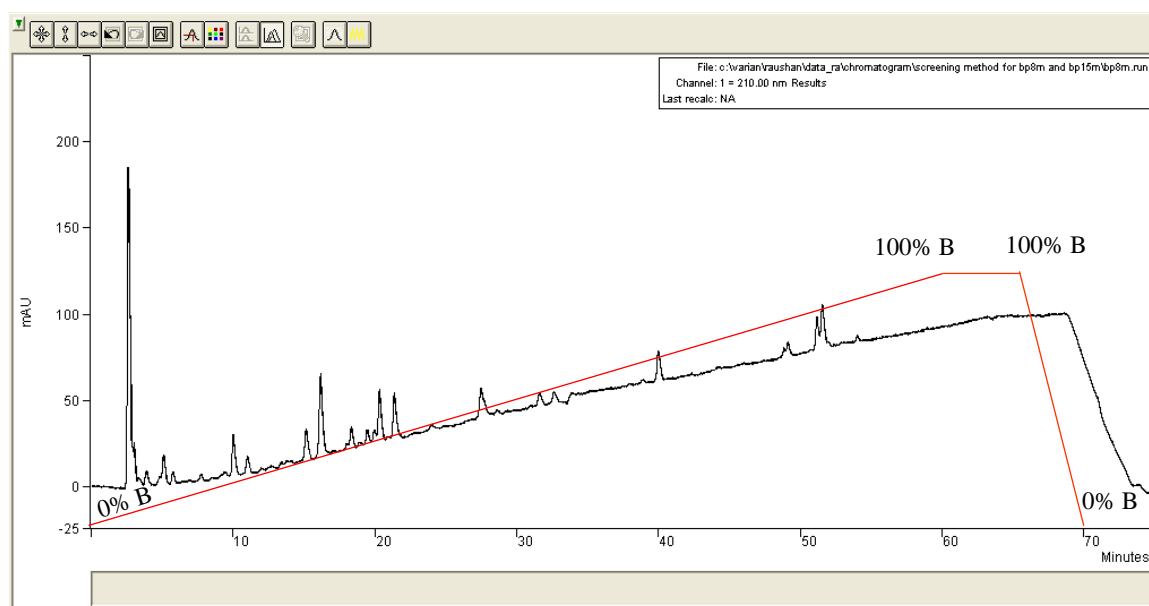
Figure 4.2 Analytical HPLC chromatogram of *Blumea lacera* CME at 210 nm

4.1.2.2 Solid phase extraction (SPE) of *Blumea lacera* CME

The extract was further fractionated using SPE column (Alltech, 60 mL, 10 g High Capacity, C-18) and eluted with a water/methanol stepwise gradient affording 6 SPE fractions, namely SPE1, SPE2, SPE3, SPE4, SPE5 and SPE6 (Table 4.1).

Table 4.1 The % yield of SPE fractions obtained from *Blumea lacera* CME

Powdered materials (g)	Extraction/Fractionation	Fraction/Sub-fraction (Water: Methanol)	Mass (g)
51.0	Maceration (100% MeOH)	Crude Methanol Extract (CME)	7.86
	SPE C-18 column (H$_2$O: MeOH)	SPE 1(100:0)	0.3
		SPE2 (80:20)	1.0
		SPE3 (65:35)	0.9
		SPE 4 (40:60)	1.7
		SPE5 (20:80)	0.5
		SPE6 (0:100)	0.2

The % yield of SPE fractions (Table 4.1) demonstrated the separation of different groups of compounds with different polarities. Of the 6 fractions, SPE4 (60% MeOH) afforded the highest yield (1.7 g) and SPE6 had the lowest yield (0.2 g). SPE1 (100% water) would comprise of highly polar constituents, whereas SPE6 (100% methanol) would contain constituents of reduced polarity.

Six SPE fractions were analyzed by analytical reversed-phase C-18 Varian HPLC column. For CME and SPE fractions, the absorbance was monitored between 190 to 400 nm at a flow rate of 1 mL/min using a linear gradient of 10 to 90% methanol for 60 min (Table 4.2). The CME (10 µL, 10 mg/mL) and each SPE fraction (20 µL, 1 mg/mL) were injected and analyzed.

Table 4.2 A general analytical HPLC screening method

Time (min)	% of solvent A	% of solvent B
0	100	0
60	0	100
65	0	100
70	100	0

Solvent A = 90% Water + 10% MeOH + 0.05% TFA; Solvent B = 90% MeOH + 10% Water + 0.05% TFA
Flow rate: 1mL/min, λ: 190-400 nm
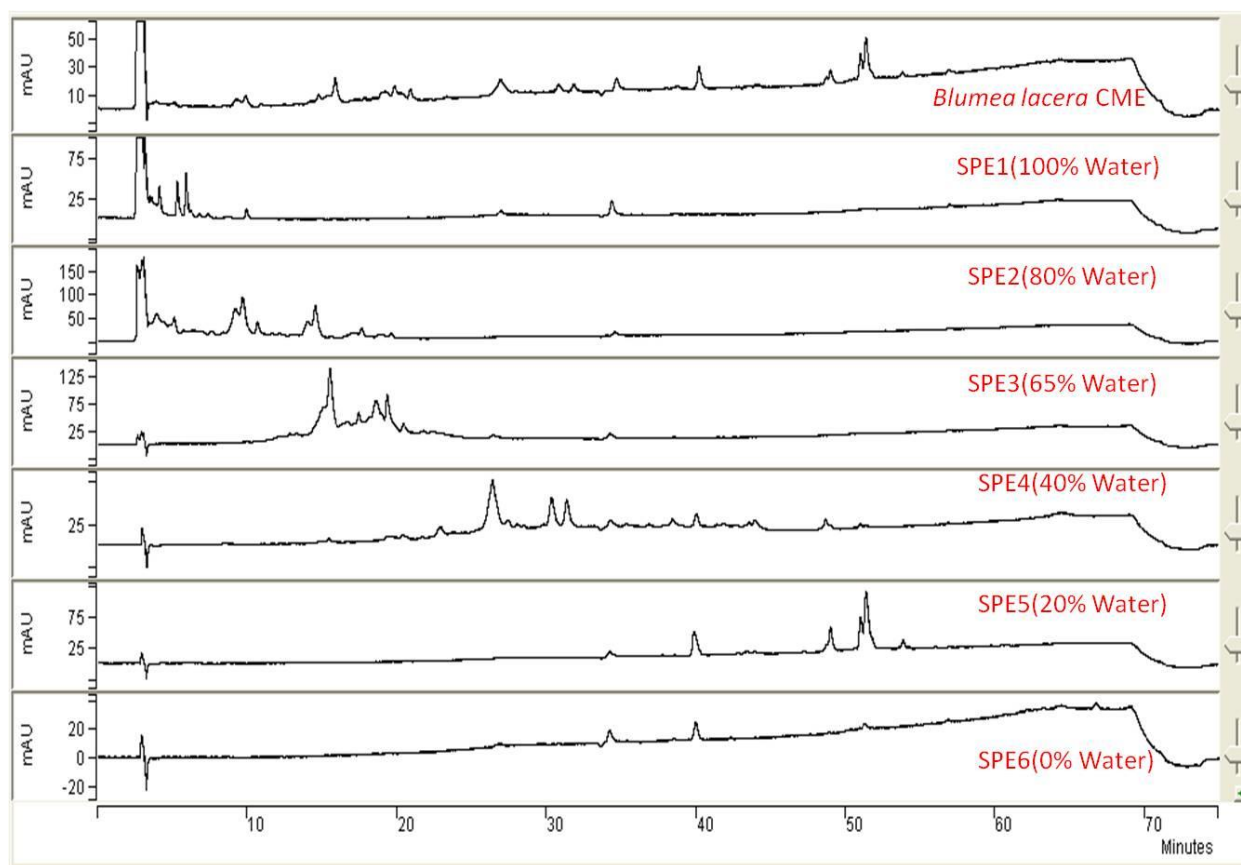
Figure 4.3 Analytical chromatograms of *Blumea lacera* CME (leaf) and SPE fractions at 210 nm

The analytical HPLC chromatograms (Figure 4.3) illustrated reasonable separation of the constituents of the CME of *Blumea lacera* according to their solubility/polarity. The majority of the compounds eluted in SPE 2 to SPE 5 within 55 min (84% MeOH), with the highest number of compounds detectable present in the SPE4 fraction. Compounds eluting in SPE2 and SPE3

fractions were of higher polarity than those eluting in SPE4 and SPE5. Moreover, few compounds eluted in SPE1.

4.1.2.3 Cytotoxic activity studies of *Blumea lacera* SPE fractions

Six SPE fractions (SPE1-100% water, SPE2-80% water, SPE3-65% water, SPE4-40% water, SPE5-20% water and SPE6-0% water) were obtained from the crude methanol extract of *Blumea lacera*.

Each of the SPE fractions were screened for cytotoxic activity using the MTT assay (Chapter two) against 3 different cancer cell lines: AGS, HT-29, MDA-MB-231 and a healthy mouse fibroblast: NIH3T3. Table 4.3 shows the results of the MTT cytotoxic activity assay and Table 4.4 represents the IC_{50} values (fraction concentration at which 50% cell growth is inhibited). From the obtained results (Table 4.3), it is evident that the cytotoxic activity of the SPE fractions is concentration dependent. Fractions SPE4, SPE5 and SPE6 showed very high cytotoxic activity ($\geq$89% cell growth inhibition) at the highest concentration tested (250 µg/mL) against all cell lines tested. These fractions also exhibited remarkable cytotoxicity (>70% cell growth inhibition) at 50 µg/mL against all cell lines except SP4 fraction which showed 57.3% cell growth inhibition at 50 µg/mL concentration against HT-29 cells. Again, at the lowest concentration (2 µg/mL), these 3 fractions showed considerable cytotoxic activity (13.3 to 71.1% cell growth inhibition) against all cell lines. The SPE3 fraction showed very low to moderate selective cytotoxicity of 5.8 to 58.3% against HT-29 and MDA-MB-231 for the concentrations tested. The SPE2 fraction selectively inhibited (87.3% cell growth inhibition at 250 µg/mL) the growth of MDA-MB-231 cells. In contrast, SPE1 showed no cytotoxicity to the cancer cell lines or to healthy mouse fibroblast.

Table 4.3 Cytotoxic activity of SPE fractions obtained from *Blumea lacera*

Cell line	Cytotoxic activity (%)															
	NIH3T3				AGS				HT-29				MDA-MB-231			
Fraction concentration (mg/mL)*	0.25	0.05	0.01	0.002	0.25	0.05	0.01	0.002	0.25	0.05	0.01	0.002	0.25	0.05	0.01	0.002
Fraction code																
SPE1	4.5±0.4	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	4.2±0.4	0±0	0±0
SPE2	11.2±0.3	10.3±0.7	3.8±0.2	0±0	0±0	0±0	8.3±1.2	9.4±1.4	25.3±6.7	19.0±0.5	4.3±0.3	21.3±1.5	87.3±4.3	60.5±5.7	19.3±2.0	16.2±0.5
SPE3	13.6±1.3	12.0±1.3	10.3±0.6	5.8±1.1	0±0	0±0	0±0	2.2±0.3	16.8±0.5	12.0±1.3	10.3±0.6	5.8±1.1	58.3±1.1	45.3±6.1	6.4±0.6	18.8±1.6
SPE4	93.1±3.4	92.5±3.9	82.6±13.1	15.5±1.2	94.2±5.7	91.6±16.8	71.3±6.2	45.7±3.4	89.5±22.1	57.3±17.1	16.9±3.5	31.7±4.0	89.7±3.9	87.4±8.4	40.0±17.2	28.4±3.5
SPE5	93.5±11.5	92.0±23.3	83.4±7.1	52.1±3.1	95.0±1.0	94.6±3.7	84.3±14.9	71.1±9.3	93.5±3.0	85.9±9.8	58.0±6.5	54.2±7.6	91.1±2.6	90.6±1.2	66.5±11.8	42.9±8.2
SPE6	93.5±5.4	93.6±1.5	74.4±21.1	30.9±12.6	94.4±2.4	92.6±5.1	68.2±8.3	44.4±0.4	93.6±3.8	84.6±28.2	46.9±6.9	36.7±5.4	90.2±1.3	89.6±4.4	66.9±6.3	23.3±5.3

SPE=Solid phase extraction　　　　* Results are mean ± standard deviation (SD), each experiment performed in triplicate

Table 4.4 Cytotoxic activity (IC_{50}) of SPE fractions obtained from *Blumea lacera*

_____

| Fraction | Cytotoxic activity (IC_{50})* (mg/mL) | | | |
| | Cell line | | | |
	NIH3T3	AGS	HT-29	MDA-MB-231
SPE 1	NA**	NA**	NA**	NA**
SPE 2	>0.25	NA**	>0.25	0.03
SPE 3	NA**	NA**	>0.25	0.136
SPE 4	0.013	0.0024	0.039	0.0094
SPE 5	0.0041	0.0002	0.0022	0.0027
SPE 6	0.018	0.0027	0.0064	0.0061

*IC_{50} (Inhibition of cell growth 50%) calculated by Probit analysis (LdP Line software, USA), each experiment performed in triplicate. NA** = No Activity shown at concentration up to 0.25 mg/mL

Among the 6 SPE sub-fractions, SPE4 and SPE5 exhibited the highest cytotoxic activity and thus were selected for isolation and purification for bioactive constituents using reversed-phase HPLC. The % of yield was taken into consideration for compound isolation and purification.

4.1.2.4 HPLC purification of *Blumea lacera* SPE fractions

4.1.2.4.1 Isolation of compounds from SPE4

The phytochemical profile of SPE4 was obtained using the general analytical HPLC method (Table 4.2). The analytical HPLC chromatogram (Figure 4.4) indicated that the majority of compounds detected eluted between 20 min (37% MeOH) and 53 min (82% MeOH) and were of 'medium' to 'low' polarity.
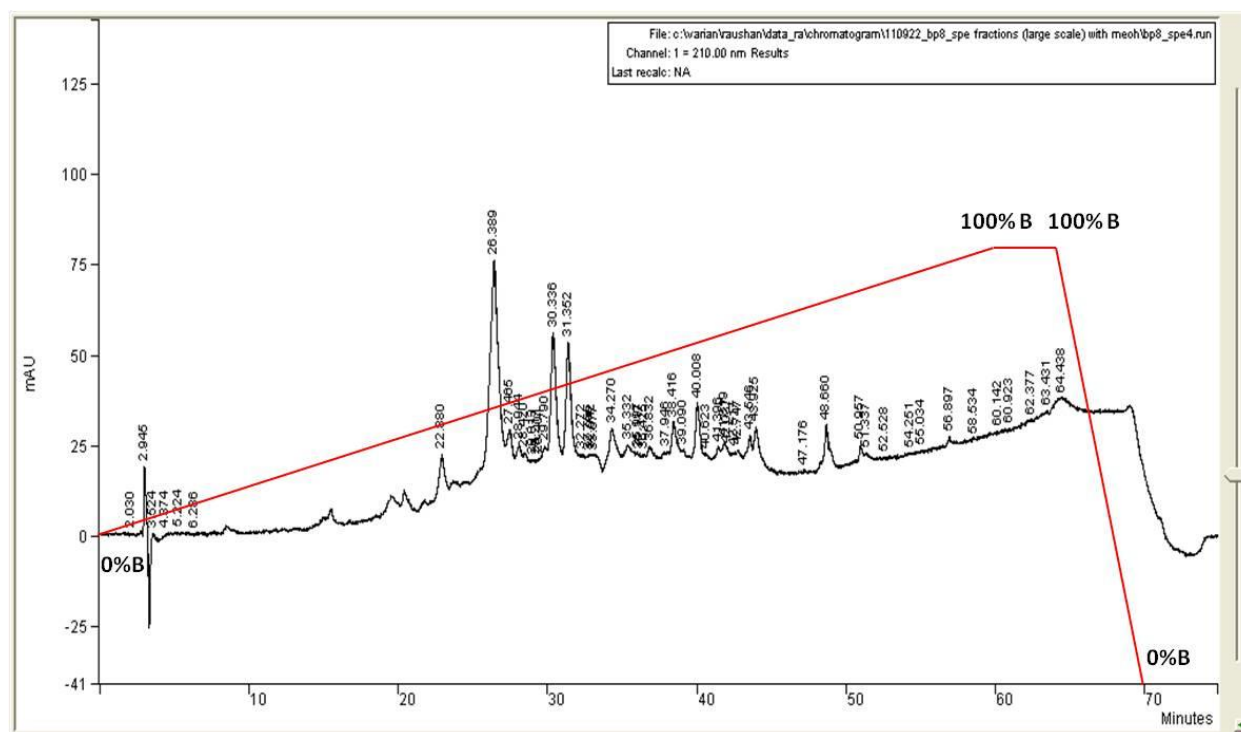
Figure 4.4 Analytical HPLC chromatogram of *Blumea lacera* SPE4 fraction at 210 nm

A reasonable separation of compounds was obtained with the general analytical HPLC method (Figure 4.4) however, it was determined that further refinement of the HPLC method was required. Considering the major peaks present in SPE4, analytical HPLC using differing gradients of MeOH and H_2O was carried out (Figure 4.5) which served for the method development for semi-preparative HPLC to obtain a better separation of compounds. Method

(Figure 4.5) produced the best separation of the peaks present in SPE4 and thus used for semi-preparative purification of the constituents.
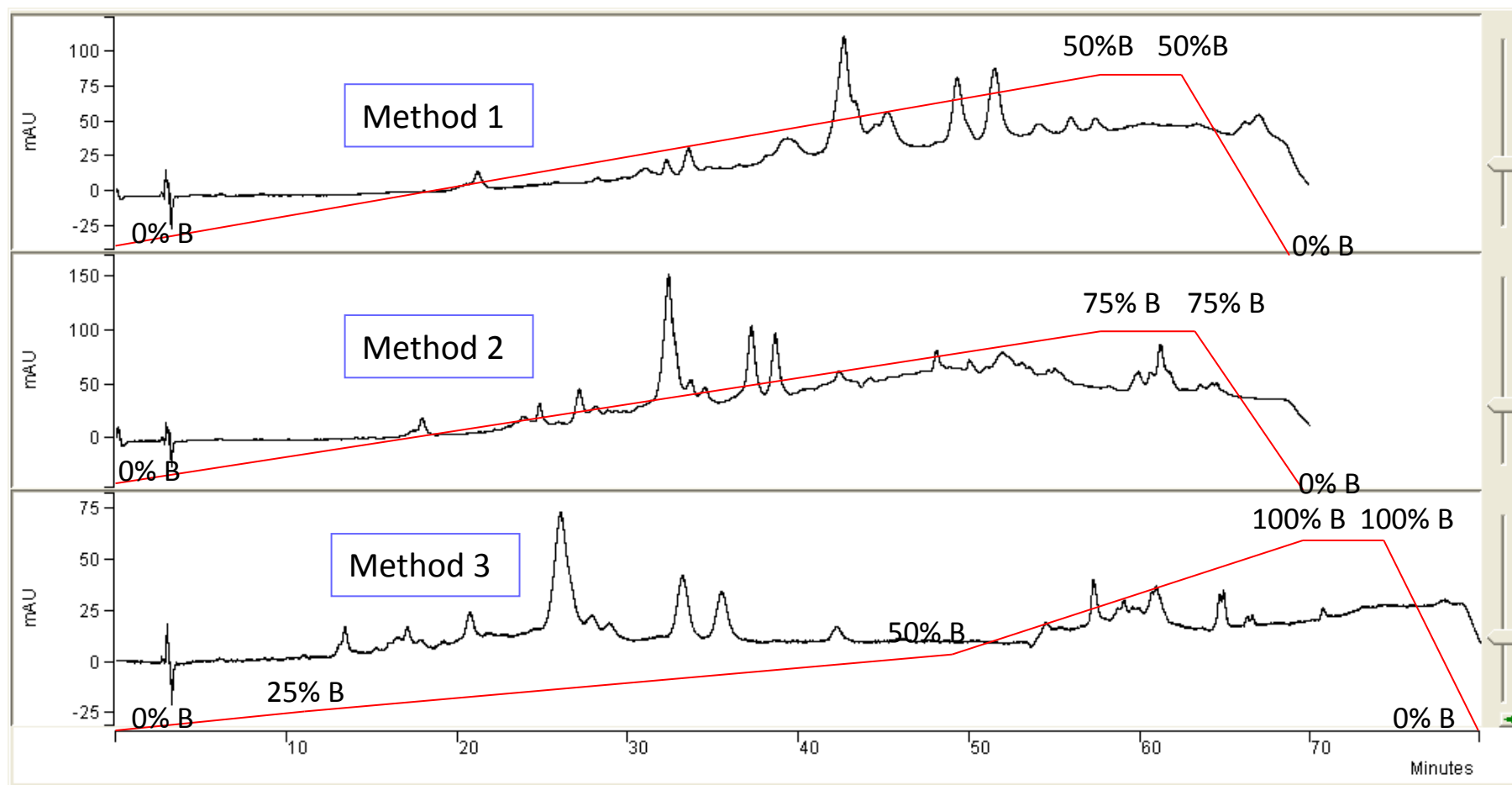
Figure 4.5 Analytical HPLC chromatograms of *Blumea lacera* SPE4 fraction at 210 nm obtained from method 1, 2 and 3

4.1.2.4.2 Semi-preparative HPLC for the purification of compounds from *Blumea lacera* SPE4

Semi-preparative HPLC isolation and purification of the SPE4 fraction obtained from CME of *Blumea lacera* leaves was conducted using a Luna 5 μm reversed-phase C-18 (150 x 21.2 mm) column. A flow rate of 13 mL/min using a gradient system of solvent A (90% water in MeOH with TFA) to solvent B (90% MeOH in water with TFA) shown below in Table 4.5. The absorbance was detected at 210 and 280 nm. The analytical HPLC method was converted to semi-preparative HPLC method by adjusting the flow rate and sample load using a conversion formula demonstrated in chapter two, section 2.8.3.2. Purity of the collected peaks from semi-preparative HPLC was confirmed by analytical HPLC analysis of each peak using the general HPLC method described in section 4.1.2.3.

Table 4.5 Semi-preparative HPLC method for the purification of SPE4 fraction

Time (min)	% of solvent A	% of solvent B
0	100	0
10	75	25
50	50	50
70	0	100
75	0	100
80	100	0

Solvent A = 90% Water + 10% MeOH + 0.05% TFA;   Solvent B = 90% MeOH + 10% Water + 0.05% TFA

Flow rate: 13 mL/min, λ: 210 and 280 nm
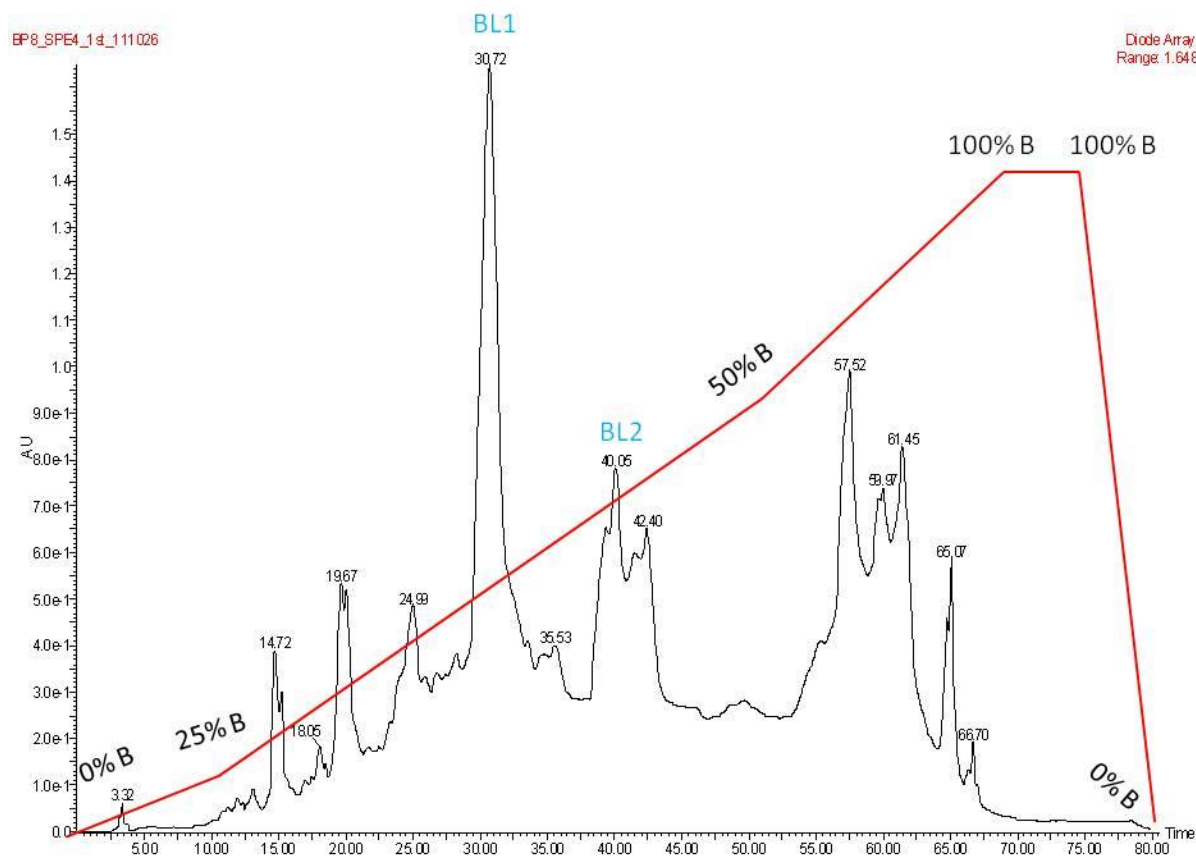
Figure 4.6 Semi-preparative HPLC chromatogram of SPE4 fraction at 210 nm

SPE4 fraction (1.5 g) was dissolved in methanol and injected (100 mg/500 μl) onto the semi-preparative column. Detection was monitored at 210 and 280 nm, with defined peaks collected according to the retention time, R_T (Figure 4.6). A total of 10 fractions were collected throughout the run. The Peak at R_T 30.72 min afforded compound **BL1** (53.3 mg) and the peak at 40.05 min produced compound, **BL2** (4.9 mg) after re-purification. Peaks at R_T 14.72 and R_T 19.67 min were evidently of a caffeoyl quinic acid derivatives type structure (indicated with ^{1}H and ^{13}C NMR spectra) but unfortunately, during re-purification these compounds degraded.

The Re-purification of the caffeoyl quinic acid derivative was done using semi-preparative HPLC. Initially, to get optimal separation of compounds (few small peaks beneath the main

peak) from the main compound (a large sharp peak) of a caffeoyl quinic acid derivative, different gradients of water: methanol were tried on analytical HPLC. Finally, caffeoyl quinic acid derivative was separated and collected on semi-preparative HPLC using the optimised method.

This fraction was then re-analysed on analytical HPLC (same conditions as initial separation) to confirm its purity. The HPLC chromatogram showed several peaks of similar height instead of a single sharp peak, indicating degradation during re-purification. The reason for the degradation is not known, but given that instability of caffeoyl quinic acid derivatives in aqueous environments has been reported (Dawidowicz and Typek 2010), it may be that this has caused the degradation also in this case.

4.1.2.4.3 Analytical HPLC method optimization of *Blumea lacera* SPE5

Based on its analytical HPLC profile (Figure 4.3), cytotoxic activity (Table 4.4) and mass (Table 4.1), SPE5 was also selected for constituent isolation. Initially, the phytochemical HPLC profile of SPE5 was obtained with a H_2O:MeOH as well as with a H_2O:ACN solvent gradient using a HPLC method (Table 4.6). The analytical HPLC profile of SPE5 (Figure 4.7) obtained with H_2O:ACN showed sharper and better resolved peaks compared to that obtained with H_2O:MeOH at 210 nm. Therefore, the compounds in the SPE5 fraction were isolated and purified using H_2O:ACN gradient as the mobile phase. The analytical HPLC chromatogram of SPE5 recorded at 210 nm (Figure 4.7) showed that all significant compounds eluted between 28 min (34.4% ACN) and 50 min (77.5% ACN) and were of 'medium to low' polarity. The major peaks required further separation though, and the general analytical HPLC method was optimized to get optimal separation of the compounds. Differing gradients of ACN and H_2O as shown in Figure 4.8 were used. The results served for the method development for semi-preparative

HPLC. Method 3 (Figure 4.8) produced optimum separation of the peaks and thus was employed for semi-preparative purification of the constituents.

Table 4.6 A general analytical HPLC screening method with H_2O:ACN

Time (min)	% of solvent A	% of solvent B
0	100	0
60	0	100
65	0	100
70	100	0

Solvent A = 90% H_2O + 10% ACN + 0.05% TFA; Solvent B = 90% ACN + 10% H_2O + 0.05% TFA
Flow rate: 1mL/min, λ: 210 and 280 nm
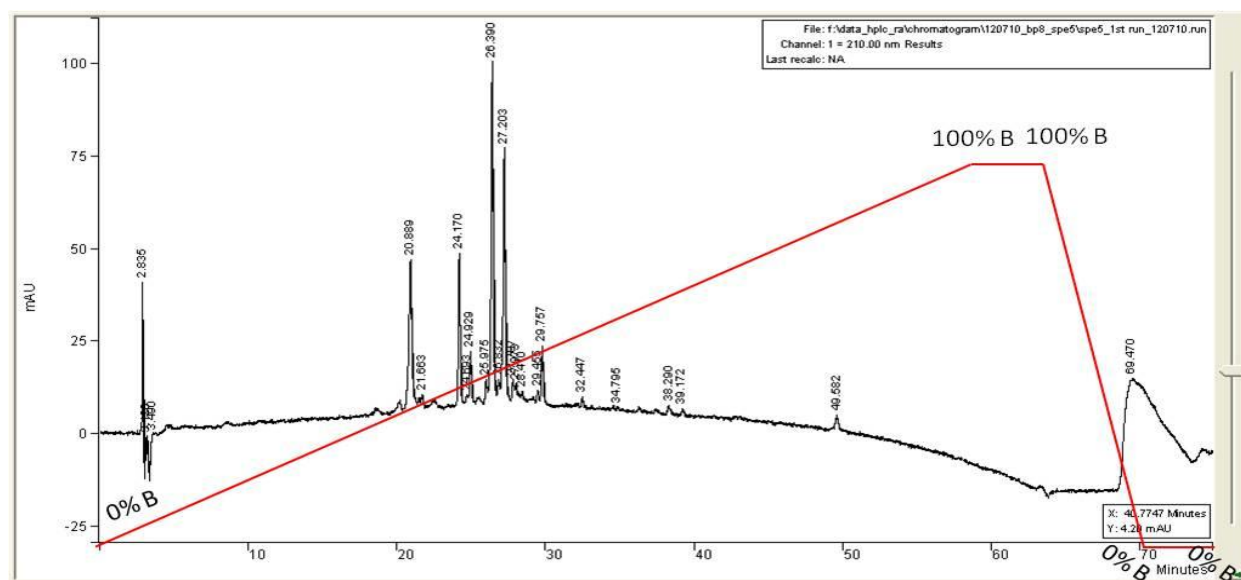
Figure 4.7 Analytical HPLC chromatogram of *Blumea lacera* SPE5 fraction at 210 nm
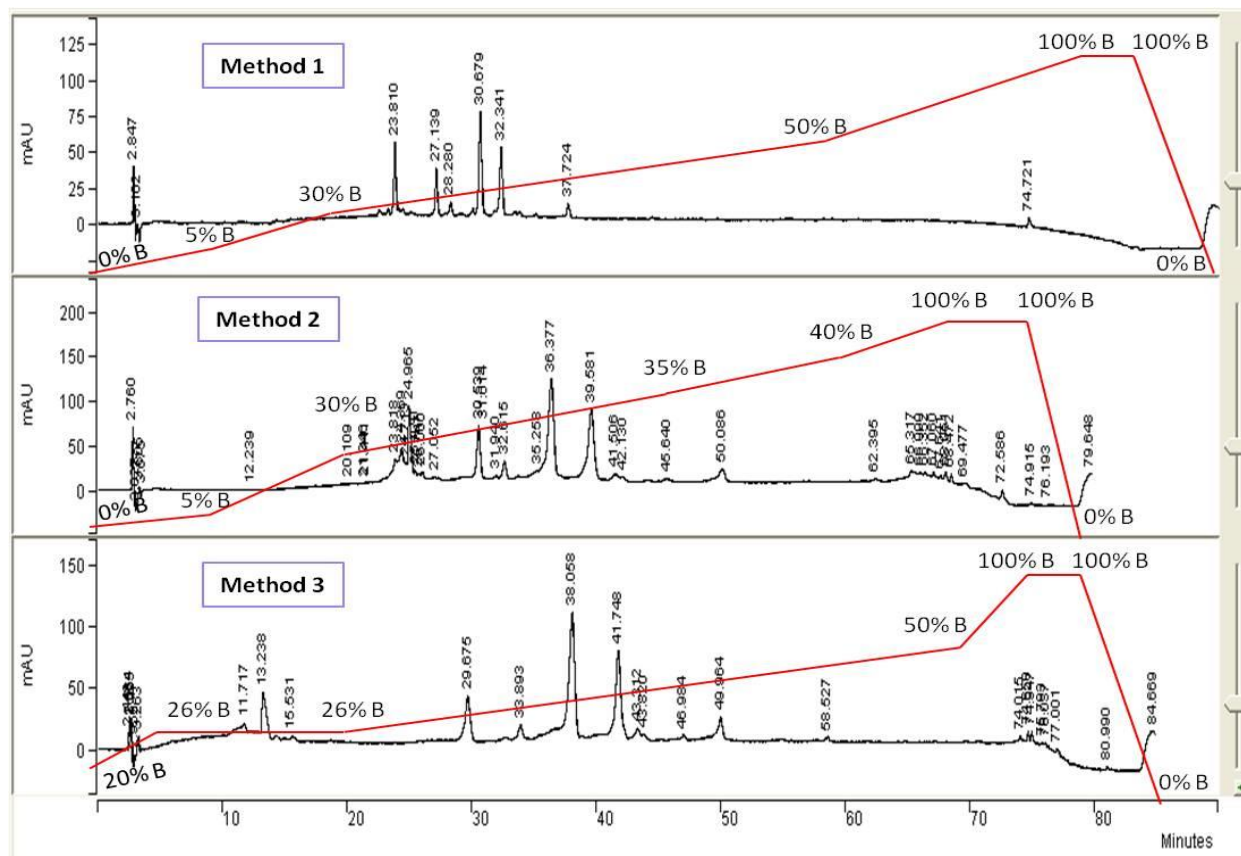
Figure 4.8 Analytical HPLC chromatograms of *Blumea lacera* SPE5 fraction at 210 nm obtained from method 1, 2 and 3

4.1.2.4.4 Semi-preparative HPLC for the purification of compounds from *Blumea lacera* SPE5

Isolation and purification of compounds from the SPE5 of crude methanol extract of *Blumea lacera* leaves was performed on a Luna 5 μm reversed-phase C-18 (150 x 21.2 mm) preparative column. The 'optimized analytical HPLC method 3' (Figure 4.8) with slight modification in run time (Table 4.7) was employed to isolate the compounds. A flow rate of 13 mL/min using a solvent gradient system of solvent A (90% water in ACN with TFA) to solvent B (90% ACN in water with TFA), with detection at 210 and 280 nm was employed for the separation. The analytical HPLC method was converted to semi-preparative HPLC method by adjusting the flow

rate and sample load using a conversion formula demonstrated in chapter two, section 2.8.3.2. Purity of the collected peaks from semi-preparative HPLC was confirmed by analytical HPLC analysis of each peak using the general HPLC method described in section 4.1.2.1.

Table 4.7 Semi-preparative HPLC method for the separation of SPE5 fraction

Time (min)	% of solvent A	% of solvent B
0	80	20
3	74	26
15	74	26
50	50	50
55	0	100
65	0	100
70	100	0

Solvent A = 90% H_2O + 10% ACN + 0.05% TFA;   Solvent B = 90% ACN + 10% H_2O + 0.05% TFA

Flow rate: 13 mL/min, λ: 210 and 280 nm
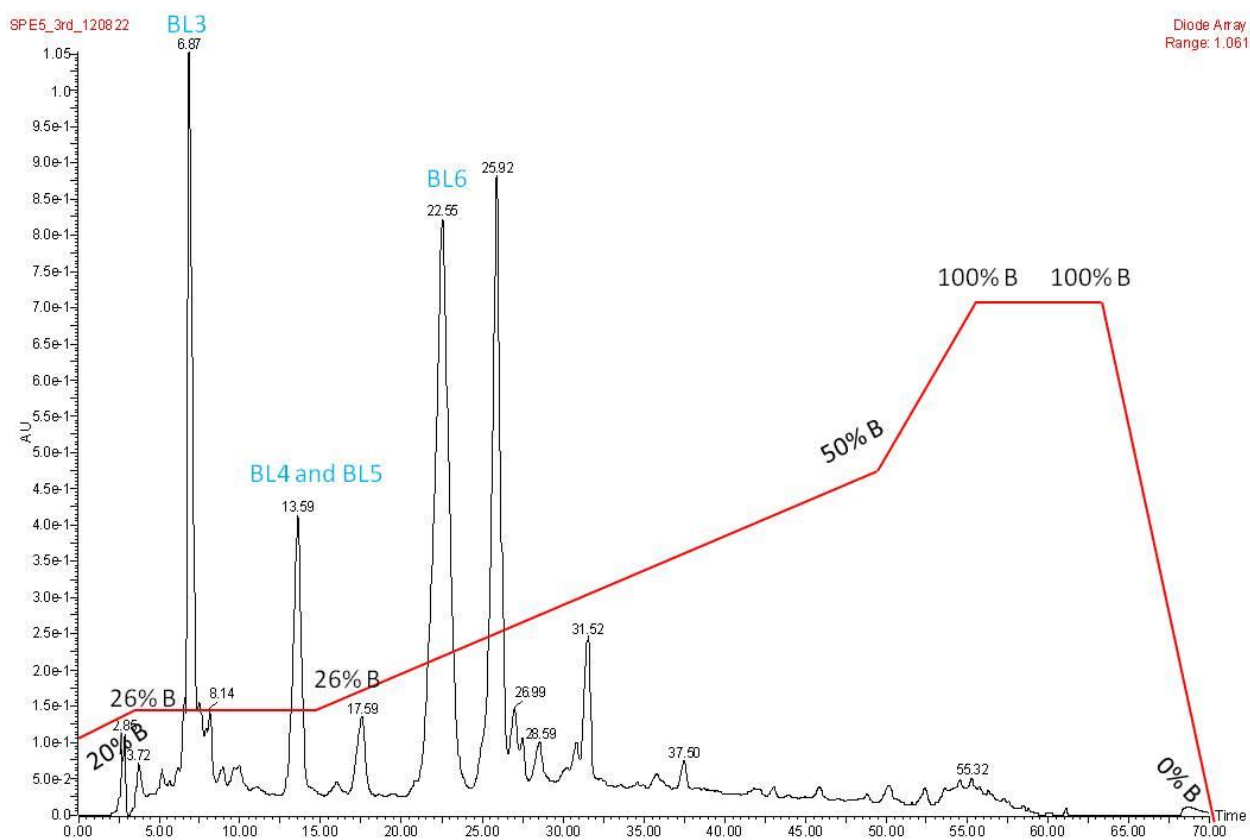
Figure 4.9 Semi-preparative HPLC chromatogram of *Blumea lacera* SPE5 fraction at 210 nm

SPE5 fraction (0.48 g) was dissolved in methanol and injected (40 mg/200 µl) onto the semi-preparative column. Detection was monitored at 210 and 280 nm, with defined peaks collected according to the retention time, R_T (Figure 4.9). Peaks were collected throughout the run. However, the collected peaks were further re-purified and their purity was confirmed by analytical HPLC using the general HPLC method (Section 4.1.2.3). Four major compounds were isolated from three peaks at R_T 6.87, 13.59, and 22.56 min. The peak at R_T 6.87 min afforded compound **BL3** (114.1 mg) and the peak at R_T 13.59 min afforded the compounds, **BL4** and **BL5** (5.0 and 1.5 mg, respectively). The peak at R_T 22.56 min afforded compound **BL6** (2.5 mg). Other peaks of SPE5 fraction underwent re-purification, but re-purified fractions were of minimal mass and still contained impurities, thus no further pure compounds were obtained. A total of 6 compounds were isolated and purified from *Blumea lacera* CME (Table 4.8).

Table 4.8 Compounds isolated from the crude methanol extract of *Blumea lacera*

Fraction	Mass (g)	Compound	Mass (mg)	Colour	Physical state
SPE4	1.7	**BL1**	53.3	Brownish-yellow	Solid
		BL2	4.9	Yellow	Solid
SPE5	0.5	**BL3**	114.1	White	Solid
		BL4	5.0	White	Solid
		BL5	1.5	White	Solid
		BL6	2.5	White	Solid

4.2 Structural characterization of compounds from SPE4 and SPE5 of *Blumea lacera*

4.2.1 Background

Nuclear magnetic resonance (NMR) spectroscopy is a powerful technique to aid in the identification and discovery of new drug molecules. Both 1D and 2D NMR (one dimensional (^{1}H and ^{13}C) and two dimensional NMR experiments (COSY, HSQC, and HMBC)) experiments have been used since 1970 and still are vital for structural elucidation of natural products (Shaikh 2010).

Mass spectrometry has also been used for several decades as a primary technique for structural elucidation of natural products as the technique is highly sensitive and provides unique molecular information. The most general ionization methods in mass spectrometry include electrospray ionization (ESI) and atmospheric pressure ionization (API). ESI (low resolution – LR-ESI-MS and high resolution HR-ESI-MS) and API can generate the ions essential for mass spectrometric analysis for greater than 90% of analytes, ranging from small molecules such as amino acids to macromolecules, such as proteins and nucleic acids (Ganguly, Pramanik et al. 2002).

4.2.2 Results and discussion

4.2.2.1 Structural characterization of flavonoid glycosides-BL1 and BL2

4.2.2.1.1 Structural characterization of BL1

BL1 (53.3 mg), was isolated as a brownish yellow amorphous powder using reversed-phase HPLC from the SPE4 fraction (H$_2$O:MeOH, 40:60) derived from the crude methanolic leaf extract (CME) of *Blumea lacera*. The structural elucidation of **BL1** was principally achieved using NMR and MS experimental data, with structure confirmed by comparison to published data (Hirose 2010). One-dimensional NMR (^{1}H and ^{13}C) along with 2D NMR spectra, (COSY,

HMBC and HSQC) were recorded. The UV spectrum of **BL1** evidenced the λ_{max} at 201, 266 and

347 nm which indicate the presence of kaempferol glycoside (Wan, Yu et al. 2011). The

molecular formula of **BL1** was established as $C_{33}H_{40}O_{19}$ from LR-ESI-MS in negative ion mode,

[M-H]$^-$ generated a quasi-molecular anion peak at *m/z* 739. The fragmentation of the peak at *m/z*

739 resulted in two additional fragments [M-146-H]$^-$ at *m/z* 593 and [M-454(triose moiety)-H]$^-$

at *m/z* 285 indicating the loss of a 6-deoxy hexose and two 6-deoxy hexose units plus one hexose

sugar unit.

The ^{1}H NMR spectrum of **BL1** (Table 4.9, Spectrum: see appendix) indicated the presence of 2

meta-coupled aromatic protons, which appeared as separate doublets (δ_H 6.18 and 6.38, *J* = 1.8

Hz) and corresponded to H-6 and H-8 protons in ring A. In addition, both two doublets at δ_H 6.89

and 8.06 both with coupling constants *J* = 9 Hz for both indicate the presence of 4 aromatic

protons in an additional ring B, which are characteristic of a 3,5,7,4'-tetrasubstituted flavone

(Hirose 2010). This pattern when compared to the literature (Hirose 2010) indicated the presence

of kaempferol as part of the structure. The ^{1}H NMR data also showed the presence of 3 anomeric

protons as doublets at δ_H 4.52, 5.22 and 5.62, with coupling constants of 1.8, 1.2 and 7.8 Hz,

respectively suggesting the presence of 3 sugar residues in the structure. The coupling constant

of *J* = 7.8 Hz for the anomeric H indicated a galactose in a β-configuration, while the two other

coupling constants of *J* = 1.2 Hz and *J* = 1.8 Hz, indicated 2 rhamnose residues with α-

configurations when compared to compound **4** [kaempferol-3-*O*-(2''6''-di-*O*-α-L-

rhamnopyranosyl)-β-D-galactopyranoside] (Hirose 2010). The methyl doublets at δ_H 0.97 and

1.17 with coupling constants of *J* = 6.0 and 6.6 Hz, respectively, were also consistent with the 2

rhamnosyl residues in compound **4** [kaempferol-3-*O*-(2''6''-di-*O*-α-L-rhamnopyranosyl)-β-D-

galactopyranoside] (Hirose 2010). Twelve oxymethines and one oxymethylene peak for one

galactose and two rhamnose residues were evident as either doublet of doublets or apparent triplet (See appendix, Figure: ^{1}H NMR spectrum of **BL1**) between δ_H 3.27-4.06 (14H) were consistent with the ring protons in the 3 sugars plus 2 C6-H's in galactose (H-2" to H-6", H-2"' to H-5"' and H-2"" to H-5"").

The ^{13}C NMR (DEPT) spectrum of **BL1** (Table 4.9, Spectrum: see appendix) displayed a total of 33 different C signals. Of these, 15 signals were attributed to the aglycone, kaempferol and 18 signals to three sugar residues, thus indicating **BL1** was a trisaccharide derivative of a flavonoid. A carbonyl carbon signal at δ_C 179.4 (C-4) and four oxygen-bearing aromatic quaternary carbons were evident at δ_C 158.4 (C-9), 163.1 (C-5), 161.3 (C-4') and 165.6 (C-7) ppm. Another 10 carbon signals of the aglycone were evident in the region of δ_C 94.6 to 158.7 ppm. The signals from the aglycone part of **BL1** were assigned to those of compound **4** [kaempferol-3-*O*-(2"6"-di-*O*-α-L-rhamnopyranosyl)-β-D-galactopyranoside] reported previously (Hirose 2010). Three anomeric carbon signals (C-1", C-1"' and C-1"") were revealed at δ_C 100.8, 101.8 and 102.6 ppm for galactopyranose, rhamnopyranose and rhamnopyranose, respectively, and were consistent with **4** [kaempferol-3-*O*-(2"6"-di-*O*-α-L-rhamnopyranosyl)-β-D-galactopyranoside] (Hirose 2010). The oxymethine carbons of galactopyranose were evident at δ_C 77.5 (C-2"), 75.7 (C-3"), 70.7 (C-4") and 75.2 (C-5") ppm, respectively, with the C-6" methylene assigned at 67.0 ppm. Oxymethine carbon signals were assigned at δ_C 72.5 (C-2"'), 72.3 (C-3"'), 74.1 (C-4"'), 69.8 (C-5"') and 72.1 (C-2""), 72.4 (C-3""), 73.8 (C-4""), 69.7 (C-5"") ppm, respectively, along with two methyl carbons for the 2 rhamnopyranose residues being evident at δ_C 17.5 (C-6"') and 17.9 (C-6"") ppm, respectively. The carbon signals for all three sugar residues (one galactopyranose and two rhamnopyranoses), sugar connections to the aglycone and the aglycone were all identical to the reported compound **4** [kaempferol-3-*O*-(2"6"-di-*O*-α-L-

rhamnopyranosyl)-β-D-galactopyranoside] (Hirose 2010). Thus, based on NMR (^{1}H and ^{13}C), MS data and published data of compound **4** [kaempferol-3-*O*-(2"6"-di-*O*-α-L-rhamnopyranosyl)-β-D-galactopyranoside] (Hirose 2010), **BL1** was characterized as kaempferol-3-*O*-(2"6"-di-*O*-α-L-rhamnopyranosyl)-β-D-galactopyranoside (Figure 4.10).

Figure 4.10 Structure of **BL1**

Table 4.9 ^{1}H and ^{13}C NMR chemical shifts for **BL1** (in CD$_3$OD, 600 and 150 MHz, respectively)

Position	δ_H (ppm) (multiplicity, J, Hz)	δ_C (ppm)
2	6.18 (*d*, 1H, *J*=1.8)	158.7
3	6.38 (*d*, 1H, *J*=1.8)	134.5
4	6.89 (*d*, 2H, *J*=9)	179.4
5	8.06 (*d*, 2H, *J*=9)	163.1
6		99.7
7		165.6
8		94.6
9		158.4
10		105.9
1'		123.0
2'		132.3
3'		116.2
4'		161.3
5'		116.1
6'		132.1
3-*O*-β-D-galactose		
1"	5.62 (*d*, 1H, *J*=7.8)	100.8
2"	3.93 (*dd*, *J*=9.6, 1.8)	77.5
3"	3.70 (*dd*, *J*=9.6, 6)	75.7
4"	3.77 (*app t*, *J*=3.7)	70.7
5"	3.64 (*m*)	75.2
6"a	3.72 (*dd*, *J*=10.2, 4.2)	67.0
6"b	3.44 (*dd*, *J*=10.1, 6.2)	
(2→1)-*O*-α-L-rhamnose		
1'''	5.22 (*d*, 1H, *J*=1.2)	102.6
2'''	4.00 (*dd*, *J*=3.6, 1.8)	72.5
3'''	3.79 (*dd*, *J*=9.6, 6.0)	72.3
4'''	3.34 (*t*, *J*=9.6)	74.1
5'''	4.06 (*dd*, *J*=9.6, 3.6)	69.8
6''' (-CH$_3$)	0.97 (*d*, 3H, *J*=6)	17.5
(6→1)-*O*-α-L-rhamnose		
1''''	4.52 (*d*, 1H, *J*=1.8)	101.8
2''''	3.57 (*dd*, *J*=3.6, 1.8)	72.1
3''''	3.50 (*dd*, *J*=9.6, 6.0)	72.4
4''''	3.27 (*t*, *J*=9.6)	73.8
5''''	3.52 (*dd*, *J*=9.6, 6.0)	69.7
6'''' (-CH$_3$)	1.17(*d*, 3H, *J*=6.6)	17.9

4.2.2.1.2 Structural characterization of BL2

BL2 (4.9 mg) was obtained as a yellow powder from the SPE4 fraction of the CME of *Blumea lacera* using reversed-phase C-18 HPLC. NMR (^{1}H and ^{13}C and HSQC), MS and UV experiments were conducted in order to elucidate the structure of **BL1**. The UV spectrum of **BL1** evidenced the λ_{max} 203, 266 and 340 nm which indicates the presence of kaempferol glycoside (Wan, Yu et al. 2011). The LR-ESI-MS negative mode spectrum of **BL2** depicted a quasimolecular ion peak [M-H]$^-$ at *m/z* 593 with fragments [M-146-H]$^-$ at *m/z* 447 and [M-146-162-H]$^-$ at *m/z* 285, indicating the aglycone loss of one 6-deoxy hexose (146 amu) and one hexose (162 amu) (Sang, Lapsley et al. 2002). Thus, the molecular formula of **BL2** determined was $C_{27}H_{30}O_{15}$.

When comparing the **BL2** spectral data with **BL1** showed that it possessed the same aglycone (kaempferol) but differed in the sugar residues. The molecular mass of **BL2** was 146 amu (rhamnopyranose) less than that of **BL1**, indicating that **BL1** had one additional 6-deoxy hexose than **BL2**.

The ^{1}H NMR spectrum (Table 4.10, Spectrum: see appendix) of **BL2** disclosed the presence of two types of aromatic protons. Protons showing highly-shielded meta-couplings were evidenced at δ_H 6.23 and 6.43 ppm as doublets (H-6 and H-8 in ring-A, respectively) whereas other aromatic protons of ring-B indicated a 1',4'-substituted system. The ^{1}H NMR also showed the presence of two sugar moieties with two anomeric protons at δ_H 5.06 (*d*, 1H, *J* = 7.8 Hz) and at 4.52 (*d*, 1H, *J* = 1.5 Hz) ppm, respectively. The ^{1}H NMR of **BL2** displayed an A ring identical to that of compound **BL1**, with the appearance of two protons at δ_H 6.23 (*d*, 1H, *J* = 2.1) and 6.43 (*d*, 1H, *J* = 2.1 Hz) ppm, assigned as H-6 and H-8, respectively. Also, evident was the 1',4'-substituted pattern in the B-ring with four proton signals at δ_H 6.91 (*d*, 2H, *J* = 9 Hz; H-3'/5')

and at 8.11 (*d*, 2H, *J* = 8.7 Hz; H-2'/6') ppm, respectively. Accordingly the aglycone of **BL2** was assigned as kaempferol (Sang, Lapsley et al. 2002). In regard to the 2 sugar residues the coupling constant of *J* = 7.8 Hz for H-1'' indicated a β-glucopyranose while the *J* = 1.5 Hz for H-1''' supported the presence of an α-rhamnopyranose, in line with previously published data for compound **4** [kaempferol 3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside] (Sang, Lapsley et al. 2002). The methyl doublet at δ_H 1.19 (*d*, 3H, *J* = 6.0 Hz), indicated a rhamnopyranose, along with eight oxymethines and one oxymethylene signals for glucopyranose and rhamnopyranose which were evident as multiplets between at δ_H 3.83-3.90 (*m*, 10H) (H-2'' to H-6'' and H-2''' to H-5''') consistent with published data for compound **4** [kaempferol 3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside] (Sang, Lapsley et al. 2002).

The ^{13}C NMR spectrum (Jmod) (Table 4.10, Spectrum: see appendix) evidenced a total of 27 different carbon signals, of which 15 carbon were attributed to the aglycone, kaempferol and 12 to two sugar moieties, which indicated that **BL2** was a disaccharide derivative of the flavonoid, kaempferol. The ^{13}C NMR of **BL2** showed a carbonyl carbon signal at δ_C 179.6 ppm (C-4) along with other signals of the aglycone in the region of δ_C 94.9 to 166.1 ppm, four oxygen-bearing aromatic quaternary carbon signals at δ_C 163.0 (C-5), 166.1 (C-7), 159.5 (C-9) and 161.6 (C-4') were identical to those of the reported compound **4** [kaempferol 3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside] (Sang, Lapsley et al. 2002). The anomeric carbon and the oxymethine carbons of glucopyranose were evident at δ_C 104.8 (C-1'') and 75.4 (C-2''), 77.6 (C-3''), 72.3 (C-4''), 77.5 (C-5''), respectively. The methylene carbon of glucopyranose (C-6'') appeared at δ_C 68.6 (+6 ppm) downfield compared to the C-6'' of the Glc monosaccharide indicating that the Glc was substituted at C-6'' with the 6-deoxyhexose sugar moiety determined to be rhamnopyarnose (Sharaf, El-Ansari et al. 2000). The anomeric carbon and oxymethine

carbon signals of rhamnopyranose were revealed at δ_C 101.3 (C-1") and 70.1 (C-2‴), 72.1 (C-3‴), 73.9 (C-4‴), 69.7 (C-5‴), respectively. The methyl carbon signal of rhamnopyranose were evident at δ_C 18.0 ppm (C-6‴). All the carbon signals from the sugar parts of **BL2** were identical to those of a published compound (Sang, Lapsley et al. 2002; Shahat, Nazif et al. 2005). Furthermore, sugar linkages to the aglycone were assigned by comparing it to linkages present in the published compound (Sang, Lapsley et al. 2002). The glucopyranose was indicated as attached at the C-3 hydroxyl of kaempferol due to a chemical shift evident in C-3 (δ_C 135.5 ppm) on the aglycone in comparison to same reported compound (Sang, Lapsley et al. 2002). The rhamnopyranose (H-1‴, δ_H 4.52) was indicated as being linked to C-6" (δ_C 68.6 ppm) according to published data (Sang, Lapsley et al. 2002). Thus, the structure assigned to **BL2** was kaempferol-3-O-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside, identical to the known reported compound **4** [kaempferol 3-O-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside] (Sang, Lapsley et al. 2002; Shahat, Nazif et al. 2005) (Figure 4.11).
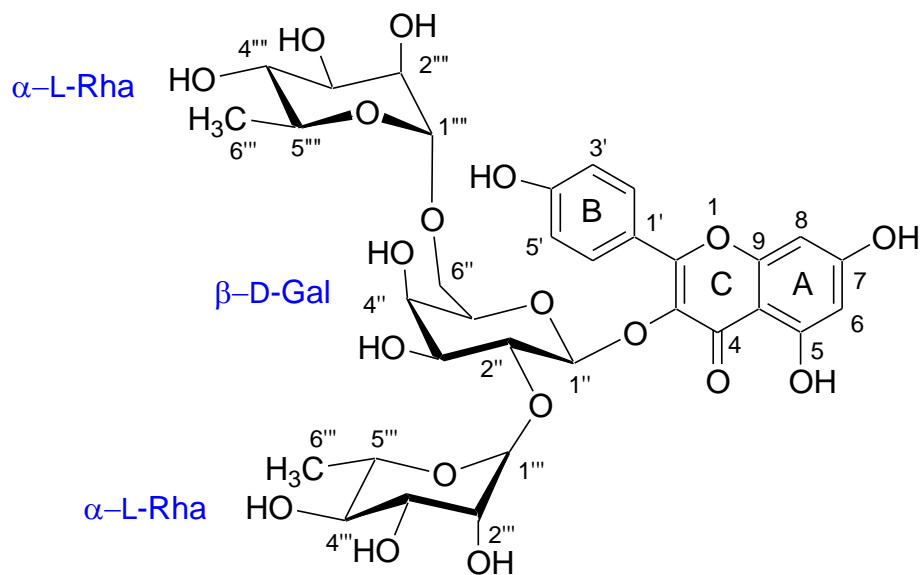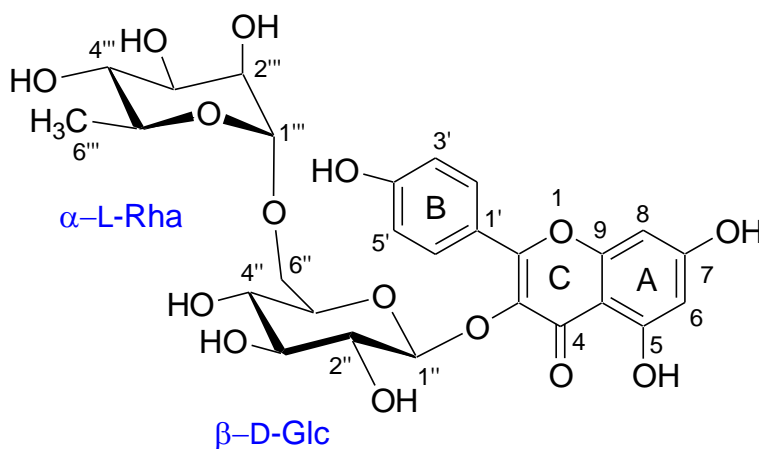

Figure 4.11 Structure of **BL2**

Table 4.10 ^{1}H and ^{13}C NMR chemical shifts for **BL2** (in CD$_3$OD, 300 and 75 MHz, respectively)

Position	δ_H (ppm) (multiplicity, J, Hz)	δ_C (ppm)
2	6.23(d,1H, J=2.1)	158.6
3	6.43 (d, 1H, J=2.1)	135.5
4	6.91 (d, 2H, J=9)	179.6
5	8.11 (d, 2H, J=8.7)	163.0
6		100.0
7		166.1
8		94.9
9		159.5
10		105.6
1'		122.7
2'		132.5
3'		116.1
4'		161.6
5'		116.1
6'		132.5
3-O-β-D-glucopyranose		
H-1"	5.06 (d, 1H, J=7.8)	104.8
H-2" to 6"	3.83-3.90 (m, 5H)	75.4
		77.6
		72.3
		77.5
		68.6
(2→1)-O-α-L-rhamnopyranose		
1'''	4.52 (d, 1H, J=1.5)	101.3
H-2''' to 5'''	3.83-3.90 (m, 4H)	70.1
		72.1
		73.9
		69.7
6''' (-CH$_3$)	1.19 (d, 3H, J=6.0)	18.0

4.2.2.2 Structural characterization of the steroidal glycoalkaloid BL3

BL3 (114.1 mg) was obtained as white powder from the SPE fraction of CME of *Blumea lacera* using reversed-phase C-18 HPLC. NMR (^{1}H, ^{13}C, COSY, HSQC, and HMBC) were recorded in order to elucidate the structure. Negative-ion mode, LR-ESI-MS generated a quasimolecular ion [M-H]$^-$ at *m/z* 1013. From HR-ESI-MS positive-ion mode the molecular formula was determined as C$_{51}$H$_{84}$NO$_{19.}$ The measured optical rotation of **BL3** was [α]$_D^{25}$= -94.4° (in MeOH) which was

consistent with that of α-solamargine $[\alpha]_D^{24} = 91.0°$ (though determined at 24°C in MeOH: CHCl$_3$=1:1) (Wanyonyi, Chhabra et al. 2002) where it had the identical aglycone structure (3-O-22αN-spirosol-5-ene) to that indicated in **BL3**. Thus, the measured optical rotation supported the assignment of **BL3**. The UV spectrum displayed λ_{max} at 238 and 202 nm. The maximum absorption at 202 nm indicated the presence of double bond (C=C) between C5 and C6 while λ_{max} at 238 nm indicated the presence of -C-NH-C in the ring E (Figure 4.12). The IR spectrum showed major absorption bands at cm^{-1}: 3380 (OH or NH), 2934 (C-H), 1669 cm^{-1} (C=C), 1200-1000 cm^{-1} (C=O).

The ^{1}H NMR spectrum of **BL3** (Table 4.11, Spectrum: see appendix) indicated the aglycone and sugar moieties in **BL3**. The ^{1}H NMR of **BL3** displayed proton signals due to seven methyl groups comprising two tertiary methyl groups at δ_H 0.86 (*s*, 3H, H$_3$-18) and 1.05 (*s*, 3H, H$_3$-19) ppm, five secondary methyl groups at δ_H 0.97 (*d*, 3H, *J* = 6.3 Hz, H$_3$-27), 1.15 (*d*, 3H, *J* = 7.2 Hz, H$_3$-21), 1.24 (*d*, 3H, *J* = 6.3 Hz, Rha1 H$_3$-1), 1.25 (*d*, 3H, *J* = 6.3 Hz, Rha2 H$_3$-1), and 1.28 (*d*, 3H, *J* = 6.0 Hz, Rha3 H$_3$-1) ppm. The three secondary methyl protons (δ_H 1.24, 1.25 and 1.28 ppm) indicated the presence of three rhamnose moieties in the glycone part of **BL3**. The ^{1}H NMR contains proton signals due to ten methylene groups at δ_H 1.58 (*m*, 2H, H$_2$-11), 1.71 and 1.77 (*m*, 1H, 1H, H$_2$-24), 1.89 (*m*, 2H, H$_2$-2), 1.98 and 2.06 (*m*, 2H, H$_2$-23), 185-1.89 (*m*, 4H, H$_2$-7, H$_2$-15), 1.85 and 1.05 (*m*, 2H, H$_2$-1), 2.46 and 2.29 (*dd*, 1H, *J* = 18, 3 Hz, and *dd*, 1H, *J* = 12.6, 3 Hz, H$_2$-4), 1.79 and 1.83 (m, 1H, 1H, H$_2$-12), 2.88 and 3.06 (*dd*, 1H, *J* = 12, 12 Hz, and *dd*, 1H, *J* = 12, 3 Hz, H-26). These ten methylene proton signals are attributed to the aglycone as the chemical shifts for them were evident in the up-field region. One olefinic proton signal at δ_H 5.39 ppm (*d*, 1H, *J* = 4.5 Hz) was indicative of a single unsaturation unit on ring-B of the aglycone. The presence of 16 oxymethine proton signals and one oxymethylene proton signal

were also evident, which were attributed to three rhamnopyranose and one glucopyranose units. Along with this one oxymethine proton signal at δ_H 4.59 ppm was attributed to ring-E of the aglycone. The ^{1}H NMR spectrum of **BL3** also evidenced the four anomeric sugar residue signals at δ_H 4.50 (*d*, 1H, *J* = 7.8 Hz), 4.83 (*d*, 1H, *J* = 1.2 Hz), 5.18 (*d*, 1H, *J* = 1.8 Hz) and 5.19 (*d*, 1H, *J* = 2.1 Hz) indicating one β-configuration (*J* = 7.8 Hz) and three other sugar units with α-configurations (*J* = 1.2, 1.8 and 2.1Hz), respectively.

The ^{13}C NMR spectrum of **BL3** (Table 4.11, Spectrum: see appendix) revealed the presence of 51 different carbon signals, among which 27 carbon signals were attributed to the aglycone and the remaining 24 indicated the presence of four hexoses. Seven methyl carbon signals were evidenced at δ_C 14.7 (C-21), 16.5 (C-18), 17.9 (Rha1 C-6), 18.0 (Rha2 C-6), 18.6 (Rha3 C-6), 18.6 (C-27), and 19.8 (C-19) ppm, respectively. Three of these methyls were consistent with the 6-deoxy-hexose rhamnose sugars. Also evident were ten methylene carbon signals at δ_C 21.9 (C-11), 28.9 (C-24), 30.7 (C-2), 33.0 (C-23), 33.1 (C-7), 33.2 (C-15), 38.5 (C-1), 39.4 (C-4), 40.3 (C-12), and 46.7 (C-26) ppm. Additionally, 16 oxymethine carbon signals between δ_C 69.0-80.8 ppm and one oxymethylene proton signal at δ_C 61.9 ppm were attributed to 3 rhamnopyranose and 1 glucopyranose units. Also one oxymethine carbon signal at δ_C 84.7 ppm was seen, which was attributed to ring-E of the aglycone part. Seven methine carbon signals at δ_C 29.3 (C-25), 32.7 (C-8), 42.8 (C-20), 51.5 (C-9), 57.6 (C-14), 62.9 (C-17), and 79.5 (C-3) ppm along with one double bonded methine carbon signal at δ_C 122.5 ppm (C-6) were also evident. The presence of four anomeric carbon signals at 100.4 (Glc C-1), 102.3 (Rha1 C-1), 102.6 (Rha3 C-1), and 103.1 (Rha2 C-1) ppm was also evident, indicating the presence of four sugar moieties.

The ^{13}C NMR spectrum of **BL3** evidenced four quaternary carbon signals at δ_C 38.0 (C-10), 42.1 (C-13), 100.2 (C-22) and 141.8 (C-5) ppm, with the carbon at δ_C 100.2 ppm (C-22), indicating

the linkage to an oxygen atom on ring-E. The carbon at 141.8 ppm (C-5) was olefinic and assigned as C-5 on ring-B of the aglycone part. The unsaturation at C-5/C-6 on ring-B of the aglycone was determined on the basis of ^{1}H and ^{13}C NMR spectral data. (Puri, Wong et al. 1993) A broad singlet δ_H 5.39 (H-6 aglycone, $J = 4.5$ Hz) in ^{1}H NMR spectrum and a downfield shift of C-5 and C-6 at δ_C 141.8 and 122.45 ppm, respectively in the ^{13}C NMR spectrum were consistent with similar reported steroidal compounds (Puri, Wong et al. 1993). Furthermore, the configuration at C-22 was determined as 22-α-N on the basis of carbon shifts at C-23, C-24 and C-26 in comparison to published ^{13}C data (Lorey, Porzel et al. 1996; Yoshikawa, Xu et al. 2007). ^{13}C NMR signals to the C-23 and C-24 in **BL3** were observed at lower fields compared with those of a corresponding 22-β-*N*-spirosolene glycosides reported, while the signal due to C-26 was observed at higher field compared with that of a corresponding 22βN-spirosolene glycosides (Lorey, Porzel et al. 1996; Yoshikawa, Xu et al. 2007). The presence of 25 *R* spiroketal moiety and an axial H-25, and equatorial configuration of the methyl at C-27 was established from proton and carbon shifts in ^{1}H and ^{13}C NMR spectra of **BL3** along with comparisons to published data (Mahato, Sahu et al. 1980). The glycosidic linkage was shown to be at C-3 of the aglycone, due to the downfield shift of C-3 at 79.45 ppm of the glycoside when compared with the free aglycone (Mahato, Sahu et al. 1980). Carbon signals due to the aglycone of **BL3** were consistent with those reported previously for **3** (solamargine) (Mahato, Sahu et al. 1980), with the exception being at C-16, which showed a downfield carbon shift at 84.7 ppm compared to that of **3** (solamargine). The ^{1}H NMR spectrum of **BL3** showed the presence of a proton signal at δ_H 4.63 as a doublet of doublets with $J = 7.5$, 7.5 Hz suggesting its assignment at C-16 which was confirmed by ^{1}H-^{1}H COSY spectrum (see appendix). By comparison to the literature it was determined that **BL3** has a steroidal glycoalkaloid structure (Ono, Takamura et al. 2007).
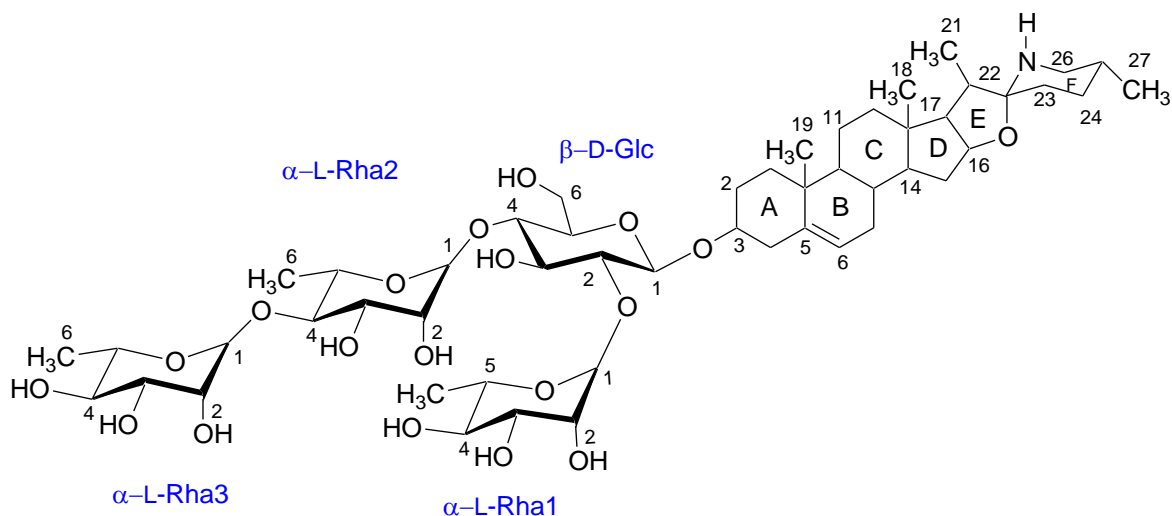
Figure 4.12 Structure of **BL3**

Table 4.11 ^{1}H and ^{13}C NMR chemical shifts for **BL3** (in CD$_3$OD, 300 and 75 MHz, respectively)

Position	δ_H (ppm) (multiplicity, J, Hz)	δ_C (ppm)
Aglycone		
1	1.85, 1.05 (*m*, 1H, 1H)	38.4
2	1.89 (*m*, 2H)	30.7
3	3.55 (*m*, 1H)	79.5
4	2.27, 2.46 (*m*, 1H, 1H)	39.4
5		141.8
6	5.39 (*br s*, 1H, *J*=4.5)	122.5
7	2.06, 1.98 (*m*, 1H, 1H)	33.0
8	1.69 (*m*,1H)	32.7
9	0.97 (*d*, 1H, *J*=6.3)	51.5
10		38.0
11	1.58 (*m*, 2H)	21.9
12	1.79, 1.83 (*m*, 1H, 1H)	40.3
13		42.1
14	2.11 (*m*, 1H)	57.6
15	1.85, 1.89 (m, 1H, 1H)	33.2
16	4.63 (*dd*, 1H, *J*=7.5, 7.5)	84.7
17	1.91(*m*, 1H)	62.9
18	0.86 (*s*, 3H)	16.5
19	1.05 (*s*, 3H)	19.8
20	2.34 (*m*, 1H, *J*=7.5)	42.8
21	1.15 (*d*, 3H, *J*=7.2)	14.7
22		100.2
23	1.85, 1.89 (*m*, 1H, 1H)	33.1
24	1.71- 1.77 (*m*, 2H)	28.9
25	1.88 (*m*, 1H)	29.3
26	2.88, 3.06 (*m*, 1H, 1H)	46.7
27	0.97 (*d*, 3H, *J*=6.3)	18.6

Table 4.11 ^{1}H and ^{13}C NMR chemical shifts for **BL3** (in CD$_3$OD, 300 and 75 MHz, respectively) (contd.)

Position	δ_H (ppm) (multiplicity, *J*, Hz)	δ_C (ppm)
Sugar Residues		
Glc		
1	4.50 (*d*, 1H, *J*=7.8)	100.4
2	3.55 (*m*, 1H)	80.8
3	4.35 (*m*, 1H)	76.6
4	3.56 (*m*, 1H)	77.9
5	3.59 (*m*, 1H)	79.1
6a, 6b	3.65, 3.80 (*m*, 1H, 1H)	61.9
Rha1		
1	5.19 (*d*, 1H, *J*=2.1)	102.3
2	3.93 (*m*, 1H)	72.3
3	3.79 (*m*, 1H)	72.8
4	3.40 (*m*, 1H)	73.8
5	4.11 (*m*, 1H)	69.7
6 (-CH$_3$)	1.24 (*d*, 3H, *J*=6.3)	17.9
Rha2		
1	5.18 (*d*, 1H, *J*=1.8)	103.1
2	3.59 (*m*, 1H)	72.3
3	4.04 (*m*, 1H)	69.0
4	3.74 (*m*, 1H)	72.9
5	3.93 (*m*, 1H)	72.1
6 (-CH$_3$)	1.25 (*d*, 3H, *J*=6.3)	18.0
Rha3		
1	4.83 (*d*, 1H, *J*=1.2)	102.6
2	3.64 (*m*, 1H)	72.3
3	3.41 (*m*, 1H)	72.3
4	3.66 (*m*, 1H)	73.9
5	3.70 (*m*, 1H)	70.4
6 (-CH$_3$)	1.28 (*d*, 3H, *J*=6.0)	18.6

Glc = Glucopyranose, **Rha** = Rhamnopyranose

The HMBC spectrum of **BL3** (Figure 4.13, Spectrum: see appendix) revealed the presence of cross peaks between the methyl protons at δ_H 0.86 ppm (H$_3$-18) and carbons at δ_C 40.3 (C-12), 57.6 (C-15), 62.9 (C-17) ppm which indicates the connection between ring-C and ring-D; proton at δ_H 0.97 ppm (H$_3$-27) and carbons at δ_C 28.9 ppm (C-24). The HMBC spectrum of **BL3** revealed correlations of the methyl protons at δ_H 1.05 ppm (H$_3$-19) to the carbons at δ_C 38.0 (C-10), 39.4 (C-4), 40.3 (C-12), 51.5 (C-9), and 141.8 ppm (C-5), indicating the connectivity of

ring-A to ring-B. The methyl protons at δ_H 1.15 (H$_3$-21) showed correlations to the carbons at δ_C 28.9 (C-24), 42.1 (C-13), 46.7 (C-26), and 100.2 (C-22) ppm indicating the connection of ring-D to ring-C as well as ring-D to ring-E. HMBC correlations between the methylene protons at δ_H 1.89 ppm (H-15) and the carbon at 57.6 (C-14) ppm, along with the methylene protons at 2.06 ppm (H-7) with the carbon at δ_C 141.8 (C-5) were evident. The methine proton at δ_H 3.55 (H-3) showed connectivity to the anomeric sugar carbon at δ_C 100.4 ppm (Glc C-1) indicating the β-Glc C-1 to be C-3 linked to the aglycone of **BL3**. In addition, connectivity of the proton at δ_H 3.55 (Glc H-2) to the carbon at δ_C 102.3 (Rha1 C-1) ppm, indicated the α-Rha1 C-1 was linked to β-Glc C-2; cross peaks between δ_H 3.56 (Glc H-4) and δ_C 103.2 (Rha2 C-1) ppm indicated the α-Rha2 C-1 was linked to β-Glc C-4; cross peaks between δ_H 3.74 (Rha2 H-4) and δ_C 102.6 (α-Rha3 C-1) ppm indicated the Rha3 C-1 was linked to Rha2 C-4. Based on ^{1}H, ^{13}C, ^{1}H-^{1}H COSY (Figure 4.15, spectrum: see appendix) and HMBC NMR spectral data of **BL3** as well as comparing with those of previously published data of compound **3** (solamargine) (Mahato, Sahu et al. 1980), the aglycone part of **BL3** was assigned as (3β, 22α, 25 *R*)-spirosol-5-ene. In summary, based on NMR spectral data and comparison to published data of compound **10** [penogenin3-*O*-α-L-rhamnopyranosyl-(1→4)-*O*-L-rhamnopyranosyl-1→4)-[*O*-α-L-rhamnopyranosyl-(1→2)]-*O*-β-D-glucopyranoside] (Ono, Takamura et al. 2007), the glycone part was assigned as 3-*O*-22αN-spirosol-5-ene (Figure 4.15).
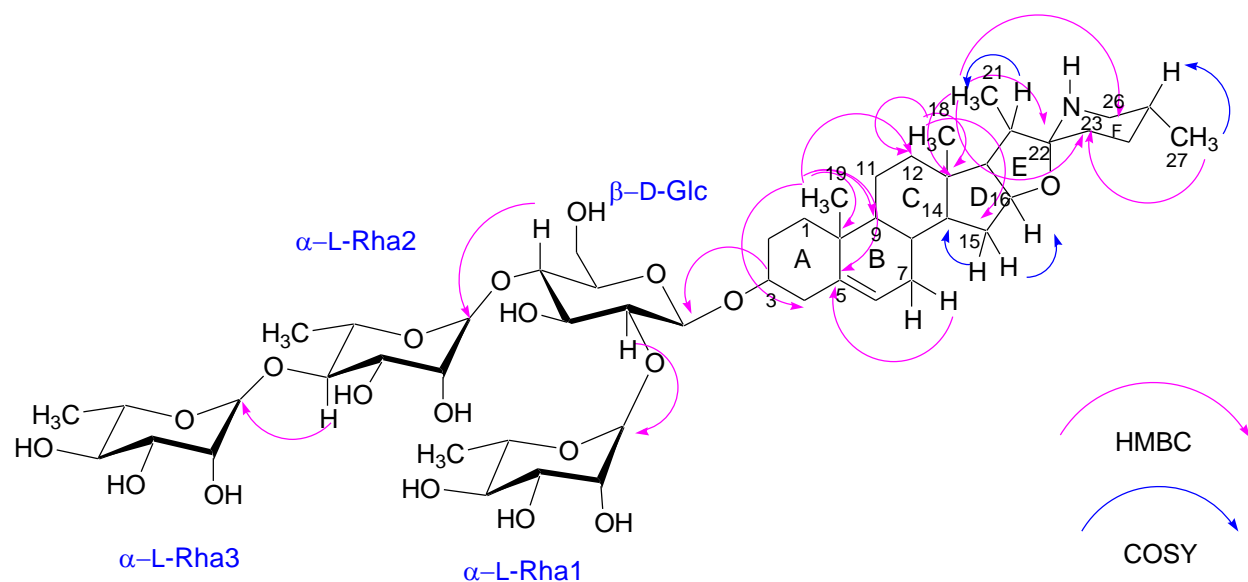
Figure 4.13 Key HMBC and ^{1}H-^{1}H COSY correlations observed in **BL3**

4.2.2.3 Structural characterization of Terpenoid glycosides-BL4, BL5 and BL6

4.2.2.3.1 Structural characterization of BL4

BL4 (5.0 mg) was obtained as a white solid from the SPE5 fraction of the CME of *Blumea lacera* using reversed-phase C-18 HPLC. NMR (^{1}H, ^{13}C, COSY, HMBC and HSQC) were recorded in order to elucidate the structure of **BL4**. The UV profile of **BL4** evidenced λ_{max} at 201 and 307 nm. The maximum absorption of UV at 201 nm indicated the presence of -C=C- in the compound, **BL4** whereas λ_{max} at 307 nm evident the presence of –CO at C-3 position. The positive ion mode LR-ESI-MS spectrum of **BL4** exhibited a quasi-molecular ion peak for [M+Na]$^+$ at *m/z* 961 together with a fragment ion peak [M-162+Na]$^+$ at *m/z* 799 indicating the loss of hexose. Thus, the molecular formula of **BL4** was determined as $C_{44}H_{74}O_{21}$.

The ^{1}H NMR spectrum of **BL4** (Table 4.12, Spectrum: see appendix) revealed the presence of terminal olefinic protons at δ_H 5.20 (*d*, 1H, *J* = 4.8 Hz, H-1a), 5.25 (*d*, 1H, *J* = 11.4 Hz, H-1b),

and also at 5.98 ppm (*dd*, 1H, *J* = 17.7 Hz, H-2) and methyl protons at δ_H 1.38 ppm (*br s,* 3H) on a quaternary carbon bearing an oxygen group. These proton chemical shifts were identical to those of geranyllinalool-3-*O*-β-D-glucopyranoside (Izumitani, Yahara et al. 1990; Terauchi 1998). The proton NMR also presented three methyls as broad singlets [δ_H 1.6 (2 x CH$_3$) and 1.79 (*br s*, 3H, CH$_3$)]. Three additional olefinic protons were seen as triplets at δ_H 5.13 (*t*, 2H, *J* = 6.9 Hz, H-6, H-10) and 5.40 ppm (*t*, 1H, *J* = 6.6 Hz, H-14 each adjacent to a methyl group. Six methylene groups [δ_H 2.02 (*m*, 8H, H$_2$-4, H$_2$-5, H$_2$-8, H$_2$-12), 2.08 (*m*, 2H, H$_2$-9) and 2.18 ppm (*m*, 2H, H$_2$-13,)] plus an oxymethylene group [δ_H 4.23, 4.32 ppm (*d*, each 1H, *J*= 11.7 Hz, H$_2$-17)] were evident. All these proton shifts indicated that 17-hydroxygeranyllinalool is present as an aglycone in the **BL4** structure (Izumitani, Yahara et al. 1990; Terauchi 1998). Four anomeric proton signals [δ_H, 4.37 (*d*, 2H, *J* = 7.8 Hz, Glc I H-1, Glc II H-1), 4.64 (*d*, 1H, *J* = 7.8, Glc III H-1) and 4.74 ppm (*s*, 1H, Rha H-1)] were exhibited indicating four sugar units were present in **BL4**. This proton NMR pattern was identical to that lyciumoside VII (compound **4**) isolated from *Lycium chinese* Mill (Terauchi 1998).

The ^{13}C NMR spectrum of **BL4** (Table 4.12, Spectrum: see appendix) confirmed the presence of an acyclic diterpene, 17-hydroxygeranyllinalool. This assignment was made by comparison of the 20 carbon signals due to eight double bond carbons [δ_C 115.8 (C-1), 144.5 (C-2), 126.0 (C-6), 135.9 (C-7), 125.8 (C-10), 135.9 (C-11), 131.3 (C-14), and 132.4 (C-15) ppm] along with one oxygenated methylene carbon (δ_C 68.2 ppm, C-17), one quaternary carbon with an oxygen atom (δ_C 81.5 ppm, C-3) to literature (Terauchi 1998). The ^{13}C NMR spectrum revealed the presence of four sugar units, three β-D-glucopyranosides and one α-L-rhamnopyranoside, having four anomeric carbon signals being evident at δ_C 99.6 (Glc I C-1), 101.1 (Glc II C-1), 104.8 (Glc III C-1) and 102.1 (Rha C-1) ppm, were consistent to those of lyciumoside VII (compound **4**)

(Terauchi 1998). The sugar linkages were also assigned by comparison of NMR data to that of lyciumoside VII (Terauchi 1998). A comparative study of ^{1}H, and ^{13}C NMR spectra of **BL4** with the published compound, capsianoside III **(5)** (Iorizzi, Lanzotti et al. 2001), indicated the presence of a 6*E*, 10*E*, 14*Z*, 17-hydroxygeranyllinalool as diterpenoid backbone. Thus, **BL4** was characterized as the known compound as 3-*O*-β-D-glucopyranosyl-17-hydroxy-6*E*, 10*E*, 14*Z*-(3*S*)-geranyllinalool17-*O*-β-D-glucopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→6)]-β-D-glucopyranoside (Figure 4.14).

Figure 4.14 Structure of **BL4**

Table 4.12 ^{1}H and ^{13}C NMR chemical shifts for **BL4** (in CD$_3$OD, 300 and 75 MHz, respectively)

Position	δ_H (ppm) (multiplicity, *J*, Hz)	δ_C (ppm)
1	5.20, 5.25 (*d*, 2H, *J*=4.8, 11.4)	115.8
2	5.98 (*dd*, 1H, *J*=17.7)	144.5
3		81.5
4	2.02 (*m*, 2H)	42.7
5	2.02 (*m*, 2H)	23.6
6	5.13 (*t*, 1H, *J*=6.9)	125.8
7		135.4
8	2.02 (*m*, 2H)	40.8
9	2.08 (*m*, 2H)	27.6
10	5.13 (*t*, 1H, *J*=6.9)	126.0
11		135.9
12	2.02 (*m*, 2H)	40.9
13	2.18 (*m*, 2H)	27.2
14	5.40 (*t*, 1H, *J*=6.6)	131.3
15		132.4
16	1.79 (*br s*, 3H)	22.0
17	4.32, 4.23 (*dd*, 2H, *J*=11.7, 11.7)	68.2
18	1.61 (*br s* 3H)	16.1
19	1.61 (*br s*, 3H)	16.2
20	1.38 (*br s*, 3H)	23.2
Glc I		
1	4.37 (*d*, 1H, *J*=7.8)	99.6
2	3.35 (*m*, 1H)	75.2
3	3.55 (*m,* 1H) [#]	77.8
4	3.80 (*m*, 1H)	71.7
5	3.55 (*m*, 1H)	77.6
6a, 6b	3.78, 3.80 (*m,* 1H, IH)	62.8
Glc II		
1	4.74 (*s*, 1H)	102.1
2	3.50 (*m*, 1H)	81.9
3	3.35 (*m*, 1H)	78.3
4	3.27 (*m*, 1H)	71.5
5	3.35 (*m*, 1H)	76.7
6a, 6b	3.98, 3.85 (*d*, 1H, *J*=10.5, *t*,1H, *J*=1.8, respectively)	67.6
Glc III		
1	4.64 (*d*, 1H, *J*=7.8)	104.8
2	3.23 (*m*, 1H)	75.9
3	3.35 (*m,* 1H) [#]	78.3
4	3.34 (*m,* 1H)	71.3
5	3.50 (*m*, 1H)	78.1
6a, 6b	3.65, 3.81 (*m,* 1H, IH)	62.8
Rha		
1	4.37 (*d*, 1H, *J*=7.8)	101.1
2	3.64 (*m,* 1H)	72.2
3	3.65 (*m*, 1H)	72.4
4	3.38 (*m*, 1H)	74.0
5	3.64 (*m*, 1H)	69.8
6 (-CH$_3$)	1.27 (*d*, 3H, *J*=6.0)	18.1

Glc = Glucopyranose, **Rha** = Rhamnopyranose; [#] assignments may be interchangeable

4.2.2.3.2 Structural characterization of BL5

BL5 (1.5 mg) was obtained as a white solid from the SPE5 fraction of the CME from *Blumea lacera* using reversed-phase C-18 HPLC. **BL5** was characterized on the basis of NMR (^{1}H, ^{13}C, ^{1}H-^{1}H COSY, HSQC and HMBC), MS data and by comparison to previously published data.

The measured optical rotation of **BL5** $[\alpha]_D^{25}$ = -34.7° (in MeOH) assisted to assign absolute configuration of **BL5** because this measured optical rotation was consistent with that of capsianoside III which had an optical rotation $[\alpha]_D^{25}$ = -28.6° (in MeOH) (Lee, Kiyota et al. 2007) where it had the identical aglycone structure 17-hydroxy-6*E*,10*E*,14*Z*-(3*S*)-geranyllinalool to that indicated in **BL5**. Thus, the measured optical rotation supported the assignment of **BL5**. The UV profile of **BL5** exhibited λ_{max} at 201 and 275 nm. The maximum UV absorption at 201 nm indicated the presence of -C=C- in the compound structure while the λ_{max} at 275 nm evident the presence of –C-OH at C-3 position of **BL5**. The HR-ESI-MS positive mode generated a quasi-molecular ion peak [M+Na]$^+$ at *m/z* 799.4087 and thus, the molecular formula of $C_{38}H_{64}O_{16}$.

The ^{1}H NMR spectrum of **BL5** (Table 4.13, Spectrum: see appendix) evidenced two terminal vinyl protons as doublets at [δ_H 5.21 (*d*, 1H, *J* = 17.4 Hz, H-1a), 5.05 (*d*, 1H, *J* = 10.8 Hz, H-1b),] plus one connected olefinic proton at 5.98 ppm (*dd*, 1H, *J* = 17.4, 10.8 Hz, H-2)] indicating a mono-substituted double bond. The presence of three methine proton signals as triplets [δ_H 5.13 (*app t*, 2H, H-6, H-10), and 5.40 ppm (*app t*, 1H, *J* = 6.6, 7.2 Hz, H-14)] each adjacent to a methylene group. Also displayed were six methylene groups as multiplets at [δ_H 1.51 (2H, H$_2$-4), 2.02 (2H x 3; H$_2$-5, H$_2$-8, H$_2$-12), 2.08 (2H, H$_2$-9), 2.18 (2H, H$_2$-13) ppm], along with an oxymethylene group (δ_H 4.31 ppm, H-17a and 4.22 ppm, H-17b) evident as a doublet of doublets (*J* = 11.4, 11.4 Hz). In addition, four methyl signals as broad singlets at [δ_H 1.79 (H-16), 1.61 (H-

18), 1.62 (H-19), and 125 (H-20) ppm] were evident. These signals clearly indicated the presence of a diterpenoid moiety in **BL5**.

Further, analysis of the ^{1}H NMR spectrum of **BL5** revealed three anomeric proton signals at [δ_H 4.35 (*d*, 1H, *J* = 7.8, Glc I H-1), 4.63 (*d*, 1H, *J* = 7.8, Glc II H-1), and 4.74 (*s*, 1H, Rha H-1) ppm] indicating the presence of three sugar units; two β-glucopyranoses units and 1 deoxyhexose (α-rhamnopyranose). The proton signals of the three sugar units of **BL5** were identical to those in **BL4** and also of the published compound capsianoside III (**1**) (Terauchi 1998; Lee, Kiyota et al. 2007).

The ^{13}C NMR spectrum of **BL5** (Table 4.13, Spectrum: see appendix) revealed the presence of 20 different carbon signals which were attributed to the aglycone. These 20 carbon signals were comprised of four methine carbons at [δ_C 146.4 (C-2), 125.8 (C-6), 126.0 (C-10), and 131.3 (C-14) ppm], one methylene carbon at [δ_C 112.2 (C-1)], three quaternary carbons at [δ_C 135.4 (C-7), 136.0 (C-11), and 132.5 (C-15) ppm], a quaternary carbon bearing a hydroxyl group at δ_C 73.9 (C-3) ppm, seven methylene carbon signals appeared at [δ_C 27.7 (C-9), 40.8 (C-8), 40.8 (C-8), 40.9 (C-12), 43.5 (C-4), 62.8 (Glc II C-6), 67.6 (Glc I C-6), and 68.2 (C-17) ppm] and four methyl carbons at δ_C 22.0 (C-16), 16.1 (C-18), 16.2 (C-19), and 27.6 (C-20) ppm. The carbon signals representing the aglycone were identical to those of the known compound capsianoside III (**1**) (Izumitani, Yahara et al. 1990; Shin, Cho et al. 2012). From the literature, the configuration at C-3 was also indicated as *S*, therefore the aglycone moiety was assigned as 17-hydroxy-6*E*, 10*E*, 14*Z*-(3*S*)-geranyllinalool.

In line with the three anomeric signals in the ^{1}H NMR spectrum, the ^{13}C NMR spectrum also displayed three anomeric carbon signals at δ_C 101.1 (Gcl I C-1), 102.1 (Rha C-1), and 104.8 (Glc II C-1) ppm.
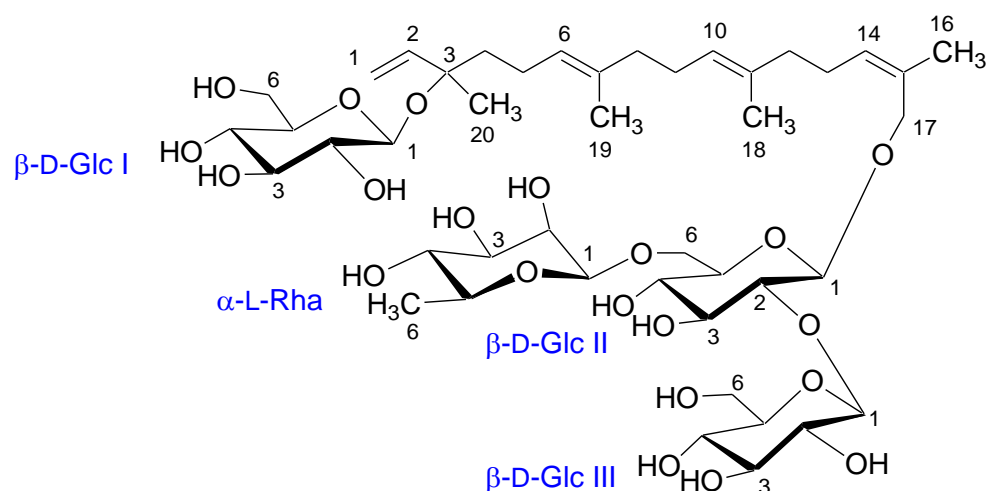
Figure 4.15 Structure of **BL5**

Table 4.13 [1]H* and ^{13}C NMR chemical shifts for **BL5** (in CD_3OD, 300 and 75 MHz, respectively)

Position	δ_H (ppm) (multiplicity, J, Hz)	δ_C (ppm)
1	5.21, 5.04 (*d*, 1H, *J*=17.4, *d*, 1H, *J*=10.8)	112.0
2	5.98 (*dd*, 1H, *J*=17.4)	144.5
3		73.9
4	1.51 (*m*, 2H)	43.5
5	2.02 (*m*, 2H)	23.7
6	5.13 (*app t*, 1H) [#]	125.8
7		135.4
8	2.02 (*m*, 2H)	40.8
9	2.08 (*m*, 2H)	27.7
10	5.13 (*t*, 1H) [#]	126.0
11		136.0
12	2.02 (*m*, 2H)	40.9
13	2.18 (*m*, 2H)	27.2
14	5.40 (*app t*, 1H, *J*=6.6, 7.2)	131.3
15		132.5
16	1.79 (*br S*, 3H)	22.0
17	4.31, 4.22 (*dd*, 2H, *J*=11.4, 11.4)	68.2
18	1.61 (*br S*, 3H)	16.1
19	1.61 (*br S*, 3H)	16.2
20	1.25 (*br S*, 3H)	27.6
Glc I		
1	4.35 (*d*, 1H, *J*=7.8)	101.1
2	3.67 (*m*, 1H)	81.9
3	3.55 (*t*, 1H, *J*=7.8, 9.0)	77.8
4	3.28 (*m*, 1H)	71.5
5	3.35 (*m*, 1H)	76.7
6a, 6b	3.97, 3.84 (*d*, 1H, *J*=10.8, *app t*,1H, *J*=1.2, respectively)	67.6
Glc II		
1	4.63 (*d*, 1H, *J*=7.8)	104.8
2	3.24 (*m*, 1H)	75.9
3	3.35 (*m*, 1H) [#]	78.3
4	3.35 (*m*, 1H)	71.3
5	3.50 (*t*, 1H, *J*=7.8, 9.0) [#]	78.2
6a, 6b	3.84, 3.67 (*m*, 1H, IH)	62.8
Rha		
1	4.74 (*s*, 1H)	102.1
2	3.67 (*m*, 1H)	72.2
3	3.67 (*m*, 1H)	72.4
4	3.38 (*m*, 1H)	74.0
5	3.67 (*m*, 1H)	69.8
6 (-CH$_3$)	1.27 (*d*, 3H, J=6.0)	18.1

Glc = Glucopyranose, **Rha** = Rhamnopyranose; [1]H* NMR: run on 600 MHz; [#]assignments may be interchangeable

The HMBC correlations for the aglycone of **BL5** (Figure: 4.16) confirmed the aglycone identity through the following correlations: δ_H 5.21 and 5.04 (H$_2$-1) to δ_C 73.9 (C-3); δ_H 1.25 (H$_3$-20) to δ_C 146.37 (C-2), 43.45 (C-4); δ_H 1.61 (H$_3$-19) to δ_C 125.8 (C-6), 135.4 (C-7), 40.8 (C-8); δ_H 2.08 (H-9) to δ_C 135.4 (C-7); δ_H 1.61 (H$_3$-18) to δ_C 136.0 (C-11), 40.9 (C-12); δ_H 2.02 (H-8) to δ_C (C-9) and δ_H 2.02 (H-12) to δ_C (C-13); δ_H 1.79 (H$_3$-16) to δ_C 131.3 (C-14), 132.5 (C-15), 68.2 (C-17); δ_H 4.31 (H-17) to δ_C 131.3 (C-14), 132.5 (C-15), 22.0 (C-16). Assignment of the aglycone of **BL5** was done on the basis of the above HMBC correlations, ^{1}H-^{1}H COSY data (Figure 4.18) and comparison with published literature (Izumitani, Yahara et al. 1990; Shin, Cho et al. 2012).
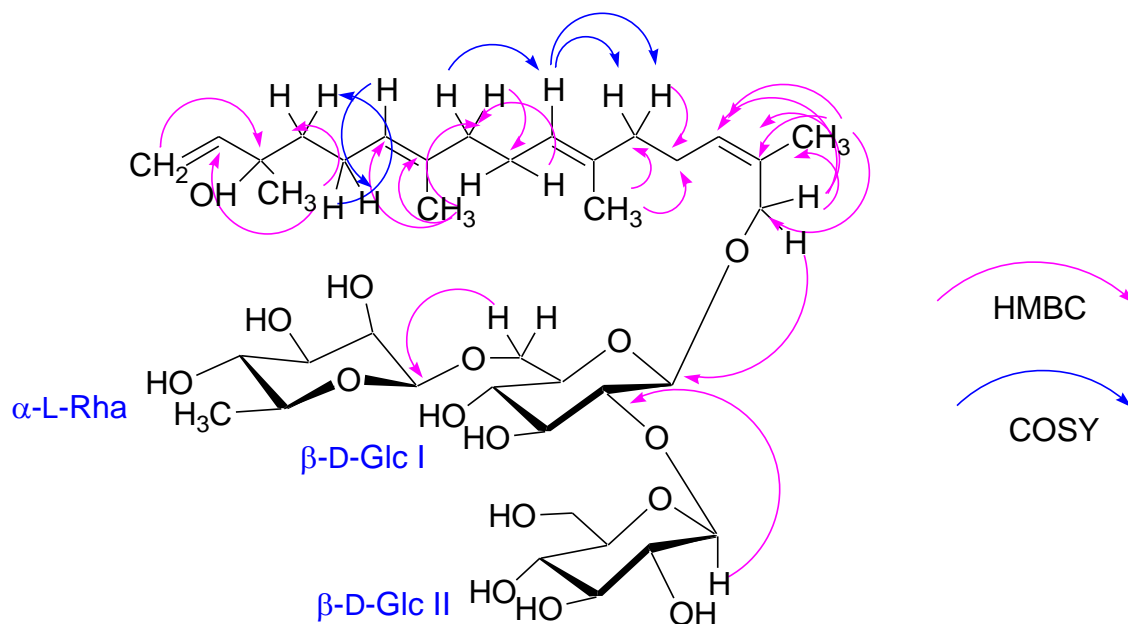
Figure 4.16 Key HMBC and ^{1}H-^{1}H COSY correlations observed in **BL5**

The HMBC correlations revealed the sugar unit connectivities as; correlation between the signal δ_H 4.22 (*d*, 1H, *J* = 7.8, H-17) of the aglycone to δ_C 101.1 (Glc1 C-1) indicating Glc I H-1 to be linked to C-17 of the aglycone; δ_H 4.63 (*d*, 1H, *J* = 7.8) to δ_C 81.9 (Glc I C-2) showing Glc II H-1 to be connected to Glc I C-2; δ_H 3.97 (Glc I H-6) to δ_C 102.1 (Rha C-1), indicating Rha C-1 to be

linked to Glc I H-6. The sugar moieties of **BL5** linked at 17-*O*- was assigned as 17-*O*-β-D-glucopyranosyl-(1→2)-α-L-rha-(1→6)-α-L-rhamnopyranoside based on ^{1}H, ^{13}C NMR data, as well as comparied to that published for capsianoside III (Lee, Kiyota et al. 2007).

BL5 has one less glucopyranosyl residue than **BL4**. It is missing the β-Glc linked to the C-3 position. This was also evident by changes in the chemical shifts assignable to C-1, C-2, C-3, and C-20 in the aglycone part of -3.6, +1.9, -7.6, and +4.4 ppm, respectively. Assignments as D or L sugars are based on consistency with published data [29]. Therefore, in summary the structure of **BL5** was assigned as novel 6*E*, 10*E*, 14*Z*-(3*S*)-17-hydroxygeranyllinalool-17-*O*-β-D-glucopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→6)]-β-D-glucopyranoside (Figure 4.15).

4.2.2.3.3 Structural characterization of BL6

BL6 (2.5 mg) was isolated as a white solid from the SPE5 fraction of the CME of *Blumea lacera* using reversed-phase HPLC. Even after extensive further purification this compound still contained impurities approximately in the ratio of 1:0.3 (**BL6**: Impurity). Thus, a tentative structure was determined, that to date has not been confirmed by LR-ESI-MS. The LR-ESI-MS negative mode generated what appeared to be a quasi-molecular ion peak [M-H]⁻at *m/z* 520.7, thus the molecular formula was determined, $C_{25}H_{45}O_{11}$. Unfortunately, the deduced tentative structure of **BL6** gave the molecular formula of $C_{27}H_{46}O_{11}$ assigned on the basis of NMR experiments (^{1}H, ^{13}C, ^{1}H-^{1}H COSY, HSQC and HMBC). The UV profile of **BL6** displayed λ_{max} at 199 and 292 nm. The maximum absorption of UV light at 199 nm indicated the presence of -C=C- in the compound structure whereas the λ_{max} at 292 nm evident the presence of –CH$_2$-OH at C-1 position of **BL6**.

^{1}H NMR spectrum of **BL6** (Table 4.14, Spectrum: see appendix) revealed the presence of three methine olefinic proton signals at δ_H 5.36 (*app t*, 1H, H-1) overlapped with δ_H 5.40 (*app t*, 1H, *J*

= 7.5, 6.3 Hz, H-9), and 5.14 (m, 1H, H-5) ppm. Also evident were six methylene protons comprising four different methylene proton signals at [δ_H 2.02 (m, 2H x 2, H$_2$-3, H$_2$-7), 2.08 (m, 2H, H$_2$-4), 2.18 (m, 2H, H$_2$-8), oxymethylene protons at δ_H 4.34 and 4.15 ppm (d, 1H, J = 11.8, 1.8 Hz, 12-Ha and d, 1H, J = 11.8, 1.3 Hz, 12-Hb) along with a methylene proton signal at δ_H 4.11 ppm (2H, 15-Ha and 15-Hb) evidently attached to a terminal hydroxyl group. Further evident were three methyl proton signals at [δ_H 1.77 (br s, 3H, H$_3$-11), 1.63 (d, 3H x 2, J = 5.4, H$_3$-13 and H$_3$-14) ppm]. Based on these proton signals and comparison with published data of compound **1** (crenulatoside E) (Magid, Voutquenne-Nazabadioko et al. 2008), the aglycone was assigned as 12-hydroxyfarnesol. Two anomeric proton signals were evident at δ_H 4.21 (d, 1H, J = 7.8, Glc C-1) and at δ_H 4.74 (br s, 1H, Rha C-1) indicating the presence of two sugar units. These were assigned as a glucopyranose and a rhamnopyranose linked to the 12-O-postion of aglycone, on the basis of comparison to compound **5** [(1-O-[α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranosyl]-(2E, 6E)-farnesol)] previously reported (Magid, Voutquenne-Nazabadioko et al. 2005).

The ^{13}C NMR spectrum of **BL6** (Table 4.14, Spectrum: see appendix) disclosed the presence of 15 different carbon signals which were attributed to the aglycone. Of these, three methine carbon signals were observed at [δ_C 121.5 (C-1), 125.9 (C-5), and 131.4 (C-9) ppm]. A further, six methylene carbon signals were evident [δ_C 40.9 (C-3), 27.1 (C-4), 40.9 (C-7), 27.1 (C-8), 67.7 (C-12)] and an oxymethylene carbon signal 67.2 (C-15) bearing terminal hydroxyl group, and three quaternary carbon signals at [δ_C 123.0 (C-2), 136.5 (C-6), and 132.1 (C-10) ppm, respectively]. **BL6** further contained three methyl carbon signals at [δ_C 21.9 (C-11), 16.0 (C-13), and 23.4 (C-14). **BL6** also displayed two anomeric carbon signals at [δ_C 102.1 (Rha C-1) and 102.3 (Glc C-1) ppm further supporting the presence of 2 sugar moieties, in addition to the

aglycone. The aglycone, sugar linkage and connectivity of the sugar moieties to the aglycone were established from the HMBC correlations and by comparison to **BL4**, **BL5** and published data (Magid, Voutquenne-Nazabadioko et al. 2005).
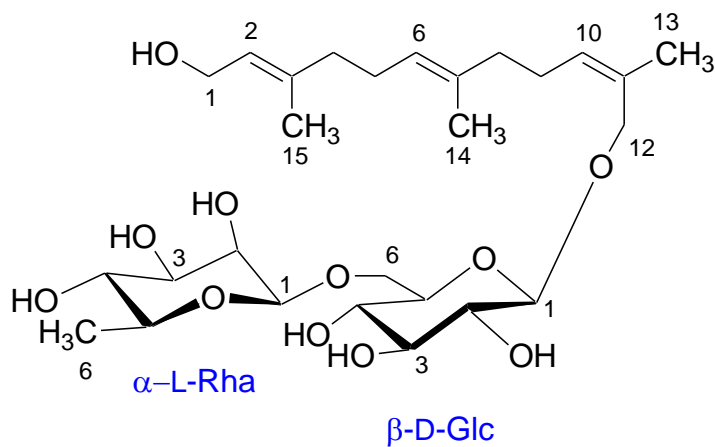
Figure 4.17 Structure of **BL6**

Table 4.14 ^{1}H and ^{13}C NMR chemical shift values for **BL6** (in CD$_3$OD, 300 and 75 MHz, respectively)

Position	δ_H (ppm) (multiplicity, J, Hz)	δ_C (ppm)	HMBC
1	5.36	121.5	
2		123.0	
3	2.02 (*m*, 2H)	40.9	C-5
4	2.08(*m*, 2H)	27.1	
5	5.14 (*m*, 1H)	125.9	
6		136.0	
7	2.02 (*m*, 2H)	40.9	C-6, C-5
8	2.18 (*m*, 2H)	27.1	
9	5.40 (*app t*, 1H, *J*=6.3, 7.5)	131.4	
10		132.1	
11	1.77 ((*br s*, 3H))	21.9	C-12, C-8
12	4.34, 4.15 (*d*, 1H, *J*=11.8, 1H, *J*=11.8)	67.7	C-9
13	1.63 (*d*, 3H, *J*=5.4)	16.0	C-6, C-5
14	1.63 (*d*, 3H, *J*=5.4)	23.4	C-1, C-2, C-3
15	3.98, 4.11(1H, 1H)	67.2	
Glc			
1	4.21 (*d*, 1H, *J*=7.8)	102.3	C-12
2	3.18 (*m*, 1H)	75.0	
3	3.35 (*m*, 1H)	78.1	
4	3.34 (*m*, 1H)	71.6	
5	3.36 (*m*, 1H)	76.8	
6a, 6b	3.94, 3.60 (1H, 1H)	67.7	Rha C-1
Rha			
1	4.74 (*br s*, 1H)	102.1	Glc C-6
2	3.69 (*m*, 1H)	72.2	
3	3.66 (*m*, 1H)	72.4	
4	3.40 (*m*, 1H)	74.1	
5	3.67 (*m*, 1H)	69.8	
6 (-CH$_3$)	1.27 (*d*, 3H, *J*=6.0)	18.1	

Glc = Glucopyranose, **Rha** = Rhamnopyranose

The HMBC spectrum of **BL6** (Table 4.14; Figure 4.20, Spectrum: see appendix) revealed the following correlations: δ_H 1.63 (H$_3$-14) to δ_C 40.9 (C-3), 121.5 (C-1), 123.0 (C-2); δ_H 2.02 (H-3, H-7) to δ_C 125.9 (C-5) and 136.5 (C-6); δ_H 1.63 (H$_3$-13) to δ_C 125.9 (C-5), 136.5 (C-6); 2.02 (H-7) to δ_C 136.5 (C-6), 125.9 (C-5); δ_H 2.18 (H-8) to 21.9 (C-11); δ_H 1.77 (H-11) to δ_C 27.1 (C-8), 131.4 (C-9), 132.4 (C-10), 67.5 (C-12). These correlations confirmed the connectivity from C-1 to C-14 of the aglycone with COSY further confirming the linkage of C-4 to C-5 and C-7 to C-8. Neither HMBC nor COSY could reveal the indicated correlation of C-15 to C-1, however this linkage was assigned by comparison with published data of compound **1** (crenulatoside E)

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((Magid, Voutquenne-Nazabadioko et al. 2008). The HMBC spectrum of **BL6** confirmed the sugar linkage with correlations between δ_H 4.21 (Glc H-1) and δ_C 67.7 (C-12) indicating that Glc H-1 was linked to C-12 of the aglycone. The correlation at δ_H 4.74 (Rha H-1) to δ_C 67.7 (Glc C-6) revealing the Rha H-1 to be linked to the Glc C-6.

Based on the ^{1}H, ^{13}C, ^{1}H-^{1}H COSY (Figure 4.17), HMBC experiments, as well as by comparison with data of a compound previously reported in the literature (Magid, Voutquenne-Nazabadioko et al. 2005; Magid, Voutquenne-Nazabadioko et al. 2008), **BL6** was assigned as novel 2*E*, 6*E*, 10*Z*-12-hydroxyfarnesol-12-*O*-β-D-glucopyranosyl-(1→6)-α-L-rhamnopyranoside (Figure 4.17).
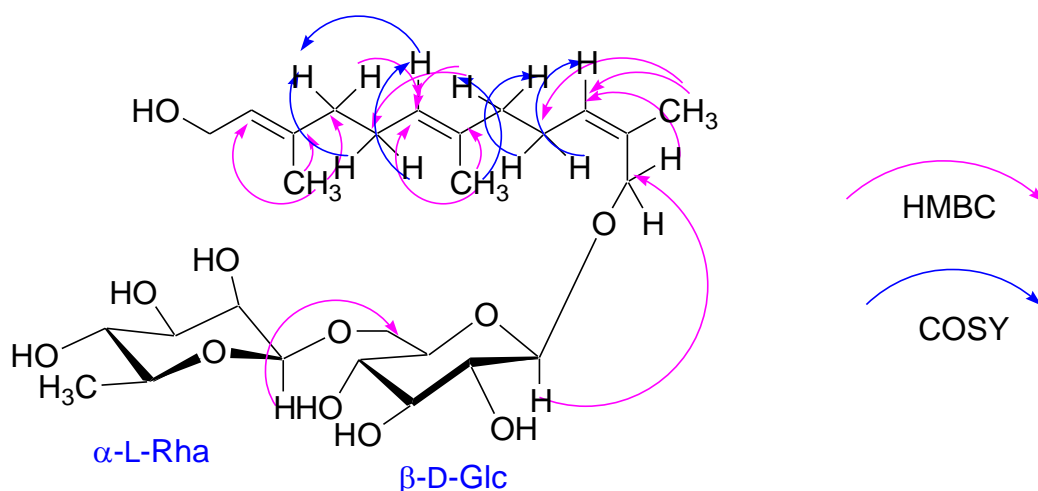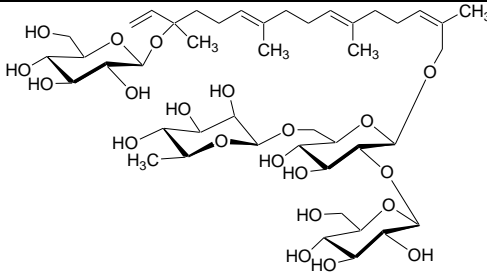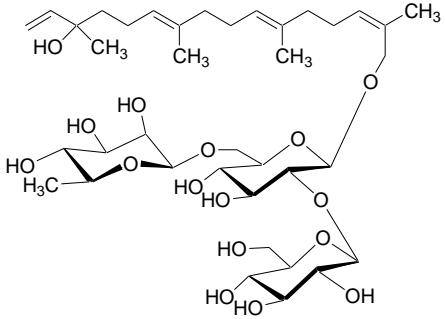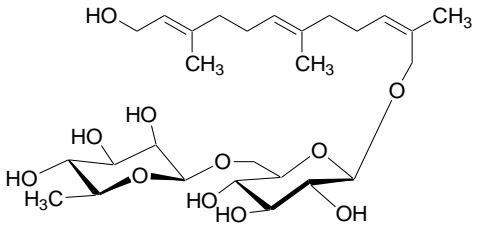
Figure 4.18 Key HMBC and ^{1}H-^{1}H COSY correlations observed in **BL6**

Table 4.15 List of compounds isolated from *Blumea lacera* in this project

Compound	Compound name	Structure	Previous report on isolation from *Blumea lacera*	Novelty
BL1	Kaempferol-3-*O*-(2"6"-di-*O*-α-L-rhamnopyranosyl)-β-D-galactopyranoside		No	Known
BL2	Kaempferol-3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside		No	Known
BL3	(25*R*)-3β-{*O*-β-D-glucopyranosyl-(1→4)-*O*-α-L-rhamnopyranosyl-(1→4)-[*O*-α-L-rhamnopyranosyl-(1→2)]-α-L-rhamnopyranosyl}-22αN-spirosol-5-ene		No	Novel

BL = *Blumea lacera*

Table 4.15 List of compounds isolated from *Blumea lacera* in this project (contd.)

Compound	Compound name	Structure	Previous report on isolation from *Blumea lacera*	Novelty
BL4	3-*O*-β-D-glucopyranosyl-17-hydroxy-6*E*, 10*E*, 14*Z*-(3*S*)-geranyllinalool 17-*O*-β-D-glucopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→6)]-β-D-glucopyranoside		No	Known
BL5	6*E*, 10*E*, 14*Z*-(3*S*)-17-hydroxygeranyllinalool-17-*O*-β-D-glucopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→6)]-β-D-glucopyranoside		No	Novel
BL6	2*E*, 6*E*, 10*Z*-12-hydroxyfarnesol-12-*O*-β-D-glucopyranosyl-(1→6)-α-L-rhamnopyranoside		No	Novel [#]

BL = *Blumea lacera*, [#]Tentative structure

CHAPTER FIVE

STEROIDAL GLYCOALKALOIDS AND THEIR CYTOTOXICITY

5.1 Background

Steroidal glycoalkaloids (SGAs) are an array of natural products with structural diversity which possess a wide spectrum of biological activities (Li, He et al. 2007; Cui 2012). SGAs are frequently found in *Solanum* species such as in *Solanum tuberosum* (potato), *Solanum lycoperiscum* (tomato), *Solanum nigrum* (blackberry nightshade) and *Solanum melongena* (eggplant) (Li 2007; Milner 2011). SGAs have also been found in *Veratrum grandifolium*, which belongs to the monocotyledonous *Lilliaceae* family (Nahar 2011). The amount of SGAs present depends on the plant species, plant part, injury and exposure to light (Patel 2013). Although steroidal glycoalkaloids are not necessary for plant growth and function, they provide defense for plants against insects, pests, herbivores and phytopathogens. They have been found to exhibit toxicity to a number of organisms such as fungi and humans in a concentration dependent manner (Milner 2011; Patel 2013).

SGAs structurally consist of two components, namely the aglycone unit and the glycosidic moieties. The amphiphilic nature of SGAs is attributed to the hydrophobic nature of the aglycone unit and the hydrophilic nature of the glycosidic moieties. The aglycone unit comprises a 27-carbon skeleton of cholestane with nitrogen incorporated into the F ring, with the glycosidic moieties attached at the 3-OH position of the aglycone (see example of α-chaconine in Figure 5.1). The aglycones of SGAs are divided into five different classes according to their structures such as; solanidanes with fused indolizidine rings, spirosolanes with an oxa-azaspirodecane system, the (22, 26)-epiminocholestanes, α-epiminocyclohemiketals, and 3-aminospirostanes. SGAs differ slightly in their structure from steroidal saponins, where the steroidal aglycone of the SGA contains nitrogen instead of oxygen at ring F. Steroidal saponins are mainly found in plants, but also occur in some marine organisms and insects (Thakur Mayank, Melzig et al, 2011).
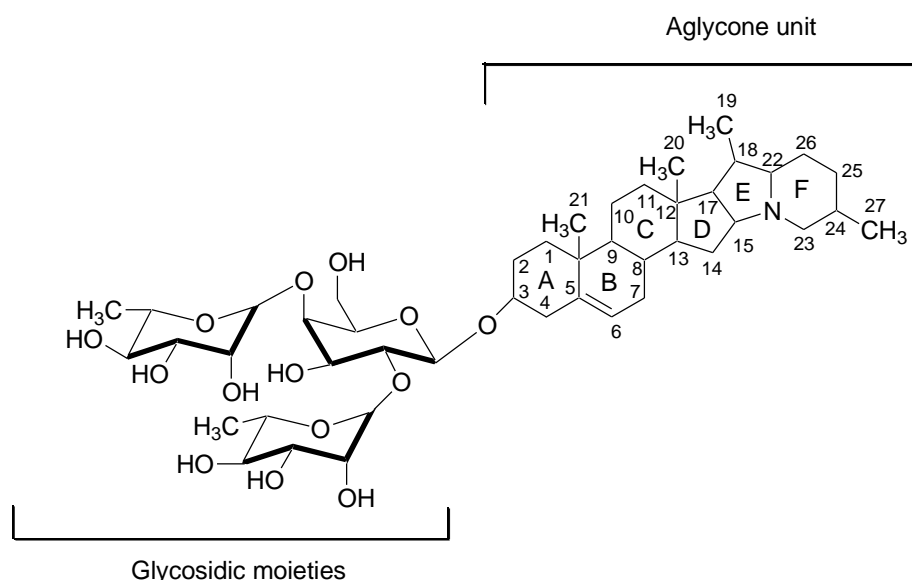
Figure 5.1 Structure of α-chaconine

The steroidal alkaloid, solasodine is being used in the pharmaceutical industry for the synthesis of steroids, such as progestagens, androgens, estrogens, norsteroids, and diuretic spirolactones (Nino, Correa et al. 2009). Research in glycobiology has revealed that glycoconjugates play an important role in the immune response, viral and bacterial infection, regulation, differentiation and development of cells, inflammation, cell adhesion and many other inter and intracellular communication processes (Nakamura 1996). SGAs are reported to possess a wide range of bioactivities. The biological activity of SGAs is attributed to various mechanisms of action; either due to the inhibition of the acetylcholinesterase (AChE), as AChE causes the termination of cholinergic transmission at the neuromuscular junction and the central nervous system or, by disruption of cell membranes and loss of cell membrane integrity through binding with sterol components of the cell membrane (Milner 2011; Mweetwa, Hunter et al. 2012).

In our study, we have isolated one novel SGA **BL3** from *Blumea lacera* leaves comprising of the aglycone, α-solasodine, and glycosidic moieties, one glucopyranose and three rhamnopyarnose moieties. As structural differences both in aglycone and glycosidic moieties influence the bioactivities of SGAs at a signicant level (Milner 2011), we have selected some SGAs analogous to **BL3** to study structure-activity relationship (SAR).

5.2 Common steroidal glycoalkaloids

More than 100 structurally different glycoalkaloids belonging to solanidane and spirosolane classes have been identified in over 350 *Solanum* species (Milner 2011; Patel 2013). A number of *Solanum* species producing common SGAs are enlisted in table 5.1., namely α-solamargine, α-solasonine, α-solanine, α-chaconine, α-tomatine and dehydrotomatine, with α-solamargine occuring in at least 100 *Solanum* species; such as, *S. nigrum* (Liu, Li et al. 2007; Chen, Li et al. 2010), *S. lycocarpum* (Schwarz, Pinto et al. 2007; Munari Carla, de Oliveira Pollyanna et al. 2013) and *S. incanum* (Li, Zhao et al. 2011; Manase, Mitaine-Offer et al. 2012). Furthermore, α-solanine and α-chaconine account for 90% of total glycoalkaloids produced in *S. tuberosum* (potato) (Yang, Paek et al. 2006; Milner 2011). Principal glycoalkaloids found in *S. lycoperiscum* (tomato) are α-tomatine and dehydrotomatine, whereas solasonine is predominantly found in *S. melongena* (eggplant) (Nahar 2011). SGAs structurally differ from each other, either by differences in the aglycone units or varying glycosidic moieties. Table 5.1 represents the structures and highlights the structural differences of the selected SGAs in our study.

The compound, α-solasodine is the aglycone for α-solasonine which contains also solatriose as the glycosidic moieties. Moreover, α-solasodine is the aglycone for α-solarmargine, but contains chacotriose as the sugar moieties.

The compound β-solamargine has β-solasodine as an aglycon rather than α-solasodine. It also contains chacotriose, but the linkages are slightly different.

Another compound with α-solasodine is the aglycone khasianine.

β-solamargine differs from khasianine not only by the stereochemistry of the amine group (22αN or 22βN) in the aglycone, but it contains also one extra rhamnose moiety in the sugar moiety as well as β-solamargine contains 22βN whereas khasianine possesses 22αN.

Another compound, β-solamarine, shares the same glycosidic residues, solatriose, with α-solanine but both contain different aglyones, tomatidenol in β-solamarine and solanidine in solanine. Similarly, α-solasonine and α-solanine possess the same sugar residues (solatriose) but contain different aglycones solasodine and solanidine, respectively (Table 5.1). The aglycone solanidine is also found in the SGA α-chaconine, and it also contains a solatriose as present in α-solanine, however, it has two rhamnose and one galactose moieties rather than one rhamnose, one galactose and one glucose residues.

Solasodine has a double bond between C4 and C5, which is absent in tomatidine. The structural differences between the SGAs play a crucial role in defining their bioactivities which will be discussed in the next section.
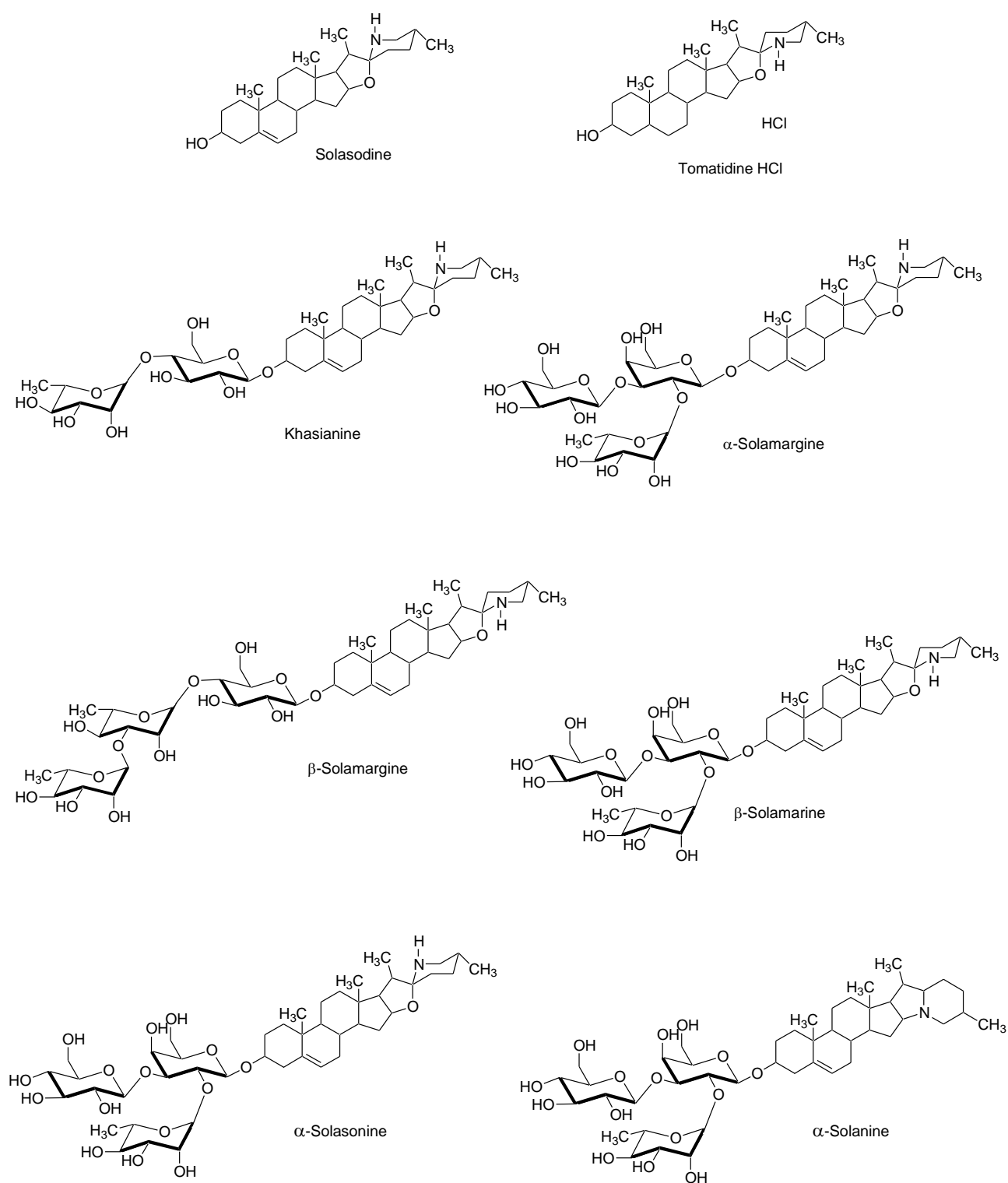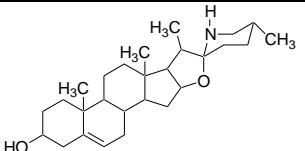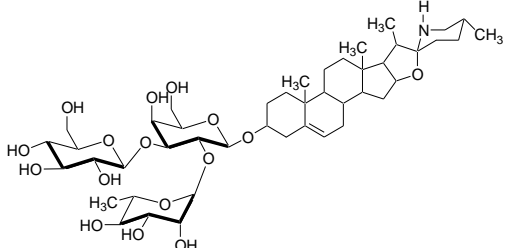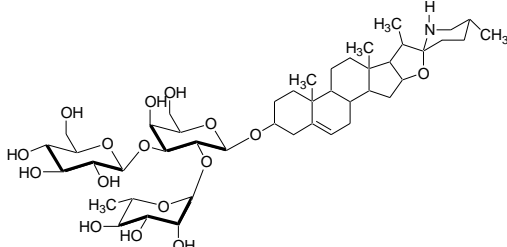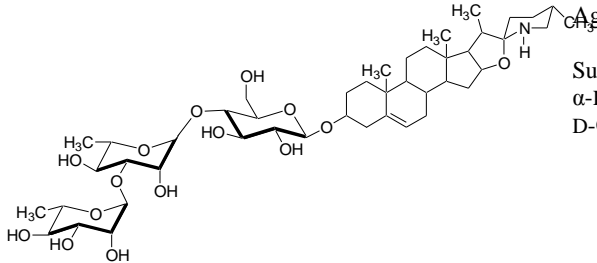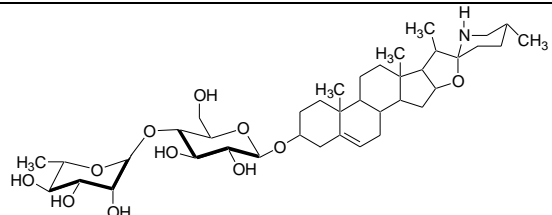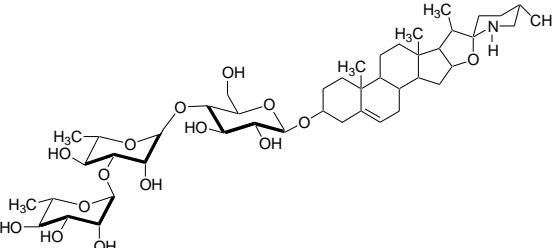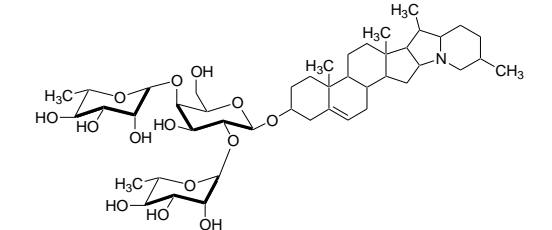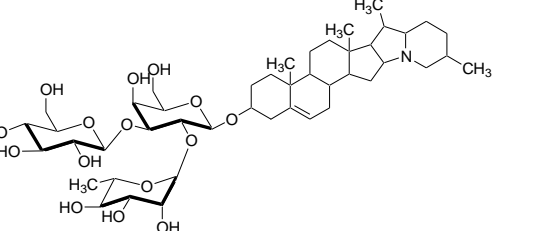
Figure 5.2 Selected steroidal alkaloids and their glycosides

Table 5.1 Structural differences of the selected steroidal glycoalkaloids

Compound	Chemical name	Plant species	Structure	Structural difference
α-solasodine	(25R)-3β-22αN-spirosol-5-ene	*Solanum flaccidum* (de Almeida and Rocca 1995); *S. indicum* (Rathore, Sharma et al. 1978).		Aglycone: α-solasodine
α-solasonine	(25R)-3β-{O-β-D-galactopyranosyl-(1→2)-[O-β-D-glucopyranosyl-(1→3)]-(O-α-L-rhamnopyranosyl}-22αN-spirosol-5-ene	*Solanum crinitum.* (Cornelius, de Carvalho et al. 2010); *S. incanum* (Liu, Liang et al. 2004).		Aglycone: α-solasodine Sugar moieties (solatriose): β-D-Glc-(1→3)-β-D-Gal-(1→2)-α-L-Rha
α-solamargine	(25R)-3β-{O-β-D-glucopyranosyl-(1→4)-O-α-L-rhamnopyranosyl-(1→2)-(O-α-L-rhamnopyranosyl}-22αN-spirosol-5-ene	*Solanum crinitum.* (Cornelius, de Carvalho et al. 2010), *S. Lycocarpum* (Munari Carla, de Oliveira Pollyanna et al. 2013).		Aglycone: α-solasodine Sugar moieties (chacotriose): α-L-Rha-(1→2)- β-D-Glc -α-L- Rha-(1→4)
β-solamargine	(25R)-3β-{O-β-D-glucopyranosyl-(1→4)-O-α-L-rhamnopyranosyl-(1→2)-(O-α-L-rhamnopyranosyl}-22βN-spirosol-5-ene	No reports found		Aglycone: β-solasodine Sugar moieties (chacotriose): α-L-Rha-(1→2)-α-L- Rha-(1→4)-β-D-Glc
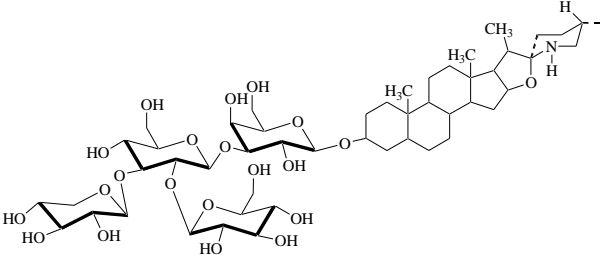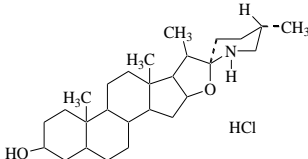
Gal: Galactose, Glc: Glucose, Rha: Rhamnose

Table 5.1 Structural differences of the selected steroidal glycoalkaloids (contd.)

Compound	Chemical name	Plant species	Structure	Structural difference
Khasianine	(25R)-3β-{O-β-D-glucopyranosyl-(1→4)-O-α-L-rhamnopyranosyl}-22αN-spirosol-5-ene	*Solanum surattense* (Ahmed, Sharif et al. 2011); *S. suaveolens* (Ripperger and Porzel 1997).		Aglycone: α-solasodine Sugar moieties: α-L-Rha-(1→4)- β-D-Glc
β-solamarine	(25S)-3β-{O-β-D-galactopyranosyl-(1→2)-[O-β-D-glucopyranosyl-(1→3)]-(O-α-L-rhamnopyranosyl}-22βN-spirosol-5-ene	*Solanum lycocarpum* (Munari Carla, de Oliveira Pollyanna et al. 2013); *S. sisymbriifolium* (Bagalwa, Voutquenne-Nazabadioko et al. 2010).		Aglycone: tomatidenol Sugar moieties (solatriose): β-D-Glc-(1→3)-β-D-Gal-(1→2)-α-L-Rha
α-chaconine	(25βH)-3β-{O-β-D-galactopyranosyl-(1→2)-[O-α-L-rhamnopyranosyl-(1→4)]-(O-α-L-rhamnopyranosylolanum-22αH-solanid-5-ene	*Solanum tuberosum* (Milner 2011)		Aglycone: solanidine Sugar moieties (solatriose): α-L-Rha-rham-(1→4)-β-D-Gal-(1→2)-α-L-Rha
α-solanine	(25βH)-3β-{O-β-D-galactopyranosyl-(1→2)-[O-β-D-glucopyranosyl-(1→3)]-(O-α-L-rhamnopyranosylolanum-22αH-solanid-5-ene	*Solanum. tuberosum* (Milner 2011) *S. nigrum* (Ji, Gao et al. 2008).		Aglycone: solanidine Sugar moieties (solatriose): β-D-Glc-(1→3)-β-D-Gal-(1→2)-α-L-Rha

Gal: Galactose**,** Glc: Glucose**,** Rha: Rhamnose

Table 5.1 Structural differences of the selected steroidal glycoalkaloids (contd.)

Compound	Chemical name	Plant species	Structure	Structural difference
α-tomatine	(22*S*,25*S*)-5α-spirosolan-3β-ylβ-D-glucopyranosyl-(1→2)-[β-D-xylopyranosyl-(1→3)]-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside	*S. lycopersicum* (Milner 2011)		Aglycone: tomatidine Sugar moieties (lycotetraose): β-D-Gal-(1→3)-β-D-Glc-(1→3)- β-D-Glc-(1→2)- β-D-Xyl-(1→3)
Tomatidine HCl	(3β,5α,22β,25*S*)-spirosolan-3-ol hydrochloride	No reports found		Aglycone: tomatidine

Gal: Galactose, Glc: Glucose, Xyl: Xylose

5.3 Bioactivity of common steroidal glycoalkaloids

SGAs are known to possess a wide range of bioactivities such as antifungal, anti-inflammatory, antiviral, antiprotozoal, anti-malarial, antibiotic, antioxidant, anticonvulsant and CNS (Central nervous system) depressant, and antipyretic effects (Table 5.2). A significant number of reports exist on the cytotoxic/anticancer activity including apoptosis of SGAs (Table 5.2). Here only cytotoxic/anticancer and antifungal activities are discussed as these are the activities that were tested in the present study.

Cytotoxic/anticancer and anifungal activities of each of the selected SGAs with their possible mechanism of action are discussed below.

5.3.1 Bioactivity of solasodine

Solasodine, the aglycone of the SGA α-solamargine and α-solasonine, was isolated from *S. nigram* and *S. dolcamra* and has drawn profound attention due to its anticancer activity (Kusano, Takahashi et al. 1987; Lin, Lu et al. 1990; Nakamura 1996; Wang, Gu et al. 2000; Esteves-Souza, Sarmento da Silva et al. 2002; Emmanuel, Ignacimuthu et al. 2006; Zha 2010; Pandurangan, Khosa et al. 2011; Cui 2012; Abreu Miranda, Tiossi et al. 2013).

Its apoptosis potential has been studied against various cancer cell lines such as colon (HCT116, HT-29), cervix (HeLa), breast (MCF-7), osteosarcoma (1547) and human hepatoma (Hep3B) cancer cells. However, it showed weak apoptosis activity in all these studies (Chang, Tsai et al. 1998; Trouillas, Corbiere et al. 2005; Koduru, Grierson et al. 2007; Bhutani, Paul et al. 2010). This weak apoptosis activity was attributed to the absence of sugar moieties. Solasodine induced apoptosis through DNA fragmentation when tested against human 1547 osteosarcoma cells (Trouillas, Corbiere et al. 2005).

Solasodine also inhibited cell growth of cervix (HeLa), breast (MCF-7), and colon (HT-29) cancer cell lines by arresting the cell cycle in the G0/G1 phase after 24 h exposure with an increase of 11.2% of G0/G phase arrest (Koduru, Grierson et al. 2007).

Furthermore, solasodine hydrochloride, a salt of a steroidal alkaloid, exhibited significant anti-tumor activity against human cervical cancer cells (HeLa) (Milner 2011). It was found to exert anticancer activity through induction of apoptosis (Ai, Liu et al. 2011).

Antifungal activity of solasodine has been reported against various fungi species such as against *Candida albicans*, *C. pseudotropicalis*, *C. famata*, *C. kefyr*, *Cryptococcus albidus*, *C. neoformans*, *Rhodotorula glutinis*, *Trichosporon cutaneum*, *Geotrichum candidum*, *Aspergillus fumigatus*, *A. candidus*, *Fonsecaea pedrosoi*, *Microsporum gypseum*, *Phialophora verrucosa*, *Sporothrix schenckii*, *Trichophyton rubrum sc.*, and *T. mentagrophytes sc* (Kusano, Takahashi et al. 1987).

5.3.2 Bioactivity of α-solasonine

The SGA α-solasonine, a glycoside of solasodine, can be found in *S. crinitum*, and *S. incanum* (Table 5.1). It has shown potent cytotoxicity against colon (HT-29, HCT-116 and SW620), breast (MCF-7), liver (HepG2), lung (PC-6), stomach (NUGC-3), ileocecal (HCT-8), Ehrlich carcinoma, mouse leukaemia (P388) and human leukaemia cells (K562) (Nakamura, Komori et al. 1996; Nakamura 1996; Esteves-Souza, Sarmento da Silva et al. 2002; Ikeda, Tsumagari et al. 2003; Lee, Kozukue et al. 2004; Li, He et al. 2007).

Antifungal activity of α-solasonine is also well reported against different fungi species (Table 5.2) such as *Cercosporella brassicae* and *Alternaria porri* (Zhao, Gao et al. 2009). Another report demonstrated the potent fungicidal effect of α-solasonine with MIC of <0.24 µg/mL against *Microsporum canis*, *M. gypseum*, *Trichophyton rubrum*, *T. mentagrophytes* (Pinto, Uchoa et al. 2011) Fungicidal activity of α-solasonine has also been reported against *Sclerotinia fructicola*, *Claviceps purpurea*, *Trichothecium roseum*, *Piricularia oryzae*, and *Fomes officinalis*, *Botrytis allii*, *Alternaria solani*, *Coniophora cerebella*, *Rhizoctonia solani*, *Schizophyllum commune* and three *Fusarium* species (Wolters 1968).

5.3.3 Bioactivity of α-solamargine

The compound α-solamargine, also a glycoside of solasodine isolated from *Solanum* species, has been found to exert significant anticancer effects against cancer cell lines such as colon, breast,

prostate, human hepatoma, lung, brain, liver, stomach, cervix and ileocecal, and human uterus cancer cells (Saijo, Murakami et al. 1982; Lin 1990; Nakamura 1996; Hu 1999; Kuo 2000; Esteves-Souza, Sarmento da Silva et al. 2002; Ikeda, Tsumagari et al. 2003; Lee, Kozukue et al. 2004; Liu 2004; Yang, Paek et al. 2006; Li 2007; Zha 2010; Sun, Zhao et al. 2011; Cui 2012; Abreu Miranda, Tiossi et al. 2013; Munari Carla, de Oliveira Pollyanna et al. 2013). It exerted a greater cytotoxic potential than cisplatin, methotrexate, 5-fluorouracil, epirubicin, and clycophosphamide against human breast cancer cells (Shiu, Chang et al. 2007). It triggers extrinsic and intrinsic apoptotic pathways of breast cancer cells through activation of caspase-3, caspase-8 and caspase-9, as well as it up-regulates the external death receptors (Kuo 2000; Shiu, Chang et al. 2007). Studies have shown that over expression of Bcl-2 and Bcl-xL, which can cause resistance to an anticancer drug such as cisplatin, can be overcome by the use of α-solamargine. It is therefore postulated, that the combination therapy of α-solamargine and cisplatin may be effective in cisplatin-resistant breast cancer (Pietras, Poen et al. 1999; Shiu, Chang et al. 2007). Moreover, α-solamargine in combination with methotrexate, 5-fluorouracil and cisplatin enhances cytotoxicity in breast cancer cells (ZR-75-1), through down-regulation of HER2/neu. HER2/neu, if over-expressed would increase resistance to methotrexate, 5-fluorouracil and cisplatin, drugs that are commonly used to treat breast carcinoma (Shiu, Liang et al. 2008). Furthermore, α-solamargine induces apoptosis and enhances susceptibility to trastuzumab and epirubicin in breast cancer cells (MCF-7 and SK-BR-3 cells) and non-cancerous breast epithelial cells (HBL-100) with low or high expression levels of HER2/neu (Shiu, Liang et al. 2009). Another report demonstrated that α-solamargine induces apoptosis on gastric cancer cells (MGC-803) through decrease of mutation p53, increase of the ratio of Bax to Bcl-2 and the activation of caspase (Ding, Zhu et al. 2013). Furthermore, the anticancer effect of α-solamargine involves triggering the gene expression of human tumor necrosis factor receptor (TNFR) I which might lead to cell apoptosis (Hsu, Tsai et al. 1996). TNFRs have been reported as important regulators in inducing apoptosis. TNFR I acts in almost every cell type and can independently transmit most biological activities of TNF-α (Kuo 2000). In addition, α-solamargine

induced apoptosis of lung cancer cells (H441, H520, H661 and H69) by phospholipid phosphatidylserine (PS) externalization in a dose-dependent manner and increased sub-G1 fraction has also been reported (Liu, Liang et al. 2004). Induction of apoptotic cell death in human hepatoma cells (SMMC-772, Hep3B) has also been reported (Chang, Tsai et al. 1998; Kuo, Hsu et al. 2000; Ding, Zhu et al. 2012). Furthermore, α-solamargine causes induction of actin disruption and down-regulation of P-glycoprotein expression in multidrug-resistant K562/A02 cells (Li, Zhao et al. 2011). It also induced apoptosis associated with p53 transcription-dependent and transcription-independent pathways in human osteosarcoma U2OS cells (Li, Zhao et al. 2011).

A number of reports have been found on antifungal activities of α-solamargine against different fungi species. Reports demonstrated very weak antifungal activity against *Cercosporella brassicae* and *Alternaria porri* (Zhao, Gao et al. 2009) and *Microsporum canis*, *M. gypseum*, *Trichophyton rubrum*, *T. mentagrophytes* and yeasts such as *Candida albicans*, *C. parapsilosis* (Pinto, Uchoa et al. 2011). Fungistatic activity of α-solamargine against *Trichophyton mentagrophytes* var granulosum, *T. mentagrophytes* var interdigitale, *T. mentagrophytes* var asteroides, *Microsporum gypseum*, *M. canis*, *Epidermophyton floccosum*, and *Candida albicans* has also been reported (Alkiewicz, Gertig et al. 1966). In addition, α-solamargine showed very little or no fungicidal effects when it was investigated against *Sclerotinia fructicola*, *Claviceps purpurea*, *Trichothecium roseum*, *Piricularia oryzae*, and *Fomes officinalis*, *Botrytis allii*, *Alternaria solani*, *Coniophora cerebella*, *Rhizoctonia solani*, *Schizophyllum commune*, and 3 *Fusarium* species (Wolters 1968).

5.3.4 Bioactivity of β-solamargine

To date, no previous reports exist either on the cytotoxic/anticancer or on other bioactivity studies of β-solamargine.

5.3.5 Bioactivity of khasianine

Khasianine, another SGA, is found in *Solanum surattense* (yellow-berried nightshade) and showed cytotoxic potential against human gastric cancer (MGC-803), human hepatocellular liver carcinoma

(HepG2), human hepatoma (Hep3B, PLC/PRF/S) and human lung adenocarcinoma (A549) cell lines (Lu, Luo et al. 2011). It showed cytotoxicity with IC_{50} values in the order 26.7 > 35.4 > 45.3 µM against A549, MGC-803, and HepG2 cancer cells, respectively (Lu, Luo et al. 2011). However, no reports were found regarding the antifungal activity of khasianine.

5.3.6 Bioactivity of β-solamarine

No biological activity reports for β-solamarine have been found in the literature.

5.3.7 Bioactivity of α-chaconine

The compound α-chaconine, a principal potato SGA, has been found to exert anticancer potential against a number of cancer cell lines such as colon, breast, gastric and others (Table 5.2). Numerous studies report on the anticancer activity of α-chaconine against human colon (HT-29, SW620), liver (HepG2) cervical (HeLa), lymphoma (U937), stomach (AGS and KATI II), breast (MCF-7), gastric (NUGC-3), lung (PC-6), prostate (LNCaP and PC-3) cancer cell lines and mouse leukaemia cells (P388) (Lee, Kozukue et al. 2004). It has been reported that liver cancer cells were more sensitive to α-chaconine than the colon cancer cells and interstingly, the anticancer activity of this compound against the liver cancer (HepG2) cells was higher than the anticancer drugs, doxorubicin and camptothecin (Lee, Kozukue et al. 2004). This compound has been reported to exert anticancer activity through the suppression of the phosphoinositide 3-kinase/AKT/NF-*k*B signaling pathway and   activation of caspase-3 activity (Shih, Chen et al. 2007).

With regards to antifungal activity of α-chaconine, it has been reported to inhibit spore generation and hyphal growth on agar as well as in liquid culture of *Alternaria brassicicola* and *Phoma medicaginis*. In addition, it also inhibited the growth of *Ascobolus crenulatus* and *Rhizoctonia solani* in liquid culture (Fewell and Roddick 1993; Fewell and Roddick 1997). Moreover, α-chaconine demonstrated strong fungicidal effects against *Cercosporella brassicae* and *Alternaria porri* (Zhao, Gao et al. 2009). In addition, α-chaconine fully suppressed penetration of fungal spores of *Phytophthora infestans* at 1-2 mg/mL (Ozeretskovskaya, Davydova et al. 1969).

5.3.8 Bioactivity of α-solanine

The compound α-solanine, a principal SGA found in potato, exhibited potent antiproliferative activity against estrogen-dependent breast (MCF-7), prostate (LNCaP and PC3), human melanoma (A2058), colon (HT-29) cervical (HeLa), liver (HepG2), lymphoma (U937), and stomach (AGS and KATO III) cancer cells (Nakamura, Komori et al. 1996; Lee, Kozukue et al. 2004; Lu, Shih et al. 2010; Zhang and Shi 2011; Ji and Gao 2012; Ji, Liu et al. 2012). Furthermore, α-solanine showed cytotoxicity against estrogen-dependent breast cancer cells (MCF-7) with an IC_{50} value of 22.08 µg/mL. This compound has been reported to exhibit antiproliferative activity through inducing apoptosis in HepG2 cancer cells, cell cycle arrest at S-phase in MCF-7 cancer cells, as well as inhibition of NF-κB activity in A2058 cancer cells (Gao, Zou et al. 2007; Lu, Shih et al. 2010; Ji, Liu et al. 2012).

Antifungal activity tests with α-solanine showed low fungicidal activity against *Cercosporella brassicae* and *Alternaria porri* (Zhao, Gao et al. 2009).

5.3.9 Bioactivity of tomatidine HCl

Tomatidine HCl is a salt of the aglycone, tomatidine. No anticancer activity reports have been found for tomatidine HCl so far, but several studies reported on the anticancer activity of the aglycone, tomatidine, itself. Moreover, tomatidine displayed dose dependent antitumor activity *in vitro* against the growth of human mammary cancer cells (BCAP) (Yang, Wang et al. 2007). However, tomatidine showed very low apoptotic activity using annexin V-FITC/PI staining by flow cytometry (Koduru, Grierson et al. 2007). Tomatidine inhibited cell growth of cervix (HeLa), breast (MCF-7), and colon (HT-29) cancer cells by blocking the cell cycle in the G0/G1 phase after 24 h exposure with an increase from 55.6% to 64.2% cells accumulation in G0/G1 phase (Koduru, Grierson et al. 2007).

Table 5.2 Pharmacological activities previously reported on steroidal glycoalkaloids

	Cytotoxic activity		Apoptosis	Antifungal activity
α-solamargine	Cell line	IC$_{50}$ (μM)	Induces apoptosis in K562/A02 cells (Li, Zhao et al. 2011)	*Cercosporella brassicae* and *Alternaria porri* (Zhao, Gao et al. 2009)
	Colon	HT-29 (2.30), HCT-15 (2.10), HCT116 (3.8), HCT-116 (4.53), SW620 (1.87) (Nakamura, Komori et al. 1996; Hu 1999; Ikeda, Tsumagari et al. 2003; Wei 2011)	Induces apoptosis in human osteosarcoma cells (U2OS) (Li, Zhao et al. 2011)	*Trichophyton mentagrophytes var granulosum, T. mentagrophytes var interdigitale, T. mentagrophytes var 168 umigates, Microsporum gypseum, M. Canis, Epidermophyton floccosum,* and *Candida albicans* (Alkiewicz, Gertig et al. 1966)
	Breast	T47D (1.9), MDA-MB-231(1.5), MCF-7 (2.1), MCF-7 (2.49), MCF-7 (8.2) (Nakamura, Komori et al. 1996; Hu 1999; Sun, Zhao et al. 2011; Wei 2011; Cui 2012); HBL-100: 2.07; SK-BR-3: 3.00 and ZR-75-1: 2.15 (Shiu, Chang et al. 2007)	Triggers apoptosis in breast cancer cells (ZR-75-1) (Shiu, Liang et al. 2008) Triggers apoptosis in human lung cancer cells (H661 and H69) to Trastuzumab and Epirubicin (Liang, Shiu et al. 2008)	
	Gastric	NUGC-3 (2.25) (Nakamura, Komori et al. 1996)	Induces apoptosis and sensitizes breast cancer cells (HBL-100, ZR-75-1 and SK-BR-3) to Cisplatin (Shiu, Chang et al. 2007)	*Microsporum canis, M. Gypseum, Trichophyton rubrum, T. mentagrophytes* and *Candida albicans, C. parapsilosis* (Pinto, Uchoa et al. 2011)
	Others	H441(3.0), H520 (6.7), H661(7.2) and H69 (5.8) (Liu 2004) Hep3B (The IC$_{50}$ values of solamargine for control, G0/G1-, M-, and G2/M-synchronized Hep3B cells were 5.76, 11.52, 4.26 and 3.57 respectively) (Kuo 2000) HeLa (6.0), HepG2 (2.5), A549 (8.0), K562 (5.2), U87 (3.2), and HL7702 (13.5), H9C2 (> 20) (Wei 2011) LNCaP (1.6), PC3 (2.1) (Hu 1999). LLCMK$_2$ (11.2) (Abreu Miranda, Tiossi et al. 2013)	Induces apoptotic cell death in nonsmall-cell lung cancer (NSCLC) (Liang, Shiu et al. 2007) Induces apoptosis on gastric cancer cells (MGC-803) (Ding, Zhu et al. 2013) Induces apoptosis in lung cancer cells (H441, H520, H661 and H69) (Liu, Liang et al. 2004)	Against *Sclerotinia fructicola, Claviceps purpurea, Trichothecium roseum, Piricularia oryzae,* and *Fomes officinalis, Botrytis allii, Alternaria solani, Coniophora cerebella, Rhizoctonia solani, Schizophyllum commune,* and 3 *Fusarium* species (Wolters 1968)

IC$_{50}$ (Inhibition of cell growth 50%), PLC/PRF/S, FHCC-98: Human hepatoma cells; KB: Squamous cancer cells; HT-29: Colon carcinoma cells; HCT-15: Colon carcinoma cells; LNCaP and PC3: Prostate cancer cells; T47D, MDA-MB-231 HBL-100, SK-BR-3 and ZR-75-1: Breast cancer cells; H441, H520, H661 and H69: Lung carcinoma cells; HeLa: Cervix carcinoma cells; A549: Lung carcinoma cells; K562: Chronic myelogenous leukemia cells; HCT116: Colon carcinoma cells; U87:Glioblastoma, brain carcinoma cells; HepG2:Hepatocellular liver carcinoma cells; HL7702 and H9C2: Normal hepatocyte; HCT-8: Ileocecal carcinoma cells; Hep3B: Human hepatoma cells; JTC-26: Human uterus cancer cells; PC-6: Lung cancer; SW620: Colon cancer; P388 : Mouse leukaemia cells; NUGC-3: Stomach cancer cells; B16F10: Murine melanoma; MO59J, U343 and U251: Human glioblastoma; ECV304: Human umbilical vein endothelial cells; A2058: Human melanoma cells; Jurkat APO-S: Human leukemia cells; U937: Lymphoma cancer cells; SGC-790, AGS and KATO III: Stomach cancer cells; LS-174: Human large intestine cancer cells; ND: Not determined; NF: Not found

Table 5.2 Pharmacological activities previously reported on steroidal glycoalkaloids (contd.)

Compound	Cytotoxic activity		Apoptosis	Antifungal activity
α-solamargine (contd.)	**Cell line**	**IC$_{50}$ (µM)**	Ttriggers apoptosis in human hepatoma cells (Hep3B) and normal skin fbroblast (Hsu, Tsai et al. 1996)	
	Others	JTC-26 (100% cell growth inhibition at 15 µg/mL) (Saijo, Murakami et al. 1982); PC-6 (3.06), P388 (1.77) (Nakamura 1996)		
		B16F10, HepG2, HeLa, MO59J, U343 and U251 (5.28 to 21.0) (Munari Carla, de Oliveira Pollyanna et al. 2013); PC-6 (3.06) (Nakamura, Komori et al. 1996)	Induces apoptosis in human hepatoma (SMMC-772) cells (Ding, Zhu et al. 2012)	
		KB (7.8), K562 (8.0), PC3 (5.9), ECV304 (ND), HL7702 (ND) (Cui 2012)	Induces apoptosis in breast cancer cells (MCF-7 and SK-BR-3 cells) and non-cancerous breast epithelial cells (HBL-100) (Shiu, Liang et al. 2009)	
		PC-12 (3.37) (Ikeda, Tsumagari et al. 2003); HCT-8 (10.63) (Li, He et al. 2007)		
			Triggers apoptosis in human hepatoma cells (Hep3B) (Kuo, Hsu et al. 2000)	
			Triggers apoptotic cell death in human hepatoma cells (Hep3B) (Chang, Tsai et al. 1998)	
β-solamargine	No reports found		No reports found	No reports found
β-solamarine	No reports found		No reports found	No reports found

IC$_{50}$ (Inhibition of cell growth 50%), PLC/PRF/S, FHCC-98: Human hepatoma cells; KB: Squamous cancer cells; HT-29: Colon carcinoma cells; HCT-15: Colon carcinoma cells; LNCaP and PC3: Prostate cancer cells; T47D, MDA-MB-231 HBL-100, SK-BR-3 and ZR-75-1: Breast cancer cells; H441, H520, H661 and H69: Lung carcinoma cells; HeLa: Cervix carcinoma cells; A549: Lung carcinoma cells; K562: Chronic myelogenous leukemia cells; HCT116: Colon carcinoma cells; U87:Glioblastoma, brain carcinoma cells; HepG2:Hepatocellular liver carcinoma cells; MCF-7: Breast carcinoma cells; HL7702 and H9C2: Normal hepatocyte; HCT-8: Ileocecal carcinoma cells; Hep3B: Human hepatoma cells; JTC-26: Human uterus cancer cells; PC-6: Lung cancer; SW620: Colon cancer; P388 : Mouse leukaemia cells; NUGC-3: Stomach cancer cells; B16F10: Murine melanoma; MO59J, U343 and U251: Human glioblastoma; ECV304: Human umbilical vein endothelial cells; A2058: Human melanoma cells; Jurkat APO-S: Human leukemia cells; U937: Lymphoma cancer cells; SGC-790, AGS and KATO III: Stomach cancer cells; LS-174: Human large intestine cancer cells; ND: Not determined; NF: Not found

Table 5.2 Pharmacological activities previously reported on steroidal glycoalkaloids (contd.)

Compound	Cytotoxic activity		Apoptosis	Antifungal activity
α-solasonine	**Cell line**	**IC$_{50}$ (μM)**	No reports found	*Cercosporella brassicae* and *Alternaria porri* (Zhao, Gao et al. 2009)
	Colon	HT-29 (Cytotoxicity mentioned as %) (Lee, Kozukue et al. 2004); HCT-116 (>5.66) (Ikeda, Tsumagari et al. 2003); SW620 (7.6) (Nakamura 1996)		*Microsporum canis, M. Gypseum, Trichophyton rubrum, T. mentagrophytes*) and yeasts (*Candida albicans, C. parapsilosis*) (Pinto, Uchoa et al. 2011)
	Breast	MCF-7 (10.97) (Nakamura 1996)		*Sclerotinia fructicola, Claviceps purpurea, Trichothecium roseum, Piricularia oryzae*, and *Fomes officinalis, Botrytis allii, Alternaria solani, Coniophora cerebella, Rhizoctonia solani, Schizophyllum commune*, and 3 *Fusarium* species (Wolters 1968)
	Gastric	NUGC-3 (13.69) (Nakamura 1996)		
	Others	LLCMK$_2$ (20.6) (Abreu Miranda, Tiossi et al. 2013); HCT-8 (11.97) (Li, He et al. 2007)		
		HepG2 (Cytotoxicity mentioned as %) (Lee, Kozukue et al. 2004)		
		PC-12 (>5.66) (Ikeda, Tsumagari et al. 2003); Ehrlich carcinoma (74.2), K562 (76.92) (Esteves-Souza, Sarmento da Silva et al. 2002)		
		PC-6 (16.29), P388 (10.60) (Nakamura 1996)		

IC50 (Inhibition of cell growth 50%), PLC/PRF/S, FHCC-98: Human hepatoma cells; KB: Squamous cancer cells; HT-29: Colon carcinoma cells; HCT-15: Colon carcinoma cells; LNCaP and PC3: Prostate cancer cells; T47D, MDA-MB-231 HBL-100, SK-BR-3 and ZR-75-1: Breast cancer cells; H441, H520, H661 and H69: Lung carcinoma cells; HeLa: Cervix carcinoma cells; A549: Lung carcinoma cells; K562: Chronic myelogenous leukemia cells; HCT116: Colon carcinoma cells; U87:Glioblastoma, brain carcinoma cells; HepG2:Hepatocellular liver carcinoma cells; MCF-7: Breast carcinoma cells; HL7702 and H9C2: Normal hepatocyte; HCT-8: Ileocecal carcinoma cells; Hep3B: Human hepatoma cells; JTC-26: Human uterus cancer cells; PC-6: Lung cancer; SW620: Colon cancer; P388 : Mouse leukaemia cells; NUGC-3: Stomach cancer cells; B16F10: Murine melanoma; MO59J, U343 and U251: Human glioblastoma; ECV304: Human umbilical vein endothelial cells; A2058: Human melanoma cells; Jurkat APO-S: Human leukemia cells; U937: Lymphoma cancer cells; SGC-790, AGS and KATO III: Stomach cancer cells; LS-174: Human large intestine cancer cells; ND: Not determined; NF: Not found

Table 5.2 Pharmacological activities previously reported on steroidal glycoalkaloids (contd.)

Compound	Cytotoxic activity		Apoptosis	Antifungal activity
α-chaconine	**Cell line**	**IC$_{50}$ (μM)**	Induces apoptosis in bovine aortic endothelial cells (BAECs) (Lu, Chen et al. 2010)	Antifungal activity against *Cercosporella brassicae* and *Alternaria porri* (Zhao, Gao et al. 2009)
	Colon	HT-29 (Cytotoxicity mentioned as %) (Lee, Kozukue et al. 2004); HT-29 (ND) (Yang, Paek et al. 2006), SW620 (1.71) (Nakamura 1996)	Induces apoptosis in prostate cancer cells (LNCaP and PC-3) (Reddivari, Vanamala et al. 2010)	Anti-fungal effect against *Ascobolus cremulatus*, *Alternaria brassicicola*, *Phoma medicaginis* and *Rhizoctonia solani* (Fewell and Roddick 1993)
	Breast	MCF-7 (Nakamura 1996)		
	Gastric	AGS (94.9% cell growth inhibition at 10 μg/mL) (Friedman, Lee et al. 2005), NUGC-3 (1.68) (Nakamura 1996)	Induces apoptosis in intestinal epithelial cells (Caco-2) (Mandimika, Baykus et al. 2007)	Against *Alternaria brassicicola* and *Phoma medicaginis*, *Ascobolus crenulatus* and *Rhizoctonia solani* (Fewell and Roddick 1997)
	Others	HepG2 (Cytotoxicity mentioned as %) (Lee, Kozukue et al. 2004), HepG2 (89.7 % cell growth inhibition at 10 μg/mL) (Friedman, Lee et al. 2005)	Induces apoptois in colon cancer cells (HT-29) (Yang, Paek et al. 2006)	Against *Aspergillus niger*, *Candida albicans* (Tukalo, Leplya et al. 1972)
		PC-6 (2.15), P388 (1.85) (Nakamura 1996)		Fungitoxic activity (Ozeretskovskaya, Davydova et al. 1969)
		LNCaP (3.52), PC3 (2.93) (Reddivari, Vanamala et al. 2010)		Fungitoxic activity (Allen and Kuc 1968)
		A549 (Shih, Chen et al. 2007)		

IC$_{50}$ (Inhibition of cell growth 50%), PLC/PRF/S, FHCC-98: Human hepatoma cells; KB: Squamous cancer cells; HT-29: Colon carcinoma cells; HCT-15: Colon carcinoma cells; LNCaP and PC3: Prostate cancer cells; T47D, MDA-MB-231 HBL-100, SK-BR-3 and ZR-75-1: Breast cancer cells; H441, H520, H661 and H69: Lung carcinoma cells; HeLa: Cervix carcinoma cells; A549: Lung carcinoma cells; K562: Chronic myelogenous leukemia cells; HCT116: Colon carcinoma cells; U87:Glioblastoma, brain carcinoma cells; HepG2:Hepatocellular liver carcinoma cells; MCF-7: Breast carcinoma cells; HL7702 and H9C2: Normal hepatocyte; HCT-8: Ileocecal carcinoma cells; Hep3B: Human hepatoma cells; JTC-26: Human uterus cancer cells; PC-6: Lung cancer; SW620: Colon cancer; P388 : Mouse leukaemia cells; NUGC-3: Stomach cancer cells; B16F10: Murine melanoma; MO59J, U343 and U251: Human glioblastoma; ECV304: Human umbilical vein endothelial cells; A2058: Human melanoma cells; Jurkat APO-S: Human leukemia cells; U937: Lymphoma cancer cells; SGC-790, AGS and KATO III: Stomach cancer cells; LS-174: Human large intestine cancer cells; ND: Not determined**;** NF: Not found

Table 5.2 Pharmacological activities previously reported on steroidal glycoalkaloids (contd.)

Compound	Cytotoxic activity		Apoptosis	Antifungal activity
α-solanine	**Cell line**	**IC$_{50}$ (µM)**	Induces apoptosis on human hepatocarcinoma cells (HepG2) (Gao, Wang et al. 2006; Gao, Zou et al. 2007; Ji, Xu et al. 2008; Ji, Gao et al. 2008; Gao, Xu et al. 2009; Ji, Gao et al. 2009; Ji and Gao 2012)	Antifungal activity *against Trypanosoma cruzi* (Chataing, Concepcion et al. 1998)
	Colon	HT-29 (84.7% after 4 hr at 100µg/mL) (Lee, Kozukue et al. 2004)		Against *Alternaria brassicicola* and *Phoma medicaginis*, *Ascobolus crenulatus* and *Rhizoctonia solani* (Fewell and Roddick 1997)
	Breast	MCF 7 (25.44) (Ji, Liu et al. 2012)		
	Gastric	AGS (86.6% cell growth inhibition at 10 µg/mL) and KATO III (Friedman, Lee et al. 2005)		Against *Ascobolus cremulatus*, *Alternaria brassicicola*, *Phoma medicaginis* and *Rhizoctonia solani* (Fewell and Roddick 1993)
	Others	HepG2 (NF) (Gao, Su et al. 2010; Ji and Gao 2012); HepG2 (79.0 % cell growth inhibition) (Friedman, Lee et al. 2005); HepG2 (89.0% after 4 hr at 100µg/mL) (Lee, Kozukue et al. 2004)		Against *Aspergillus niger*, *Candida albicans*, and other fungi (Tukalo, Leplya et al. 1972)
				Fungitoxic activity (Ozeretskovskaya, Davydova et al. 1969)
		PC6 (18.09), P388 (9.55) (Nakamura, Komori et al. 1996); HeLa, U937 (Lee, Kozukue et al. 2004); HepG2, SGC-7901, LS-174 (Gao, Zou et al. 2007)		Fungitoxic activity (Allen and Kuc 1968)
		PC3 (Zhang and Shi 2011); PC3 (17.28) (Reddivari, Vanamala et al. 2010); A2058 (Lu, Shih et al. 2010)		
		LNCaP (18.43), Jurkat APO-S (8.5 mM) (Rosenkranz and Wink 2008)		
Khasianine	Gastric	MGC-803 (35.4) (Lu, Luo et al. 2011)	Induces very weak apoptosis on human hepatoma cells (Hep3B) (Chang, Tsai et al. 1998)	No reports found
	Others	A549 (26.7), HepG2 (45.3) (Lu, Luo et al. 2011)		

C$_{50}$ (Inhibition of cell growth 50%), PLC/PRF/S, FHCC-98: Human hepatoma cells; KB: Squamous cancer cells; HT-29: Colon carcinoma cells; HCT-15: Colon carcinoma cells; LNCaP and PC3: Prostate cancer cells; T47D, MDA-MB-231 HBL-100, SK-BR-3 and ZR-75-1: Breast cancer cells; H441, H520, H661 and H69: Lung carcinoma cells; HeLa: Cervix carcinoma cells; A549: Lung carcinoma cells; K562: Chronic myelogenous leukemia cells; HCT116: Colon carcinoma cells; U87:Glioblastoma, brain carcinoma cells; HepG2:Hepatocellular liver carcinoma cells; MCF-7: Breast carcinoma cells; HL7702 and H9C2: Normal hepatocyte; HCT-8: Ileocecal carcinoma cells; Hep3B: Human hepatoma cells; JTC-26: Human uterus cancer cells; PC-6: Lung cancer; SW620: Colon cancer; P388 : Mouse leukaemia cells; NUGC-3: Stomach cancer cells; B16F10: Murine melanoma; MO59J, U343 and U251: Human glioblastoma; ECV304: Human umbilical vein endothelial cells; A2058: Human melanoma cells; Jurkat APO-S: Human leukemia cells; U937: Lymphoma cancer cells; SGC-790, AGS and KATO III: Stomach cancer cells; LS-174: Human large intestine cancer cells; ND: Not determined; NF: Not found

Table 5.2 Pharmacological activities previously reported on steroidal glycoalkaloids (contd.)

Compound	Cytotoxic activity			Apoptosis	Antifungal activity
Solasodine	Cell line	IC$_{50}$ (µM)		Induces weak apoptotic response on colon cancer cells (HCT116) (Bhutani, Paul et al. 2010)	Antifungal activity against *Candida albicans*, *C. pseudotropicalis*, *C. famata*, *C. kefyr*, *Cryptococcus albidus*, *C. neoformans*, *Rhodotorula glutinis*, *Trichosporon cutaneum*, *Geotrichum candidum*, *Aspergillus 173 umigates*, *A. candidus*, *Fonsecaea pedrosoi*, *Microsporum gypseum*, *Phialophora verrucosa*, *Sporothrix schenckii*, *Trichophyton rubrum sc.*, and *T. mentagrophytes sc* (Kusano, Takahashi et al. 1987).
	Colon	SW620 (8.46) (Nakamura 1996).		Exhibits very low apoptotic effect on Hela cells (Koduru, Grierson et al. 2007).	
	Breast	MCF-7 (ND) (Cui 2012)			
	Gastric	NUGC-3 (3.55) (Nakamura 1996)		Solasodine hydrochloride inducing apoptosis activity in cervical cancer HeLa cells (Li and Liu 2008; Ai, Liu et al. 2011)	
	Others	PLC/PRF/S (9.07), KB (9.04) (Lin, Lu et al. 1990)			Antifungal effect against on the genetic substance of *Saccharomyces cerevisiae* GL7 and *Prototheca wickerhamii* (Wang, Gu et al. 2000).
		KB (ND), K562 (ND), PC3 (13.6), ECV304 (ND), HL7702 (ND) (Cui 2012).		Induces weak apoptotic effect on human 1547 osteosarcoma cells (Trouillas, Corbiere et al. 2005)	
		PC3 (>25) (Zha 2010)			
		LLCMK$_2$ (82.2) (Abreu Miranda, Tiossi et al. 2013)		Induces apoptosis to a lesser extent on human hepatoma cells (Hep3B) (Chang, Tsai et al. 1998)	
		Ehrlich carcinoma (ND), K562 (ND) (Esteves-Souza, Sarmento da Silva et al. 2002)			
		PC-6 (10.27), P388 (5.27) (Nakamura 1996)			
Tomatidine HCl	No reports found			Tomatidine exhibites very low apoptotic effect on Hela cells (Koduru, Grierson et al. 2007)	

IC50 (Inhibition of cell growth 50%), PLC/PRF/S, FHCC-98: Human hepatoma cells; KB: Squamous cancer cells; HT-29: Colon carcinoma cells; HCT-15: Colon carcinoma cells; LNCaP and PC3: Prostate cancer cells; T47D, MDA-MB-231 HBL-100, SK-BR-3 and ZR-75-1: Breast cancer cells; H441, H520, H661 and H69: Lung carcinoma cells; HeLa: Cervix carcinoma cells; A549: Lung carcinoma cells; K562: Chronic myelogenous leukemia cells; HCT116: Colon carcinoma cells; U87:Glioblastoma, brain carcinoma cells; HepG2:Hepatocellular liver carcinoma cells; MCF-7: Breast carcinoma cells; HL7702 and H9C2: Normal hepatocyte; HCT-8: Ileocecal carcinoma cells; Hep3B: Human hepatoma cells; JTC-26: Human uterus cancer cells; PC-6: Lung cancer; SW620: Colon cancer; P388 : Mouse leukaemia cells; NUGC-3: Stomach cancer cells; B16F10: Murine melanoma; MO59J, U343 and U251: Human glioblastoma; ECV304: Human umbilical vein endothelial cells; A2058: Human melanoma cells; Jurkat APO-S: Human leukemia cells; U937: Lymphoma cancer cells; SGC-790, AGS and KATO III: Stomach cancer cells; LS-174: Human large intestine cancer cells; ND: Not determined**;** NF: Not found

5.4 Structure-activity relationship (SAR) of steroidal glycoalkaloids (SGAs)

SGAs comprise two main subunits; the aglycone unit and the glycosidic moieties. Studies indicate that both, the aglycone and glycosidic moieties play crucial roles in exerting biological effects (Wang, Zhang et al. 2007; Man, Gao et al. 2010; Gao, Li et al. 2011). There are a number of SGAs bearing the same aglycone unit but having different sugar moieties. This difference in sugars is recognized as contributing to a difference in their biological activities (Milner 2011) and thus, investigations into SARs could be carried out by chemical modifications of glycosidic residues or by testing SGAs with different sugar moieties. Similarly, it is reported that the aglycone linked to the sugar moiety is of paramount importance for bioactivities, thus the difference of the aglycone in a series of glycoalkaloids with the same sugar residues will also provide important SAR information (Milner 2011).

5.4.1 SAR investigations into anticancer activity

SAR is the study of relationship between drug's structure and its biological activity, and thus, SAR investigations are critical to developing new drugs with the most potent bioactivity and least side effects. Therefore, SAR investigations into anticancer of SGAs including isolated new SGA **BL3** were undertaken in the present study.

A number of studies have been carried out to look into the SAR analysis on anticancer activity of SGAs (Nakamura, Komori et al. 1996; Chang 1998; Li, He et al. 2007; Cui 2012). The impact of sugar moieties present in SGAs on their anticancer potential was investigated by testing α-solasonine, α-solamargine and 6-*O*-sufated solamargine and their acid hydrolytic products against human ileocecal cancer cells (HT-8).
The SGAs α-solasonine, and α-solamargine showed more cytotoxicity than their hydrolytic products and 6-*O*-sufated solamargine, indicating that the nature, number, as well as order of the sugar residues attached to the aglycone have a profound impact on their antiproliferative potential (Li, He et al. 2007). Another study reports the SAR of natural glycoalkaloids with 6-*O*-sulfated modification of α-chaconine and α-solanine and found a marked reduction in anticancer activity upon removal of sugar moieties (Zhao, Li et al. 2006).
Significance of the rhamnose moiety in α-solamargine was investigated by comparing its anticancer effect to khasianine where an extra rhamnose is attached at position 2' of the glucose in α-solamargine. The apoptotic

potential of α-solamargine was enhanced 4 fold due to the additional 2' rhamnose moiety, as the additional rhamnose reportedly affects the dihedral angle of the glycosidic bond and, thus provide more stability to α-solamargine (Chang 1998). Another SAR investigation revealed the crucial role of the glycosidic moieties in producing cytotoxic effects where β-chacotriosyl containing two rhamnose and one glucose moieties (as present in α-solamargine) showed more cytotoxic activity than the β-solatriosyl containing one rhamnose, one glucose and one galactose moieties as well as other sugar linkages in prosapogenins. They also found that the rhamnosyl-(1→2)-glucosides (as present in **BL3** and α-solamargine) were much more effective in exerting anticancer activity than the rhamnosyl-(1→4)-glucosides (as present in khasianine) (Nakamura, Komori et al. 1996).

As indicated, both α-chaconine and α-solanine possess the same aglycone, solanidine, but different sugar residues with different linkage, such as chacotriose (as present in α-chaconine) and solatriose (as present in α-solanine), respectively (Table 5.1). In a number of studies, higher activities have been observed with α-chaconine compared to α-solanine which demonstrated the importance of rhamnose moieties and linkage for its potency (Roddick 1989; Wierenga and Hollingworth 1992; Blankemeyer, Atherton et al. 1995; Friedman, Henika et al. 1996; Blankemeyer, White et al. 1997; Roddick, Weissenberg et al. 2001). Similarly, when comparing α-solamargine with α-solasonine (same aglycone), α-solamargine (Figure 5.2) displayed consistently greater potency in several studies in various biological systems than α-solasonine (Roddick, Rijnenberg et al. 1990; Friedman, Rayburn et al. 1991; Blankemeyer, McWilliams et al. 1998; Li, He et al. 2007). Moreover, α-solasonine and its aglycone, solasodine, were tested for their anticancer activities against murine Ehrlich carcinoma and human K562 leukaemia cells where α-solasonine was significantly active, whereas its aglycone, solasodine, was found inactive thus confirming the importance of sugar moieties for providing anticancer potential (Esteves-Souza, Sarmento da Silva et al. 2002).

The mechanism of action behind the influence of sugar moieties on biological activities involves the participation of sugar moieties in binding with receptor sites of cell membranes (Keukens, De Vrije et al. 1992; Keukens EAJ 1995; Keukens, de Vrije et al. 1996). On the other hand, reports exist such as the *in vitro* anticancer activity study of α-SGA, α-chaconine and α-solanine, and their hydrolysis products with

colon and liver cancer cells showing comparable cancer cell growth inhibition (Lee, Kozukue et al. 2004). Another study showed that the aglycones solanidine and solasodine exhibited cytotoxic effects similar to their glycoalkaloids α-chaconine and α-solanine. In addition, the aglycones solanidine and solasodine reportedly showed similar cancer cell growth inhibition to their α-SGAs, α-solasonine and α-solamargine (Lee, Kozukue et al. 2004). This indicates that cell growth inhibition is dependent on the biological system as well as on the mode of examination (*in vitro* or *in vivo*) (Milner 2011).

5.4.2 SAR investigations into antifungal activity

SAR is the study of relationship between drug's structure and its biological activity, and thus, SAR investigations are critical to developing new drugs with the most potent bioactivity and least side effects. Therefore, SAR investigations into antifungal activities of SGAs including isolated new SGA **BL3** were undertaken in the present study.

A number of reports have been found on SAR investigations of antifungal activities of α-solamargine, α-chaconine, α-solasonine, α-tomatine, α-solanine and solasodine (Table 5.2). When comparing the antifungal activity of several SGAs against *Cercosporella brassicae* and *Alternaria porri* the fungicidal effects of the compounds were ranked in the order of α-tomatine > α-chaconine > α-solamargine > α-solasonine > α-solanine (Zhao, Gao et al. 2009). The SGA, α-tomatine, displayed the highest fungicidal activity which may be attributed to the number as well as the type of sugar moieties (two glucose, one galactose and one xylose moieties) and the sugar linkages. The ranking of the activity above suggests that the aglycone is of lesser importance as α-solamargine and α-solasonine possess the same aglycone (solasodine), as do α-chaconine and α-solanine (solanidine as aglycone). More importantly, α-tomatine contains four sugar moieties, whereas α-chaconine, α-solamargine, α-solasonine and α-solanine possess only three sugar moieties. The sugar moieties of the latter differ such as α-chaconine contains one galactose and two rhamnose moieties, α-solamargine contains one glucose and two rhamnose moieties whereas both α-solasonine and α-solanine bear one glucose, one galactose and one rhamnose moieties. As α-tomatine, which lacks rhamnose and with a different aglycone showed the higher antifungal activity than the other SGAs with at least one rhamnose moiety, it indicates that the rhamnose moieties are less important in exerting antifungal activity than in

exhibiting anticancer activity of SGAs. Another study, however, reported that α-solasonine had a higher fungicidal effect than α-solamargine (Pinto, Uchoa et al. 2011).

5.5 Bioactivity studies of the novel steroidal glycoalkaloid

5.5.1 A brief introduction to the novel compound BL3

A novel SGA named (25*R*)-3β-{*O*-β-D-glucopyranosyl-(1→4)-*O*-α-L-rhamnopyranosyl-(1→3)-[*O*-α-L-rhamnopyranosyl-(1→2)]-α-L-rhamnopyranosyl}-22αN-spirosol-5-ene (**BL3**) was isolated from the methanolic extract of *Blumea lacera* leaf. The compound was isolated using semi-preparative HPLC followed by solid phase extraction (SPE). The structure was elucidated using spectroscopic methods including 1D and 2D NMR experiments (^{1}H, ^{13}C, COSY, HSQC and HMBC) and HRMS (Chapter four, Section 4.2.2.2).

5.5.2 Structural differences of the selected SGAs to the novel compound BL3

SGAs differ from each other either by having different aglycones or possessing different glycosidic moieties linked to the aglycone (Figure 5.3). The novel compound **BL3** possesses the aglycone solasodine and four sugar moieties; three rhamnose residues and a galactose moiety attached at the 3-OH position of the aglycone. In our present study, seven SGAs; solasodine, α-solasonine, β-solamargine, khasianine, β-solamarine, α-solanine and α-tomatidine HCl were selected for SAR analysis based on structural similarity to the isolated novel SGA **BL3** and commercial availability. For example, α-solamargine as not been included as it could not be purchased and therefore could not be tested for activity. Figure 5.3 demonstrates the structural differences of the selected SGAs tothe novel **BL3**.
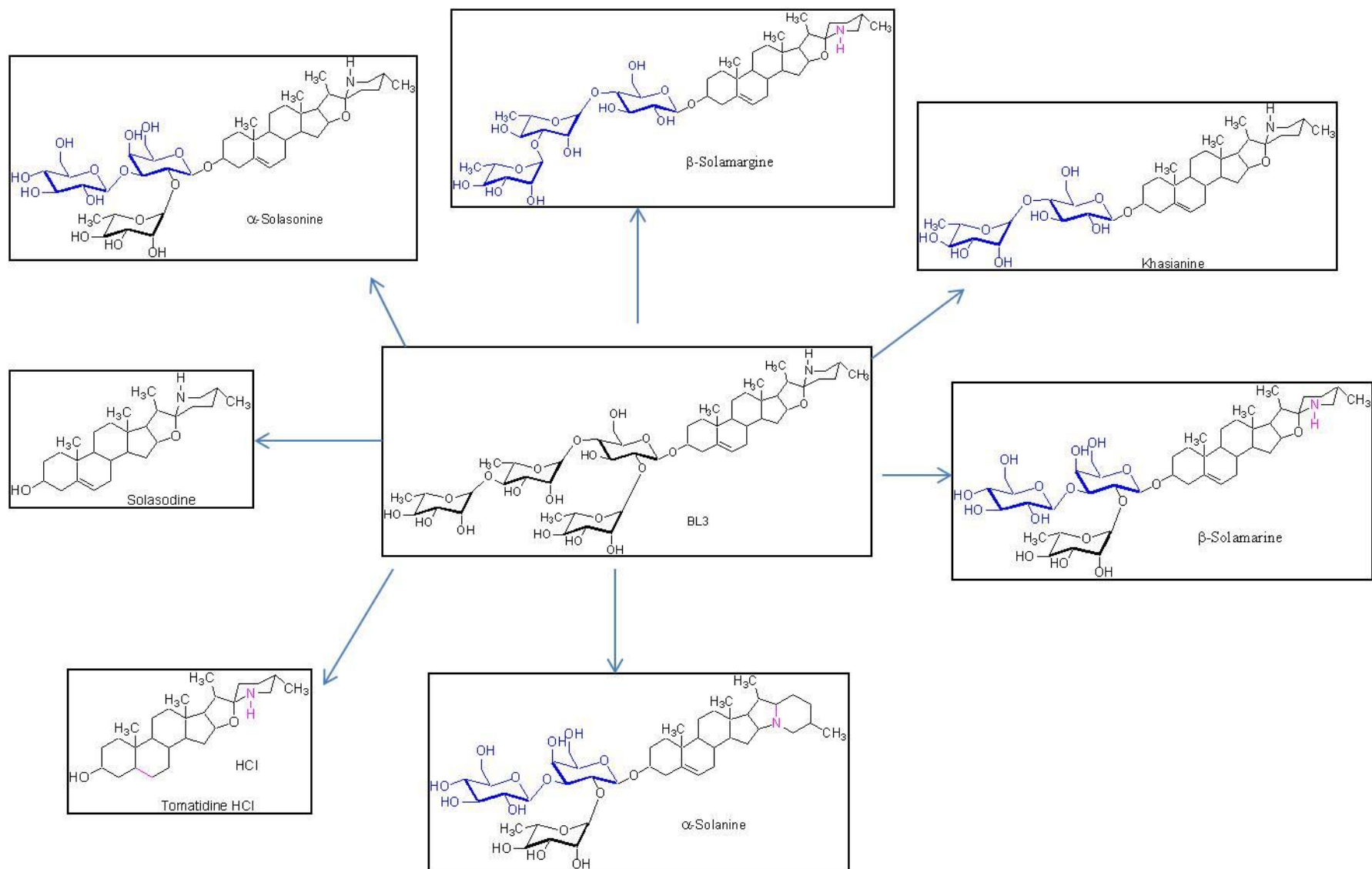
Figure 5.3 Structural differences (indicated as coloured) of the selected steroidal glycoalkaloids compared to the novel compound **BL3**

BL3 and solasodine

The novel SGA **BL3** possesses solasodine as its aglycone.

BL3 and α-solasonine

The novel SGA **BL3** contains the same aglycone as α-solasonine (α-solasodine), but differs in number, linkages, and type of glycosidic residues at the 3-OH position of the aglycone. Thus, the novel compound **BL3** and α-solasonine only differ in their sugar residues.

BL3 and β-solamargine

The novel SGA **BL3** contains a very similar aglycone to β-solamargine (α-solasodine vs. β-solasodine). **BL3** contains three rhamnose moieties with different linkages and one central glucose residue attached at the 3-OH position of the aglycone, whereas β-solamargine, which has only two rhamnose moieties and one glucose residue attached at the 3-O position of the aglycone. Thus, the novel SGA **BL3** and β-solamargine possess only slightly different aglycones, but different sugar residues.

BL3 and khasianine

The novel SGA **BL3** and khasianine share the same aglycone (α-solasodine). **BL3** contains three rhamnose moieties and a central glucose attached at the 3-OH position of the aglycone whereas khasianine has only one rhamnose 4-linked to a single glucose which is attached to the aglycone at 3-O position. Thus, the novel SGA **BL3** and khasianine only differ in their sugar residues.

BL3 and β-solamarine

The novel SGA **BL3** contains a different aglycone (α-solasodine) to β-solamarine (tomatidenol). **BL3** possesses 3 rhamnose and one central gluose moieties attached at the 3-OH position of the aglycone whereas β-solamarine has one rhamnose, one glucose, and one galactose residues linked to its aglycone. Thus, the novel SGA **BL3** and β-solamarine possess different aglycones as well as different sugar residues.

BL3 and α-solanine

The novel SGA **BL3** contains a different aglycone (α-solasodine) to α-solanine (solanidine). **BL3** possesses a tetrasaccharide (three rhamnose and one central glucose moiety), whereas α-solanine contains a trisaccharide (one rhamnose, one glucose and one galactose). Thus, α-solanine and the novel SGA, **BL3** possess different aglycone as well as different sugar residues.

BL3 and tomatidine HCl

The novel SGA **BL3** contains the aglycone, solasodine, whereas tomatidine HCl is a salt of the aglycone tomatidine. **BL3** possesses three rhamnose and one central glucose moietie attached at the 3-OH position of the aglycone, whereas tomatidine HCl, being a salt of the aglycone, does not possess any sugar moieties.

Impact of structure variation on the cytotoxic activity studies of these SGAs is discussed in section 5.5.3.1.

5.5.3 Cytotoxic activities of the novel SGA BL3 and other SGAs in this study

The literature search showed potent cytotoxic/anticancer activity of SGAs (Table 5.2). Therefore, **BL3**, a novel SGA isolated from *Blumea lacera* leaf extract (see chapter four, section 4.1.2.3.4), along with 7 other known SGAs (purchased), which are analogous to **BL3**, were tested for their cytotoxic/anticancer effects in our study to gain SAR information for this activity. These SGAs were tested for their cytotoxic potential against two healthy cell lines namely, a normal mouse fibroblast (NIH3T3) and a monkey kidney cell line (VERO) as well as against four cancer cell lines including one gastric adenocarcinoma (AGS), one colon adenocarcinoma (HT-29), and two breast ductal carcinoma (estrogen-dependent, MCF-7 and estrogen-non-dependent, MDA-MB-231) cell lines, using the MTT assay (Chapter two, Section 2.5). The results are summarised in the Figures 5.4 to 5.11 and in the Table 5.3.

5.5.3.1 Results and discussion of cytotoxicity tests of BL3 and the selected SGAs

A total of 8 SGAs including the novel compound **BL3** have been tested for their cytotoxic activity against different cancer as well as healthy cell lines at different concentrations. All thsese SGAs showed concentration dependent cytotoxicity (Figure 5.4 to 5.11).

Figure 5.4 Percent (%) of cell growth inhibition of the novel SGA **BL3** against the tested cell lines

Figure 5.5 Percent (%) of cell growth inhibition of solasodine against the tested cell lines

Figure 5.6 Percent (%) of cell growth inhibition of SGA α-solasonine against the tested cell lines
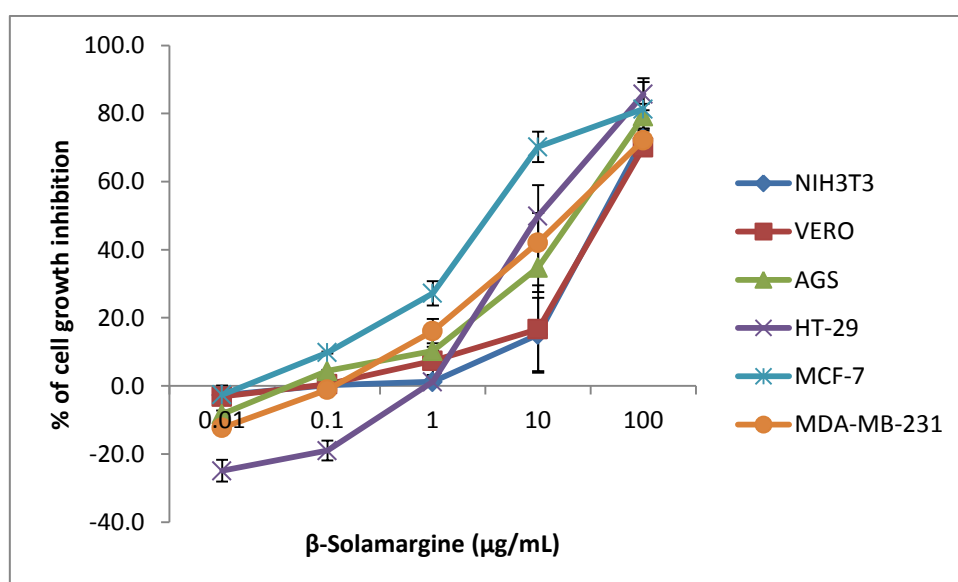
Figure 5.7 Percent (%) of cell growth inhibition of SGA β-solamarine against the tested cell lines
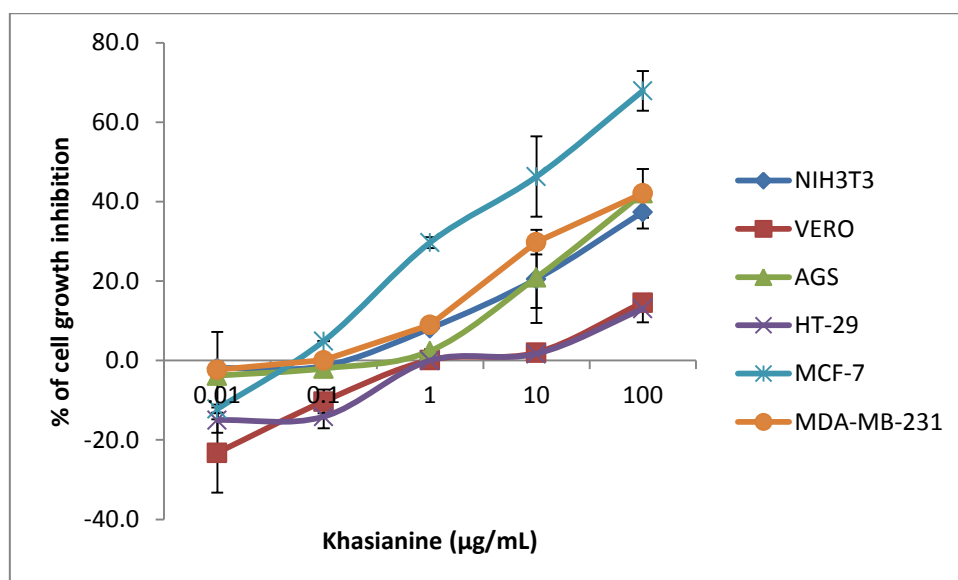
Figure 5.8 Percent (%) of cell growth inhibition of SGA khasianine against the tested cell lines
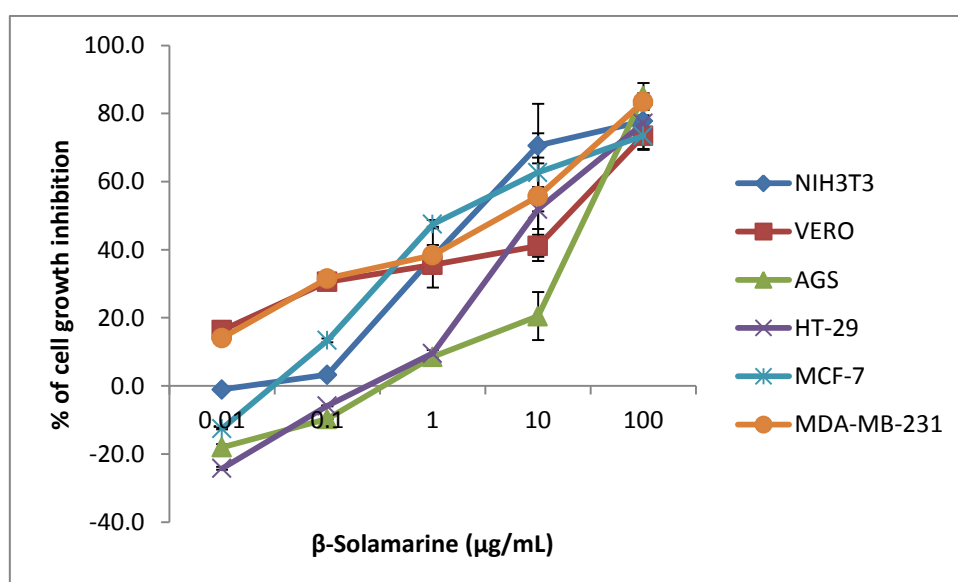
Figure 5.9 Percent (%) of cell growth inhibition of SGA β-solamarine against the tested cell lines
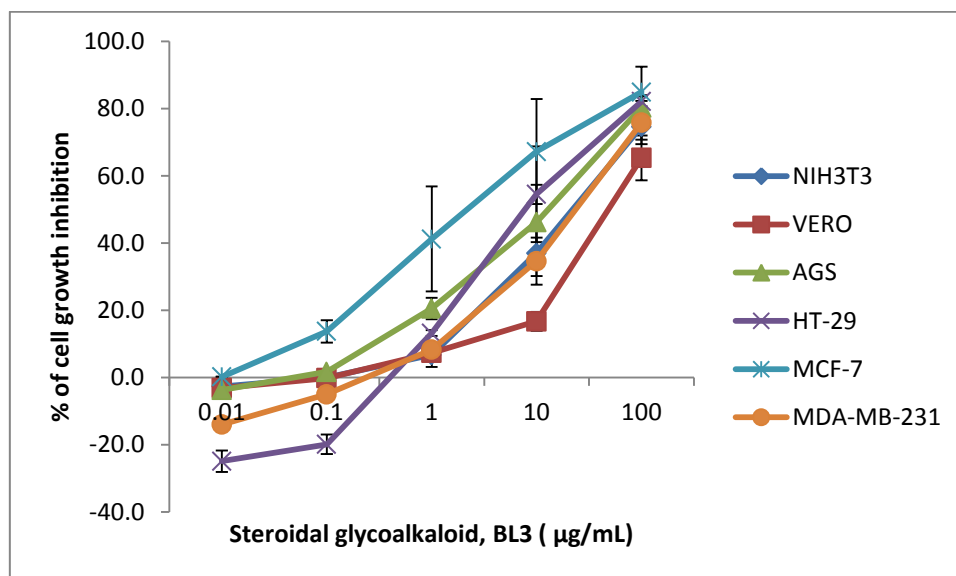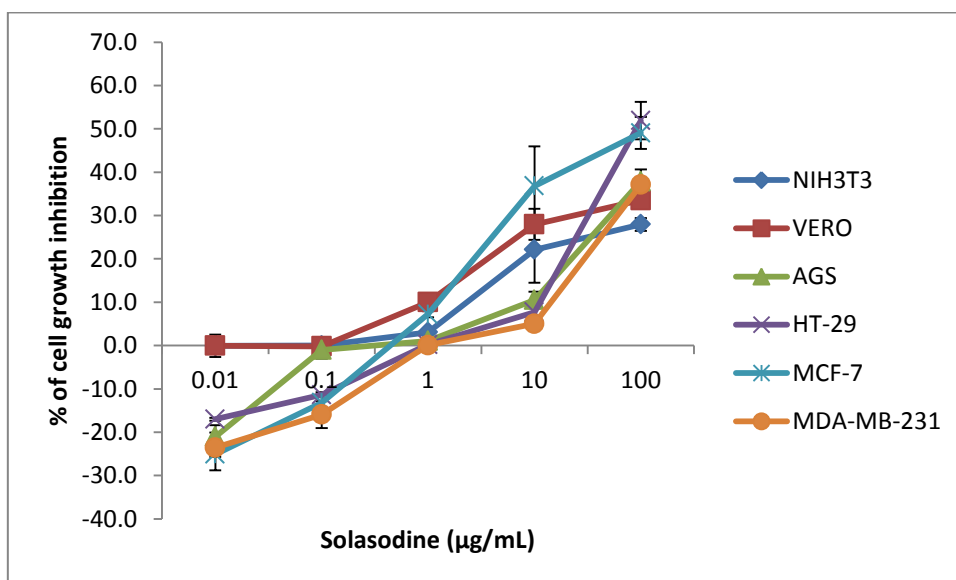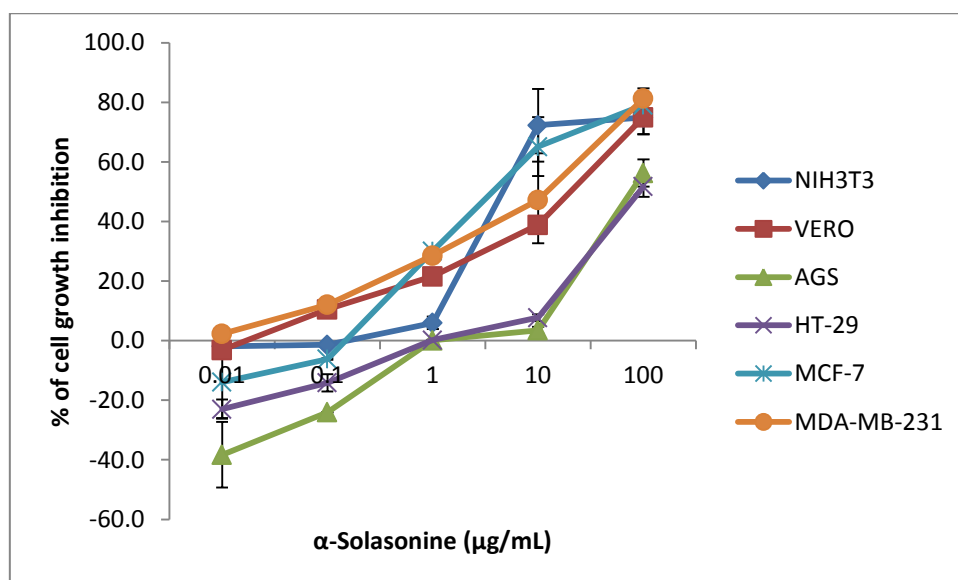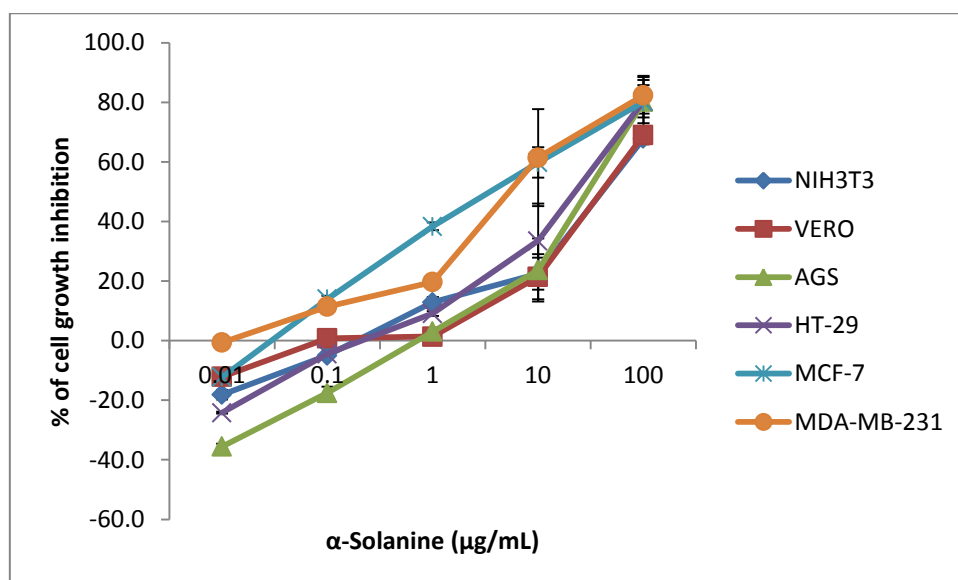
Figure 5.10 Percent (%) of cell growth inhibition of SGA α-solanine against the tested cell lines
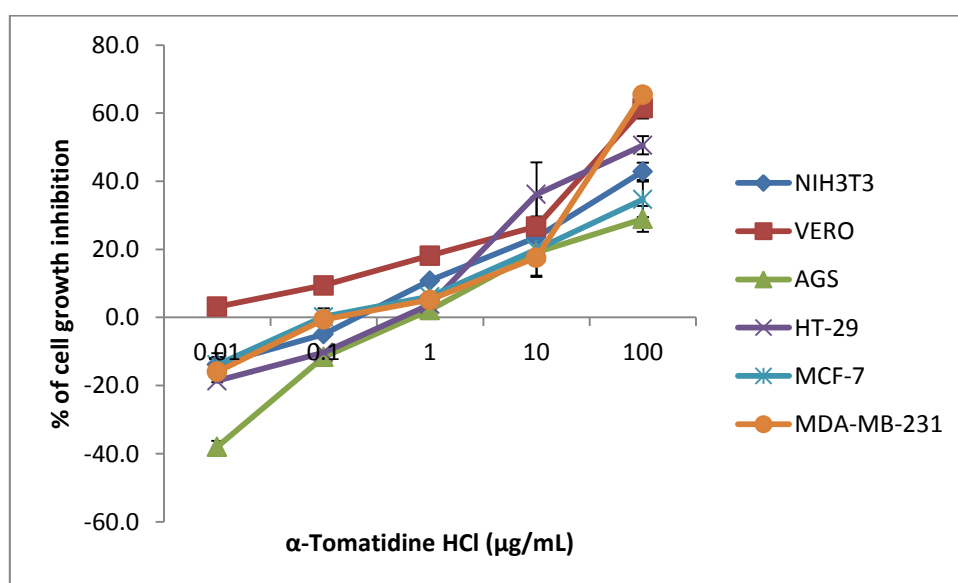
Figure 5.11 Percent (%) of cell growth inhibition of tomatidine HCl against the tested cell lines

The calculated IC_{50} values for these SGAs are presented in Table 5.3.

Table 5.3 Cytotoxic activity (IC_{50}) of the selected steroidal glycoalkaloids and steroidal alkaloids

Compound	Cytotoxic activity (IC_{50})* (µM)					
	VERO P35	NIH3T3	AGS	HT-29	MCF-7	MDA-MB-231
BL3 (Novel)	24.03	22.33	11.20	10.83	2.62	13.72
Solasodine	>241.76	>241.76	>241.76	>241.76	178.63	>100
α-solasonine	16.65	11.18	94.32	105.09	5.09	8.36
β-solamargine	53.94	47.60	21.19	16.36	5.01	20.68
Khasianine	>138.52	>138.52	>138.52	>138.52	19.89	>138.52
β-solamarine	8.32	5.08	26.08	15.90	4.10	2.55
α-solanine	42.39	44.66	31.56	22.39	4.57	6.94
Tomatidine HCl	120.72	>221.19	>221.19	144.32	>221.19	110.11
Cycloheximide (positive control)	24.45	26.44	8.53	11.02	181.87	22.39

IC_{50}* (Inhibition of cell growth by 50%) calculated by probit analysis (LdP Line software, USA), data was generated by experiments performed in triplicates.

Amongst all 8 SGAs tested, and looking at the results across all cell lines tested, the novel SGA **BL3**, displayed a high cytotoxic potential, only slightly lower than β-solamarine. Importantly, **BL3** was less toxic to the healthy cell lines tested. The compounds, α-solanine, β-solamargine and α-solasonine were slightly less toxic overall, but still showed significant activity against at least one or two cell lines. In contrast, much less cytotoxic were the compounds khasianine, solasodine and tomatidine HCl. It is difficult to establish very specific SARs from our results, however, it is obvious that the glycosides tested are more cytotoxic than just the aglycones, as solasodine and tomatidine HCl display the least activity against all cell lines. This confirms results from a number of studies which had reported that SGAs are more cytotoxic than their respective aglycones alone (Nakamura, Komori et al. 1996; Esteves-Souza, Sarmento da Silva et al. 2002). One previous study stated that the type, number, as well as order of the sugar residues attached to the aglycone, have a

significant influence on the anticancer potential of SGAs (Li, He et al. 2007). Especially the significance of number of sugar moieties was shown when α-solamargine was found to be 4 fold more apoptotic than khasianine, most likely due to the presence of an additional rhamnose moiety (Chang, Tsai et al. 1998). Our result confirm that the number of sugar moieties play a role as the less active SGA khasianine has only two sugar moieties, whereas the more active SGAs; β-solamarine, α-solanine, β-solamargine, α-solasonine, as well as **BL3**, comprise of three or four sugars.

Our study also indicates that the type of aglycone does not impact significantly on the cytotoxic activity overall, as in our study compounds with different aglycones i.e. **BL3** and β-solamarine, had overall similar cytotoxic activities. Amongst all 8 SGAs tested, the novel SGA **BL3** had the highest cytotoxic potential against estrogen-dependent breast cancer cells (MCF-7) with the IC_{50} value of 2.62 μM. Similarly, β-solamarine, α-solanine, β-solamargine and α-solasonine were all very toxic to MCF-7 cells with IC_{50} values of 4.10, 4.57, 5.01 and 5.09 μM, respectively.

A number of studies have reported on the cytotoxic activity of α-solamargine against estrogen-dependent cancer cells (MCF-7) where it showed cytotoxicity with IC_{50} values of 2.49 (Nakamura, Komori et al. 1996), 8.2 (Sun, Zhao et al. 2011; Cui 2012) and 2.1 (Wei 2011) μM. We tested the synthetic compound β-solamargine for which no activity data exist, thus this is the first report on cytotoxic activity. Structually β-solamargine and α-solamargine differ slightly in their aglycones (β-solasodine vs α-solasodine), but also in one of the linkages in the sugar moiety (Table 5.1). This difference does not seem to influence the cytotoxicity of the compounds as in our study β-solamargine's IC_{50} of 5.01 μM falls among the reported results for α-solamargine. Again, cytotoxic activity of α-solasonine was also reported against MCF-7 cells where it exhibited cytotoxicity with an IC_{50} value of 10.97 μM (Nakamura 1996). In contrast, in our study α-solasonine showed double the cytotoxicity (IC_{50} of 5.09 μM) than the reported result.

In addition, the cytotoxic activity of α-solanine against MCF-7 cells was reported with an IC_{50} value of 25.44 μM (Ji, Liu et al. 2012). In contrast, our study showed a significantly higher cytotoxic activity with an IC_{50} value of 4.57 μM which is further supported by our apoptosis and cell cycle analysis of this compound (chapter 5, section 5.5.4 and 5.5.5, respectively).

No reports were found on the cytotoxicity study of β-solamarine against MCF-7 so far, though this SGA exhibited high cytotoxic potential in our study.

BL3 had the second highest cytotoxic activity against colon cancer cell line (HT-29) with an IC_{50} value of 10.83 μM. Similarly, β-solamargine and β-solamarine were toxic to HT-29 with IC_{50} values of 15.90, and 16.36 μM, respectively.

BL3 contains α-solasodine rather than β-solasodine as the aglycone and contains one additional rhamnose moiety, as well as some different sugar linkages in comparison to β-solamargine and β-solamarine (Table 5.1). The impact of sugar moieties in SGAs on the anticancer potential of these compounds has been reported previously where α-solasonine and α-solamargine were more cytotoxic than their hydrolytic products and 6-*O*-sulphated solamargine, indicating that the nature, number and linkages of the sugar moieties exert paramount importance on their anticancer potential (Chang, Tsai et al. 1998).

Another study reported that α-solamargine showed 4 fold higher anticancer activity than that of khasianine due to the presence of one additional rhamnose moiety at the 2'position of glucose in α-solamargine (Chang, Tsai et al. 1998). Thus, our results support previously published findings on SAR of SGAs.

The SGA α-solamargine has been reported to inhibit the growth of colon cancer cells (HT-29) effectively with an IC_{50} of 2.49 μM (Hu 1999). Another report demonstrated the antiproliferative potential of α-solamargine on HT-29 where α-solamargine has been found to inhibit 82% cell growth at a concentration of 100 μg/mL (Lee, Kozukue et al. 2004). Although structurally slightly different (Table 5.1), β-solamargine showed 86% cell growth inhibition at the highest concentration

of 100 µg/mL against HT-29 colon cancer cells (Figure 5.9) which is consistent with findings for α-solamargine (Lee, Kozukue et al. 2004). The same researchers reported that α-solanine displayed 85% cell growth inhibition of HT-29 (Lee, Kozukue et al. 2004), which is very similar to our result of 80%. In contrast, in our study α-solasonine showed only 52% cell growth inhibition of HT-29 at 100 µg/mL, whereas prior research reported on 82% at the same concentration (Lee, Kozukue et al. 2004).

BL3 displayed high cytotoxic effects against gastric cancer cells (AGS) with an IC_{50} value of 11.20 µM. Less effective were β-solamargine, β-solamarine and α-solanine with IC_{50} values of 21.19, 26.08 and 31.56 µM, respectively.

The test results reveal the impact of the number of sugar moieties and to some extent the influence of the rhamnose moiety on the cytotoxic activity of SGAs. The novel compound **BL3** with three rhamnose residues showed the highest cytotoxic activity (Table 5.1), whereas the second most poetent compound, β-solamargine, has two rhamnose moieties (Table 5.1) and β-solamarine and α-solanine contain only one rhamnose each (Table 5.1), thus showed lower cytotoxic effects than **BL3** and β-solamargine. This study results are consistent with previous reports where the influence of the number of sugar moieties as well as the impact of the rhamnose moiety were investigated (Chang, Tsai et al. 1998; Li, He et al. 2007).

SGA, α-solanine has been reported to inhibit the growth of gastric cancer cells (AGS) by 87 % at a concentration of 100 µg/mL (Friedman, Lee et al. 2005). Similarly, in our study α-solanine inhibited AGS cancer cell growth by 80% at the same concentration. However, no reports have been found on the cytotoxic activity of β-solamargine and β-solamarine so far, thus cytotoxic activity against gastric cancer cells is reported here for the first time.

BL3 had less cytotxicity (IC_{50}: 13.72 µM) to estrogen non-dependent breast cancer cells (MDA-MB-231). In contrast, β-solamarine showed highest toxicity to MDA-MB-231 with an IC_{50} value of

2.55 μM. In addition, α-solanine and α-solasonine were also more toxic to MDA-MB-231 cells than **BL3** presenting IC_{50} values of 6.94 and 8.36 μM, respectively.

No reports were found on the cytotoxicity of α-solanine and α-solasonine so far against MDA-MB-231 breast cancer cells. However, to date no reports were found on the cytotoxic activity of β-solamarine against any cell line. Thus, this is the first report on the cytotoxic activity of β-solamarine against the cell lines tested.

The cytotoxicity study of SGAs reports the potent cytotoxicity of the novel compound **BL3**, as well as SAR investigations using selected SGA analoguess to **BL3** which may be due to **BL3** possessing a higher number of rhamnose moieties (three rhamnose) relative to β-solamargine, β-solamarine and α-solasonine. This study furthermore reports on the cytotoxic activity of β-solamarine, β-solamarine and α-tomatidine HCl against AGS, HT-29, MCF-7 and MDA-MB-231 for the first time where β-solamarine and β-solamarine exhibited very strong cytotoxic effects against MCF-7 breast cancer cells, whereas, α-tomatidine HCl was non-toxic to all cell lines tested.

Inhibiting tumor growth has been a continuous effort in cancer treatment. Tumor growth can be arrested either through inhibition of cancer cell growth or through induction of cancer cell death (Koduru, Grierson et al. 2007). In our study, potent cytotoxic effect of the novel compound, **BL3** as well as some other selected SGAs against MCF-7 cells demonstrate a possible function of these SGAs in tumor cell growth inhibition of breast cancer cells. To understand the molecular mechanisms underlying the cytotoxic effects of **BL3** and other SGAs against MCF-7 cells, apoptosis and cell cycle analysis were undertaken. These tests will also help with the understanding of SAR of SGAs including the novel SGA **BL3**.

5.5.4 Apoptosis assay of the novel SGA BL3 and the selected SGAs

Apoptosis, the prevalent form of programmed cell death, plays a central role in the development and homeostasis of multi-cellular organisms. Disregulation of the apoptotic process can cause many debilitating diseases in human including cancer (Shi 2005). SGAs have been reported to induce

apoptosis in different cancer cells including estrogen-dependent cancer cells (MCF) (Table 5.2). The novel SGA **BL3** showed significant cytotoxic activity against MCF-7 cells with an IC_{50} of 2.62 µM (Table 5.3). Similar to **BL3**, other selected SGAs also exhibited significant cytotoxic effects against breast cancer cells (Table 5.3). To determine whether **BL3** induces apoptosis, as well as to reveal SARs apoptotic activity against MCF-7 cells at the IC_{50} concentration was studied for the cytotoxic SGAs; **BL3**, β-solamargine, β-solamarine, α-solasonine and α-solanine (Table 5.3) (Chapter two, Section 2.6.1). The activity was compared to the known anticancer drug paclitaxel (Saunders, Lawrence et al. 1997), which served as a positive control.

5.5.4.1 Results and discussion of apoptosis assay of the novel SGA BL3 and the selected SGAs

Flow cytometry measurements were performed using annexin V-FITC and PI staining. The selected SGAs including the novel **BL3** induced apoptosis. The results are summarised in figures 5.12 and 5.13.
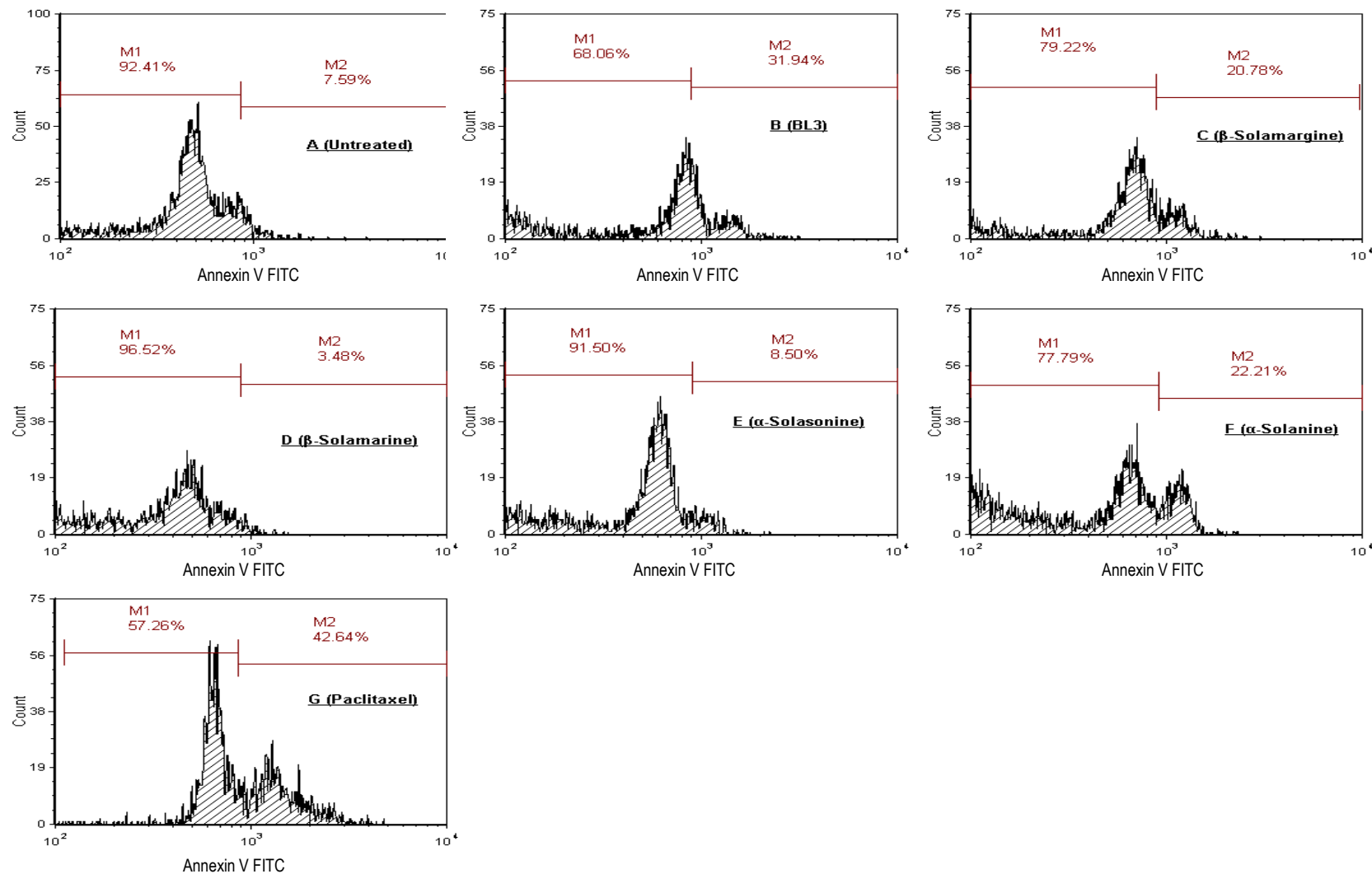
Figure 5.12 DNA histogram plots of MCF cells with and without treatment of SGAs after 24 h obtained by annexin V-FITC staining flow cytometry: untreated cells (A), **BL3** (B), β-solamargine (C), β-solamarine (D), α-solasonine (E), α-solanine (F) and paclitaxel (G). M1 indicates viable cells (AV$^-$) whereas M2 indicates apoptotic cells (AV$^+$)
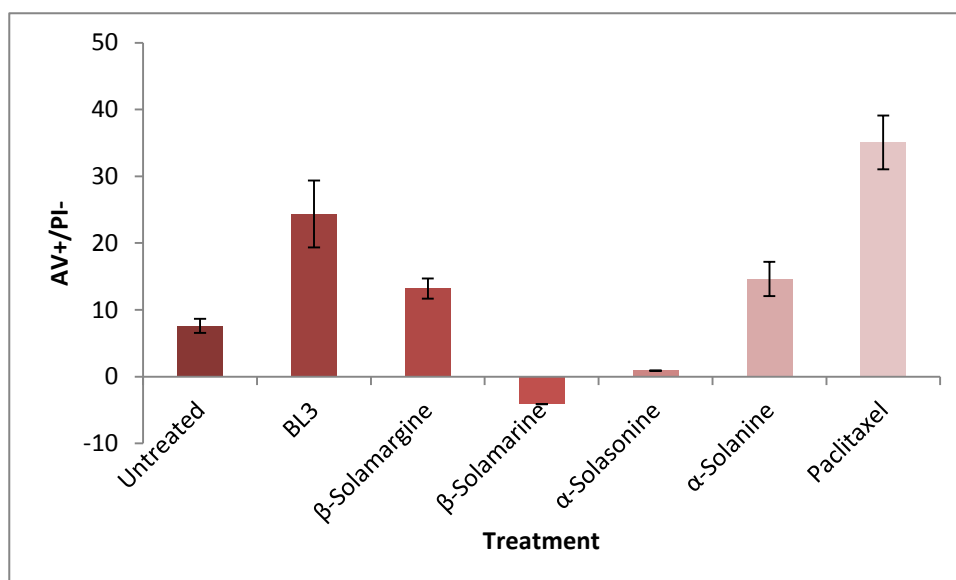
Figure 5.13 Representative diagram of SGAs (conc. = IC$_{50}$) induced apoptosis (AV$^+$/PI$^-$) in MCF-7 cell after 24 h treatment measured by annexin V-FITC staining flow cytomtery. Paclitaxel was used as positive control at 20 ng/mL

Treatment of MCF-7 cells with **BL3** at IC$_{50}$ of 2.62 μM for 24 h showed the highest apoptotic (AV$^+$/PI$^-$) effects (24%) among all SGAs tested. Although its activity was still below that observed for the positive control paclitaxel (35% AV$^+$/PI$^-$) (Figure 5.13).

The compounds α-solanine (IC$_{50}$: 4.10 μM) and β-solamargine (IC$_{50}$: 5.01 μM) exhibited some degree of apoptosis with 15% (AV$^+$/PI$^-$) and 13% (AV$^+$/PI$^-$), respectively (Figure 5.13). Although the apoptotic effects of **BL3** were not significant, they do compare to α-solanine and β-solamargine and could be due to the presence of four sugar moieties such as one glucose and three rhamnose (Figure 5.5). The impact of the number and linkages of the sugar moieties on ther cytotoxic activity or apoptosis has previously been reported against different cancer cell lines (Chang, Tsai et al. 1998; Li, He et al. 2007).

In contrast, β-solamarine showed no apoptosis though it was significantly cytotoxic with IC$_{50}$ of 4.10 μM. This result suggests that this potent cytotoxicity of β-solamarine may be attributed to effects on other mechanisms such as cell cycle or signal transduction pathways (Noori and Hassan

2012). The SGA α-solasonine showed only 0.9% apoptosis (AV^+/PI^-) which was also negligible (Figure 5.15). The positive control, paclitaxel induced apoptosis by (35% AV^+/PI^-) (Figure 5.13).

Following 48 h of treatment of MCF-7 cells with the selected SGAs neither an increase in apoptosis (% AV^+/PI^-) nor an increase in the number of necrotic cells (% AV^+/PI^+) was observed for any of the SGAs. This could be due to the consumption of the whole sample in 24 h by the cells. However, the positive control, paclitaxel, showed increased apoptotic effects (AV^+/PI^-) from 35 % to 49.5 % after 24 and 48 h, respectievly.

That α-solanine induces apoptosis has been reported for human hepatoma cells (Table 5.1). For α-solamargine reports exist on inducing apoptosis and enhancing the susceptibility to the anticancer drugs trastuzumab and epirubicin on MCF-7 and other breast cancer cells (SK-BR-3) (Shiu, Liang et al. 2009), but also on colon, gastric, liver, lung and other cell types (Table 5.2). However, no reports have been found on investigations into the apoptosis of β-solamargine, β-solamarine and α-solasonine. Thus, this is the first report on their apoptosis activity.

5.5.5 Results and discussion of cell cycle analysis of the novel SGA BL3 and the selected SGAs

Cell cycle analysis by measurement of DNA content using propidium iodide staining flow cytometry is one of the recognised techniques to explore how cytotoxic compounds provide their effect. It shows the distribution of cells in three major phases of the cycle; G1, S and G2/M, as it can measure apoptotic cells with fractional DNA content. The fluoroscence intensity of the stained cells at certain wavelengths correlate with the quantity of DNA in the cells (James W. 2011).

In order to further explore the mechanism by which the novel compound **BL3** and other cytotoxic SGAs, inhibit cell proliferation a cell cycle analysis by propidium iodide staining was performed (Chapter two, Section 2.6.2). Paclitaxel, a known anticancer drug was used as positive control at 20 ng/mL concentration.

The effects of **BL3** and the other SGAs on the cell cycle of MCF-7 breast cancer cells after 24 h incubation in comparison to no treatment and paclitaxel are shown in the Figures 5.14 and 5.15.

In the present study (paclitaxel, showed a significant increase in the percentage of cells at the G2/M phase, which correlates to a decrease in the percentage of cells at the G1 phase relative to no treatment, indicating that paclitaxel induced G2/M cell cycle arrest. This result is supported by another cell cycle study previously performed on MCF-7 with paclitaxel for 48 h (Saunders, Lawrence et al. 1997).
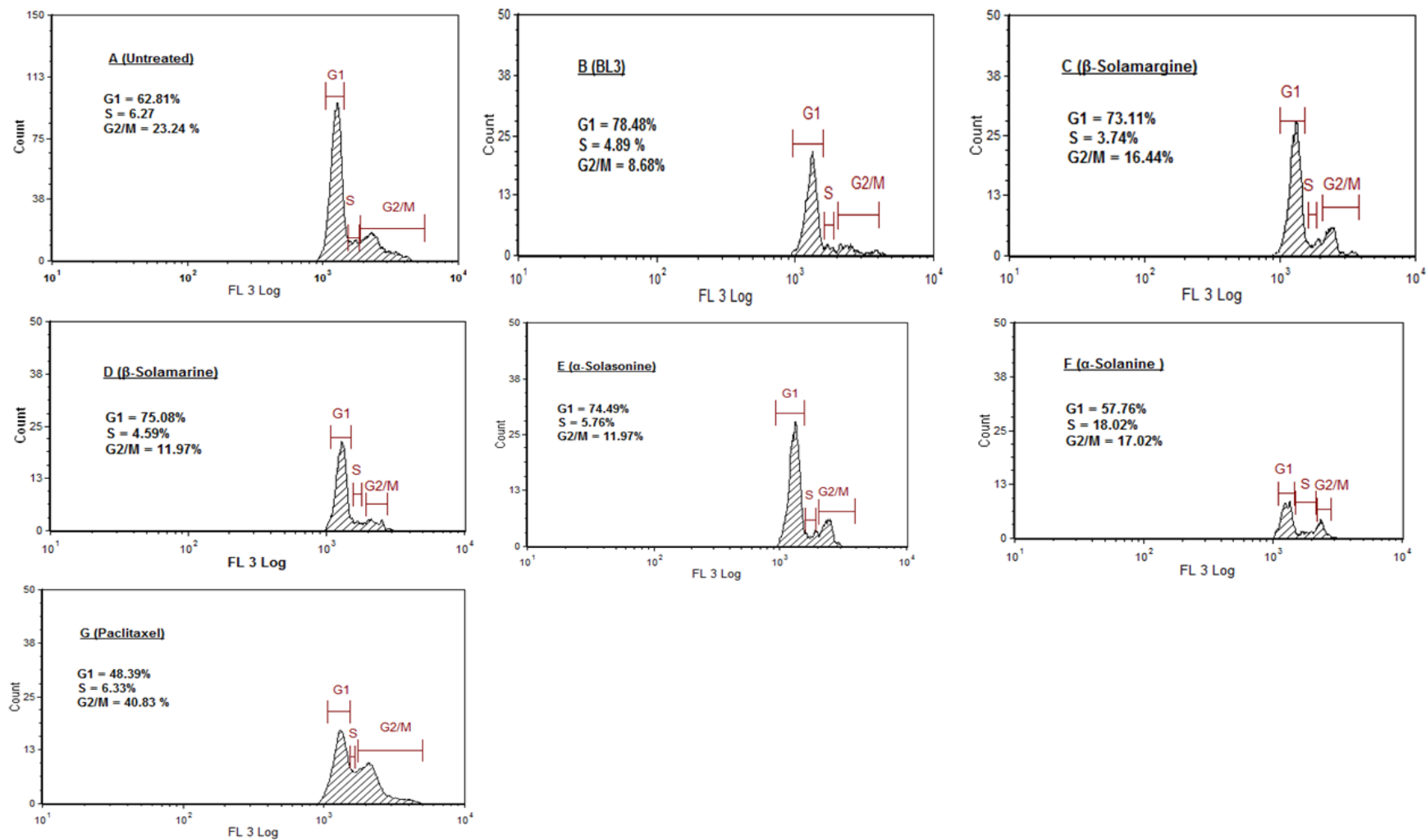
Figure 5.14 Histogram plots of cell cycle analysis of MCF-7 cells after 24 h of incubation: untreated cells (A), **BL3** (B), β-solamargine (C), β-solamarine (D), α-solasonine (E), α-solanine (F) and paclitaxel (G). The histogram is showing distribution of cells in three different phases G1, S and G2/M
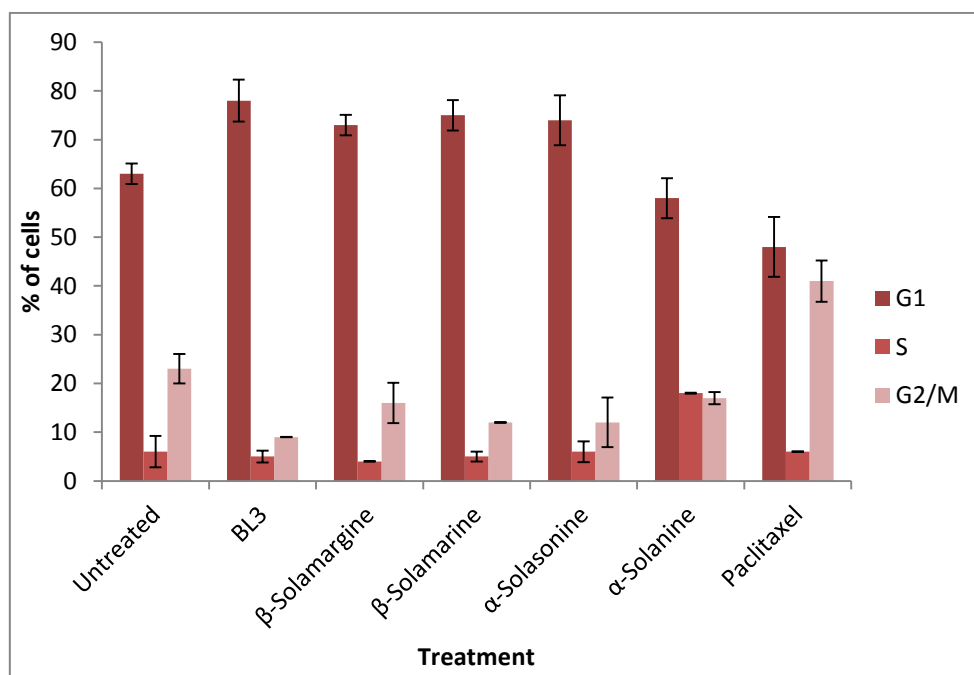
Figure 5.15 Cell cycle analysis of MCF-7 cells following 24 h incubation with **BL3** and other SGAs

The histogram of untreated cells shows a cell distribution of 62.81% in G1 phase, 6.27% in S phase and 23.24% in G2/M phase. In comparison to untreated cells, the percentage of MCF-7 cells at the G1 phase is increased to 78 % following treatment with **BL3**, which corresponds to a decrease in the S phase (to 4.9%) and G2/M phase (to 8.7%), indicating the arrest of the cell cycle at G1 (G0/G1) phase. Similarly, the decrease of MCF-7 cells in the G1 phase following treatment with β-solamargine, β-solamarine or α-solasonine (G1 = 73%, 75% and 74%) also indicates cell cycle arrest at G1 phase

A clear SAR analysis is difficult given that **BL3 a**nd β-solamargine, β-solamarine or α-solasonine arrest cells in G1 to a similar extent, however, the slightly stronger effect for **BL3** could be due to the presence of the largest number of sugar residues (Figure 5.5).

This study provides the first report on the cell cycle effects of β-solamargine, β-solamarine, and α-solasonine, as no previous research reports exist about the cell cycle effects of these compounds.

However, α-solamargine has been reported to arrest lung cancer cells (H441, H520, H661 and H69) in their sub-G1 phase (Liu, Liang et al. 2004).

The histogram of α-solanine clearly shows that in the presence of α-solanine there was a significant increase in the percentage of MCF-7 cells arrested in S phase (18.1%), with a corresponding decrease in the percentage of cells observed in the G1 phase (57.8%) and G2/M phase (17%), compared to untreated cells (62.8% and 23.2%, respectively). The effect of α-solanine arresting the S phase of the cell cycle in MCF-7 cells to produce antiproliferative activity correlates with previous findings (Ji, Liu et al. 2012).

Effects of SGAs on MCF-7 cells were observed (4 x magnification) (Figure 5.16) during apoptosis and cell cycle analysis. The images of the breast cancer cells treated with SGAs and positive control (B-G) indicate a decrease in number of cells relative to untreated cells (A). In D (cell treated with β-solamarine) and G (paclitaxel) similar pattern of cell morphology change is observed.
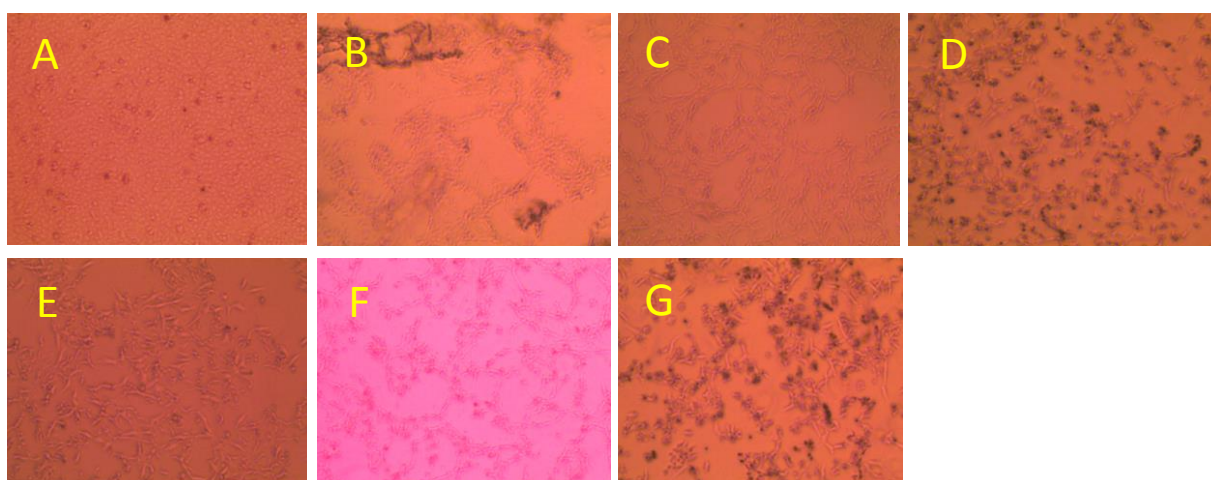
Figure 5.16 Microscopic views (4 x magnifications) of MCF-7 estrogen-dependent breast cancer cells tested for apoptosis analysis after 24 h of incubation with: untreated cells (A), **BL3** (B), β-solamargine (C), β-solamarine (D), α-solasonine (E), α-solanine (F) and paclitaxel (G)

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5.5.6 Results and discussion of antifungal activities of the selected steroidal glycoalkaloids

Search for new antifungals as well as look into their SAR studies led us to the investigations into antifungal activities of the isolated novel SGA, **BL3** and its analogues. Since there are a number of reports on the antifungal activity of SGAs as well as on their SAR investigations against various fungi species (Table 5.2; Sub-section 5.4.2), **BL3**, β-solamargine (SM), khasianine (KHS) and solasodine (SLS) were tested against four microfungi namely, *Candida albicans* (*C. albicans*), *Aspergillus niger* (*A. niger*), *Aspergillus fumigatus* (*A. fumigatus*), *Trichophyton mentagrophytes* (*T. mentagrophytes*) in order to explore their antifungal potential. The antifungal assay was developed and used as described by (Brechbühler 2013).

At a concentration of 20 µg/mL none of the compounds displayed a significant inhibitory effect and standard deviations are very high for all compounds tested against all fungi. A summary of all inhibitory activities of SGAs against all four microfungi is shown in Figure 5.17.
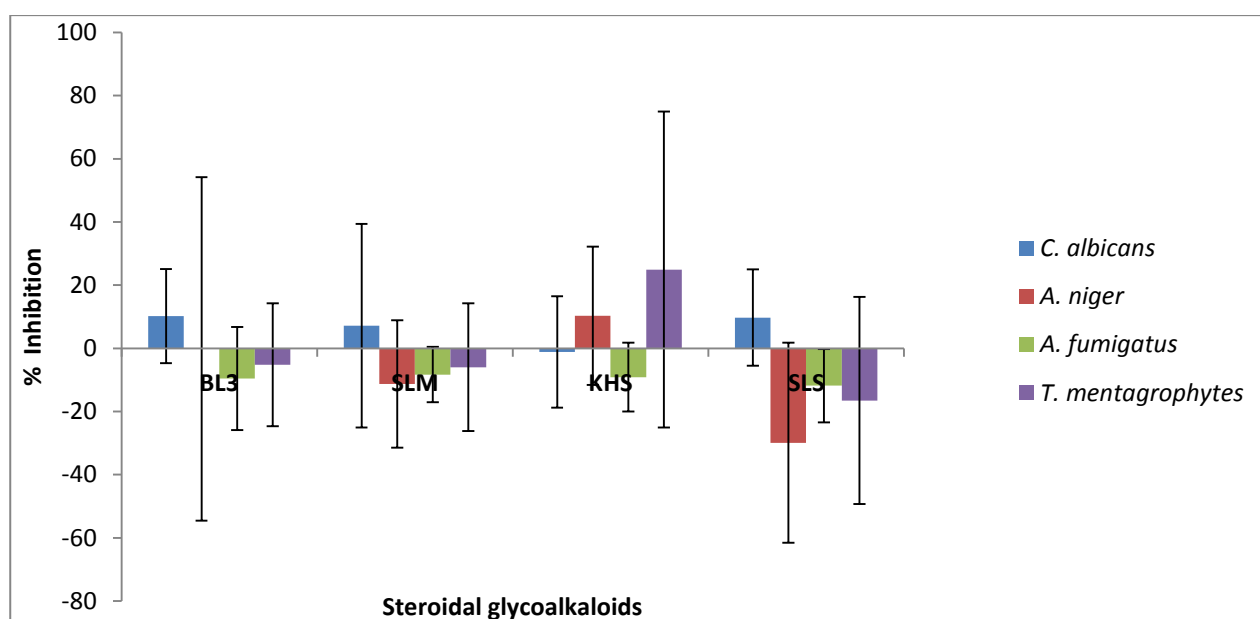
Figure 5.17 Antifungal activities of steroidal glycosides (20 µg/mL) determined using broth microdilution assay

Solasodine has been reported to exhibit very weak fungicidal activity when it was studied against various fungi species such as against *Candida albicans*, *C. pseudotropicalis*, *C. famata*, *C. kefyr*,

Cryptococcus albidus, *C. neoformans*, *Rhodotorula glutinis*, *Trichosporon cutaneum*, *Geotrichum candidum*, *Aspergillus fuumigates*, *A. candidus*, *Fonsecaea pedrosoi*, *Microsporum gypseum*, *Phialophoraverrucosa*, *Sporothrix schenckii*, *Trichophyton rubrum sc.*, and *T. mentagrophytes sc* (Kusano, Takahashi et al. 1987). In our study solasodine was found to be inactive against the tested fungi species may be due to different experimental conditions such as pH, media and temperature.

So far, no studies have reported on the antifungal effects of β-solamargine and khasianine and on their SAR studies. However, a number of reports have been found on α-solamargine against various fungi species but in every study this SGA showed very weak antifungal activity (Wolters 1968; Zhao, Gao et al. 2009). On the other hand, SGAs containing different aglycone (tomatidine in α-tomatine; solanidine in α-chaconine) have been reported to exert strong antifungal activity than SGAs containing solasodine as aglycone such as in α-solamargine (Fewell and Roddick 1993; Fewell and Roddick 1997). In contrast, SAR investigations into antifungal activity of α-solamargine and other SGAs against *Cercosporella brassicae* and *Alternaria porri* revealed the 3rd highest fungicidal effect of α-solamargine as was shown in the order of α-tomatine > α-chaconine > α-solamargine > α-solasonine > α-solanine (Zhao, Gao et al. 2009).

CHAPTER SIX

TERPENOID GLYCOSIDES AND THEIR CYTOTOXICITY

6.1 Background

Terpenoids, also referred to as terpenes, are the largest class of natural products with over 40,000 chemical structures (Rivas, Parra et al. 2013). They are produced by a wide variety of plants and animals and represent a rich reservoir of candidate compounds for drug discovery and development (Paduch, Kandefer-Szerzen et al. 2007; Huang, Lu et al. 2012). They frequently occur in fruits, vegetables and flowers. In 2002, the worldwide sales of terpene-based pharmaceuticals were approximately US $12 billion. Of these pharmaceuticals, the anticancer drug taxol® and the antimalarial compound, artemisinin are two of the most renowned terpene-based drugs (Guangyi Wang 2005).

Terpenoids are formed of five-carbon isoprene units (C_5H_8) and based on the number of building blocks, terpenoids are commonly classified as monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), triterpenes (C_{30}), tetraterpenes (C_{40}) and polyterpenes consisting of a long chain of many isoprene units (Figure 6.1).

Over 1000 monoterpenoids have been identified in nature (Salminen, Lehtonen et al. 2008). For example, α-pinene, thymol and geranol have been isolated from a number of plant species (Figure 6.1). Over 7000 sesquiterpenoids have been characterized with sesquiterpene lactones being the most abundant sesquiterpenoids (Salminen, Lehtonen et al. 2008). Farnesol and artemisinin are two widely known sesquiterpenoids isolated from *Commelina communis* (for example) and *Artemisia annua*, respectively (Qi, Wang et al. 2007; Rivas, Parra et al. 2013) (Figure 6.1). Moreover, thousands of diterpenoids have been identified from terrestrial and marine organisms (Lanzotti 2013). Acanthonic acid, tanshinone IIA and triptolide are examples of diterpenoids obtained from *Acanthopanax koreanum*, *Salvia miltiorrhiza* and *Tripterygium wilfordi,* respectively (Guangyi Wang 2005; Rivas, Parra et al. 2013) (Figure 6.1).
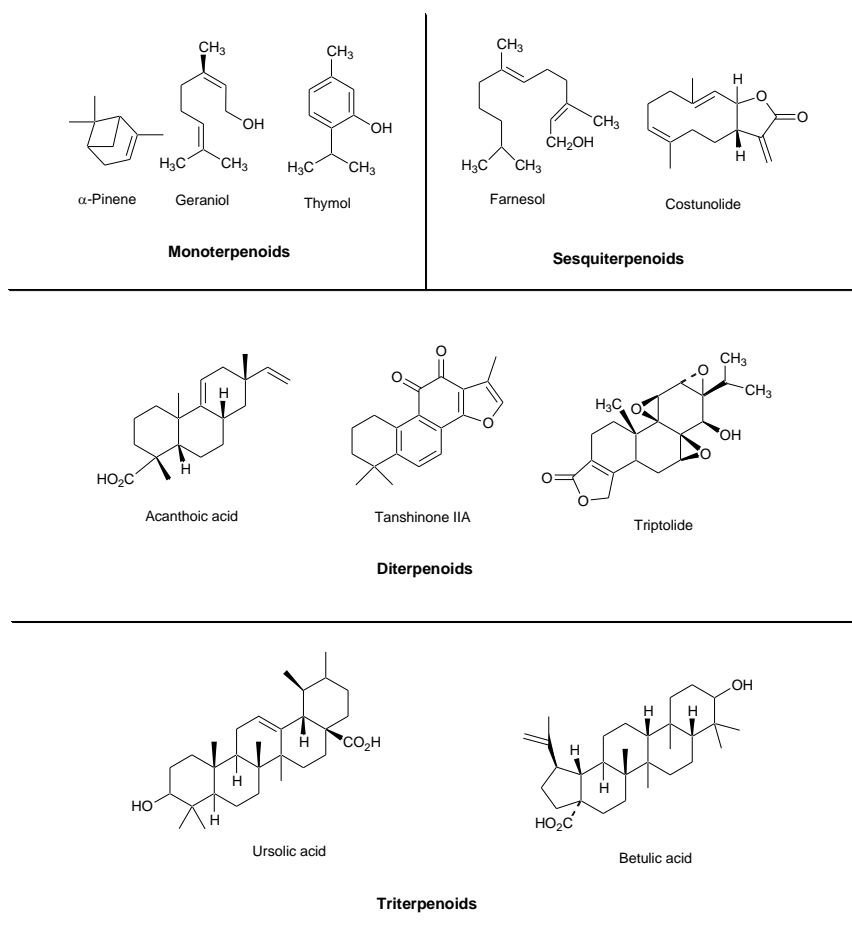
Figure 6.1 Some common terpenoids of different sub-classes
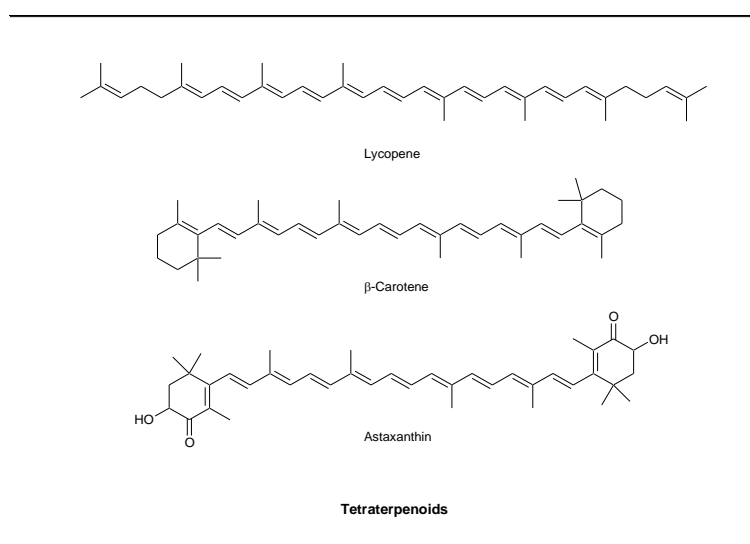
Figure 6.1 Some common terpenoids of different sub-classes (contd.)

Triterpenoids are the largest family of terpenoid natural products, with approximately 300 new triteprnoids characterized each year (Kuo, Qian et al. 2009). Ursolic acid, betulinic acid and lupeol are the triterpenoids that are commonly present in various plant species (de Carvalho and da Fonseca 2005; Lanzotti 2013) (Figure 6.1).

Carotenoids are the most common tetraterpenoids and comprise more than 600 known chemical structures (Huang, Lu et al. 2012). Lycopene is the most common carotenoid, terpenoid found in tomato and other red vegetables. Another common carotenoid terpenoid is β-carotene, which is predominantly found in carrots, but also in many other fruits with orange colour, whereas, astaxanthin is commonly found in salmon, shrimp and crabs (Salminen, Lehtonen et al. 2008).

6.2 Bioactivities of terpenoids

Terpenoids have been reported to be useful in the prevention and treatment of several human diseases, including cancer. They have also been found to possess a wide range of biological activities such as antimicrobial, antifungal, antiviral, anti-hyperglycemic, anti-inflammatory, and antiparasitic (Paduch, Kandefer-Szerzen et al. 2007). Anticancer and antifungal activities of terpenoids are thoroughly described below as these activities have been tested in this study for our isolated compounds. Importantly, inflammation has potential to cause cancer or exacerbate the cancer states. A number of terpenoids have been reported to provide anticancer effects through inhibition of inflammation caused by inhibition of NF-kB activity (Huang, Lu et al. 2012).

6.2.1 Anticancer activity

Recent progress and efforts into the research and development of anticancer drugs has afforded the identification of a variety of terpenoids from natural products (Huang, Lu et al. 2012).

Monoterpenoids (e.g. (+)-perillic acid), sesquiterpenoids (e.g. artemisinin), diterpenoids (e.g. andrographolide), triterpenoids (e.g. cucurbitacin) and tetraterpenoids (lycopene) have shown immunomodulatory and anticancer activities (Kuo, Qian et al. 2009; Kuttan, Pratheeshkumar et al. 2011; Huang, Lu et al. 2012).

Terpenoids were reported to inhibit cancer cell proliferation and induce apoptosis through the inhibition of various oncogenic and anti-apoptotic signalling pathways as well as suppression or nuclear translocation of various translocation factors, including NF-kB (Kuttan, Pratheeshkumar et al. 2011).

Dietary monoterpenes may be helpful in the prevention and treatment of cancer, which has been suggested in epidemiological studies (Paduch, Kandefer-Szerzen et al. 2007). D-limonene, a monocyclic terpenoid available in many citrus oils of lemon, orange, lime and grape fruit has been shown to possess chemo-preventive and therapeutic potential against many human cancers, such as breast, liver, skin, lung, colon, fore-stomach, prostate and pancreatic cancers in a dose dependent fashion. D-limonene provides anti-carcinogenic effects by preventing the interaction of carcinogens with the DNA in the initial phase and induces apoptosis in the later phase. It also inhibits the prenylation (the addition of hydrophobic molecules to proteins which in turn facilitates protein–protein binding in the cell membrane) of proteins which regulates the cell growth (Paduch, Kandefer-Szerzen et al. 2007).

Sesquiterpene lactones are the active constituents of a variety of medicinal plants used traditionally for treating inflammation. Artemisinin is a sequiterpene trioxane lactone isolated from the Chinese medicinal herb *Artemisia annua*. Artemisinin derivatives such as dihydroartemisinin and artesunate have shown antiproliferative activities against leukemic, breast, ovarian, prostate, colon, hepatocellular, gastric, skin and lung cancer cells (Huang, Lu et al. 2012). They are also reported to be effective against multidrug-resistant cancer cells and

provide similar anticancer potency in the parent and multidrug-resistant cancer cells. Artemisinin derivatives produce their antitumor activity through cleavage of the iron- or heme-mediated peroxide bridge (Huang, Lu et al. 2012).

A number of diterpenoids has been reported to provide anticancer activity. For example, andrographolide is a bicyclic diterpenoid lactone and the main active constituent of the plant *Andrographis paniculata*. It has been reported to exert potent anticancer activity against gastric, liver, lung, and breast cancer cells (Qi, Wang et al. 2007). The antiproliferative potential of andrographolide is due to apoptosis, cell cycle inhibition, and enhancement of antitumor activity of lymphocytes (Qi, Wang et al. 2007). It also potentiated the cytotoxic activity of 5-fluorouracil against human hepatoma cells (SMMC-7721) by inducing apoptosis (Kuttan, Pratheeshkumar et al. 2011).

Triterpenoids comprise the largest subclass of terpenoids tested for antiproliferative activity. For instance, cucurbitacins are highly oxidized cucurbitane type triterpenoids which are widely distributed in plants. Over 100 cucurbitacins have been reported to date to possess strong anticancer activity against various cancer cells. Cell cycle arrest, mainly G2/M phase, was noted with S phase arrest possible based on cell types and the specific cucurbitacin. Cucurbitacins have also been reported to dramatically inhibit tumor invasion and metastasis *in vivo* (Huang, Lu et al. 2012).

Anticancer activity of tetraterpenoids is well reported. For instance, lycopene's anticancer potential has been tested in clinical studies and in a phase II clinical trial. Lycopene alone or in combination with soy isoflavones delayed progression of hormone-dependent as well as hormone-nondependent prostate cancer (Huang, Lu et al. 2012). Lycopene most likely exerts its anticancer activity through scavenging reactive oxygen species (ROS) and inhibition of matrix metalloproteinase-2 (MMP2) and u-PA (urokinase-type plasminogen activator) (Huang, Lu et al. 2012).
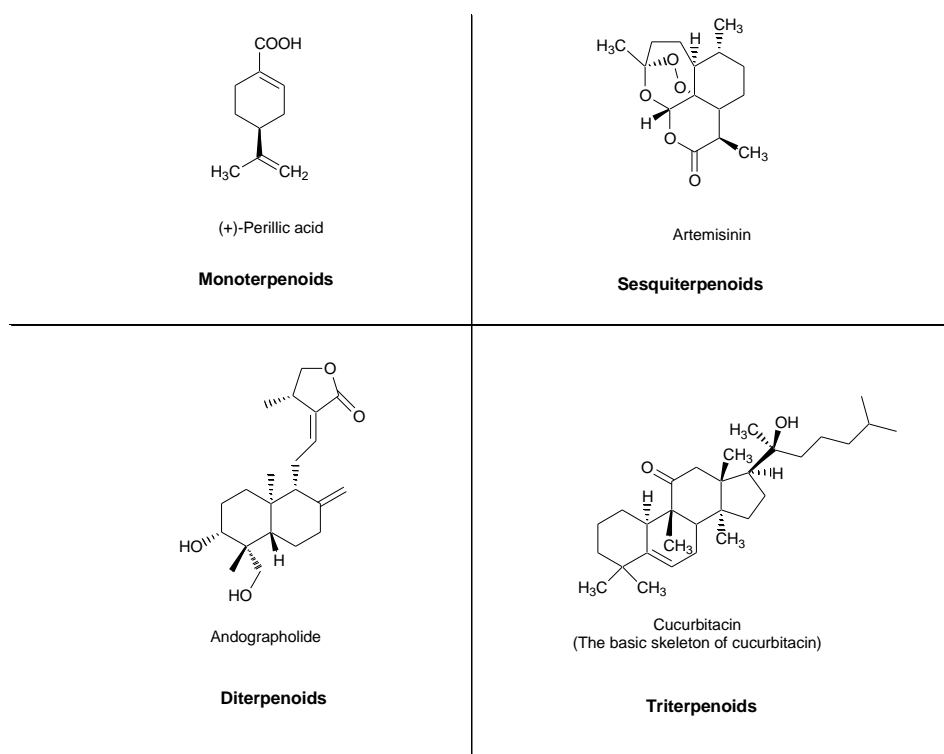
Figure 6.2 Chemical structures of selected terpenoids with anticancer activities

6.2.2 Antifungal activity

Mono-, sesqui-, di- and triterpenoids, but not tetraterpenoids have been reported to exhibit antifungal activity against different fungi species. Monoterpenes such as terpinen-4-ol, α-pinene, β-pinene, 1,8-cineole, linalool, and α-terpineol have shown activities against *C. albicans*, *C. krusei*, *C. tropicalis*, *Trichophyton mentagrophytes*, *T. rubram*, and *Microsporum gypseum* (Paduch, Kandefer-Szerzen et al. 2007). Carvone has shown effects against *Saccharomycetes* and mildew (Paduch, Kandefer-Szerzen et al. 2007), as well as against *Candida albicans* (de Carvalho and da Fonseca 2005).

Sesquiterpenoids for example khusinoldiolmonobrosylate, N-khusilidene-*p*-methoxy aniline, epikhusinol, N-khusilidene-*p*-fluoroaniline, and N-khusilidene-*p*-bromoaniline have been investigated for their fungicidal effects against *Vetiveria zizanoides*, *Alternaria alternata* and *Fusarium oxysporum* (Kaushal and Chahal 2005). In addition, antifungal activities of (+)-

curcuphenol, (+)-curcudiol, solavetivone, lubimin, lubiminoic acid, aethione and lubiminol have also been reported against *Absidia ramosa*, *Aspergillus niger*, *Botrytis cinerea*, *Cladosporium cucumerinum*, *Fusarium oxysporum*, *Penicillium expansum*, *Rhizopus oryzae*, and *Trichoderma harzianu* (Nagaoka, Goto et al. 2001; Gaspar, Feio et al. 2004).

Moreover, the natural diterpenoids salvic acid and acetylsalvic acid, as well as the semisynthetic diterpenoids propanoylsalvic acid, and butanoylsalvic acid inhibited the mycelial growth of *Botrytis cinerea*. Acetylsalvic acid was found to demonstrate fungitoxic effect by altering membrane permeability (Mendoza, Espinoza et al. 2009).

The triterpenoids cycloartenone β-amyrin acetate and friedelin, isolated from *Heliotropium ellipticum*, were found to possess broad spectrum antifungal activity against *Aspergillus niger*, *A. flavus*, *Rhizoctonia phaseoli* and *Penicillium chrysogenum* (Jain, Singh et al. 2001).

6.3 Terpenoid glycosides

A significant number of terpenoid glycosides have been identified from natural sources where sugar residues are linked to the active terpenoid groups. Glycosidic residues are essential for the activity of many terpenoid, although it is reported in some cases to only improve the pharmacokinetic parameters (Rivas, Parra et al. 2013). Terpenoid glycoside subgroups mimic those of the terpenoids; monoterpenoid glycosides, sesquiterpenoid glycosides, diterpenoid glycosides, and triterpenoid glycosides.

Acyclic diterpenoid glycosides are discussed in detail below since our study on *Blumea lacera* afforded isolation and structural elucidation of two acyclic diterpenoid glycosides (**BL4** + **BL5**) along with a sequiterpenoid glycoside (**BL6**).

6.3.1 Diterpenoid glycosides

Diterpenoid glycosides are diterpenoids with one or more sugar residues linked to a diterpenoid. Diterpenoid glycosides are found as acyclic and cyclic forms based on whether

the aglycone, diterpenoid, is present as a ring structure or an open chain. Although a significant number of acyclic diterpenoid glycosides have been isolated, only a few of them have been studied for their bioactivities. Table 6.1 provides a list of acyclic diterpenoid glycosides and their bioactivities for those isolated to date from plants (unless otherwise stated). Lyciumoside I   exhibited appreciable inhibition of *Helicobacter pylori* when antimicrobial activity of lyciumosides I, II and III have been investigated on *Micrococcus flavus*, *Helicobacter pylori*, *M. flavus*, *Bacillus mycoides*, and *Cotyuebacterium xerosis* (Terauchi 1998).

Table 6.1 Acyclic diterpenoid glycosides isolated and their bioactivities studied previously

Compounds	Plant species	Anticancer/cytotoxic activity	Other bioactivities	References
6*E*,10*E*,14*Z*-(3*S*)-17-hydroxygeranyllinalool17-*O*-α-L rhamnopyranosyl(1→4)-[α-L-rhamnopyranosyl-(1→6)]-β-D-glucopyranoside (1, capsianoside XVIII) and capsianoside F (2)	*Capsicum annuum*	No reports found	Antioxidant activity	(Shin, Cho et al. 2012)
Tetra-acetylated oligosaccharide diterpene	*Cupania vernalis*	No reports found	No reports found	(Cavalcanti, Teles et al. 2001)
Lyciumoside IV-IX ; (lyciumoside VII: **BL4**)	*Lycium chinese*	No reports found	No reports found	(Terauchi 1998)
Virescenosides V (1), W (2), and X (3)	*Acremonium striatisporum* (**Marine fungus**)	No reports found	No reports found	(Afiyatullov, Kalinovsky et al. 2006)
Capsianosides I, II, III (1), C (2), D (3), E (4) and F (5)	*Capsicum annuum*	No reports found	No reports found	(Lee, Kiyota et al. 2007)
Lyciumosides I, II and III	*Lycium chinese*	No reports found	Antimicrobial activity	(Terauchi 1998)
Capsianosides VI, G and H	*Capsicum annum*	No reports found	No reports found	(Yahara, Kobayashi et al. 1991)
Capsianosides XVII (1), V methyl ester (2) , capsianosides I (1) and II (2)	*Capsicum annuum*	No reports found	No reports found	(Lee, Kiyota et al. 2008)
Lyciumosides I-III (5-7)	*Lycium chinese*	No reports found	No reports found	(Yahara, Shigeyama et al. 1993)
3*S*, 6*E*, 10*E*, 14*Z* -tetramethyl-3,14,15-trihydroxyhexadeca-6,10,14-triene 3-*O*-β-D-glucopyranoside, named lyciumoside X (3), phytol (1) and lyciumoside III (2).	*Trigonotis peduncularis*	No reports found	No reports found	(Song, Yang et al. 2008)
Capsianoside VII (3), capsianoside III (5) and capsianoside II (6)	*Capsicum annuum*	No reports found	No reports found	(Iorizzi, Lanzotti et al. 2001)

Table 6.1 Acyclic diterpenoid glycosides isolated and their bioactivities studied previously (contd.)

Compounds	Plant species	Anticancer/cytotoxic activity	Other bioactivities	References
Capsianosides (1-4) , capsianoside III (5), capsianoside V (6) and capsianoside I (7)	*Capsicum annuum*	No reports found	Antioxidant activity (Compounds; 1, 5 and 6)	(De Marino, Borbone et al. 2006)
3β-[(α-L-arabinopyranosyl)oxy]-19α hydroxyolean-12-en-28-oic acid 28-β-D-glucopyranoside (4), 3β-[(α-L-arabinopyranosyl)-oxy]-19α-hydroxyurs-12-en-28-oic acid 28-β-D-glucopyranoside (ziyu glycoside I, 5), 3β,19α-hydroxyolean-12-en-28-oic acid 28-β-D-glucopyranoside (6) and 3β,19α-dihydroxyurs-12-en-28-oic acid 28-β-D-glucopyranoside (**7**).	*Sanguisorba officinalis*	No reports found	Hemostatic activities	(Sun, Zhang et al. 2012)
Attenoside (3), lyciumoside IV (1) and lyciumoside II (2)	*Manduca sexta*	No reports found	No reports found	(Jassbi, Zamanizadehnajari et al. 2006)
Capsianoside XIII and capsianoside XV, 3-*O*-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl 17,19-dihydroxy-6*E*,10*E*,14*Z*-(3*S*)-geranyllinalool 17-*O*-β–D-glucopyranosyl-(1→4)-α-L-rhamnopyranosyl-[α-L-rhamnopyranosyl-(1→6)]-β-D-glucopyranoside and 3-*O*-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl 6*E*,10*E*,14*Z*-(3*S*)-17-hydroxy-geranyllinalool 17-*O*-[3-*O*-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl- 6*E*,10*E*,14*Z*-(3*S*)-13'*R*,19'-dihydroxy-geranyllinalool-16'-oyl (16'→2)-[α-L-rhamnopyranosyl-(1→3)]-α-L-rhamnopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→6)]-β-D-glucopyranoside	*Capsicum chinense*	No reports found	No reports found	(Lee, El-Aasr et al. 2009)
Capsianosides XIII (2), XV (3), IX (4), XVI (5), X (6) and VIII (7) , and capsianoside II (1)	*Capsicum annuum*	No reports found	No reports found	(Lee, Kiyota et al. 2006)
Capsianosides A-F and I-V	*Capsicum annuum*	No reports found	No reports found	(Izumitani, Yahara et al. 1990)
Capsianosides I, II, III, IV, and V and (2) and capsianosides A, B, C, D, and E	*Capsicum annuum*	No reports found	No reports found	(Yahara, Izumitani et al. 1988)

6.4 Isolated terpenoid glycosides from *Blumea lacera*

6.4.1 Introduction to the isolated terpenoid glycosides (BL4, BL5 and BL6) from *Blumea lacera*

In this study three terpenoid glycosides, **BL4** (3-*O*-β-D-glucopyranosyl 17-hydroxy-6*E*, 10*E*, 14*Z*-(3*S*)-geranyllinalool 17-*O*-β-D-glucopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→6)]-β-D-glucopyranoside), **BL5** (6*E*, 10*E*, 14*Z*-(3*S*)-17-hydroxy geranyllinalool 17-*O*-β-D-glucopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→6)]-β-D-glucopyranoside) and **BL6** (2*E*, 6*E*, 10*Z*-12-hydroxy farnesol 12-*O*-β-D-glucopyranosyl-(1→6)-α-L-rhamnopyranoside) were isolated from *Blumea lacera* leaf extract (Table 6.2, Figure 6.3). **BL4** is a known compound but this is the first time that it has been isolated and reported from *Blumea lacera*. Of them, **BL5** and **BL6** are novel compounds. The compounds were isolated and purified using semi-preparative HPLC followed by SPE and the structures were elucidated using spectroscopic methods NMR (^{1}H, ^{13}C, COSY, HSQC and HMBC) and HRMS (Chapter IV). Table 6.2 summarises information on the isolated terpenoid glycosides from *Blumea lacera* leaf extract.

Table 6.2 Terpenoid glycosides isolated from *Blumea lacera*

Compounds	Colour	Compound name	Molecular formula	Novelty
BL 4	White	3-*O*-β-D-glucopyranosyl 17-hydroxy-6*E*, 10*E*, 14*Z*-(3*S*)-geranyllinalool 17-*O*-β-D-glucopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→6)]-β-D-glucopyranoside	$C_{44}H_{74}O_{21}$	First time for this plant
BL 5	White	6*E*, 10*E*, 14*Z*-(3*S*)-17-hydroxy geranyllinalool 17-*O*-β-D-glucopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→6)]-β-D-glucopyranoside	$C_{38}H_{64}O_{16}$	Novel
BL6	White	2*E*, 6*E*, 10*Z*-12-hydroxy farnesol 12-*O*-β-D-glucopyranosyl-(1→6)-α-L-rhamnopyranoside	$C_{27}H_{46}O_{11}$	Novel

BL: *Blumea lacera*

3-O-β-D-Glucopyranosyl 17-hydroxy-6E, 10E, 14Z-(3S)-geranyllinalool 17-O-β-D-glucopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→6)]-β-D-

6E, 10E, 14Z-(3S)-17-hydroxy geranyllinalool 17-O-β-D-glucopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→6)]-β-D-

2E, 6E, 10Z-12-Hydroxy farnesol 12-O-β-D-glucopyranosyl-(1→6)-α-L-rhamnopyranoside (**BL6**)
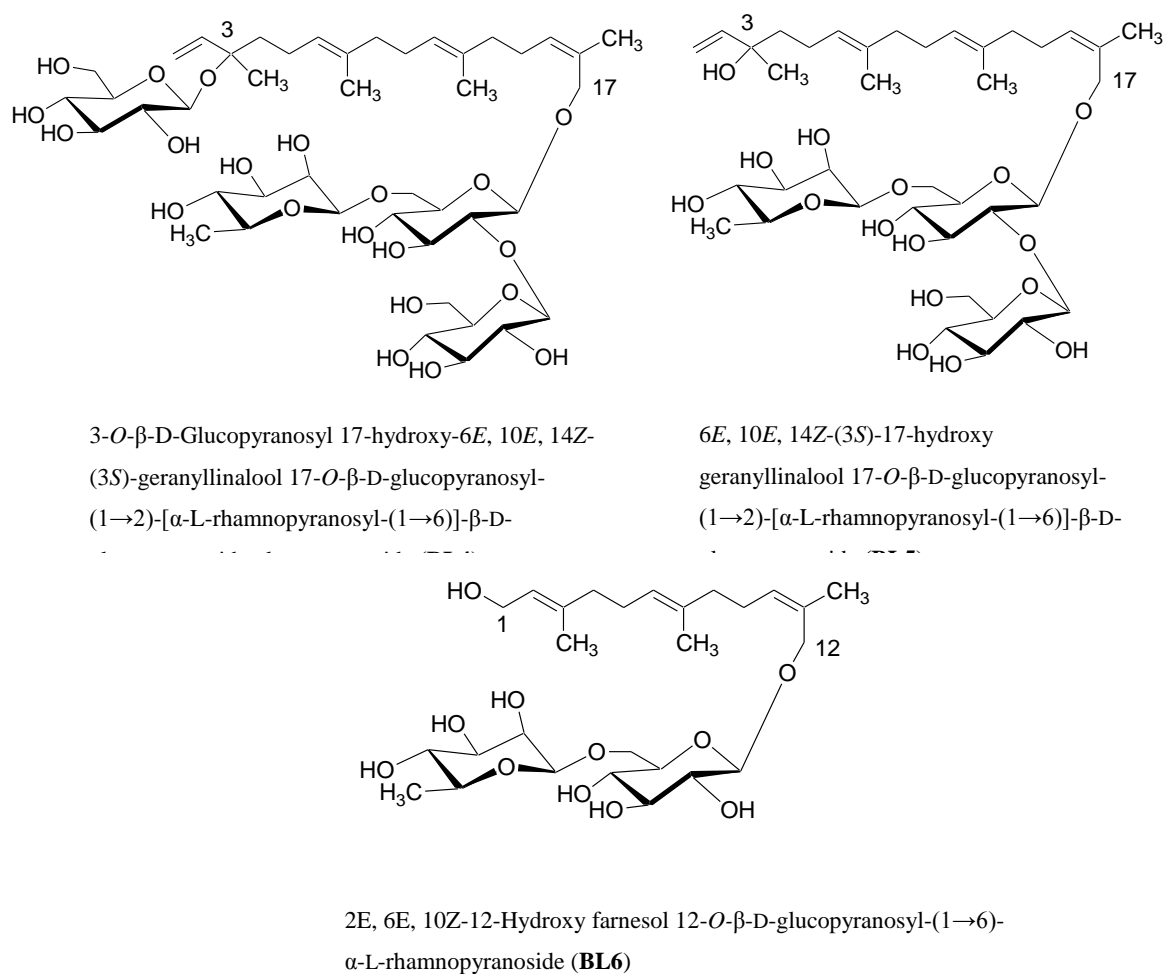
Figure 6.3 Terpenoid glycosides (**BL4, BL5** and **BL6**) isolated from *Blumea lacera*

6.4.2 Bioactivity studies on BL4, BL5 and BL6 from *Blumea lacera*

6.4.2.1 Cytotoxic activity tests on BL4, BL5 and BL6

The three isolated terpenoid glycosides (**BL4**, **BL5** and **BL6**) were tested for their cytotoxic potential against two healthy cell lines, namely a normal mouse fibroblast (NIH3T3) and a monkey kidney cell line (VERO), as well as against four cancer cell lines; one gastric adenocarcinoma (AGS), one colon adenocarcinoma (HT-29), and two breast ductal carcinoma (MCF-7 and MDA-MB-231) cell lines (Section 2.5).

6.4.2.1.1 Results and discussion of cytotoxic activity tests of BL4, BL5 and BL6

Cytotoxicity results (% of cell growth inhibition) of **BL4**, **BL5** and **BL6** are summarized in the figures 6.4 to 6.6. IC_{50} values of the isolated terpenoid glycosides are summarized in the Table 6.3.
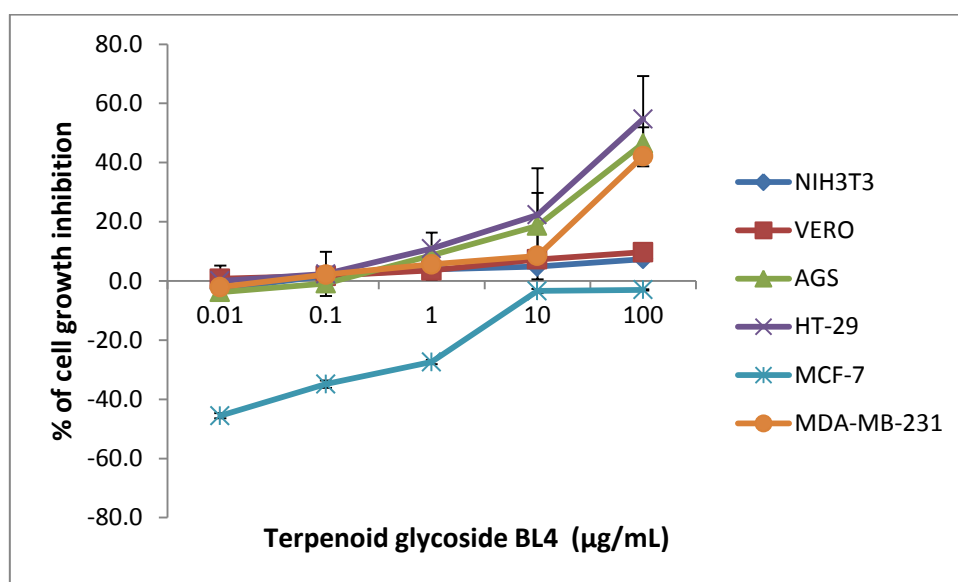
Figure 6.4 Percent (%) of cell growth inhibition of terpenoid glycoside **BL4** against the tested cell lines
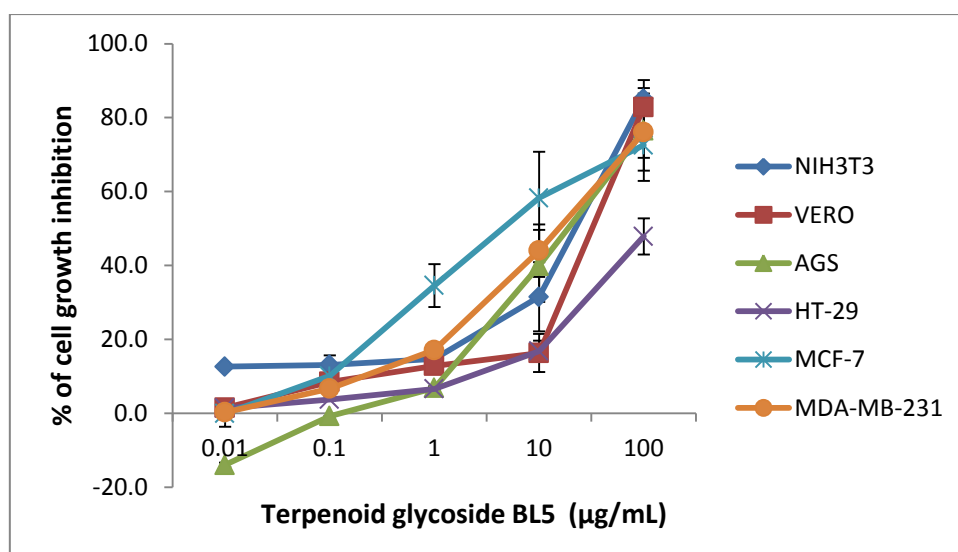
Figure 6.5 Percent (%) of cell growth inhibition of terpenoid glycoside **BL5** against the tested cell lines
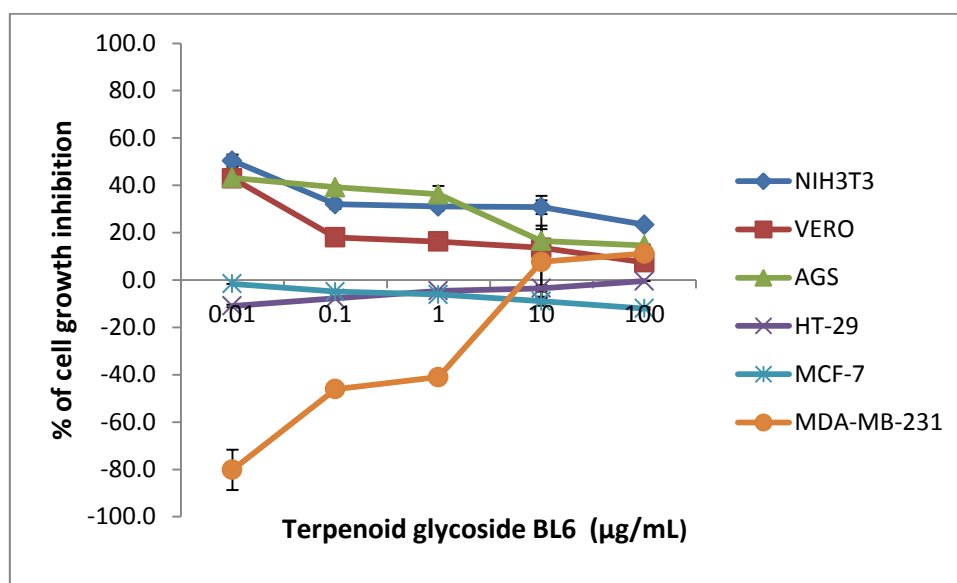
Figure 6.6 Percent (%) of cell growth inhibition of terpenoid glycoside **BL6** against the tested cell lines

Table 6.3 Cytotoxic activity (IC_{50}) of terpenoid glycosides isolated (**BL4, BL5** and **BL6**) from *Blumea lacera*

Compound	Cytotoxic activity (IC_{50})* (μg/mL)					
	VERO	NIH3T3	AGS	HT-29	MCF-7	MDA-MB-231
BL4	>100	>100	>100	81.6	>100	>100
BL5	25.50	33.10	19.90	>100	6.66	13.95
BL6	NC*	NC*	NC*	NC*	NC*	NC*
Cycloheximide (positive control)	6.88	7.44	2.40	3.10	51.17	6.30

IC_{50}* (Inhibition of cell growth by 50%) calculated by probit analysis (LdP Line software, USA), data was generated by experiments performed in triplicates.

NC*: No cytotoxic activity at highest concentration tested

No reports were found on the anticancer/cytotoxic activity of the isolated diterpenoid glycosides (**BL4**, **BL5** and **BL6**). **BL4** (lyciumoside VII) has been isolated previously from another plant *Lycium chinese* (Terauchi 1998) interestingly though, it has not been tested for any bioactivity so far. There are a number of acyclic diterpene glycosides; lyciumosides I, II and III, capsianoside I, capsianoside III, and capsianoside V which are structurally close to **BL4** and **BL5** have been isolated from *Lycium chinese* and *Capsicum annuum*, respectively (Terauchi 1998 ; De Marino, Borbone et al. 2006).

Here we report findings that **BL4** was non-toxic to all cell lines tested, except for colon cancer cells (HT-29), although this activity was low (IC$_{50}$ of 81.6 µg/mL). In contrast, the novel compound, **BL5**, showed significant cytotoxicity against all cell lines except for HT-29. In fact, **BL5** showed the highest and second highest cytotoxicity recorded against breast cancer cells, MCF-7 and MDA-MB-231 (IC$_{50}$ = 6.66 µg/mL and IC$_{50}$ of 13.95 µg/mL, respectively). This activity was against MCF-7 cancer cells almost 8 times higher than that of the known cytotoxic compound, cycloheximide. Importantly, **BL5** was less toxic to healthy cells, NIH3T3 and VERO than to breast cancer cells (MCF-7 and MDA-MB-231). In contrast, **BL6** was non-toxic to all cell lines tested. Interestingly, compound **BL6** displayed concentration-dependent cell proliferative activity instead of cytotoxic effects against NIH3T3, VERO, AGS and MCF-7 cells (Figure 6.6).

No reports exist on the cytotoxic/anticancer activity of terpenoid glycosides, although terpenoids have been widely reported for their anticancer potential. For example, andrographolide, a bicyclic diterpenoid lactone isolated from *Andrographis paniculata* has been reported to exert potent anticancer activity against gastric, liver, lung, and breast cancer cells (Qi, Wang et al. 2007). In addition, artemisinin is a sequiterpene trioxane lactone isolated from the Chinese medicinal herb *Artemisia annua* with antiproliferative activities against various cancer cells including breast cancer cells, MDA-MB-231 reported (Huang, Lu

et al. 2012). Cucurbitacins, being triterpenoids are also well reported to exert anticancer activity against various cancer cells (Huang, Lu et al. 2012).

Structurally, **BL4** and **BL 5** are very similar. The difference between the two compounds is that a glucopyranose sugar unit is attached at the C-3 position of the aglycone in **BL4**, whereas in **BL5** a free hydroxyl group is present at this position. This difference could account for the significant cytotoxic activity detected for **BL5**. It has been reported, that structural modifications at C-3 leads to alteration of anticancer activity as well as selectivity of the terpenoid derivatives. This was demonstrated for the pentacyclic triterpenoid, betulinic acid (Kuo, Qian et al. 2009).

The structure of **BL6** differs from **BL4** and **BL5** in aglycone and sugar moieties. **BL6** contains a hydroxymethyl group instead of a terminal double bond at the C-1 as well as lacking one glucopyranose sugar residue C2 linked from the central β-D-Glc. It may be that both structural differences account for the cell proliferative activity observed for **BL6**.

Terpenoids have been reported to inhibit cancer cell proliferation through apoptosis induction, cell cycle arresting as well as suppression or nuclear translocation of various translocation factors including NF-kB (Kuttan, Pratheeshkumar et al. 2011; Huang, Lu et al. 2012). As **BL5** displayed significant cytotoxic effects out of the three terpenoid glycosides, it was decided that the mechanism behind this cytotoxicity would be investigated further by undergoing apoptosis and cell cycle analysis.

6.4.2.2 Apoptosis study of BL5

Apoptosis, the prevalent form of programmed cell death, plays a central role in the development and homeostasis of multi-cellular organisms. Disregulation of the apoptotic process can cause many debilitating diseases in humans including cancer (Shi 2005).

Anticancer agents with antiproliferative as well as apoptosis-inducing potential are promising candidates for cancer treatment (Shaikh 2010).

Because the isolated compound **BL5** showed significant cytotoxic activity with an IC_{50} of 6.66 µg/mL against estrogen-dependent breast cancer cells MCF-7 (Table 6.4), it was investigated for apoptosis inducing potential against MCF-7 cells at its IC_{50} (6.66 µg/mL) concentration of studies for 24 and 48 h using annexin V-FITC and propidium iodide staining flow cytometry (Chapter two, Section 2.6.1) were employed, with the activity compared to the known anticancer drug, paclitaxel (Saunders, Lawrence et al. 1997) which served as a positive control.

6.4.2.2.1 Results and discussion on apoptosis study of BL5

Flow cytometry measurements were performed using annexin V-FITC and PI staining. The **BL5** induced apoptosis results are summarised in Figures 6.7 and 6.8.
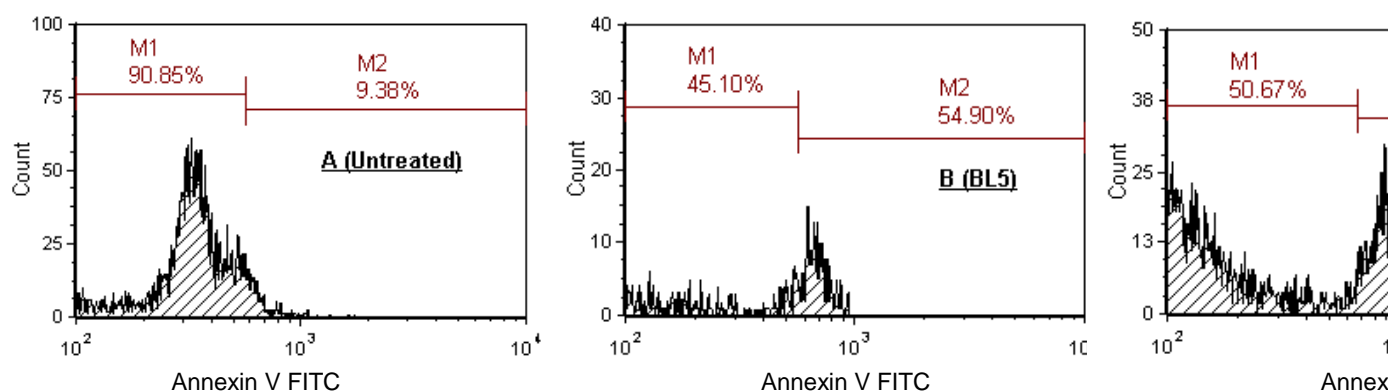
Figure 6.7 DNA histogram plots of MCF cells with and without treatment of **BL5** after 24 h obtained by annexin V-FITC staining flow cytometry: untreated cells (A), **BL5** (B), paclitaxel (C). M1 indicates viable cells (AV$^-$) whereas M2 indicates apoptotic cells (AV$^+$)
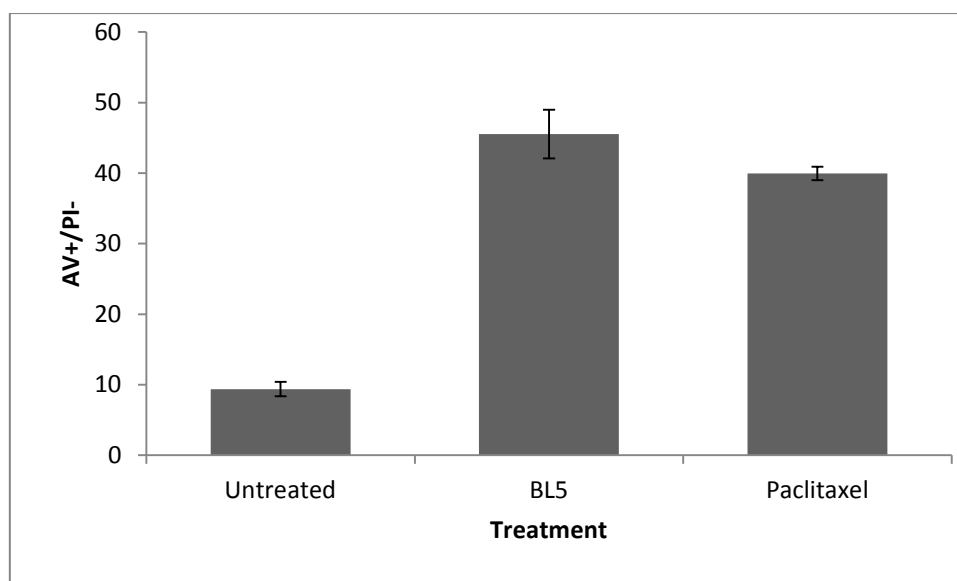
Figure 6.8 Representative diagram of **BL5** (conc. = IC_{50}) induced apoptosis (AV^+/PI^-) in MCF-7 cells after 24 h treatment measured by annexin V-FITC staining flow cytomtery. Paclitaxel was used as positive control at 20 ng/mL

Treatment of MCF-7 cells with **BL5** at IC_{50} of 6.66 µg/mL for 24 h showed 45.5% AV^+/PI^- (apoptosis) (Figure 6.8). The **BL5** induced-apoptosis was slightly higher than that observed for the positive control, paclitaxel (39.9% AV^+/PI^-) (Figure 6.8). Following 48 h of treatment of MCF-7 cells with **BL5** neither an increase in apoptosis (% AV^+/PI^-) nor an increase in the number of necrotic cells (% AV^+/PI^+) was observed. This could be due to the consumption of the whole sample in 24 h by the cells. A, the positive control, paclitaxel showed increased apoptosis (AV^+/PI^-) from 39.9% to 50.3% for 24 to 48 h though the concentration used was 20 ng/mL.

No previous apoptotic studies of diterpenoid glycosides analogous to **BL5** were found in the literature. However, the diterpenoid, andrographolide has been reported to exert antitumor activity against lymphocytes is due to apoptosis (Qi, Wang et al. 2007). Andrographolide also enhanced the cytotoxic activity of 5-fluorouracil against human hepatoma cells (SMMC-7721) through apoptosis (Kuttan, Pratheeshkumar et al. 2011).

6.4.2.3 Cell cycle analysis of BL5

Cell cycle analysis by measurement of DNA content using propidium iodide staining flow cytometry is a recognised technique to explore how anticancer compounds produce their effect. Cell cycle analysis shows the distribution of cells in three major phases; G1, S and G2/M, as it can measure apoptotic cells with fractional DNA content. The fluorescence intensity of the stained cells at certain wavelengths correlate with the quantity of DNA in the cells (James W. 2011).

Based on the significant cytotoxic effect of **BL5** on MCF-7 cells (Table 6.4), a cell cycle analysis was performed against MCF-7 cells at the IC_{50} 6.66 µg/mL concentration for 24 h (Chapter two, Section 2.6.2). The known anticancer drug paclitaxel served as a positive control (Saunders, Lawrence et al. 1997).

6.4.2.3.1 Results and discussion on cell cycle analysis of the novel terpenoid glycoside BL5

The effect of **BL5** on the cell cycle of MCF-7 cancer cells after 24 h incubation in comparison to no treatment and paclitaxel is shown in the Figures 6.9 and 6.10.
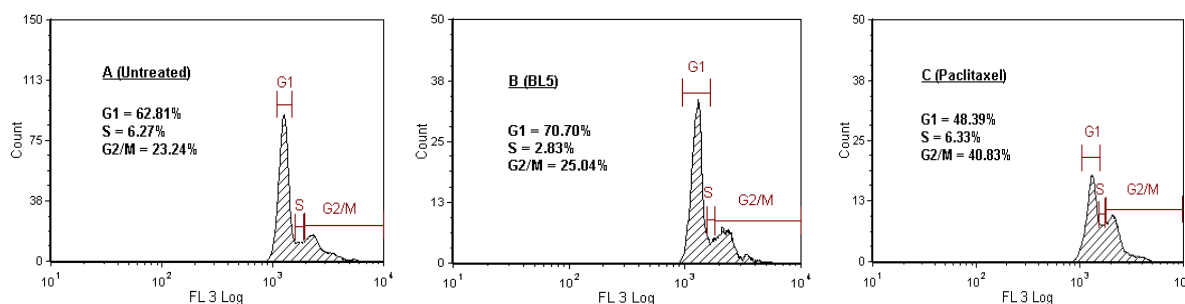
Figure 6.9 Histogram plots of MCF-7 cells tested for cell cycle analysis after 24 h of incubation: untreated cells (A), **BL5** (B), and paclitaxel (C). The histogram shows cells distribution in three different phases G1, S and G2/M
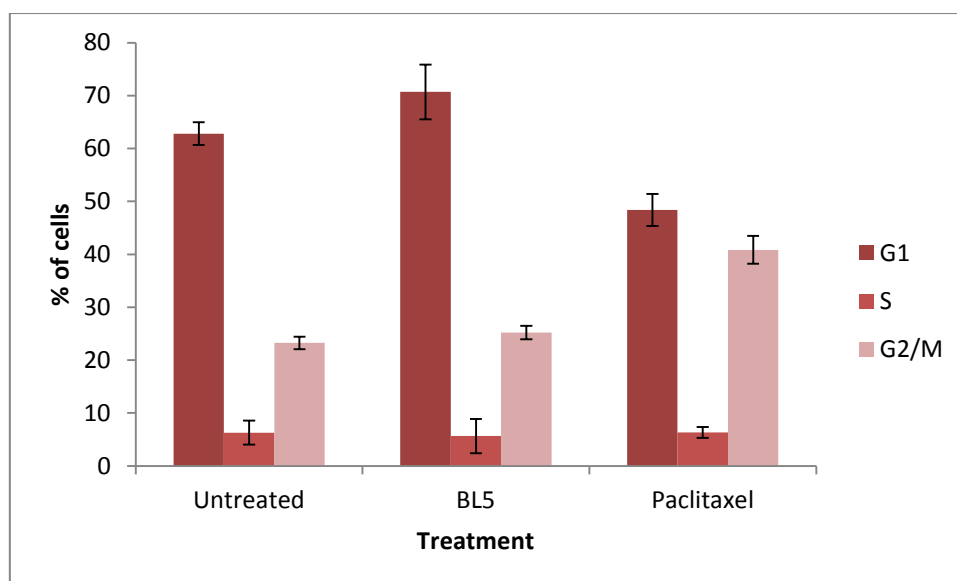
Figure 6.10 Cell cycle effects of **BL5** on MCF cells following 24 h incubation

The effect of **BL5** on the cell cycle of MCF-7 cancer cells after 24 h incubation in comparison to no treatment and paclitaxel (positive control) is shown in Figure 6.9. The histogram of untreated cells shows a cell distribution of 62.81% in G1 phase, 6.27% in S phase and 23.24% in G2/M phase. In contrast the histogram of **BL5** shows that 70.70% of cells are at G1 phase, 2.83% at S phase and 25.04% at G2/M phase. In comparison to untreated cells, although not significant, the percentage of cells at the G1 phase in **BL5** is increased, which is accompanied by a decrease in the S phase, indicating only a slight arrest of the cell cycle at G1 (G0/G1) phase. This result suggests that the significant cytotoxic activity of **BL5** may be attributed to mechanisms such as apoptosis induction or effects on signal transduction pathways (Noori and Hassan 2012). Given that **BL5** has shown 45.5% AV^+/PI^- (apoptosis) the significant antiproliferative activity can at least partially be attributed to its apoptosis-inducing capacity.

No previous studies of terpenoid glycosides affects on the cell cycle have been identified. However, a report exists on the cell cycle effects of a natural sesquiterpene lactone against MCF-7 cells, where this compound induced G0/G1 phase arrest (Noori and Hassan 2012).

Another study reported that cucurbitacin (triterpenoid) arrested G2/M phases and in some cases it arrests S phase (Huang, Lu et al. 2012). In the present study, a known anticancer drug, paclitaxel, was used as positive control and it showed a significant increase in the percentage of cells at the G2/M phase, which correlates to a decrease in the percentage of cells at the G1 phase relative to no treatment, indicating that paclitaxel induced G2/M cell cycle arrest. This result is supported by another cell cycle study previously performed on MCF-7 with paclitaxel for 48 h (Saunders, Lawrence et al. 1997).

Effects of diterpenoid **BL5** on MCF-7 cells were observed (4 x magnification) (Figure 6.11) during apoptosis and cell cycle analysis. The images of the breast cancer cells treated with **BL5** and positive control, paclitaxel (A-C) indicate a decrease in number of cells relative to untreated cells (A). In image B (cells treated with **BL5**) apoptotic body has shown to be formed.
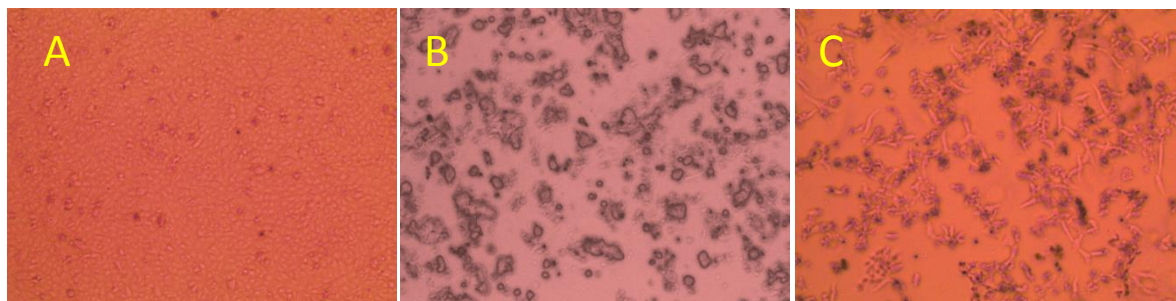
Figure 6.11 Microscopic views (4 x magnifications) of MCF-7 estrogen-dependent breast cancer cells tested for cell cycle analysis for 24 h of incubation where: untreated cells (A), **BL5 (**B) and paclitaxel (C)

6.4.2.4 Antifungal activity of BL4 and BL5

Increased resistance to current antifungals necessitated the search for new, safer and more potent antifungals to combat fungal infections. Also, there are a number of reports that have demonstrated the antifungal activity of terpenoids against various fungi species (Gaspar, Feio et al. 2004; Mendoza, Espinoza et al. 2009). On this basis **BL4** and **BL5** were selected for

antifungal potential studies. **BL6** was not included in this study as its structure was still unresolved.

The antifungal assay was developed and used as described by (Brechbühler 2013).

Noteworthy reports are that salvic acid and acetylsalvic were found to inhibit the mycelial growth of *B. cinerea* (Mendoza, Espinoza et al. 2009), whereas no reports have been found on the fungicidal activity of diterpenoid glycosides to date.

Reports exists that we are aware of on the antifungal activity of the known terpenoid glycoside **BL4**. Given that **BL5** was a novel compound, both isolated terpenoid glycosides (**BL4** and **BL5**) were tested against four micro-fungi namely*, C. albicans*, *A. niger*, *A. fumigatus* and *T. mentagrophytes*. Neither **BL4** nor **BL5** exhibited antifungal activity against the micro-fungi tested at 20 µg/mL concentration. Furthermore, standard deviations were very high for the two inactive terpenoid glycosides, but were low for other, active compounds tested. A summary of the fungal growth inhibitory activities of the terpenoid glycosides (**BL4** and **BL5**) against all four micro-fungi is shown in the figure 6.12.
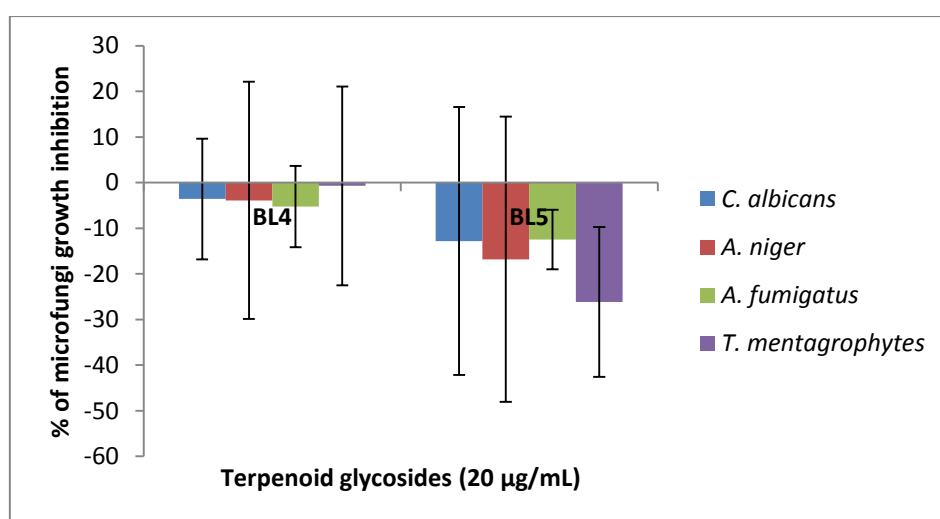
Figure 6.12 Antifungal activities of isolated two terpenoid glycosides (**BL4** and **BL5**) from *Blumea lacera* determined using broth microdilution assay

CHAPTER SEVEN

FLAVONOID GLYCOSIDES AND THEIR CYTOTOXICITY

7.1 Background

Flavonoids represent a large group of polyphenolic compounds comprising more than 10,000 individual structures (Yao, Jiang et al. 2004; Agati, Azzarello et al. 2012). They are the most common and widely distributed secondary metabolites naturally occurring in plants. Major sources of flavonoids in human diets are onions, spinach, cauliflower, broccoli, carrots, tomatoes, parsley, apples, oranges, plums, apricots, grapes, berries, cherries, black tea, cumin, and red wine (Yao, Jiang et al. 2004; Veitch and Grayer 2008; Tsuchiya 2010; Veitch and Grayer 2011). Flavonoids have been isolated from plants belonging to a wide range of plant families. For example, kaempferol, one of the most common flavonoids, has been isolated from a significant number of plants including *Indigofera suffruticosa*, *Lactuca scariola*, and *Lilium candidum* belonging to Leguminosae, Asteraceae, and Liliaceae, respectively (Calderon-Montano, Burgos-Moron et al. 2011). Another common and widely distributed flavonoid, quercetin, has also been isolated from a significant number of plants including, *Pterogyne nitens*, *Acrostichum aureum*, and *Cassia nodosa*, belonging to Fabaceae, Pteridaceae and Caesalpiniaceae, respectively (Mei, Zeng et al. 2006; Regasini, Vellosa et al. 2008; Hung, Cuong et al. 2011).

Flavonoids are synthesized by plants and are responsible for transporting electrons during photosynthesis. They provide antioxidant activity against UV light, act as bactericidal, fungicidal, insecticidal, and antiviral agents in plants (Graf, Milbury et al. 2005).

Structurally, flavonoids are composed of two aromatic rings, designated-A and -B, and an oxygenated heterocyclic ring C (Figure 7.1). Based on the connection position of the rings-B to-C as well as on the degree of saturation, methylation, oxidation and hydroxylation of the ring-C, flavonoids are divided into six sub-classes: flavonols, flavones, flavanones, flavan-3-ols, isoflavones, and anthocyanidins (Figure 7.2).
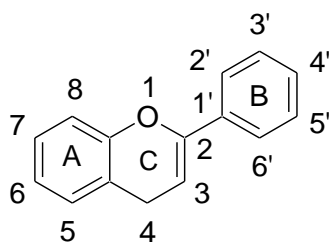
Figure 7.1 Basic structure and numbering system of flavonoids
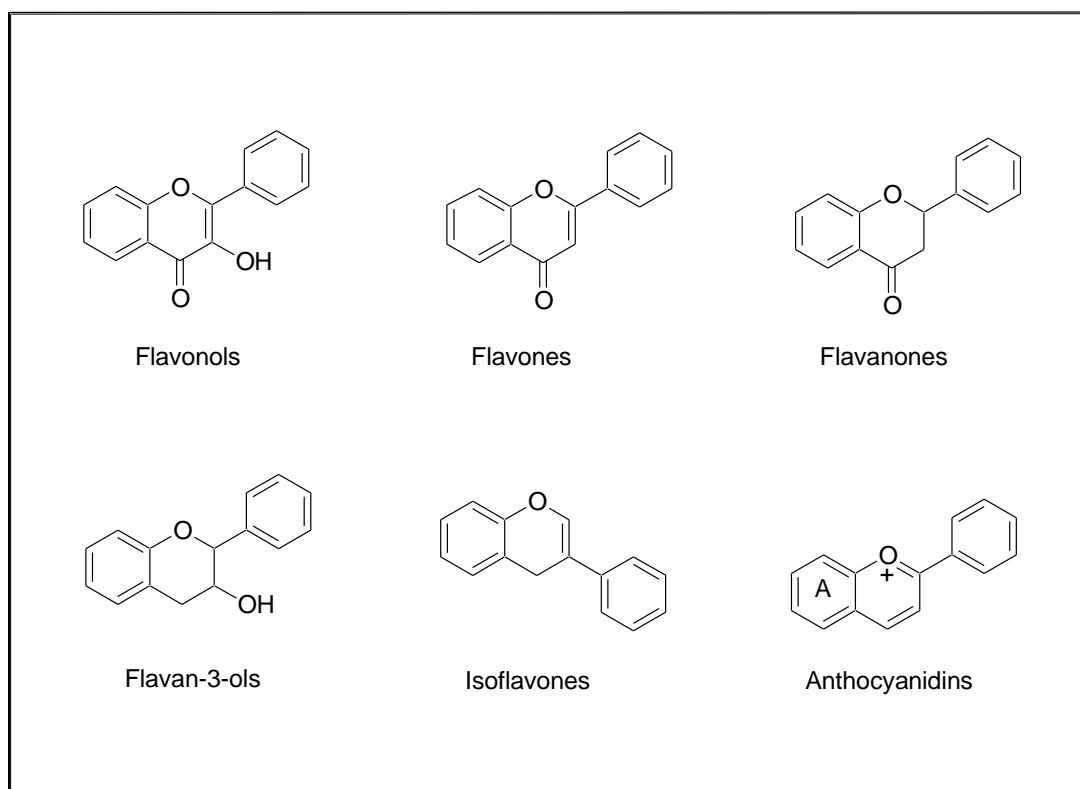
Figure 7.2 Subclasses of flavonoids, namely flavonols, flavones, flavanones, flavan-3-ols, isoflavones and anthocyanidins

Table 7.1 represents two examples of each sub-class of flavonoids. Kaempferol and quercetin are the most common flavonoids which belong to the flavonols. Apigenin and luteolin belong to the flavone type flavonoids, catechin and epigallocatechin are flavanols and hesperidin and naringenin, flavanones. Genistein and daidzein are isoflavones, with cyanidin and malvidin, anthocyanidins (Table 7.1).

Table 7.1 Common flavonoids of sub-classes and their sources
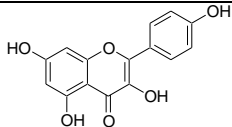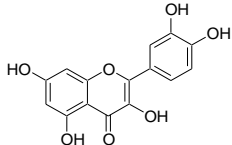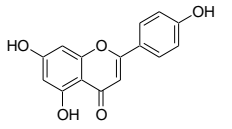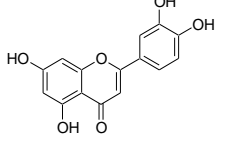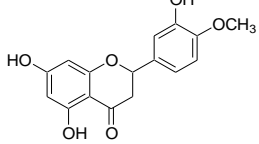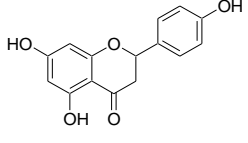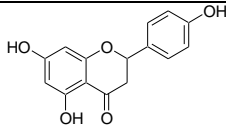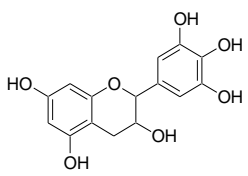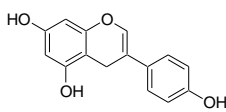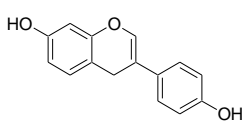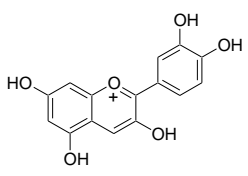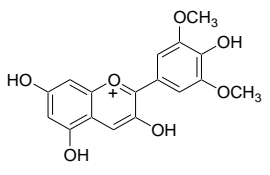
Flavonoid sub-class	Compound	Structure	Food source	Plant source	Reference
Flavonols	Kaempferol		Onions, raw broccoli, tomatoes, apples, cherries, berries, grapes, black tea, fruit juice, and wine	*Indigofera suffruticosa*, *Lactuca scariola*, *Lilium candidum*	(Yao, Jiang et al. 2004; Graf, Milbury et al. 2005; Calderon-Montano, Burgos-Moron et al. 2011)
	Quercetin			*Pterogyne nitens*, *Acrostichum aureum*, *Cassia nodosa*	(Yao, Jiang et al. 2004; Graf, Milbury et al. 2005; Mei, Zeng et al. 2006; Regasini, Vellosa et al. 2008; Hung, Cuong et al. 2011)
Flavones	Apigenin		Cereals, parsley, celery, fruits, flowers, and thyme	*Matricaria recutita*, *Artemisia lavandulaefolia*	(Yao, Jiang et al. 2004; Graf, Milbury et al. 2005; Ma, Zhou et al. 2012; Roberts, Allen et al. 2013)
	Luteolin			*Cakile maritime*, *Matricaria recutita*	(Yao, Jiang et al. 2004; Graf, Milbury et al. 2005; Haggag, Kamal et al. 2011; Roberts, Allen et al. 2013)
Flavanones	Hesperitin		Citrus fruits, lemon, oranges, grapes, peppermint, and cumin	*Citrus unshiu*, *Citrus sinensis*	(Yao, Jiang et al. 2004; Graf, Milbury et al. 2005; Inoue, Tsubaki et al. 2010; Al-Ashaal and El-Sheltawy 2011)
	Naringenin			*Lippia gracilis* Schauer, *Soymida febrifuga*	(Rao, Gupta et al. 1979; Yao, Jiang et al. 2004; Graf, Milbury et al. 2005; Guimaraes, Gomes et al. 2012)

Table 7.1 Common flavonoids of sub-classes and their sources (contd.)

Flavonoid sub-class	Compound	Structure	Food source	Plant source	Reference
Flavan-3-ols (flavanols)	Naringenin		Apples, hops, black tea, beer, and red wine	*Lippia gracilis* Schauer, *Soymida febrifuga*	(Rao, Gupta et al. 1979; Yao, Jiang et al. 2004; Graf, Milbury et al. 2005; Guimaraes, Gomes et al. 2012)
	Epigallocatechin			*Lindera akoensis*, *Neolitsea aciculata*	(Yao, Jiang et al. 2004; Graf, Milbury et al. 2005; Kim, Hyun et al. 2013; Yang, Huang et al. 2013)
Isoflavones	Genistein		Soy milk and tofu	*Trifolium pratense*, *Momordica dioica*	(Giachi, Manunta et al. 2002; Yao, Jiang et al. 2004; Graf, Milbury et al. 2005; Kale and Laddha 2012)
	Daidzein			*Momordica dioica*, *Genista morisii*	(Giachi, Manunta et al. 2002; Yao, Jiang et al. 2004; Kale and Laddha 2012)
Anthocyanidins	Cyanidin		Blue berries, raspberries, flowers, red onions, and red wine	*Oryza sativa*, *Peristrophe roxburghiana*	(Yao, Jiang et al. 2004; Graf, Milbury et al. 2005; Yang, Zhang et al. 2012; Tanaka, Nakanishi et al. 2013)
	Malvidin			*Daucus carota*, *Lycium ruthenicum*	(Ravindra and Narayan 2003; Yao, Jiang et al. 2004; Graf, Milbury et al. 2005; Ouyang, Ji et al. 2012)

7.2 Flavonoid glycosides

Flavonoid glycosides are polyphenolic secondary metabolites comprising a flavonoid as the aglycone and a sugar moiety. Most naturally occurring flavonoid glycosides are *O*- and *C*-glycosides of flavones and flavonols (Veitch and Grayer 2008; Veitch and Grayer 2011). From 2004-2009, a total of 110 new flavone glycosides and 350 flavonol glycosides were isolated from different plant species (Veitch and Grayer 2008; Veitch and Grayer 2011).

7.2.1 Flavone-*O*- and -*C*-glycosides

Flavone-*O*-glycosides have sugar moieties connected to the 3-*O*-position of the flavone aglycone. Up to 2009, 752 flavone glycosides have been reported, with a total of 113 new flavone-*O*-glycosides during 2004 and 2009 reported (Veitch and Grayer 2008; Veitch and Grayer 2011). In *C*-glycosides the sugar residues are *C*-linked at the 3 position of the aglycone, flavone. Table 7.2 presents some representative examples of flavone-*O*- and -*C*-glycosides and their sources.

7.2.2 Flavonol-*O*-glycosides

Flavonol-*O*-glycosides are glycosylated polyphenolic compounds where sugar moieties are 3-*O*-linked to the flavonol aglycone. At least 1662 flavonol-*O*-glycosides in total have been reported up to 2010, comprised of 458 different kaempferol-*O*-glycosides and 363 quercetin-*O*-glycosides (Veitch and Grayer 2011). Table 7.2 presents some representative examples of flavone-*O*-glycosides, flavone-*C*-glycosides and flavonol-*O*-glycosides and their sources.

Table 7.2 Some representative flavonoid glycosides and their sources (Veitch and Grayer 2011)

Flavonoid glycosides	Compound (s)	Plant	Plant part	Family
Flavone-*O*-glycosides	6-hydroxy-5-methoxyflavone-6-*O*-β-glucopyranoside and	*Casimiroa edulis*	Leaf	Rutaceae
	5,7,4'-trihydroxyflavone (apigenin)-7-*O*-(4,6-di-*O*-acetyl-β-glucopyranoside)	*Chamomilla recutita*	Floret	Asterceae
	5,7,3',4'-tetrahydroxyflavone (luteolin)-7-*O*-β-glucopyranosyl-(1→2)[α-rhamnopyranosyl-(1→6)]-β-glucopyranoside	*Schlerochiton vogetii*	Whole plant	Acanthaceae
Flavone-*C*-glycosides	6,7,4'-trihydroxyflavone-3-*C*-β-glucopyranoside	*Abutilon pakistanicum*	Aerial part	Malvaceae
	5-hydroxy-7-methoxyflavone-(chrysin7-methylether)-6-*C*-β-glucopyranoside	*Sphaeranthus indicus*	Aerial part	Asteraceae
	5,7,4'-trihydroxy-6,3'-dimethoxyflavone-8-*C*-β-glucopyranoside (verticilliside)	*Enicostema verticillatum*	Whole plant	Gentianaceae
Flavonol-*O*-glycosides	3,5,7,4'-tetrahydroxyflavone (kaempferol)-3-*O*-β-glucopyranosyl-(1→4)-[α-rhamnopyranosyl-(1→6)]-β-glucopyranoside	*Lamium amplexicaule*	Aerial part	Lamiaceae
	3,5,7,3',4'-pentahydroxyflavone (quercetin)-3'-α-rhamnopyranoside	*Oenothera speciosa*	Aerial part	Onagraceae
	3,7,3',5'-tetrahydroxy-4'-methoxyflavone-3-*O*-β-D-glucopyranoside	*Vernomia mapirensis*	Aerial part	Asteraceae

7.3 Kaempferol and kaempferol glycosides

As the present study afforded isolation of two kaempferol glycosides, **BL1** and **BL2** from *Blumea lacera,* kaempferol and its glycosides are therefore described in details here.

Kaempferol [3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one] is a yellow coloured flavonol, commonly available in many plants used in traditional medicines and in plant-derived foods (Table 7.1). For instance, kaempferol has been identified and isolated from *Ginko biloba*, *Moringa oleifera* and *Sophora japonica*. It is also widely distributed in foods such as broccoli, cabbage, kale, beans, leek, tomatoes, strawberries, grapes, and black tea (Calderon-Montano, Burgos-Moron et al. 2011). Kaempferol glycosides are the glycosylated derivatives of kaempferol and are also found in many plants used as traditional medicines (Calderon-Montano, Burgos-Moron et al. 2011). Table 7.3 shows a number of examples of kaempferol glycosides isolated from different medicinal plants.

7.4 Bioactivity of kaempferol glycosides

A significant number of studies on kaempferol glycosides exist and have reported a wide range of biological activities such as cytotoxic/anticancer, anti-inflammatory, antioxidant, antimicrobial, antifungal, antiviral, immunomodulatory, analgesic, anti-ulcer, anxiolytic, and molluscidal activities (Table 7.3). Here only cytotoxic/anticancer and antifungal activities are discussed as these are the activities that were tested in the present study.

Cytotoxic/anticancer activity

Several epidemiological studies have revealed the relationship between the consumption of kaempferol-rich foods and the reduction in risk of developing different types of cancers such as lung, gastric, pancreatic and ovarian (Calderon-Montano, Burgos-Moron et al. 2011). Several kaempferol glycosides isolated from different plant species have been evaluated for their cytotoxic/anticancer activity against various cancer cell lines including blood (MT-4),

lung (NCI-H187), epidermoid carcinoma (KB), breast (MCF-7, T47D, and Sk-Br-3), pancreatic (PANC1), prostate (DU145), human embryonic lung fibroblast cell (HELF), human colon (DLD), human oral epithelium (KB), human cervical (Hela), human hepatoma (Hepa) and mouse lung (A-549) tumor cell lines (Table 7.3). Kaempferol and its glycosides are indicated as producing their anticancer potential through apoptosis, induction of caspase-3, caspase-7 and caspase-9 activities (Calderon-Montano, Burgos-Moron et al. 2011), while, its anticancer activity is attributed to its antioxidant activity. Furthermore, the anti-inflammatory effects of kaempferol and its glycosides play a vital role in preventing cancer, in that they likely block release of inflammatory mediators such as chemokines, cytokines (TNF-α and IL-1β), and transcription factors (NF-$_K$B) which in turn promote the development of cancers (Calderon-Montano, Burgos-Moron et al. 2011).

Antifungal activity

Flavonoid-rich plants have been used in traditional medicines to treat various infectious diseases including microbial invasion (Calderon-Montano, Burgos-Moron et al. 2011). A number of publications have reported antifungal activity of various kaempferol glycosides against a number of fungi species, including *Candida albicans*, *C. krusei*, *Fusarium oxysporum* f.sp., *Aspergillus niger*, and *Canthium* spp. (Bisignano, Sanogo et al. 2000; Subramani, Gunasegaran et al. 2004; Galeotti, Barile et al. 2008; Orhan, Oezcelik et al. 2010) (Table 7.3). For example the report indicating that kaempferol and kaempferol-3-*O*-(2"-*O*-galloyl)-β-D-glucoside inhibited fungal cell division through inhibition of chitin synthase II, which is responsible for the synthesis of chitin, a vital component of fungal cell wall integrity (Calderon-Montano, Burgos-Moron et al. 2011).

Table 7.3 Bioactivity studies reported on kaempferol glycosides (isolated from plants)

Compound	Plant (part)	Cytotoxic/anticancer activity	Other bioactivities	Reference
BL1: Kaempferol-3-*O*-(2″,6‴-di-*O*-α-rhamnopyranosyl)-β-galactopyranoside	*Chenopodium quinoa* (seed)		Antioxidant activity	(Hirose 2010)
BL2: Kaempferol-3-*O*-α-L-rhamnopyranosyl-(1 → 6)-β-D-glucopyranoside [1]/kaempferol-3-*O*-rutinoside	*Carthamus tinctorius* (petals)	Cytotoxic effects against human leukaemia (MT-4) cells Cytotoxicity against mouse lung cancer (A549) cells Cytotoxic activity against human small lung-cancer (NCI-H187), epidermoid carcinoma (KB) and breast cancer (BC) cells	Antibacterial, antifungal and antiviral activity Antifungal activity Antifungal activity Antimicrobial activity Antioxidant activity	(Plumb, Price et al. 1999; Bisignano, Sanogo et al. 2000; Han, Bang et al. 2004; Subramani, Gunasegaran et al. 2004; Cimanga, Kambu et al. 2006; Wirasathien, Pengsuparp et al. 2006; Orhan, Ergun et al. 2007; Yoon, Han et al. 2007; Galeotti, Barile et al. 2008; Orhan, Oezcelik et al. 2010; Sashidhara, Singh et al. 2011; Zhao 2011)
Kaempferol-3-β-D-glucopyranoside	*Phyllanthus emblica* (fruit)	Anticancer effects against breast cancer (MCF-7) and human embryonic lung fibroblast (HELF) cells	Immunomodulatory activity	(Liu, Zhao et al. 2012)
Kaempferol-3-*O*-(2-*O*-acetyl-α-L-rhamnopyranoside; kaempferide-3-*O*- α -L-rhamnopyranoside, and kaempferol-3-O-α-L-arabinofuranoside	*Excoecaria agallocha* (leaves)	Cytotoxicity against human pancreatic (PANC1) and prostate (DU145) cancer cells		(Rifai, Arai et al. 2011)
Drabanemoroside; kaempferol-3-*O*-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranose)	*Draba nemorosa* (seeds)	Cytotoxicity against mouse small lung cancer (A549) and melanoma (SK-Mel-2) cells		(Moon, Abdur Rahman et al. 2010)

BL: *Blumea lacera*

Table 7.3 Bioactivity studies reported on kaempferol glycosides (isolated from plants) (contd.)

Compound	Plant (part)	Cytotoxic/anticancer activity	Other bioactivities	Reference
Kaempferol-3-*O*-β-D-galactopyranosyl-(1→2)-β-D-xylopyranoside	*Asclepias syriaca* (dried biomass)	Cytotoxicity tested against three breast cancer (MCF-7, T47D, and Sk-Br-3) and one normal breast (Hs578Bst) cells		(Araya, Kindscher et al. 2012)
Kaempferol-3-glucoside, and kaempferol-7-glucoside	*Podocarpus fasciculus* (stems and leaves)	Cytotoxicity against colon cancer (DLD), oral epithelium carcinoma (KB), cervical carcinoma (Hela), human hepatoma (Hepa, and mouse lung carcinoma (A-549) cells		(Kuo, Hwang et al. 2008)
Kaempferol-7-*O*-glucoside	*Cassia nodosa* (root, stem, leaves, flowers and pods)	No reports found	Antimicrobial (bactericidal and fungicidal) effect	(Singh, Sharma et al. 2011)
Kaempferol-7-*O*-glucoside	*Cakile maritime*	No reports found	Antioxidant and molluscidal activities	(Shams, Radwan et al. 2010)
3'-(3",7"-dimethyl- 2",6"-octadiene)-8-*C*-β-D-glucosyl-kaempferol-3-*O*-β-D-glucoside	*Sida cordifolia* (aerial parts)	No reports found	Analgesic and anti-inflammatory activities	(Sutradhar, Rahman et al. 2006)
Kaempferol-3,7-*O*-α-L-di-rhamnopyranoside, and kaempferol-3-*O*-(4- β -xylopyranosyl)-α-L-rhamnopyranoside-7-*O*-α-rhamnopyranoside	*Chenopodium moquinianum* (flowering parts)	No reports found	Anti-ulcer activity	(Hawas, Ibrahim et al. 2009)

Table 7.3 Bioactivity studies reported on kaempferol glycosides (isolated from plants) (contd.)

Compound	Plant (part)	Cytotoxic/anticancer activity	Other bioactivities	Reference
Kaempferol-3,7-*O*-di-rhamnoside (kaempferitrin), kaempferol-3-pentosylhexoside; kaempferol-3-*O*-glucoside (astragalin), and kaempferol-3-*O*-rhamnoside; kaempferol-3-*O*-(6-*p*-coumaroyl)-glucoside (tiliroside)	*Tilia americana* (flowers and bracts)	No reports found	Anxiolytic-like response	(Aguirre-Hernandez, Gonzalez-Trujano Ma et al. 2010)
Kaempferol-3-*O*-β-D-xylopyranosyl-(1→2)-α-L-arabinofuranosyl-7-*O*-α-L-rhamnopyranoside; kaempferol-3-*O*-β-D-xylopyranosyl-(1→2)-α-L-rhamnopyranosyl-7-*O*-α-L-rhamnopyranoside; kaempferol-3-*O*-β-D-glucopyranosyl-(1→2)-α-L-arabinofuranosyl-7-*O*-α-L-rhamnopyranoside; kaempferol-3-*O*-α-L-rhamnopyranosyl-(1→2)-α-L-arabinofuranosyl-7-*O*-α-L-rhamnopyranoside; kaempferol-3-*O*-β-D-apiofuranosyl-(1→2)-α-L-arabinofuranosyl-7-*O*-α-L-rhamnopyranoside; kaempferol-3-*O*-β-D-glucopyranosyl-(1→2)-α-L-rhamnopyranosyl-7-*O*-α- L -rhamnopyranoside; kaempferol-3-*O*-β-D-glucopyranosyl-(1→4)-α-L-rhamnopyranosyl-7-*O*-α-L- rhamnopyranoside; kaempferitrin, and kaempferol 7-*O*-α-L-rhamnopyranoside	*Cinnamomum osmophloeum* (twigs)	No reports found	Anti-inflammatory effect	(Lin and Chang 2012)
Kaempferol-3-O-{α-L-rhamnopyranosyl(1→6)-[α-L-rhamnopyranosyl(1→2)]}-β-D-galactopyranoside	*Hemerocallis fulva* (flowers)	No reports found	Antioxidant activity	(Lin, Lu et al. 2011)

Table 7.3 Bioactivity studies reported on kaempferol glycosides (isolated from plants) (contd.)

Compound	Plant (part)	Cytotoxic/anticancer activity	Other bioactivities	Reference
Kaempferol-3-*O*-β-glucopyranoside-4′-*O*-β-xylopyranoside; kaempferol-3-*O*-[4″″-*O*-acetyl-α-L-rhamnopyranosyl-(1→6)]-[β-D-glucopyranosyl-(1→2)]-β-D-glucopyranoside; kaempferol-3-*O*-[4″″-*O*-acetyl-α-L-rhamnopyranosyl-(1→6)]-[β-D-xylopyranosyl-(1→2)]-β-D-glucopyranoside; kaempferol-3-*O*-[α-L-rhamnopyranosyl-(1→6)]-[β-D-xylopyranosyl-(1→2)]-β-D-glucopyranoside; kaempferol-3-*O*-[α-L-rhamnopyranosyl-(1→6)]-[β-D-glucopyranosyl-(1→2)]-β-D-glucopyranoside; kaempferol-3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside; kaempferol-3-*O*-β-D-xylopyranosyl-(1→2)-β-D-glucopyranoside (leucoside), and kaempferol-3-*O*-β-D-glucopyranosyl-(1→2)-β-D-glucopyranoside	*Diplotaxis harra* (whole plant) *Camellia oleifera* (seed cake)	No reports found	Antiviral screening against foot-and-mouth disease virus types A and O	(Kassem, Afifi et al. 2013)

7.5 Bioactivity studies of isolated kaempferol glycosides from *Blumea lacera*

7.5.1 A brief introduction to the isolated kaempferol glycosides from *Blumea lacera*

Two known flavonoid glycosides, kaempferol 3-*O*-(2″,6‴-di-*O*-α-rhamnopyranosyl)-β-galactopyranoside (**BL1**) and kaempferol-3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside, also known as kaempferol-3-*O*-rutinoside or nicotiflorin (**BL2**) were isolated from a crude methanolic extract (CME) of *Blumea lacera* leaves for the first time. These compounds were isolated using semi-preparative HPLC followed by solid phase extraction (SPE) and characterized structurally using 1D and 2D NMR spectroscopy (^{1}H, ^{13}C and HSQC) as well as mass spectrometry (ESI-MS). They were both obtained as yellow solids.

Kaempferol 3-*O*-(2″,6‴-di-*O*-α-rhamnopyranosyl)-β-galactopyranoside (**BL1**)

Kaempferol-3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (**BL2**)
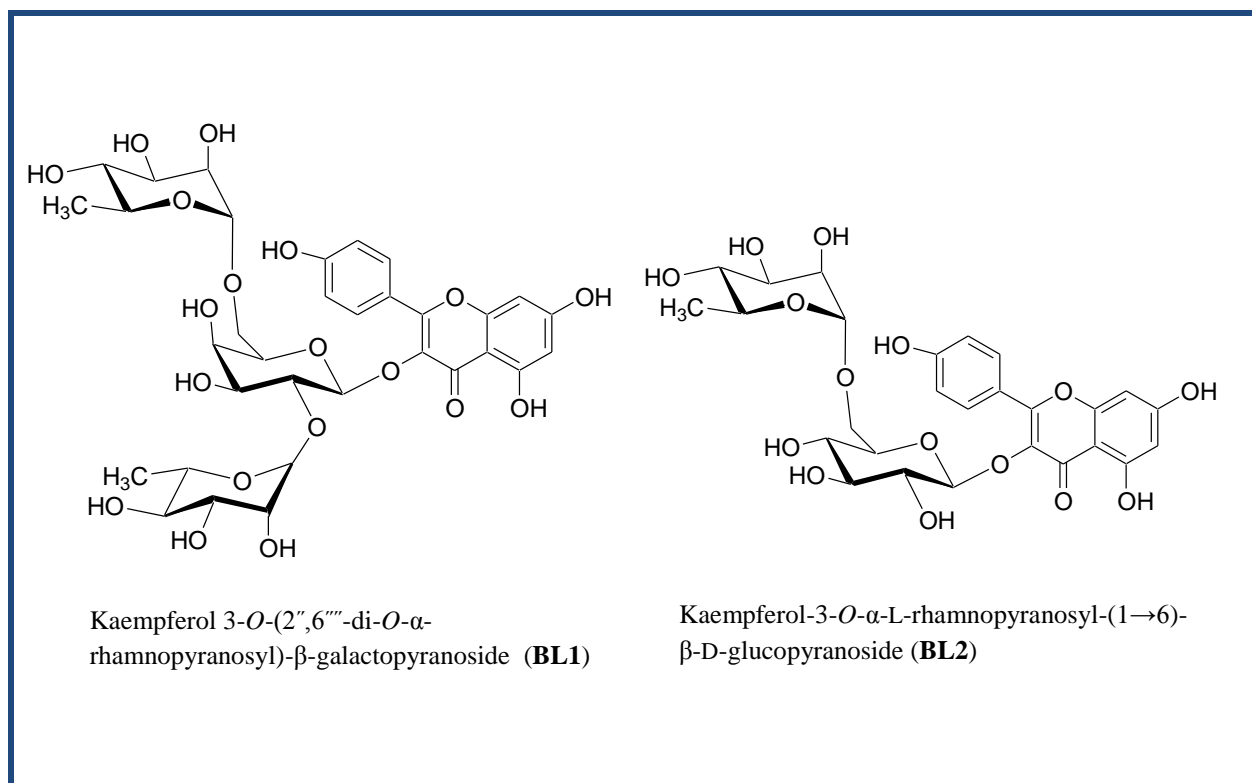
Figure 7.3 Structures of compounds, **BL1** and **BL2** isolated from the methanolic extract of *Blumea lacera* leaves

7.5.2. Cytotoxic activity of isolated kaempferol glycosides

7.5.2.1 Results and discussion of cytotoxic activity tests on the isolated kaempferol glycosides BL1 and BL2

No previous studies are reported on the cytotoxic activity of **BL1**, but previous cytotoxicity screening of **BL2** against various cancer cell lines has been undertaken (Table 7.3) (Cimanga, Kambu et al. 2006; Wirasathien, Pengsuparp et al. 2006; Zhao, Gao et al. 2011).

In our study the cytotoxic activity of the isolated kaempferol glycosides (**BL1** and **BL2**) were investigated against two healthy cell lines namely, a normal mouse fibroblast (NIH3T3) and a monkey kidney cell line (VERO), as well as against four cancer cell lines including a gastric (AGS), a colon (HT-29), and two breast (MCF-7, estrogen-dependent and MDA-MB-231, estrogen non-dependent) cancer cell lines, using the MTT assay (see section 2.5) (Yaacob Nik, Hamzah et al. 2010). Varying concentrations of each compound (0.01, 0.1, 1.0, 10 and 100 μg/mL) were used for testing their cytotoxic activity. Cytotoxic activity results for compound, **BL1** and **BL2** are presented in Figure 7.4 and 7.5.
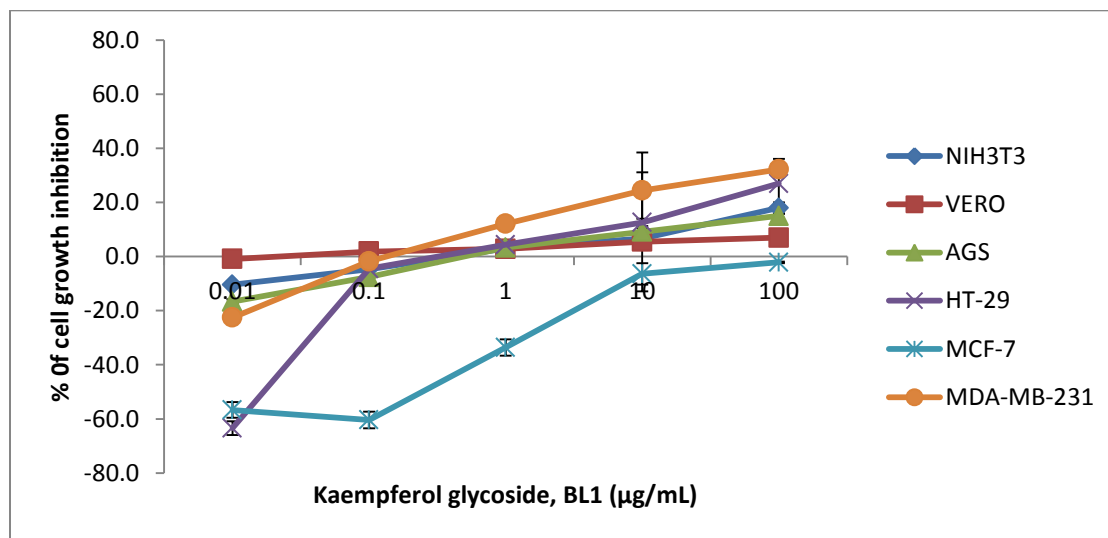
Figure 7.4 Percent (%) of cell growth inhibition of **BL1** against the selected cell lines

BL1 was nontoxic to all the cell lines tested at the concentrations of 0.01 and 0.1, µg/mL, whereas it showed very weak to weak cytotoxicity (2.8 to 32.2 % cell growth inhibition) at concentrations from 1.0 to 100 µg/mL against all cell lines tested except MCF. **BL1** was the most toxic to the estrogen non-dependent breast cancer cell line, MDA-MB-231 (though only 32.2 % cell growth inhibition at the highest concentration of 100 µg/mL), whereas it showed no cytotoxicity against the estrogen-dependent breast cancer cell line, MCF-7 even at the highest concentration of 100 µg/mL. No previous reports were found on the cytotoxicity studies of **BL1**.
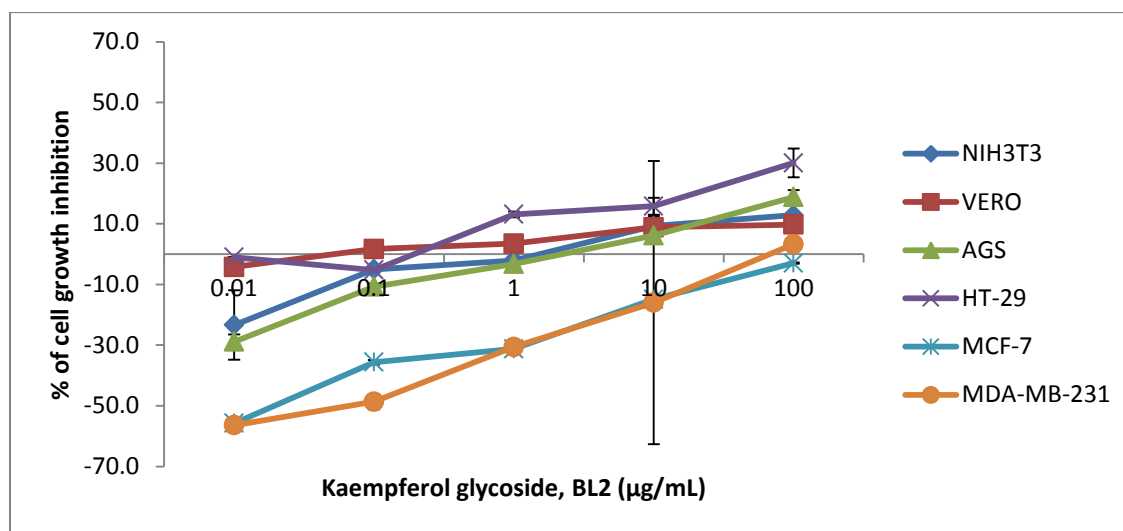
Figure 7.5 Percent (%) of cell growth inhibition of **BL2** against the selected cell lines

BL2 displayed no cytotoxicity against all cell lines tested at the lowest concentration of 0.01 µg/mL, whereas it exhibited very weak to weak cytotoxic effects (1.7 to 30.0 % cell growth inhibition) at concentrations of 0.1 to 100 µg/mL against all cell lines tested, except MCF-7. This compound was the most toxic to HT-29 colon cancer cells, showing 30% cell growth inhibition at the highest concentration of 100 µg/mL. Similarly, like **BL1**, **BL2** was also non-toxic to the MCF-7 estrogen-dependent breast cancer cell line, even at the highest concentration of 100 µg/mL.

Previous studies have reported that **BL2**, was non-toxic against mouse lung (A549), human small-cell lung (NCI-H187), human breast (BC), and human epidermal (KB) cancer cell lines (Wirasathien, Pengsuparp et al. 2006; Zhao 2011). In addition, only weak cytotoxic activity (IC_{50} = 72.5 µg/mL) was reported on this compound against human T-cell leukaemia (MT-4) cells (Cimanga, Kambu et al. 2006). Our study confirmed the lack of cytotoxicity that was reported previously for **BL2** against the breast cancer cell line. This study indicates that **BL2** was inactive against the estrogen-dependent breast cancer cells, MCF-7. This was not indicated in the previous publication on the cytotoxicity of this compound against breast cancer cell lines (Wirasathien, Pengsuparp et al. 2006).

The lack of cytotoxicity of **BL1** and **BL2** may in part be due to the presence of the sugar moieties, given that previous studies reported flavonoid glycosides to be less active than their aglycones alone (Tasdemir, Kaiser et al. 2006).

Cycloheximide was used as a positive control in our study at the same concentrations as those used for **BL1** and **BL2**. Figure 7.6 represents the cytotoxicity of cycloheximide against the selected cell lines.
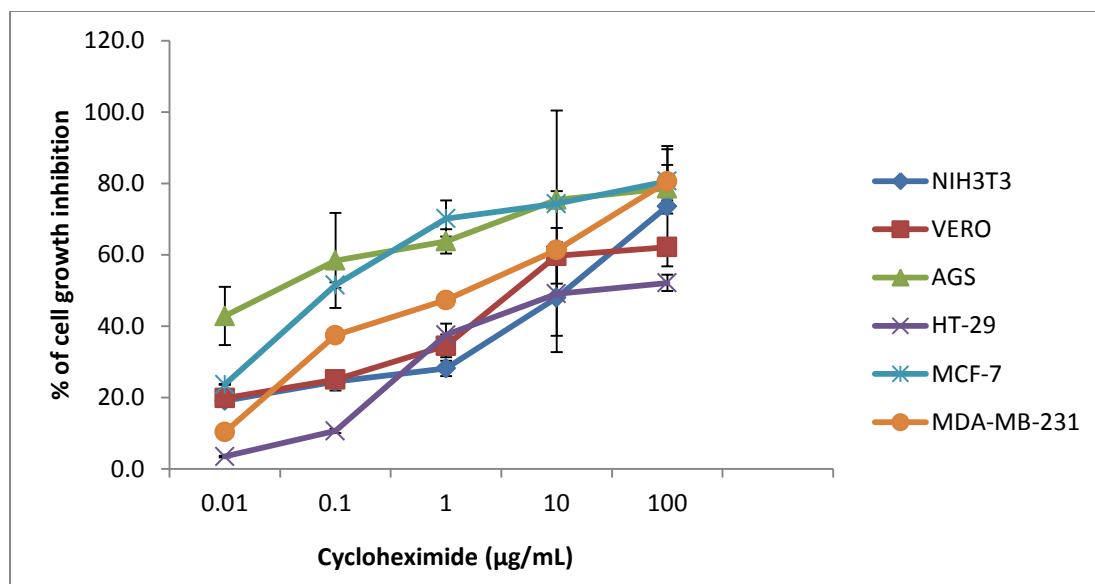
Figure 7.6 Percent of cell growth inhibition of cycloheximide (positive control) against the selected cell lines

7.5.3 Antifungal activity of the isolated kaempferol glycosides-BL1 and BL2 from *Blumea lacera*

Natural products with more potent antifungal activity are always in great demand to treat the fungal infections. Thus, the antifungal activity of **BL1** and **BL2** were stuied against four fungi species; *C. albicans*, *A. niger, A. fumigatus,* and *T. mentagrophytes* and results are summarized in the Figure 7.7. The antifungal assay was developed and used as described by (Brechbühler 2013).

7.5.3.1 Results and discussion of antifungal activity tests of BL1 and BL2

For **BL1**, no previous reports on antifungal activity exist. Previous studies report on the antifungal activity of **BL2** against *Candida albicans*, *C. krusei* (Orhan, Oezcelik et al. 2010), *Fusarium oxysporum* f.sp. (Galeotti, Barile et al. 2008), *Aspergillus niger* and *Canthium* spp. (Subramani, Gunasegaran et al. 2004). We therefore investigated the isolated flavonoid glycosides, **BL1** and **BL2** against four micro-fungi, namely *C. albicans*, *A. niger, A. fumigatus,*

and *T. mentagrophytes* using an initial concentration of 20 µg/mL. Neither **BL1** nor **BL2** displayed significant inhibitory effects with standard deviations being very high for all compounds tested against all fungi. A summary of the inhibitory activities of the two flavonoid glycosides, **BL1** and **BL2** against all four micro-fungi is shown in Figure 7.7.
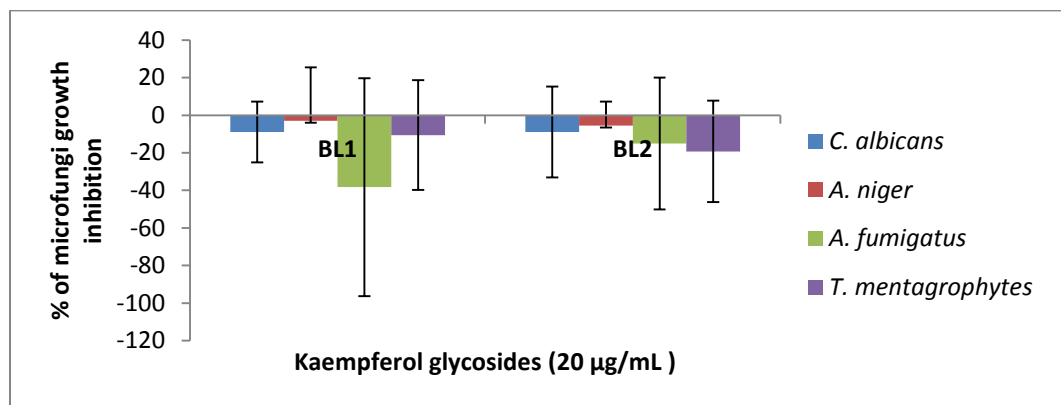
Figure 7.7 Antifungal activity of kaempferol glycosides isolated from *Blumea lacera* determined using broth microdilution assay

No previous reports were found on antifungal activity studies of **BL1** so far. On the other hand, in contrast to our study, where we detected no antifungal activity for **BL2** at 20 µg/mL, a separate study reported strong antifungal activities against *C. albicans* and *C. krusei* with MICs of 16 and 32 µg/mL, respectively (Orhan, Oezcelik et al. 2010). Another study reported that compound, **BL2** showed fungicidal activity against different *Fusarium oxysporum* f. sp (Galeotti, Barile et al. 2008). Variation in experimental conditions (pH, buffer, incubation temperature) may have influenced the conflicting antifungal activity of **BL2** against the same fungal strain, *A. albicans*. For example for *A. albicans* an incubation temperature of 25°C was used in this study, while 35°C was used in the study of Orhan et al (Orhan, Oezcelik et al. 2010). Although it had not been possible to repeat the antifungal tests of **BL2** under similar conditions to Orhan et al. 2010, these experiments have been included as future experiments (chapter 8; section 8.2).

CHAPTER EIGHT

CONCLUSION AND FUTURE DIRECTIONS

8.1 Conclusions

Plants used in traditional medicines are a proven source of new lead compounds for anticancer drug discovery. This PhD project was focused on the bioactivity-guided isolation and structural elucidation of novel anticancer compounds from the Bangladeshi medicinal plant *Blumera lacera* and subsequently a focused determination of the mechanisms of action and cytotoxic/anticancer potential of these isolated compounds. At the outset of the project nineteen Bangladeshi medicinal plants were selected as possible candidates for screening. This selection was based on their traditional medicinal uses, limited previous pharmacological and phytochemical research and the availability of a suitable quantity of plant material. The selected plants had been used traditionally as antitumor/anticancer, anti-infective and anti-inflammatory agents, as well as treatments for other diseases. Methanolic extracts of the selected plants were screened for their cytotoxic potential using the MTT assay against two healthy cell lines (mouse fibroblast, and monkey kidney) and four human cancer cell lines (gastric, colon, one estrogen-dependent and one estrogen non-dependent breast). The cytotoxicity screening data correlated the traditional use of some of the medicinal plants as anticancer agents as well as identifying several new plants with potent cytotoxic activity. *Blumea lacera* had been assayed previously in our research laboratory and when compared to results for the nineteen plants assayed demonstrated outstanding cytotoxicity. Hence, it was selected for a focused investigation in this research work. *Blumea lacera* was collected from Bangladesh and belongs to the family Compositae/Asteraceae. It has been used traditionally as an astringent, stimulant, anthelmentic, antimicrobial, anti-inflammatory and diuretic agent. A literature review on *Blumera lacera* reported very limited research relating to biological activity. Thus, bioassay-guided phytochemical investigation of *Blumea lacera* led to the isolation of six compounds (**BL1**-**BL6**).

BL1 (kaempferol-3-*O*-(2"6"-di-*O*-α-L-rhamnopyranosyl)-β-D-galactopyranoside) and **BL2** (kaempferol-3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside) were isolated and identified as known flavonoid glycosides, although these had not previously been isolated from this plant. **BL3** ((25*R*)-3β-{*O*-β-D-glucopyranosyl-(1→4)-*O*-α-L-rhamnopyranosyl-(1→4)-[*O*-α-L-rhamnopyranosyl-(1→2)]-α-L-rhamnopyranosyl}-22αN-spirosol-5-ene) was identified as a novel steroidal glycoalkaloid. While **BL4** (3-*O*-β-D-glucopyranosyl-17-hydroxy-6*E*, 10*E*, 14*Z*-(3*S*)-geranyllinalool 17-*O*-β-D-glucopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→6)]-β-D-glucopyranoside) was identified as a known terpenoid glycoside, although not previously isolated from this plant. **BL5** (6*E*, 10*E*, 14*Z*-(3*S*)-17-hydroxygeranyllinalool-17-*O*-β-D-glucopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→6)]-β-D-glucopyranoside) and **BL6** (2*E*, 6*E*,10*Z*-12-hydroxyfarnesol-12-*O*-β-D-glucopyranosyl-(1→6)-α-L-rhamnopyranoside) were identified as novel terpenoid glycosides, although the structure of **BL6** requires absolute confirmation.

The cytotoxic potential of all six compounds was investigated using the MTT assay against two healthy cell lines and four different human cancer cell lines. **BL1** and **BL2** were found to be non-toxic to all tested cell lines. **BL3** showed the highest cytotoxic potential against MCF-7 estrogen dependent breast cancer cells (IC$_{50}$: 2.66 µg/mL), while **BL4** exhibited weak cytotoxic activity (IC$_{50}$: 81.6 µg/mL) against HT-29 gastric cancer cells. **BL5** displayed significant cytotoxicity against MCF-7 cells (IC$_{50}$: 6.66 µg/mL) and estrogen non-dependent cancer cells MDA-MB-231 (IC$_{50}$: 13.95 µg/mL). In contrast, **BL6** showed cell proliferative effects against the cell lines tested. Cell proliferative affects of **BL6** result from variation in the sugar moieties and aglycone in relation to **BL4** and **BL5**. In comparison to **BL4** and **BL5**, **BL6** contains a hydroxymethyl group instead of terminal double bond at C-1, as well as lacking one glucopyranose sugar residue

in comparison to **BL4** and **BL5**. Though **BL4** is a known terpenoid glycoside, no reports exist on its cytotoxic activity.

The SAR of steroidal glycoalkaloids (SGAs) in relation to their cytotoxic activity has been well reported. **BL3** a novel SGA was thus investigated for its SAR along with selected SGAs structurally similar to **BL3** (β-solamargine, β-solamarine, α-solasonine, α-solanine, solasodine and tomatidine.HCl). The SAR study revealed that **BL3** showed the highest cytotoxic potential (IC$_{50}$ of 2.66 µg/mL), indicating that the number and nature of the sugar moieties along with the aglycone structure are important for activity.

Given the significant cytotoxic potential of **BL3** and **BL5** the mechanism of their cytotoxicty was investigated further. The apoptosis-inducing potential of **BL3** and **BL5** in MCF-7 cells was investigated using double staining annexin V-FITC and PI assays. Both **BL3** (24.35% AV$^+$/PI$^-$) and **BL5** induced apoptosis (45.5% AV$^+$/PI$^-$), with **BL5** inducing a higher apoptosis than paclitaxel (39.9% AV$^+$/PI$^-$).

Further, selected SGAs were investigated for apoptosis SAR in relation to **BL3**. The following order was determined: **BL3** (24.35 % AV$^+$/PI$^-$) > β-solamargine (13.35 % AV$^+$/PI$^-$) > α-solanine (4.62 % AV$^+$/PI$^-$). The results indicated that the number and linkages of the sugar moieties along with the structure of the aglycone affect apoptotic potential.

Cell cycle analysis by measuring DNA content using propidium iodide (PI) staining flow cytometry is a recognised technique to explore the mechanism by which anticancer compounds induce their effect. **BL3** and **BL5** were investigated as to their affects on the arrest of cell cycle phases (G1, S and G2/M) in MCF-7 cells using PI staining flow cytometry. **BL3** arrested the G1 phase (cell population was increased in the G1 phase by 15.67 % compared to untreated cells),

whereas **BL5** showed no considerable arrest of any of the three phases of the cell cycle in MCF-7 cancer cells compared to untreated MCF-7 cells.

In addition, structurally related SGAs were investigated for SAR by cell cycle analysis. Results showed that β-solamargine, β-solamarine, and α-solasonine also arrested the G1 phase. Therefore in relation to **BL3** the following order was determined: **BL3** (15.67 %) > β-solamarine (12.27%) > α-solasonine (11.68%) > β-solamargine (10.30%) compared to untreated cells. These results further highlighted the requirement of possessing sugar moieties, the specificity of the sugar-linkage as well as specificity in the aglycone.

Moreover, **BL1**, **BL2**, **BL3**, **BL4**, and **BL5** and some selected SGAs were investigated for antifungal activities; however none of the tested compounds displayed antifungal effects.

In conclusion, this PhD project was focused on advancing the knowledge and understanding of the pharmacology and phytochemistry of Bangladeshi medicinal plants in relation to the discovery of novel anticancer compounds. The project has resulted in the characterization of six different compounds, including three novel structures. Two of these novel compounds (**BL3** and **BL5**) possessed potent selective cytotoxicity and apoptosis-inducing potential against MCF-7 cells. While one of these (**BL3**) also displayed significant cell cycle arresting potential.

This PhD project has made an important contribution towards understanding the traditional use of selected Bangladeshi medicinal plants and in particular *Blumera lacera*. The identification of novel and known selectively-cytotoxic compounds from the methanolic extract of this plant indicate that Bangladeshi plants are an exciting source of interesting bioactive molecules and are of future promise for on-going anticancer drug discovery.

8.2 Future directions

Future research should focus on:

1. SPE2 and SPE3 both exhibited selective cytotoxicity to estrogen non-dependant breast cancer cells (MDA-MB-231) and had considerable mass. But work on the isolation of constituents from these fractions was not performed due to time constraints, thus, further studies into isolation of constituents from SPE2 and SPE3 fractions would be a focus of future work.

2. It had not been possible to repeat the antifungal activity study of **BL2** under similar conditions to Orhan et al. 2010 due to time constraints. Thus, the antifungal activity study of **BL2** under similar conditions to Orhan et al. 2010 will be performed in future.

3. Assessment of additional cytotoxic activity of **BL1**-**BL6** against other human cancer cell lines.

4. Investigation into additional molecular mechanisms of apoptosis-induction for **BL3** and **BL5**, for example caspase activity.

5. Further toxicity studies on **BL3** and **BL5** are required before further investigation of biological activities.

6. Evaluation of therapeutic potential for **BL3** and **BL5**.

7. Investigation into the anti-inflammatory and antioxidant potential of **BL3** and **BL5,** as these activities are related to anticancer activity.

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APPENDIX

Appendix i
Spectroscopic data of BL1
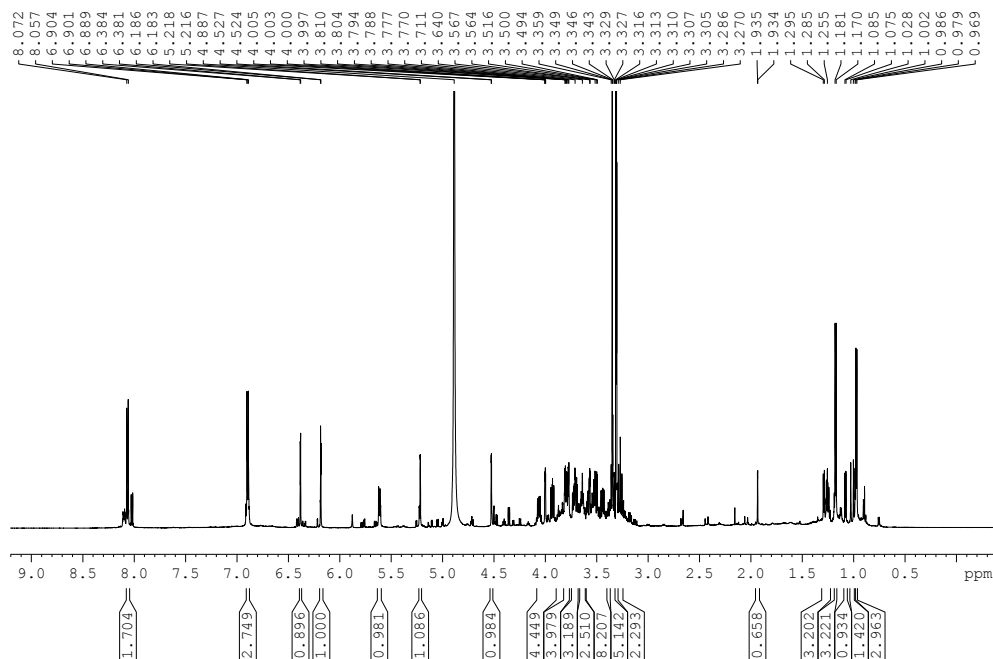
^{1}H NMR spectrum of **BL1** (CD$_3$OD at 600 MHz)
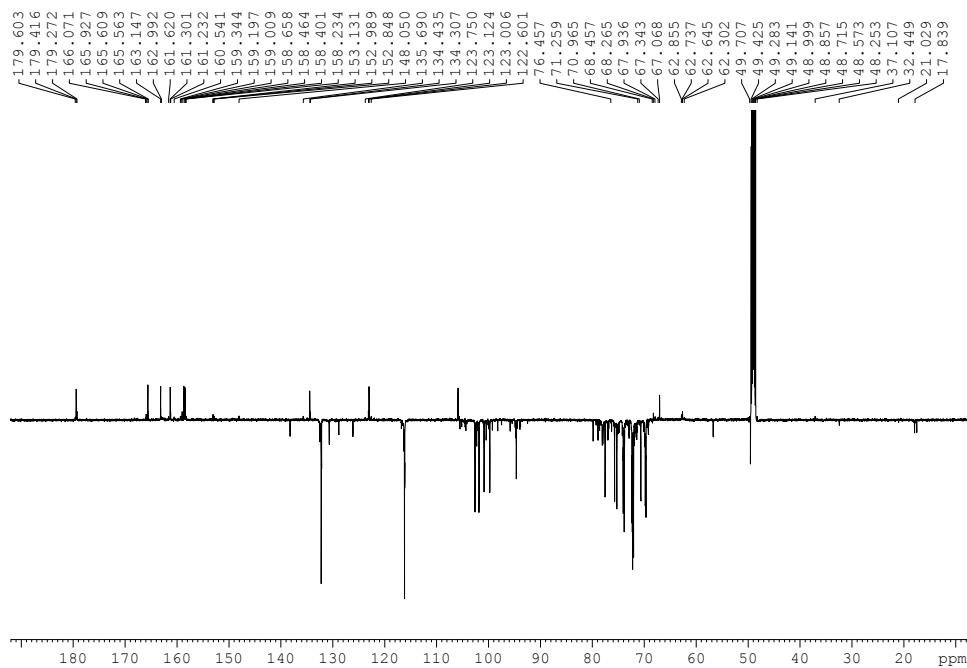
^{13}C (DEPT) NMR spectrum of **BL1** (CD$_3$OD at 150 MHz)
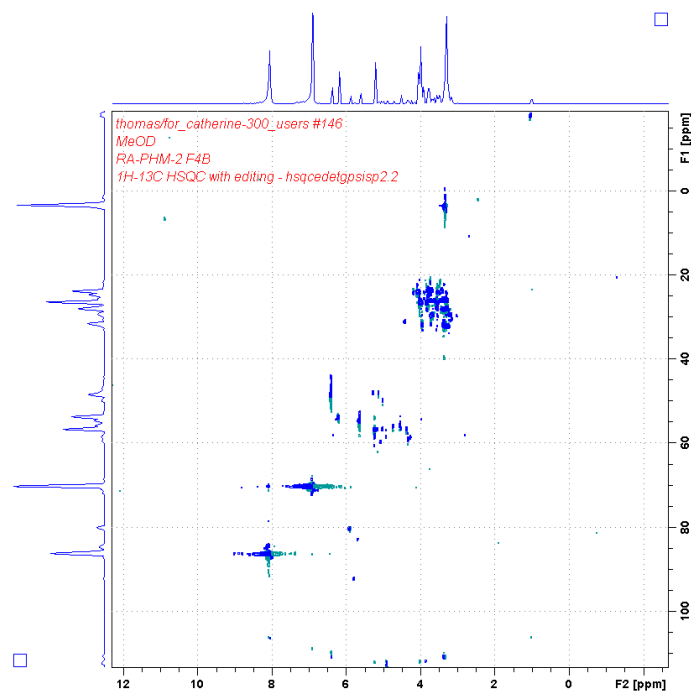
HSQC spectrum of **BL1** (CD₃OD at 600 MHz)

Appendix ii
Spectroscopic data of BL2
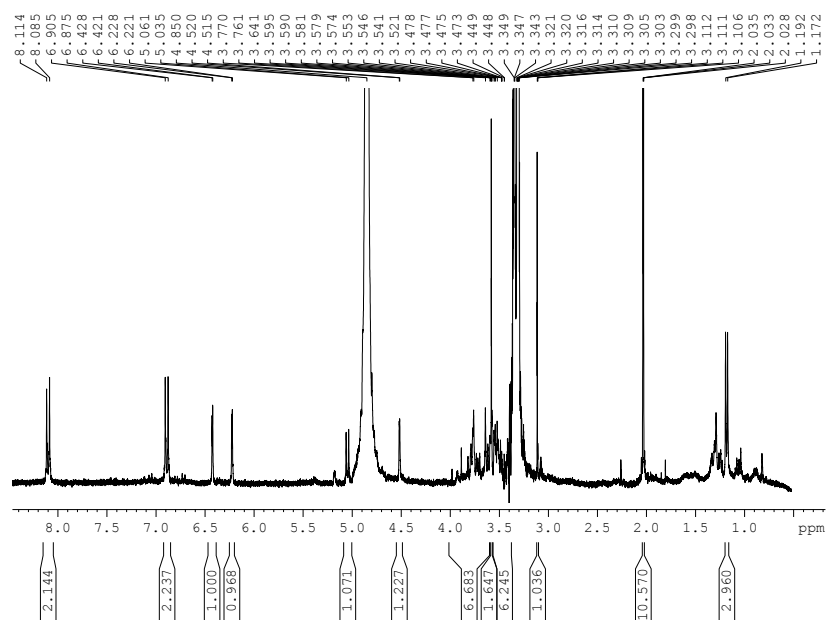
¹H NMR spectrum of BL2 (CD₃OD at 300 MHz)

The numbers along the top of the spectrum read:
179.642, 177.507, 174.831, 168.168, 167.743, 166.136, 163.075, 162.071, 161.660, 158.830, 158.599, 136.140, 135.548, 132.461, 132.358, 122.653, 121.997, 120.492, 120.110, 119.110, 116.608, 116.138, 115.476, 108.942, 105.636, 105.507, 102.980, 102.800, 101.925, 101.436, 100.471, 99.976, 94.863, 93.622, 77.640, 77.563, 76.688, 75.389, 75.061, 73.871, 73.466, 73.273, 72.964, 72.842, 72.295, 72.077, 71.337, 70.147, 69.729, 68.610, 67.401, 40.421, 25.726, 17.963

^{13}C (Jmod) NMR spectrum of **BL2** (CD$_3$OD at 300 MHz)

RA-PHM2 #39
MeOH
F5B-r

HSQC spectrum of **BL2** (CD$_3$OD at 300 MHz)

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Appendix iii
Spectroscopic data of BL3
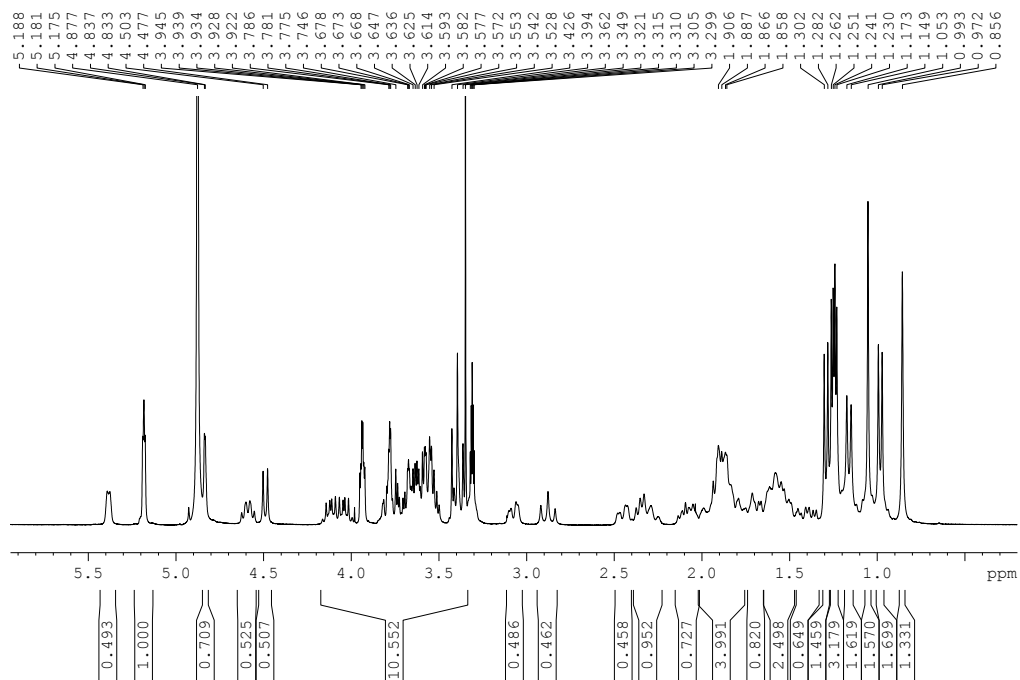
^{1}H NMR spectrum of **BL3** (CD$_3$OD at 300 MHz)
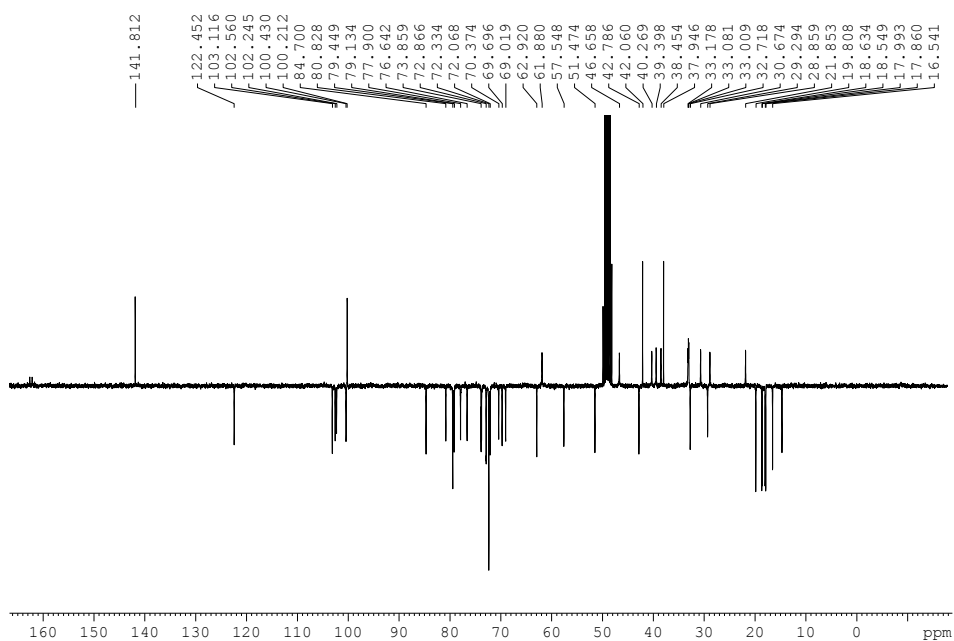
^{13}C (Jmod) NMR spectrum of **BL3** (CD$_3$OD at 75 MHz)
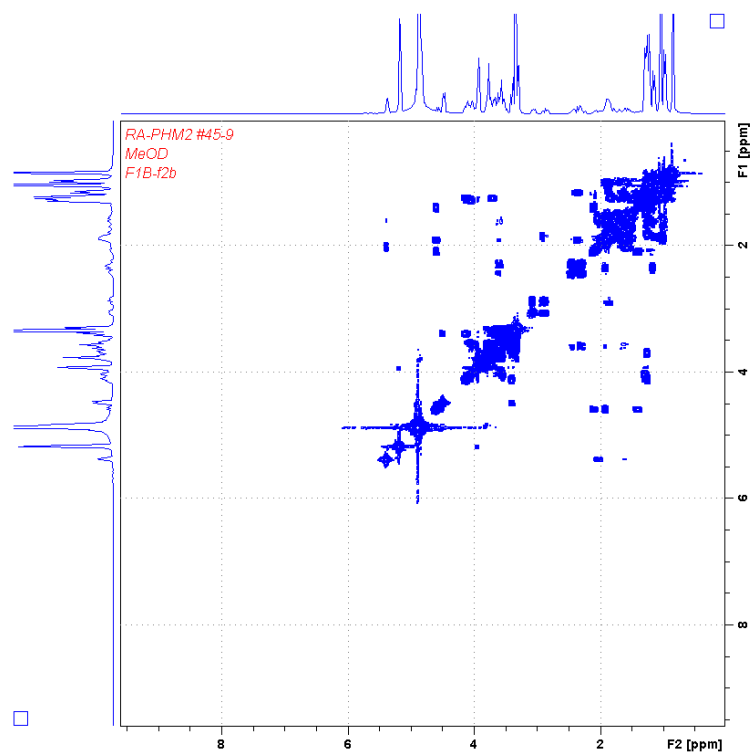
^{1}H-^{1}H COSY spectrum of **BL3** (CD$_3$OD at 300 MHz)
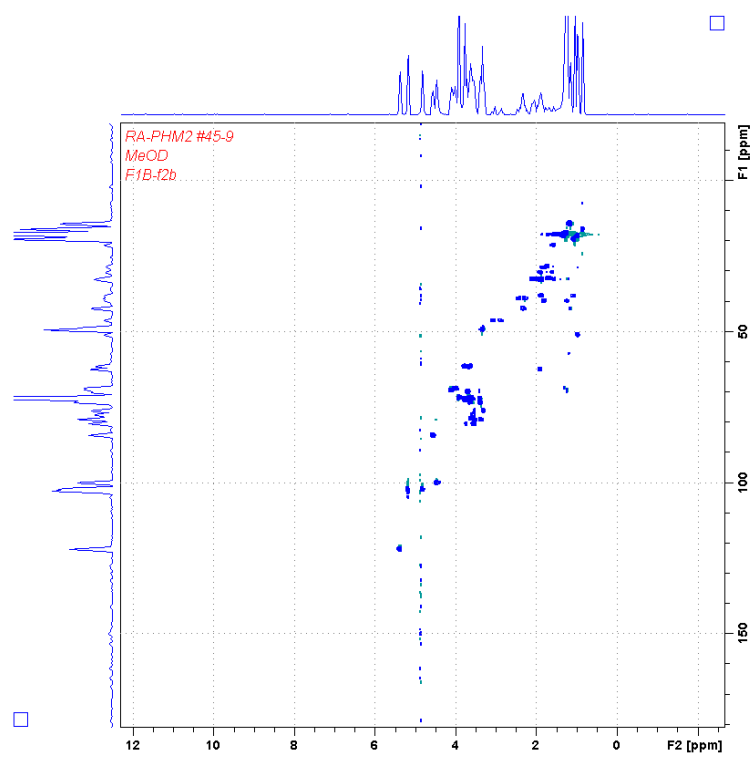
HSQC spectrum of **BL3** (CD$_3$OD at 300 MHz)
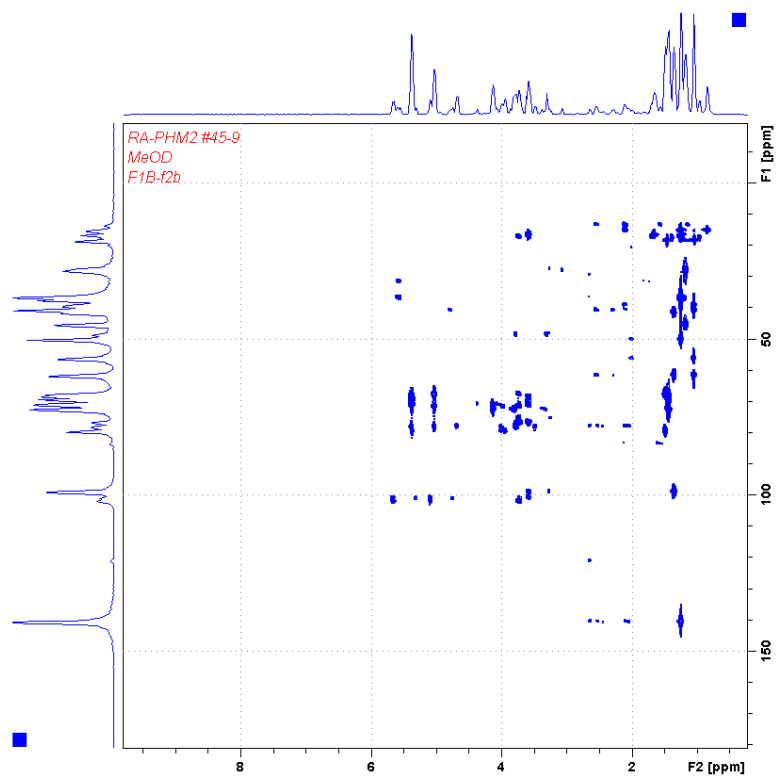
HMBC spectrum of **BL3** (CD₃OD at 300 MHz)
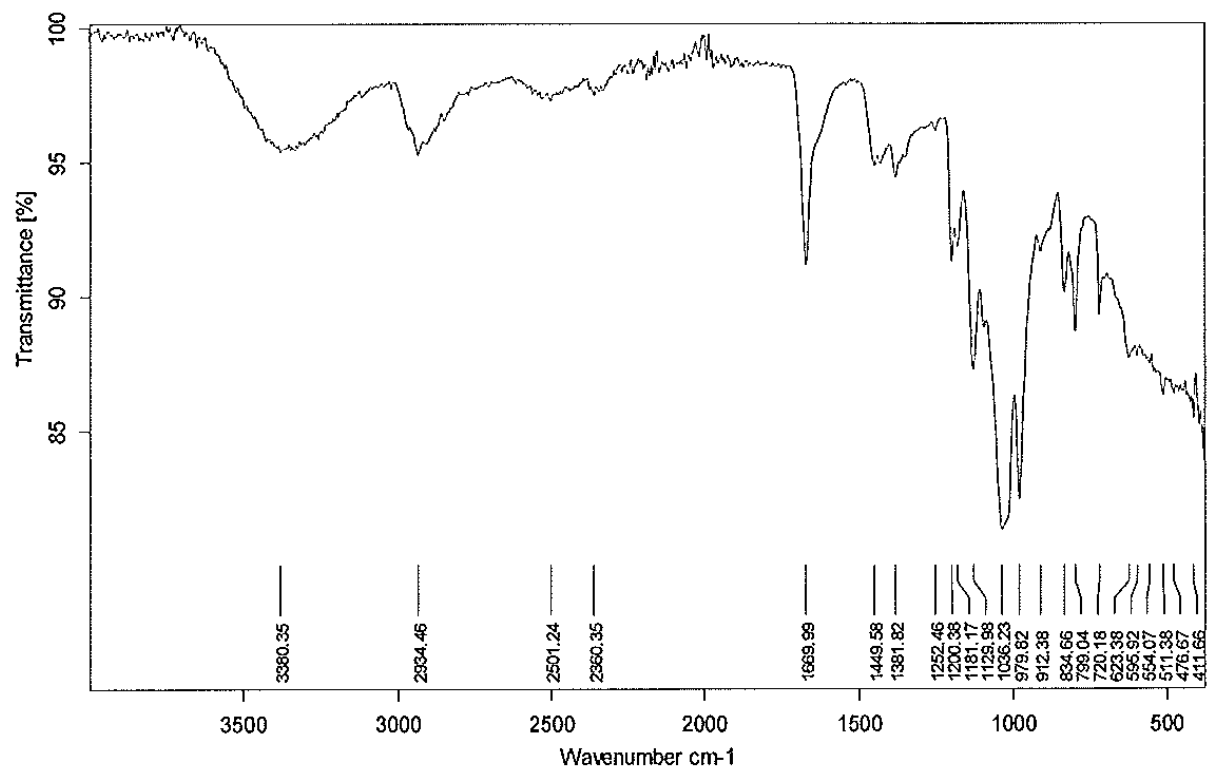
IR spectrum of BL3

Appendix iv
Spectroscopic data of BL4
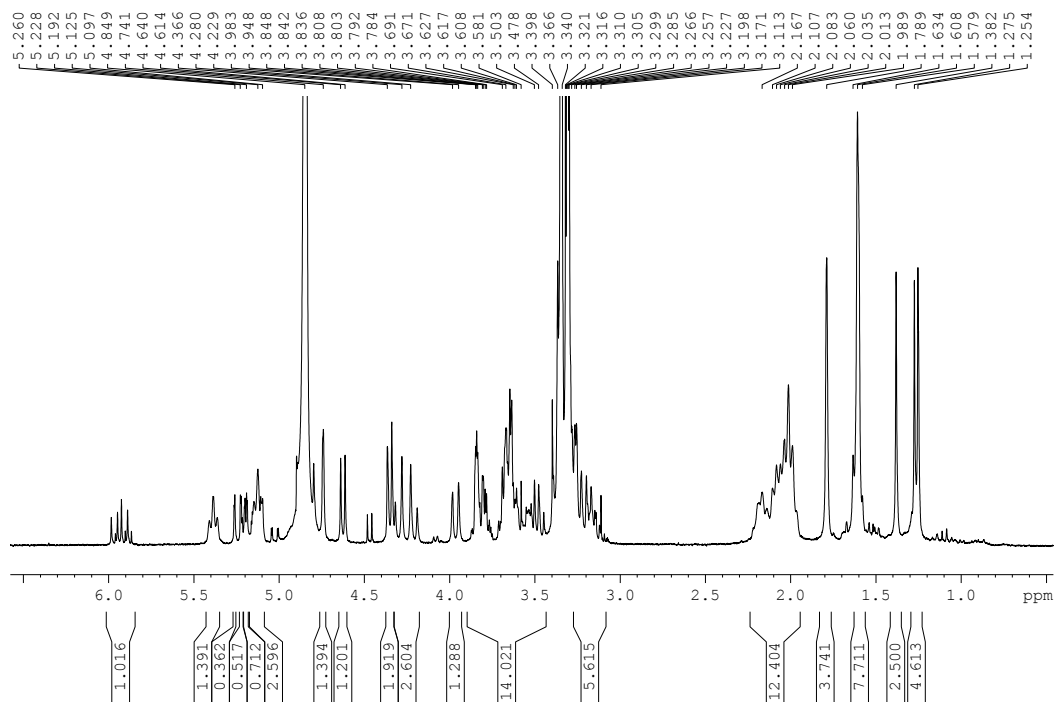
^{1}H NMR spectrum of **BL4** (CD$_3$OD at 300 MHz)
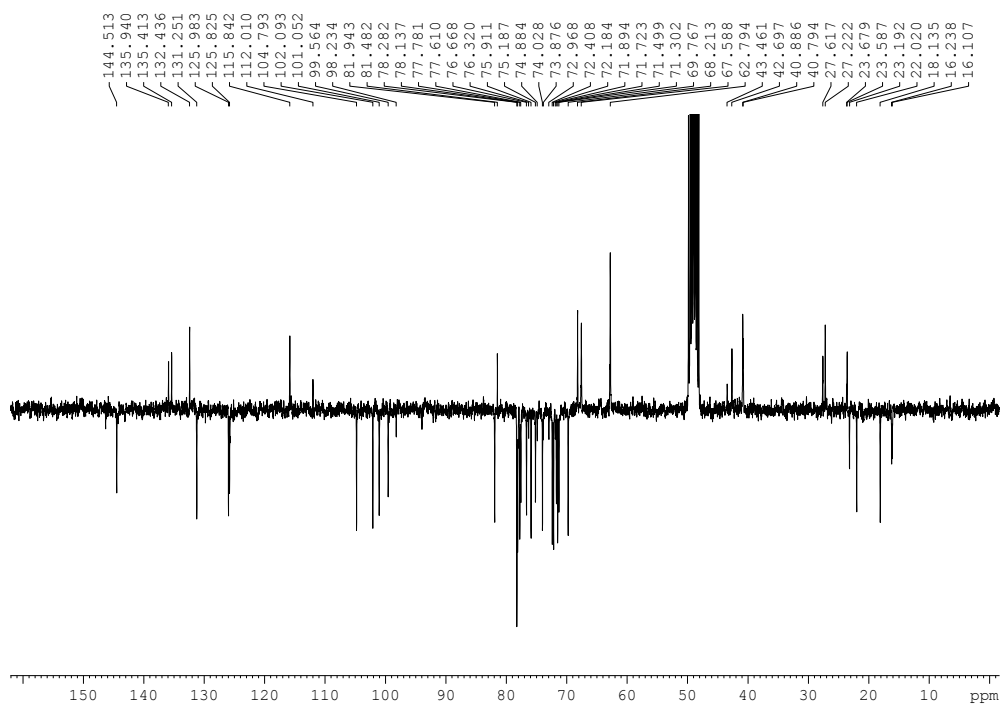
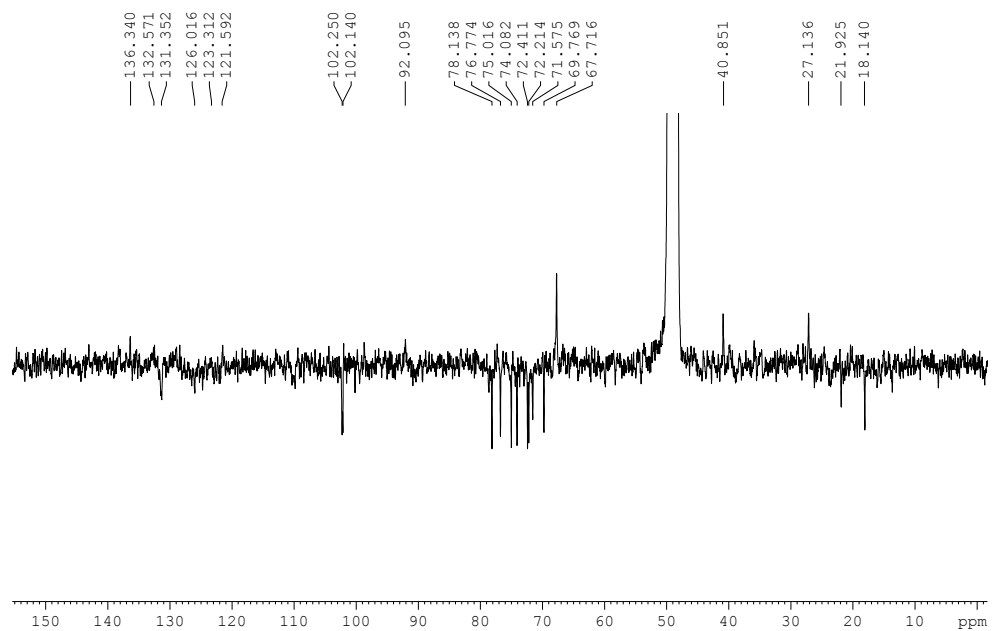^{13}C (Jmod) NMR spectrum of **BL4** (CD$_3$OD at 75MHz)
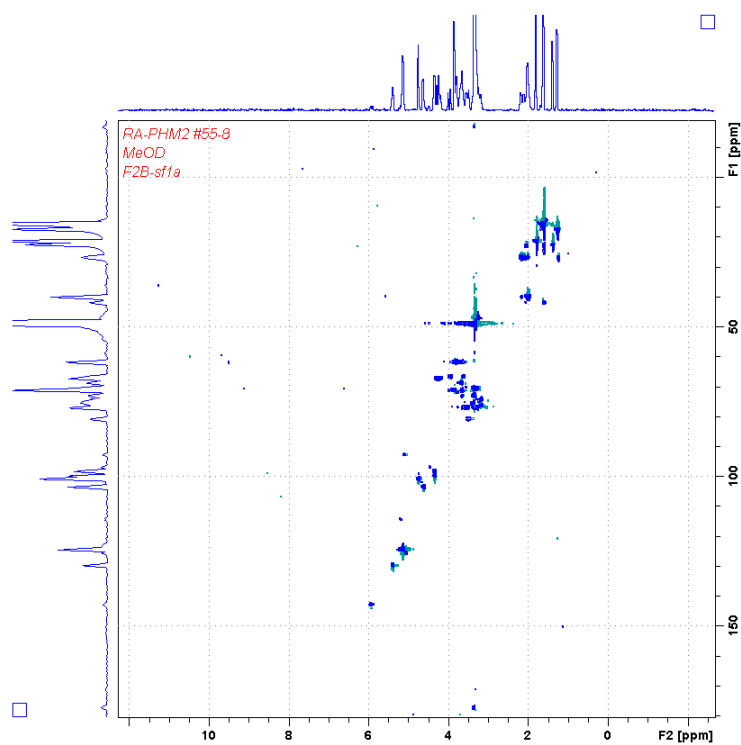
HSQC spectrum of BL4 (CD₃OD at 300 MHz)

Appendix v
Spectroscopic data of BL5
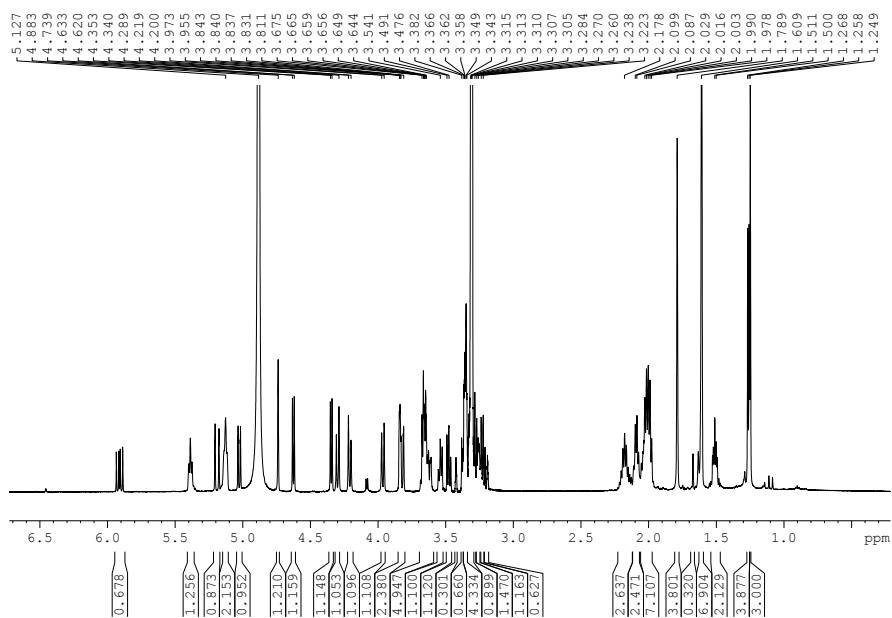
¹H NMR spectrum of BL5 (CD₃OD at 600 MHz)
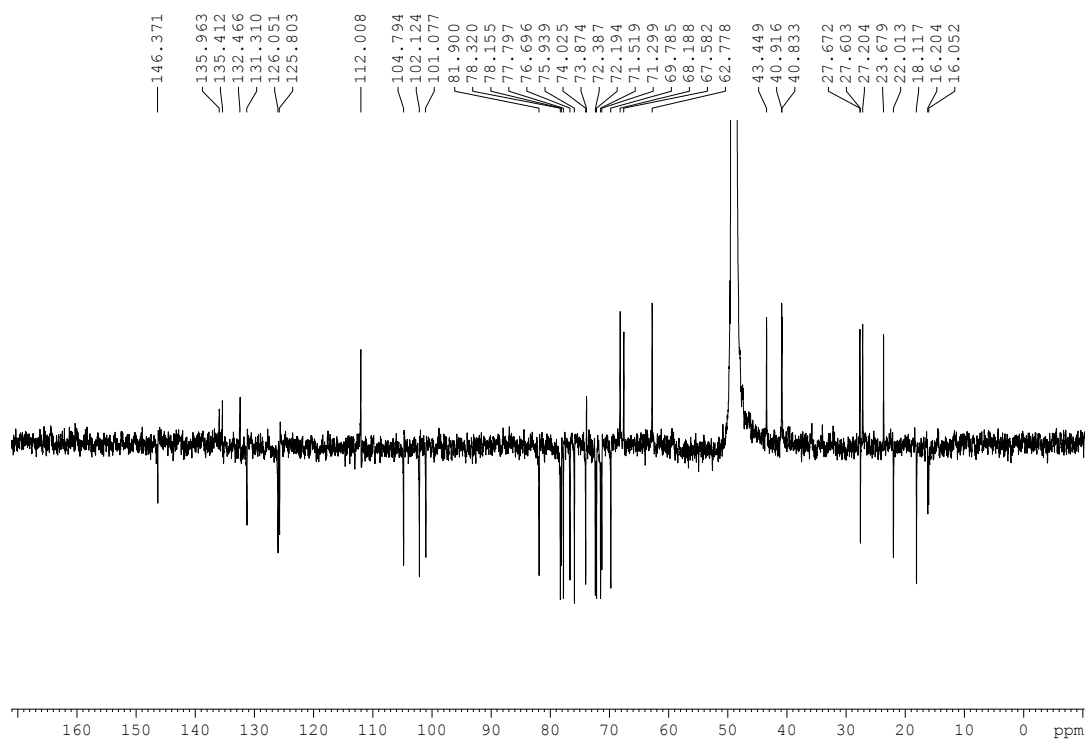
^{13}C (Jmod) NMR spectrum of **BL5** (CD$_3$OD at 75 MHz)
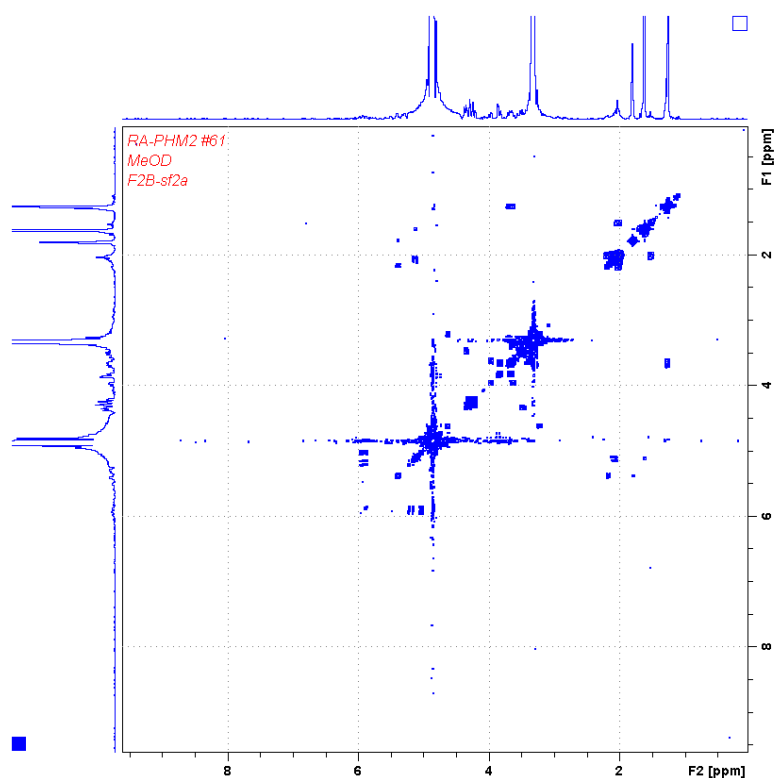
^{1}H-^{1}H COSY spectrum of **BL5** (CD$_3$OD at 300 MHz)
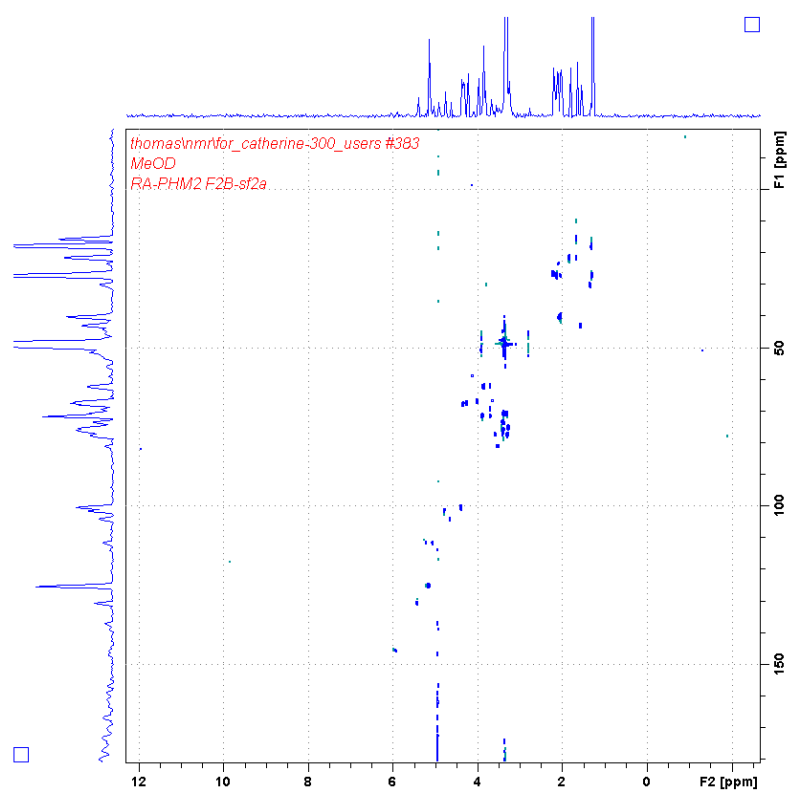
HSQC NMR spectrum of **BL5** (CD$_3$OD at 300 MHz)

HMBC spectrum of **BL5** (CD$_3$OD at 300 MHz)
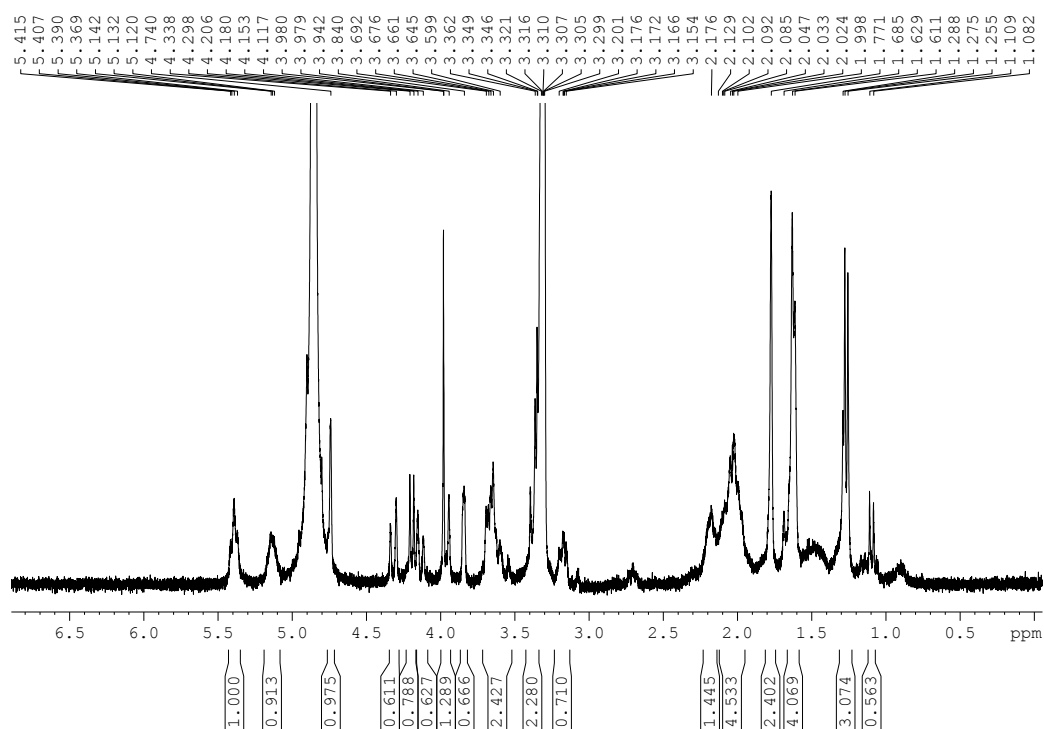
^{1}H NMR spectrum of **BL6** (CD$_3$OD at 300 MHz)

^{13}C (Jmod) NMR spectrum of **BL6** in (CD$_3$OD at 75 MHz)

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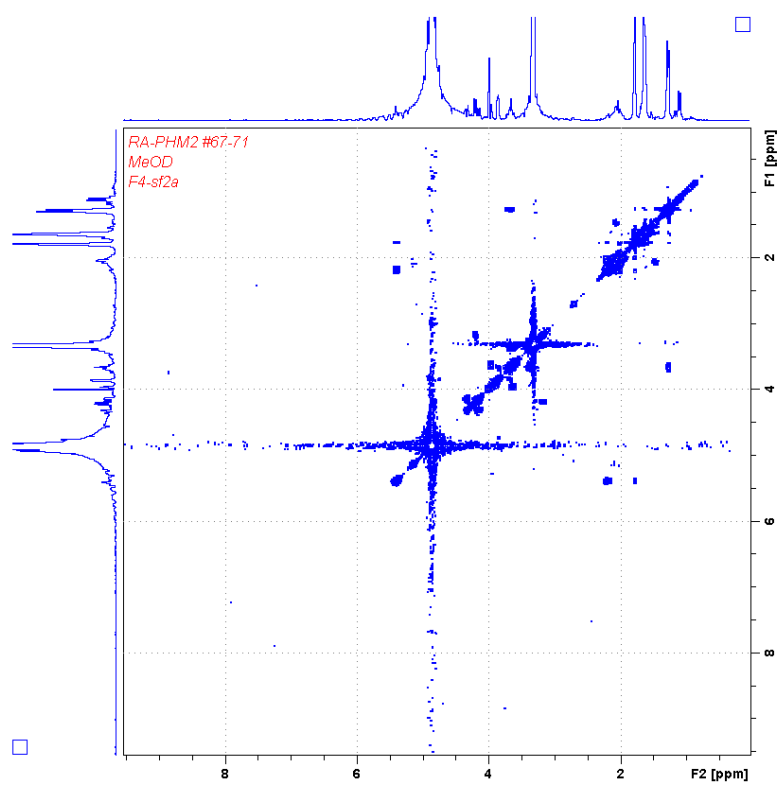
¹H-¹H COSY spectrum of **BL6** (CD₃OD at 300 MHz)

HSQC spectrum of **BL6** (CD₃OD at 300 MHz)

HMBC spectrum of **BL6** (CD$_3$OD at 300 MHz)