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EVALUATING THE ANTI-PROLIFERATIVE EFFECTS OF METHANOL AND BUTANOL EXTRACTS OF *LOBOSTEMON FRUTICOSUS* ON A PANCREATIC CANCER CELL LINE ASPC-1

Malangu Sibusiso Blose

A Dissertation submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfillment of the requirements of the degree of Masters of Science.

February 2017

DECLARATION

I declare that **"EVALUATING THE ANTI-PROLIFERATIVE EFFECTS OF METHANOL AND BUTANOL EXTRACTS OF *LOBOSTEMON FRUTICOSUS* ON A PANCREATIC CANCER CELL LINE ASPC-1"** to be my own, unaided work. It is being submitted for the Degree of Master of Science at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other university.

Malangu Sibusiso Blose

Signature Blose on the

20th Day of FEBRUARY 2017

RESEARCH OUTPUT

Poster presentation:

Malangu Blose, Lesetja Raymond Motadi**.** EVALUATING THE ANTI-PROLIFERATIVE EFFECTS OF METHANOL AND BUTANOL EXTRACTS OF *LOBOSTEMON FRUTICOSUS* ON A PANCREATIC CANCER CELL LINE ASPC-1. MBRT Conference 2015. University of Witwatersrand, Johannesburg. 03 December 2015

Malangu Blose, Lesetja Raymond Motadi. CYTOTOXIC EFFECTS OF EUPHORBIA MAURITANICA AND KEDROSIS HIRTELLA EXTRACTS AGAINST LUNG CANCER. SASBMB conference, Worcester, Cape Town, 6-9 July 2014.

Oral presentation:

Malangu Blose, Lesetja Raymond Motadi**.** EVALUATING THE ANTI-PROLIFERATIVE EFFECTS OF METHANOL AND BUTANOL EXTRACTS OF *LOBOSTEMON FRUTICOSUS* ON A PANCREATIC CANCER CELL LINE ASPC-1. National Research Foundation (NRF) intern conference. Birchwood Hotel, Johannesburg. 19 February 2015

ABSTRACT

Cancer has become a problematic fatal disease in developing and industrialised countries with pancreatic cancer as the seventh leading cause of cancer-related deaths, with an average survival rate of less than 5%. Environmental risk factors associated with pancreatic cancer include smoking, obesity, diet, alcohol etc. Furthermore, pancreatic cancer is commonly diagnosed at a late stage where its response to current anti-cancer agents is poor. Consequently, with South Africa being a 3rd world country and the cost of chemotherapy being so high, this has led to us trying to identify new, cheaper therapeutics for cancer cells. A majority (80%) of the South African population relies on traditional medicines, hence in this study we aimed to assess *Lobostemon fruticosus* for anti-proliferative effects on pancreatic cancer cell line (AsPC-1). This was achieved by the use of methanol and butanol extracts of *L. fruticosus* to screen for induction of apoptosis and inhibition of cell proliferation. The plant was collected, dried, crushed and dissolved in butanol and methanol to obtain experimental extracts. Cytotoxicity of the plant on Aspc-1 was determined using MTT Assay, xCELLigence and cell cycle analysis. MRC-5 cell line was used as a positive control cell line. *L. fruticosus* extracts induced cell death at IC_{50} of 60µg/ml (methanol extract) and 50µg/ml (butanol extract) at 48hour treatments on AsPC-1 cell line. Western Blots showed that the methanol and butanol extracts of *L. fruticosus* led to slight upregulation of the apoptotic gene p53 in AsPC-1 cell line, which was further confirmed by FACS apoptosis detection. Cell cycle analysis further showed the plant extracts do promote cell cycle arrest. LC/MS of the extracts gave spectra of active compounds presumed to play a role in induction of apoptosis on the pancreatic cancer cell line.

The data obtained implies that the methanol and butanol extracts of *L. fruticosus* does have, to a certain extent, growth inhibiting and apoptosis inducing potential on the pancreatic cancer cell line.

KEYWORDS: *Lobostemon fruticosus*, Pancreatic Cancer, methanol extract, butanol extract, AsPC-1

DEDICATION

I dedicate this dissertation to my mother Duduzile Happiness Blose, her sister Zodwa Winsley Benga and my sister Nkosinomusa Sijabulisiwe Blose

ACKNOWLEDGEMENTS

It all would not have been possible if it were not the Lords will. May His will be done.

I would like to express my sincere gratitude to my Supervisor Dr. L.R Motadi for the opportunity to work on this project, his constant motivation, guidance, support and input throughout this project.

I would also like to extend my gratitude to my Co-supervisor Dr. B.T Letsolo for not allowing me to give up and for always providing a shoulder to cry on and my advisor Prof. M Ntwasa for guidance and support.

My appreciation goes to the National Research Foundation (NRF) for providing financial support for this project.

I thank my friends and colleagues Sindiswa Lukhele, Lungile Ndlovu, Vincent Hlatshwayo, Pontsho Moela and Mpho Choene who made the hours spent in the lab easier.

Heartfelt gratitude goes to my parents Duduzile Blose, Nkosinathi Blose and Zodwa Benga, my siblings Nkosinomusa Blose, Simphiwe Blose and Khanyisile Sithole for always pushing me to be a better person and for always letting me shine.

Last but not least, Thank you Esethu Duduzile Blose for being my reason to keep pressing on even at the toughest times

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LIST OF ABBREVIATIONS

ABBREVIATION	DESCRIPTION
AIDS	Acquired ImmunoDeficiency Syndrome
AsPC-1	Pancreatic adenocarcinoma cell line
ATCC	American Type Culture Collection
BCA	Bicinchoninic Acid assay
BCL 2	B-cell lymphoma 2
BRCA 1	Breast Cancer 1 gene
BRCA 2	Breast Cancer 2 gene
CDKN2A	Cyclin-Dependent Kinase Inhibitor 2A
CO2	Carbon Dioxide
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
E2F	Eukaryotic transcription factor 2
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FITC	Fluorescein Isothiocynate
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
HRP	Horse Radish Peroxide
IC 50	Half Maximal inhibitory concentration
KRAS	Kirsten Rat Sarcoma Viral Oncogene Homolog
L. FRUTICOSUS	*Lobostemon fruticosus*
MDM 2	Murine Double Minute 2
MTT	3-4,5-dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromide
MRC 5	Medical Research Council 5 (human Lung Fibroblast)
PAGE	PolyAcrylamide Gel Electrophoresis

PanIN	Pancreatic intraepithelial neoplasia
PBS	Phosphate buffered saline
PC	Pancreatic Cancer
PCR	Polymerase chain reaction
PEN/STREP	Penicillin/Streptomycin
PI	Propidium iodide
RB	Retinoblastoma
RBBP 6	Retinoblastoma Binding Protein 6
RIPA	Radioimmunoprecipitation assay
QPCR	Quantitative Polymerase chain reaction
SDS	Sodium Dodecyl Sulfate
SMAD 4	Similar to Mothers Against Decapentaplegic 4
TAXOL	Paclitaxel
TCRP	Time-dependant Cellular Response Profiles
TOP-1	Topoisomerase-1
WHO	World health organisation
5-FU	5-Fluorouracil

CHAPTER 1: INTRODUCTION

1.1 Pancreatic Cancer

1.1.1 Epidemiology

Pancreatic cancer (PC) is considered as less common compared to other cancers such as that of the lung, breast, liver, prostate etc. However, in 2007 it was the fourth leading cause for cancer-related deaths worldwide (Herman et al, 2007). In 2002, the disease had 170000 new cases worldwide (Ghandirian et al, 2003). In 2010 it was reported to be the tenth most common form of disease (Kennedy et al, 2012) where 43000 cases were reported. The death of 37000 of those cases, among other things, contributed to it being ranked as the fourth leading cause of cancer- related deaths worldwide (Kennedy et al, 2012). Ghandirian et al (2003) further reported that annually approximately 2.1% of all new cancer cases worldwide were PC. In 2012, Pancreatic cancer ranked as the seventh most common cause of cancer related deaths worldwide with it being responsible for 331 000 deaths per year (Globocan 2012)

Studies on PC have gained a position of growing importance due to its increasing incidence and poor prognosis. According to Pandol et al (2012), an estimated number of new PC cases was 277000 worldwide with number of deaths estimated at 266000. This suggests an alarming 96% incidence/mortality rate in 2012 and 99% incidence/mortality rate in 2013 (Lan et al, 2013). Moreover, conventional treatments such as chemotherapy, surgery, radiation and combination therapy had little effect on the treatment of PC (Li et al, 2004). This may be attributed to its tendency to enter metastasis at an early stage in tumour growth and progression thus making it an aggressive cancer. In addition, only 25% of patients diagnosed with PC survive a year after diagnosis and only 6% survive 5 years (Kennedy et al, 2012).

1.1.2 Risk factors

The risk factors for PC can be categorised into three segments namely environmental and lifestyle, demographic and genetic risks.

Demographic risk factors include age, sex and ethnic group. Over 80% cases are diagnosed in individuals aged between 60 and 80 years with approximately 10% of PC patients developing tumours at ages below 50 (Li et al, 2004; Lowenfels and Maisoneuver, 2006). This can be linked to loss of cellular function (Campisi et al, 2011). PC is also more common in males and this can be associated with occupational risk factors and lifestyle such as higher smoking rates in men than women (Ghadirian et al, 2003; Pandol et al, 2012).

Amongst the 10% of cases diagnosed at ages below 50, 0.1% had family history and genetic factors as common risk factors (Yeo and Lowenfels, 2012, pandol et al, 2012). Moreover, PC affects various race and ethnic groups differently. It has high incidence rates in African American population compared to other populations (Lowenfels and Maisonneuve, 2006; Li et al, 2004; Yeo and Lowenfels, 2012). This high incidence rate can be explained by an increased prevalence of risk factors amongst the population. Similarly, The Jewish population of Ashkenazi ancestry have a higher risk for PC compared to other religious groups. Reasons for this may not be clear but it is suspected that it is due to a high prevalence in mutations within the BRCA1 and BRCA2 genes (Kim et al, 2009; Yeo and Lowenfels, 2012). These mutations are also associated with breast, gastric, ovarian and bile duct cancers. The susceptibility of Jewish populations to PC suggests that it could be hereditary, genetic and/or a result of inherited germline mutation. It was reported that those with a family history of PC are at greater risk of developing the disease. In addition, pre-existing conditions such as pancreatitis familial breast cancer, diabetic mellitus or long standing type 1 and type 2 diabetes, non-polyposis colorectal cancer, gastrectomy, among others, may lead to the development of PC (Li et al,2004; Yeo and Lowenfels,2012; Pandol et al, 2012). Furthermore, abnormal glucose metabolism and higher insulin resistance are greatly associated with PC risk (Pandol et al, 2012).

The environmental risk factors probably remain responsible for a majority of PC cases and these include smoking, occupational exposure, alcohol consumption, food habits or dietary factors. Smoking is a common risk factor for many cancers including PC. In fact, in 2004 it was reported to account for approximately 25% - 29% of PC incidence (Li et al, 2004; Pandol et al, 2012). Cigarettes

contain nicotine and numerous other carcinogens such as N-nitrosamines, polycyclic aromatic hydrocarbons, benzo(α)pyrene, β-napthylamine, methylfluoranthenes and acrylamines. These carcinogens are transported to the pancreas via the bloodstream. The carcinogens then bind to DNA forming adducts which increase the risk of somatic mutations and cancer development (Yeo and Lowenfels, 2012). According to Pandol et al (2012), moderate use of alcohol may not be associated with an increased risk of PC but heavy drinkers exhibit a 22% increased risk. Body Mass Index (BMI) is also linked to development of PC in the sense that overweight and obese individuals with a BMI > 30 are at risk of developing the disease. This might be an indication that an increased metabolic syndrome caused by obesity, accumulation of fats and high calorie diets is also a risk factor for PC.

1.1.3 The cell cycle and regulatory genes

Numerous genetic changes take place in the transition of normal cells to cancer cells. The cell cycle has led to a better understanding of development and progression of cancer. It regulates DNA replication, DNA repair and genome segregation leading to the generation of a *bona fide* copy of a cell. The cell cycle is made up of four general stages, as seen in figure 1.1, the G_1, S, G_2 and M phase (Hartwell and Kastan, 1994; Kennedy et al, 2012). Gap 1 (G_1) is where the cell grows in size. DNA replication takes place in the S-phase. The cell then progresses to Gap 2 (G_2) where the cell prepares for mitosis. The M-phase is where mitosis (chromosome segregation) takes place. Following cell division certain cells may go through a state of quiescence known as G_0 (figure 2) (Kennedy et al, 2012; Hartwell and Weinert, 1989; Collins et al, 1997). The cell cycle machinery controls the cell cycle by interpreting incoming signals that either promote cell proliferation, senescence or apoptosis. This control takes place in the restriction point and is highly involved in deciding the fate of the cell as it interprets incoming signals related to cell regulation (Hartwell and Weinert, 1989; Kennedy et al, 2012).

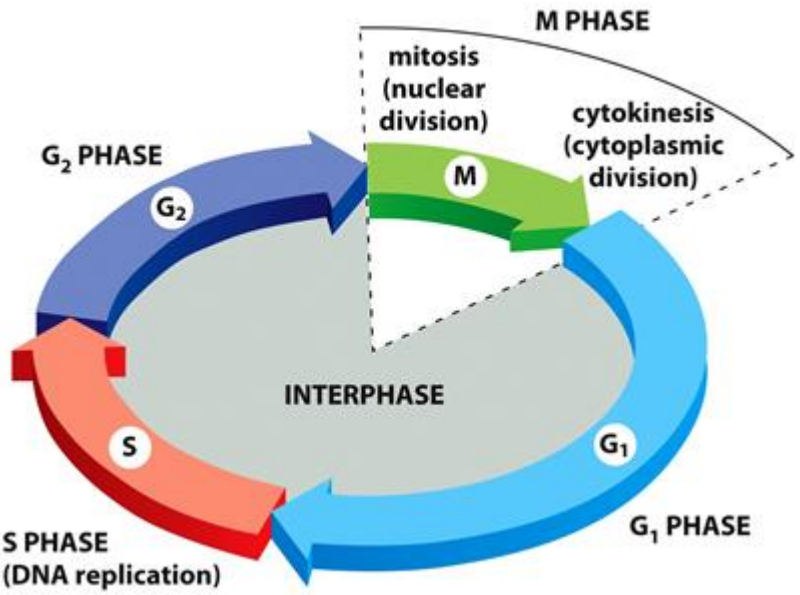


Figure 1.1: The cell cycle. Image adapted from https//www.oregonstate.edu.

Cancer is a genetic disease (Vogelstein and Kinzler, 2004) and mutations in oncogenes and tumour-suppressor genes result in tumorigenesis. Oncogene activation and tumour suppressor gene mutations promote tumour formation or suppression by stimulating cell generation or inhibiting cell death or cell cycle arrest (Vogelstein and Kinzler, 2004). P53 is known as a well-studied tumour suppressor and is found mutated in many human cancers (Speidel, 2009; Farnebo *et al.*, 2010). Inactivation of the tumour suppressor genes, CDKN2A and P53 and activation of oncogenes, forms part of the characteristics of pancreatic cancer (Hildago, 2010).

CDKN2A suppresses tumour formation by inhibiting Cyclin-d Dependent Kinases (CDK). This leads to phosphorylation of Retinoblastoma (RB) (transcription factor). In activation of CDKN2A then leads to upregulation of CDK, this promotes upregulation of RB. RB is an important gene in the cell cycle progression and plays an essential role in development and cell differentiation (ahlnder and Bosco, 2009). Its function is mainly dependent on its ability to inhibit E2F transcription factor resulting in progression of the cell from the G0/G1 phase to the S-phase of the cell cycle (Vogelstein and Kinzler, 2004; Hildago, 2010).

P53, known as TP53 in humans, was the first tumour suppressor gene to be identified. It was first described in 1979 (Vogelstein *et al*, 2000) and according to Green and Kroumer (2009) it has an inactivating mutation in most cancers. As mentioned earlier, p53 is a well-studied tumour suppressor gene which plays an important role in numerous signalling pathways such as cell cycle arrest, senescence and apoptosis. It regulates these processes by transactivating a range of cellular genes involved in their regulation (Vermeulen et al, 2003; Farnebo et al; 2010). Regulation of the stability, conformation, expression and activity of p53 gene is highly linked to the MDM2 protein (Balint and Vousden, 2001).

When cells are exposed to stress such as exogenous mutagens, oncogene activation, telomere damage and hypoxia, which are stresses that influence post-translational modification and interaction of p53 with cofactors, p53 induces cell cycle arrest through a p21-mediated pathway giving the cells a chance to repair the damaged DNA (Freeman, Wu, and Levine., 1999). p21 gene encodes an inhibitor of CDKs and is responsible for the onset of cell cycle arrest at G1/G2 phase (Moela P, 2013). However, should the cell fail to repair DNA damage during the cell cycle arrest, p53 protein induces irreversible arrest of the cell cycle and/or apoptosis through the mitochondrial-mediated pathway. Briefly, cytoplasmic p53 protein translocates to the mitochondrial outer membrane inducing permeabilization of the membrane known as mitochondrial outer membrane permeabilization (MOMP). This process stimulates several pro-apoptotic BCl_2 members including Bax, Puma, Noxa, and Bid and inhibits the transcription of anti-apoptotic genes. This activates the caspase cascade resulting in apoptosis (Speidel, 2009; Farnebo et al., 2010). In addition, cytoplasmic and nuclear p53 play a role in other biological processes such as ageing and longevity (Green and Kroemer, 2009).

1.1.4 The pancreas

The pancreas is a cylindrical organ on the body found between the stomach and the spine. It is made up of three sections, the head, body and the tail). It is an essential organ that plays a major role in hormonal and digestive systems. It has an endocrine and exocrine function. The endocrine pancreas is made up of cells called the islets of Langerhans (Figure 1.2). These cells secrete peptide hormones

known as glucagon, insulin, somatostatin and pancreatic polypeptide which regulate energy metabolism (Williams J, 2014). Less than 10% of PCs occur in this region. However, the endocrine region may contribute to PC progression through its ability to secrete hormones that regulate cell fate and by providing a capillary network for metastasizing tumour cells (Kennedy et al, 2012).

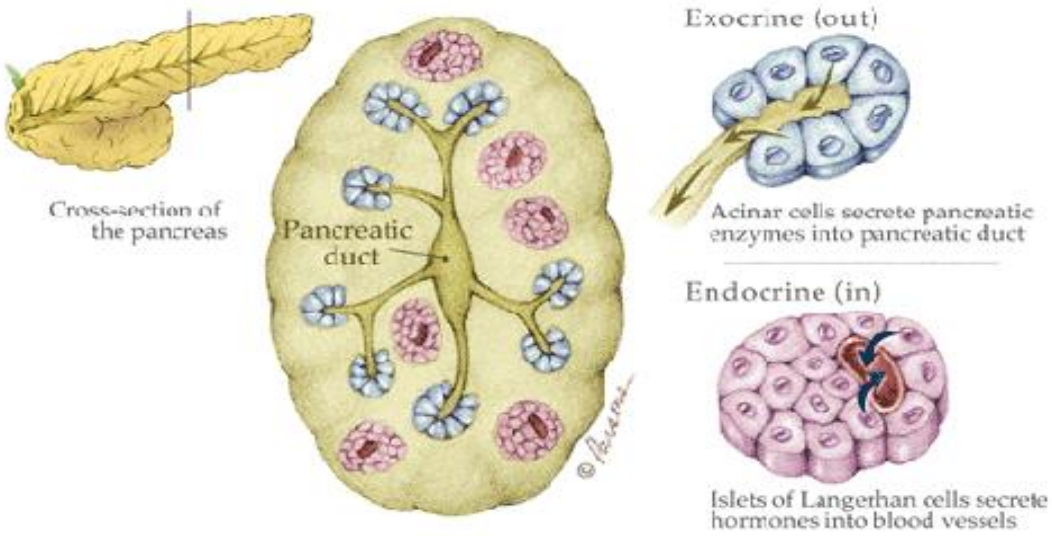


Figure 1.2: image of the pancreas showing the endocrine and exocrine regions.(Image adapted from https://beyondthedish.wordpress.com)

Over 90% of PC tumours occur in the exocrine region. The major role of exocrine pancreas is nutrient digestion. The exocrine pancreas consists of acinar and ductal cells (Figure 1.2). The acinar cells secrete pancreatic juices with digestive pro-enzymes. This juice is then transported to the duodenum through the ductal system (Shih et al, 2013; Kennedy, 2012). Moreover it was noted that the pancreatic juices also contain bicarbonate ions which are responsible for neutralising acid that comes from the stomach. The acinar cells also secrete mucin proteins, which are overexpressed in most cancers. Consequently, these cells are regarded as the potential culprits from which pancreatic ductal adenocarcinomas or PC arise (Kennedy et al, 2012).

1.1.5 The development of pancreatic cancer

PC is generally develops from pre-existing lesions. According to Vincent et al (2011), most common pre-existing lesions that generally progress to an invasive pancreatic adenocarcinoma are called Pancreatic intraepithelial neoplasia (PanIN). The formation of neoplastic lesions is characterised by a

columnar epithelium. At an advanced stage, the lesion is termed papillary hyperplasia. This stage of lesion formation can take numerous forms but it is usually distinguished by a dense mucosa (Hilgers and Kern, 1999; Li et al, 2004). Other precursor neoplasia include intraductal papillary mucinous neoplasms, branch duct neoplasms and mucinous cystic neoplasms (Vincent et al, 2011). These neoplastic lesions become carcinomas when they start to invade through the wall of the duct. This invasion generally brings forth an enflamed collagenous reaction. This reaction is known as desmoplasia. The desmoplasia provides a scaffold, growth factors, angiogenesis factors and immune modulators for the nourishment of cancer cells thus regulating PC tumour formation (Pandol et al, 2012; Hilgers and Kern, 1999).

Pancreatic cancer lesions have mutations on the KRAS2 gene. While colon or ovarian epithelial cells with the mutation on the KRAS2 gene progress to the formation of non-cancerous lesions, this may not be the case with pancreatic cancer (Vogelstein and Kinzler, 2004). Consequently, transcription of the mutated KRAS2 gene results in the production of a defected protein, leading to activation of proliferative signalling pathways in pancreatic cancer (Hildago, 2010).

1.1.6 Treatment of pancreatic cancer

As mentioned earlier, a large number of patients with pancreatic cancer are diagnosed when at metastatic stages and this has led to the cancer being associated with the worst survival, with figures of survival rate still below 3% (Beger et al, 2003). Moreover, pancreatic cancer cells are known to express a multidrug resistance associated protein which contributes to the cancer's resistance to treatment (Khan et al, 2013). Tumour size, lymph node involvement, stage of infiltration into marginal tissue and presence of metastasis serve as the key markers in induction and choice of treatment (Pierantoni *et al*, 2008). The complete removal of the tumour by surgical resection is associated with increased chances of survival in patients with a tumour size < 2cm. Due to its early metastasis, less than 15% of pancreatic cancer patients can be treated by curative surgery. Furthermore, long-term survival of ≥5 years post-surgery is limited to a very low number of cases. This is because the efficiency of surgery is limited by certain factors such as occurrence of liver

metastases, distant metastases, tumour infiltration into mesenteric vessel walls and more (Pierantoni *et al*, 2008). Assessment of pancreatic cancer patients following surgery gives valuable prognostic information. Promising surgery generates negative resection margins. The presence of positive resection margins, poor tumour differentiation and a large cancer (amongst other things) are characteristics of poor prognosis. In addition, the detection of certain markers such as SMAD4, BRAC2 and TP53 also forms part of the means of detecting outcomes of curative surgery (Borredy and Srivastava, 2013; Vince et al, 2011). With 20-25% survival rate of patients that have undergone curative surgery or resection, further treatments are still needed to improve the treatment of pancreatic cancer (Pierantoni et al, 2008).

Adjuvant and neoadjuvant therapy has improved the outcomes of surgical removal of pancreatic cancer tumours. This form of treatment could either be chemotherapy, radiotherapy or a combination of the two. It is administered before or after surgery depending on the stage of the disease (Pierantoni et al, 2008).

Metastatic pancreatic cancer is reported as one of the most chemotherapy and radiation resistant tumours (Hermann et al, 2007). Commonly used chemotherapeutic agents are 5-Fluorouracil (5-FU) and gemcitabine. Other chemotherapeutic drugs include doxorubicin and mitomycin C, cisplatin, oxaliplatin, capecitabine, irinotecan, epirubicin and more (Pierantoni et al, 2008). Though they may provide an improved quality of life, these agents have a response rate below 20% in patients (Rathos et al, 2012). Moreover, other treatments include radiotherapy and combinational therapy (Beger et al, 2003; Rosenburg, 1997). Radiotherapy may have shown positive effects on benign cancers but it may have negative effects on surrounding tissues (Rathos et al, 2012; Vincent et al, 2011).

1.1.7 Justification

Numerous trials are on-going to improve the outcome on pancreatic cancer patients (Pierantoni et al, 2008).The short fall in current treatments (inadequacy, costs and availability of currently used treatments, or unavailability of efficient treatments) on pancreatic cancer influences numerous ongoing trials aimed at improving the outcome on pancreatic cancer patients. This includes in-depth

studies on the use of alternative treatments, such as traditional plants on cancer with the hope of finding treatments with high efficiency and low costs. This study aims to investigate the efficiency of *Lobostemon fruticosus*, an indigenous traditional plant, for efficiency as an anticancer agent.

1.2 Traditional Plants

Nature has played a role in providing treatments for a number of ailments for thousands of years (Mukherjee et al, 2001). According to the World Health Organisation (WHO), 80% of the world's population relies on traditional medicines. In most cases this is generally based on cultural and economic factors. In Africa, dependence on traditional medicines from traditional plants is influenced by limited access to allopathic medicines and the pressing need to fight against pandemics such as HIV/AIDS (Mahomoodally, 2013). Despite a few problems such as inefficient registration and regulation of traditional remedies, lack of understanding of the use of these remedies and a high demand for conventional medicines, more and more developed countries seem to be venturing into the use of traditional plants for their primary health care needs due to the continued need for development of improved drugs. There may be some growth in the documentation of the use of traditional plants medically; however, this has been a practice within the African population for decades (Mahomoodally, 2013; Sawadogo et al, 2012).

Africa has a huge plant biodiversity with over 5000 species being used medicinally. The location of the African continent within the Tropical and Subtropical regions is assumed to contribute to the accumulation of a wide variety of essential secondary metabolites by the African plants. These metabolites play a role in the healing process (Mahomoodally, 2013).

Plants contain a range of phytochemicals that elicit biological activities. Based on these activities, three major groups of anticancer agents have been identified. These are: (1) inhibitors of carcinogen formation by production of nitrosoamines from secondary amines, (2) blocking agents which prevent carcinogens from binding to their target sites and (3) anti-progression agents (Mukherjee et al, 2001). These phytochemicals are known as secondary metabolites. These metabolites may act individually or synergistically to cause an immune reaction.

A larger percentage of secondary metabolites generally isolated from plants include sesquiterpenes, diterpenes and triterpenes. Understanding of the mechanisms of these terpenes highlights the prospects of pre-clinical studies that can be aimed at producing natural and cost effective anti-cancer drugs (Sawadago et al, 2012).

In several countries certain traditional healers have claimed the ability to treat cancer using traditional medicines. This has led to an increased interest in studies focusing on the treatment of cancer using traditional plants. Figure 1.3 shows several cancer types (studied by Sawadago et al in 2012) in which the effects of traditional plant extracts have been tested. Some of these have displayed high sensitivity to these plant extracts. This suggests that certain plant extracts do have cytotoxicity against cancer cell lines.

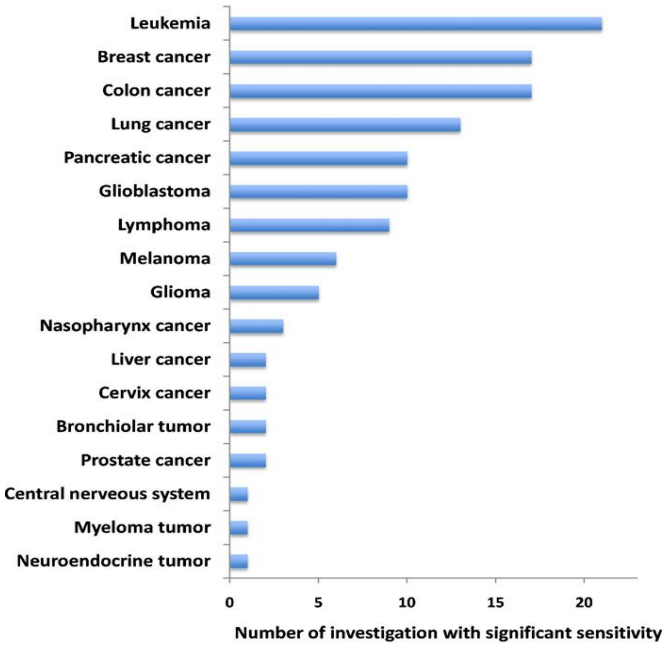


Figure 1.3: A study based overview of the frequency of certain types of cancers that are sensitive to traditional plants. (Sawadago et al, 2012)

Food and Drug Administration (FDA) approved Medicines derived from traditional plants have been in use since the 1950s.Vinblastine and Vincristine were the first anti-cancer agents used clinically.

These compounds were derived from *Catharanthus roseus* (Thafeni et al, 2012; Cragg and Newman, 2005; Mukherjee et al, 2001). Other examples of plant derived anti-cancer agents include Taxol (Paclitaxel), Taxotere (Docetaxe), Navelbine, Etoposide, Teniposide, Topotecan and Irinotican (Mukherjee et al, 2001). The use of traditional plants is increasingly being accepted and plants are presumed to offer a huge potential in improving health (Mahomoodaly, 2013). An example of a plant used traditionally to treat ailments and improve health is *Lobostemon fruticosus*.

1.2.1 *Lobostemon fruticosus*

Lobostemon fruticosus is an evergreen perennial bush commonly known as the Pyjama Bush. In vernacular, it is known as Agdaegeneesbos or Douwurmbos. It is a member of the *Boraginaceae* family of plants. It is endemic to the floristic kingdom and is found in the Western Cape Province (Rief and van Wyk, 1997). It is a shrub that has densely hairy branches and leaves and it can grow up to 1m in height. *L. fruticosus* has blue-green leaves that have a succulent, rough leathery texture and it produces long funnel-shaped blue, pink and white flowers (Levyns, 1934). A crude extract of *L. fruticosus* that has been extracted using vegetable oil or butter is traditionally used to treat ringworm, eczema, wounds, sores, syphilis and other dermatological disorders. It was also reported that aqueous extracts of *L. fruticosus* have been administered traditionally to remedy gynaecological discomforts (Levyns, 1934). Little is known about the chemical makeup of *L. fruticosus* but Levyns (1934) identified the presence of cyclitols, tannins, naphthoquinone derivatives, pyrrolizidine alkaloids, phenolic acids, saponins and reducing sugars within this plant. Some of the secondary metabolites found in this plant are known to have anticancer activity (Cragg and Newman, 2005).



Figure 1.4: An image of *Lobostemon Fruticosus* adapted from www.strangewonderfulthings.com.

Not many studies have been done on the effects of *L. fruticosus* on cancer more specifically, pancreatic cancer. Therefore it would be interesting to investigate the effects that this traditional / medicinal plant could have on pancreatic cancer.

1.3 Hypothesis

L. fruticosus has been reported to have certain metabolites that have anti-inflammatory and antioxidant activity, and cause an immune reaction. Furthermore, reports on anticancer activity on some of the compounds in *L.fruticosus*, such as 4-Hydrocoumarins in Lung cancer has lead us to hypothesise that it also has anticancer activity against pancreatic cancer. Moreover, we hypothesised that *L. fruticosus* may have anti-proliferative effects on the pancreatic cancer cell line AsPC-1.

1.4 Aim

The aim of the study was to evaluate the anti-proliferative activity of methanol and butanol extracts of *Lobostemon fruticosus* on pancreatic cancer cell line, AsPC-1.

1.5 Objectives

The objectives of the project were:

- Examining cell growth inhibition capabilities of methanol and butanol extracts of *Lobostemon fruticosus* by treating AsPC-1 cells with the extracts and analysing the effects using MTT assay and xCELLigence.

- Evaluating the ability of plant extracts to promote cell cycle arrest using flow cytometry.

- Assessing the apoptotic effects of the extracts on the AsPC-1 cells using flow cytometry
- Analysing the expression of apoptotic genes by measuring protein expression using western blot technique
- Identifying key chemical components in the plant extracts using LC/MS

CHAPTER 2: METHODS AND MATERIALS

2.1 Materials

2.1.1 Cell culture

A pancreatic cancer cell line, AsPC-1, was used as the test cell line, a primary source of protein and as a basis for the assessment of growth and morphology following treatments. A lung fibroblast cell line, MRC5, was used as a control cell line to ensure that the methanol and butanol extracts of *Lobostemon Fruticosus* do not have adverse effects on the normal cells. All cells were purchased from ATCC.

2.2.2 Extracts

Lobostemon fruticosus was collected at the Fernkloof nursery in Hermanus, Western cape, South Africa.

2.1.3 Control treatments

Paclitaxel (Taxol)

Paclitaxel, commonly known as Taxol, is a promising anti-tumour agent that was isolated from *Taxus brevifolia* in the 1960s. It is used in the treatment of breast, ovarian, lung, oesophageal, pancreatic, prostate and other cancers including Karposis Sarcoma (Singla et al, 2002). This drug affects cell proliferation by promoting formation of tubulin dimmers, by stabilising microtubule formation, thus shifting the equilibrium between dimers and polymers. This affects mitosis resulting in the formation of abnormal cytoskeletal structures (Belotti et al, 1996). Subsequently, cells exposed to Taxol become arrested in mitosis. Furthermore, Taxol may also possess properties that induce apoptotic cell death (Milross et al, 1996; Fan, 1999). This has been characterised in a number of tumours by observing features such as paclitaxel-induced chromosome condensation, cytoplasmic blebbing, cell shrinkage and DNA Fragmentation (Fan, 1999).

Camptothecin

Camptothecin (Calbiochem®) is a natural anti-proliferative agent which was isolated from the bark of a Chinese tree called Camptotheca acuminate. It is a quinolone alkaloid that which was discovered in the early 1970s. Its derivatives include irinotecan and topotecan (Pommier, 2006). Camptothecin inhibits topoisomerase 1 (TOP1) by stabilising the cleavable complex which is a covalent reaction intermediate. The agent only targets TOP1 and leads to apoptotic cell death within minutes of exposure (Pommier, 2006; Li and Lui, 2001). It binds irreversibly to DNA-Topoisomerase 1 complex preventing re-ligation of DNA resulting in apoptosis (Moela, 2013; Li and Lui, 2001).

2.1.4 Antibodies

In this study Primary antibodies for p53, BCl2, MDM2, RBBP6 and beta-actin (reference gene) were used for western blot

2.2 Methods

2.2.1 Plant preparation

L. fruticosus plant was dried at room temperature for 3 weeks. The plant (root, stem and leaves) was crushed. 10 g of plant powder was dissolved in 100 ml of Methanol and 100 ml of Butanol. The butanol and methanol mixtures were left overnight on a shaker at 150 rpm. The extracts were filtered and solvent was evaporated in an incubator at 25°C under low pressure using a Büchi rotavapor R-205 (Büchi Labortechnik AG, Switzerland) and the remaining dry extract was weighed (289.6 mg methanol and 96.2mg butanol) and dissolved in 1ml DMSO (Merck) making up a final concentration of 289.6 mg/ml methanol extract and 96.2 mg/ml butanol extract. These were then stored in -20^{0}C as stock extracts.

2.2.2 Cell culture

The AsPC-1 and MRC5 cells were maintained in Dulbecco's Modified Eagles Media (DMEM) containing 2 mM L-glutamine, supplemented with FBS (10 %) and Pen/Strep (1%) (Highveld Biological). The cells were cultured in 25 cm^3 flasks and incubated at 37^0C, 95% humidity and 5% CO_2. Cultures were allowed to reach 70% to 80% confluency as a monolayer and were split every 2 days. When splitting the cells, the media was removed and the cells were washed twice with PBS. The cells were then trypsinised with 500 µl of trypsin (0.025%) at 37ºC until cells were detached. Trypsinization was stopped by adding 5 ml of complete DMEM. A fraction of these cells is transferred to a new flask and incubated.

2.2.3 MTT

The MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) assay is an *in vitro* cytotoxicity assay used extensively to measure viable cells. MTT is a water soluble tetrazolium salt. During the assay, the tetrazolium salt is converted to an insoluble formazan which is purple in colour. The conversion involves a cleavage of the tetrazolium ring by succinate dehydrogenase found in mitochondria of viable cells. This results in the formation of formazan crystals which are impermeable to the cell membrane. The crystals dissolve in the presence of dimethyl sulphoxide (DMSO) and the optical density of the suspension can be measured at 560 nm. This way an increase and decrease in viable cells can be measured.

Applications of the MTT assay include: sensitivity testing on new drugs, drug screening on cell lines, testing of drug combinations on cell lines and studies of cross resistance between related and unrelated drugs (van Meerloo *et al.*, 2011; Fotakis and Timbrell, 2005).

Application: For the purpose of this study, this technique was used to evaluate the cytotoxic effects of methanol and butanol extracts of *L. fruticosus* on AsPC-1 and to determine the inhibitory concentration (IC_{50}). A cell density of 5000 cells in 90 µl of media per well was seeded in 96-well plates following a cell count using haemocytometer. After 24 hours AsPC-1 cells were treated with a range of concentrations of the butanol (25 µg/ml, 50 µg/ml, 75 µg/ml) and methanol (50 µg/ml, 60

µg/ml and 70 µg/ml) extracts which were prepared from the stocks by diluting with DMEM. Experiment controls were DMEM only (Blank), DMSO treated cells, untreated cells in DMEM (negative control) and camptothecin (3 µM) and Taxol (1 µM) treated cells (positive control). Cells were treated for 48 hours. 10 µl of MTT solution was added into each well and the plate was incubated for 4 hours at 37^0C in the dark. DMSO (90 µl) was then added to dissolve formed formazan crystals. An end point measurement of viability was taken at an absorbance of 570 nm with a Multiscan and assessed using the following formula to calculate the cytotoxicity of each concentration:

$$\%\ Cell\ viabilty = \frac{OD\ (treated) - OD\ (blank)}{OD\ (untreated) -\ OD\ (blank)} \times 100$$

$$\%Cell\ death = 100 - \%\ Cell\ viability$$

2.2.4 xCELLigence

xCELLigence is an assay used in the quantitative monitoring of cell proliferation, viability and cytotoxicity in real time and in vitro cell culture. This technique allows label free assessment of cell viability, proliferation and cytotoxicity. It provides a physiological state of the cells using impedance as readout. The technique also allows for the calculation of time dependant physiological inhibitory concentration IC_{50} (Urcan *et al.*, 2009). The impedance measurement is displayed as cell index (CI) value. It provides time-dependant cellular response profiles (TCRP). xCELLigence also allows the measurement of optimal cytotoxic effect when a compound is screened for cytotoxic capabilities (Ke *et al.*, 2011).

Application: This technique was used to determine the IC50 of methanol and butanol extracts of *L. fruticosus* on AsPC-1 and MRC-5 cell line. It was also used for real-time and continuous monitoring of cell growth and cell death following treatment with the extracts. To condition the plate, 100 µl of DMEM was added into each well and the plate was inserted into the xCELLigence machine for approximately 30 minutes. Following that, 10000 cells per well were seeded by adding 100 µl of cell suspension in each well in E-16 plates which have gold cell-sensor electrodes at the bottom for 24 hours. Various concentrations of butanol (25 µg/ml, 50 µg/ml, 75 µg/ml) and methanol (50 µg/ml, 60 µg/ml and 70 µg/ml) extracts, were added 24 hours later onto the seeded cells to evaluate cellular response following treatment. The experiment was run in an xCELLigence machine at 37^0C, 5% CO_2 and 95% humidity for a period of 48 hours. Camptothecin (3µM) and Taxol (1µM) were used as a positive control and untreated cells in media as negative control. The program was set to run for 250 sweeps at 15 minute intervals.

2.2.5 Flow cytometry

Early features of apoptosis include a change in plasma membranes. In cells undergoing apoptosis the phosphotidylserine (PS), a membrane phospholipid, is translocated from the inner layer to the outer layer of the plasma membrane. This exposes PS to extracellular activity (Alabsi *et al.*, 2013)

Flow cytometry can be utilised for quantitative analysis of the number of viable, apoptotic and necrotic cells. This is achieved by analysing the rate of uptake and retention of certain dyes by cells. Apoptotic cells reject all dyes generally used for cell viability assays such as Propidium iodide (PI). When the PI enters receptive cells it intercalates with DNA material and gives off a red fluorescence (Vernes *et al.*, 2000). Annexin binding assay allows the detection of early apoptosis. Annexin V binds to phosphotidylserine displayed in the surface of apoptotic cells. The combined use of PI and Annexin V distinguishes between viable, apoptotic and necrotic cells (Vernes *et al.*, 2000; Alabsi *et al.*, 2012).

Application: Flow cytometry was adopted to identify the effect of methanol and butanol extracts of *L. fruticosus* on the cell cycle of AsPC-1 and MRC-5 cells and to identify the type of cell death these extracts induced on the cells. Cultured AsPC-1and MRC-5cells were seeded and treated with optimal (IC50) concentrations of the butanol (50 µg/ml) and methanol (60 µg/ml) extracts for 48 hours. Camptothecin (3 µM) treated, Taxol (1 µM) treated cells, untreated cells as well as MRC-5 cells served as controls.

Cell cycle analysis

Basically, treated and untreated cells were harvested by trypsinization and washed with PBS. The cells were then fixed in 30 µl of cold PBS and 70 µl of cold 100% ethanol. The suspension was kept at -20ºC until needed for analysis. For analysis the cells were stained with 200 µl of FxCycle™ PI/RNAse staining solution (Life Technologies) and analysed using BD Accuri™ Flow cytometer.

Apoptosis detection

Briefly, treated and untreated cells were harvested by trypsinization and washed twice with PBS. Following that 200 µl of Annexin V binding buffer (annexin V-FITC Apoptosis Detection Kit, Biolegend®) was added. A 100 µl of the cell suspension was transferred to an Eppendorf and mixed with 5 µl of FITC Annexin V and 10 µl of Propidium Iodide solution. The mixture was then kept at room temperature, in the dark for 15 minutes. Annexin V Binding Buffer (100 µl) was added into each tube and the cells were analysed using BD Accuri™ Flow cytometer.

2.2.6 Western blotting

Western blotting also known as immunoblotting or protein blotting is a technique widely used in cell and molecular biology. It is used to detect the presence of a specific protein or multiple proteins within a sample. The technique is based on three elements i.e. (1) separation of proteins by size using SDS-PAGE chromatography, (2) the efficient transfer of separated proteins to a solid membrane support and (3) specific detection of the target protein. Western blotting is useful for comparing of target proteins or when assessing how a particular protein responds to certain stimuli (Moore, 2009).

Advantages in the use of this method include: (a) wet membranes are flexible and easy to handle, (b) transferred proteins are readily available to ligands, (c) membranes with transferred protein bands can be stored and used for multiple analyses (Moore, 2009; Kurein and Scofield, 2005).

Application: The Western blotting technique was employed for the detection of the expression of apoptotic and regulatory gene (p53) at protein level. AsPC-1 cells were cultured and treated with the optimal concentrations of butanol (50 µg/ml) and methanol (60 µg/ml) extracts of *L. fruticosus* for 48 hours.

Protein extraction

Following that, protein was harvested from the cells using RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 10% sodium dodecylsulfate (SDS), and 3 µl/ml aprotinin and 5 µg/ml leupeptin in PBS, pH 7.4). Briefly, the cells were washed twice with PBS (1X) and 500µl RIPA buffer was introduced. The cell flasks were kept on ice for 5 minutes with gentle swirling. The lysate was then scraped off the flask surface and centrifuged at 13400 rpm for 15 minutes. The obtained protein was then quantified using Pierce® BCA Protein Assay Kit and used in an SDS-PAGE chromatography.

SDS-PAGE chromatography

A SDS-PAGE gel with Stacking gel (4%) and resolving gel (12%) was prepared. The protein was denatured at 95ºC for 5 minutes. Subsequently, 30 µg of the denatured protein was loaded into each well and the gel was run at 100 Volts for 1 hour.

Blotting

Thereafter, the protein was transferred onto a nitrocellulose membrane overnight by means of a wet electro-transfer method at 30 Volts. Transfer was confirmed by staining the membrane with Ponceau Stain [(0.1 %(w/v) Ponceau S in 5 %(v/v) acetic acid)] for 45 minutes. Following this, the membrane was incubated in 5% blocking milk for an hour which was followed by an overnight incubation with primary antibody (1:1000 dilutions) at 4ºC. Following 1 hour incubation with a HRP-Linked secondary antibody the membrane was exposed to Pierce® ECL Western Blotting

Chemiluminescence Substrate to strengthen the signal produced and the blots were imaged using a ChemiDoc™.

2.2.8 LC/MS

Due to is ability to identify metabolites which have not been derivatized, HPLC/MS or LC/MS has gained popularity over the past decade or so (Schultz et al, 2013). According to Korfmacher (2005), The LC/MS system is composed of an autosampler, an HPLC system, an ionization source and a mass spectrometer. Furthermore the technique has two major approaches known as targeted and untargeted analysis (Schultz et al, 2013). These two approaches differ, with the untargeted analysis being of interest to this particular project. This is largely due to the fact that this approach enables analysis and identification of known and unknown metabolites in a sample. This serves beneficial, especially in medical research (drug discovery in cancer research to be more specific), by providing a source of novel metabolites that can serve as therapeutic agents (Schultz et al, 2013). This interface between LC and MS is responsible for transfering analytes from a liquid phase to a gas phase. This is followed by analysis by a mass spectrometer (lee and Kerns, 1999). The coupling of the resolving power of LC with the identifying ability of MS has benefited research immensely.

Application: The methanol and butanol extracts were sent to University of Fort Hare for LC/MS analysis. The technique was done using Triple TOF 5600. Obtained results were used on mass bank (MS library Mass Bank) to determine the unknown compounds.

CHAPTER 3: RESULTS

The continuous use of *L. fruticosus* as a traditional remedy for certain ailments such as those mentioned in chapter 1, encourages the investigation of its effects as well as the chemical make-up of the plant. In this study, a few experimental tests were conducted to screen *L. fruticosus* for anticancer activity. The investigation was based on the hypothesis that *L. fruticosus* may have anti-proliferative effects on the pancreatic cancer cell line AsPC-1 since it has been reported to have anti-tumour effects and certain metabolites that cause an immune reaction. The conducted experiments were MTT Assay, Flow Cytometry, xCELLigence, Western Blotting and LC/MS.

3.1 Cytotoxicity Assays

3.1.1 MTT assay

MTT Assay was used to determine the cytotoxic activity and IC_{50} (Half Maximal inhibitory concentration) of *L. fruticosus* on pancreatic cancer cell line, AsPC-1. The cell viability of AsPC-1 post treatment with a range of concentrations of 25-75 µg/ml butanol and 50-70 µg/ml methanol extracts was measured and the results recorded in figure 3.1 were obtained. Cells that were untreated were used as a negative control considered as 100% viability. The methanol and butanol plant extracts of *L. fruticosus*, in this study, showed antiproliferative effect on AsPC-1 cells in a concentration-dependant manner . These extracts had a half maximal inhibitory concentration (IC_{50}) of 50 µg/ml with a 39% cell viability (butanol) and 60 µg/ml with a 33% cell viability (methanol) following 48hour treatment of the AsPC-1 cells (figure 3.1) Moreover, DMSO (0.06%) treated cells showed a cell viability of 93% (AsPC-1). The positive controls used gave expected results of 44% and 34% cell viability for camptothecin and taxol respectively on the AsPC-1 pancreatic cancer cell line.

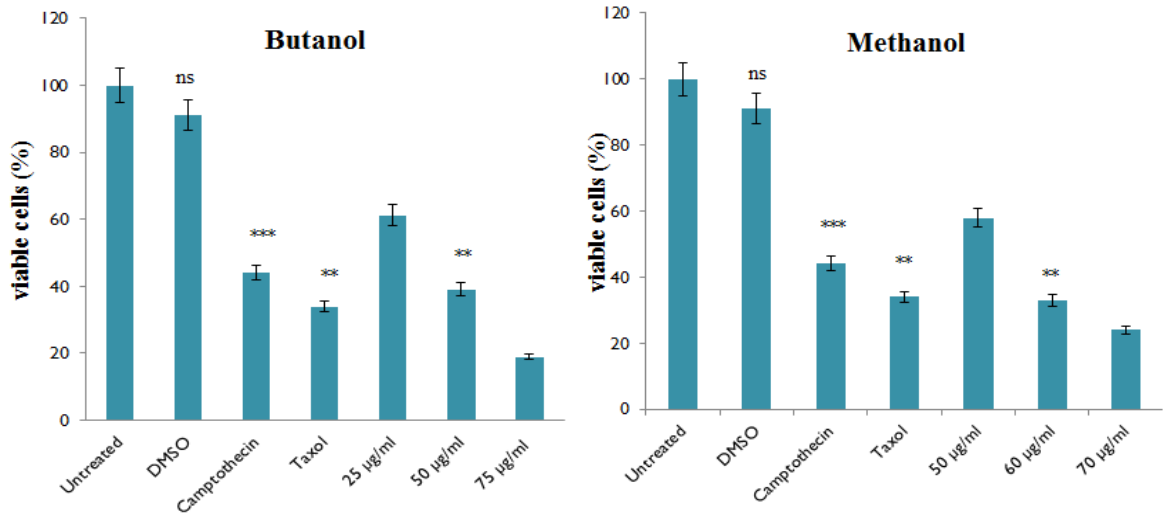


FIGURE 3.1: Cell viability Assay of AsPC-1cell line following treatment with various concentrations of Butanol extract and Methanol extract for a period of 48 hours. DMSO (0.06%), Taxol (1 µg/ml) and Camptothecin (1.5µM) were included as controls. Data was expressed as the mean ± SD. * indicates p≤0,05; ** indicates p≤0,01; *** indicates p≤0,001 and ns indicates p≥0,05.

3.1.2 xCELLigence

To monitor real time cell proliferation and adhesion and to further confirm the obtained half maximal inhibitory concentrations of butanol and methanol extracts, the xCELLigence system was used. The MRC-5 lung fibroblast was used as a positive control to see if there was induction of apoptosis in response to stimuli by *L. fruticosus*. A standard curve was used to determine how much cells to seed in each well. 10000 cells per well were seeded for both AsPC-1 and MRC-5 cell lines. Cells were allowed to grow for 24 hours before being treated with butanol and methanol extracts of *L. fruticosus*. A gradual increase in cell index of AsPC-1 cells is noticed over 24 hours (figure 3.2 A). As expected, in figure 3.2 B the cell index of the untreated MRC-5 increases at a slower rate compared to that of the AsPC-1. However, Treatment of the AsPC-1 and MRC-5 cells with camptothecin and taxol gave cell indices that were lower than that of the untreated cells at 20 hours of treatment exposure. Similarly, figure 3.2 also shows that the growth rate of AsPC-1 and MRC-5 cells treated with the butanol (50 µg/ml) and methanol (60 µg/ml) extracts remains lower than that of the untreated cells for about 16 hours of exposure to treatments.

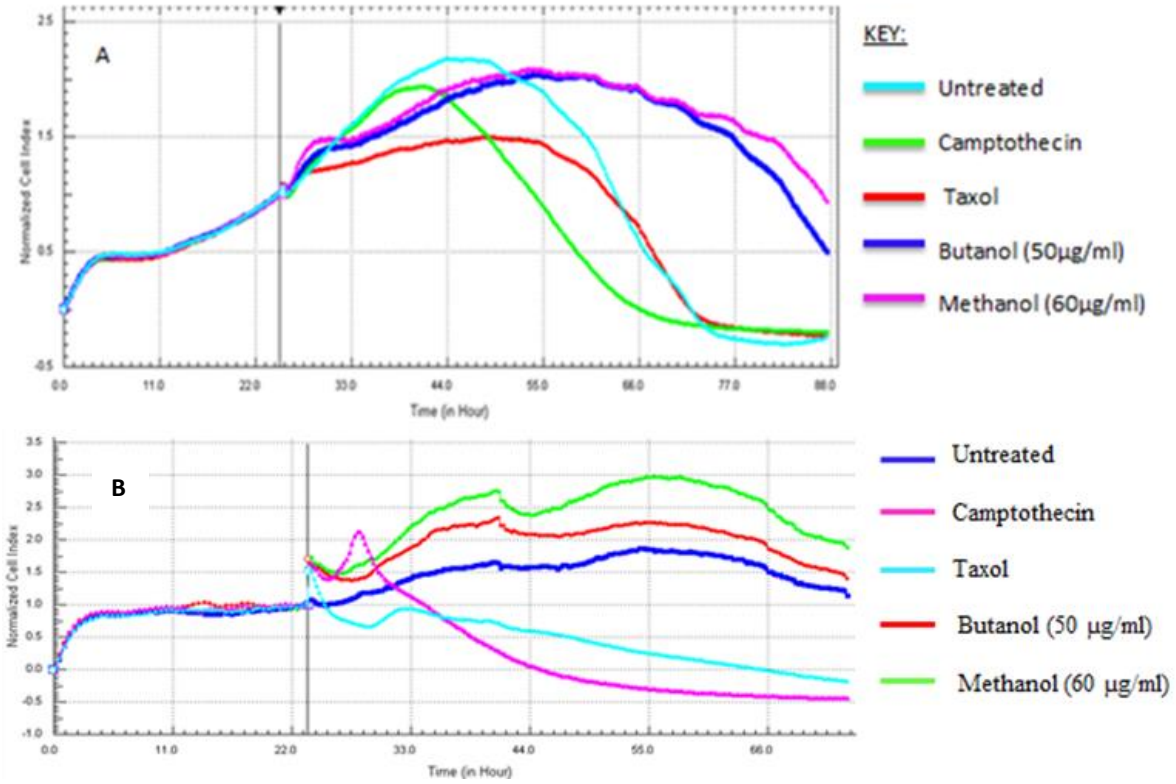


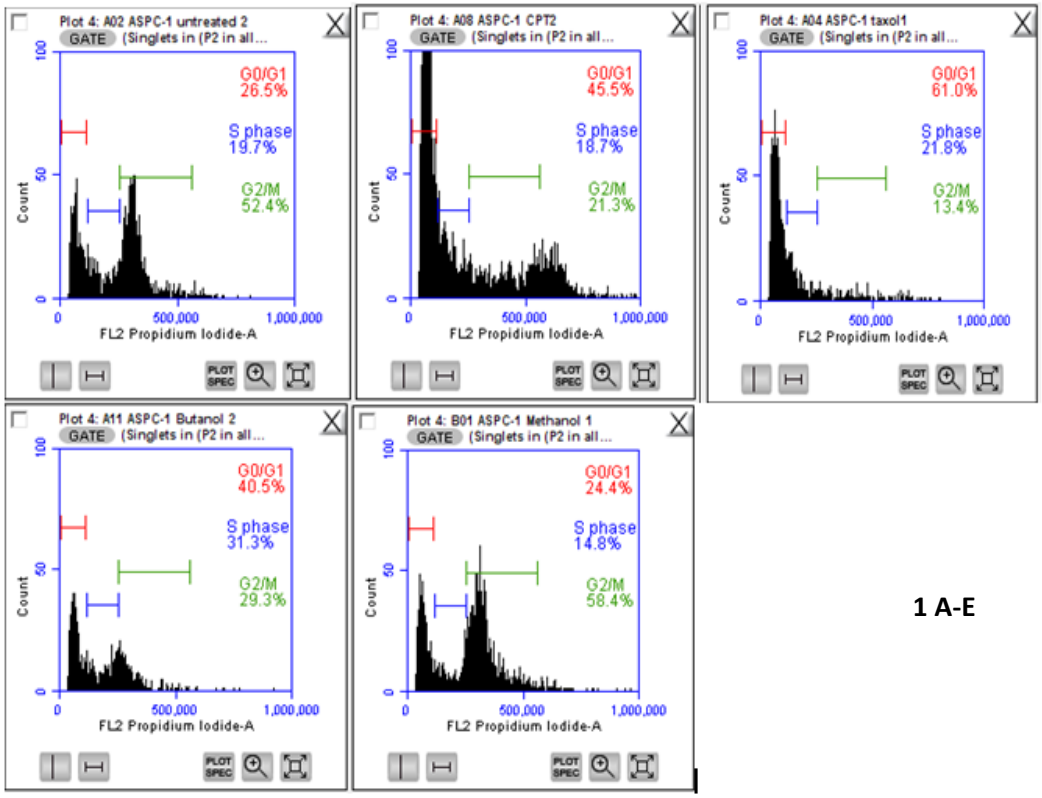
FIGURE 3.2: Plots of Normalised cell index vs Time from monitoring of real-time cell proliferation and adhesion of *L. fruticosus* on **(A)** AsPC-1 pancreatic cancer cell line and (**B)** MRC-5 lung fibroblast following 48hours of exposure to treatments.

In addition, figure 3.2 displays that the cell index of the butanol and methanol extract treated AsPC-1 and MRC-5 have a pattern similar to that of untreated cells. The butanol and methanol treated AsPC-1 cells begin to respond to the inhibitory effects of *L. fruticosus* at around 48 hours of exposure, whereas the MRC-5 cells respond at around 55 hours of exposure.

3.2 Flow Cytometry

3.2.1 Cell cycle analysis

The cell cycle progression was analysed to see if the methanol and butanol extracts of *L. fruticosus* would cause cell cycle arrest or cell progression through the stages of the cell cycle. Post exposure to treatments for 48 hours the cells were prepared as mentioned in chapter 2 (section 2.2.5.2) and analysed using flow cytometry. Figure 3.4 (1) indicates 47.8% AsPC-1 cells populated in the G2/M phase in untreated cells. Similarly 50.7% AsPC-1 cells treated with methanol populated in G2/M phase. However, only 24.2% AsPC-1 cells treated with the butanol extract resonated in the G2/M phase which was similar to the low percentages of taxol (9.8%) and camptothecin (11.5%) treated cells foung in the G2/M phase. Methanol extract treated cells had a high percent of cells in the S-phase compared to butanol extract treated cells (figure 3.4 (2)). The data was collected in triplicates (n = 3) and standard deviation was calculated from the mean (mean ± SD), the changes in cell cycle distribution were plotted on a bar graph figure 3.4 (2).



1 A-E

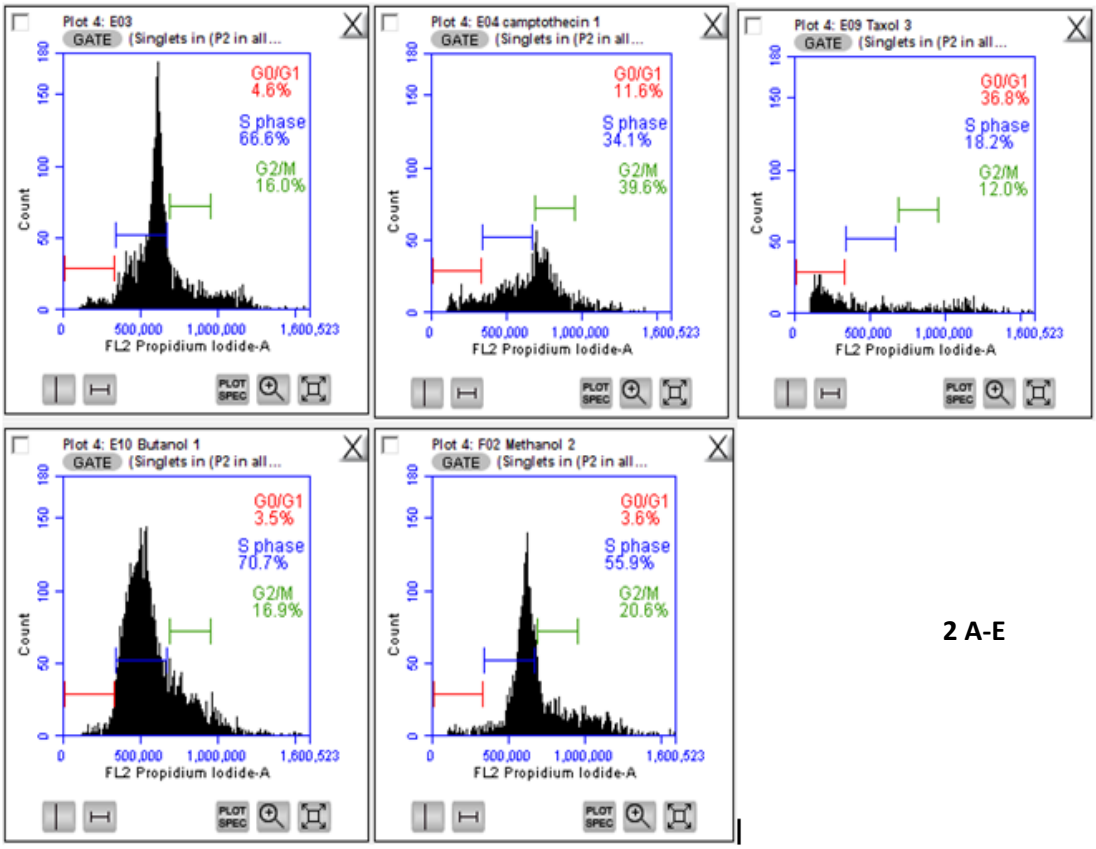


Figure 3.4: Histograms of AsPC-1(1A-E) and MRC-5 (2A-E) cells indicating cell progression and inhibition from G0/G1 to the S and G2/M phase of cell cycle. (A) untreated, (B) cells treated with Campthothecin, (C) cells treated with Taxol, (D) cells treated with Butanol extract and (E) cells treated with Methanol extract for 48 hours and stained with Annexin V and PI.

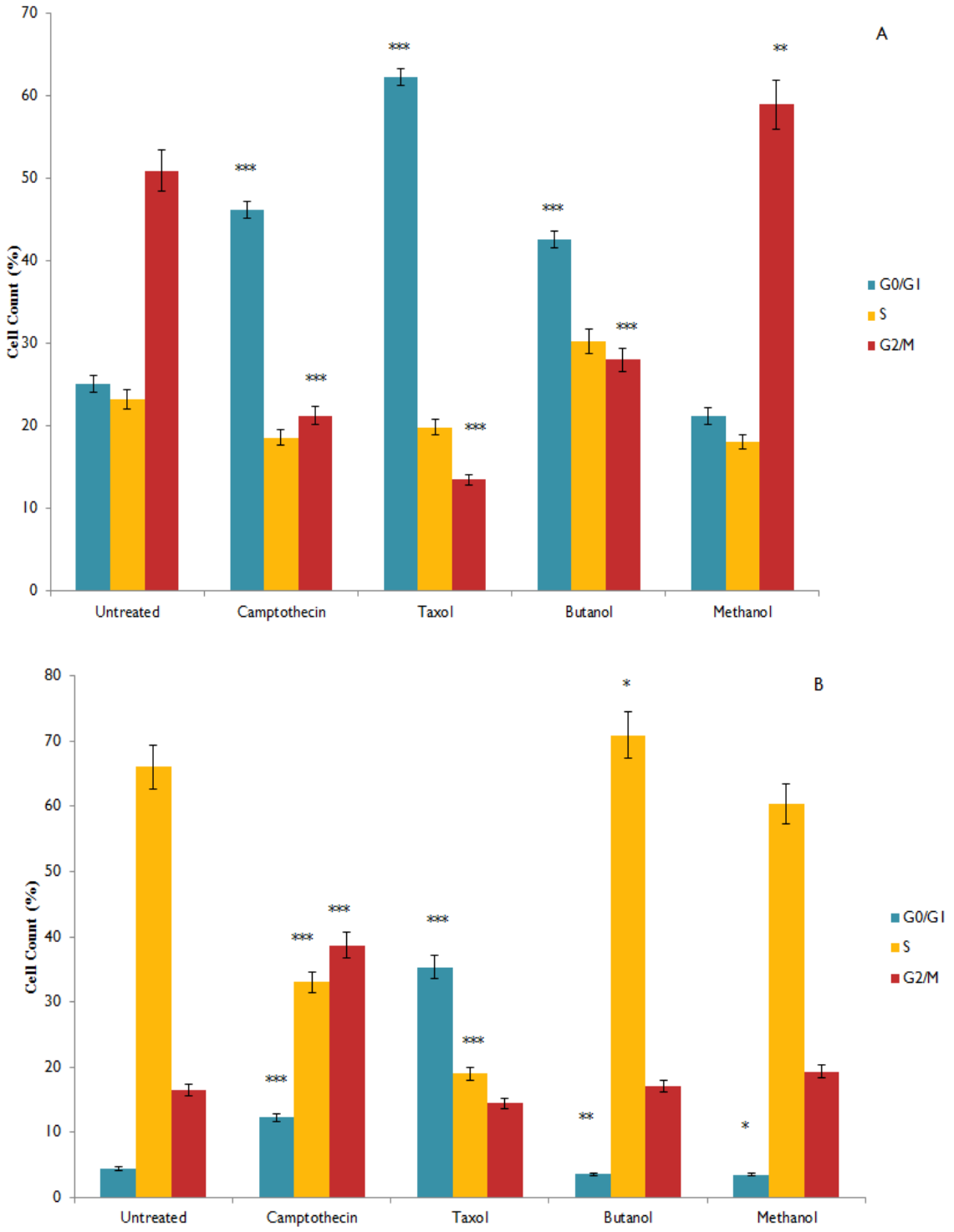
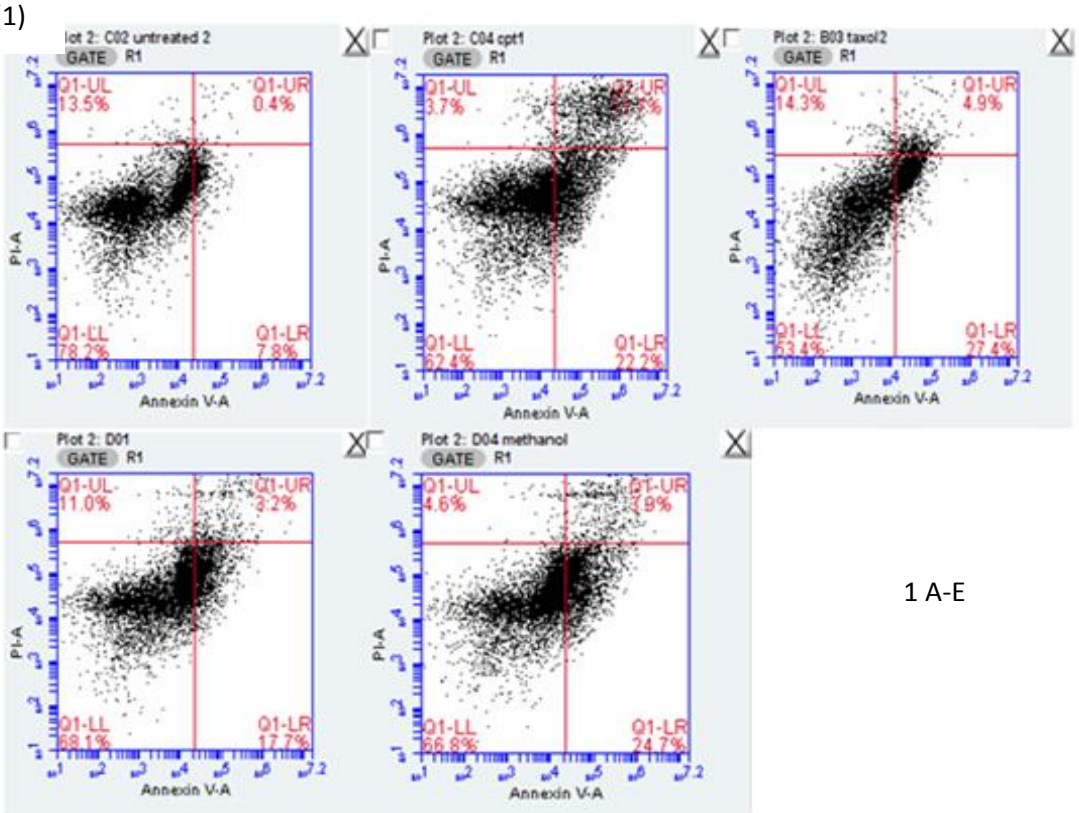


Figure 3.5: Statistical representation of cell population in stages of the cell cycle of treated (A) AsPC-1 and (B) MRC-5 cells,* indicates $p>0.05$, ns indicates $p\geq0.05$.

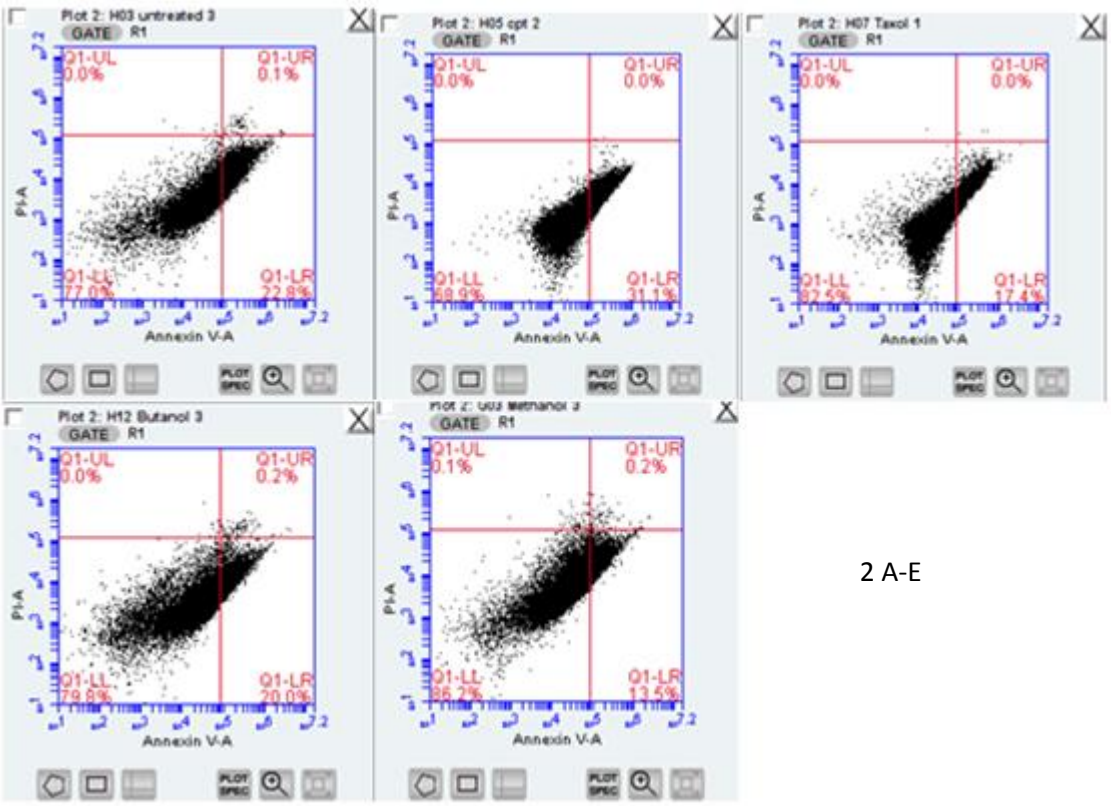
3.2.2 Apoptosis detection

AsPC-1 and MRC-5 cells were analysed for occurrence of apoptotic activity. Cells were treated with 1.5µM of camptothecin, 1.0µg/ml of Taxol, 50µg/ml of the Butanol extract and 60µg/ml of the methanol extract. During the analysis, cells were stained with PI and Annexin V as described in chapter 2 (section 2.2.5) and the changes in the amount of each dye taken up by the cells was measured. Apoptosis observed was at 20.9% in AsPC-1 cells treated with the butanol extract and 28.6% in AsPC-1 cells treated with the methanol extract as compared to that of untreated cells which is 8.2% (figure3.6). The data was collected in triplicates for reliability of results (n = 3) and standard deviation was calculated from the mean (mean±SD), the various levels of apoptosis were plotted on a bar graph Figure 3.3 (2).

(1)



1 A-E



2 A-E

Figure 3.6:Representation of analysis of apoptosis occurance in ASPC-1 (1A-E) and MRC-5 (2A-E) cells. (1) Scatter plots of Apoptosis in AsPC-1, (A) untreated, (B) cells treated with Campthothecin, (C) cells treated with Taxol, (D) cells treated with Butanol extract and (E) cells treated with Methanol extract, with viable cells (Q1-LL), cell death (Q1-UL), early (Q1-LR) and late (Q1-UR) stages of apoptosis.

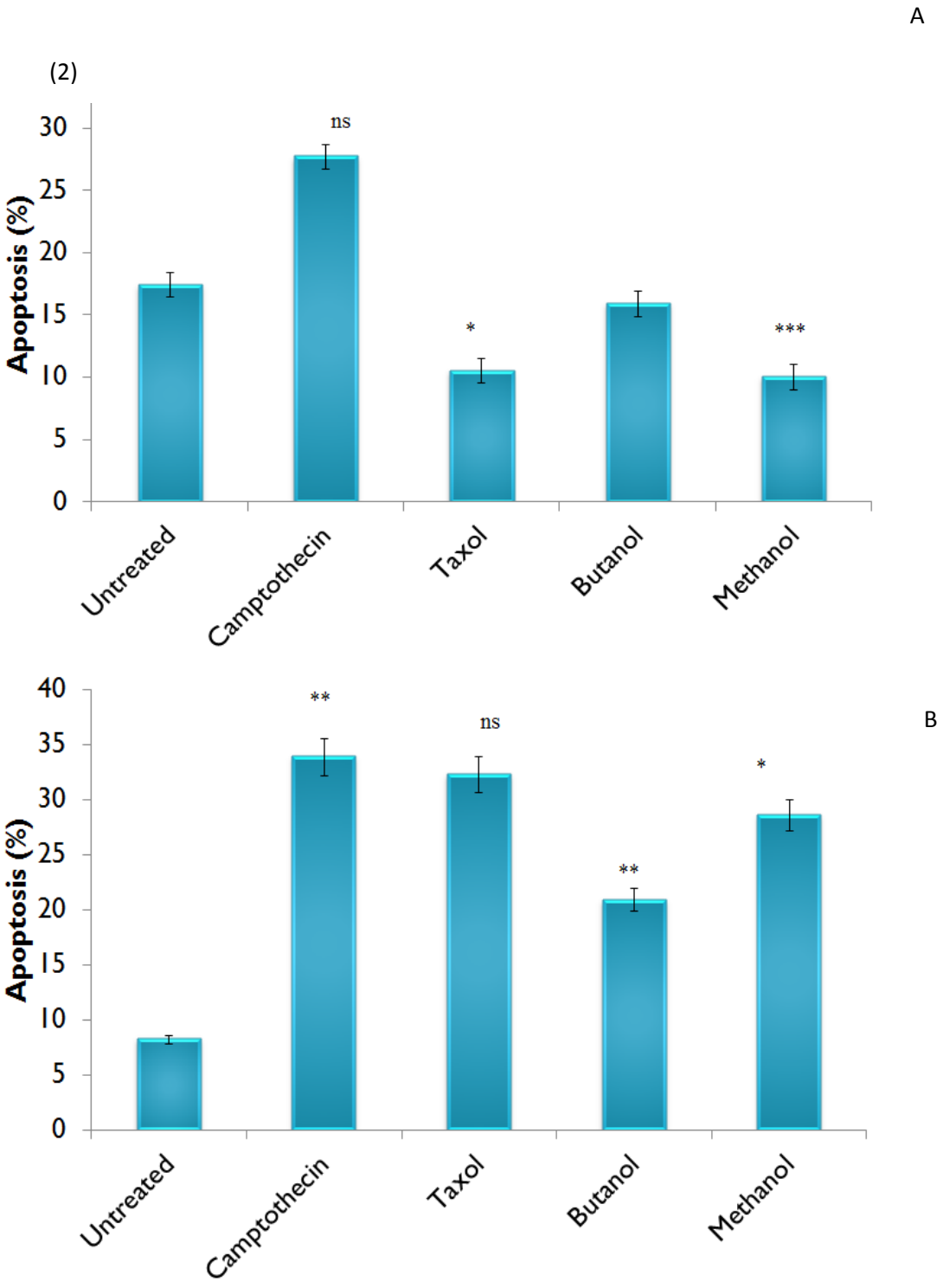


Figure 3.7: Statistical representation of apoptotic (A) AsPC-1 and (B) MRC-5 cells when treated with methanol and butanol extracts of lobostemon fruticosus. The data was collected in triplicates for reliability of results (n = 3) and standard deviation was calculated from the mean (mean±SD), ** indicates $p \leq 0.01$;* indicares $p \leq 0.05$; ns indicates not significant.

3.3 Western Blot Analysis of Protein Expression

Having seen that the methanol and butanol extracts of *Lobostemon fruticosus* cause a population of some cells in the G0/G1 phase as well as the G2/M phase and a certain degree of apoptosis on AsPC-1 cells, analysis at protein level was necessary. The cells were treated for 48 hours with methanol (60µg/ml) and butanol (50µg/ml) extracts, camptothecin (1.5µM) and taxol (1µg/ml). Protein expression was analysed using western blotting as described in chapter 2 (section 2.2.6) and the results seen in figure 10 were obtained. The expression of β-actin and p53 was measured in AsPC-1 cells. The expression of p53 in untreated AsPc-1 cells was at 13%. A significant increase in p53 expression was observed in camptothecin and taxol treated cells with 28% and 45% respectively. Furthermore, methanol extract treated cells induced a higher expression of p53 which was at 21% as compared to that of butanol extract treated cells which was 15%.

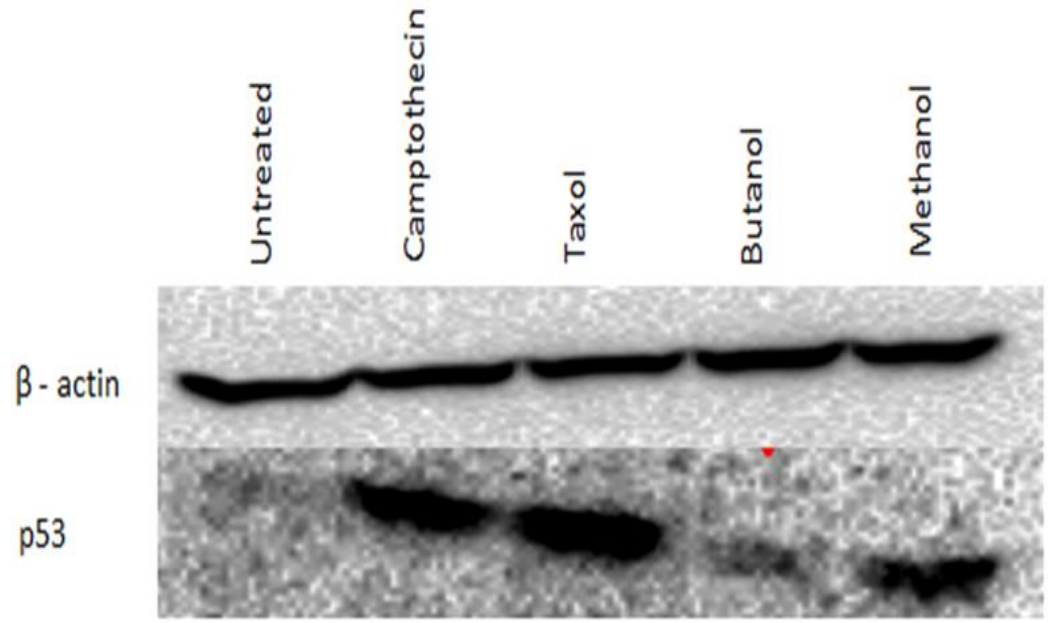


Figure 3.9: Protein expression in AsPC-1 where 30µg of β-actin and p53 extracted from AsPC-1 cells exposed to treatments for 48 hours and was run in SDS-PAGE and western blotting was measured. Protein from untreated cells was used as a control.

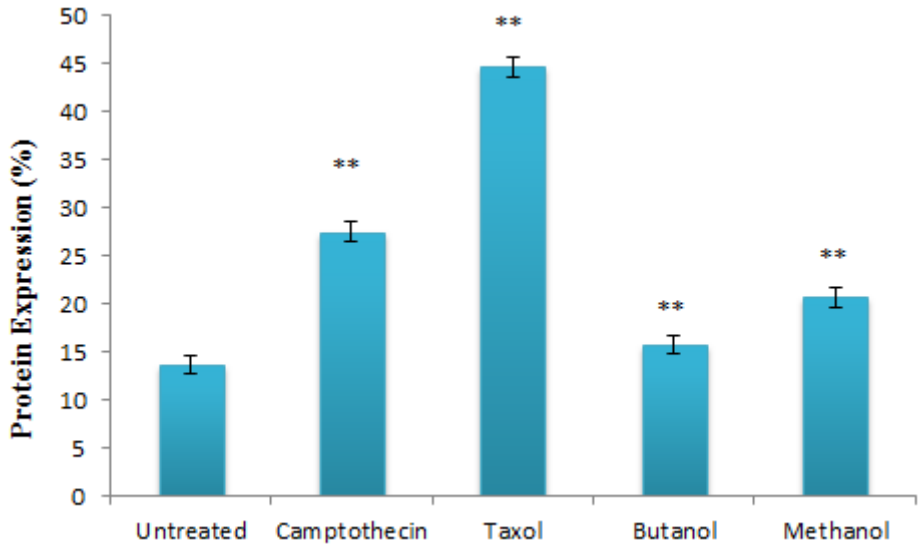


FIGURE 3.10: Protein levels on bands estimated by densitometric method on chemidoc. p53 expression vs treatments presentation of statistical analysis of western blot protein expression in AsPC-1 pancreatic cancer cell line. ** indicates $p \leq 0.01$

3.4 LC/MS

To identify active compounds in *L. fruticosus* HPLC coupled to MS was used to analyse the methanol and butanol extracts. These extracts of *L. fruticosus* were analysed using the quadropole-time-of-flight liquid chromatography- mass spectrometry tandem (Q-TOF LC-MS/MS). A number of molecules were detected from the analysed samples and were presented as peaks on a spectrum of intensity vs time (min) (figure 3.11 and 3.15). Each compound had a unique mass/charge ratio and retention time. Figure 3.12; 3.13 and 3.14 presents peaks of Justicidin B, Flavocomellin and Fisetin, respectively, based on their retention time and mass/charge ratio. Similarly figure 3.16; 3.17 and 3.18 presents the same for 4-Hydroxycoumarin; Hydroxylamino-4,6-dinitrotoluene and Sarpagine respectively.

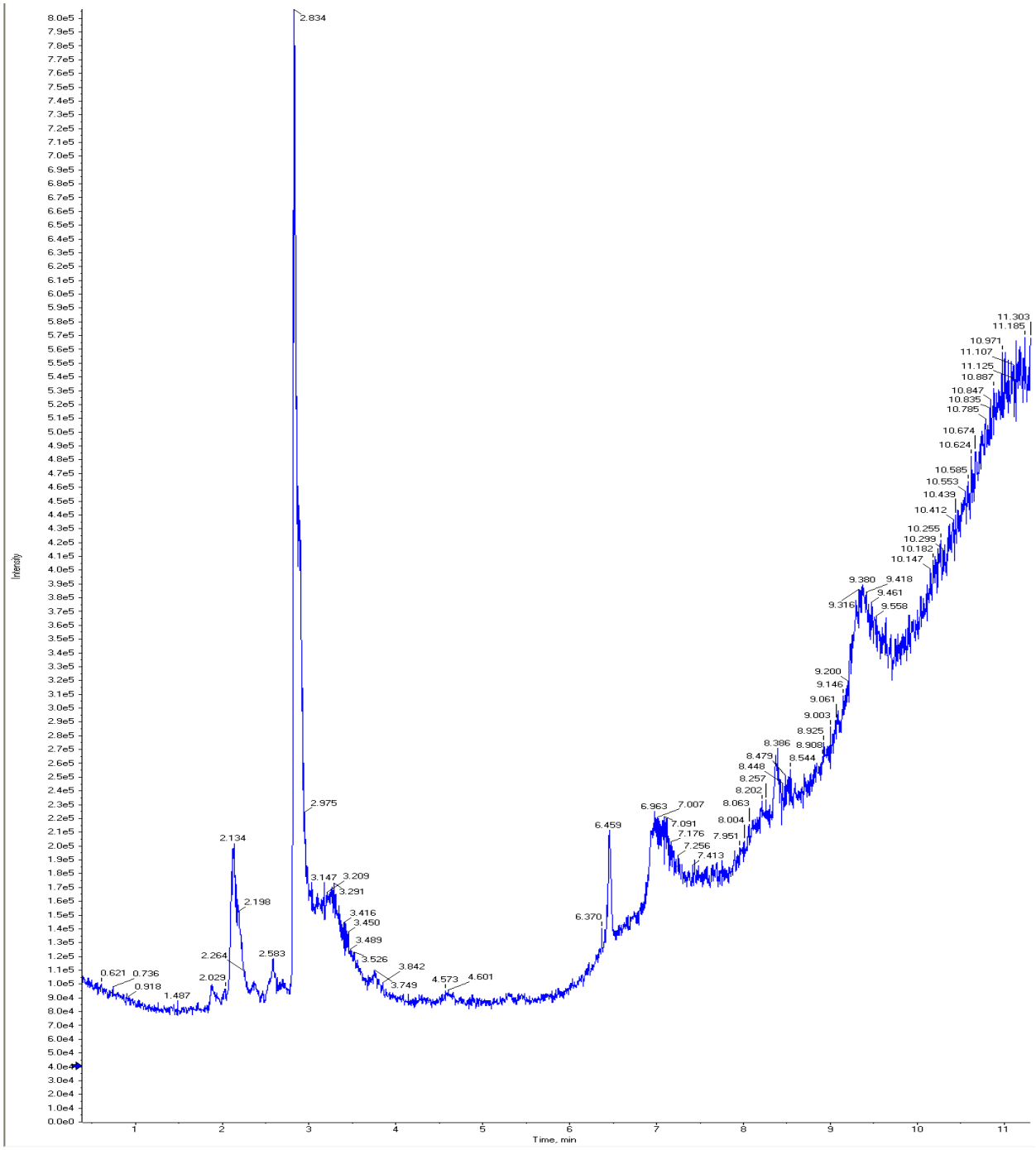


Figure 3.11: LC/MS chromatogram of Time vs Intensity of entire profile of butanol extract of *Lobostemon fruticosus*.

Justicididn B, one of the active compounds identified in the butanol extract of *L. fruticosus*. The compound peaked with a mass-charge- ratio of 365.1048 m/z (figure 3.12 B) and at a retention time of 2.11 min (Figure 3.12 A). figure 3.5 B also shows a peak at 366.1083 m/z which is an isotope of justicidin B. Justicidin Bhas a molecular mass of 364.348 Da and was first isolated from *Justicia* spp.

(Acanthaceae) and *Haplophyllum* spp. (Rutaceae) it has further been isolated from different *Phyllanthus* species (Euphorbiaceae) (Vasilev et al, 2006).

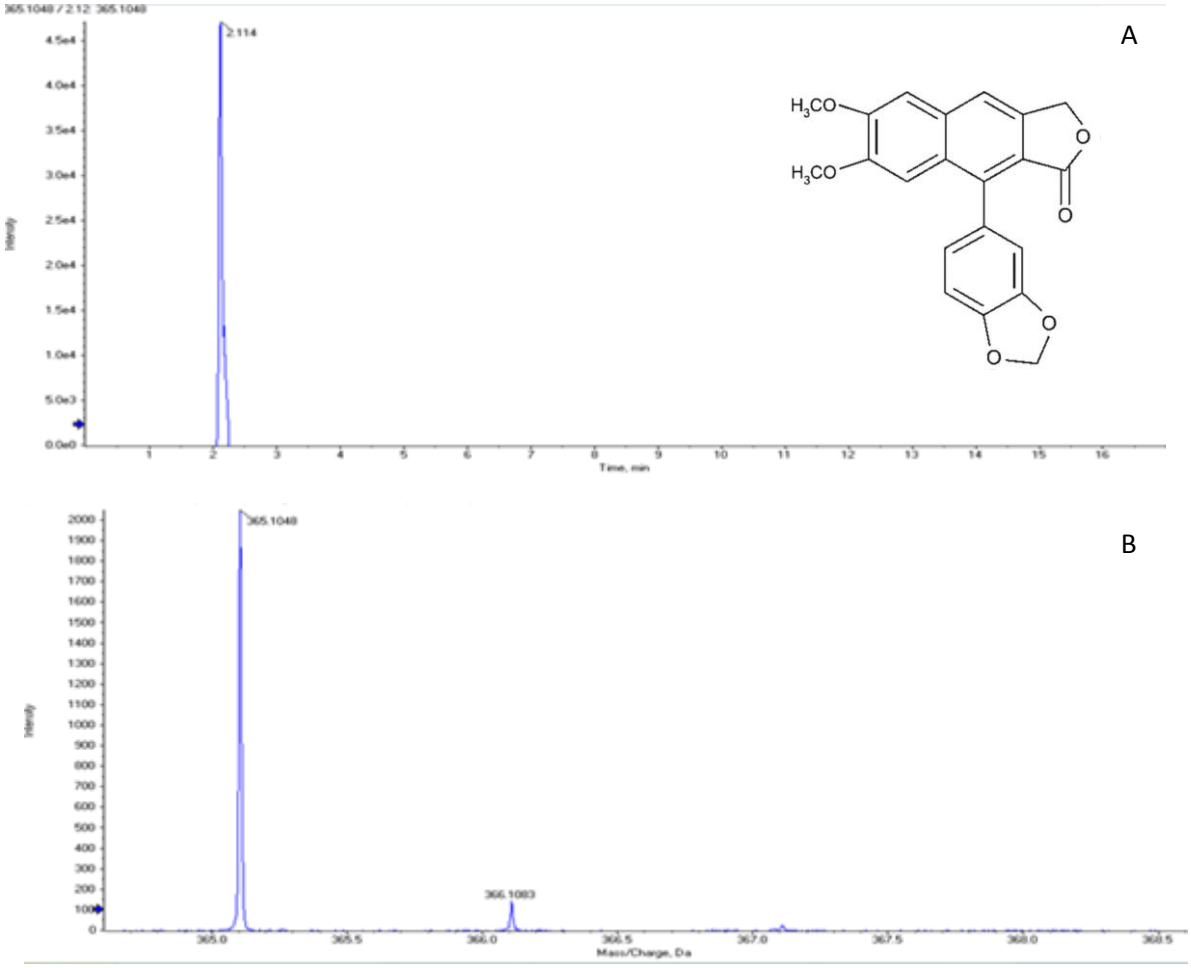


Figure 3.12: chromatogram and compound structure of justicidin B. (A) retention time of 2.11 at which the compound peaks (mins) and structure of the compound. (B) peaks of compound and isotopes based on mass-charge ratio (m/z).

Amongst other compounds, Flavocommelin was detected from the butanol extract. This compound is amongst a huge variety of flavonoids found in the plant kingdom (Oyama and Kondo, 2004). This compound peaks with a retention time of 2.83 min (figure 3.13 C) and a mass-charge-ratio of 631.3403 m/z (figure 3.13 D) and a molecular mass of 608,544 Da. An isotope to flavocommelin can also be seen on figure 3.13 D resonating with a mass-charge-ratio of 632.3446 m/z.

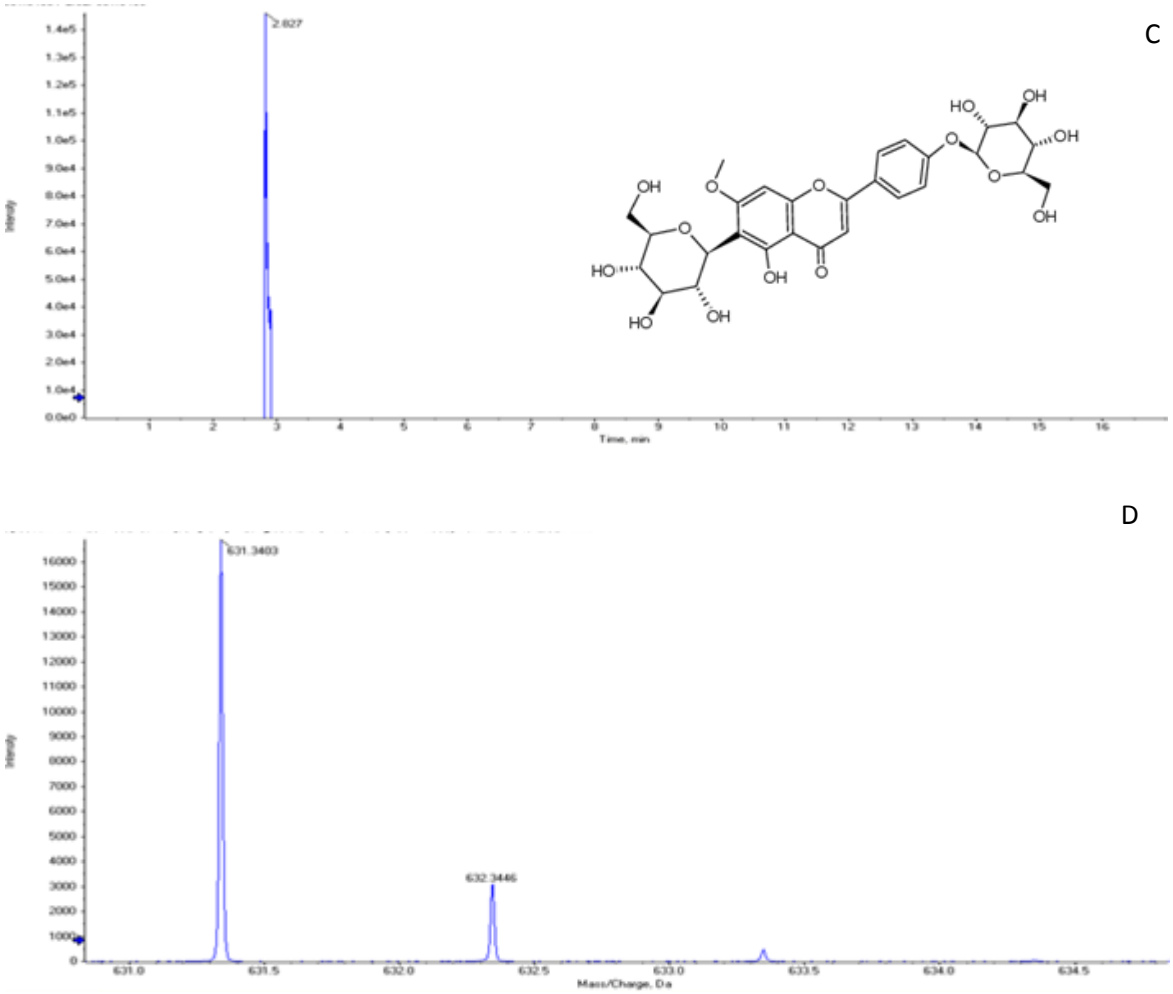


Figure 3.13: Chromatogram of flavocommelin. (C) Display of retention time at which the compound peaks and the compound structure, (D) Spectrum of mass/charge ratio of compound and its isotopes.

Fisetin, a bioactive flavonol molecule commonly found in fruits and vegetables. Fisetin had the retention time of 3.75 min and a mass/ charge ratio of 157.083 m/z. This secondary compound has a molecular mass of 286.236 Da and posseses antioxidant, anticancer and neuroprotective activities, amongst others (Khan et al, 2013).

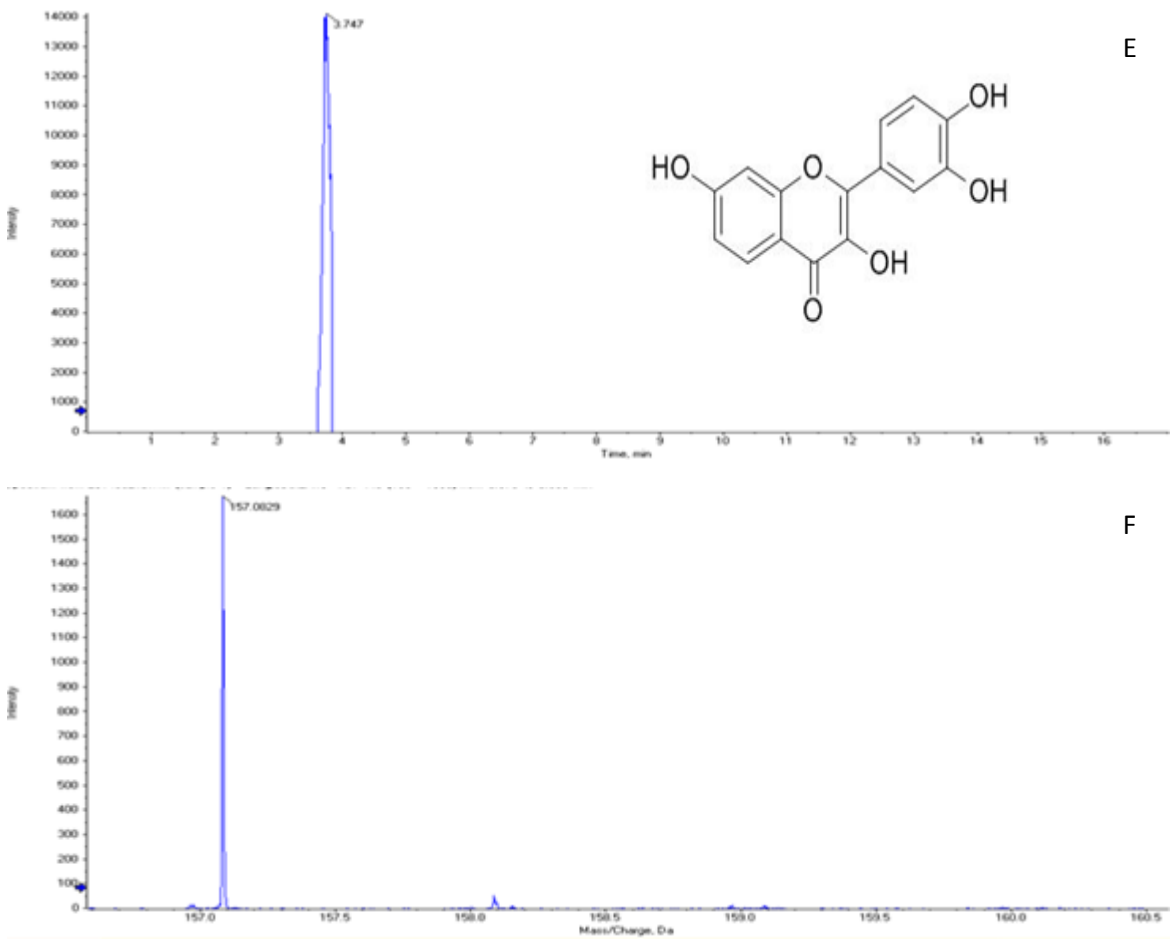


Figure 3.14: Chromatogram of fisetin. (E) Spectrum of retention time (3.75 mins) at which Fisetin peaks and the compound structure. (F) Peaks of Fisetin and its isotopes based mass/charge ratio.

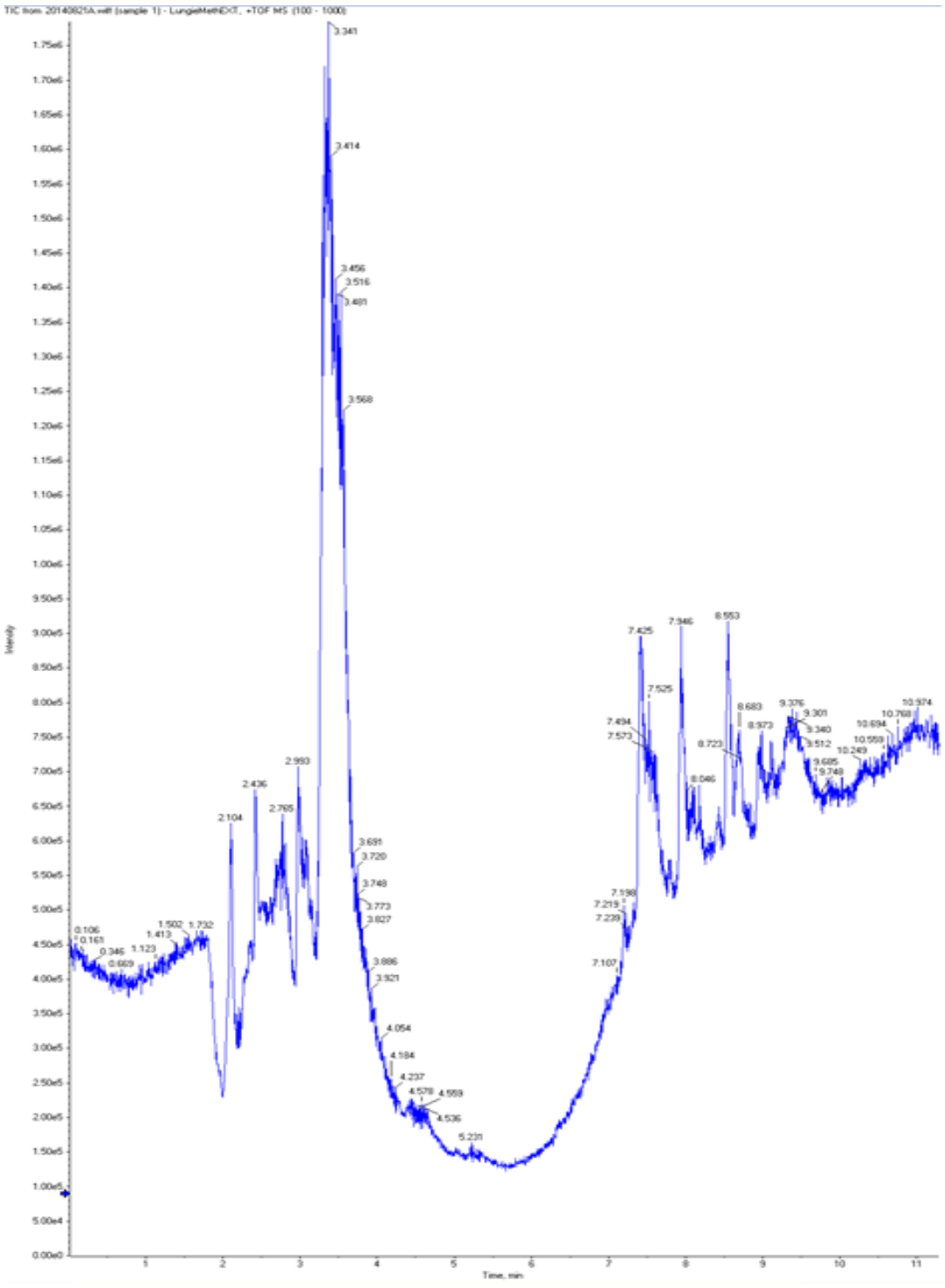


Figure 3.15: LC/MS chromatogram of Time vs Intensity of entire profile of methanol extract of *L. fruticosus*.

From the methanol extract a few compounds were also extracted (Figure 3.15). Amongst these compounds was 4-hydroxycoumarin. This is an antioxidant which is found occurring naturally in plant families such as Umbelliferae, Rutaceae, Leguminosae and more (Nishiyama et al, 2001). 4-

Hydroxycoumarin resonated at 10.27 mins and had a mass charge ratio of 376.0991 m/z with a molecular mass of 162.14 Da.

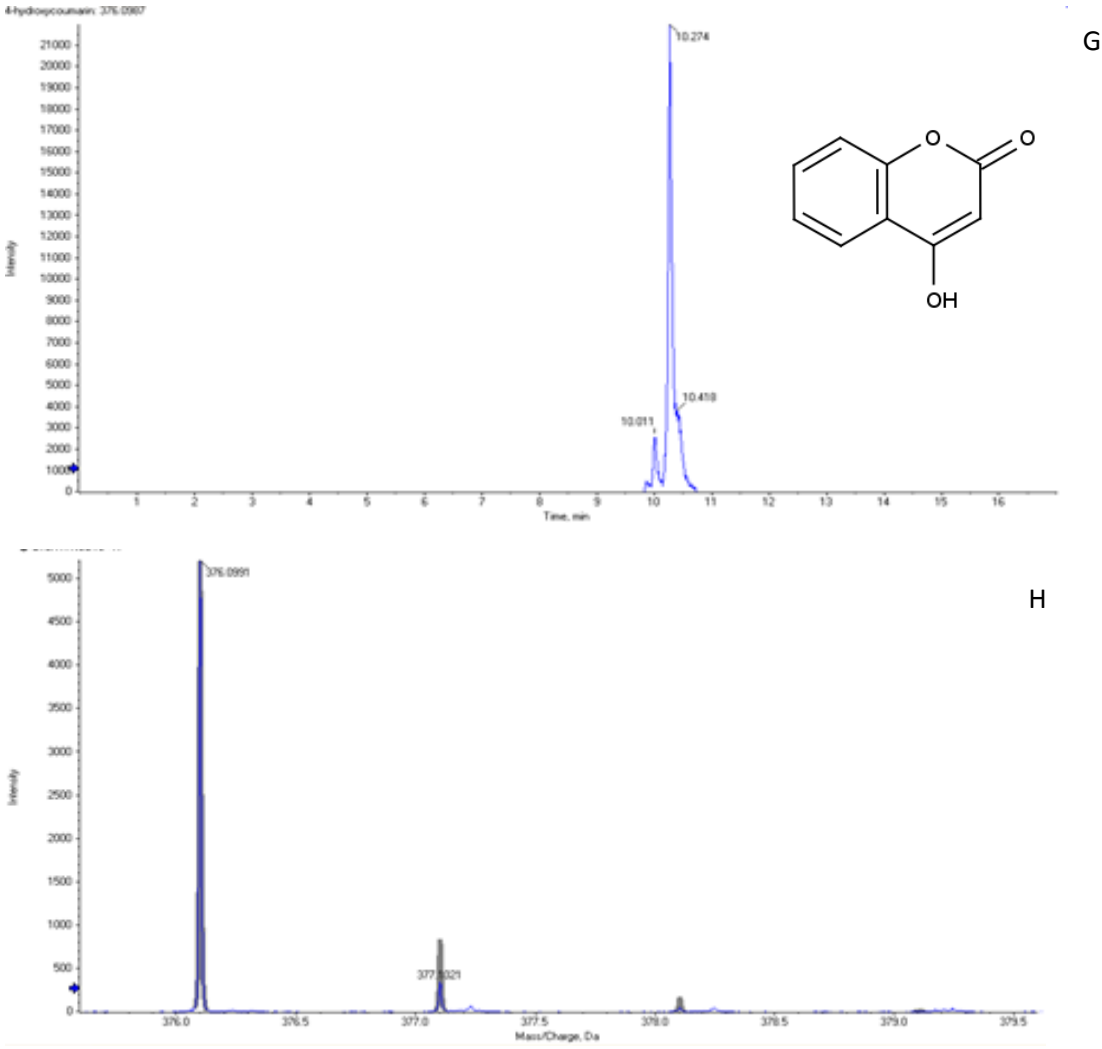


Figure 3.16: chromatogram of 4-Hydroxycoumarin. (G) Shows retention time and structure of eluted compound. (H) Shows the Mass/Charge Ratio of the eluted compound and isotope.

Furthermore, 2-Hydoxylamino-4,6-dinitrotoluene was eluted from the methanol extract (Figure 3.16). This compound belongs to the toluene family, has a molecular mass of 213.15 Da and eluted at 10.27 mins and has a mass charge ratio of 376.0991 m/z.

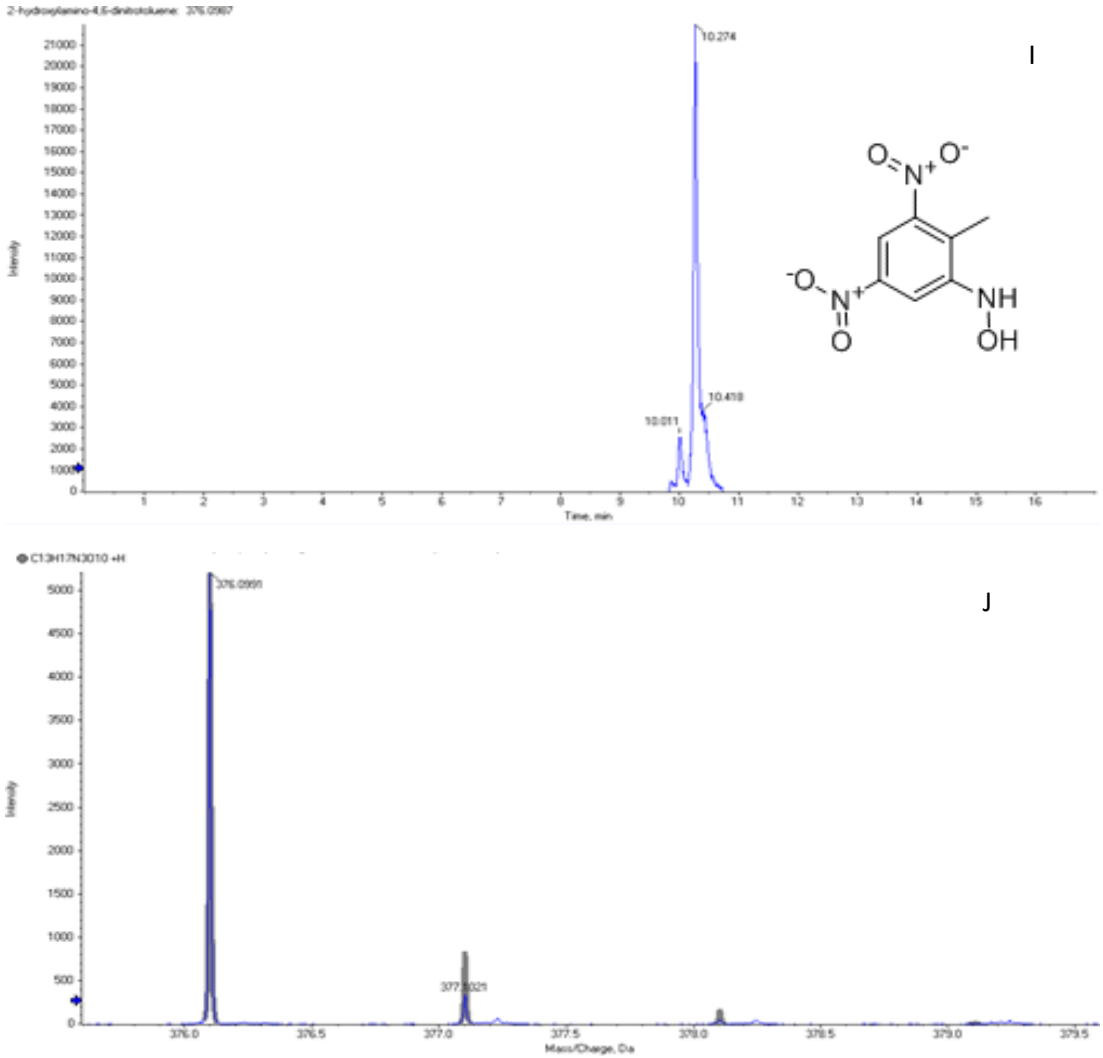


Figure 3.17: chromatogram of 2-Hydroxylamino-4, 6-dinitrotoluene. (I) Retention time of 10.27 min and structure of eluted compound. (J) Spectrum of eluted compounds based on Mass/charge Ratio.

Sarpagine was also eluted from the methanol extract of *L. fruticosus* (Figure 3.17). It is an alkaloid which has a retention time of 10.27 and a mass charge ratio of 209.0808 m/z. Furthermore, Sarpagine has a molecular mass of 310.39 Da.

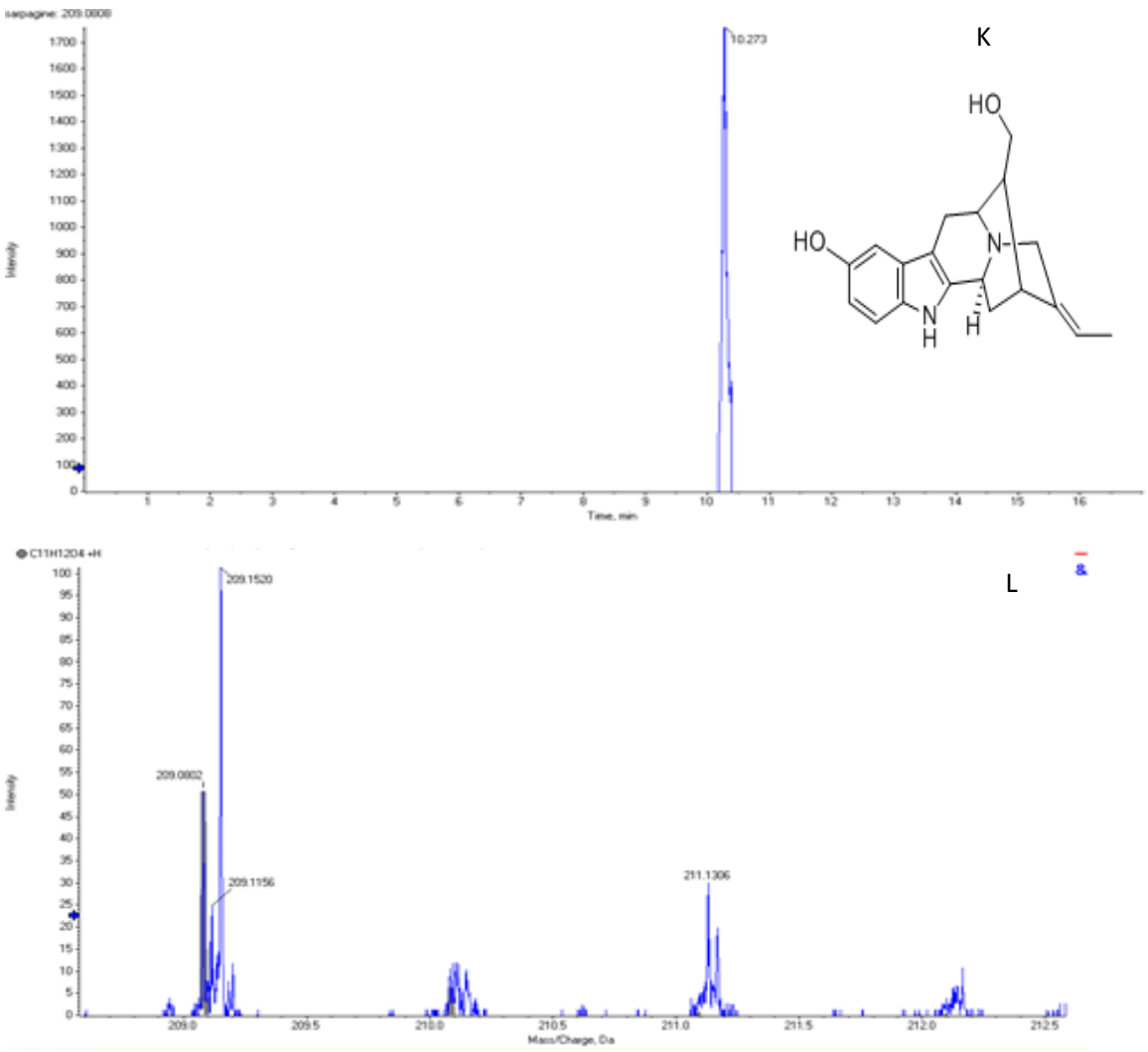


Figure 3.18: Chromatogram of Sarpagine. (K) Retention Time at which Sarpagine peaked and compound structure. (L) Peaks of compound and isotopes based on Mass/Charge Ratio.

CHAPTER FOUR: DISCUSSION AND CONCLUSION

4.1 DISCUSSION

Traditional plants have played a vital role in medicine for decades (Efferth et al, 2007). It is the presence of natural bioactive agents within natural plants and the common use of the plants medically has led to an increased interest in finding anti-proliferative agents in traditional plants (Efferth et al, 2007). As mentioned earlier, the economic state and certain cultural factors has resulted in 80% of the worlds' population relying on traditional plants for medicinal purposes. Furthermore, studies have shown that traditional plants which have been screened for anticancer/ anti-proliferative activity have been reported to be safer, cheaper and readily available compared to modern anticancer medication (Mthembu N, 2013).

This project was designed to screen *L. fruticosus* for antiproliferative activity towards a pancreatic cancer cell line AsPC-1. Furthermore, the project aimed to study the mode of cell death induced by *L. fruticosus* on AsPC-1. The first objective was to identify whether methanol and butanol extracts of *L. fruticosus* have cytotoxic effects on the pancreatic cancer cell line AsPC-1cells. This objective was fulfilled using the MTT Assay as well as the xCELLigence. The importance of the MTT assay was to elucidate the half maximal inhibitory concentration of methanol and butanol extracts of *L. fruticosus*, which is the concentration that induces 50% cell death on the AsPC-1 cell line. As reported in chapter 3, DMSO was used in the MTT assay as a control to eliminate false positive results. Furthermore, the false positive results were eliminated by evaporating the solvent during extraction and also diluting the compounds to a 0.0001% of solvent residue present in treatment. Cells treated with DMSO and untreated cells showed a cell viability that was high. The untreated cells were used as 100% viability. Taxol and Camptothecin were both used as positive controls because of their known effect on cancer cells and also for the mere fact that they are derivatives of plants so they will show and corroborate the antiproliferative function of our extracts. Although the exact mechanism of Taxol is unclear, it has been established as an anti-microtubule agent which inhibits microtubule depolymerisation leading to formation of stable microtubules. As mentioned earlier (Chapter 2), this disrupts the normal

progression of mitosis in the cell cycle (Fan, 1999). Thus taxol, as expected, showed growth inhibition activity with a cell viability of 34% at the IC50 concentration on the AsPC-1 cell line. Much like Taxol, Camptothecin also showed an expected growth inhibition on the AsPC-1 cell line, giving a cell viability of 44% at the IC_{50} concentration. Camptothecin is an inhibitor known to enter the cell and target Topoisomerase1 (Top 1) which is responsible for DNA unwinding (Pommier, 2006). As presented in chapter 3, the methanol and butanol extracts were able to reduce cell viability on the AsPC-1 cells. The findings suggest that at 50 µg/ml of the butanol and 60 µg/ml of the methanol extracts display cytotoxic effects. This is similar to the findings by Ndlovu L, 2015 (unpublished) which indicated that *L. fruticosus* extracts had cytotoxic effects on the lung adenocarcinoma A549. The difference in the IC_{50} concentrations between the methanol and butanol extracts could be explained by varying in the compounds extracted by the two solvents. The concentration of cell proliferation inhibition at cellular level is higher but that may be as a result of the plant having shown to be pro-cancerous which in many cases has resulted in DNA damage that initiates a cell response leading to cell death.

Lung fibroblast cells (MRC-5) were used as a control cell line to evaluate the effect of the extracts at the IC_{50} concentrations. The continuous monitoring of adherent AsPC-1 and MRC-5 cells using xCELLigence was done to further confirmed that the extracts have cytotoxic or inhibitory effects on the pancreatic cancer cell line AsPC-1. The cell index of camptothecin and taxol decreases tremendously within 24 hours of exposure to treatment for both the AsPC-1 and MRC-5 cell lines. A decrease in the cell index might be an indication of growth inhibition. The decrease in the cell index of untreated AsPC-1 suggests that the cells may have reached well capacity. However, AsPC-1 cells exposed to IC_{50} concentrations of methanol and butanol extracts showed a decrease in cell index much later than that of the untreated and positive control treated cells. Because the xCELLigence system is based on measurement of the impedance that is affected by cell adherence (Gumulec *et al*,2013), this result suggests that there was not enough impedance perhaps due to the fact that the plant might have initiated proliferation. On the other hand, methanol and butanol treated MRC-5 cells behaved

similarly to the untreated cells. This was interesting to note as it implies that the plant extracts does not have an inhibitory effect on the normal lung fibroblast (MRC-5). These results however are similar to other studies that used xCELLigence in antiproliferation studies which showed that xCELLigence only read cells that were attached onto the well rather than all inhibited cell proliferation (Choene and Motadi,2016)

It is important to evaluate proliferative capacity within the context of cell function and cell cycle progression (Kennedy *et al*, 2012). Therefore, after seeing that the methanol and butanol extracts did have late or delayed inhibitory effect on the pancreatic cancer cell line AsPC-1 using the MTT assay and xCELLigence, it was necessary for us to assess if the extracts were able to cause cell cycle arrest and at which stage of the cell cycle would the cells resonate. Propidium iodide (PI) is used in cell cycle analysis to stain double stranded DNA. The cells are permeabilised by fixing and the PI then gains access and intercalates with the double stranded DNA. The intensity of the stained cells can then be measured using flow cytometry. A high percentage of untreated AsPC-1 cells resonated in the G2/M phase. Similarly, a high percentage of methanol extract treated cells resonated in the G2/M phase. This could signify that the cell cycle was halted during mitosis. This, however, was not the case with the taxol, camptothecin and Butanol extract treated AsPC-1 cells as most cells in these treatments resonated in the G0/G1 phase. Depending on the G0/G1 phase check point, cells that populate in this phase with DNA damage either undergo quiescence/ senescence or apoptosis. This is a result of an increase in the expression of the p53 protein which transcriptionally activates p53-dependent genes (Hartwell and Kastan,1994; Collins et al, 1997). Contrarily, a high percentage of untreated MRC-5 cells populated in the S-Phase. This was also the case with the methanol and butanol extract treated cells. The S-phase is responsible for DNA replication (Kennedy et al, 2012; Hartwell and Kastan, 1994; Suryadinata et al, 2010). These findings then further confirm that *L. fruticosus* induced insignificant apoptosis-related cell cycle arrest in the MRC-5 cell line as most cells were undergoing continuous DNA division. Furthermore, comparison of these cells to those treated with camptothecin and taxol shows that most cells remained alive despite 48 hour exposure to *L.*

fruticosus. This experiment further supported our study that perhaps the plant extracts might induce cell death might be inhibitory towards cell proliferation especially seeing that the butanol extract behaved similarly to taxol and camptothecin treated cells.

The percentage of AsPC-1 cells that populated in the G0/G1 phase called for detection of occurrence of apoptosis using flow cytometry. As mentioned earlier, apoptosis, also known as programmed cell death, is a gene regulated form of cell death (Renehan et al,2001). Cells are stained with annexin v and PI. Once more, the PI intercalates with double stranded DNA. The annexin v binds to phosphatidyl serine generally expressed on the outer membrane of cells undergoing apoptosis (Taylor et al,2008). The coupling of the two dyes ensures detection of early and late apoptosis. A higher percentage of apoptosis occurred in treated AsPC-1 cells compared to untreated cells, with an even much higher percentage in camptothecin treated cells. It was interesting to see that a significant degree of apoptosis occurred in methanol and butanol treated AsPC-1 cells. This could indicate that *L. fruticosus* does induce apoptosis on the pancreatic cancer cell line AsPC-1. Moreover, apoptosis may have occurred in Taxol, Butanol extract and methanol extract treated MRC-5 cells but it was significantly lower than that of the untreated MRC-5 cells except for the camptothecin treated cells. This could have been caused by the concentration of camptothecin being harsh on the MRC-5 cells. However, the results obtained in apoptosis detection are contradictory to those obtained from cell cycle assay where cells arrested at G1/G0 and G2/M phase. This cell cycle arrest is not always associated with apoptosis because once the cells are repaired they can re-enter the cell division process.

As mentioned previously in chapter 1, P53 is a transcription factor which accumulates in response to cellular stress. It is known as a prominent tumour suppressor. This is based on the fact that this gene is found mutated in numerous human tumours (Volgestein *et al*, 2000). Furthermore p53 gene controls whether the fate of a cell is apoptosis, cell senescence, cell cycle arrest etc. Numerous times its anticancer activity is highly linked to induction of apoptosis (speidel, 2009; Farnebo et al, 2010). In normal cells however, p53 expression levels are low (Hinds and Weinberg, 1994). The expression

levels of p53 were thus measured at protein level to assess the impact of *L. fruticosus* on apoptosis induction via p53-mediated pathway on the AsPC-1 cell line. Figure 3.5 is a presentation of the results obtained when the western blot technique was done. It was interesting to see that the expression levels of p53 in treated AsPC-1 cells remained well above that of the untreated AsPC-1 cells. This was an indication that all treatments did cause changes in the expression of p53. Similar to results obtained by Rudolf et al (2011), camptothecin, which we used as a positive control, caused an increase in the expression of p53. Likewise, taxol also caused an increase in the expression of p53 in the AsPC-1 cells, which is similar to findings by Rakovitch et al, 1999. More interestingly so, the butanol and methanol extracts showed significant upregulatory effect on the expression of p53.

Following our discovery of a certain degree of anti-proliferation properties shown by *L. fruticosus* extracts, phytochemical screening using Q-TOF LC/MS, was done to identify the compounds at play within the butanol and methanol extracts. These compounds include but is not limited to Justicidin B, Flavocommelin, Fisetin, Hydrocoumarin, Hydroxylamino-4,6-dinitrotoluene and Sarpagine. Figure 3.10 to 3.17 are spectrums of these compounds. In many cases compounds associated with anticancer properties include antioxidants and flavonoids as reported in many studies (Sawadogo et al,2012). In addition, antioxidants are known to scavenge free radicals and an imbalance between antioxidants and reactive radicals has been highly implicated in numerous diseases such as diabetes mellitus (Khan et al, 2013). Furthermore, flavonoids are responsible for decreasing the accumulation of drugs within the body (Khan et al, 2013). Secondary compounds are believed and have been proven to have numerous biological activities. In many cases pre-cancerous and anti-cancer compounds within a plant extract work in synergy to prevent cancer. Compound confidence was obtained from a baseline ABSciex library by comparison of mass charge ratio, retention time and isotope pattern of eluted compounds. *L. fruticosus* presents potential to be an anticancer plant.

4.2 CONCLUSION

From this study, extracts of *L. fruticosus* have shown contradicting results with MTT showing some inhibition of cell proliferation in the pancreatic cancer cell line while xCELLigence was unable to show any form of inhibition. Observation from cell death analysis have shown cell cycle arrest with a contradiction between butanol extract treated cells which were arrested in the G0/G1phase and methanol extract treated cells which were arrested in the G2/M phase. The two phases have different requirements with one looking at damaged cells at onset and the other focuses on microtubules that attach to chromosomes resulting in cell division. Apoptosis detection also gave contradictory results with MRC-5 cell line undergoing apoptosis while slight apoptosis is observed in AsPC-1cell lines. One of the genes commonly affected in cancer cell lines is p53. In the methanol extract treated AsPC-1 cells p53 was over-expressed as compared to untreated cells, whereas expression of p53 was moderate in the butanol extract treated AsPC-1 cells. This over expression of p53 might support both cell cycle arrest and apoptosis depending on the degree of cellular damage.

In conclusion, the methanol and butanol extracts of *L. fruticosus* to some extent do present potential anticancer properties, however, selected compounds need to be isolated and tested.

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