Anti – Cancer effects of *Momordica charantia in- vitro*

By

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Abstract

A multitude of plants have been used extensively for the treatment of cancers throughout the world. In many parts of the world, especially in poor countries, this may be the only form of cancer therapy. Much research has been focused on the scientific evaluation of traditional anti-cancer drugs from the tropical plant; *Momordica charantia* (MC) is one of them and it has been used frequently as an anti-cancer agent. The green leaves, fruits, seeds and stems of *M. charantia* composed of many different proteins and steroids that are chemically active. These proteins are α and β momorcharins which possess anti-cancer and anti-HIV properties similar to crude water and methanol soluble extracts of *M. charantia*. This study investigated the anti cancer effect of either the crude water and methanol soluble extract of *M. charantia,* α and β and α, β momorcharins based on dose-dependent, time-dependent on the viability of 1321N1, Gos-3, U87-MG, Sk Mel, Corl -23, Weri Rb-1 and L6 cell lines employing different concentrations of each extract or drug. In addition, the study measured the effect of either temozolomide or vinblastine alone or combining each with either the crude water soluble extract of *M. charantia* or α β momorcharin measuring cell viability in the different cell lines. Furthermore, the present study investigated the cellular mechanism(s) via which the different anti-cancer agents were able to induce cell death measuring the activities of caspase - 3 and caspase - 9, the release of cytochrome c and intracellular free calcium concentrations $[Ca^{2+}]_i$.

The results have shown that the crude water soluble extract of *M. charantia* can evoke both time-course at (800 μg) and dose-dependent (200 μg - 800 μg) decreases in cell viability with maximal increases with 800 μg over a period of 24 hrs following incubation. Either the crude methanol soluble of *M. charantia* (200 μg - 800 μg), alpha

or beta momorcharin (200 µM - 800 µM) had little or no effect on the viability of the different cell lines. In contrast, either alpha, beta momorcharin (200 µM - 800 µM), temozolomide (80 µM - 320 µM) or vinblastine (10 µg - 40 µg) can evoke significant ($p < 0.05$) decrease in cell viability, similar to the crude water soluble extract of *M. charantia*. The results also show that combining either temozolomide (240 µM) or vinblastine (40 µg) with either (800 µg) of the crude water- soluble extract of *M. charantia* or (800 µM) of alpha, beta momorcharin can result in significant decreases in cell viability for each cell line but these effects were neither additive or synergetic compared to the individual effect of temozolomide or vinblastine. The result of this study have also shown that either the crude water-soluble extract of *M. charantia* (800 µg) or (800 µM) of alpha, beta momorcharin can elicit marked and significant ($p < 0.050$ increases in the activities of caspase - 3 and caspase - 9 in all the cell lines. Similarly, both the crude water soluble extract of *M. charantia* and alpha, beta momorcharin can stimulate the release of cytochrome-c and elevated $[Ca^{2+}]_i$ in the different cancer cell lines compared to untreated cell lines. Together, the results of the study have shown that either the crude water soluble extract of *M. charantia* or alpha, beta momorcharin can exert their anti-cancer effects (cell death) on cancer cell lines by increasing the activities of caspase - 3 and caspase - 9 and by releasing cytochrome-c and elevating $[Ca^{2+}]_i$ in the cancer cells. These findings implicate the role of apoptosis and cellular Ca^{2+} homeostasis in cancer cell death. Moreover, they confirm the beneficial use of extracts of *M. charantia* to treat cancers.

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Declaration

I declare that this thesis has been composed by myself and that, whilst registered as a candidate for the degree of Doctor of Philosophy. I have not been a registered candidate for any other award or by any other awarding body.

Gunasekar Manoharan

List of Abbreviation

AA	Anaplastic Astrocytoma
AG	Angiocentric Glioma
AGT	O6-alkylguanine-DNA alkyltransferase
ATP	Adenosine Triphosphate
BSA	Bovine Serum Albumin
CAD	Caspase-Activated Deoxyribonuclase
CNS	Central Nervous System
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DT	Diffusion Tensor
ECACC	European Collection of Cell Cultures
EGFR	Epithelial Growth Factor Receptor
EMEM	Eagle's Minimal Essential Medium
ER	Endoplasmic Reticulum
FBS	Fetal Bovine Serum
fMRI	Functional Magnetic Resonance Imaging

GBM	Glioblastoma Multiform
HBSS	Hank's Buffered Salt Solution
ICAD	Inhibitor of Caspase Activated Deoxyribonuclase
IP	Intra-Pulmonary
IP_3	Inositol Triphosphate
IP3RS	Inositol 1, 4, 5-Triphosphate Receptors
MC	Momordica Charantia
mM	Milli Molar
μM	Micro Molar
MMR	Miss Match Repair
MRI	Magnetic Resonance Imaging
MSI	Magnetic Source Imaging
MTIC	(3-methyl)-triazen-1-yl-imidazole-4-Carboxamide
MW	Molecular Weight
NADH	Nicotineamide Adenine Dinucleotide Reduced

NADPH	Nicotineamide Adenine Dinucleotide Phosphate
NSCLC	Non-small Cell Lung Carcinomas
PARP	(Poly (ADP-ribose) polymerase)
PBS	Phosphate Buffer Solution
PDT	Photodynamic therapy
PET	Positron Emission Tomography
PMA	Pilomyxoid Astrocytoma
PNA	p-nitroanilide
RPM	Rotation Per Minute
S.E.M.	Standard Error of the Mean
SCLC	Small Cell Lung Carcinomas
SPECT	Single Proton Emission Tomography
SPSS	Standard Error of the Mean
TMZ	Temozolomide
UV	Ultra-Violet
WHO	World Health Organisation

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Chapter One

Introduction and Literature Survey

1. Introduction

A multitude of plants have been identified and used for the treatment of different diseases throughout the world, especially in poor countries. Much research has been focused on the scientific evaluation of traditional drugs from the tropical plant; *Momordica charantia* (MC) has been commonly or frequently used as an anti-cancer agent and anti-diabetic agent and it is often described as food of medicine (Heinrich and Bremner, 2006). MC is commonly known as either bitter melon or bitter gourd. Bitter gourd grows in all tropical parts of the world and it is cultivated throughout South America, Asia and Africa. The plant is a slender climbing annual vine with long-stalked leaves and yellow, solitary male and female flowers borne in the leaf axils (Bailey *et al*. 1985). It is related to squash and cucumber plants. The fruit looks like a warty gourd. The young fruit is emerald green, turning to orange-yellow when ripe (see figure 1.1).

The Latin name momordica means "to-bite" referring to the jagged edges of the leaves, which appear as if they have been bitten. All parts of the plant, including the fruit, the stem and seed taste very bitter.

In botanical terms, the plant is referred as

   a. Family: Cucurbitaceae

   b. Genus: Momordica

   c. Species: charantia

   d. Synonyms: Momordica chinenus, Momordica elegans, Momordica indica, Momordica operculata, Momordica sinenuns and Silyos fauriei.

   e. Common Names: Bitter Melon, papailla, melaode sao caetano, bittergourd, balsam apple, balsam pear, karela, ku kua karela, kor-kuly, ku gua, para-aki, salsamino, Soru, Sorossis borossieb, pare, peria La at, peria.

Figure 1.1: Fruits of *Momordica charantia*

(Adapted from Herbal secrets of the Rainforest, 2nd edition 2007)

1.1 Tribal and herbal Medicine uses

In the Amazon, local people grow bitter melon in their gardens for food and medicines (Singh *et al.* 2004). They add the fruit for bitter or sour flavor by parboiling it first with a dash of salt to remove some of the bitter taste (Basch *et al.* 2003; Abhishek *et al,* 2004: Akhtar, 1982). It is used as leaf tea for the treatment of diabetes, to expel intestinal gas, to promote menstruation and as an antiviral treatment for measles, hepatitis and feverish conditions (Akhtar *et al*, 1991:Heinrich and Bremner. 2006). It is also used typically for sores, wounds, infections and also

internally and externally to treat for worms and parasites. MC is also used for the treatment of cancer tumours (Ahmed *et al*, 2004; Lee.1998). In the last few decades, several hundreds of studies that have been carried with MC using modern tools and they have credited MC with anti-diabetic, anti-viral, anti-tumour, anti-leukemic, anti-bacterial, anthelmintics, antimutagenic, antimycobacterial, antioxidant, antiulcer, anti-inflammatory and hypochlolestrolemic, hypoglyceridemic, hypotensive, immunostimulant and insecticidal properties (Ahmed *et al*, 1999: Alessandra *et al,* 2008: Ng *et al.* 1987; Raman and Lau. 1996; Basch *et al.* 2003). MC seems to have universal medicinal properties for the treatment of different diseases. Some of the phytochemical constituents are shown in the Table 1.1.

Source	Phytochemicals present	References
Plant body	Momorcharins, momordenol, momordicilin, momordicins,momordicinin,momordin, momordolol, charantin, charine, cryptoxanthin, cucurbitins, cucurbitacins, cucurbitanes,cycloartenols,diosgenin, elaeostearic acids, erythrodiol, galacturonic acids,gentisicacid,goyaglycosides, goyasaponins and multiflorenol,	Husain *et al*.1994; Xie *et al*. 1998; Yuan *et al*. 1999; Parkash *et al*. 2002; Murakami *et al*. 2001.
Plant leaves	Glycosides, saponins, alkaloids, fixed oils, triterpenes, proteins and steroids	Raman and Lau, 1996
Fruit	Momorcharins, momordicine, charantin, polypeptide- p insulin, ascorbigee, amino acids, aspartic acid serine, glutamic acid, threonine, glutamic acid, threonine, alanine and g-amino butyric acid. Other constituents include pipecolic acid, luteolin and a number of fatty acids such as lauric, myristic, palmitic, palmitoleic, stearic,oleic, linoleic, linolenic acid	Lolitkar and Rao, 1966; Yuwai *et al*. 1991 http://www.tropil ab.com
Seeds	Urease, amino acids–valine, threonine methionine, isoleucine, leucine, phenylalanine.	Orlovskaya *et al*. 2007

Table 1.1: Some phytochemicals and constituents of the different parts of

Momordica charantia.

1.2 Plant chemicals with their anti-cancer effect

Since ancient times, plant and herbal preparations have been used as traditional medicines to treat a number of diseases. *M. charantia* has been a very popular source of traditional medicines for several aliments (Grover *et al.* 2001). A number of preliminary studies both *in vitro* as well as *in vivo* with crude extract of *M. charantia* have shown anti-cancer activity against lymphoid leukaemia, lymphoma, choriocarcinoma, melanoma, breast cancer, skin tumour and prostatic cancer (Licastro *et al.* 1980; Ng *et al.* 1994; Ganguly *et al.* 2000; Sun *et al.* 2001). An aqueous extract of *M. charantia* was shown to inhibit the growth of prostatic adencarcinoma (Claflin *et al.* 1978). The chronic treatment with hot water extract of *M. charantia* inhibited uterine adenomyosis and mammary tumour growth in mice (Nagasawa *et al.* 2002; Singh *et al.* 1998) demonstrated maximal anti-carcinogenic activity in the peel of *M. charantia.*

Several studies have reported that the water-soluble extract of *M. charantia* can exert anti-cancerous activity through inhibition of DNA, RNA and cellular protein synthesis (Licastro *et al.* 1980; Zhu *et al.* 1990; Tsao *et al.* 1990; Chang *et al.* 2008; Terenzi *et al.* 1999). The fruit juice of *M. charantia* has been found to increase glucose up take by several tissues *in vitro* and moreover, it can increase the storage of glycogen by the liver (Welihinda *et al.* 1986). The water-soluble extract of the *M. charantia* can significantly reduce blood glucose concentrations in type-1 diabetic rats (Ahmed *et al.* 1999). The *in vivo* anti-cancer activity of a crude water-soluble extract *M. charantia* was shown to inhibit tumour formation in CAB/H mice which had been given i.p injections of 1.0×10^5 CBA/ DI tumours cells. The extract also inhibited tumour formation in DBA/2 mice which following i.p injections (Catherine *et al.* 1983). The water-soluble crude extract is cytotoxic to the CBA/DI cells in culture and the toxicity is dose-dependent and requires

24 hrs before it becomes apparent (Catherine *et al.* 1983). However, the cells exposed to the water-soluble extract for 30 min and washed and incubated for 24 hrs also shows a decrease in cell viability (Ali *et al*, 1993: Takemoto *et al.* 1982). There are also increasing evidences that the increased consumption of *M. charantia* intake can reduce the risk of various pathological events including cancer, high blood pressure and diabetes (Goodwin and Brodwick, 1995; Steinmetz and Potter, 1996).

A number of preliminary *in vitro* and *in vivo* studies with the water-soluble extract of *M. charantia* and its various purified fractions have shown anti-cancer activity against human bladder carcinomas and breast cancers (Amila *et al*, 2000; Zhu, 1990). Recent, subcritical water extract has become an alternative for extraction of herbal plants (Ayala *et al.* 2001). The subcritical water extract of *M. charantia* potentially shows anti-tumour, anti carcinogenic and anti-inflammatory effects (Lee *et al.* 2003; Horax *et al.* 2005). The dried fruit of *M. charantia* can be pulverized into a fine powder in a grinder and stored at 4°C. This powder and the extracts of the powder can be employed for chemotherapeutic studies (Zhu, 1990; Pitipanponga *et al.* 2007). Previous studies employed three methods of extraction, namely sub critical water extraction, solvent extraction and Soxhlet apparatus extraction method in order to obtain maximal activity from the powder (Takemoto *et al.* 1982; Parichat and Artiwan, 2008).

MC contains a number of biologically active plant-based chemicals including triterpenes, proteins and steroids (Zhu, 1990), alkoloids, inorganic lipids and phenolic compounds (Grover *et al.* 2004). Some proteins in bitter melon including MAP-30, MRK29, alpha-momocharin, beta-momocharin and momordicin (see figure 1.2) and they have the ability to treat tumours and HIV (Yuan *et al.* 1999; Luetrakul. 1998). A steroid called charantin is found mainly in the aerial parts of MC and it has been shown to possess anti-diabetic properties (cakici *et al.* 1994).
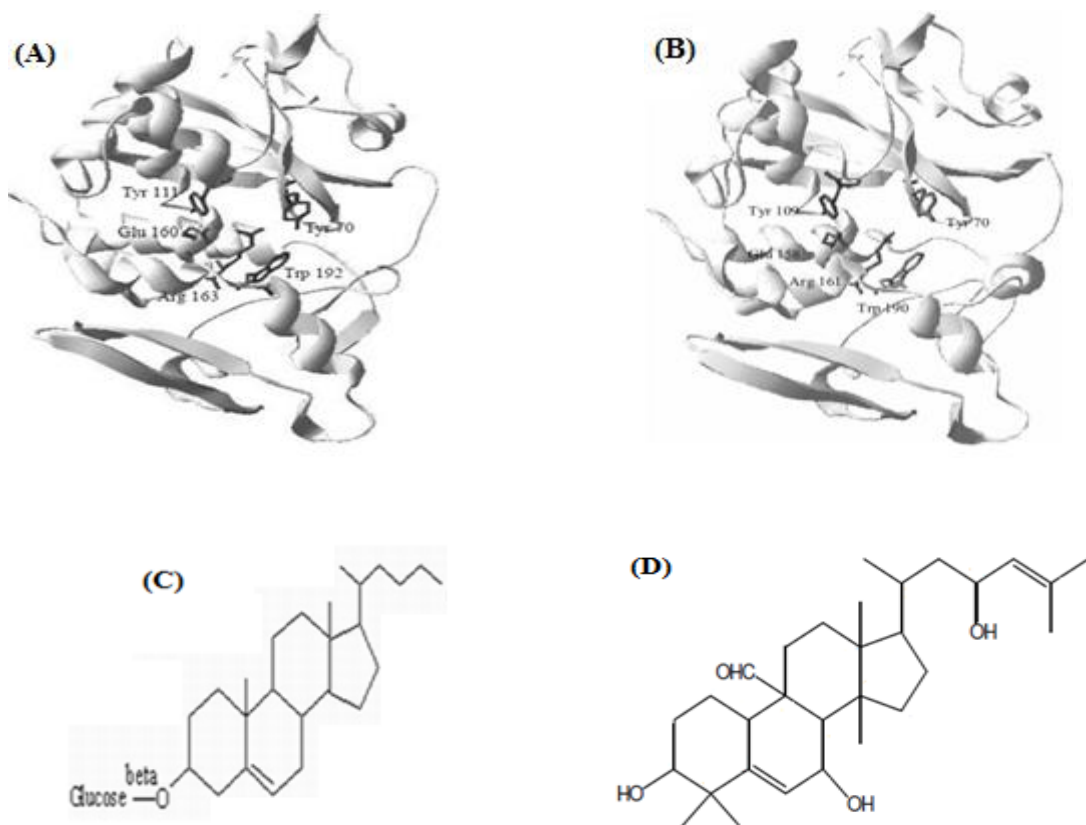
Figure 1.2: Chemical structures of (A) Alpha-momorcharin, (B) Beta-momorcharin, (C) Charantin and (D) Momordicin. (Adapted from Herbal secrets of the Rainforest, 2nd edition, 2007)

The phenolic compounds extracted from MC using a solvent extraction procedure were reported to exhibit anti-oxidant activity (Au *et al,* 2000; Horax *et al.* 2005). The ripe fruit of MC has been shown to exhibit some remarkable anti-cancer effects, especially leukemia (Asli and Alaattin, 2007; Satish-kumar *et al.* 2010).

There is much evidence that MC can be used effectively to treat cancer. MC and its extracts have been shown to inhibit cancer and tumour formation (Cunnick *et al.* 1990). A phytochemical in MC has been clinically demonstrated to inhibit an enzyme named guanylate cyclase. This enzyme is thought to be linked to the pathogenesis and replication of psoriasis, leukemia and cancer (Herbal secrets of the Rainforest, 2nd edition, 2007) and clinical trials have found much evidence that MC

can improve immune cell function in people with cancer (Cunnick *et al.* 1990; Yuan *et al.* 1999). Several *in vivo* studies have demonstrated cytostatic and anti-tumour activity of the entire plant of bitter melon (Ayala and Castro 2001; Cunnick *et al.* 1990; Zheng *et al*. 1999). A previous study has reported that a water-soluble extract of MC can inhibit the growth of rat prostate carcinoma and moreover, a hot water extract of the entire plant of MC can inhibit the development of mammary tumours in mice (Bailey and Day 1989; Cunnick *et al.* 1990). Similarly, numerous *in vitro* studies have demonstrated the anti-cancerous and anti-leukemic activities of MC against several commercial cell lines including human leukemia, liver cancer, melanoma and solid sarcomas (Zheng *et al*. 1999). A chemical analogue of a protein isolated from MC was developed and named MAP-30. It was reported that MAP-30 could inhibit prostate tumour growth. Several phytochemicals extracted from MC have been documented with cytotoxic activities and they include a group of ribosome-inactivating proteins named (α.β momocharins, momordin and cucurbitacin B) (Pongnikorn *et al.* 2003; Satish-kumar *et al.* 2010). Tables 1.2 and 1.3 summarie the anti-cancer activities for the different chemical extracts of MC isolated from different parts of the plant and tested on different animal species with positive results.

Part- origin	Activity Tested For	Types Extract	Test Model and route of administration	Dosages	Notes/Organism tested	Results	References
Fruit (India)	Antitumuor Activity	Aqueous Ext	Mice (Oral)	Not stated	Protected against the development of skin tumour or and increased life expectancy.	Active	Ganguly *et al.* 2000
Fruit (India)	Antitumuor Activity	Aqueous Ext	Mice (Oral)	Not stated	Reduced carcinogen-induced lipid peroxidation in the liver and DNA damage in lymphocytes	Active	Ganguly *et al.* 2000
Fruit (India)	Antitumuor Activity	Aqueous Ext	Mice (Oral)	Not stated	Activated liver enzymes glutathione-S-transferase, glutathione peroxidase and catalase which were depressed after carcinogen exposure	Active	Ganguly *et al.* 2000
Fruit(USA)	Antitumuor Activity	H_2O Ext	Mouse (IP)	100.0 μg/ml	Cells-CBA/D1.	Active	Jilka *et al.*1983
Fruit (USA)	Antitumuor Activity	H_2O Ext	Mouse (IP)	Not stated	LEUK-L1210. Drug was preincubated with tumour cell line *in vitro.*	Active	Jilka *et al.*1983
Fruit (USA)	Antitumuor Activity	H2O Ext	Mouse (IP)	Not stated	LEUK-P388. Drug was pre incubated With tumour cell line *in vitro.*	Active	Jilka *et al.*1983
Fruit (China)	Cytotoxic Activity	Not stated	Cell Culture	Not stated	Ca-755.	Active	Yeung *et al.*1984

Table 1.2: Biological and anti-cancer activities of some extracts of *Momordica charantia* (Adapted from Herbal secrets of the Rainforest, 2nd edition, 2007)

Part –Origin	Activity Tested For	Types Extract	Test Model and route of administration	Dosages	Notes/Organism tested	Results	References
Fruit (India)	Carcinogenesis Inhibition	H_2O Ext	External Mouse	100.0 mcg	vs. DMBA-initiated, croton oil promoted skin tumours.	Active	Singh *et al.*1998
Fruit Pulp (India)	Carcinogenesis Inhibition	H_2O Ext	External Mouse	100.0 mcg	vs. DMBA-initiated, croton oil promoted skin tumours.	Active	Singh *et al.*1998
Fruit Peel (India)	Carcinogenesis Inhibition	H_2O Ext	External Mouse	100.0 μg	vs. DMBA-initiated, croton oil promoted skin tumours.	Active	Singh *et al.* 1998
Seed (India)	Carcinogenesis Inhibition	H_2O Ext	External Mouse	100.0 mcg	vs. DMBA-initiated, croton oil promoted skin tumours.	Active	Singh *et al.* 1998
Fruit (Japan)	Tumor Promotion Inhibition	MEOH Ext	Cell Culture	200.0 mcg	Virus-*Epstein-barr*vs. 12-o-hexadecanoylphorbol-13-acetate-induced epstein-barr virus activation.	Inactive	Koshimizu *et al.*1988
Fruit (India)	Cell Proliferation Inhibition	Protein Fraction	Cell Culture	Not stated	Hep2 cells. Sea urchin ova.	Active	Raman *and Lau,*1996
Fruit (USA-CA)	Cytotoxic Activity	H_2O Ext	Cell Culture	CD10= 50.0 mcg/ml	Cells-CBA/D1	Active	Jilka *et al.*1983

Table 1.3: Biological and anti-cancer activities of some extracts of (*Momordica charantia*) (Adapted from Herbal secrets of the Rainforest, 2nd edition, 2007)

1.4 Anti-cancer activity

Cancer is a term, which is used to refer to a number of conditions where the cells begin to grow and reproduce in an uncontrollable way. Sometimes a cancer begins in one part of the body and then spreads to other parts of the body. This process is known as metastasis (Kleihues *et al*. 2002; Lewandowicz *et al*. 2000). Cancer is a common condition and moreover, it is deemed as a serious health problem, both in the UK and across the world. It is estimated that 7.6 million people in the world died of cancer in 2009. In the UK cancer is responsible for 126,000 deaths per year (Cancer Research UK, 2007).

Cancer occurs when the genes in a cell become abnormal and the cell divides and grows uncontrollably (see figure 1.3). Cell division is normal, but when this process is uncontrolled a mass of tissue called a growth or tumour is formed (Cancer Research UK, 2007).

Malignant tumours are cancerous and also can spread widely to other parts of the body by entering either the blood stream or lymphatic system as well as harming nearby tissues and the organs. This rapid growth of cancerous cells is known as malignant tumour. These cells can then invade and destroy healthy tissues, including organs. In addition, it can harm a number of vital organs at the same time (Merol *et al*. 2006)
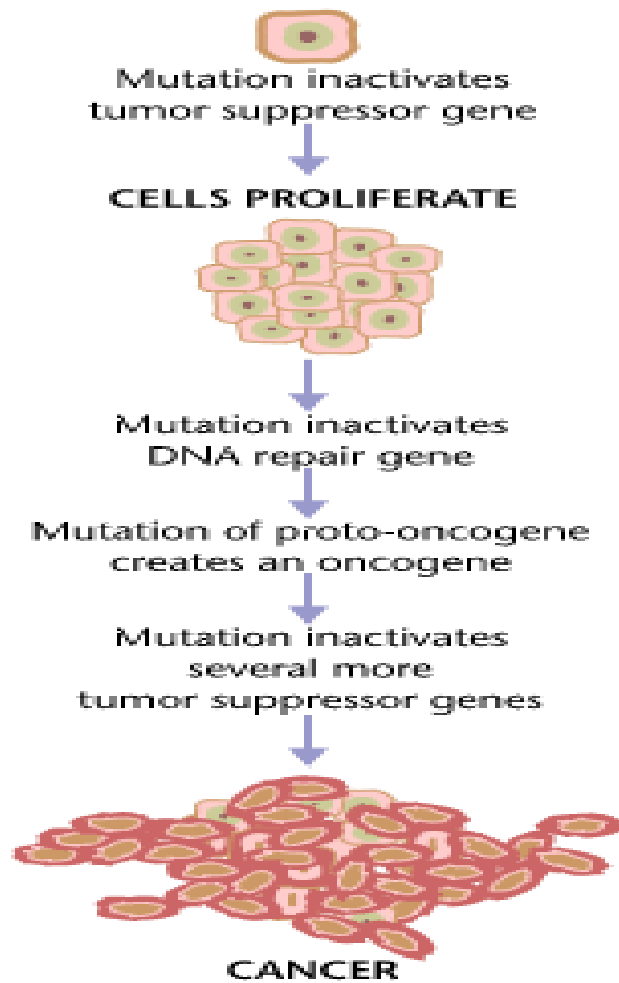
Figure 1.3: A schematic diagram showing the development of a cancer

(Adapted form bio.miami.edu, January 2006)

Types of cancers:

There are different types of cancers in the body. They include anal, bladder, breast, cervical, colon, endometrial, oesophageal, kidney, leukaemia, liver, lung, lymphoma, ovarian, pancreatic, penile, prostate, skin, stomach cancer and several others. Almost any part of the body can be infected with cancers (Roberts and Rudee. 1988). All the cells in the body have similar structures and share a majority of their functions. Cancers may be categorized based on the functions and locations

of the cells from which they originate (see figure 1.3). The following terms are commonly used to distinguish tumours of different origin.

- **Carcinoma**- is a tumour, which is derived from epithelial cells that line the surface of our skin and organs. The digestive tract and airways are also lined with epithelial cells. This is the most common cancer type and represents about 80-90% of all cancer cases reported (Wen and Keasri. 2008).

- **Sarcoma**- is a tumour, which is derived from muscle, bone, cartilage, fat and connective tissues.

- **Leukemia**- is a cancer, which is derived from white blood cells or their precursors. The cells that form both white and red blood cells are located in the bone marrow.

- **Lymphoma**- is a cancer of bone marrow and it is derived from cells that affect the lymphatic system.

- **Myeloma**- is a cancer involving the white blood cells and it is responsible for the production of antibodies (B lymphocytes or B-cells).

1.6 Causes of cancer

There are many causes of cancers and they include mutation of the genes that control cell growth, chemicals, radiations, preservatives etc (Merlo *et al.* 2006). Gene mutation is found in every cell in the body and it regulates all of its activities. Cancers are caused by damage to the DNA. The body is usually able to repair damaged DNA, but it is unable to do so in cancer cells (Goodarz *et al.* 2005). In most cases, people damage their DNA via their lifestyle habits, which include unbalanced diet, smoking, stress, exposure to ultraviolet radiation (UV) from the sun and to substances known as carcinogens in the environment. Some carcinogens

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include benzene, asbestos, formaldehyde, which are specific for skin cancers and in most cases these can be prevented with care and by following health and safety regulations. Smoking causes the majority of lung cancers, but scientists have long known that tobacco contributes to a number of other forms of the disease (Roberts *et al.* 1988). Each type of cancer is caused by different factors, which are well established, while others are uncertain and unknown (Goodarz *et al.* 2005).

1.7 Introduction to CNS and CNS tumours
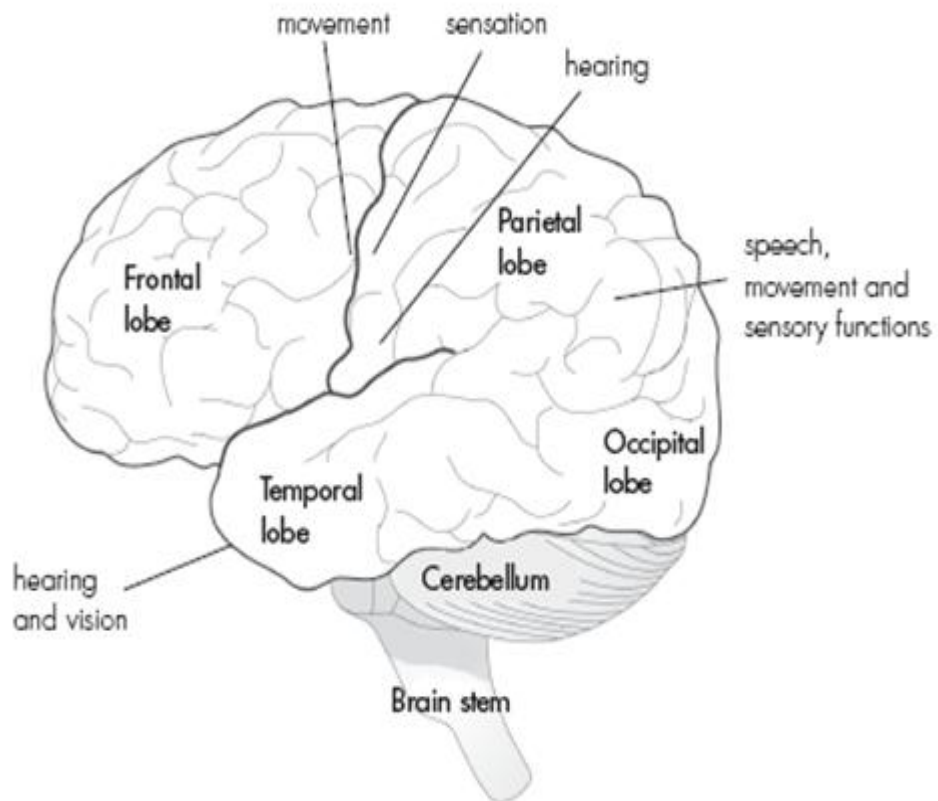
Figure 1.4: Diagram showing the lobes and functions of the brain

(Adaptedfrom-http://www.cancerbackup.org.uk/Cancertype/Brain/General/Thebrain#2006)

The brain is the most important part of the central nervous system (CNS). It is divided into several lobes, the cerebellum and the brain stem (see figure 1.4). The different parts of the brain are associated with different functions of the body. For example, each lobe or area of brain has one or more specific functions such as vision, smell, taste, hearing and balance (see figure 1.4). Brain tumours are composed of cells that exhibit uninhibited growth in brain. There are nearly 100 types of brain tumours. They are generally named after the type of cells they develop from. The tumour cell arising in glial cells (supportive nerve cells in brain) is called glioma. Glioma can develop in any part of the brain or its nerves and covering tissues. They can be benign (non-cancerous, meaning that they do not spread elsewhere or invade surrounding tissue) or malignant (cancerous). The two major types of brain tumours are primary and secondary. Primary brain tumours start in the brain. Secondary brain tumours start in another part of the body, and then spread to the brain. A glioma is a primary brain tumour, accounting for 45% of cancers that begin in brain cells (Goodarz *et al.* 2005).

Central nervous system (CNS) tumours are very common and they can cause death of the patients. CNS tumours are lumps of abnormal growth of cells and can be benign and malignant.

CNS tumours are of two types: -

1. Begnin tumours

They are also called primary tumour which can grow in the brain or the spinal cord itself and they do not spread to other organs or tissues of the body. They are not usually life-threatening cancers and moreover, they grow very slowly.

2. Malignant tumours

These are also called secondary tumours of the body, which usually spread to other parts of the body including organs and tissues. They generally tend to grow very fast and invade nearby tissues.

Eventually, more than half of the CNS tumours are benign. They can cause substantial morbidity, but according to some literature, malignant primary brain tumours are life threatening and they are the leading cause of death in children and the third leading cause of death in adolescents and adults aged 15 to 34 yrs (Kimmel *et al*. 1987). Some common symptoms of CNS tumours in the patients include headache, seizures and altered mental status. The imaging technique such as Magnetic Resonance Imaging (MRI) scan can help to define the anatomical extent of the tumours. Biopsy is normally performed on most occasions for the conformation of diagnosis in order to treat CNS tumours. Benign tumours are usually curable with either surgical resection or radiation. Radiation therapy includes stereotactic radiation. However, the majority of patients having malignant brain tumours benefit from chemotherapy either at the initial diagnosis or at tumours recurrence. Metastases to the brain remain a frequent and morbid complication of solid tumours but are frequently controlled with either surgery or radiation therapy. Unfortunately, the mortality rate from malignant brain tumours remains high, despite initial disease control. Therefore, there is an urgent need in finding remedies from the natural sources without any side effects. In order to find the remedies of tumour treatment, it is necessary to identify the grade of tumour. Therefore, the World Health Organization (WHO) grading system was established in order to detect and cure the tumour at different stages by various treatment identified (Kimmel *et al*. 1987; Nelson and Cha, 2003).

1.7.1 The new WHO Classification of tumours affecting the central nervous system

In 1993, the WHO ratified a new wide-ranging classification of neoplasms affecting the CNS (WHO grade, 2007). The classification of brain tumours is based on the abnormal growth of a specific cell type. The tumour classification dictates the choice of therapy and predicts prognosis. The new WHO system is particularly useful in this regard with only a few notable exceptions for example, either all or almost all gemistocytic astrocytomas are actually anaplastic and hence named grade III or even IV rather than grade II has been designated by the WHO system. The WHO classification also provides a corresponding grading system for each type of tumour. In this grading system, most named tumours are of a single defined grade. The new WHO classification provides the standard for communication between different Centres in the United States and around the world. An outline of this classification is provided below.

1.7.2 Neuro-epithelial Tumours of the CNS

Astrocytic tumours [glial tumours--categories I-V, below] may also be sub-classified as invasive or non-invasive, although this is not formally part of the WHO system. The non-invasive tumour types are indicated below. Categories in italics are also not recognized by the new WHO classification system, but are in common use.

1. Astrocytoma (WHO grade II)

   1. Variants: protoplasmic, gemistocytic, fibrillary, mixed

2. Anaplastic (malignant) astrocytoma (WHO grade III)

   1. *Hemispheric, diencephalic,optic,brain stem,cerebellar*

3. Glioblastoma multiforme (WHO grade IV)

   1. Variants: giant cell glioblastoma, gliosarcoma.

1.8 Gliomas

Gliomas are the commonest primary tumours of the brain and they comprise about 2% of all newly diagnosed cancers every year in the UK (The National Institute for Health and Clinical Excellence Cancer Service Guidance, 2007). Overall, the incidence of malignant gliomas roughly equals that of leukemia. Gliomas form about 50% of all intracranial tumours in the adult, of which half are malignant gliomas. There are two main types of brain tumours: those that start in the brain (primary) and those that spread from cancer to somewhere else in the body (secondary). Primary brain tumours that appear to derive from cells of neuroglial origin are referred to as glioma (Nikkhah *et al.* 1992). According to the (WHO) grading system, gliomas are assigned a malignancy grade of I–IV based on the appearance of certain pathological features (Laws *et al.* 2003). The malignant transformation of astrocytes, oligodendrocytes or their cells gives rise to a tumour that is called a glioma (Noda *et al.* 2001). Gliomas account for 40-60 percent of the primary brain tumours.

There are several kinds of gliomas and they include astrocytomas, which grow anywhere in the brain or spinal cord. Brain stem gliomas, arise in the lowest part of the brain. Ependymomas develop inside the brain, in the lining of the ventricles.

Oligodendrogliomas, usually grow in the cerebrum (very rare, representing just 3% of all primary brain tumours). An advanced astrocytoma is called glioblastoma and it represents 23% of all primary brain tumours (Laws *et al.* 1993).

1.8.1 Epidemiology

Brain tumors always have been one of the most devastating diseases because they are so difficult to treat, much less cure. But now scientists are on track toward finding what may be definitive treatments for the most virulent of these tumors. These may be broadly classified into primary brain tumours that start in the brain and secondary brain tumours that spread to the brain from another site. The worldwide annual incidence rate of primary malignant brain tumours ranges from 5.8 per 100,000 for males and 4.1 per 100,000 for females in developed countries (Jain *et al*. 2008). Gliomas are the most common forms of primary brain tumours accounting for 80% of the cases (Schwartzbaum *et al*. 2006; DeAngelis*,* 2001). Gliomas are essentially tumours, which arise from the glial cells present in the brain, which provide support and nutrition. They also produce myelin, and maintain homeostasis in the nervous system. The incidence of glioma in the UK is about 21 per 100,000 (Poberskin and Chadduck, 2000). In the USA, 13,000 deaths and 18,000 new cases of primary malignant brain tumours occur annually of which glioma is responsible for approximately 77% cases (Schwartzbaum *et al.* 2006).

1.8.2 Classification and Grades

Depending on their cell of origin, gliomas are classified into three main types including astrocytoma, oligodendroglioma, and ependymoma. The WHO system further grades these, on the basis of histological degrees of malignancy with Grade I being the least malignant to Grade IV being the most malignant (Louis *et al*. 2007). The degree of malignancy is based on either the presence or absence of

increased cellularity, nuclear atypia, mitosis, endothelial proliferation and necrosis. Grade I and Grade II tumours are considered to be low grade gliomas while Grade III and Grade IV are high-grade gliomas. Low-grade tumours are usually circumscribed and grow slowly over a period of time while high-grade tumours are comparatively aggressive having poor prognosis (Chandana *et al*. 2008). Some of the low-grade gliomas undergo malignant transformation to high-grade neoplasms with time and age. High-grade gliomas are the most common type of brain tumours in the adult age group and represent a major cause of morbidity and mortality in neurologic practice (Benjamin *et al*. 2003). Glioblastoma multiform (GBM) and anaplastic astrocytoma (AA) are the most malignant and aggressive high-grade glioma (WHO Grade IV and III, respectively), having a combined incidence of 5-8/100,000 population (Avgeropoulos and Batchlor, 1999). In 2007, the WHO classification of tumours of the nervous system revised and added three new tumours (Table 1.3): angiocentric glioma (AG), pilomyxoid astrocytoma (PMA), and pituicytoma (Louis *et al*. 2007; Brat *et al*. 2007).

WHO DESIGNATION	GRADE
Astrocytic Tumours	
Pilocytic Astrocytoma	I
Pilomyxoid Astrocytoma	II
Diffuse Astrocytoma	II
Anaplastic Astrocytoma	III
Glioblastoma Multiforme	IV
Oligodendroglial Tumours	
Oligodendroglioma	II
Anaplastic oligodendroglioma	III
Ependymal Tumours	
Subependymoma	I
Ependymoma	II
Anaplastic ependymoma	III
Mixed and other common types of glioma	
Angiocentric glioma	I
Oligoastrocytoma	II
Anaplastic oligoastrocytoma	III

Table 1.3: WHO classification of Gliomas

(Adapted from NICE cancer service guidance 2007)

1.8.3 Mutations leading to infiltrative astrocytic tumours

Molecular studies have identified some of the genetic changes that underlie the pathologic differences among astrocytic tumours. Progression in tumour grade is associated with an ordered accumulation of mutations (see figure 1.5). Approximately, 33% of low grade infiltrating astrocytomas (St. Anne/Mayo Grade 2) have mutations detected in the p53 gene on chromosome 17p. Anaplastic astrocytomas (Grade 3) - whether found in pre-existent low grade astrocytomas or detected *de novo*-have a similar incidence of p53 mutations but, in addition, show a loss of heterozygosity on chromosome 19q in more than 40% of cases (Louis *et al*. 2007). Progression from astrocytoma to anaplastic astrocytoma also involves mutations in other tumour suppressor genes including the retinoblastoma gene on chromosome 13q (see figure 1.5). Finally, glioblastomas have the same incidence of these genetic aberrations and in addition 70 percent have lost heterozygosity for chromosome 10 and one third have amplification of the epidermal growth factor receptor gene. Many of these correlations have been defined largely through work at the MGH Molecular Neurooncology Laboratory (Brat *et al*. 2007).
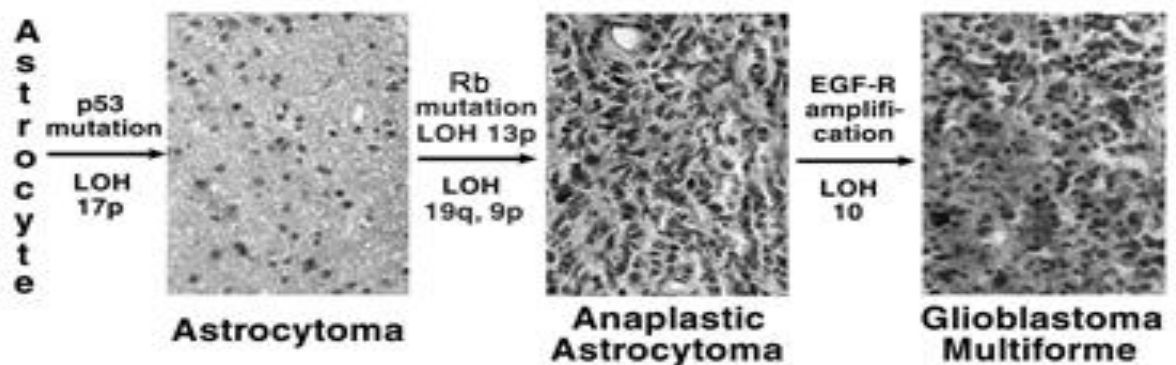
Figure1.5: Diagram showing the progression (arrows) of astrocytoma to glioblastoma multiform (Adapted from stke.sciencemag.org).

1.9 Astrocytoma

Astrocytes are star-shaped neuroglial cells that provide structural supports for the neurones and maintain electrolytes and neurotransmitter homeostasis in the brain. The majority of gliomas in both adults and children are astrocytomas originating from astrocytes (Yarbro *et al.* 2005). Tumours are graded to describe their degree of malignancy where each grade is associated with a clinically distinct prognosis (Pagano *et al.* 2004). By histological appearance, astrocytes can be characterized into two main types: namely fibrillary and protoplasmic (Miller *et al.* 2004). The distinction between the two is based on the shape of their cell processes and on the presence of intra cytoplamic fibrils.

Astrocytes are present in the white matter of the brain (Kettenmann *et al.* 1995). All these types of astrocytes are found in different sub-types of astrocytoma. The major histological subtypes of low-grade astrocytomas include pilolytic, fibrillary, gemistocytic and protoplasmic astrocytoma. Low-grade astrocytomas are more frequent in children than adults (Vinken and Bruyn. 2002). The astrocytoma accounts for the majority of malignant gliomas in adults and graded into four groups according to the WHO grade classification 2007 (See table 1.4).

GRADING OF ASTROCYTOMAS		
WHO grades	**Designation**	**Histological Criteria**
I	Pilocytic astrocytoma	Rosenthal fibres + piloid cells
II	Diffuse astrocytoma	Nuclear atypia
III	Anaplastic astrocytoma	Cellular anaplasia, mitoses
IV	Glioblastoma	Anaplasia, mitoses, vascular endothelial proliferation and necrosis

Table 1.4: WHO grading of gliomas.

In the cerebral hemispheres, astrocytomas form 25-30% of all gliomas. In the cerebellum, they make up 30% of the gliomas in children. In adults, grade I is uncommon and comprise a distinct entity called pilocytic astrocytoma which occurs most commonly in the cerebellum in children. Grade II astrocytomas account for approximately 10-15% of all astrocytic brain tumours with an incidence rate of 1.4 new cases / million people a year (Bigner *et al.* 1998). Grade II astrocytomas affect any region of CNS but predominately occur in the cerebrum followed by the brain stem and spinal cord (Reiser *et al.* 2007). Grade II astrocytomas are well differentiated slowly growing as astrocytic glioma (Louis *et al.* 2007). Grade II astrocytomas exhibit hyper-cellularity and cellular plemorpheins. Microscopically, these tumours exhibit low and moderate cellularity. Microcystic degeneration and occasional nuclear is a typical

feature of these tumours. Mitotic activity is generally absent or extremely rare (Black and Loeffler, 2004).

1.9.1 Grade III

Anaplastic astrocytoma represents 10% of all intra-cerebral tumours and about 25-30% of all gliomas (Yarbro *et al.* 2005). Grade III gliomas generally appear between the fourth and fifth decades of life, which are more common in males than in females (Moore *et al.* 1998). Grade III astrocytomas show strong mitotic activity along with hyper-cellularity and anaplasia. Grade III astrocytomas may be defined as an in filtrating lesion with either focal or dispersed anaplasia or marked proliferative potential. When the tumour shows either vascular endothelial proliferation and or the presence of necrosis, it is graded as Grade IV glioblastomas (Sawyer *et al.* 1991; Reiser *et al.* 2007; Louis *et al*. 2001). Treatment is based on the location of the tumour and how far it has progressed. The treatment of Grade III requires a multidisciplinary approach, which includes maximal surgical debulking of the tumour followed by radiation therapy, with chemotherapy during or post radiotherapy (Gilbert *et al.* 2007).

1.9.2 Grade IV (Glioblastomas: GMBs)

Grade IV astrocytomas are generally referred to as malignant astrocytomas. Glioblastomas are the most frequent brain tumours accounting 6-075% of astrocuticory tumours (Ricci. 1999; Louis *et al*. 2007). Grade IV GMBs are highly anaplastic infiltrating tumours, which invade and destroy surrounding brain tissues in patient's age of 50-70 years (Morantz and Walsh, 1993). They commonly arise within the cerebral hemispheres, basal ganglia and thalamus (Bradley *et al.* 2004). Molecular biology studies suggest two types of glioblastomas, primary (de novo) and secondary (evolving from a pre-existing low grade glioma) (Louis *et al*. 2007).

Primary tumours exhibit frequent occurrence of epithelial growth factor receptor (EGFR) gene amplification, whereas the secondary tumours show more frequent P53 gene mutation (Barnet. 2006; Louise, 2008). Prominent micro vascular proliferation, endothelial hyperplasia, tumoural haemorrhage and tissue necrosis are essential diagnostic features of GBMs (Barnett, 2006; Louise, *2008*).

1.10 Diagnosis of gliomas

Early clinical diagnosis of glioma can be challenging. After a clinical suspicion the diagnosis is made by imaging with CT scans' or Magnetic Resonance Imaging (MRI). MRI is more sensitive than CT scan and enables the visualization that might not be detected by CT scan (Kaye. 2005). Recently, the role of Diffusion Tersor Imaging (DTI) to study the peritumoural region of glioma has been proven to be useful for diagnosis (Lu *et al.* 2004). MR Spectroscopy (MRS), diffusion and perfusion MR (DPMR), functional MRI (fMRI), Magnetic-Source Imaging (MSI) and Diffusion Tersos Imaging (DTI) have all been extremely helpful in the assessments of the nature and extent of gliomas and their relation with eloquent brain areas (Fan *et al.* 2006).

Magnetic Resonance Spectroscopy (MRS) can reveal chemical and physiological changes which may occur in brain tumours and it can also help to determine the presence and ratio of tissue metabolites (Bernstein *et al.* 2007). MRS is used to grade gliomas before treatment. These include the study of peritumoural regions helping to guide biopsies, to identify radiotherapy targets, to distinguish between tumour recurrence and radiation necrosis and monitor patients post treatment (Butowski *et al.* 2006; Fan *et al.* 2006). The evaluation of the tissue samples obtained from biopsies also plays a vital role in definitive diagnosis. Detection of tumour residue in either post operative or post radiotherapy MRI /CT scan can be difficult and 18-GDG-PET (18f-

flucrodeoxyglucose positron emission tomography) has proven to be useful in this situation. The accuracy of 18-FDG PET is reported to be as high as 87% compared to 60.9% for CT /MRI scan (Fan *et al.* 2006).

Positron emission tomography (PET) and Single proton emission tomography (SPET) are additional metabolic imaging modalities, which aid in glioma diagnosis. These techniques involve intravenous administration of radioactive tracers, which are detected by scanners followed by tomographic image reconstruction (Miller *et al.* 2004). Recent application of PET involves mapping of tumour receptors for either various radiolabelled chemotherapeutic agents or for monoclonal antibodies (Miller *et al*. 2004). Dynamic imaging techniques, such as capillary permeability mapping, are being used in studies of new drugs that target specific molecular features of gliomas (Simpson *et al.* 1993).

1.10.1 Use of fluorescent dyes to detect, locate and help with treatment of brain tumours

The use of fluorescent optical methods for brain tumour diagnostics was evaluated according to the difference of florescence spectra. Injection of 5-amainolevulinic acid (ALA) induces fluorescence when applied to *in vivo* detection of spectral signatures of endogenous porphyries in malignant tissues. Tsien (2010) used synthetic molecules called activatable cell penetrating peptides (ACCPs) and microscopic nanoparticles to develop probes carting fluorescent and magnetic tags (Tsien, 2010). These tags make tumours visible to MRI scanning and allow the tumour to 'glow'. The ACCP nanoparticles probe enabled to visualize areas of tumours, which are buried beneath other tissues. Only a single injection of the nanoparticle- based probe containing the fluorescent and magnetic tag examines the tumour before and after surgery techniques. There are many advantages to molecularly guided cancer surgery techniques. Probes

can be used in staging cancer, particularly in prostate cancer, and can be used in a variety of tumour types. In addition, probes can be used in laparoscopic and robotic surgery, where surgeons cannot feel the tumour (Tsien, 2010).

1.11 Treatment of gliomas.

The treatment of malignant gliomas includes surgery, chemotherapy and radiotherapy (Lonardi *et al.* 2005). Depending on the site of the lesion and the condition of the patient, surgery can include gross total excision of the tumour using image guidance or may be restricted to biopsy. The treatment remains challenging whilst advances in each of these disciplines have improved both the quality of life and survival of the patients (Liu *et al.* 2008). The main goal of the surgery is to resect the tumour completely. However, the in filtering nature of gliomas and their ill-defined margins make this goal very difficult (Wen and Kesari, 2008). Surgery is definitely the first modality in glioma management. Maximal resection is performed whenever possible and it has positive outcome in patients with both low-grade and malignant gliomas (Lamborn *et al.* 2004).

A meta-analysis has concluded that for the population as a whole, the extent of resection is not predictive of outcome, but radical resection may be useful in a small subset of young patients with favorable functional status and histology, as a mean of increasing survival and quality of life (Quigley and Maroon, 1991). A study from the Eastern co-operative oncology and radiation therapy group showed a positive correlation between survival and extent of resection. A retrospective review on three consecutive trials revealed a longer median survival for maximal resection of 11.3 months as compared to biopsy alone of 6.6 months (Chang *et al.* 1983). Many recent advances in surgical techniques have helped to achieve the aim but have not shown to make any significant differences in survival time (Keles and Berger, 2004). The main advances include pre-

operative functional imaging, fusion of functional and anatomical imaging for preoperative neuro-navigation intra operative functional mapping and the use of intra operative MRI (Rampling *et al.* 2004).

Technical advances in neuro-imaging like MRI, MR spectroscopy and surgical technology like image guided surgery, intra operative ultrasound and, CT have improved and maximized tumour resection, while decreased procedure - related morbidity (Rampling *et al.* 2004).

Magnetic Source Imaging (MSI) is useful in the surgical decision making for lesions adjacent to functionally important brain areas (Ganslandt *et al.* 2004). The integration of Functional Magnetic Resonance Imaging (fMRI) data into neuro-navigation is a useful concept to assess the risk of a new motor deficit after surgery (Nimsky *et al.* 2004). The adjuvant strategies include radiotherapy and chemotherapy, which are employed before tumour progression (Keles and Berger, 2004).

1.11.1 Radiotherapy

Radiation therapy is one of the most vital systems to treat gliomas. Standard radiation treatment of glioma includes whole brain irradiation of 60GY with conventional external beam radiotherapy following surgery in fractionated doses (Laperrier *et al.* 2002). High radiation doses using standard fractionation may pose a greater risk of radiation injury to normal brain without any survival benefits (Ricci. 1999). Only 10% of patients with glioblastomas and 44% of those with Grade III gliomas will survive for more than 2 years following their diagnosis (Burger *et al.*1985).

Radio-immunotherapy is a beneficial treatment technique as it aims at selectively destroying tumour cells by using radio-labelled monoclonal antibodies which target specific antigens expressed only by tumour cells (Westphal *et al.* 2003) while sparing

normal tissues. Diffusion tensor (DT) magnetic resonance imaging to track fibers is being used to help image guided tumour resection with decreased morbidity

1.11.2 Chemotherapy

Approximately, 45% of brain tumour patients treated with nitrosourea drugs respond clinically (Kornblith and Szypko, 1978). Despite treatment with surgery and radiotherapy, their inevitable recurrence makes high-grade gliomas the most devastating neoplasms, causing death. A number of randomized clinical trials have been tried to assess the role of chemotherapy in the improvement of survival for glioma patients (Lonardi *et al.* 2005). Various chemotherapeutic agents have been administrated before (neo-adjuvant), concomitantly or post radiotherapy (Stewart *et al.* 2001). The most influential randomized study was undertaken by the National Cancer Institute of Canada, which led to the establishment of concurrent temozolomide and radiation therapy (Stupp *et al.* 2005). The two major classes of chemotherapeutic drugs that are being used currently in the treatment of gliomas include the alkylating agents and microtubule modulations.

1.12 Alkylating agents

Alkylating agents were the first compounds identified to treat cancer (Espinosa *et al.* 2003). These compounds react directly with electron rich atoms in biological molecules to form covalent bonds. The chemotherapeutic and cytotoxic effects are directly related to the alkylation of DNA (Goeffrey *et al.* 2003). Temozolomide (TMZ) is an orally administrated alkylating agent and has excellent penetration power into the blood brain barrier. TMZ is the current standard treatment for anaplastic astrocytomas. TMZ is also being tested in combination with spectrum of other drugs extensively in low-grade gliomas (Berger *et al.* 2007). Nitrosoureas are other commonly used drugs in the

salvage treatment of gliomas as they are highly lipid soluble and readily cross the blood brain barrier (Walker *et al.* 1978). The main nitrosureas employed in glioma treatment are nimustine, carmustine and lomustine (Espinosa *et al.* 2003).

1.12.1 Alkylating antineoplastic agents

An alkylating anti-neoplastic agent is an alkylating agent used in cancer treatment and it attaches an alkyl group (C_nH_{2n+1}) to DNA. The alkyl group is attached to the guanine base of DNA, at the number 7 nitrogen atom of the imidazole ring. Alkylating agents were the first compounds to be identified for the treatment of neoplasms (Espinosa *et al.* 2003). Since cancer cells, in general, proliferate faster and with less error correcting than healthy cells, they are more sensitive to DNA damage (Berger *et al.* 2007). The alkylating agent drugs lead to an alteration in DNA structure by interfering in the transcription and replication process. Alkylating agents are used to treat several cancers. However, at the same time, they are also toxic to normal cells (cytotoxic) in the body. This can lead to damage, in particular in cells that divide frequently, as those in the gastrointestinal tract, bone marrow and ovaries, which can cause loss of fertility and other complications. The primary goal of these drugs, as stated above, is to either kill or inhibit proliferation of abnormal cells (Berger *et al.* 2007). This is best accomplished by administering the highest dose possible (one that does not endanger the life of the patient). Since these agents may present with severe toxicity, there are several agents whose dose may be limited by their toxicity. Since many of these agents act by different mechanisms, combination therapy is common. Additionally, since these drugs act by inhibiting cell division, the most common side effects are those that occur in areas of the body where cell replication occurs. These side effects may represent the dose limiting toxicity or some other toxic effect may limit the dose of a particular anti-neoplastic.

Anti-neoplastics, while sharing many of the same mechanisms of action, may exhibit varying degrees of efficacy in different neoplasias (Bernstein *et al.* 2007). This may represent differences in the growth cycle of the specific neoplasia or they can result from different uptake mechanisms that may limit the amount of drug that reaches the nucleus of the neoplastic cell. Anti-neoplastic drugs enter the cell by different mechanisms including active transport (often at sites for amino acids or other cell constituents such as choline) and passive diffusion. Changes in the uptake of the drug represent one form of resistance that may develop to anti-neoplastic therapy. Other forms of resistance include increases in glutathione production, which may serve as the site of drug action, reducing its effect on DNA, increased efficiency in DNA repair, increased metabolism of the drug and failure to express the p53 gene.

1.12.2 Temozolomide (TMZ)

Temozolomide (Temodal) is an alkylating agent derived from dacarbazine and first synthesised in 1984 (Stevens *et al.* 1993; Friedberg. 2001). Temozolomide (trade name: Temadol in Europe, Temador in the USA) is a new chemotherapy agent that has generated considerable interest as a treatment for glioma. Temozolomide is an interesting drug in part because its development (in England) was based on chemosensitivity assays using a wide variety of cultures of brain-tumour cells. Surprisingly, the other common chemotherapy agents used for brain cancer were developed, not on their effect in the laboratory on cultured brain tumour cells, but on cell cultures from other types of cancer. It is recommended for the treatment of patients with malignant gliomas showing recurrence or progression after standard therapy. FDA in the USA has approved TMZ for the treatment of glioma. It is easier to administer than other chemotherapeutic regimes for this indication and is given orally, once a day for 5

days in a 28-day cycle. It has high bioavailability and crosses the blood-brain barrier where it is spontaneously hydrolysed to its active form (Newlands *et al.*1992). It is toxic to cancer cells due to inhibition of tumour cell DNA replication. In a clinical trail, the combination of TMZ and radiotherapy showed more significant effect than radiotherapy alone. The 2 year survival rate was 10.4% with radiation alone and 26.5% with radiation and TMZ (Stupp *et al.* 2005). Its half-life is 1.83 hours (Newlands *et al.* 1992).

1.12.3 Structure of Temozolamide (TMZ)

Temozolomide (8-carbamoyl-3-methylimidazo [5,1-d]-1,2,3,5-tetrazin-4(3H)-one) is a bicyclic heterocycle and is chemically classed as an imidazotetrazinone (see figure 1.6) (Stevens *et al.* 1984). The defining characteristic of this class of compound is an imidazole ring that is fused with a tetrazinone ring system that contains three adjacently bonded nitrogen atoms.
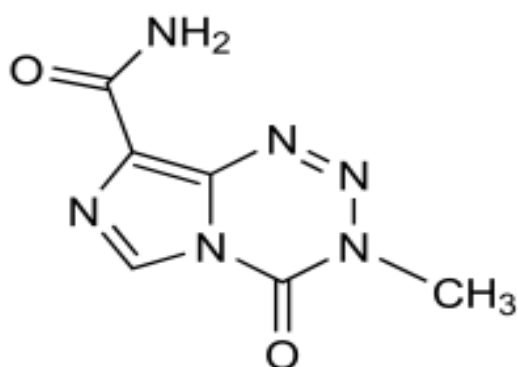
Figure1.6: Chemical structure of TMZ (Taken from Stupp *et al.,* 2005)

(Adapted from http://www.medicinescomplete.com/mc/clarke/images/clk1564c001.gif 2009)

TMZ is a 3-methyl derivative of mitozolomide. It is structurally similar to dacarbazine (DTIC), both producing the common active metabolite (3-methyl)-triazen-1-yl-imidazole-4-carboxamide (MTIC).

1.12.4 Mechanism of action of TMZ

To the malignant cells, the main mechanism for cytotoxicity of TMZ seems to be methylation of DNA (see figure 1.7). The effect of the water at highly electropositive c4 position of TMZ helps to convert it into methyldiazonium cation and AIC (Clark *et al.* 1990). Methylation occurs commonly at N7 position of guanine followed by methylation at O3 position of adenine and O6 position of guanine. The hypothesis is that the MMR cannot find the correct partner opposite to O6 MG. This in turn leads to the long lived nicks in DNA. These nicks accumulate and persist into subsequent cell cycle. Thus, replication of daughter cells are inhibited and eventually G2/M phase is blocked this leads to apoptosis of cells (Aoki *et al*, 2003; Taverna *et al.* 2000).
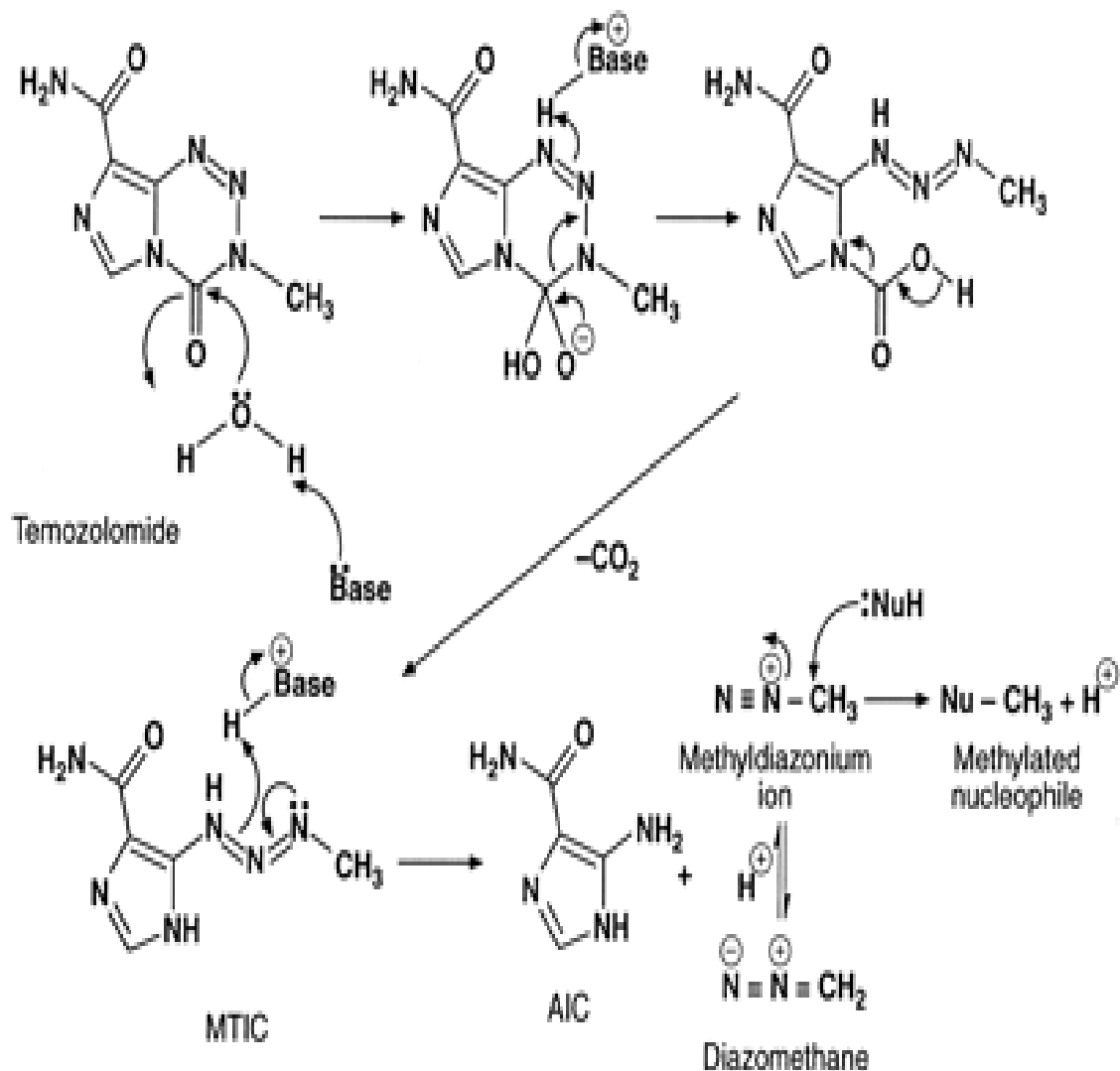
Figure 1.7: Mechanism of action of TMZ

(Adapted from http://theoncologist.alphamedpress.org/cgi/content/full/5/2/144/F1)

1.12.5 Mechanism of Resistance of TMZ

AGT, MMR pathway and PARP are the three different mechanisms of resistance to the TMZ.

a) AGT (O6-alkylguanine-DNA alkyltransferase): AGT plays the major role in resistance to the TMZ. The alkyl group is removed from the O6 position of

guanine and this in turn reverses the cytotoxicity lesion of the TMZ. BNCU and DTIC are correlated with the AGT levels (Tisdale, 1987).

b) MMR (miss match repair) pathway

The mutations in the one or more protein complexes make MMR pathway deficiency. Thus, cells can tolerate to the methylation and cytotoxic effects of the TMZ. This leads to the unrepaired O6 MG adducts produced by the TMZ causing DNA replication to pass the O6 MG adducts without apoptosis (Wedge *et al.*1996).

c) PARP (Poly (ADP-ribose) polymerase)

Base excision repair pathway or PARP is another mechanism of resistance. The cells treated with the TMZ induced an increase in PARP activity which is thought to be involved in the nucleotide excision repair (Wedge *et al.*1996). The cells deficient in the excision repair plays a major in the repairing of N7 methyl guanine and O3 methyl adenine adducts in resistance to TMZ (Tisdale *et al.*1985).

1.12.6 Introduction of Vinblastine

The Vinca alkaloids have become clinically useful since the discovery of their anti-tumour properties in 1959 (Nederman *et al.*1984). Vinblastine is a chemotherapeutic drug that belongs to the class of microtubule depolymerising agents and binds specifically to tubulin, inhibiting its polymerization and the subsequent association of microtubules (Yang *et al.* 2010). Vinblastine is mainly used to treat bladder cancer and to a lesser extent to treat other cancers including lymphoma and Kaposls sarcoma (BNF 58 edition, Perry book source 2007).

The anti tumour drug, vinblastine was analysed on the human tumour cell lines U-118 MG (glioma) and HTh 7 (Thyroid cancer). The cells were cultured as monolayers and exposed to different concentrations of 0.1, 1.0, 10 μg/ml of vinblastin for 15 min, 2 hrs,

24 hrs, respectively. The drug was found to induce a significant delay in cell growth (Nederman *et al.*1984).

Renal cell cancer (RCC) is characteristically unresponsive to chemotheraphy (Repchinsky *et al*. 2006). Vinblastine has been found to be one of the most consistently active agents, including objective responses in 7 % to 15 % of patients when administered as a single agent or in combination with other chemotherapeutic agents (Wen *et al.*2006, Robert and Rudee 1998). Vinblastine has some immunosuppressant effects. The vinca alkaloids are sometimes referred to as cycle phase specific (Stevens *et al.* 1993).

Some recent experiments have shown that mouse L-cells exposed to vinblastine in vitro accumulate in mitosis and concomitantly lose their proliferative capacity (Takemoto *et al.*1980). When such cells were exposed to vinblastine for one generation time, most of the cells lose their ability to produce colonies (Binet *et al.*1990).

1.12.7 Structure of vinblastine

Vinblastine sulfate has the molecular formula of $C_{46}H_{58}O_9N4 \bullet H_2SO_4$ and it is a dimeric alkaloid containing both indole and dihydroindole moieties (see figure 1.8). The molecular weight of vinblastine is 909.07. Vinblastine sulfate is a white to off-white powder and it is freely soluble in water, in methanol and slightly soluble in ethanol. It is insoluble in benzene, ether and naphtha.
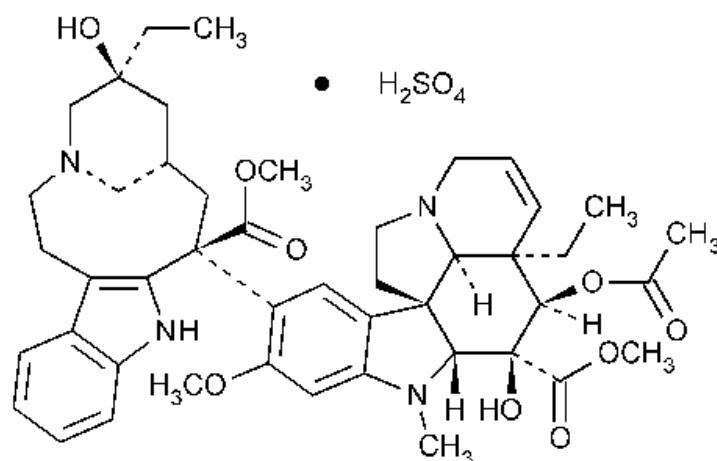
Figure 1.8: Chemical structure of vinblastine (Adapted from lookchem.com)

1.12.8 Mechanism of action of vinblastine

The mechanism of action of the Vinca alkaloids was initially thought to involve depolymerization of spindle microtubules and induction of paracrystalline tubulin-Vinca alkaloid arrays. At relatively high concentrations (micromolar), the Vinca alkaloids inhibit microtubule polymerization (Binet *et al*. 1990; Jordan and Wilson. 1999; Kruczynski *et al*. 1998). However, they also have a more subtle and powerful action on microtubules. They inhibit their dynamics at concentrations below those required to inhibit polymerization (Jordan *et al*. 2004; Yang *et al.* 2010

; Dhamodharan *et al*.1995). For example, low concentrations of vinblastine (8–32 nM) block mitosis in BSC-1 cells in association with suppression of microtubule dynamics, in the absence of appreciable changes in microtubule mass or spindle microtubule organization (Dhamodharan *et al*. 1995). Vinblastine inhibits chromosome congression at the prometaphase movement of chromosomes to the spindle equator and the transition from metaphase to anaphase, by binding with high affinity to microtubule ends and suppressing microtubule dynamics (Jordan and Wilson, 2004; Yang *et al.* 2010).

1.13 Lung carcinoma

Lung cancer is a disease of uncontrolled cell growth in tissues of the lung. This growth may lead to metastasis, which is the invasion of adjacent tissue and infiltration beyond the lungs. The primary lung cancers are carcinomas, derived from epithelial cells. Lung cancer is the most common cause of cancer-related death rates in men and women (Gorlova *et al.* 2007). Lung cancer remains the first cancer killer in 2009, claiming over 161,000 lives in UK, three times as many men as prostate cancer. Death rate is 39 % more than 2003 (Lung Cancer Alliance).

The main types of lung cancers are:

A. Non-small cell lung carcinomas (NSCLC)

B. Small cell lung carcinomas (SCLC; figure 1.9)
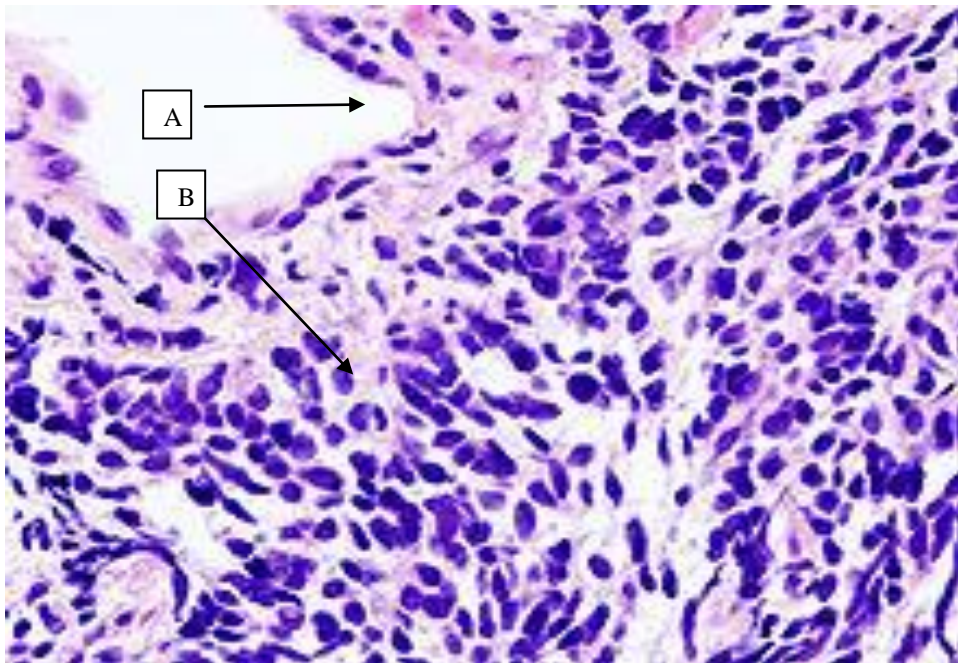
Figure 1.9: (a) Enlarged, irregular alveolar spaces. (b) Small cell lung carcinoma cells (600×452 pixels)

(Adapted from http://radiographics.rsna.org/content/22/6/1473.full 2004)
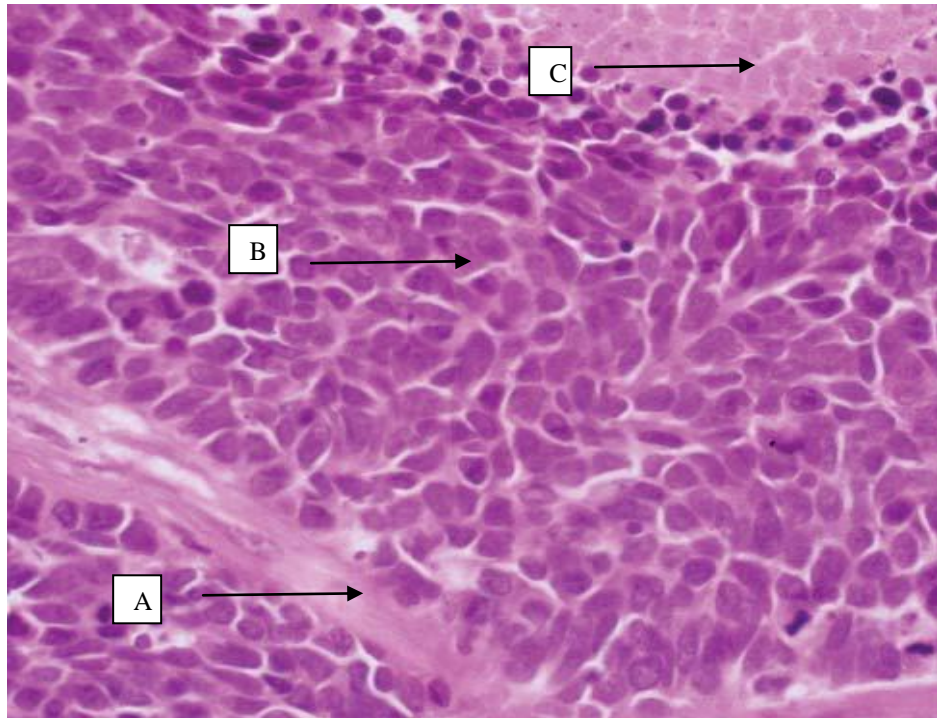
Figure 1.10: (a) Enlarged, irregular alveolar spaces. (b) Non-small lung carcinoma cells.

(c) Normal Lung cells (750 × 600 pixels)

(Adapted from http://radiographics.rsna.org/content/27/5/1777.full 2004)

1.13.1 Non-small cell lung carcinomas (NSCLC; figure 1.10)

NSCLC is sometimes treated with surgery, while SCLC usually responds better to chemotherapy and radiation (Vaporciyan *et al.* 2000). NSCLC are grouped together because their prognosis and management are similar (see figure 1.10). The three main sub-types are a. squamous cell lung carcinomas, b. adenocarcinomas and c. large cell lung carcinomas (Travis, 2002).

1.13.2 Squamous cell lung carcinomas

Squamous cell lung carcinomas usually start at the central bronchus and accounting for 25 % of lung cancers (Travis, 2002). A hollow cavity and associated necrosis are

commonly found at the centre of the tumours. Generally, squamous cell lung cancers often grow more slowly than other cancer types (Vaporciyan *et al.* 2000).

1.13.3 Adenocarcinomas

Adenocarcinomas usually originate in peripheral lung tissues and account for 40% of non-small cell lung cancers (Travis, 2002). Most cases of adenocarcinomas are associated with smoking (Subramanian *et al.* 2007). A subtype of adenocarcinoma, the bronchiole-alveolar carcinoma, is more common in female non-smokers and it may respond differently to treatment (Raz *et al.* 2006).

1.13.4 Large cell lung carcinomas

Large cell lung carcinomas are a heterogeneous group of indifferent malignant neoplasms originating from transformed epithelial cells in the lung (Blachhall *et al.* 2007). Large cell lung carcinoma comprise between 5% - 10% of all lung cancers. Large cell lung carcinomas are differentiated from small cell carcinomas primarily by the larger size of the anaplastic cells, a higher cytoplasmic to nuclear size ratio chromatin (Blachhall *et al.* 2007). At the cellular level, large cell lung carcinomas have a higher proliferation rate (Edelman *et al.* 2006). Large cell lung carcinomas are more frequently present as a peripheral tumour as opposed to typical, which are generally central in location. Garcia (2007) reported that two thirds of large cell lung carcinomas are present in the periphery of the lung parenchyma. Patients with large cell lung carcinomas are less likely to present with symptoms such as cough, haemoptysis or post obstructive pneumonia (Etienne *et al.* 2002).

1.13.5 Small cell lung carcinomas (SCLC)

Small cell lung carcinomas are less common (see figure 1.9). They were formerly referred as 'oat cell' carcinomas (Raz *et al.* 2006). Small cell lung carcinomas are strongly associated with smoking (Barbone *et al.* 1997). The cancer cells contained dense neurosecretory granules (neuroendocrine hormones) which give the tumour an endocrine paraneoplastic syndrome association (Rosti *et al.* 2006). Small cell lung carcinomas are more sensitive to chemotherapy and radiation therapy. Small cell lung carcinomas have been distinguished into limited and extensive stage disease (Borbone *et al.* 1997).

1.13.6 Diagnosis of lung carcinomas

Performing a chest radiograph is the first step suggested for lung cancer (Raz *et al.* 2006). Bronchoscope and CT scan may provide the necessary information. Either bronchoscope or CT guided biopsy is often used to identify the tumour type (Edelman *et al.* 2006). Sputum cytological examinations combined with other screening examinations have an important role in the early detection of lung cancer (Fan *et al.* 2009). The accurate differentiation of large cell lung carcinoma from other subtitle requires careful review of the pathologic specimen. Large cell lung carcinomas are frequently diagnosed preoperatively. The diagnosis of large cell lung carcinoma was ascribed to these tumours. In addition, neuroendocrine differentiations must also be confirmed by light microscopy and subsequently distinguished from typical carcinoma, atypical carcinomas and absence of necrosis or mitosis rate (Travis, 2002). Large cell lung carcinomas can be confirmed by immune-histochemical staining for neuroendocrine markers such as chrommogranin, synaptophysin, neuro- specific enloase and neuroendocrine differentation can be determined using ultra structurally

using electron microscopy (Krewski *et al.* 2005). Positron emission tomography (PET) is also used to determine the disease is localized (Strand *et al.* 2010).

1.13.7 Treatments of lung carcinomas

The treatments of SCLC and NSCLC are based on the cancer specific cell types and how far they have invaded other tissues and organs of the body. The most common treatments are surgery, chemotherapy and radiation therapy (Schiller *et al.* 2009). Surgery is usually only an option in non-small cell lung carcinomas limited to one lung. Treatment procedures include:-

Wedge resection   -   Removal of part of a lobe

Segmentectomy   -   Removal of an anatomic division of a particular lobe of the lung

Lobectomy   -   Removal of a one lobe

Bilobectomy   -   Removal of a two lobes

Pneumonectomy   -   Removal of a whole lung

In patients with adequate respiratory reserve, lobectomy is the preferred option (El-Sherif *et al.* 2007; Fernando *et al.* 2005).

a. Chemotherapy treatment

Small cell lung carcinomas and non-small cell lung carcinomas are treated primarily with chemotherapy and radiation. The combination regimen depends on the tumour type. Non-small cell lung carcinomas are often treated with either cisplatin or carboplatin and in combination with gemcitabine, paclitaxel, docetaxel, etoposide, or vinorelbine (Clegg *et al.* 2002). In small cell lung carcinomas, cisplatin and etoposide are most commonly used (Murray *et al.* 2006). Combination either with carboplatin, gemcitabine, paclitaxel, vinorelbine, topotecan or irinotecan is also used (Araujo *et al.* 2009). Radiotherapy is often given together with either chemotherapy. For both non-small cell lung carcinomas and small cell lung carcinomas patients are given smaller

doses of radiation to the chest as this may be used for symptom control (palliative radiotherapy). Unlike other treatments, it is possible to deliver palliative radiotherapy without confirming the histological diagnosis of lung cancers. Brachytherapy (localized radiotherapy) may be given directly inside the airway when the cancer affects a short section of the bronchus (Raben *et al.*1997). This treatment is used when it is not possible to operate on lung cancer due to blockage of a large airway lobe (Celebioglu *et al.* 2002).

1.14 Skin melanomas

The skin is the major organ of the body and it is made up of several layers including the epidermis and the dermis (see figure 1.11A). The main function of the skin is to protect the inner tissues of the body from external insults including chemicals, sun light, radiations, burns and others. Constant exposure to some of these external insults can lead to skin cancers. One such cancer is melanoma, which is the most dangerous form of skin cancer (Figure 1.11B). Melanomas are least common, with 10,672 new cases diagnosed in 2009. The statistics show that 5,697 cased were diagnosed in women and 4,975 in men. In 2009, there were 491 deaths in the UK, around 80 % occur in people aged 60 years and over. In their advanced states, they can cause serious illness and even death (Sabal and Tsai*,* 2008).

Skin melanomas

Structure of the Skin
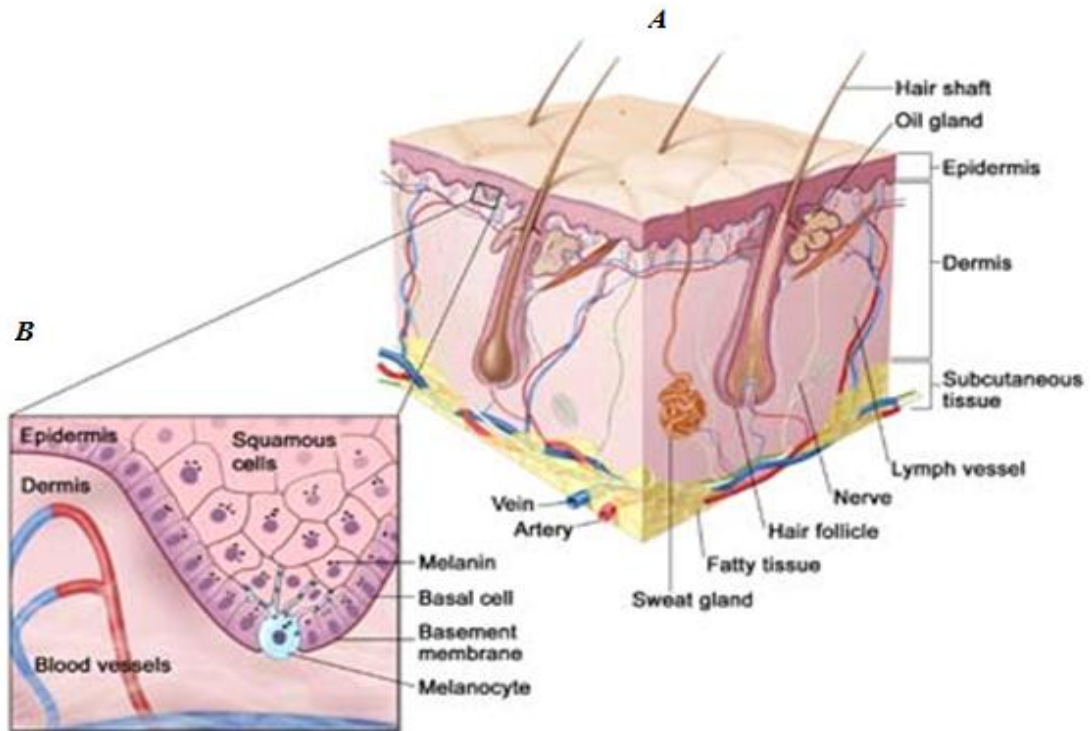
Figure 1.11: Diagrams showing (A) the structure of the skin and (B) the formulation of

skin melanoma.

(Adapted from http://img.Webmed.com/ March, 2005)

Melanomas are the most serious form of skin cancer. However, if they are recognized

and treated early, they can be cured by almost 100%. Melanomas of the skin are one of

the most rapidly increasing malignancies in both young and old patients because

patient's body may be less able to repair damage caused by exposure to ultraviolet (UV)

rays and intense intermittent sun exposure (Gachon *et al.* 2005). New methods of

imaging and targeting therapy are widely sought. Radio- iodinated methylene blue is a

promising tracer showing selective uptake in human pigmented melanoma cells (Sabal and Tsai. 2008). At present, the examination of melanomas is usually followed by biopsy of suspicious lesions and is the gold standard (Sabal and Tsai, 2008).

1.14.1 Classification of skin melanomas

Skin melanomas classified as-

A. **Radial melanoma** (about 70% of skin melanoma cases) – appears from dysplastic lesions. Melanoma progress takes up to 5 years and it can be detected by using the ABCD rule, specific for elderly ages. In early phases, melanomas spread in the upper layer of skin – epidermis (see figure 1.11). Later, it enters a vertical growth phase, after which the cancer becomes dangerous as it starts to invade the inner tissues (Cancer Research UK, 2007).

B. **Nodular melanoma** (about 15% of cases) – This type of melanoma rises very rapidly and it is the most aggressive type of skin cancers and it grows vertically up and down. Thus, there is a danger from the beginning that the melanoma may spread into inner tissues of the body. This type of melanoma appears to be either dark black, blue, grey or red with smooth borders (Cancer Research UK, 2007).

C. **Lentigoous malignant melanoma** -This type of melanoma usually appears in the head and the neck areas and it is bigger than 3 centimetres with a non symmetrical lesion. It takes a long time to progress – up to 20 years. Most cases usually occur in a patient of 70 over or under years of age (Cancer Research UK, 2007).

D. **Acral lentigous melanoma** – It is the most common type of melanoma for dark skinned people. It appears on the palms of the hands and the feet- especially

under the nail of the first finger. It takes a short time to progress from 3 to 36 months and it is most common among people who are 60 years of age and over (Cancer Research UK, 2007).

E. **Unclassified** – There are other types of carcinomas, which represent about 5% of cases. Generally speaking, all melanomas grow in two phases including horizontal-radial and vertical. In the radial phase, the lesion usually spreads in the epidermis and papillary dermis without metastases (see figure 1.11). In this period, the patient can easily cured. When the growth enters the vertical phase, the lesions usually spread in to deeper tissues through dermis and other deeper layers of skin. In this area, they usually begin to form metastases (Jemal *et al*. 2009; Rigel and Carucc,. 2003; Cockburn *et al*. 2009; Gachon *et al*. 2005).

1.14.2 Diagnosis of Skin Melanomas

Dermoscopy is a technique whereby the dermatologist is able to examine a lesion using a hand-held instrument called a dermoscope. This is equipped with a special polarizing light and standard magnifying optics. The lesion may or may not be placed in a fluid medium for better visualization. The technique allows evaluation of specific, minute pigment patterns, enhancing differential diagnosis (Morris *et al.*2008). Melafind is another non-invasive instrument that can assist in early diagnosis of melanomas by providing physicians with a recommendation for whether a suspicious lesion should be biopsied. It uses light of multiple wavelengths to capture images of suspicious skin lesions and compares the data against a database of melanomas and benign lesions (Vries *et al*. 2003).

Siascopy spectrophotometric intracutaneous analysis system (SSIAS) is yet another imaging process that may allow the Physician to distinguish the difference between

benign moles and melanomas. Like Melafind, it employs multiple wavelength illumination of clinical and dermoscopic images, but requires the Physician's interpretation. In contrast, Melafind provides diagnosis in a completely automated system (Lipsker *et al.* 1999). Other non-invasive diagnostic techniques are also in development and they include Confocal Scanning Screening (CSS), which is used to check people who are unaware of signs or symptoms (Vries *et al.* 2003).

Some melanomas may involve a total body examination by a Health Professional. Recently, various technologies have been introduced including digital photography to process where suspicious lesions are mapped on a whole body image (mole mapping). These methods identify lesions, which could be either melanomas or other forms of skin cancers (Vries *et al.* 2003).

Treatments

a. Radiotherapy for skin cancer

Skin cancer can be treated with both radiotherapy and chemotherapy. Radiotherapy uses high energy rays to kill cancer cells. It can be used to treat skin cancers that cover a large area or on an area of the body that is difficult to operate on or where the cosmetic result may be poor. It can also be used in people who are not fit enough for surgery. Radiotherapy may be given after surgery to lower the risk of the cancer coming back or to treat skin cancers that have invaded other areas of the body (Legha *et al.* 1989).

b. Photodynamic therapy (PDT) for skin cancer

Photodynamic therapy or PDT is a relatively new type of treatment. It is a treatment with a chemical that makes the skin cells sensitive to light. When the area to be treated is exposed to laser light, the cells die off (Hiller *et al.* 2001). PDT is an alternative

option to surgery. It is best used in cases where a lot of surgery is required. It is not suitable for deeper skin cancers because the light cannot penetrate far enough into the skin. PDT is not recommended for squamous cell skin cancers because there is too high a risk of the cancer returning. PDT is now available on the NHS for Bowen's disease, basal cell skin cancers and actinic keratosis (solar keratosis) (Agarwal *et al.* 2002).

Chemotherapy

Either chemotherapy tablets or injections are only used in certain circumstances for skin cancer. More often, chemotherapy creams are used. Either solar or actinic keratosis can sometimes develop into squamous cell skin cancer if it is not treated. Chemotherapy cream containing 5FU is a common treatment. Bowen's disease is also sometimes treated with chemotherapy cream. Chemotherapy tablets or injections are only used for skin cancers that have spread. This treatment is mostly used to relieve symptoms in cancers that cannot be cured (Legha *et al.*1989).

1.15 Chemical structures of compounds of *M. charantia* with some commercial available anti-cancer drugs for comparison.

M.charantia has many different chemical components, which help medicinally either alone or when combined. Charantin has a molecular weight of 9.7 kDa and it is the belief that charantin is the active parent agent of *M. charantia* (Taylor, 2002). Figures 1.12 and 1.13 show the chemical structures of some medicinal compounds including charantin (MW 548), momordicine, α momorcharin (MW 576), β momorcharin (MW 576.), diterpene (MW 397), triterpenoids (MW 532) extracted from *M.charantia* compared to some commercially available anti-cancer drugs including vincristine (MW 923), vinblastine (MW 909.), temozolomide (MW 194), cisplatine (MW 300) and pacitaxel (MW 853).

A. Diterpene
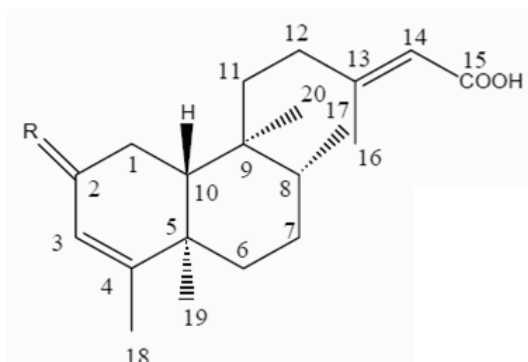
B. Vinblastine
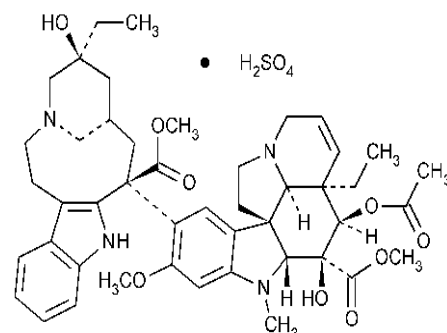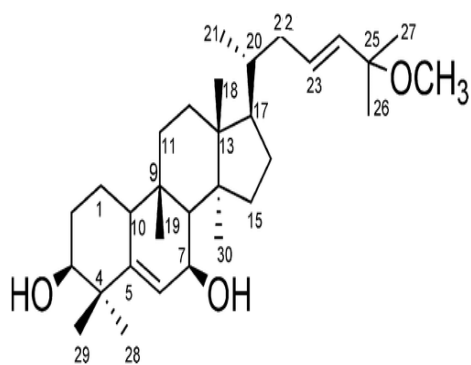
C. Triterpenoids
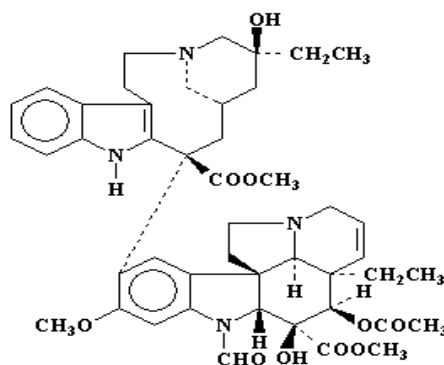
D. Vincristine
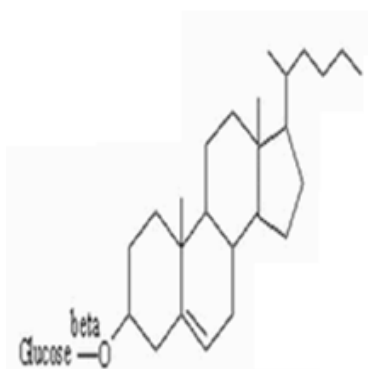
E. Charantin

F. Pacitaxel
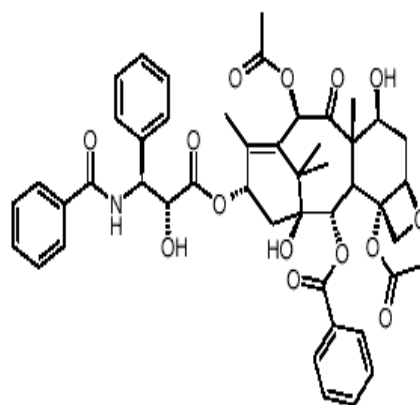
Figure 1.12: Chemical structures of (A) Diterpene, (B) Vinblastine, (C) Triterpenoids, (D) Vincristine, (E) Charantin and (F) Pacitaxel.

A. Momordicine
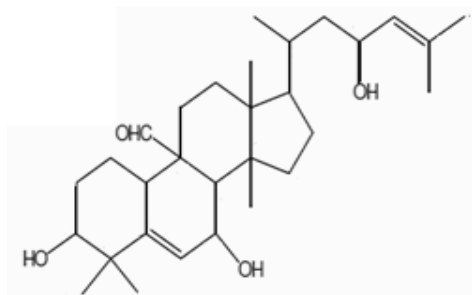
B. Temozolomide
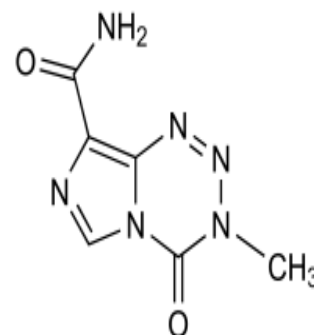
C. Alpha momorcharin
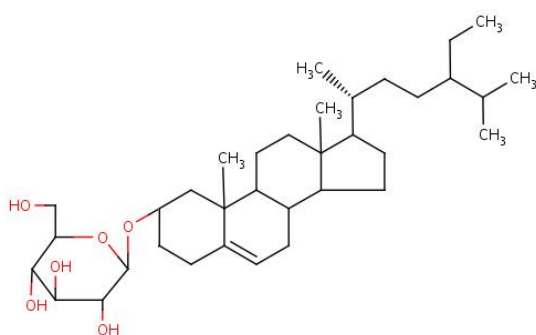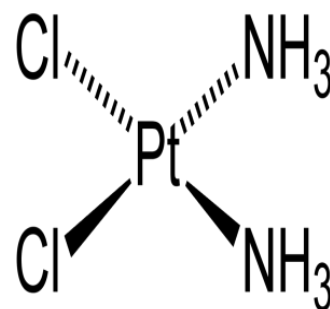
D. Cisplatin

E. Beta-momorcharin
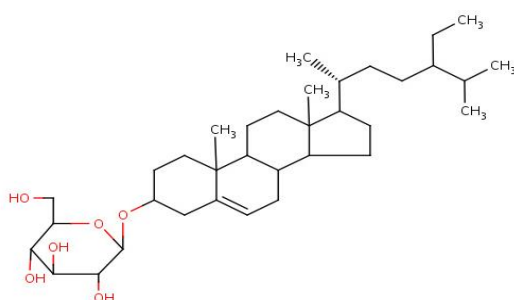
Figure 1.13: Chemical structures of (A) Momordicin, (B) Temozolomide, (C) Alpha momorcharin (D) Cisplatin and (E) Beta momorcharin.

1.16 Cellular and sub-cellular mechanisms associated with cell death

1.16.1 Introduction

There are different mechanisms via which chemotherapeutic drugs can exert their effects on cancer cell death. These include apoptosis involving permanent damage to the mitochondria leading to the release of cytochrome-c, caspase-3, caspase-9 and cellular calcium over load (Kerr *et al.* 1972). This study measured the activities of cytochrome c, caspase-3 and caspase- 9 as well as intracellular free calcium concentrations $[Ca^{2+}]_i$ in different treated and untreated cancer cell lines compared to L6 cell line. Thus, emphasis will be placed on processes which involve the metabolism of these cellular markers and the methods involved in their measurements.

Apoptosis was originally described as a mechanism of controlled or programmed cell death (Kerr *et al.* 1972). Apoptosis is very common in cells and tissues with hematopoietic activity and in organs with high proliferative activity. Apoptosis has been implicated in the progression of a number of pathological conditions, including cancers, AIDS and autoimmune diseases (Ameisen *et al.* 1995).

Apoptosis is characterized by a variety of cellular changes including loss of membrane phospholipid asymmetry, mitochondrial swelling and DNA cleavage (Vermes *et al.*1995; Darzynkiewicz *et al.*1992). The result of these changes is a form of cell death that avoids the normal inflammatory response associated with necrosis.

1.16.2 Cytochrome-c and its assay

Cytochrome-c is a small heme protein found loosely associated with the inner membrane of the mitochondrion. Cytochrome-c is a highly soluble protein, unlike other cytochromes with a solubility of about 100 g/L (Tris-acetate-phosphate medium) and is

an essential component of the electron transport chain. Cytochrome-c is capable of undergoing oxidation and reduction (Vidal *et al.* 2002).

1.16.3 Role of apoptosis

Cytochrome-c is an intermediate in apoptosis, which is a controlled form of cell death in the process of development or in response to infection or the induction of DNA damage of any apoptotic programme in cell free extracts (Chandra *et al.* 2002; Johnstone *et al.* 2002). NADPH-cytochrome c reductase (NADPH cytochrome P450 reductase, EC 1.6.2.4) is a flavoprotein localized in the endoplasmic reticulum (ER) of the cell. It transfers electrons from NADPH to several oxygenases**.** The most important of which is the cytochrome P450 family of enzymes, which are responsible for xenobiotic metabolism (Shen *et al.* 1993; Plonne *et al.* 1999). NADPH-cytochrome c reductase is widely used as an ER marker-3 and as a biomarker of ecological pollution and dietary lipid uptake (Vidal *et al.* 2002; Hochgraf *et al.*1997). Cytochrome-c is released by the mitochondria in response to pro-apoptic stimuli. Normally calcium levels are elevated and this in turn is preceded by the release of cytochrome-c from the mitochondria. The small amounts of releasable cytochrome-c lead to an interaction with the inositol triphosphate (IP_3) receptor on the ER causing it to release calcium. The increase in cellular free calcium triggers a massive release of cytochrome-c which then maintains ER calcium release through the inositol 1, 4, 5-triphosphate receptors (IP3RS). ER calcium release can reach cytotoxic levels and cause calcium overloading.

The release of cytochrome-c in turn enhances the activity of caspase-9, a cysteine protease. Cytochrome-c assay kit is designed to measure the NADPH cytochrome-c reductase activity in cell and in purified microsomes of the ER. The cytochrome-c measurement is based on a colorimetric assay that monitors the reduction of cytochrome-c by NADPH- cytochrome-c reductase in the presence of NADPH. The

reduction of cytochrome-c results in the formation of distinct bands in the absorption spectrum and the increase in absorbance at 550 nm is measured with time (Vermillion and Coon, 1974).

1.16.4 Caspase-3 and its assay

Caspases (Cysteine-requiring aspartate proteases) are a family of proteases that mediate cell death. It is also important to the process of apoptosis. Caspase-3 is a member of the CED-3 subfamily of caspase and is one of the critical enzyme of apoptosis. Caspase-3 processes procaspase 2, 6, 7 and 9 and specifically cleaves most of caspase- related substrates. Many key proteins including nuclear enzyme activate poly (ADP-ribose), and polymerase (PARP) (Nicholson *et al.* 1995), the inhibitor of caspase-activated deoxyribonuclase (ICAD) gelsolin and fodrin, which are the proteins, involved in the apoptosis regulation (Sakahira *et al.*1998). This cleavage is part of the mechanism leading to cell death. Caspase-3 plays a central role in mediating nuclear apoptosis including chromatin condensation and DNA fragmentation as well as cell blebbing. The activity of caspase- 3 in cells and tissues of the body is either a marker or indicator of cell death (Porter and Janicke, 1999).

Caspase-3 colorimetric assay is based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp p-nitroanilide (AC-Devo-PNA) by Caspase-3, resulting in the release of the p-nitroanilide (PNA) moiety. The p-nitroanilide has a high absorbance at 405 nm ($\sum^{mM} = 10.5$). The concentration of PNA released from the substrate is calculated from the absorbance values at 405 nm or from a calibration curve prepared with defined PNA solutions.

Caspase 3

Ac-DEVD-pNA ⟶ Ac-DEVD + pNA
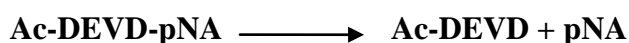
Caspase-3 is a crucial component of the apoptotic machinery in many cell types. The activation of caspase-3 is a central event in the process of apoptosis (Thornbery and Littlewood, 1998; Wolf *et al.* 1999).

1.16.5 Caspase – 9 and its assay

There are four different apoptotic pathways, which are associated with cell death leading to the elevation in caspase-3 and caspase-9. They include.

A. Caspase -9 and caspase-3 dependent apoptotic pathway.

B. Caspase -9 and caspase-3 independent apoptotic pathway.

C. Caspase -9 independent and caspase -3 dependent apoptotic pathway.

D. Caspase-9 dependent and caspase -3 independent apoptotic pathway (Hakem *et al*.1998).

Caspase-9, also known as ICE –Lap6, Mch6, is an upstream proenzyme in the cascade of enzymatic reactions required to induce cellular apoptosis (Duan *et al.* 1996). During the release of mitochondrial cytochrome-c, and caspase-9 activated following their association with the protein complex of Apaf and cytochrome-c. The active caspase-9 is generated through protein phosphoylation events and the optimal cleavage recognition sequence for caspase-9 is Leu-Glu-His-Asp-7 (LEHD). The caspase-9 is associated with both pro-apoptotic and anti-apoptotic proteins as well as its regulatory function during embryonic development. This suggests that caspase-9 is a key regulatory enzyme of apoptosis (Thornberry *et al.*1997; Petty *et al*. 1995). The Caspase-Glo® 9 assay is a homogenous luminescent assay that measures caspase-9 activity. Addition of a single Caspase-Glo®-9 reagents in an 'add-mix measure' formant resulting in cell lysis. This

is followed by caspase cleavage of the substrate and the generation of a glow type luminescent signal produced by the luciferase reaction. The signal generated is proportional to the amount of caspase activity present. Figure 1.14 shows the process involved in the measurement of caspase-9 in cells and tissues.
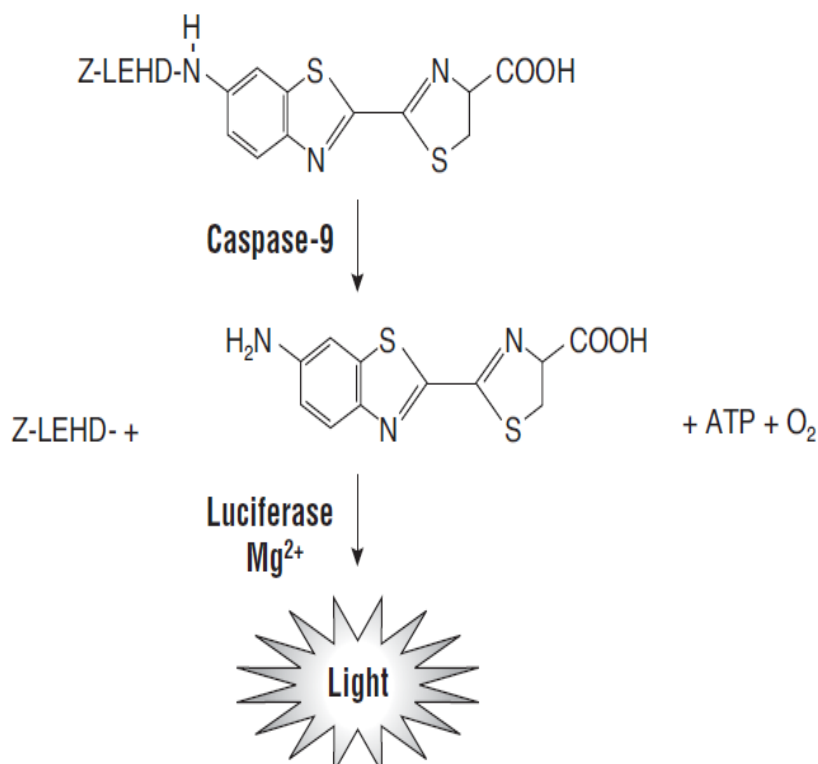
Figure 1.14: Caspase-9 cleavage of the luminogenic substrate containing the LEHD sequence. (Adapted from www.promega.com/automethods/ April 2004)

1.16.6 Luminescent assay

Luminescent assay for ATP was developed to measure cell viability. The high level of ATP will produce a high level of luminescence and this indicates the number of viable cells. A lower luminescence value is an indication of a lower level of ATP release from cells due to cell death or low cell viability. The assay is more sensitive and accurate. The colorimetric assay for ATP has simplified the sensitivity testing. The sensitivity of the assay is limited by the number of cells and there ATP levels. The assay is most

efficient from 100 to 200 cells per wells. The ATP assay is based on the principle of bioluminescence and measures ATP in live cell. The process involves the interaction of a substrate luciferin with ATP in the presence of an enzyme extracted from the firefly called luciferase, which produces light. Firefly, luciferase extracted from Photinus pyralis is a 62 KDa enzyme responsible for the yellow green bioluminescence of this species (Tisi *et al*. 2002). Firefly luciferase is high-sensitivity bio-detection. Its light emitting assay is fast, rapid and accurate and moreover, it can generate consistent results very quickly. Firefly luciferase is a single polypeptide chain that catalyzes the moo-oxygenation of the compound of beetle luciferin (LH_2) in an ATP-dependent fashion to give oxyluciferin (LO) (Tisi *et al*. 2002). The nascent LO is in the excited state that delays to its ground state releasing a photon (Jeffrey *et al.*1986).

$$\textbf{LH}_2 + \textbf{ATP (Mg}^{2+}) + \textbf{O}_2 = \textbf{LO} + \textbf{CO}_2 + \textbf{PPi} + \textbf{AMP} + \textit{hv}$$
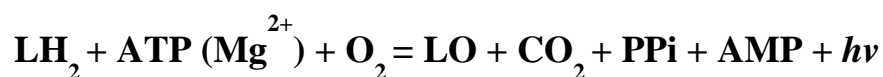
Figure 1.15 (Adapted from www.promega.com/automethods/ May 2009)

The reaction (Figure 1.15) is extremely efficient with a photon emitted for nearly every LH_2 molecule consumed, giving a quantum yield of close to 0.9 as shown in figure 1.14 (Tisi *et al.* 2002). The emitted photon is measured quantitatively in a luminometer, and thus, the luminescence is directly proportional to the amount of ATP and hence, the number of live cells (Di *et al.*2002).

Another ATP assay is the CellTiter-Glo™ Luminescent Cell Viability Assay (Promega®) which is a homogeneous method of determining the number of viable cells in culture based on quantification of the ATP present, which signals the presence of metabolically active cells. The CellTiter-Glo® Assay is designed for use with 96 and 384 multi well-plate formats, making it ideal for automated high-through put screening, and cell proliferation and cytotoxicity assays. The CellTiter-Glo® Assay relies on the

properties of a proprietary thermo stable luciferase (Ultra-Glo™ Recombinant luciferase), which generates a stable glow-type. Luminescent signal improves performance across a wide range of assay conditions. The luciferase reaction is shown below in figure 1.16.
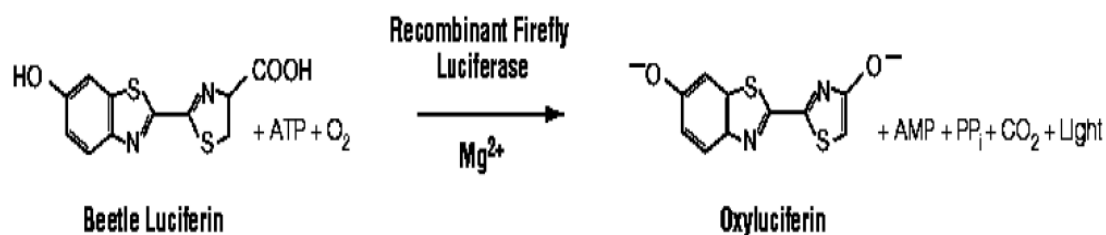
Figure 1.16: Ultra-Glo™ Recombinant luciferase reaction

(Adapted from www.promega.com/automethods/ May 2009)

The half-life of the luminescent signal resulting from this reaction is greater than five hours. This extended half-life eliminates the need for reagent injectors and provides flexibility for continuous or batch-mode processing of multiple plates (Promega, USA).

1.16.7 MTS   (Colorimetric cell viability assays)

MTS assay is most popular colorimetric assay in which the compound 3-(4, 5-dimethyl thiazol-2yl)-5,(3-carboxymethoxyphenyl)-2(4sulfophenyl)-2H(tetrazolium) is converted to a coloured insoluble formazan product by mitochondrial enzymes. The end product is solubilised in either alcohol or detergent and the absorption measured at 490 nm (Lewandowicz *et al.* 2000; Nikkhah *et al.* 1992). The amount of colour produced is directly proportional to the number of live cells. MTS assay has been used in several investigations for the measurement of chemo-sensitivity testing in malignant gliomas (Nikkhah *et al.* 1992). The enzymatic reactions (see figure 1.17) are based on either the

oxidase and dehydrogenase reactions, which are determined either calorimetrically or fluorimetrically using different reagents including oxidative chromogenic dyes or reductive chromogenic dyes. Tetrazolium salts are one of the reductive chromogenic dyes employed in the measurement and it is a very sensitive detection system.
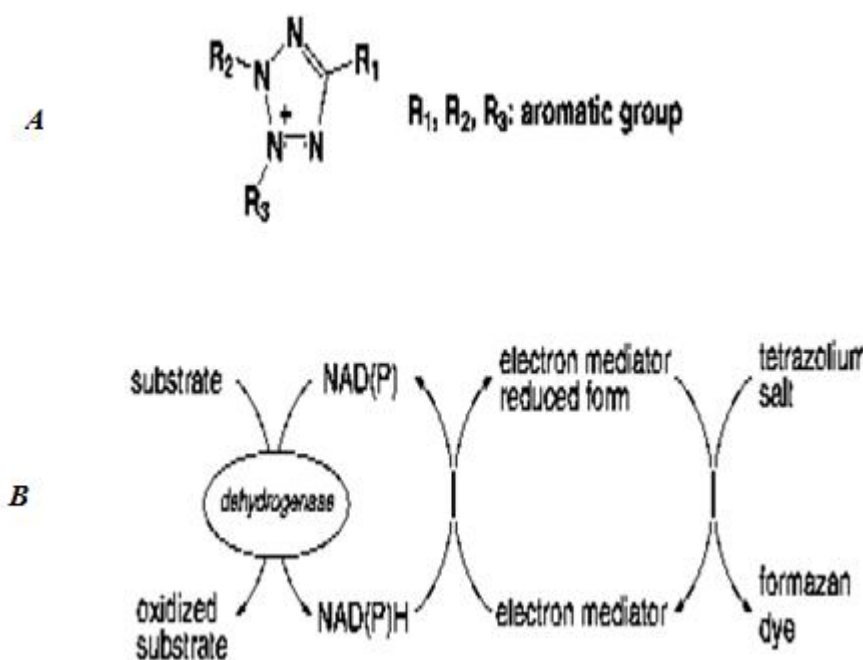
Figure 1.17: (a) Structure of tetrazolium salt and (b) electron transfer mechanism for

MTS assay   (Adapted from www.promega.com/automethods/ April 2007)

The tetrazolium salt is generally reduced through the reaction directly with either

NADH (nicotineamide adenine dinucleotide reduced form) or NADPH (nicotineamide

adenine dinucleotide phosphate reduced form), which is generated, from either NAD or

NADP by the reaction of dehydrogenase and its substrates, lactate dehydrogenase and

lactic acid. Therefore, the tetrazolium salt is utilized for the determination of

dehydrogenase activity (Berridge *et al.* 1996). The CellTiter 96 AQueous is a cell

proliferation assay solution, which is based on a colorimetric method for the

determination of viable cells in proliferation or in cytotoxicity assay. The assay solution contains a novel tetrazolium compound [3-(4,5-dimethylthiazol-2yl)- 5- (3-carboxymethoxyphenyl)-2–(4-sulfophenyl)-2H-tetrazolium:MTS] and an electron-coupling reagent (phenazine ethosulfate; PES). PES has enhanced chemical stability, which allows it to be combined with MTS to form a stable solution. MTS tetrazolium compound is bio- reduced by cells into a coloured formazan product in tissue culture medium. Figure 1.18 below shows the MTS formazan reaction.
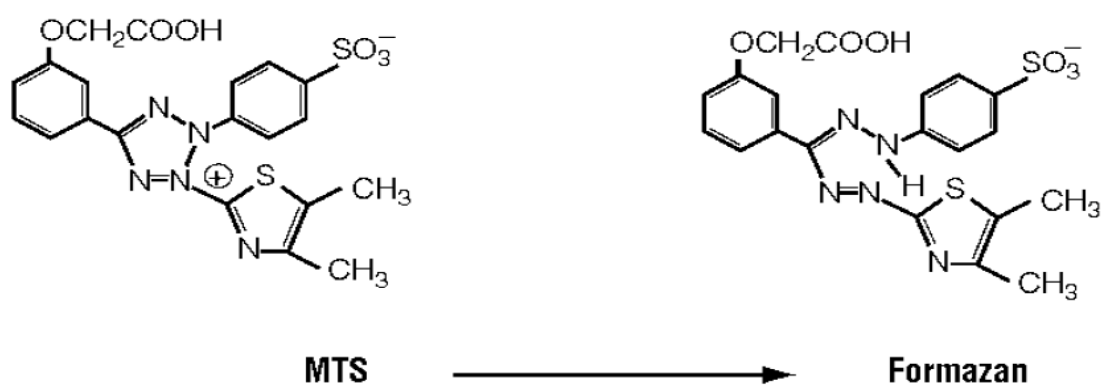
Figure 1.18: The molecular structure of MTS and formazan

(Adapted from www.promega.com/automethods/ April 2007)

The amount of the formazan product is measured at an absorbance range of 490 nm and this is directly proportional to the number of living cells in culture. The MTS formazan product is soluble in tissue culture medium.

1.16.8 Calcium (Ca^{2+})

Calcium is the most important physiological cation in cellular regulation (Koolman *et al*. 2005; Johansson *et al*. 2003). It is the trigger, the promoter and the regulator and moreover, a ubiquitous intracellular signalling molecule which controls a wide range of

cellular processes including secretion, membrane transport, contraction, cell proliferation, gene transcription and even cell death (Bootman and Berridge. 1995; Clapham, 2007). In un-stimulated pancreatic acinar cells, as well as in other cells, the free intracellular calcium concentration $[Ca^{2+}]_i$ is between 50–100 nM. In order to maintain this low resting level, a variety of pumps and uptake systems are present in the plasma membrane and in intracellular organelles to buffer (Ca^{2+}) (Williams *et al*. 1993). Calcium mobilisation is dependent upon intracellular calcium stores as well as extracellular calcium medium $[Ca^{2+}]_o$. An increase in cellular calcium originates from two major sources:

a. The release of Ca^{2+} from intracellular stores (ER), which is rapid. Calcium release from the ER after IP_3 generation is crucial for exocytosis. Similarly the release of Ca^{2+} from SR is crucial in muscle contraction.

b. The influx of Ca^{2+} from extracellular source with the aid of IP_3 by the depletion of an intracellular pool (Putney, 1989; Irvine, 1989).

Both of these mechanisms give rise to high-localised (Ca^{2+}) signals. Many agonists can promote an influx of Ca^{2+} from the extracellular medium by the following processes:

a. Opening of voltage-operated Ca^{2+} channels

b. Phosphorylation of Ca^{2+} channel proteins by cAMP and cGMP.

c. Receptor-operated Ca^{2+} channels in which modulation of channel activity does not involve cytosolic signal molecules but a direct control by receptor channel coupling G- proteins (Berridge and Irvine, 1989).

1.16.9 Calcium signalling

The intracellular level of $[Ca^{2+}]_i$ in resting cells is maintained within a normal range of 50 -100 nM. Ca^{2+} homeostasis is tightly controlled to prevent and overcome problems of cytotoxicity due to its very low diffusibility in the cytoplasm. Distributed throughout the cytoplasm is an extensive array of Ca^{2+} pumps (Irigoin *et al*. 2009; Carafoli, 1994). There are two main intracellular mechanisms which increase $[Ca^{2+}]_i$. They include

a. Ca^{2+} release from internal stores (eg ER, SR, mitochondria) via intracellular calcium channels, activated via the ryanodine receptors (RYRs), and the inositol 1, 4, 5- triphosphate receptor (IP$_3$R).

b. Ca^{2+} entry from the outside $[Ca^{2+}]_o$ through a variety of channels such as the VOCs, ROCs or store- operated channels (SOCs).

Cytosolic Ca^{2+} homeostasis in resting cells is achieved by balancing the leak of Ca^{2+} (entering from the outside of from the stores) by the constant removal of Ca^{2+} using pumps either on the plasma membrane or on the internal stores. These pumps ensure that cytoplasmic $[Ca^{2+}]_i$ remains low and that the stores are loaded with signal Ca^{2+}. In most cells, it is the internal stores (eg ER, SR, mitochondria) which provide most of the signal calcium (Streb *et al*. 1983). Activation of the IP$_3$Rs is also regulated by cytosolic (Ca^{2+}) concentrations. Intact and internally per-fused cells have evidently shown that an increase in $[Ca^{2+}]_i$ can inhibit IP$_3$ -evoked Ca^{2+} release (Wakui *et al*. 1990; Parker and Ivorra. 1990) where opening of the IP$_3$R is enhanced by low concentration of Ca^{2+}. This is crucial in the generation of complex patterns of Ca^{2+} signals seen in many cells (Bootman and Lipp, 2001). Unlike the RYR, where Ca^{2+} can act as the sole trigger for its activation, IP$_3$ Rs usually requires the simultaneous presence of Ca^{2+} and IP3 (Berridge, 1987).

1.16.10 Calcium over load and cell death

Many studies have demonstrated mitochondrial Ca^{2+} overload as the link between complement deposition and the observed changes in mitochondrial physiology and the triggering of programmed cell death (PCD) (Irigoin *et al*. 2009). The mitochondrial Ca^{2+} overload is responsible for the increased O(2)(*-) production (Jimenez and Hernandez-Cruz, 2001). If cytosolic Ca^{2+} rise is not accompanied by the accumulation of the cation in the mitochondrion and consequent production of O(2)(*-), cells usually die by necrosis instead of PCD. For example, membrane attack complex assembly (MACA) on the parasit in cell surface allows Ca^{2+} entry in the cell and its accumulation in the mitochondrion can lead to O2 production. This in turn constitutes a PCD signal (Jimenez and Hernandez-Cruz, 2001).

Based mainly on *in vitro* observations, one currently popular model consistent with excitotoxic apoptosis, proposes that mitochondrial Ca^{2+} overload triggers an injury response, possibly through a mitochondrial permeability transition (MPT) that leads to the loss of permeabilization of the inner mitochondrial membrane, swelling of the matrix and outer membrane rupture, followed by the release of apoptogenic proteins (Bernardi *et al*. 2001). Such mechanisms present a paradox, in that the destruction of mitochondria impairs the ATP supplies that are essential for activation of energy-dependent apoptotic pathways (Gogvadze *et al.* 2004). It has also been suggested that only a subpopulation of mitochondria undergoes a permeability transition and releases apoptogens, whereas the remaining, undamaged mitochondria respire normally and produce ATP (Simpson *et al*. 2002; Kabir *et al.* 1999; Jimenez and Hernandez-Cruz , 2001).

Loss of Ca^{2+} homeostasis, often in the form of cytoplasmic increases, leads to cell injury. Depending upon the cell type and the intensity of Ca^{2+} toxicity, the ensuing

pathology can be either reversible or irreversible (Gogvadze *et al.* 2004). Although multiple destructive processes are activated by Ca^{2+}, lethal outcomes are determined largely by Ca^{2+} induced mitochondrial permeability transition (Johansson *et al.* 2003; Goldstein *et al.* 2000). This form of damage is primarily dependent upon mitochondrial Ca^{2+} accumulation, which is regulated by the mitochondrial membrane potential (Gogvadze *et al.* 2004). Retention of the mitochondrial membrane potential during Ca^{2+} favours mitochondrial Ca^{2+} uptake and overload, resulting in mitochondrial permeability transition and cell death. In contrast, dissipation of mitochondrial membrane potential reduces mitochondrial Ca^{2+} uptake, retards mitochondrial permeability transition, and delays death, even in cells with large Ca^{2+} increases. The rates of mitochondrial membrane potential dissipation and mitochondrial Ca^{2+} uptake may determine cellular sensitivity to Ca^{2+} toxicity under pathological conditions, including ischemic injury (Gogvadze *et al.* 2001).

1.17 Working hypothesis

Is it possible to use naturally occurring plant-based medicines to treat cancer? Are they effective enough to kill the cancer cells compared to commercially available anti-cancer drugs? Is it possible to use a higher dose of the plant-based medicines with a low to moderate dose of the commercially available anti-cancer drug in order to obtain maximal and effective treatment without adverse side effects to the body? By what cellular and sub cellular mechanism(s) do these anti-cancer compounds exert their effects on cell death?

1.18 Aims

The main aim of this study was to investigate the effects of extracts and isolated compounds of *M. charantia* as well as commercially available drugs vinblastine and temozolomide on cancer cell viability and to determine their modes and cellular mechanism(s) of action as chemotherapeutic agents in cancer therapy.

1.18.1 Objectives

1) To isolate crude water and methanol soluble extracts of *M. charantia* using different extraction, analytic and biochemical methods.

2) To use a tissue culture technique to measure the cytostatic and anti-tumour activity by *in vitro* study employing in the cell culture medium.

3) To determine the time-course effects of the crude water-soluble extract of *M. charantia* on cancer cell viability.

4) To investigate the dose-dependent toxic effects of the extracts and isolated and purified compounds (alpha momocharin, beta momocaharin, alpha, beta

momocharin) of *M. charantia,* and vinblastine and temozolomide on cancer cell viability.

5) To investigate the combined effects of the crude water-soluble extract of *M. charantia* and alpha, beta momocharin with either temozolomide or vinblastine.

6) To investigate the modes and cellular and molecular mechanisms of action of each the crude water-soluble extract of *M. charantia* and alpha, beta momocharin   in inducing cell death measuring caspase-3 and caspase-9 activities, cytochrome-c release and cytosolic calcium.

Chapter Two

Materials and Methods

Materials and Methods

2.1 Materials

2.1.1 Consumables required for tissue culture

1. Cell lines:

   1321N1 (ECACC, UK), Gos-3 (DSM2, Germany), U87-MG (ECACC, UK), L6,

   WERI-Rb1 (ECACC, UK), SK-Mel (DSM2, Germany), Corl-23 (ECACC, UK)

2. Chemicals and Reagents:

   1. Ethanol, methanol and isopropanol, hexane (Fisher Scientific, UK).

   2. Cell titer-Glo luminescent cell viability Assay kit (Promega, UK).

   3. MTS assay (Promega, UK).

   4. Fura-2 (AM) (Sigma, UK).

   5. Caspase-3 assay kit (Sigma, UK).

   6. Cytochrome-c assay kit (Sigma, UK).

   7. Caspase-9 assay kit (Promega, USA).

3. Drugs

   1. Crude water and methanol soluble extracts of *M.charantia*.

   2. α and β momocharin (IMAM Pharmaceuticals, China)

   3. α momocharin (IMAM Pharmaceuticals, China)

   4. β momocharin (IMAM Pharmaceuticals, China)

   5. Temozolomide (Sigma, UK)

   6. Vinblastine (Sigma, UK)

2.1.2 Media and supplements required for the cell culture

DMEM (Doulbeco's Modified Eagle's Medium), MEM (Minimal Essential Medium), RPMI-1640 (Roswell Park Memorial Institute), Foetal bovine serum (FBS), Trypsin, L-Glutamine, Non-essential amino acid (NEAA) and Sodium pyruvate (all the products bought from Lonza, UK).

2.1.3 Equipment and Materials used in the study

New Brunswick Scientific (CO281R) - Water jacketed CO_2 incubator, Water bath , Laminar flow hood, Inverted phase contrast microscopes, Weighing balance, Tecan plate reader, Refrigerator, Freezer (-20°C), Deep Freezer (-80°C), Centrifuge machine, Electrical aspirator, Vortex mixture, 2, 20, 200 and 1000 μl micro pipettes and pipette tips, Motorized Pipette Controller, 75 cm^2/25 cm^2 Culture flasks, sterile forceps, aluminium foil, 5 and 10 ml disposable plastic pipettes, 15 ml and 50 ml centrifuge tubes, Cryovials, "Mr. Frosty" freezing container and liquid nitrogen, DMSO, clear 96-well white plates, 0.22 μm and 0.44 μm sterile filters, syringes, pasteur pipettes, Universal bottles, funnel 5 ml Volumetric flask, sterile spatula and clear bottom white 96 well plates (Nunc or Grenier).

2.1.4 Composition of medium for cell lines

i. **1321N1** Dulbecco's Modified Eagle Medium (DMEM-500 ml)**,** 10% Foetal bovine serum (FBS-50 ml)**,** 2 mM L-glutamine (5 ml).

ii. **U-87 MG** Minimal Essential Medium (MEM-500 ml), 10% Foetal bovine serum (FBS-50 ml), 2 mM L-glutamine (5 ml),1% Non Essential Amino Acids (NEAA- 5 ml), 1 mM Sodium Pyruvate (5 ml).

iii. **Gos-3** Dulbecco's Modified Eagle Medium (DMEM-500 ml), 10% Foetal bovine serum (FBS- 50 ml), 4 mM L-glutamine (10 ml).

iv. **L6 muscle cell line** Dulbecco's Modified Eagle Medium (DMEM-500 ml), 10% Foetal bovine serum (FBS-50 ml), 4 mM L-glutamine (10 ml).

v. **Sk Mel** Minimal Essential Medium (MEM-500 ml), 10% Foetal bovine serum (FBS-50 ml), 1mM Sodium Pyruvate (5 ml), 1% Non Essential Amino Acids (NEAA- 5 ml).

vi. **Corl-23** RPMI-1640 (Roswell Park Memorial Institute), 2 mM L-glutamine (5 ml). 10% Foetal bovine serum (FBS-50 ml).

vii. **Weri-Rb-1** RPMI-1640 (Roswell Park Memorial Institute), 2 mM L-glutamine (5 ml). 10% Foetal bovine serum (FBS-50 ml).

2.1.5 Ethical consent

Ethical and health and safety consents were granted by the Ethics Committee of the School of Pharmacy and Biomedical Sciences at University of Central Lancashire (UCLAN).

2.2 Methods

2.2.1 Extraction of crude water-soluble extract of *M. charantia*

The unripe green fruits of *M. charantia* were obtained from the local market and subsequently cleaned and cut into small pieces (Karunanayake *et al.*1984). Approximately one kilogram of chopped green fruit was liquidized in distilled water for 5-10 min using a blender. The juice was then kept in a hot water bath for 2 hrs at the temperature of 67°C. The fruit juice was centrifuged at 5000 RPM (Beckman, UK) for 30 min. The suspension was removed and filtered through Whatmann filter paper (No: 4 Whatmann, UK). The filtered green sample was then transferred to the 1000 ml round bottom rotating flask. The flask was then connected to the Rota evaporator machine through a clamp. The rotating flask was then heated by partial emersion in a hot water

bath at a temperature of 40°C. A typical 120 rpm speed was used for the flask rotation. The rota evaporated sample was then scrapped using spatula and dried overnight in an oven at 43°C. This crude water-soluble extract (powder) was stored at 2°C for further use.

2.2.2 Extraction of crude methanol soluble extract of *M. charantia*

The unripe green fruits of *M.charantia* were obtained from the local market and cleaned and cut into small pieces (Karunanayake *et al.*1984) and oven dried at 50°C about 48 hrs until it reached a constant weight. The dried sample was then pulverized with a grinder into fine homogenous powder, which was stored at 2°C until used. An amount 40 gm of the fine ground sample was extracted with 300 ml of methanol in a flask placed in an ultrasonic bath, containing water and the temperature was maintained at 65°C for 90 min. The sample was cooled to room temperature and centrifuged at 2000 rpm for 20 min. The suspension was removed and double filtered through Whatmann filter paper (No: 4 Whatmann, UK). The filtered sample was then transferred to the 500 ml round bottom rotating flask. The flask was then connected to the Rota evaporator machine through a clamp. The rotating flask was then heated by partial emersion in a hot water bath at a temperature of 50 °C. A typical 100 rpm speed was used for the flask rotation. The Rota evaporated sample was then scrapped using spatula and stored at 2°C until used.

2.2.3 Extraction method for either of alpha or beta momorcharin

In this study alpha, beta or alpha beta momorcharin was purchased as a purified compound from IMAM International Group Pharmaceutical Company in China. According to the literature, the Company extracted, isolated and purified each compound using the following procedure. The whole fruit of bitter gourd was ground and homogenized in 2 mM sodium phosphate buffer, pH 7.5. The resulting slurry was

then stirred for 3 hrs to extract the crude proteins (Feng *et al*. 1996). The insoluble component from crude proteins was removed by the filtration and centrifugation at 30,000 x g for 1 hour at 48°C. By using 2 mM sodium phosphate buffer, pH 7.5, the crude protein solution was dialysed. The dialysed protein sample was applied to DEAE Sepharose column equilibrated with 2 mM sodium phosphate buffer at pH 7.5. The unbound proteins was then applied to Mono-S column which was equilibrated by 2 mM sodium phosphate buffer at pH 7.5 and eluted by 0.5 m of NaCl (Fong *et al*.1996).

The fraction corresponding to either alpha and beta or alpha, beta momorcharin, which was confirmed the N-glycoside activity RNA, was concentrated and dialysed against 20 mM Tris-HCl buffer, pH 7.8. The chromatography was performed on Bio Logic DuoFlow system (BioRad, Hercules, CA) at 48°C. The purity of alpha and beta or alpha, beta momorcharin was examined by SDS-PAGE and gel filtration chromatography. The concentration of alpha momorcharin was determined by spectrophotometrically using optical absorbance at A280 nm.

2.2.4 Extraction method of beta momorcharin

The whole fruit of bitter gourd was ground and homogenized in 2 mM sodium phosphate buffer, pH 7.5. The resulting slurry was then stirred for 3 hrs to extract the crude proteins (Fong *et al.* 1996). The insoluble components from crude proteins was removed by the filtration and centrifugation at 30,000 x g for 1 hour at 48°C. By using 2 mM sodium phosphate buffer, pH 7.5 the crude protein solution was dialysed. The dialysed protein sample was applied to DEAE Sepharose column equilibrated with 2 mM sodium phosphate buffer at pH 7.5. The unbound protein was then applied to Mono-S column, which was equilibrated by 2 mM sodium phosphate buffer at pH 7.5 (Fong *et al.* 1996).

The fraction corresponding to beta momorcharin, which was confirmed by the N-glycoside activity RNA was concentrated and dialysed against 20 Mm Tris-hydrochloride buffer, pH 7.8. The chromatography was performed on BioLogic DuoFlow system (BioRad, Hercules, CA) at 48°C. The purity of beta momorcharin was examined by SDS-PAGE and gel filtration chromatography. The concentration of beta momorcharin was determined spectrophotometrically using A280 nm.

2.3 Cell culture

2.3.1 Passaging of the Cancer cell lines and Control cell line

The culture medium, phosphate buffer solution (PBS), and trypsin (sterile) were removed from the fridge at 4^oC and subsequently placed in the water bath at 37^oC for 30 min in order to equilibrate. The Laminar flow hood was turned on for 15 min, prior to start of the experiment, in order to purge the air inside the cabinet and to reach the maximum cleanliness.

The different cancer and normal cell lines were incubated at 37°C incubator in an atmosphere of 5% CO_2 in air. The cells were examined under the inverted contrast microscope to note the both confluence and general health of the cells. The flask was passaged when the cells had reached 70-80% confluence.

The medium was aspirated from the cultured flask and was washed with sterile PBS (5 ml if 75 cm^2 flask and 2 ml if 25 cm^2 flask) in order to remove any traces of serum from the cells. This prevented the serum from inactivating the trypsin which was used to detach adherent cells from the cell clump. Trypsin solution (2 ml if 75 cm^2 flask or 1 ml if 25 cm^2 flask) was pipetted in the flask and incubated at 37°C in an incubator in an atmosphere of 5% CO_2 in air for 3-5 mins until the cells began to detach. The detachment was confirmed by observing at intervals under an inverted microscope. The

cells were left in the trypsin solution for the correct length of time. If the cells were left for a longer period of time then this would lead to damage of the cells. A volume of 3 ml complete growth medium was then added to the flask to inactivate the trypsin and the cells were pipetted up and down to break up any large cell aggregates. The cell suspension was transferred from flask into 15 ml centrifuge tube and centrifuged at 1000 rpm for 5 min. Following centrifugation, the supernatant was aspirated and the cells were pellet at the bottom of the centrifuge tube. Based upon the cell pellet density volumes of 1 ml to 3 ml fresh medium were suspended in the centrifuge tube. The cell pellet was flicked properly in the medium containing 20 µl of trypsinised cell suspension and 80 µl of tryphan blue (used to detect dead cells in the cell suspension 1:5 ratio). The contents were mixed well together and a haemocytometer test was performed using 1 ml of cell suspensions. This process helped to assess the total number of the cell suspension present in the centrifuge tube and which was required to make 1 or 2 flasks and to do 96 well plates. Thereafter, the cells were frozen in liquid nitrogen depending on the number of cells present per ml. The cell suspension was divided in either one or several flasks (depending on the cell density) and fresh growth medium (10 ml to 12 ml if 75 cm^2 flask and 5 ml if 25 cm^2 flask) was added to the flasks. These were then placed in a 5% CO_2 incubator to continue cell growth.

2.3.2 Cell Counting Method

A volume of 20 µl of cell suspension and 80 µl of tryphan blue (1:5 ratio) were pipetted into a microcentrifuge tube and mixed together. A coverslip was gently pushed over the chambers of a haemocytometer and 20 µl of cell suspension was slowly pipetted against each short side of the coverslip so that the suspension could spread into each chamber. The haemocytometer was placed onto the stage of an inverted phase contrast microscope and focused on the central 25 squares of one chamber. The numbers of cells

in these squares were counted. These steps were repeated for the other chambers. The average number of cells in the centre grid (1 mm^2) of each chamber was calculated. This number was multiplied by 10^4 to obtain the number of cells per 1 ml of suspension. The total number of cells was calculated by multiplying the number of cells per 1 ml by the total volume of the cell suspension.

2.3.3 Cryopreservation of cells

The cell suspension obtained during passaging was divided into stocks and they were either stored for long periods or cryopreserved. This involved slowly freezing the cells down in culture medium supplemented with a cryoprotectant, which helped to protect the cells from rupture due to formation of ice crystals. The most commonly used cryoprotectant employed in this study was dimethyl sulphoxide (DMSO). The cell suspension of 900 μl was mixed with 100 μl of DMSO. Together, they contained approximately 1 million cells, which were subsequently frozen at -80°C. The cell populations normally survived the freezing process with high cell viability. This was done by the use of a freezing medium that consisted of the normal culture medium supplemented with 10% DMSO. However, it was common practice to increase the proportion of serum in the medium up to 90%. The most important principle of cryopreservation involved the cells being frozen down slowly at a rate of 1-3°C and thawed out quickly in a 37°C water bath. The vial container was also frozen in a bath of isopropyl alcohol called "Mr. Frosty", which mediated a 1°C/min cooling of the cryovials in a -80°C freezer prior to storage at a temperature below -135°C in either vapour or liquid phase nitrogen. The isopropyl alcohol could only be used 5 times before it needed replacing.

2.4 Cell Viability Experiments

2.4.1 Preparation and application of crude water-soluble and methanol soluble

extracts of *M. charantia* on the cancer and L6 cell lines.

An amount of 30 mg of the crude water-soluble extract of *M. charantia* was initially

dissolved in 500 µl of phosphate buffer by continuous stirring and with the brief use of

a sonicator water bath. This was made up to 5 ml by adding 4.5 ml of the cell medium.

The water-soluble crude extract stock solution was transferred to a 10 ml syringe and

sterile filtered using 0.22 µm filters into other sterile 10 ml Universal bottles. These

stock solutions were stored in a sealed tube in the fridge until required. Once removed

from the fridge, the prepared crude water-soluble extract of *M. charantia* solutions were

gently warmed in water bath at 37°C in order to ensure that the water-soluble and

methanol soluble crude extract was mixed complete in solution, before aliquoting.

Volumes of 34 µl, 68 µl, 102 µl, 136 µl contained 200 µg, 400 µg, 600 µg, and 800 µg

of the crude water-soluble extract of *M. charantia* respectively. Different concentration

of either water or methanol soluble crude extract in cell medium was transferred in

triplicate using a Gilson pipette to 96 well plates to give a final volume of 200 µl to the

treated cell wells. An equivalent volume of 200 µl of the medium was added to the

control (untreated) well with cells. In this study, both time course and dose-dependent

experiments were performed. The time-course experiments were done over a period of

48 hours, where the dose dependent experiments were done during incubation period of

24 hrs.

2.4.2 Time-course experiments

Cell suspensions of either 1321N1, Gos-3, U87-MG, Sk Mel, Corl -23, Weri Rb-1 or L6

obtained during passaging were diluted (1:10 ratio). The required volumes of cell

suspensions and fresh medium volumes were calculated based on the number of wells to be plated. The dilution was done such that 200 μl of each suspension contained 2500 cells. After seeding the cells, the plates (transparent 96 well plates) were incubated for 6, 12, 18, 24 and 48 hrs at 37°C in a 5% CO_2 incubator. After each time period the media from 96 well plates were tipped out completely on sterile tissue paper. The drug stock solution was transferred to a 10 ml syringe and sterile filtered using 0.22 μm filters into another sterile 15 ml centrifuge tube. A volume of 100 μl of cell medium (sterile filtered) contains 800 μg of crude water-soluble extract of *M. charantia* was then added to the 96 well plates. The 96-well plates were incubated for 6, 12, 18, 24 and 48 hours at 37°C in a 5% CO_2 incubator. In another series of control experiments, the different cancer cell lines were incubated in 96 well plates for the same period but in the absence of any extract of *M. charantia.* Following every 6 hrs of incubation, the plate for either test and control was removed from incubator for 30 min. MTS procedure was carried out in the transparent 96 well plates (Griner, UK). A volume of 20 μl of MTS assay sample was added to wells containing both treated and untreated cells. The ratio resulted in a final concentration of 317 μg/ml MTS assay in the assay wells. Each plate was then placed in the incubator at 37°C in a 5 % CO_2 atmosphere for 90-120 min. The absorbance's was measured at a 492 nm wavelength, and 30 sec of shaking time and 30 sec for the settling time in the plate reader. The absorbances of the crude extract alone at different time points in the medium were also measured as background sequence. These values were subtracted from the test values at the different time points. The same was also done for the dose-dependent experiments or a combination of extracts with drug.

2.4.3 Dose - dependent experiments

In this series of experiments, the experimental procedure was the same except that the test cells were incubated with different concentrations of each extract for 24 hrs. Initial

experiments established maximum cell viability at 24 hrs of incubation with the crude water-soluble extract of *M. charantia.*

Cell suspensions of either 1321N1, Gos-3, U87-MG, Sk Mel, Corl -23, Weri Rb-1 or L6 obtained during passaging were diluted (1:10 ratio). The required volume of cell suspension and fresh medium volume were calculated based on number of wells to be plated. The dilution was done such that 200 µl of the suspension contained 2500 cells. After seeding the cells, the plates (transparent 96 well plates) were incubated for 24 hrs at 37°C in a 5% CO_2 incubator. Control 96 well plates were incubated for 24 hrs in the absence of extracts. After 24 hrs the media from 96 well plates were tipped out completely on sterile tissue paper. The drug stock solution was transferred to a 10 ml syringe and sterile filtered using 0.22 µm filters into another sterile 15 ml centrifuge tube. These stock solutions were stored in a sealed tube in the fridge until required. A volume of 100 µl of cell medium (sterile filtered) contained extract concentrations of 200 µg, 400 µg, 600 µg, 800 µg in the 96 well plates. After the supplement of the crude extract, the 96-well plate was again incubated for 24 hrs at 37°C in a 5% CO_2 incubator.

Following 24 hrs of incubation, each plate was removed from incubator for 30 min. MTS procedure was carried out in the transparent 96 well plates (Griner, UK). A volume of 20 µl of MTS assay sample was added to wells containing both treated and untreated cells. The ratio resulted in a final concentration of 317 µg/ml MTS assay in the assay wells. The plate was then placed in the incubator at 37°C in 5 % CO_2 atmosphere for 90-120 min. The absorbance was measured at a 492 nm wavelength by initial 30 sec of shaking time and 30 sec of settling time of the plate in the plate reader.

2.4.4 Preparation and application of either alpha or beta momorcharin and alpha, beta momorcharin on the cancer and L6 cell lines.

Amounts of 14.51 mg, 29.25 mg, 43.53 mg, 58.50 mg, and 72.57 mg of either alpha or beta momorcharin and alpha, beta momorcharins (9.7 kDa) were weighed out separately in 5 ml universal vials and initially dissolved in 500 µl of phosphate buffer by continuous stirring and with the brief use of a sonicator water bath. These were then made up to 5 ml by adding 4.5 ml of the cell medium to give concentrations 200 µM, 400 µM, 600 µM, 800 µM, 1000 µM, respectively. The drug (extract) stock solution was transferred to a 10 ml syringe and sterile filtered using 0.22 µm filters into another sterile 10 ml Universal bottles. These stock solutions were stored in a sealed tube in the fridge until required. Once removed from the fridge the prepared drug (extract) solutions were gently warmed in water bath at 37°C in order to ensure that the alpha, beta momorcharins or alpha and beta momorcharin was in a complete solution, before aliquoting. Volumes of 40 µl, 80 µl, 120 µl, 160 µl, 200 µl contained 200 µM, 400 µM, 600 µM, 800 µM, 1000 µM, respectively. Different concentrations of either alpha beta momorcharins and alpha, beta momorcharin were transferred in triplicate using a Gilson pipette to 96 wells plate and the volume made to 200 µl by adding the cell media to both treated and control cell wells. Both control (untreated) and treated with (either alpha, beta and alpha, beta momorcharin) 96 well plates were incubated for 24 hrs. After the incubation period cell viability was measured as described above.

2.4.5 Dose dependent effects of either TEM or vinblastine on cancer cell line viability

In this series of experiments, different cancer cell lines (1231N1, Gos-3, U87-MG, Weri Rd-1, Corl-23, Sk Mel) and healthy L6 muscle cell line were incubated with the different concentrations of either TEM (80 - 320 µM) or vinblastine (10 - 40 µg) for 24

hours. Control cell lines were also incubated for the same period of time but without any TEM or vinblastine. At the end of the incubation period, cell viability of each cell line was measured using the MTS assay as described below.

2.5 Combined effects of either temozolomide or vinblastine with the crude water-soluble extract of *M. charantia* or alpha, beta momorcharin

Since both the commercial anti-cancer drugs and either the crude water-soluble extract of *M. charantia* or alpha, beta momorcharin could elicit significant decreases in cell viability, it was decided to investigate the combined effects of these anti-cancer agents (drug + extract) on cell viability. Different cancer cell lines (1231N1, Gos-3, U87-MG, Weri Rd-1,Corl-23, Sk Mel) and healthy L6 muscle cell line were incubated with either temozolomide (240 µM) and the water-soluble extract of *M. charantia* (800 µg) or temozolomide (240 µM) and (800 µM) of the alpha, beta momorcharin for 24 hours. Similarly, each cell was incubated with either vinblastine (30 µg) and the water-soluble extract of *M. charantia* (800 µg) or vinblastine (30 µg) and (800 µM) of the alpha, beta momorcharin for 24 hours. Control cell lines were also incubated for the same time but without any drug or extract. At the end of the incubation period, cell viability of each cell line was measured using the MTS assay.

2.5.1 Measurement of caspase-3 assay

The six different cancer cells lines were incubated with either 800 µg crude water-soluble extract of *M. charantia* or 800 µM alpha, beta momorcharin. The induced apoptosis cell suspension contained roughly 10^7 cells. A sample of non-induced cells for a zero-time (control) also contained 10^7 cells. The induced and non-induced cells were incubated for 24 hrs at 37°C in 5 % CO_2 atmosphere. The induced cells and the control cells were transferred to 15 ml centrifuge tube and centrifugation at 600 x *g* for 5 min at

4 °C. The supernatant was removed by gentle aspiration for both induced and control cell suspensions. The cells were then pelleted and washed once with 1 ml of phosphate buffer solution (PBS) and centrifuged at 5000 rpm for 5 min and the supernatant was completely removed by gentle aspiration. The centrifuged cell pellets were then treated with 1 X lysis buffer at a concentration of 100 µl per 10^7 cells, and the cells were incubated on a ice for 15-20 min. The lysed cells were centrifuged at 16,000 to 20,000 x g for 10 to 15 min at 4 °C and the supernatants were transferred to new 1 ml tube and subsequently frozen in liquid nitrogen and stored in aliquots at -70°C for further use (see table 2.1 for reaction scheme). All the values were expressed as µmol/min/ml.

2.5.2 Determination of protein concentration by Bradford assay

The protein concentration of each sample was measured based on the method of Bradford (Bradford, 1976). Bovine serum albumin (BSA) stock solution was diluted with double distilled water concentrations of 2 µg/ml, 3 µg/ml, 4 µg/ml, 5 µg/ml, 6 µg/ml, 7 µg/ml, 8 µg/ml, 9 µg/ml and 10 µg/ml for the standard solutions. In flat bottom transparent 96 well plates volumes of 20 µl of dye reagent and 80 µl of either BSA or protein sample added. The mixtures were rota mixed for 30-40 sec and kept in an incubator for 10 min. The absorbance of each standard was measured at 595 nm wavelength recorded and generated as a standard curve. The protein concentration for each sample was determined by extrapolating the intersection of the standard curve at a point corresponding 2 to 10 µg/ml. All values were expressed as µg/ml.

Test Samples	Cell lysate	Caspase-3 5µg/ml	1x Assay buffer	Caspase-3 inhibitor Ac-DEVD-CHO 200 µM	Caspase-3 substrate Ac-DEVD-pNA 2mM
Reagent blank	-----	-----	90µl	-----	10 µl
Non-induced cells	5 µl	-----	85 µl	-----	10 µl
Non-induced cells + inhibitor	5 µl	-----	75 µl	10 µl	10 µl
Induced cells	5 µl	-----	85 µl	-----	10 µl
Induced cells + inhibitor	5 µl	-----	75 µl	10 µl	10 µl
Caspase -3 positive control	-----	5 µl	85 µl	-----	10 µl
Caspase -3 positive control + inhibitor	-----	5 µl	75 µl	10 µl	10 µl

Table 2.1: Reaction scheme for 96 well plate microassay method.

(Adapted from www.Sigmaaldrich.com, April 2006)

2.5.3 Calibration curve of P-nitroaniline (PNA)

A series of p-nitroaniline solutions was prepared at a concentration range of 10 to 200 µM by diluting the p-nitroaniline stock solution in the 1 X Assay Buffer (see table 2.2 for reaction scheme). A volume 100 µl in duplicate was added into 96 well plates, including 100 µl of assay buffer as a blank. The absorbance was measured at 405 nm.

The calibration curve was plotted using absorbance values versus the concentrations of the p-nitroaniline solutions.

μM p-Nitoaniline	μmol p-Nitroaniline
10	0.001
20	0.002
50	0.005
100	0.01
200	0.02

Table 2.2: Reaction scheme for calibration curve of p-nitroaniline

Calculation.

Caspase 3 activity was calculated in μmol pNA released per min per ml of cell lysate or Positive control based on the formula:

$$\text{Activity, μmol pNA/min/ml} = \frac{\text{μmol pNA} \times d}{t \times v}$$

Where:

v - Volume of sample in ml, d - Dilution factor, t - Reaction time in minutes.

2.5.4 Measurement of Caspase -9 activity
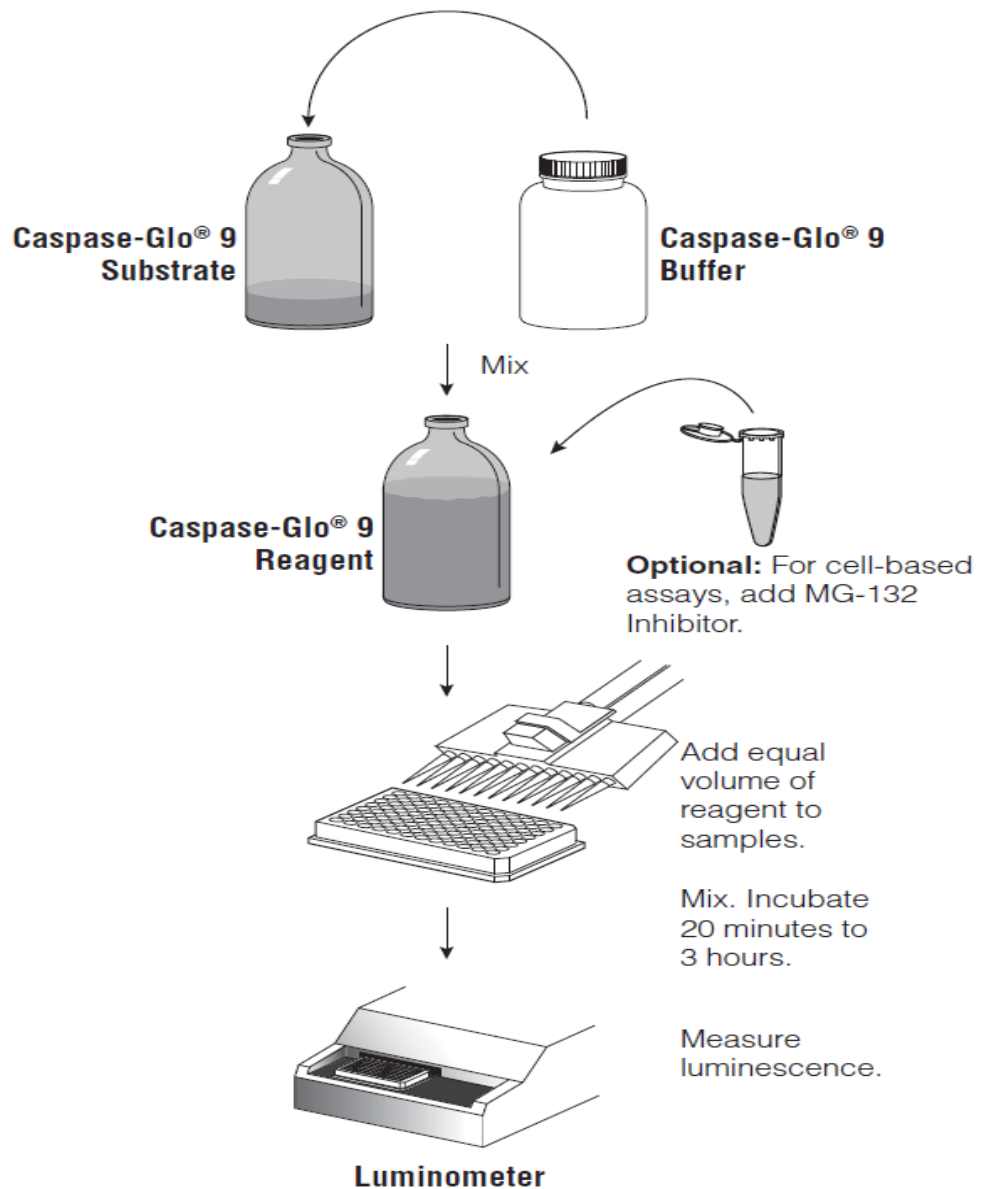
Figure 2.1: Schematic diagram of the Caspase-Glo® 9 Assay protocol

(Adapted from www.promega.com/automethods/2004)

The six different cancer cells lines and L6 cell line were incubated with either 800 μg

crude water-soluble extract of *M. charantia* or 800 μM alpha, beta momorcharin. The

induced apoptosis cell suspension contained roughly 10^7cells. A sample of non-induced

cells for a zero-time control also contained 10^7cells. The induced and non-induced cells

were incubated for 24 hrs at 37°C in 5 % CO_2 atmosphere. Prior to starting the assay the prepared sample of Caspase-Glo® 9 reagents was equilibrated to room temperature. After 24 hr prior to the experiment, each plate (96 well plates GRE 96 fb) was removed from the incubator for 30 min to equilibrate to room temperature (see the figure 2.1 for protocol). A volume of 100 µl of Caspase-Glo® 9 assay reagent was added to each well of a white walled 96 well plate containing 100 µl of blank and treated cells in the culture medium. The plate was then covered with the aluminium foil. The covered plate was then mixed gently by using a plate shaker at 300-500 rpm for 2 min. The plate was then incubated at room temperature for 30 min. The Caspase-Glo® 9 assay was carried in the absence of light. The Luminescence was measured on a Techan Plate reader with the appropriate settings for the plate. The software used for the Luminescence assay was XFLUORHGENIOSPRO version V 4.53. The plate type used from the software was specified as GRE 96 fb Pdf. The temperature was stated to be 20 - 23°C. All the values were expressed as µmol/min/ml.

2.5.5 Measurement of cytochrome-c release

The six different cancer cells lines and L6 cell line were incubated with either 800 µg crude water-soluble extract of *M. charantia* or 800 µM alpha, beta momorcharin. The induced apoptosis cell suspension contained roughly 10^7 cells. A sample of non-induced cells for a zero-time control also contained 10^7 cells. The induced and non-induced cells were incubated for 24 hrs at 37°C in 5 % CO_2 atmosphere. The induced cells and the control cells were transferred to 15 ml centrifuge tube and centrifugation at 1000 x *g* for 5 min at 4 °C. The supernatant was removed by gentle aspiration of both induced and control. The cell pellets were then washed once with 1 ml of PBS. The microsomal pellet was subsequently obtained by centrifuging at 12,000 x *g* supernatant for 1 hour at 100,000 x *g* and the supernatant was removed completely by gentle aspiration. The

centrifuged cell pellets were then treated with 1 X lysis buffer at a concentration of 100 µl per 10^7 cells, and the cells were incubated on ice for 15-20 min. The lysed cells were centrifuged at 16,000 to 20,000 x g for 10 to 15 min at 4°C and the supernatants were transferred to new 1 ml tube and subsequently frozen in liquid nitrogen and stored in aliquots at -70°C for further use. Table 2.3 showed the experimental protocol for the cytochrome-c assay. A volume of 950 µl of the working solution (9 mg of cytochrome-c to 20 ml of assay buffer to get a concentration of 0.45 mg/ml, 36 mM) was made up by adding 9 mg of cytochrome-c to 20 ml of the assay buffer in a 1 ml cuvette. A volume of 50 µl of the test sample was added to 1 ml cuvette containing the working solution. For the sample, which had interference from cytochrome-c oxidase activity, a volume of 20 µl of cytochrome-c oxidase inhibitor solution was used for the positive control reaction. The positive control was obtained by diluting an aliquot of the cytochrome-c reductase (NADPH) 10-fold with the enzyme dilution buffer. Each set of reactions required a total of 75 ml of the diluted positive control. A volume of 100 µl NADPH solutions was added to start the reaction. The blank reaction was measured by the value given by the reagents alone without enzyme present.

Test samples	Working Solution	Enzyme Dilution Buffer	Sample or Positive Control	NADPH Solution O.85 mg/ml	Inhibitor solution
Blank	950 µl	50 µl	-----	100 µl	-----
Unknown sample	950 µl	50 - X µl	X µl	100 µl	-----
Unknown sample with interference	950 µl	30- X µl	X µl	100 µl	20 µl
Positive control (10-fold dilution)	950 µl	25 µl	25 µl	100 µl	-----
Positive control (10-fold dilution)	950 µl	-----	50 µl	100 µl	-----

Table 2.3: Reaction scheme outline to measure cytochrome–c activity

(Adapted from www.Sigmaaldrich.com, June 2009)

X= Volume of unknown sample

Calculation:

Unit definition: One unit could reduce 1.0 µmole of oxidized cytochrome-c in the presence of 100 µM, NADPH per minute at pH 7.8 at 25°C

$$\text{Units/ml} = \frac{\Delta A_{550}/\text{min} \times \text{dil} \times 1.1}{21.1 \times \text{Enzvol}}$$

$\Delta A_{550}/\text{min} = \Delta A_{sample} - \Delta A_{blank}$

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dil = the dilution factor of the original enzyme sample

Enzvol = volume of the enzyme sample (ml)

21.1 = extinction coefficient (EmM) for reduced cytochrome c

1.1 = Reaction volume (ml)

The reaction time is longer than 1 minute; divide the ΔA_{550} by the reaction time to obtain

ΔA_{550} / min. All the values for cytochrome -c were expressed as units/ml.

2.5.6 Measurement of intracellular free calcium $[Ca^{2+}]_i$ using a fluorescence micro plate reader.

Measurement of $[Ca^{2+}]_i$ was performed as previously described in the literature (Robinson *et al.*2004). The measurement of $[Ca^{2+}]_i$ was performed by seeding a density of 10,000 cells/well in black 96 well plates (Griner, UK) in 100 µl of growth medium. At sub confluence, the cell culture medium was then replaced with FBS free medium for 24 hr in order to synchronize cells into a non-proliferation stage. The cell were then loaded with 25 µM Fura-2 acetoxylmethyl ester (AM) in cell medium at 37°C for 30 min followed by 30 min at temperature to minimize dye leakage and sequestration into intracellular organelles. After loading, the cells were washed 2-3 times with 200 µl of Hank's Buffered Salt Solution (HBSS) to remove excess fluorescent dye. The cells were then treated with either crude water-soluble extract of *M. charantia* (800 µg) or with 800 µM of alpha, beta momorcharin for different durations (0 min - 420 min) in a calcium free medium. The cells were washed twice with 200 µl HBSS and then a volume of 100 µl HBSS /wells was added to each well. The same procedure was carried out for the untreated cells. The intensity of the fluorescence's of Fura-2 AM was

measured at emission wavelengths of 340 nm. The relative changes in calcium concentration using Fura-2 AM were determined by calculations of

$$[[Ca^{2+}]_i = K_d \, \beta \, (f - f\,min) \, / \, (f\,max - f).$$

K_d, the Ca-Fura-2 dissociation constant, β the fluorescence intensity ratio, excited at 380 nm without and with Ca; f min and f max (Robinson *et al.,* 2004). All values were expressed as ratio units of the Fura-2 AM fluorescent intensity.

2.6 Statistical Analysis

All control and test data collected from the different experiments were analysed using Statistical Package for Social Sciences (SPSS) version 17, Student's − t test and ANOVA test. Data obtained were expressed as mean ± standard deviation (S.D). Each experiment was repeated for 4-6 times in duplicate (6 for cell viability and 4 for cell signalling) to ensure the accuracy of results. A value of ($p < 0.05$) was taken as significant.

Chapter Three

Effects of different extracts of the fruit *Momordica charantia*, alpha beta momorcharin and commercial anti-cancer drugs on cancer cell line viability *in vitro*

3. Introduction

The present study investigated the anti-cancer effects of two extracts (the crude water-soluble extract and methanol soluble) of the fruit of *M. charantia,* and three isolated and purified proteins from *M. charantia* (α momorcharin, β momorcharin and α,β momorcharin) and two commercially available anti-cancer drugs namely vinblastine and temozolomide, either alone or in combination on different cancer cell lines *in vitro* measuring cell viability compared to healthy L6 skeletal muscle cell line.

3.1 Methods

As described in Chapter 2, section 2.4.

3.2 Results

3.2.1 Morphology of untreated and treated cell lines

Figure 3.1 shows confocal images of the morphology of (A) untreated, (B) treated 1321N1 cell line with 800 µg of the crude water-soluble extract of *M. charantia* and (C) treated 1321N1 cell line with 800 µM of the α,β momorcharin. 1×10^{6} cells/mL cells were treated for 24 hours. The results in figure 3.1A show the normal structure of numerous 1321N1 cells growing in the medium. In contrast, figure 3.1B/C shows the same cells line following 24 hours of incubation with either crude water-soluble extract of *M. charantia* or with α, β momorcharin. However, in this case, the numbers of cells have markedly decreased and structurally, they look different from untreated cells (figure 3.1A). They have lost their neuronal-like structures and they seem to have a donought shape.

A

B

C

Figure 3.1: Confocal images, at 40X magnification, showing the morphology of (A) untreated (B) treated 1321N1 cell line with either 800 µg of the crude water-soluble extract of *M. charantia* or treated with 800 µM of the α, β momorcharin (C). Cells were treated for 24 hr. Micrographs are typical of 6 such different experiments and they were assessed for cell viability. Note the black arrows indicate membrane blebbing of individual cells under going apoptosis and cells detached from the surface and from one another, decrease in cell viability, the donought-like structure and the loss of neuronal like glands following treatment with the crude water-soluble extract of *M.charantia* or with α, β momorcharin

Figure 3.2 Figure 3.2 shows confocal images of the morphology of (A) untreated and treated Gos-3 cell line with 800 µg of the crude water-soluble extract of *M. charantia* (B) treated Gos-3 cell line with 800 µM of the α, β momorcharin (C). The result in figure 3.2A shows the normal structure of numerous Gos-3 cells growing in the medium. In contrast, figure 3.2B/C shows the same cell line following 24 hours of incubation with either *M. charantia* or α, β momorcharin, respectively but in this case the treated cells seem to decrease in number. In addition the treated cells seem to shrink and they developed a round shape and they have lost their neuronal-like structure.

Figure 3.3 shows confocal images of the morphology of (A) untreated and treated U87-MG cell line with either 800 µg of the crude water-soluble extract of *M. charantia* (B) or treated or with 800 µM of the α, β momorcharin (C). The results in figure 3.3A show the normal structure of numerous U87-MG cells growing in the medium. In contrast, figure 3.3B/C shows the same cell line following 24 hours of incubation with either *M. charantia* or with α, β momorcharin, respectively. Here, the numbers of cells have decreased markedly and structurally, they look different from untreated cells. Typically, they seem to have a donought like (ring) structure and they have lost their neuronal-like features.

Figure 3.2: Confocal images at 40X magnification showing the morphology of (A) untreated (B) treated Gos-3 cell line with either 800 μg of the crude water-soluble extract of *M. charantia* or treated with either 800 μM of the α, β momorcharin (C). Cells were treated for 24 hrs. Micrographs are typical of six such different experiments and they were assessed for cell viability. Note the black arrows indicate decrease in cellular density, condensation, fragmentation, the number and structure of the treated cells in B and C compared to untreated cells in A.
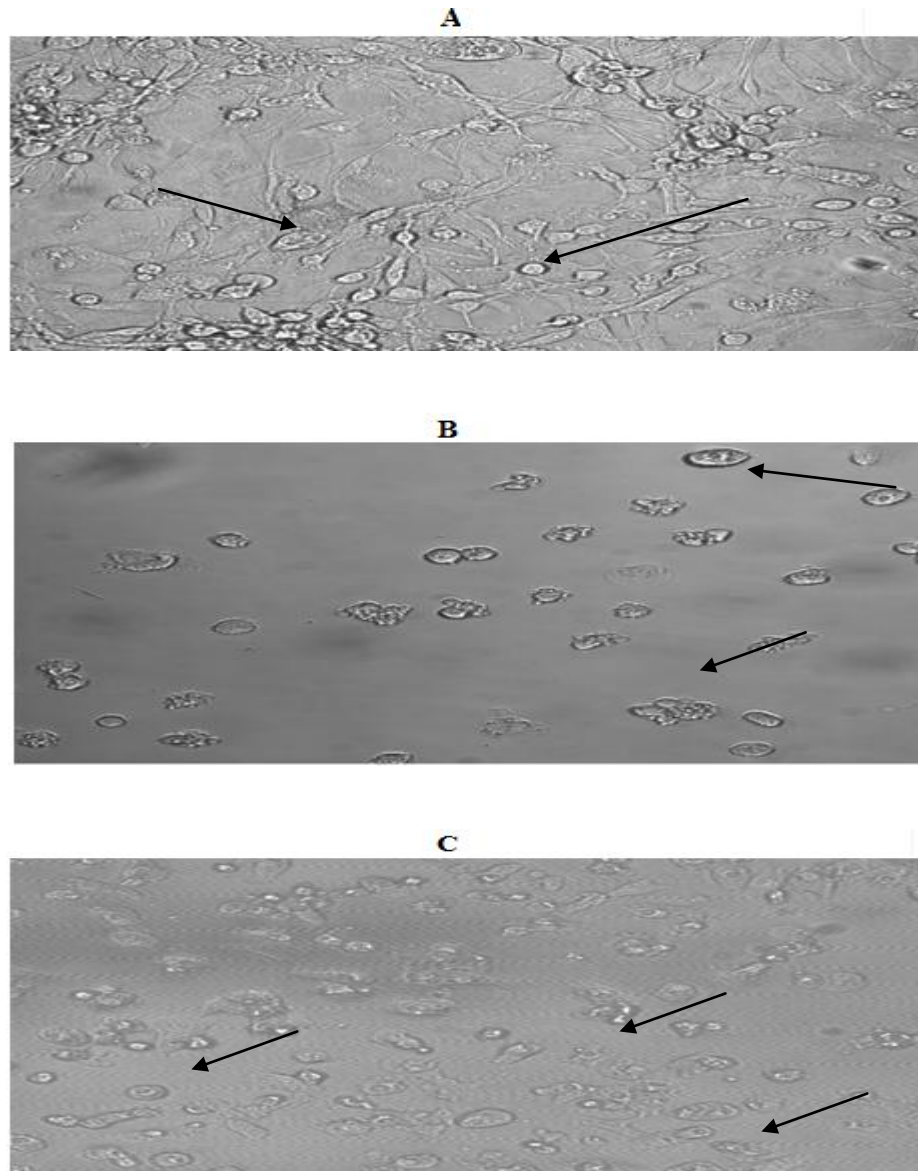
A

B

C

Figure 3.3: Confocal images at 40X magnification showing the morphology of (A) untreated (B) treated U87-MG cell line with either 800 µg of the crude water-soluble extract of *M. charantia* or treated with 800 µM of the α, β momorcharin (C). Cells were treated for 24 hrs. Micrographs are typical of six such different experiments and they were assessed for cell viability. Note the black arrows indicate decrease in cellular density, condensation, fragmentation, the number and structure of the treated cells in B and C compared to untreated cells in A.

Figure 3.4 shows confocal images of the morphology of (A) untreated and treated Sk-Mel cell line with either 800 µg of the crude water-soluble extract of *M. charantia* (B) or with 800 µM of the α, β momorcharin (C). The results in figure 3.4A show the normal structure of numerous Sk-Mel cells growing in the medium. In contrast, figure 3.4B/C shows the same cell line following 24 hours of incubation with either *M. charantia* or with α,β momorcharin, respectively. Here, the numbers of cells have decreased significantly compared to control and structurally, they look different from untreated cells. They seem to clump together which is typical of apoptosis or cell death.

Figure 3.5 shows confocal images of the morphology of (A) untreated and treated Corl-23 cell line with either 800 µg of the crude water-soluble extract of *M. charantia* (B) or with 800 µM of the α, β momorcharin (C). The results in figure 3.5A show the normal structure of numerous Corl-23 cells growing in the medium. In contrast, figure 3.5B/C shows the same cell line following 24 hours of incubation with either *M. charantia* or with α, β momorcharin, respectively. Here, the numbers of cells seem to decrease and structurally, they look smaller and they seem to separate from one another.

Figure 3.4: Confocal images, at 40X magnification, showing the morphology of (A) untreated (B) treated Sk-Mel cell line with either 800 µg of the crude water-soluble extract of *M. charantia* or with 800 µM of the α,β momorcharin (C). Cells were treated for 24 hrs. Micrographs are typical of six such different experiments and they were assessed for cell viability. Note the black arrows indicate membrane blebbing of individual cells under going apoptosis and cells detached from the surface and from one another, decrease in cell viability the reduction in number and structural difference in treated (B/C) compared to untreated (A) cells.
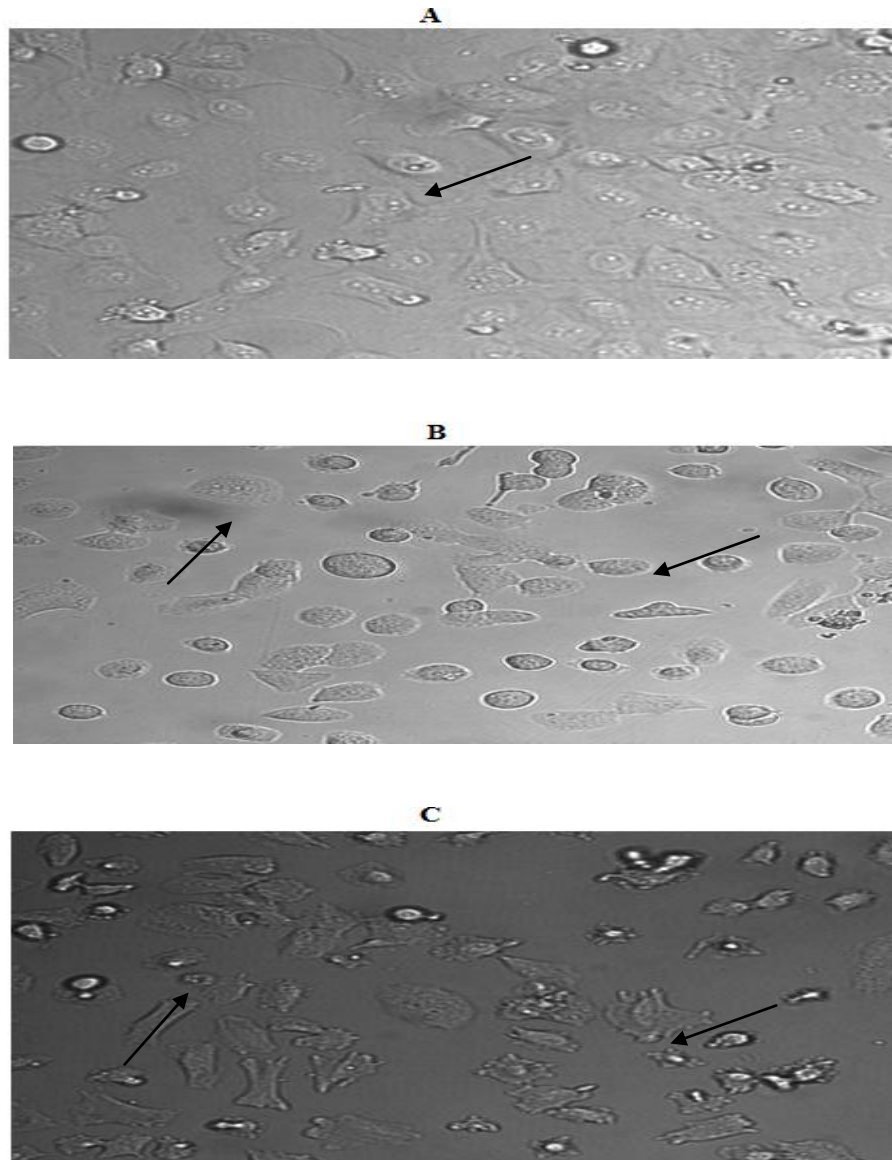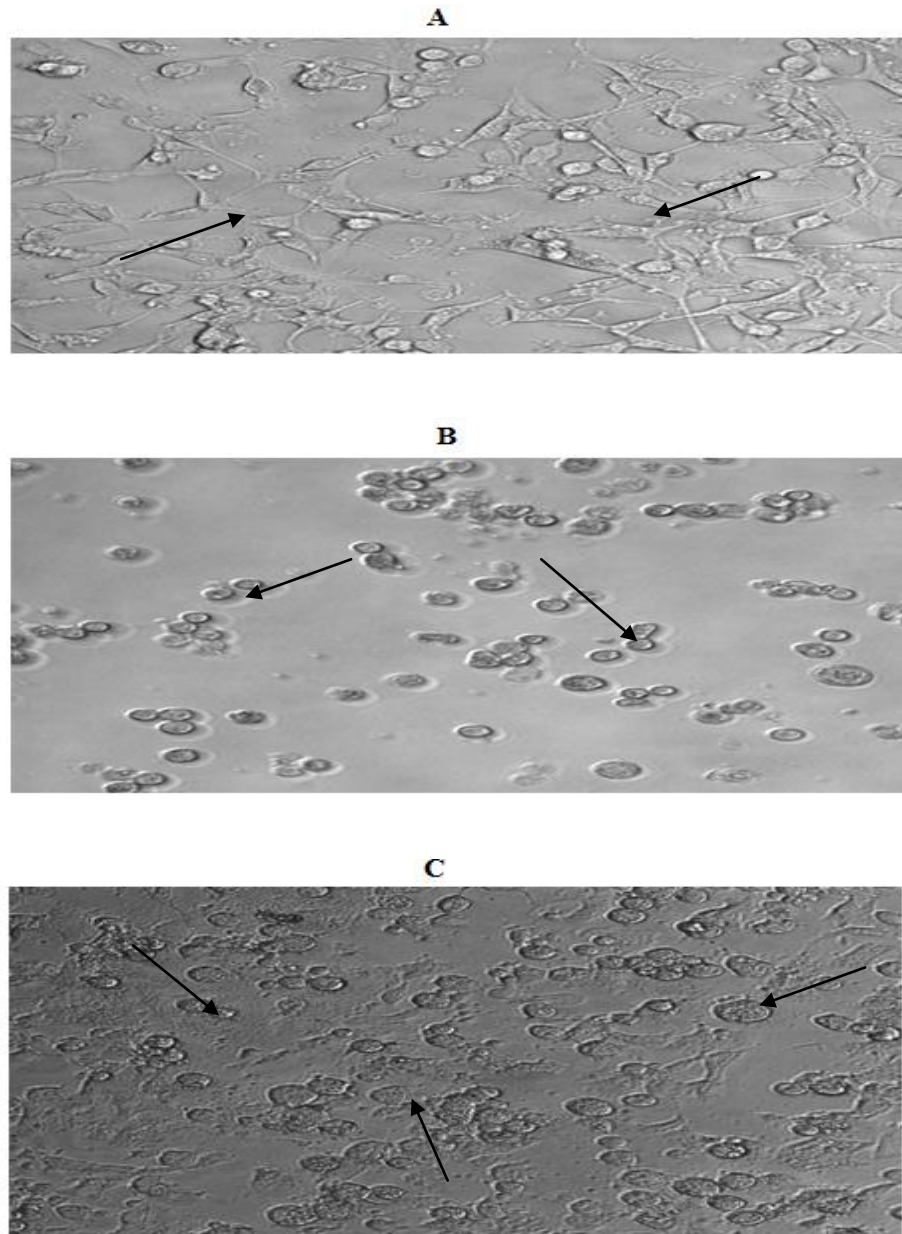
A

B

C
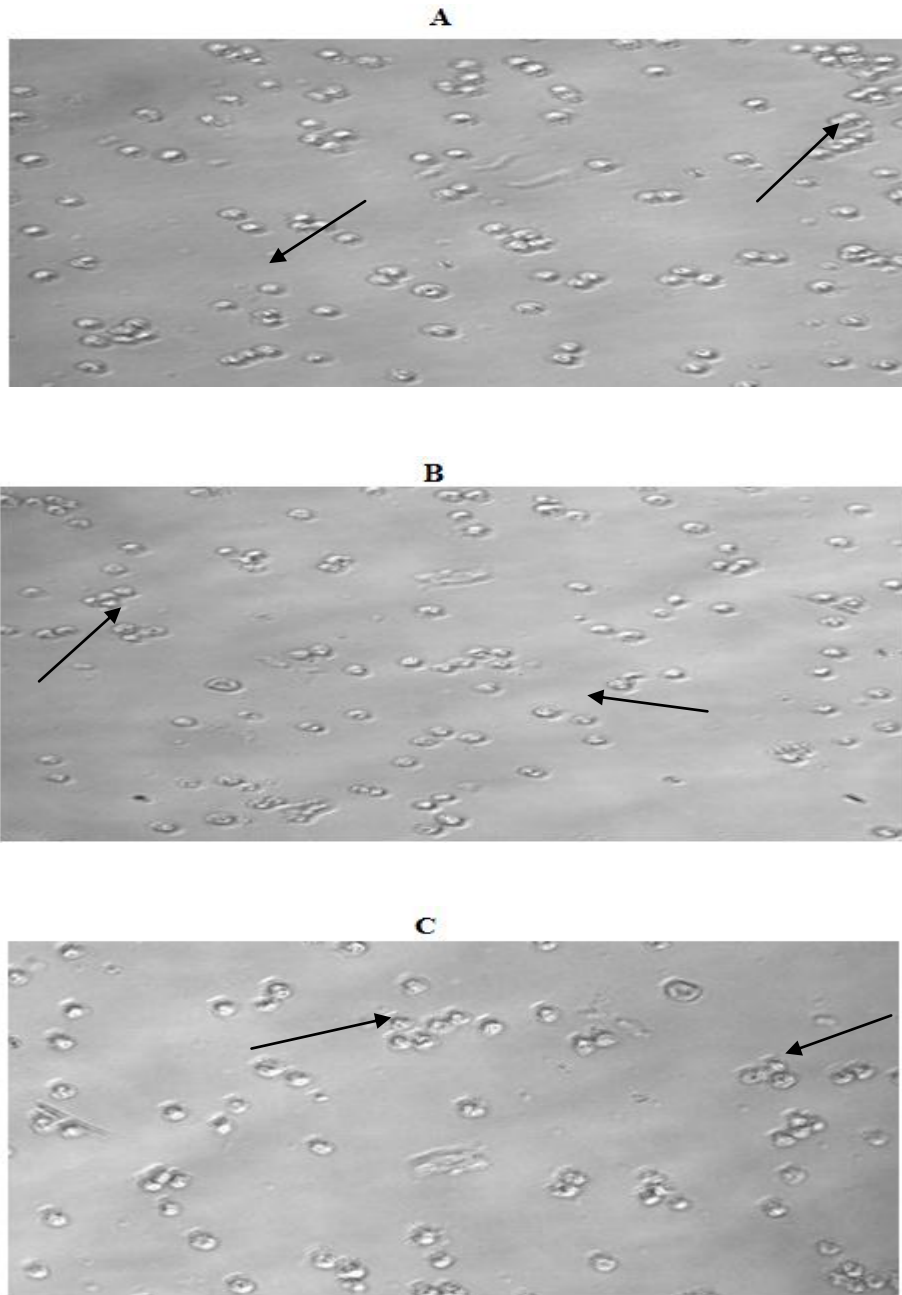
Figure 3.5: Confocal images, at 40X magnification, showing the morphology of (A) untreated (B) treated Corl-23 cell line with either 800 µg of the crude water-soluble extract of *M. charantia* or with 800 µM of the α, β momorcharin (C). Cells were treated for 24 hrs. Micrographs are typical of six such different experiments and they were assessed for cell viability. Note the black arrows indicate decrease in the number of treated cells, cellular density, condensation, fragmentation and blebbing of and their structural difference (B/C) compared to untreated cells (A).

Figure 3.6 shows confocal images of the morphology of (A) untreated and treated WeriRb-1 cell line with either 800 µg of the crude water-soluble extract of *M. charantia* (B) or with 800 µM of the α, β momorcharin (C). The results in the figure 3.6A show the normal structure of numerous WeriRb-1 cells growing in the medium. In contrast, figure 3.6B/C shows the same cell line following 24 hours of incubation with either *M. charantia* or with α, β momorcharin, respectively. Here, the number of cells seems to decrease and structurally, they look different from untreated cells. Several cells seem to clump together which is typical of cell death during apoptosis.

Figure 3.7 shows confocal images of the morphology of (A) untreated and treated L6 skeletal muscle cell line with either 800 µg of the crude water-soluble extract of *M. charantia* (B) or with 800 µM of the α, β momorcharin (C). The results in the figure 3.7A show the normal structure of numerous L6 cells growing in the medium. Similarly, figure 3.7B/C shows the same cell line following 24 hours of incubation with either *M. charantia* or with α, β momorcharin, respectively. Here, the number of cells seemed to be the same and with the similar structure.
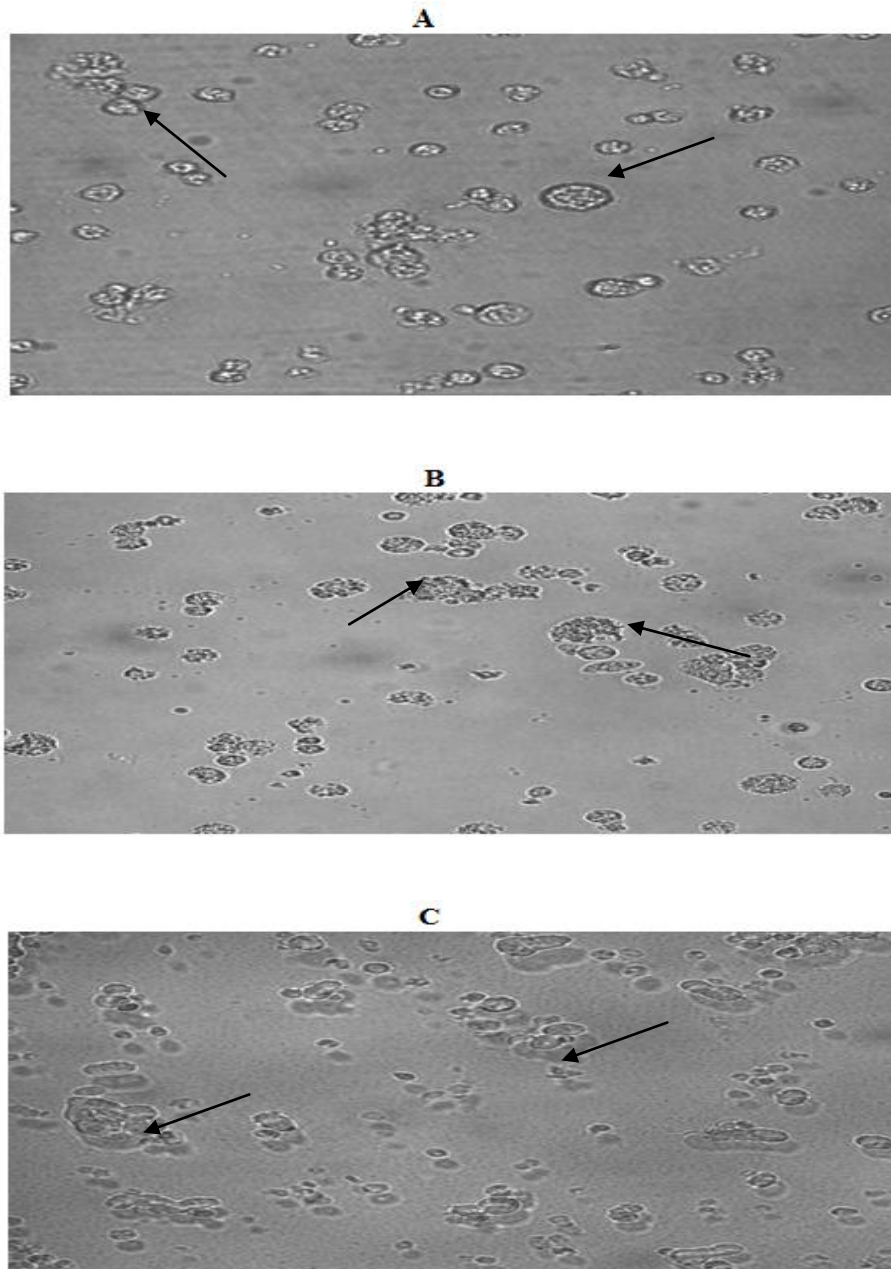
Figure 3.6: Confocal images, at 40X magnification, showing the morphology of (A) untreated (B) treated Weri Rb-1 cell line with either 800 µg of the crude water-soluble extract of *M. Charantia* or with 800 µM of the α,β momorcharin (C). Cells were treated for 24 hrs. Micrographs are typical of six such different experiments and they were assessed for cell viability. Note the the black arrows indicate decrease in number and the clumping to the treated cells (B/C) compared to untreated cells (A).
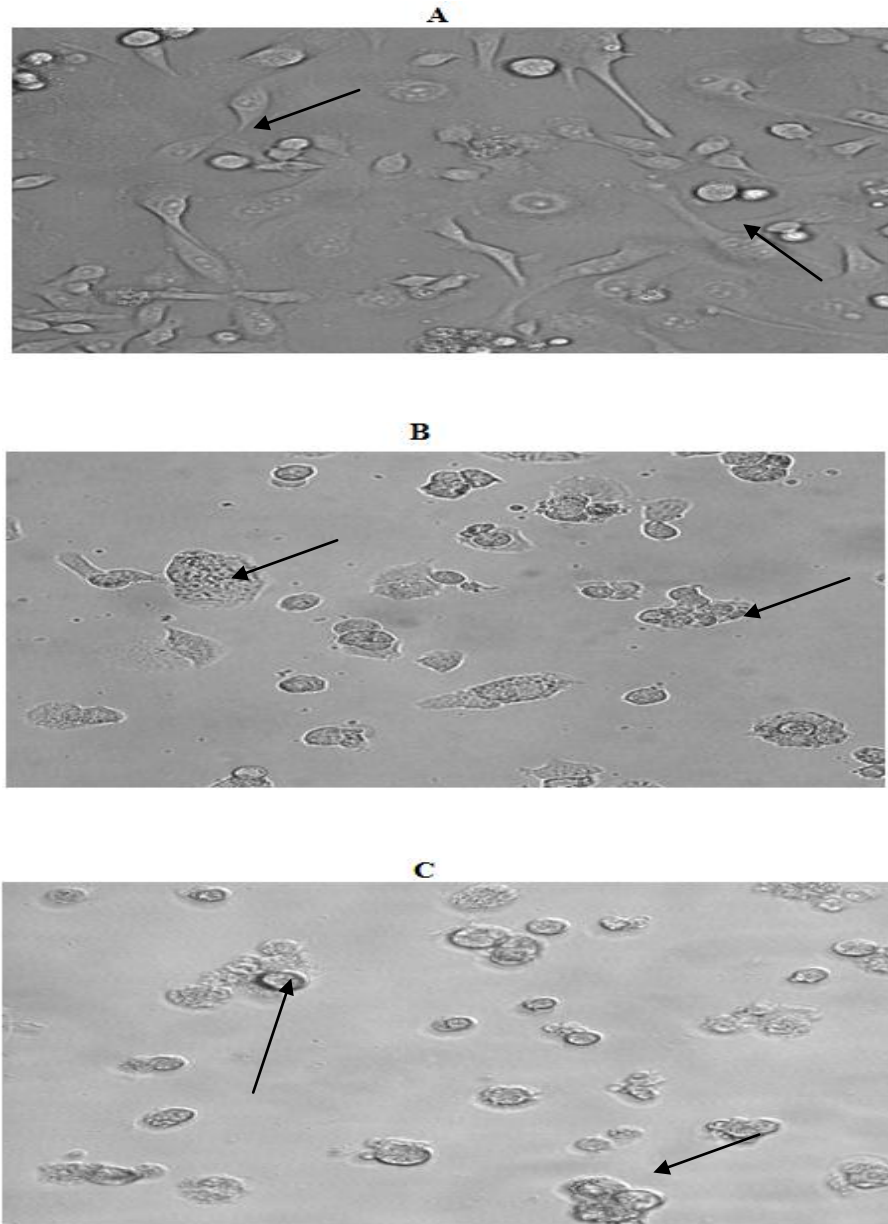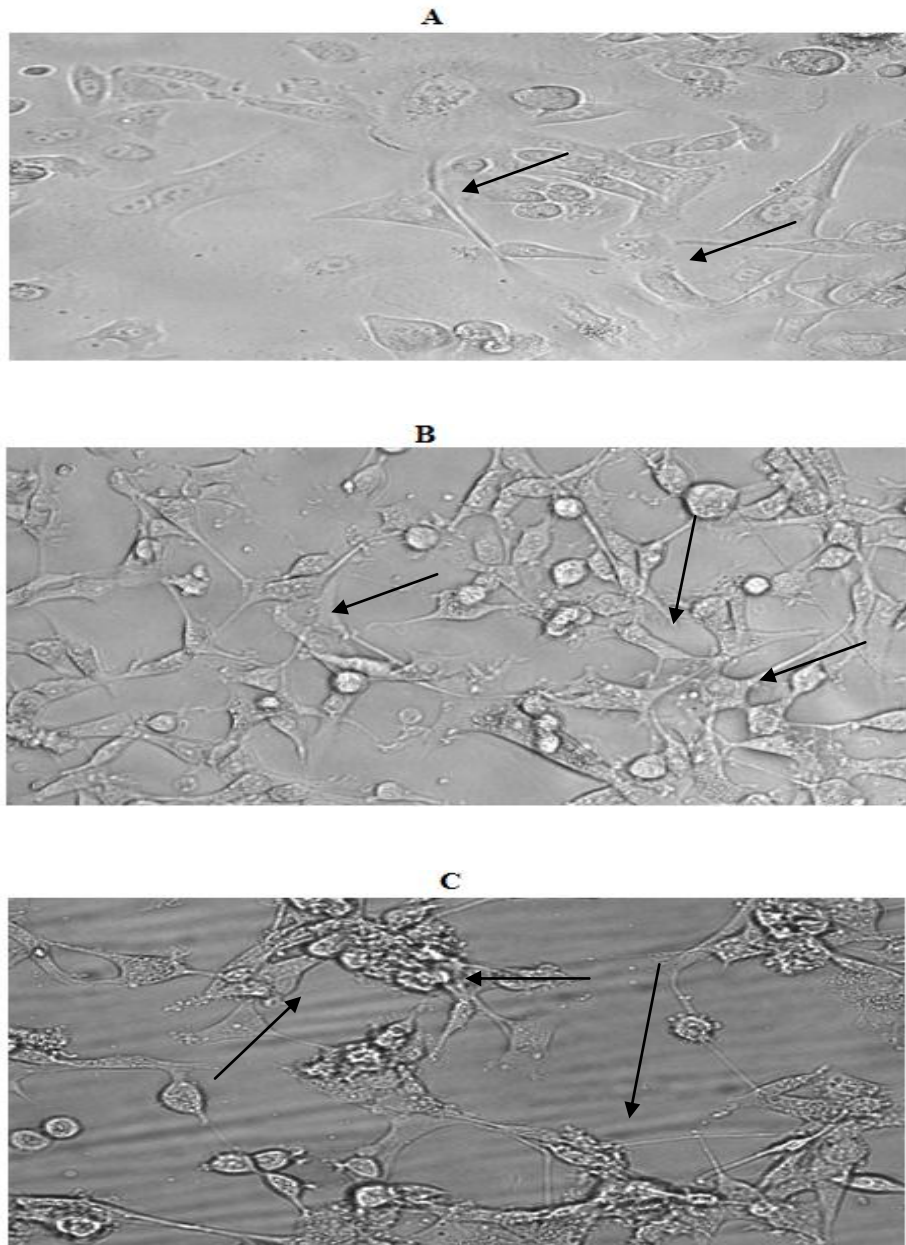
Figure 3.7: Confocal images, at 40X magnification, showing the morphology of (A) untreated (B) treated L6 muscle cell line with either 800 µg of the crude water-soluble extract of *M. charantia* or with 800 µM of the α, β momorcharin (C). Cells were treated for 24 hr. Micrographs are typical of 4-6 such different experiments and they were assessed for cell viability. Note that back arrows indicate the cells growing normaly attached to the surface of the flask in both untreated and treated cells look similar.

3.2.2 Time-course effects of the crude water-soluble extract of *M. charantia* on cell viability

Figure 3.8 shows the time-course effects of 800 µg of the crude water-soluble extract of *M. charantia* on the viability of different cancer cell lines (1231N1, Gos-3, U87-MG, Weri Rd-1, Corl-23, Sk Mel) and healthy L6 muscle cell line. Each cell line was incubated with the extract for 6, 12, 18, 24 and 48 hours. Also shown in the figure 3.8 is healthy L6 skeletal muscle cell line incubated with both extracts for the same time for comparison. All the cells were treated for up to 48 hours. The results show that in all six different cancer cell lines (1321N1, Gos-3, U87-MG, Sk Mel, Corl -23, Weri Rb-1), the crude water-soluble extract of *M. charantia* can evoke marked and significant ($p < 0.05$) decreases in the cell viability (cell death) compared to untreated cells. From these initial time- course experiments, it was established that cell viability (cell death) decreased to its maximal level after 24 hours of incubation with the crude water-soluble extract of *M. charantia*. No further sygnificant effect on cell viability was observed at 48 hours of incubation compared to 24 hrs. The incubation time of 24 hours was employed in all the dose dependent experiments of this study. The results also presented in figure 3.8 also show that the crude water-soluble extract of *M. charantia* had little or no effect on the death of the healthy L6 skeletal muscle cell line.
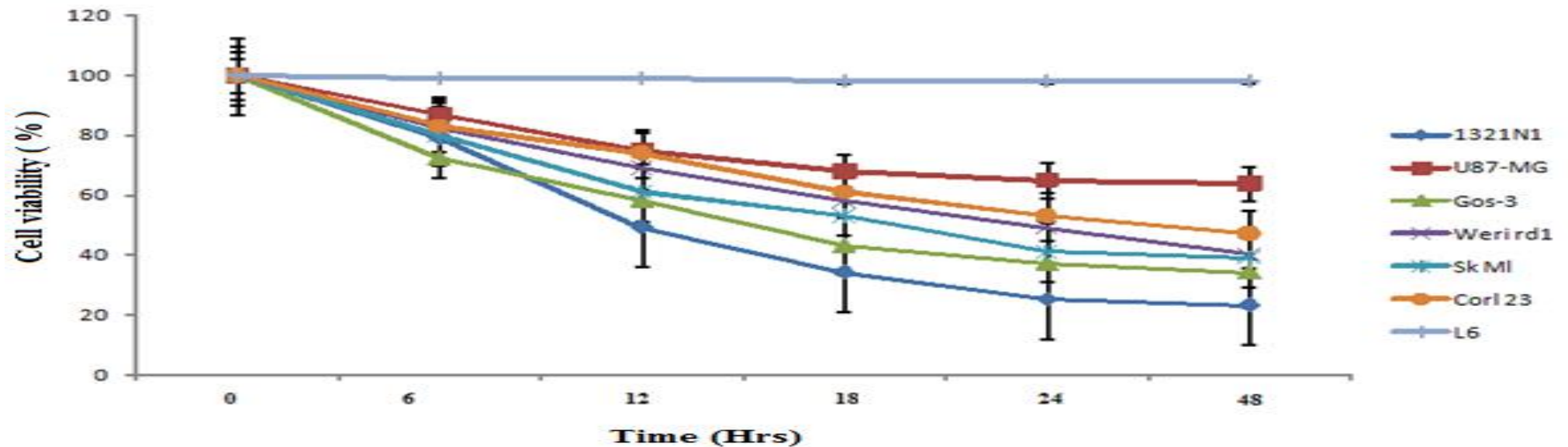
Figure 3.8: The time-course effects of 800 µg of crude water-soluble extract of *M. charantia* on the viability of six different cancer cell lines (1321N1, Gos-3, U87- MG, Sk Mel-2, Corl -23, Weri Rb-1) and on healthy L6 muscle cell line for comparison. Cell lines incubated at the same time point without any extract are also shown in the figure for comparison. Solid straight line shows the 100 % values for each time point. Cell viability for each time point was expressed as percentage of the respective control (no extract, but only cells in medium) at each time point of incubation. Each cell line was incubated with the crude water-soluble extract of *M. charantia* for up to 48 hrs. All Data are mean ± SD, n = 6 different experiments in duplicate; * $p < 0.05$ for all test samples compared to control (100 %).

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3.3.3 Dose-dependent effects of the crude water and methanol soluble extracts of *M. charantia*, α momorcharin, β momorcharin and α, β momorcharin on cell viability.

Figure 3.9 shows the effects of different concentrations (200 - 800 µg) of the crude water-soluble extract of *M. charantia* on the viability of the six different cancer cell lines and on healthy L6 skeletal muscle cell line employed in this study. Also shown in the figure 3.9 are the untreated six different cancer cell lines and healthy L6 skeletal muscle cell line for comparison. All the cells were treated with the crude water-soluble extract of *M. charantia* for 24 hours. Control (untreated) cell lines were also incubated for 24 hrs but without any extract. The results show that in all six different cancer cell lines (1321N1, Gos-3, U87-MG, Sk Mel, Corl -23, Weri Rb-1), the crude water-soluble extract of *M. charantia* evoked marked and significant ($p < 0.05$) decreases in the cell viability (cell death) compared to untreated cells (100% viability). These effects of the crude extract were dose-dependent with maximal cell death occurring with 600 µg and which was not significantly $p > 0.05$ different from 800 µg. In contrast, the crude water-soluble extract of *M. charantia* had a little or no effect on the death of healthy L6 skeletal muscle cell line for comparison. The result also show that the crude extract was more effective in killing 1321N1, Sk Mel and Corl-23 cell lines compared to its effect on Gos-3 and U87-MG cell lines.
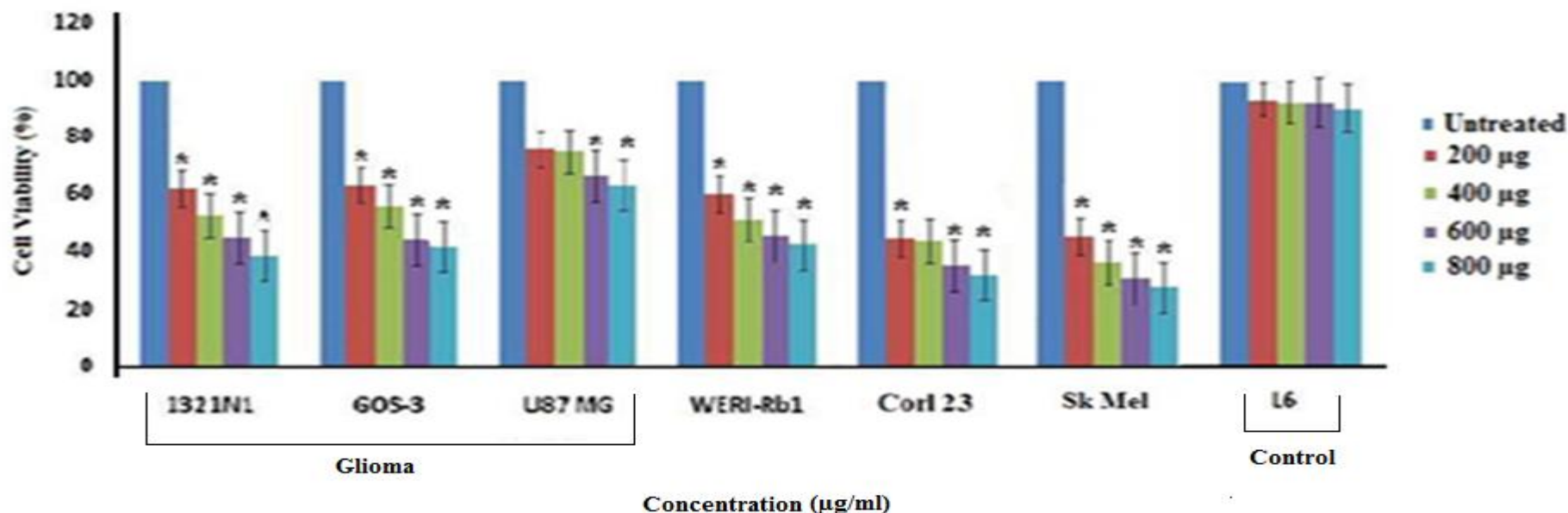
Figure 3.9: Bar charts showing the effects of different concentrations (200 - 800 μg) of the crude water-soluble extract of *M. charantia* on the viability of six different cancer cell lines (1321N1, Gos-3, U87- MG, Sk Mel-2, Corl -23, Weri Rb-1) and healthy L6 muscle cell line for comparison. The untreated (no crude extract) cell line for each (first bar chart) is also shown as 100% in the figure for comparison. Each cell line was incubated with the crude water-soluble extract of *M. charantia* for 24 hrs. Data are mean ± SD, n = 6 different experiments in duplicate; (* $p<0.05$) for untreated (100 % viability) compared to the treated cells for the different concentrations. Note the pronounced anti-cancer effects of the crude water-soluble extract of *M. charantia* on 1321N1, SkMel and Corl-23 compared to the other cancer cell lines.

Figure 3.10 shows the effect of different concentrations (200 - 800 µg) of the crude methanol soluble extract of *M. charantia* on the viability of the six different cancer cell lines and healthy L6 muscle cell lines for comparison. All the cells were treated for 24 hours with the crude methanol soluble extract of *M. charantia*. Control cell lines were also incubated for 24 hrs but without any extract. The results show that in all six different cancer cell lines (1321N1, Gos-3, U87-MG, Sk Mel, Corl-23, Weri Rb-1), the crude methanol soluble extract of *M. charantia* can evoke only a very small decrease in cell viability, but the decrease was not significantly different from the viability (100%) of control cells lines except for 1321N1 in which high doses of 600 µg and 800 µg evoked a small, but significant ($p < 0.05$) decrease in cell viability compared to the control. Similarly, administration of crude methanol soluble extract of *M. charantia* to healthy L6 cell lines did not have any sygnificant effect on the cell viability. The cell viability was not significantly different ($p > 0.05$) from the untreated cell lines compared to the treated cell lines using the methanol extract.

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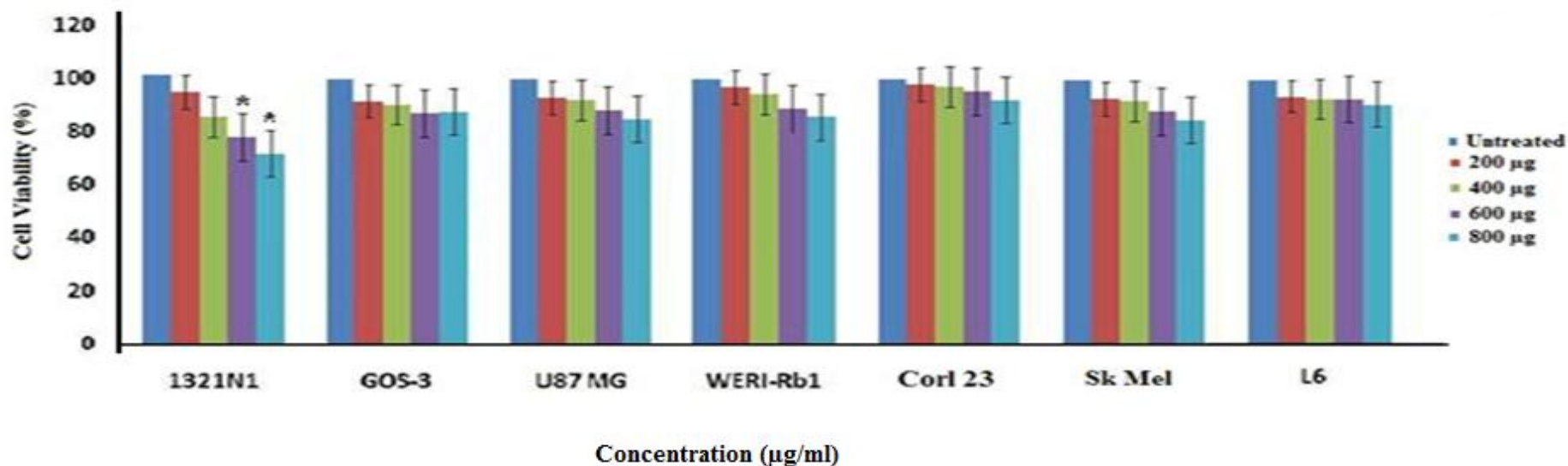
Figure 3.10: Bar charts showing the effects of different concentrations (200 - 800 μg) of the crude methanol soluble extract of *M. charantia* on the viability of six different cancer cell lines (1321N1, Gos-3, U87- MG, Sk Mel-2, Corl -23, Weri Rb-1) and healthy L6 muscle cell line for comparison. The untreated (no methanol extract) cell line for each (first bar chart) is also shown as 100% in the figure for comparison. Each cell line was incubated with crude methanol soluble extract of *M. charantia* for 24 hrs. Similarly, each control cell line was incubated in the medium alone for 24 hrs. Data are mean ± SD, n= 6 different experiments in duplicate; * $p > 0.05$ for untreated (100 % viability) compared to the treated cells for the different concentrations except for 1321N1 in which * $p < 0.05$ for 600 μg and 800 μg compared to control.

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Figure 3.11 shows the effect of different concentrations (200 - 800 μM) of α, β momorcharin on the viability of six different cancer cell lines and healthy L6 muscle cell lines for comparison. All the cells were treated for 24 hours. Control cell lines were also incubated for 24 hrs but without any α, β momorcharin. The results show that in all six different cancer cell lines (1321N1, Gos-3, U87-MG, Sk Mel, Corl - 23, Weri Rb-1) α, β momorcharin evoked marked and significant (p < 0.05) decreases in the cell viability (cell death) compared to untreated cells (100% viability). These effects of α, β momorcharin were also dose-dependent with maximal cell death occurring at 800 μM of α, β momorcharin. In contrast, α, β momorcharin had little or no effect on the death of healthy L6 skeletal muscle cell line. The results presented in Figure 3.11 are more or less similar to the results obtained with the crude water-soluble extract of *M. charantia* (see figure 3.9). Again α, β momorcharin was more effective in killing Sk Mel and Corl -23 cell lines compared to Gos-3 and U87-MG and 1321N1 cell lines.

Figure 3.11: Bar charts showing the effects of different concentrations (200 - 800 µg) of α, β momorcharin on the viability of six different cancer cell lines (1321N1, Gos-3, U87- MG, Sk Mel, Corl -23, Weri Rb-1) and healthy L6 muscle cell line for comparison. The untreated (no α, β momorcharin) cell line for each (first bar chart) is also shown as 100% in the figure for comparison. Each cell line was incubated with α, β momorcharin for 24 hrs. Each control cell line was also incubated for 24 hrs but with no α, β momorcharin. Data are mean $\pm$ SD, n = 6 different experiments in duplicate; * $p < 0.05$ for untreated (100 % viability) compared to the treated cells for the different concentrations. Note the pronounced effect of α, β momorcharin on Sk Mel and Corl -23 cell lines compared to other cell lines.

Figure 3.12 shows the effect of different concentrations (200 - 800 μM) of α momorcharin on the viability of the six different cancer cell lines and healthy L6 muscle cell line for comparison. All the cells were treated for 24 hours. Control cell lines were also incubated for 24 hrs but without any α momorcharin The results show that in all six different cancer cell lines (1321N1, Gos-3, U87-MG, Sk Mel, Corl -23, Weri Rb-1), α momorcharin was less effective in killing the cells compared to either the crude water-soluble extract of *M. charantia* (Figure 3.9) or α,β momorcharin (figure F.11). Similarly, administration of α momorcharin to healthy L6 cell line did not have any effect on the cell viability. However, α momorcharin had little or no effect on 1321N1 cell line, but it was slightly more effective on the other five cancer cell lines especially at high concentrations. In some cases, the cell viability (cell death) reached significant ($p < 0.05$) level compared to untreated cells.

Figure 3.13 shows the effect of different concentrations (200 - 800 μM) of β momorcharin on the viability of the six different cancer cell lines and healthy L6 muscle cell line for comparison. All the cells were treated for 24 hours. Each control cell lines was also incubated for 24 hrs but with no β momorcharin. The results show that in all six different cancer cell lines (1321N1, Gos-3, U87-MG, Sk Mel, and Corl 23, Weri Rb-1) low doses of β momorcharin had little or no effect on 1321N1, Gos-3 and U87- MG cell lines. However, at high concentrations of 600 μM and 800 μM, β momorcharin evoked small, but significant ($p < 0.05$) dose-dependent decreases in cell viability compared to control. Similarly, in Sk Mel, Corl -23 and Weri Rb-1, β momorcharin evoked significant ($p < 0.05$) dose dependent decrease in cell viability for all concentrations tested compared to control cell lines. These effects of β momorcharin were more pronounced than α momorcharin (see figure 3.12) but less effective compared to either the crude water-soluble extract of *M.charantia* (see figure 3.9) and α, β momorcharin (see figure 3.11).

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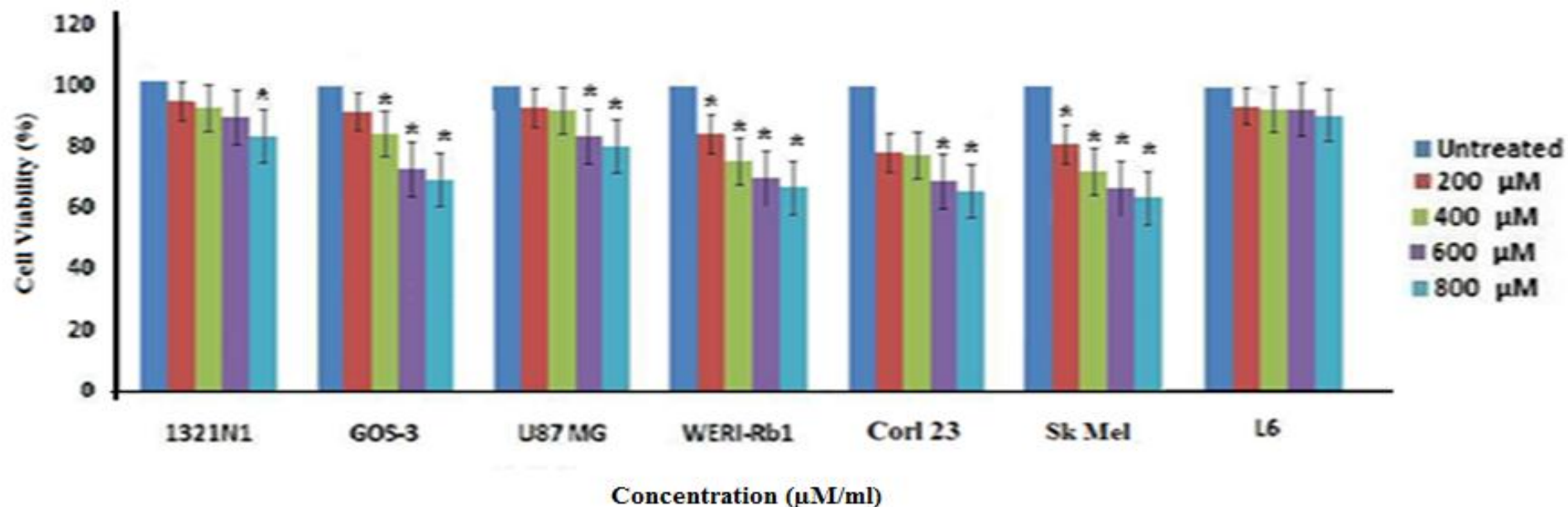
Figure 3.12: Bar charts showing the effects of different concentrations (200 - 800 μM) of α momorcharin on the viability of the six different cancer cell lines (1321N1, Gos-3, U87- MG, Sk Mel, Corl- 23, Weri Rb-1) and healthy L6 muscle cell line for comparison. The untreated (no α momorcharin) cell line for each (first bar chart) is also shown as 100% in the figure for comparison. Each cell line was incubated with α momorcharin for 24 hrs. Each control cell lines were also incubated for 24 hrs but with no α momorcharin. Data are mean ± SD, n = 6 different experiments in duplicate; * p<0.05 for untreated (100 % viability) compared to the treated cells for the different concentrations. Note that α momorcharin is less effective in killing the cancer cell lines.

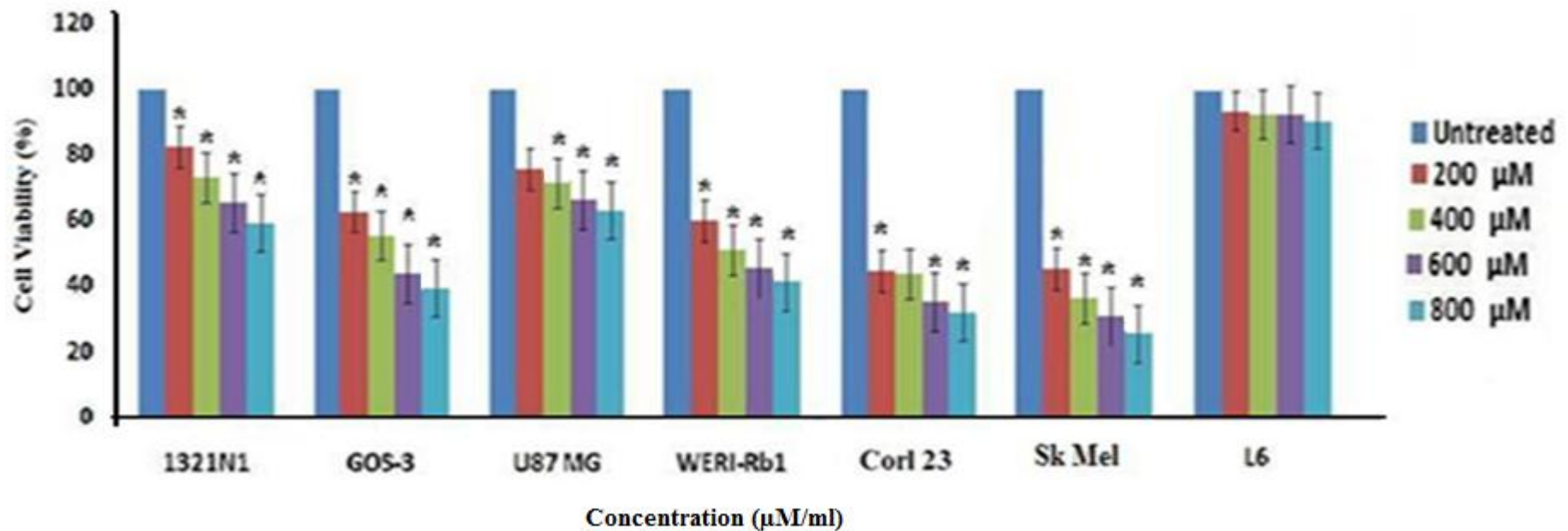Figure 3.13: Bar charts showing the effects of different concentrations (200 - 800 μM) of β momorcharin on the viability of six different cancer cell lines (1321N1, Gos-3, U87- MG, Sk Mel, Corl 23, Weri Rb-1) and healthy L6 muscle cell line for comparison. The untreated (no β momorcharin) cell line for each (first bar chart) is also shown as 100% in the figure for comparison. Each cell line was incubated with β momorcharin for 24 hrs. Each control cell lines was also incubated for 24 hrs but with no β momorcharin. Data are mean ± SD, n = 6 different experiments in duplicate; * p<0.05 for untreated (100 % viability) compared to the treated cells for the different concentrations. Note that β momorcharin was slightly more effective as an anti-cancer agent in Sk Mel cell line compared to the other cell line

3.3.4 Dose-dependent effects of vinblastine and temozolomide on cell viability

Figure 3.14 shows the effects of different concentrations (10 - 40 µg) of vinblastine on the viability of the six different cancer cell lines and on healthy L6 skeletal muscle cell line employed in this study. Also shown in the figure 3.14 are the untreated six different cancer cell lines and healthy L6 skeletal muscle cell line for comparison. All the cells were treated with vinblastine for 24 hours. Each control cell lines were also incubated for 24 hrs but with no vinblastine. The results show that in all six different cancer cell lines (1321N1, Gos-3, U87-MG, Sk Mel, Corl -23, Weri Rb-1), vinblastine can evoke marked and significant ($p < 0.05$) decreases in the cell viability (cell death) compared to untreated cells (100% viability). These effects of the vinblastine were dose-dependent with maximal cell death occurring with 40 µg. Similarly, vinblastine significantly ($p < 0.05$) decreased the viability of healthy L6 skeletal muscle cell line compared to untreated L6 cell line but mainly at a high doses. The results also show that vinblastine was more effective in killing 1321N1, Gos-3, Sk Mel and Corl -23 cell lines. It has less effective on U87-MG cell line, which seems to be more resistant to the drug. The surprised finding in this study was that vinblastine could also kill healthy L6 skeletal muscle cell compared to the crude water-soluble extract of *M. charantia* (see figure 3.9) and α, β momorcharin (see figure 3.11) which had no detectable effect on the viability of L6 cell line.
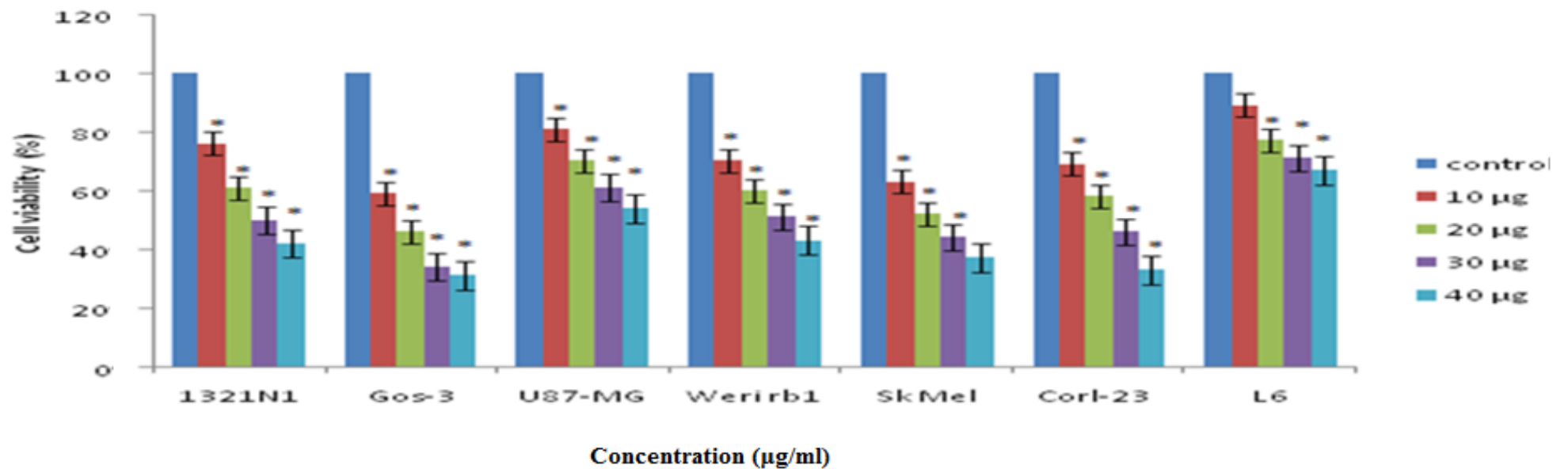
Figure 3.14: Bar charts showing the effects of different concentrations (10 - 40 µg) of vinblastine on the viability of six different cancer cell lines (1321N1, Gos-3, U87- MG, Sk Mel-2, Corl -23, Weri Rb-1) and healthy L6 muscle cell line. The untreated (no vinblastine) cell lines for each (first bar chart) is also shown as 100% in the figure for comparison. Each cell line was incubated for 24 hours either with or without vinblastine. Data are mean ± SD, n = 6 different experiments in duplicate; * $p < 0.05$ for untreated (100 % viability) compared to the treated cells for the different concentrations. Note that vinblastine was more potent on viability of Gos-3 and Corl-23 compared to the others. Moreover, it was more effective at 40 µg. The drug also killed healthy L6 muscle skeletal muscle cell lines at high concentrations.

Figure 3.15 shows the effects of different concentrations (80 - 320 μM) of temozolomide on the viability of the six different cancer cell lines and on healthy L6 skeletal muscle cell line employed in this study. Also shown in the figure 3.15 are the untreated six different cancer cell lines and healthy L6 skeletal muscle cell line for comparison. All the cells were incubated for 24 hours either with or without temozolomide. The results show that in all six different cancer cell lines (1321N1, Gos-3, U87-MG, Sk Mel, Corl -23 and Weri Rb-1) temozolomide evoked marked and significant (p < 0.05) decreases in the cell viability (cell death) compared to untreated cells (100% viability). These effects of the temozolomide were dose-dependent with maximal cell death occurring with 320 μM. Similarly, temozolomide evoked a significant (p < 0.05) decrease in viability of healthy L6 skeletal muscle cell line but this was less compared to the cancer cell lines. Nevertheless, the values reach significant levels (p < 0.05) compared to control (untreated) L6 cells. This effect of temozolomide on L6 muscle cells was dose-dependent. The result also show that the temozolomide was more effective in killing 1321N1, Gos-3, Sk Mel, Weri Rb-1 and Corl -23 cell lines. It was less effective on and U87-MG cell line. Comparing the effects of temozolomide with vinblastine (see figure 3.14). The results clearly show that vinblastine was more effective than temozolomide in killing cancer cells.
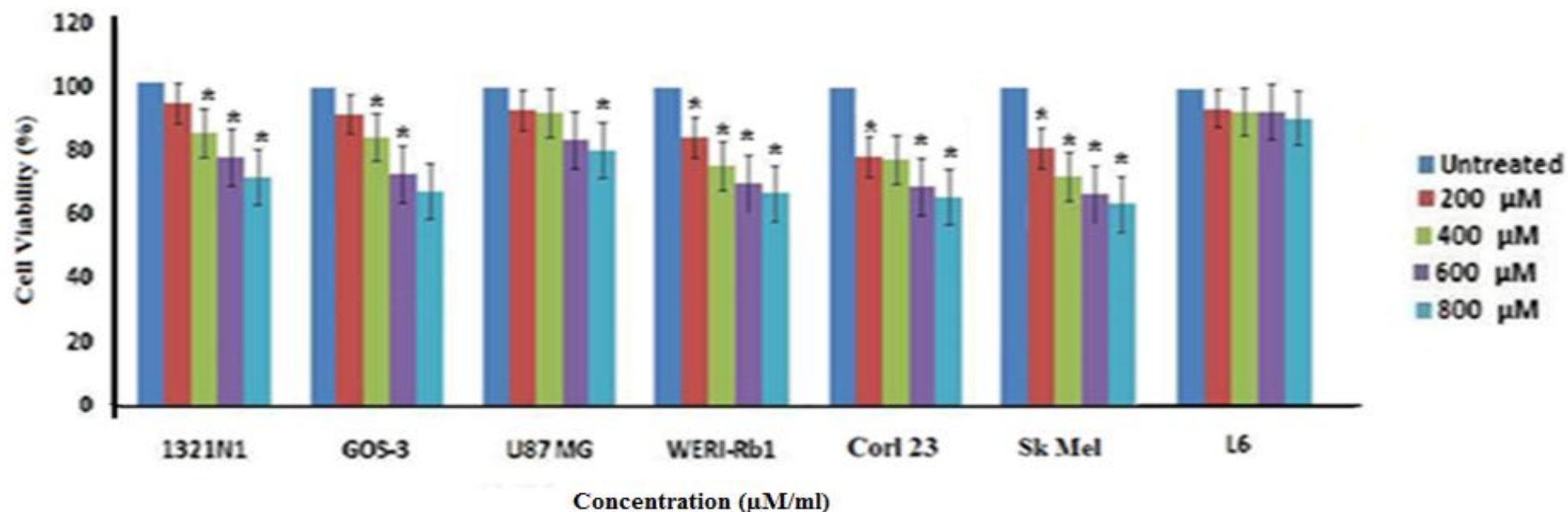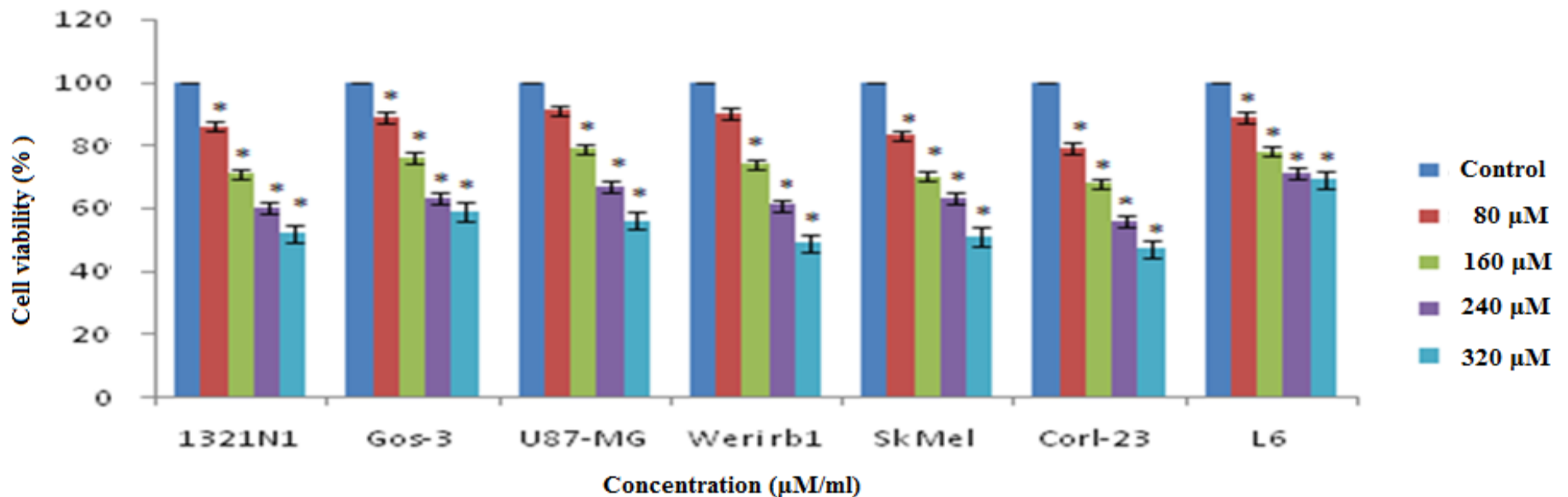
Figure 3.15: Bar charts showing the effects of different concentrations (80 - 320 μM) of temozolomide (TEM) on the viability of six different cancer cell lines (1321N1, Gos-3, U87- MG, Sk Mel, Corl 23 and Weri Rb-1) and healthy L6 muscle cell linen for comparison. The untreated (no temozolomide) cell lines for each (first bar chart) is also shown as 100% in the figure for comparison. Each cell line was incubated for 24 hours either with or without temozolomide. Data are mean ± SD, n = 6 different experiments in duplicate; * p < 0.05 for untreated (100 % viability) compared to the treated cells for the different concentrations. Note that temozolomide was more effective in killing Corl-23 cell line compared to the others. Temozolomide also killed healthy L6 skeletal muscle cell line, but it was less effective compared to cancer cell lines.

3.3.5 Combined effects of crude water-soluble extract of *M. charantia* with either vinblastine or temozolomide

Figure 3.16 shows the effect of either vinblastine (40 µg) alone or the crude water-soluble extract of *M. charantia* (800 µg, a high dose) alone or a combination of vinblastine (40 µg) with the crude extract soluble extract of *M. charantia* (800 µg) on the viability of the six different cancer cell lines and on healthy L6 skeletal muscle cell line employed in this study. Also shown in the figure 3.16 are the untreated six different cancer cell lines and healthy L6 skeletal muscle cell line for comparison. All the cells were treated with either vinblastine or the crude water-soluble extract of *M. charantia* or combined drugs (drug + crude extract) for 24 hours. Control cell lines were also incubated for the same time. The results show that in all six different cancer cell lines (1321N1, Gos-3, U87-MG, Sk Mel, Corl -23, Weri Rb-1) either vinblastine, or crude water-soluble extract of *M. Charantia* can evoke marked and significant $p < 0.05$ decreases in the cell viability (cell death) compared to untreated cells (100% viability). However, when vinblastine was combined with the crude water-soluble extract of *M. charantia,* there was a further decrease in cell viability. These values were significantly ($p < 0.05$) different compared to either untreated cells (100%) or cell treated with either vinblastine or crude water-soluble extract of *M. charantia.*

Similarly, vinblastine combined with the crude water-soluble extract of *M. charantia* evoked significant ($p < 0.05$) decrease in the death of healthy L6 skeletal muscle cell line. The results also show that combined drugs (drug + crude extract) were more effective in killing 1321N1, Gos-3, Sk Mel, Weri Rb-1 and Corl -23 cell lines. It was less effective on U87-MG cell lines.

Figure 3.16: Bar charts showing the effect of either of 40 μg vinblastine alone or 800 μg of crude water-soluble extract of *M. charantia* alone or a combination of vinblastine (40 μg) and the crude extract soluble extract of *M. charantia* (800 μg) on the viability of six different cancer cell lines (1321N1, Gos-3, U87- MG, Sk Mel-2, Corl -23, Weri Rb-1) and health L6 muscle cell line for comparison. The untreated (no vinblastine, crude extract, combined drugs) cell line for each (first bar chart) is also shown as 100% in the figure for comparison. Each cell line (with or without drug) was incubated for 24 hrs. Data are mean ± SD, n = 6 different experiments in duplicate; *p < 0.05 for untreated (100 % viability) compared to the treated cells for the different concentrations. Also ** p < 0.05 for the combined effect of vinblastine + crude extract soluble extract of *M. charantia* compared to the individual effect.

Figure 3.17 shows the effect of either 40 μg of the vinblastine, 800 μM of α,β momorcharin alone or combining α,β momorcharin (800 μM) with vinblastine (40 μg) on the viability of the six different cancer cell lines and on healthy L6 skeletal muscle cell line employed in this study. Also shown in the figure 3.17 are the untreated six different cancer cell lines and healthy L6 skeletal muscle cell line for comparison (100 % viability). All the cells were treated with either vinblastine, α,β momorcharin or combined drugs (vinblastine + α,β momorcharin) for 24 hours. Control cell lines were also incubated for the same time of 24 hours. The results show that in all six different cancer cell lines (1321N1, Gos-3, U87-MG, Sk Mel, Corl -23 and Weri Rb-1) either vinblastine, αβ momorcharin or combined drugs (vinblastine + α,β momorcharin) can evoke marked and significant ($p < 0.05$) decreases in the cell viability (cell death) compared to untreated cells (100% viability). In all the cell lines vinblastine was more effective on cell viability compared to α β momorcharin alone. Moreover, the results also show that when vinblastine was combined with α, β momorcharin they were slightly more effective in killing 1321N1, U87-MG, Sk Mel and Corl -23 cell lines. In contrast, they were less effective on Gos-3 cell line. The result also show that either vinblastine or a combination of vinblastine with α, β momorcharin can elicit a significant decrease of L6 skeletal muscle cell line compared to untreated cells or treated with α β momorcharin alone.
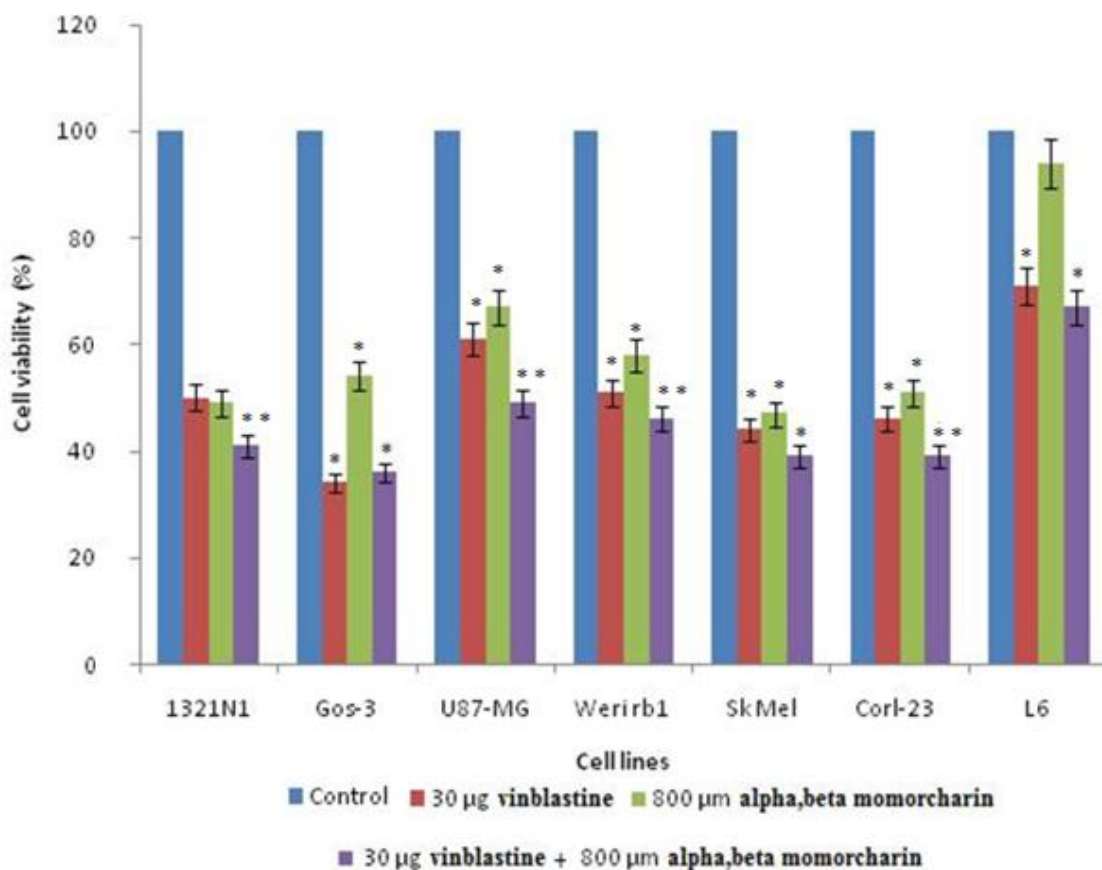
Figure 3.17: Bar charts showing effect of either 40 μg of the vinblastine alone or 800 μM α,β momorcharin alone or combining α,β momorcharin (800 μM) with vinblastine (40 μg) on the viability of six different cancer cell lines (1321N1, Gos-3, U87- MG, Sk Mel, Corl -23 and Weri Rb-1) and healthy L6 muscle cell line for comparison. The untreated (no vinblastine, α,β momorcharin, or combined drugs) cell line for each (first bar chart) is also shown as 100% in the figure for comparison. Each cell line was incubated for 24 hrs either with or without drugs. Data are mean ± SD, n = 6 different experiments in duplicate; * $p < 0.05$ for untreated (100 % viability) compared to the treated cells for the different concentrations. Similarly, ** $p < 0.05$ for the combined effect of vinblastine + α,β momorcharin compared with either vinblastine or α,β momorcharin alone. Note that vinblastine or combination of vinblastine with α β momorcharin can also decrease the viability of healthy L6 skeletal muscle cell line.

Figure 3.18 shows the effects of 240 µM of the temozolomide alone, 800 µg of crude water-soluble extract of *M. charantia* alone or a combination crude extract soluble extract of *M. charantia* (800 µg) with temozolomide (240 µM)) on the viability of the six different cancer cell lines and on healthy L6 skeletal muscle cell line employed in this study. Also shown in figure 3.18 are the untreated six different cancer cell lines and healthy L6 skeletal muscle cell line for comparison. All the cells were treated with either temozolomide alone, crude water-soluble extract of *M. charantia* alone or a combination of both (temozolomide + crude extract) for 24 hours. Control cell lines were also incubated for the same time of 24 hours. The results show that in all six different cancer cell lines (1321N1, Gos-3, U87-MG, Sk Mel, Corl -23, Weri Rb-1) either temozolomide, the crude water-soluble extract of *M. charantia* or combined drugs (temozolomide + crude extract) can evoke marked and significant $p < 0.05$ decreases in the cell viability (cell death) compared to untreated cells (100% viability). Similarly, temozolomide alone, but neither the crude extract soluble extract of *M. charantia* nor a combination of both, can evoke a significant ($p < 0.05$) decrease in the viability of healthy L6 skeletal muscle cell line compared to untreated cell. The results also show that a combination of temozolomide with the crude extract was more effective in killing 1321N1, Gos-3, Sk Mel and Corl -23 cell lines. They are less effective on Weri Rb-1 U87-MG cell lines.
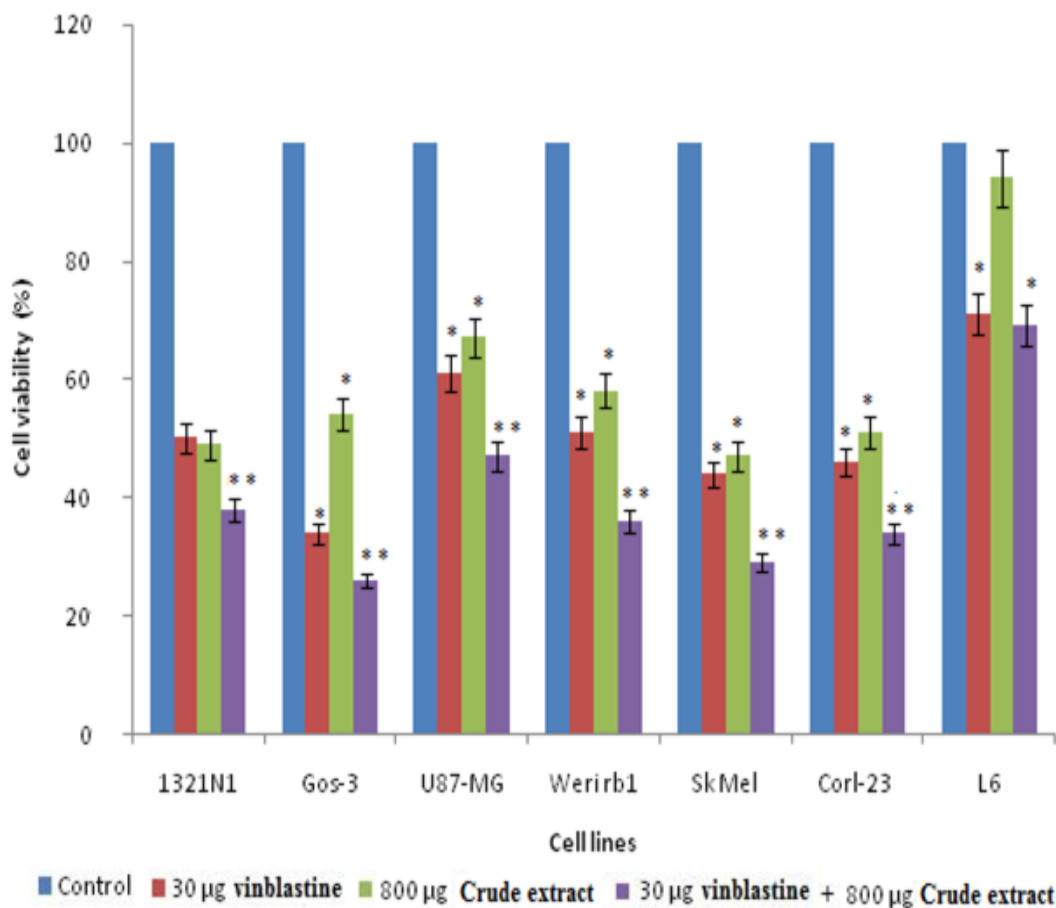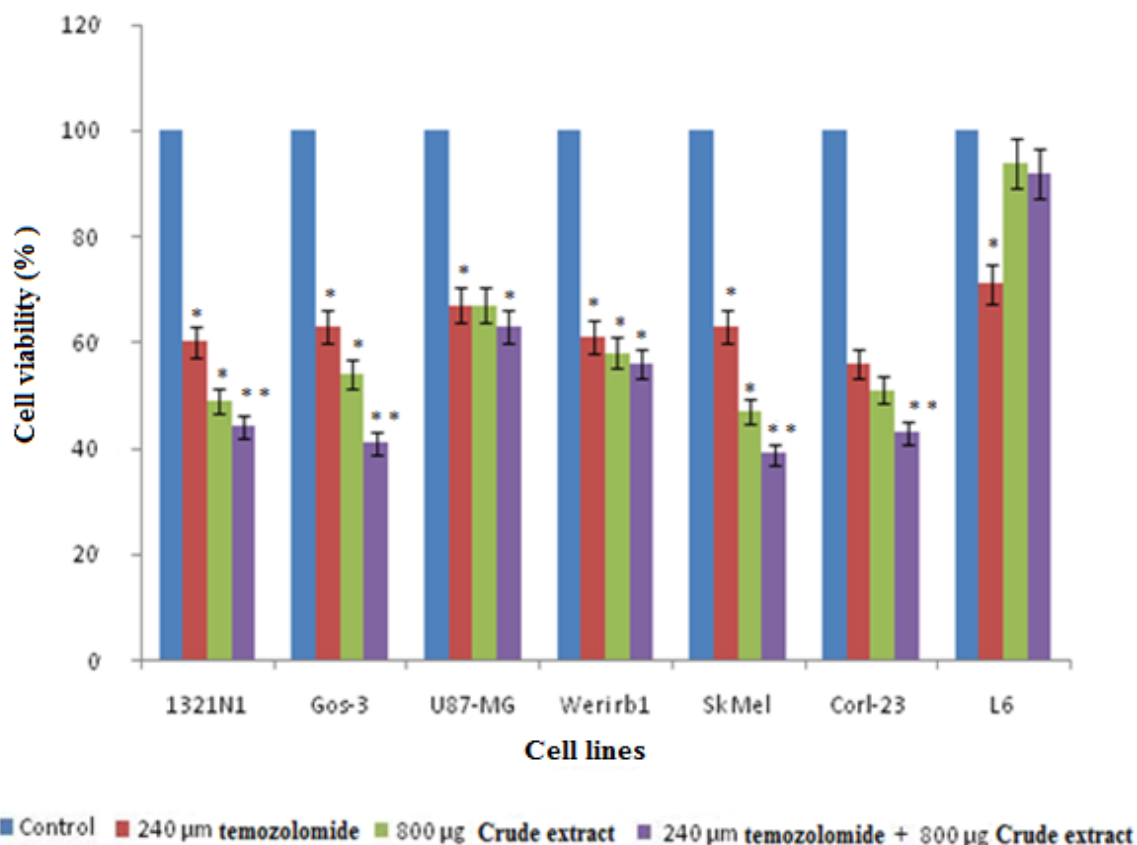
Figure 3.18: Bar charts showing effect of either temozolomide 240 μM, or the crude water-soluble extract of *M. charantia* 800 μg and a combination of the crude extract soluble extract of *M. charantia* (800 μg) with temozolomide (240 μM) on the viability of six different cancer cell lines (1321N1, Gos-3, U87-MG, Sk Mel, Corl -23, Weri Rb-1) and healthy L6 muscle cell line for comparison. The untreated (no temozolomide or crude extract) cell line for each (first bar chart) is also shown as 100 % in the figure for comparison. Each treated and untreated cell line was incubated with for 24 hrs. Data are mean ± SD, n = 6 different experiments in duplicate; * p < 0.05 for untreated (100 % viability) compared to the treated cells either with temozolomide or with the crude water-soluble extract of *M. charantia* or when they were combined; ** p <0.05 for the combined effect of temozolomide and the crude water-soluble extract of *M. charantia* compared to each alone.

Figure 3.19 shows the effect of either temozolomide 240 μM alone, or α,β momorcharin 800 μM alone or a combination of α,β momorcharin (800 μM) with temozolomide (240 μM) on the viability of the six different cancer cell lines and on healthy L6 skeletal muscle cell line employed in this study. Also shown in figure 3.19 are the untreated (no temozolomide or α, β momorcharin) six different cancer cell lines and healthy L6 skeletal muscle cell line for comparison. All the cells were treated with either temozolomide, α,β momorcharin or a combination of temozolomide with α,β momorcharin for 24 hours. Control cell lines were also incubated for the same time. The result shows that in all six different cancer cell lines (1321N1, Gos-3, U87-MG, Sk Mel, Corl -23, Weri Rb-1) either temozolomide, α,β momorcharin or a combination of temozolomide with α,β momorcharin can evoke marked and significant ($p < 0.05$) decreases in the cell viability (cell death) compared to untreated cells (100% viability). Similarly, either temozolomide or a combination of temozolomide with α,β momorcharin can evoke significant ($p < 0.05$) decreases in the viability of healthy L6 skeletal muscle cell lines. The results also show that a combination of temozolomide with α,β momorcharin was slightly more effective in killing 1321N1, Gos-3, Weri Rb-1, Sk Mel and Corl-23 cell lines compared to either temozolomide or α,β momorcharin alone. However, temozolomide with α,β momorcharin seem to be more effective on U87-MG cell line.
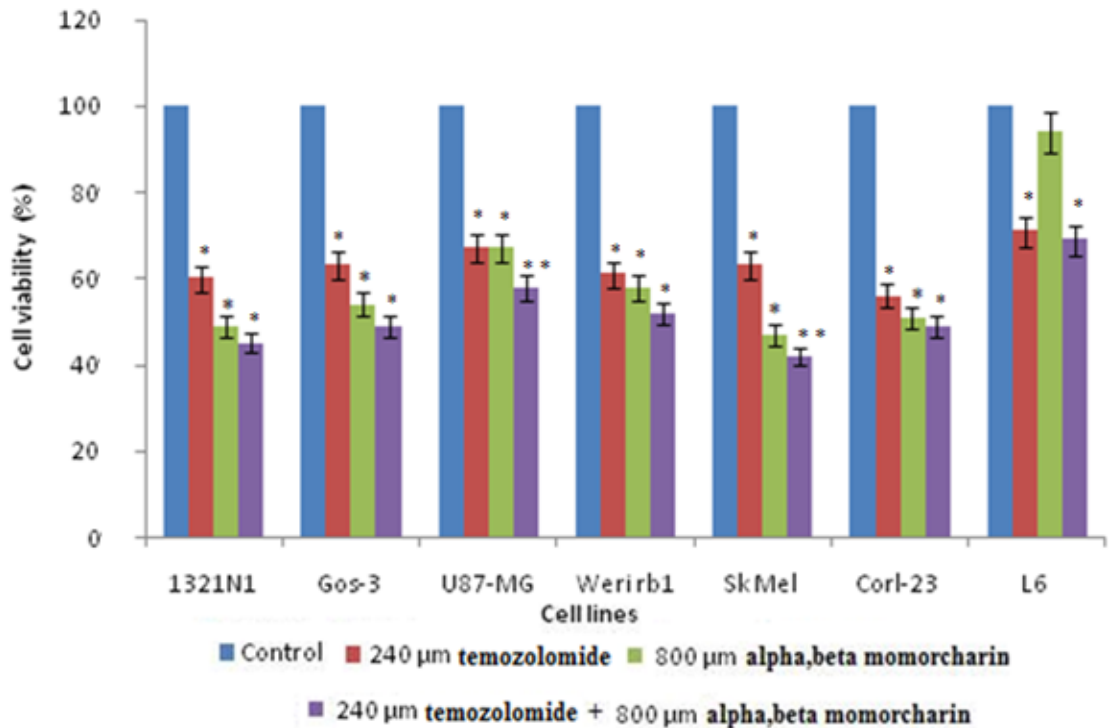
Figure 3.19: Bar charts showing effect of of the temozolomide 240 μM alone or of α,β momorcharin 800 μM alone or a combination of α,β momorcharin (800 μM) and temozolomide (240 μM) on the viability of six different cancer cell lines (1321N1, Gos-3, U87- MG, Sk Mel, Corl -23, Weri Rb-1) and healthy L6 muscle cell line for comparison. The untreated (no temozolomide, α,β momorcharin, or combined α,β momorcharin 800 μM and temozolomide 240 μM) cell line for each (first bar chart) is also shown as 100% in the figure for comparison. Each treated or untreated cell line was incubated for 24 hrs. Data are mean ± SD, n = 6 different experiments in duplicate; * $p < 0.05$ for untreated (100 % viability) compared to the treated cells for the different concentrations. Note that the combined effect of temozolamide with α,β momorcharin was more or less the same as α,β momorcharin alone, except for 1321N1, U87-MG, WeriRb-1 and Sk Mel cells lines, where there was only a small decrease in cell viability; ** $p < 0.05$ for either temozolomide or α,β momorcharin compared to the combination of both.

3.4 Discussion

A number of preliminary studies have shown anti-cancer activity of *M. charantia* against lymphoid, leukaemia, lymphoma, choriocarcinoma, melanoma, breast, skin and prostatic cancers (Licastro *et al.*1980; Ng *et al.*1994; Sun *et al.* 2001). Maximal anti-carcinogenic activity was demonstrated following chronic treatment with the hot water extract of *M. charantia* in uterine adenomyosis and mammary tumour growth in mice (Nagasawa *et al.* 2002; Singh *et al.*1998).

The beneficial anti-cancer effects of the crude water-soluble extract of *M. charantia* have been reported previously by several other investigators (Ganguly *et al.* 2000; Jilka *et al.*1983; Singh *et al.*1998; Raman and Lau, 1996; Takemoto *et al.*1982; Yeung *et al.* 1988). Moreover, a number of preliminary studies have been conducted using the crude preparation of *M. charantia*. In all these studies, the chemical profile of the extract was not reported in the investigations. Nevertheless, some studies have demonstrated marked biological activities of several compounds including charantin, MAP 30, momordicin, alpha, beta momorcharin extracted from *M. charantia* (Grover *et al.* 2004).

In other studies, alpha, beta and alpha, beta momorcharins have been isolated and purified from *M. charantia* and they were tested on viability of different cancer cell line. The crude water-soluble extract of *M. Charantia* was found to be cytotoxic *to CBA/D* cells in culture and the toxicity was dose dependent. However, the cells exposed to the water-soluble extract for 30 min and washed and incubated for 24 hrs also show a decrease in cell viability (Takemoto *et al.*1982). The results of this study have also shown that alpha, beta momorcharin can produce similar anti-cancer effects on the viability of each cell line compared to the crude water-soluble extract of *M. charantia*.

However, either alpha or beta momorcharin, the two different and distinct proteins extracted from *M. charantia* (compared to alpha, beta momorcharins) was less effective in killing the different cancer cell lines employed in this study compared to alpha, beta momorcharin or the crude water-soluble extract of *M. charantia* alone.

It has been previously reported that alpha, beta momorcharin, a type-1 ribosome inactivating protein (RIP), isolated and purified form *M. charantia* possesses a number of anti-cancer activities including inhibition of tumour growth and anti-HIV (Leung *et al*.1987). Similarly, it was also reported that either individual alpha momorcharin or beta momorcharin had excellent cytotoxicity of tumour cells (Xiong *et al*. 2010). These later findings contrast with the result for either alpha or beta momorcharin obtained in this study.

Second, the present study also investigated the possibility of combining a high dose of the extract of *M. charantia* or alpha, beta momorcharin with a low to moderate dose of commercially available anti-cancer drugs, vinblastine and temozolomide on the viability of each cell line. The rationale was that the commercially available anti-cancer drug in high concentration not only kill cancer cells, but also healthy cells in the body (Nagasawa *et al*. 2002). If a low to moderate dose of either temozolomide or vinblastine can be combined with a high dose of the crude water-soluble extract of *M. charantia* to produce maximal anti-cancer effect, without killing healthy cells, then they should be safer in treating cancer.

The results of the present study have shown that either vinblastine or temozolomide can significantly decrease the viability of 1321N1, Gos-3, U87-MG, Sk Mel, Corl -23, Weri Rb-1 cancer cell lines. Both anti-cancer drugs also decreased the viability of healthy L6 skeletal muscle cell line. The effect of each drug was dose–dependent with maximal

effect occurring at 40 μg for vinblastine and 360 μM for temozolomide (Friedberg *et al.* 2001). The results of this study also show that combining a moderate to a high dose of either vinblastine or temozolomide with a high dose of either the crude water-soluble extract of *M. charantia* or with alpha, beta momorcharin only produce a small, but significant decrease in the viability of each cancer cell line compared to the effect of either temozolomide, vinblastine, the crude water-soluble extract of *M. charantia* or alpha, beta momorcharin alone. This small decrease in cell viability of each cell line was significant, but it was neither additive nor synergetic compared to the separate effect of each. This was a rather surprising result in this study. Nevertheless, plant-based medicines have been shown to exert little or no effects on the cancer cells and tissues when they combine with drugs like, cisplatin, temozolomide, vinblastine or 5-fluorouracil (Omar, 2007). These commercially available drugs not only kill the cancer cells but also kill normal cells. This also depends on the type of cancer and concentrations of the drugs employed by the physician (Hong Jie, 2005). However, further search must find a safer plant based-medicine to treat cancer.

The question, which now arises, is: How do these different extracts exert their effect on cell death? The morphological data for this study have clearly shown marked changes in structure of each cancer cell line comparing treated with untreated cell lines for 24 hrs with either the crude water-soluble extract of *M. charantia* or alpha, beta momorcharin. The morphology of each glioma cell line seemed to alter, losing their normal structures following incubation with either the crude water-soluble extract of *M. charantia* or with alpha, beta momorcharin. Moreover, each cell line seems to aggregate forming clumps of cells following treatment for 24 hrs. In some cases, they seem to possess a donought-like structure similar to red blood cells. These abnormal structural features are typical of either cell necrosis or apoptosis (Kerr *et al.* 1972). These data for the morphology study

presented in figures 3.1 - 3.6 of this study clearly show that either the crude water-soluble extract of *M. charantia* or alpha, beta momorcharin can induce marked structural changes in each cancer cell line leading to morphology of the dead cells or a decrease in cell viability. In contrast, they had little or no effects in the cell viability of healthy L6 skeletal muscle cell line. Like this study, pervious workers have also demonstrated successfully that either the crude water-soluble extract of *M. charantia* or alpha, beta momorcharin can also kill different cancer cells (Chang *et al.* 1983, Terenzi *et al*. 1999).

Previous studies (Ahmad, 1999; Cummings *et al*. 2004) have investigated the synergistic effect of the crude water-soluble extract of *M. charantia* with insulin measuring titrated glucose uptake in L6 skeletal muscle cell lines, It was shown that high doses (100 - 200 µg) of the crude water-soluble extract inhibited the effect of insulin on glucose uptake. The higher the dose of the crude water-soluble extract, the lower was the glucose uptake into skeletal muscle cell line. However, when a low dose of insulin was combined with a low dose (5 µg - 10 µg) of the crude water-soluble extract of *M. Charantia,* there was a synergistic increase in glucose uptake into L6 skeletal muscle cell line (Ahmad, 1999). The present study only employed a very high dose of the crude water-soluble extract of *M. charantia* and a moderate to high dose of either temozolomide or vinblastine to investigate their combined effects on cancer cell viability. It is worthy to investigate low doses of both the extract and the commercial anti-cancer drug on the viability of each cell line.

In this study, the methanol soluble extract of *M. charantia* failed to elicit any decrease in the viability of each cancer cell line except for a small decrease in the viability of 1321N1 cell line, but only at high concentrations. This may due to the fact that the methanol extraction denatures the active ingredient. Similarly, either alpha or beta

momorcharin was less effective in decreasing cell viability (cell death) compared to the effect of either alpha, beta momorcharin alone or the crude water-soluble extract of *M. charantia* alone. Again, it is possible that both the alpha and beta moieties are required on the momocharin protein to make it more active or potent as an anti-cancer drug. On examination of the effect of either the crude water-soluble extract of *M. charantia* or alpha, beta momorcharin, the data show that each possesses more or less similar potency for each concentration employed in this study. Together, the results have clearly indicated that the active ingredient(s) of the crude water-soluble extract of *M. charantia* is possibly alpha, beta momorcharin. Further studies are required to isolate the active components of the crude water-soluble extract and then purify and characterize its chemical properties, structures and anti-cancer effects.

3.5 Conclusion

In conclusion, the results of this study have clearly demonstrated that either the crude water-soluble extract of *M. charantia* or the isolated and purified protein of *M. charantia,* namely alpha, beta momorcharin can evoke significant decreases in cancer cell viability (an increase in cell death) without killing healthy cell line like L6 skeletal muscle cell line. These effects were both time and dose-dependent with maximal effect occurring after 24 hrs at a dose of 800 µg for the crude water-soluble extract of *M. charantia* or 800 µM for alpha, beta momorcharin. Either temozolomide or vinblastine with maximal effect of 360 µM and 40 µg respectively can also elicit dose-dependent decreases in cancer cell viability. Combining either temozolomide or vinblastine with either the crude water-soluble extract of *M. charantia* or alpha, beta momorcharin had no additive or synergetic effect on the viability of each cell line compared to the effect of either alone. It is concluded that extracts of *M. charantia*

possess anti-cancer properties since they can induce cell death. However, further experiments are required to investigate the mechanism(s) whereby these extracts can induce cell death. These results are presented in the next chapter of this study.

Chapter Four

Cellular mechanisms associated with decreased cancer cell viability

4. Introduction

Chapter three of this study describes the anti-cancer effects of both the crude extract of *M.charantia* and alpha beta momocharin. This chapter of the study was specifically designed to investigate the cellular mechanisms whereby either the crude water-soluble extract of *M. charantia* or alpha, beta momorcharin can induce cell death measuring activities of caspase-3 and caspase-9, the release of cytochrome-c and elevation in intracellular free calcium concentrations in the different cancer cell lines.

4.1 Methods

As described in chapter 2, section 2.5.

4.2 Results

4.2.1 Measurements of caspase-3 and caspase-9 activities in untreated and treated cells

Figure 4.1 shows caspase -3 activity in (A) cancer and L6 cell lines treated with 800 μg of the crude water-soluble extract of *M. charantia* for 24 hours and (B) cell lines incubated alone with medium but without any crude extract for the same duration of 24 hrs. The results show that caspase -3 activity decreased significantly ($p < 0.05$) in both treated and untreated cell lines compared to control caspase -3 activity without any cells (assay kit control). The decrease was much more pronounced in untreated cells compared to treated cells. Comparing cells treated with the crude water-soluble extract of *M. charantia* to untreated (no extract), the results (treated – untreated cell lines) also show a significant ($p < 0.05$) increase in caspase -3 activity. The results also show a significant ($p < 0.05$) increase in caspase -3 activity in healthy L6 skeletal muscle cell line compared to untreated cell lines.

Figure 4.2 shows the percentage difference (treated – untreated cells lines) in caspase -3 activity in all six different cancer cell lines (1321N1, Gos-3, U87-MG, Sk Mel, Corl- 23 and Weri Rb-1) and healthy L6 muscle cell line following incubation with 800 μg of the crude water-soluble extract of *M. charantia.* The caspase-3 activity in each control, untreated cell line was expressed as 100 % and the value for the respective treated cell line was expressed as a percentage increase of the control (untreated) value. The difference (treated minus untreated activity) in the activity of caspase-3 is plotted for each cell line in Figure 4.2. The data are obtained from figure 4.1 and they show significant ($p < 0.05$) increases in caspase-3 activity in all the cell lines. The results show that caspase-3 activity was much more pronounced in Gos-3 cell line compared to the other cell lines.

Figure 4.1: Bar charts showing caspase-3 activity in six different cancer cell lines (1321N1, Gos-3, U87-MG, Sk Mel, Corl- 23 and Weri Rb-1) and healthy L6 muscle cell line incubated with 800 μg of the crude water-soluble extract of *M. charantia* for 24 hours (A) and cell lines incubated alone in the medium without any crude extract for the same duration of 24 hrs (B). Assay kit control caspase-3 activity is shown in the figure for comparison. Data are mean ± SD, n = 4 different experiments in duplicate. Note that caspase-3 activity increased significantly (* p < 0.05) in treated cells compared to untreated cells. However, caspase-3 activity in both treated and untreated cells significantly (* p < 0.05) decreased compared to control caspase -3 value. This decrease was much more pronounced in the untreated cells compared to treated cells.

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Figure 4.2: Bar charts showing the percentage difference (treated − untreated cell lines) or increase in caspase-3 activity in six different cancer cell lines (1321N1, Gos-3, U87-MG, Sk Mel, Corl- 23 and Weri Rb-1) and healthy L6 muscle cell line incubated with 800 μg of the crude water-soluble extract of *M. charantia* for 24 hours. Caspase-3 activity for each untreated cell line incubated alone in the medium without any crude extract for the same duration of 24 hrs was taken as 100 %. The caspase-3 activity for each reated cell line was expressed as a percentage increase of untreated control value (100 %). Data are taken from figure 4.1 and they represent mean ± SD, n = 4 different experiments in duplicate. The results show significant (* $p < 0.05$) increases in caspase -3 activity for all the cell lines even in L6 cell line when treated with crude water-soluble extract of *M. Charantia* compared to untreated cell lines.

Figure 4.3 shows caspase-9 activity in (A) cell lines treated with 800 μg of the crude water-soluble extract of *M. charantia* for 24 hours and (B) the same cell lines incubated alone with medium but without any crude extract for the same duration of 24 hrs. The results show that the crude extract can elicit significant ($p < 0.05$) increases in caspase - 9 activity in 1321N1, Gos-3, U87-MG, Sk Mel, Corl-23, Weri Rb-1 cancer cell lines compared to untreated cell line for each. There was little or no significantly increase in caspase-9 activity in either Sk Mel or L6 healthy skeletal muscle cell lines compared to the respective untreated cell lines.

Figure 4.4 shows the percentage difference (treated − untreated cell lines) or increase in caspase-9 activity in all six different cancer cell lines (1321N1, Gos-3, U87-MG, Sk Mel, Corl- 23 and Weri Rb-1) and healthy L6 muscle cell line. The Caspase - 9 activity for each untreated cell line was taken as 100 %. This value was substracted from the treated cells caspase-9 activity with 800 μg of the crude water-soluble extract of *M. charantia*. The results show marked and significant increase in caspase-9 activity in all the cell lines, even in L6 cell line stimulated with the crude water soluble extract of *M. Charantia.* More caspase-9 activity was seen in 1321N1, U87-MG, and Weri Rb-1 compared to the other cell lines.
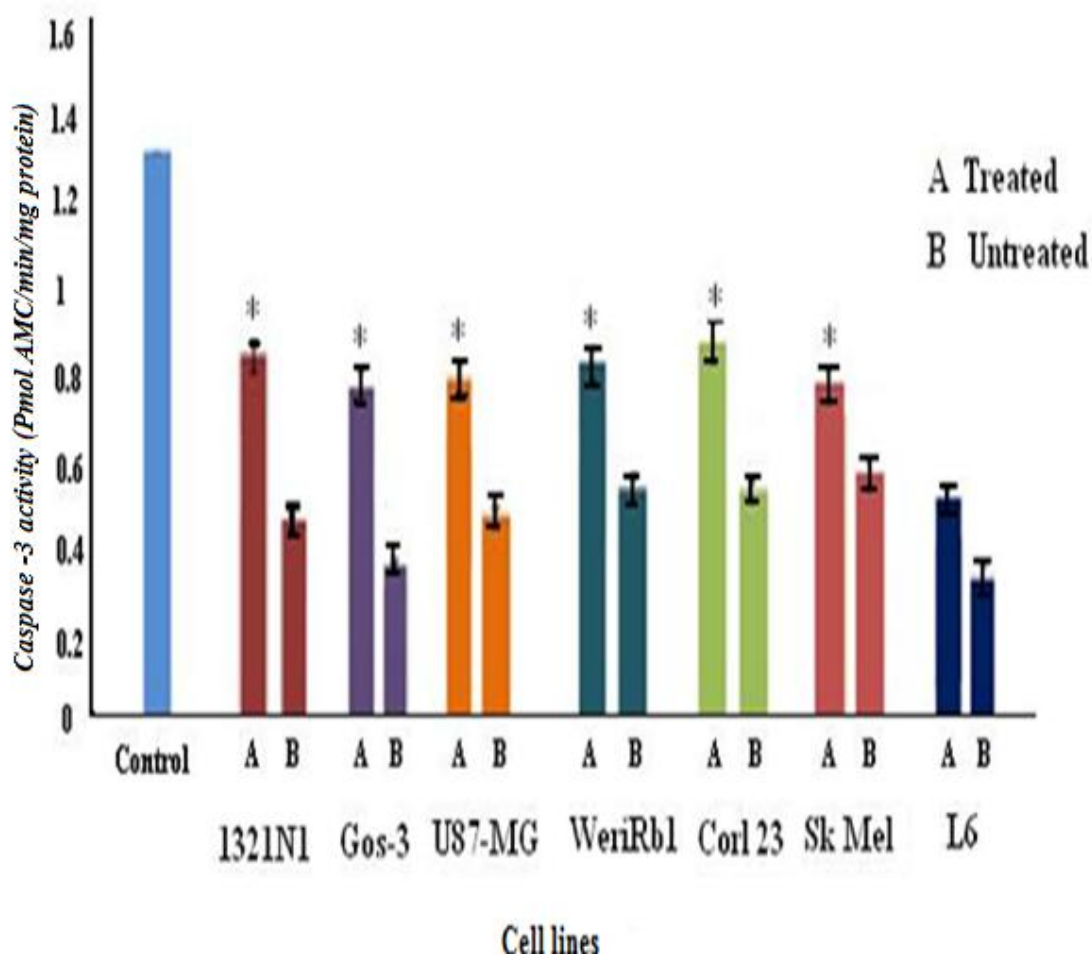
Figure 4.3: Bar charts showing caspase-9 activity in the six different cancer cell lines (1321N1, Gos-3, U87-MG, Sk Mel, Corl- 23, Weri Rb-1) and healthy L6 muscle cell line following incubation with 800 μg crude water-soluble extract of *M. charantia* for 24 hrs (A). The caspase-9 activity for the untreated cell lines (no extract) incubated in the medium alone for 24 hrs is also shown in the figure for comparison (B). Data are mean ± SD, n = 4 different experiments in duplicate. The results show that caspase-9 activity increased significantly (* $p < 0.05$) in treated (A) 1321N1, Gos-3, U87-MG, Corl- 23 and Weri Rb-1 cell lines compared to their respective untreated control (B). The crude water-soluble extract of *M. charantia* had little or no significant effect on caspase -9 activity in Sk Mel skin cancer and L6 skeletal cell lines.
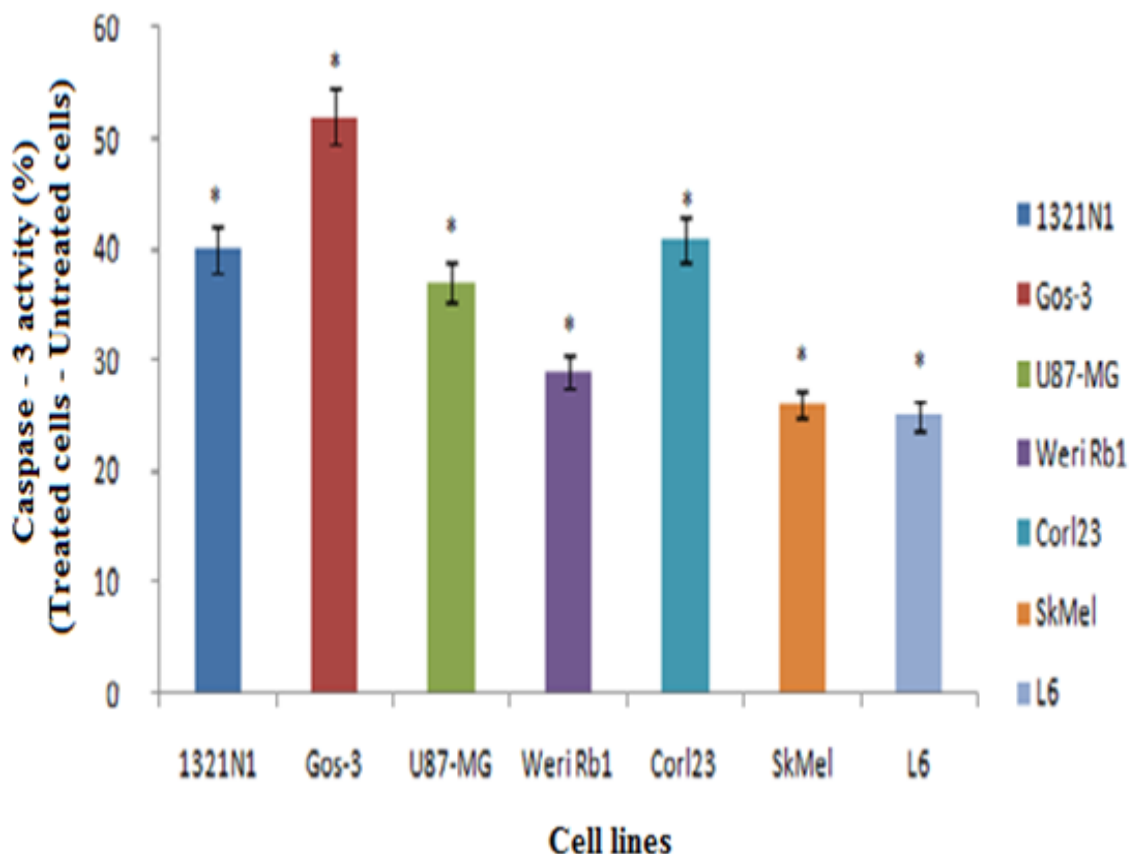
Figure 4.4: Bar charts showing the percentage difference (treated - untreated cell lines) in caspase-9 activity in six different cancer cell lines (1321N1, Gos-3, U87-MG, Sk Mel, Corl- 23 and Weri Rb-1) and healthy L6 muscle cell line incubated with 800 μg of the crude water-soluble extract of *M. charantia* for 24 hours. Caspase-9 activity for each untreated cell line was taken as 100 % and this caspase -9 activity for each treated cell line was expressed as percentage increase. This was then substracted from the untreated (100 % control value). Data are mean ± SD, n = 4 different experiments in duplicate. The results show marked and significant increases in caspase-9 activity in all the cell lines, even in L6 cell line stimulated with the crude water soluble extract of *M. Charantia* compared to untreated control.

4.2.2 Measurement of cytochrome – c activity in untreated and treated cell lines

Figure 4.5 shows the activity of cytochrome-c in (A) cell lines incubated with 800 µg of the crude water-soluble extract of *M. charantia* for 24 hours and (B) cell lines incubated alone with medium, but without any crude extract for the same duration of 24 hrs. The control response of cytochrome-c is also shown in the figure for comparison. The results show that the crude water-soluble extract of *M. charantia* can evoke significant ($p < 0.05$) increases in cytochrome-c activity in all the cell lines (1321N1, Gos-3, U87-MG, Sk Mel, Corl -23, Weri Rb-1 and L6) compared to untreated cell lines. The increase in cytochrome-c activity in 1321N1, Gos-3, U87-MG, Corl-23 and Weri Rb-1 cancer cell lines were much more pronounced compared to control cytochrome-c activity with Sk Mel and L6 cell lines.

Figure 4.6 shows the percentage difference (treated - untreated cell lines) or increase in cytochrome-c activity in all six different cancer cell lines (1321N1, Gos-3, U87-MG, Sk Mel, Corl- 23 and Weri Rb-1) and healthy L6 muscle cell line treated with 800 µg of the crude water-soluble extract of *M. charantia*. Cytochrome - c activity in each untreated cell line was taken as 100 %. The cytochrome-c activity for each treated cell line was expressed as a percentage of the respective control and then substracted from the untreated control value. The results in figure 4.6 show marked and significant ($p < 0.01$) increases in cytochrome – c activity in all the cell lines including L6. However, cytochrome – c activity was much more pronounced in 1321N1, Gos-3, U87-MG, Corl-23 and Weri Rb-1 compared to L6 and Sk Mel cell lines.
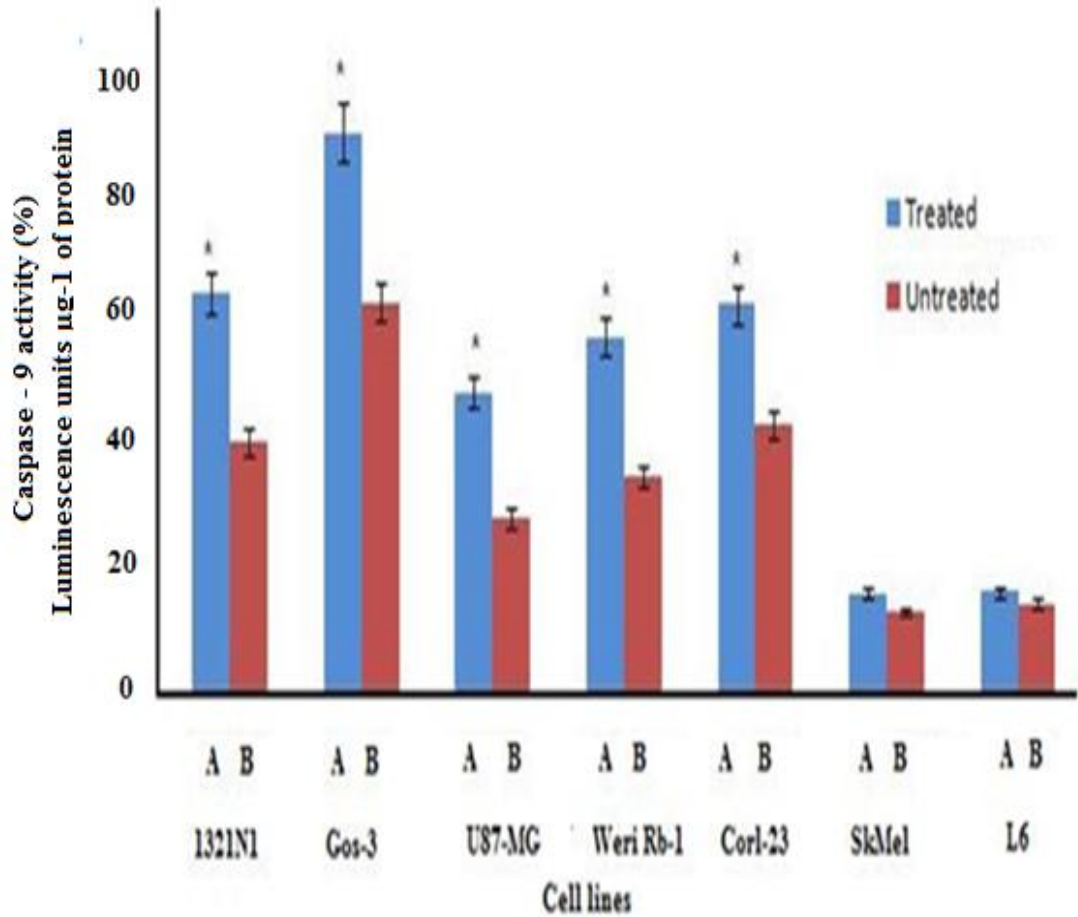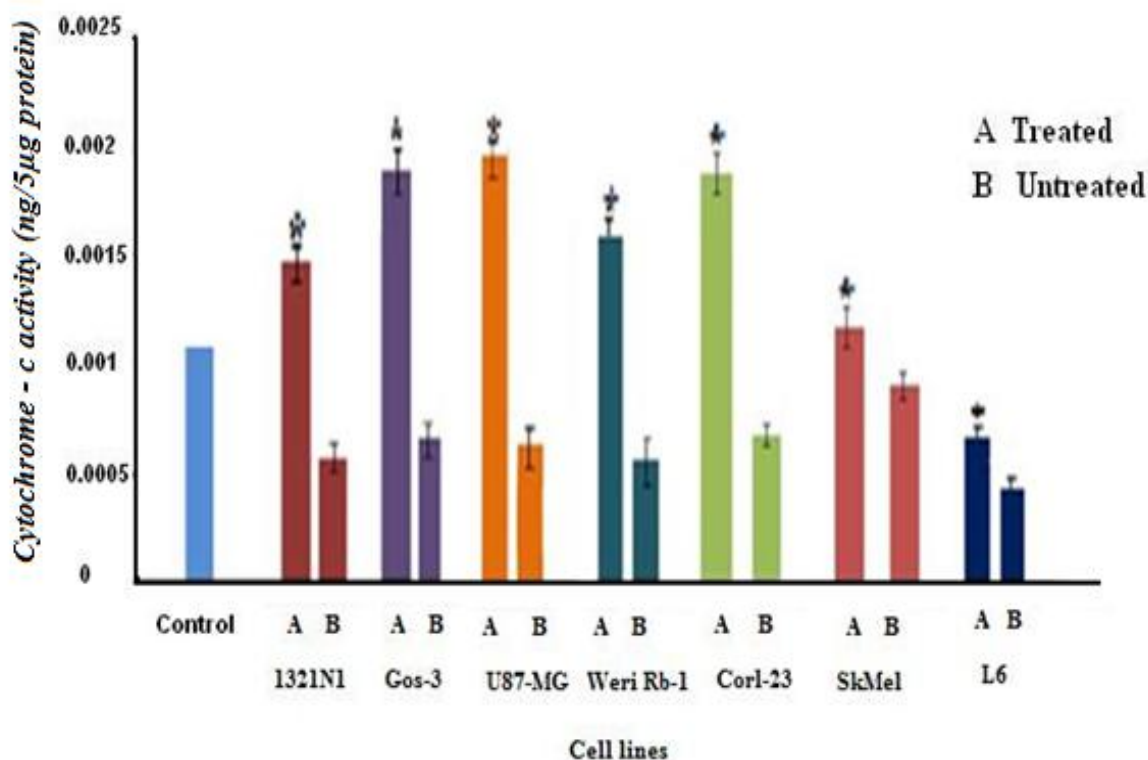
Figure 4.5: Bar charts showing cytochrome-c activity in (A) six different cancer cell lines (1321N1, Gos-3, U87-MG, Sk Mel, Corl- 23, Weri Rb-1) and healthy L6 muscle cell line following incubation with 800 μg crude water-soluble extract of *M. charantia* for 24 hrs. The cytochrome-c activity (B) for the untreated (no extract) cell lines is also shown for comparison. Similarly, the control background cytochrome-c activity in the absence of any cells is also shown in the figure. Data are mean ± SD, n = 4 different experiments in duplicate. Note the siginificantn (* $p < 0.05$) increases in cytochrome-c activity in treated cells compared to untreated cells. For 1321N1, Gos-3, U87-MG, Sk Mel, Corl- 23 and Weri Rb-1 cancer cell lines, cytochrome-c activity was significantly (* $p < 0.05$) higher compared to the assay kit control activity. In Sk Mel and L6 skeletal muscle cell lines, cytochrome-c activity increased significantly (* $p < 0.05$) for treated compared to untreated cells but these values were either the same (Sk Mel cell line) or less (L6 cell line) than the assay kit control cytochrome-c activity.
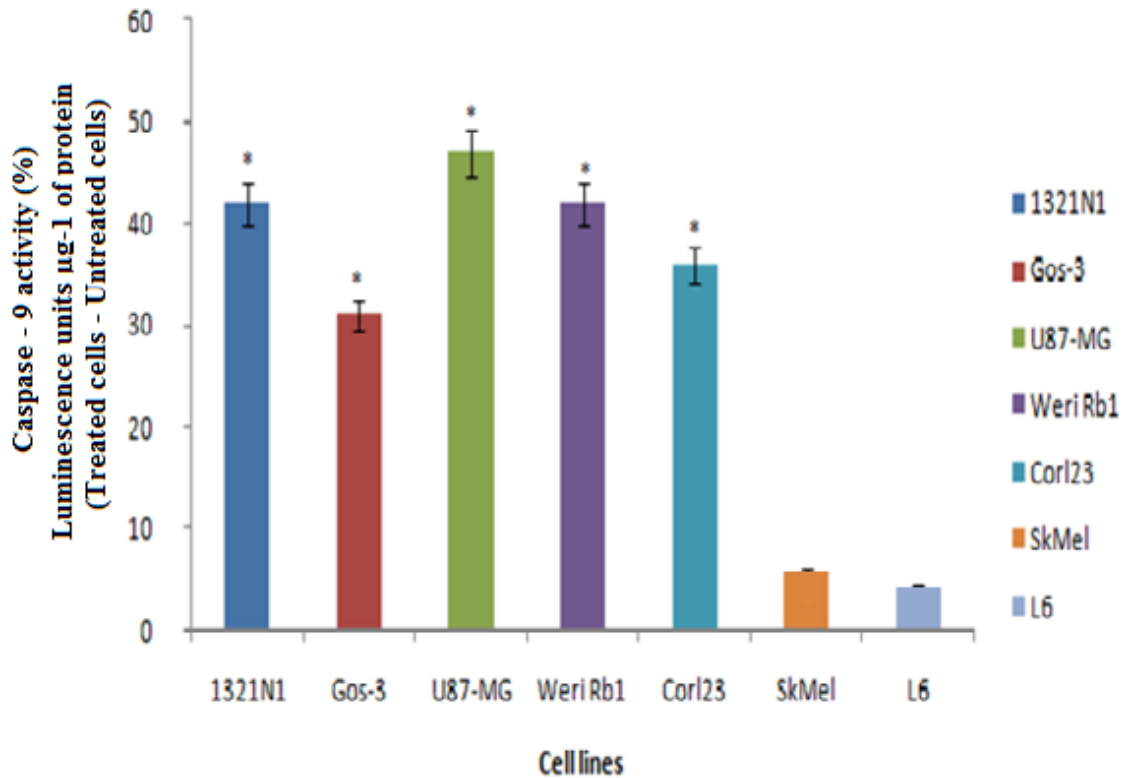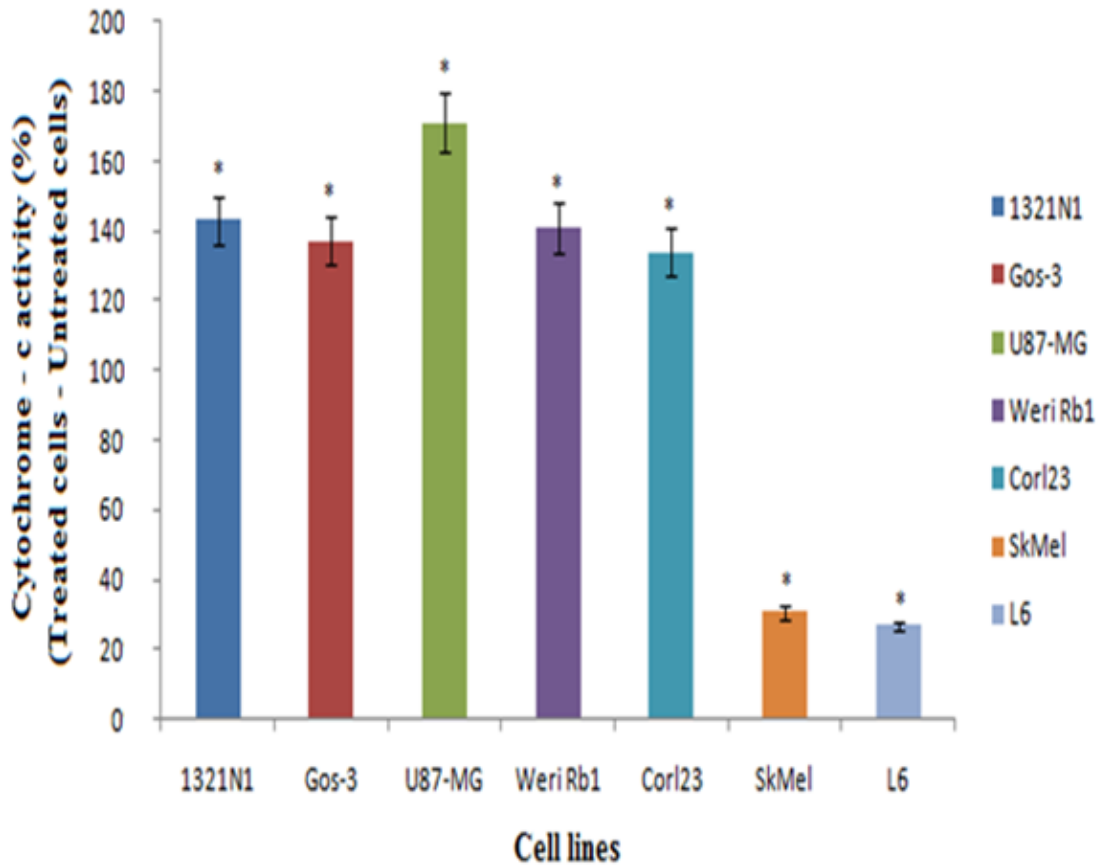
Figure 4.6: Bar charts showing the percentage difference (treated − untreated cell lines) in cytochrome-c activity in six different cancer cell lines (1321N1, Gos-3, U87-MG, Sk Mel, Corl- 23 and Weri Rb-1) and healthy L6 muscle cell line incubated with 800 µg of the crude water-soluble extract of *M. charantia* for 24 hours. Cytochrome – c activity in each cell line was taken as 100 %. The value for each treated cell line was expressed as a percentage of control and the value was substracted from the 100 % untreated control value and expressed as a percentage increase in cytochrome-c activity. Data are taken from Figure 4.6 and they are expresses as mean ± SD, n = 4 different experiments in duplicate. Note that cytochrome-c activity increased significantly (* $p < 0.05$) in all cell lines including L6 compared to the untreated control value.

4.2.3 Time-course measurements of intracellular free [Ca $^{2+}$]$_i$ in untreated and treated cells

In this study, basal (mean ± SD) [Ca^{2+}]$_i$ at the start of any incubation (0 min) was 0.17 ± 0.09 ratio units (intensity), n = 82. Figure 4.7 shows the time-course changes (solid diamonds) in intracellular free calcium concentrations [Ca^{2+}]$_i$ expressed as fluoresences ratio units, in (A) 1321N1 and (B) Gos-3 cancer cell lines following incubation with 800 μg of the crude water-soluble extract of *M. charantia*. The changes in [Ca^{2+}]$_i$ in untreated (control) 1321N1 and Gos-3 cancer cell lines (solid squares) are also shown in the figure for comparison. The result show that incubation of the two cancer cell lines with the crude water-soluble extract of *M. charantia* can result in significant (p < 0.05) time- dependent increases in [Ca^{2+}]$_i$ in both cancer cell lines compared to control (untreated) cells. Maximal increase [Ca^{2+}]$_i$ was attained after 420 min of incubation. In control untreated cell line for each, [Ca^{2+}]$_i$ remained more or less stable in both cell lines after 420 mins.

Figure 4.7: Time-course changes in $[Ca^{2+}]_i$ expressed as fluorescence ratio units (intensity) in Fura-2 AM loaded (A) 1321N1 and (B) Gos-3 cell lines (solid diamonds) following incubation with 800 µg of the crude water-soluble extract of *M. charantia* for 420 min. The fluorescence intensity for untreated cells (solid squares) at different time points are also shown for comparison. Data are mean ± SD, n = 6; (* $p < 0.05$) for treated compared to untreated cells for the time points of 120 - 420 min in A and in B for time points of 240, 300, 360 and 420 min. Note gradual time-course increases in $[Ca^{2+}]_i$ following treatment with crude water-soluble extract of *M. charantia* compared to control.

Figure 4.8 shows the time-course changes $[Ca^{2+}]_i$ in (A) U87- MG and (B) Sk Mel cancer cell lines following incubation with 800 µg of the crude water-soluble extract of *M. charantia*. The changes in $[Ca^{2+}]_i$ in untreated (control) U87-MG and Sk Mel cancer cell lines are also shown in figure 4.8 for comparison. The data show that incubation of the Sk Mel cancer cell line with the crude water-soluble extract of *M. charantia* can result in significant ($p < 0.05$) time-dependent increases in $[Ca^{2+}]_i$ compared to control (untreated) cells with maximal increases in $[Ca^{2+}]_i$ after 420 min of incubation. In control, untreated Sk Mel cancer cell line $[Ca^{2+}]_i$ remained more or less stable up to 420 min. In contrast, in U87-MG cell line $[Ca^{2+}]_i$ increased slightly in both untreated and treated cells up to 360 min. In the control (untreated) cells, $[Ca^{2+}]_i$ remained stable for 360 - 420 min. However, in treated U87-MG cells there was a small but significant ($p < 0.05$) increase $[Ca^{2+}]_i$ compared to untreated cells after 360 min of incubation with the crude water-soluble extract of *M. charantia.*

Figure 4.9 shows the time-course changes in $[Ca^{2+}]_{i,}$ in (A) Corl-23 and (B) Weri Rb-1 cancer cell lines (solid squares) following incubation with 800 µg of the crude water-soluble extract of *M. charantia*. The changes in $[Ca^{2+}]_i$ in untreated (control) Corl-23, Weri Rb-1 cancer cell lines (solid triangle) are also shown in figure 4.9 for comparison. The data show that incubation of the two cancer cell lines with the crude water-soluble extract of *M. charantia* can result in significant ($p < 0.050$ time-dependent increases in $[Ca^{2+}]_i$ in both cancer cell lines compared to control (untreated) cells with maximal increases in $[Ca^{2+}]_i$ at 420 min. In untreated control, $[Ca^{2+}]_i$ remained more or less stable in both cell lines after 420 min of incubation.
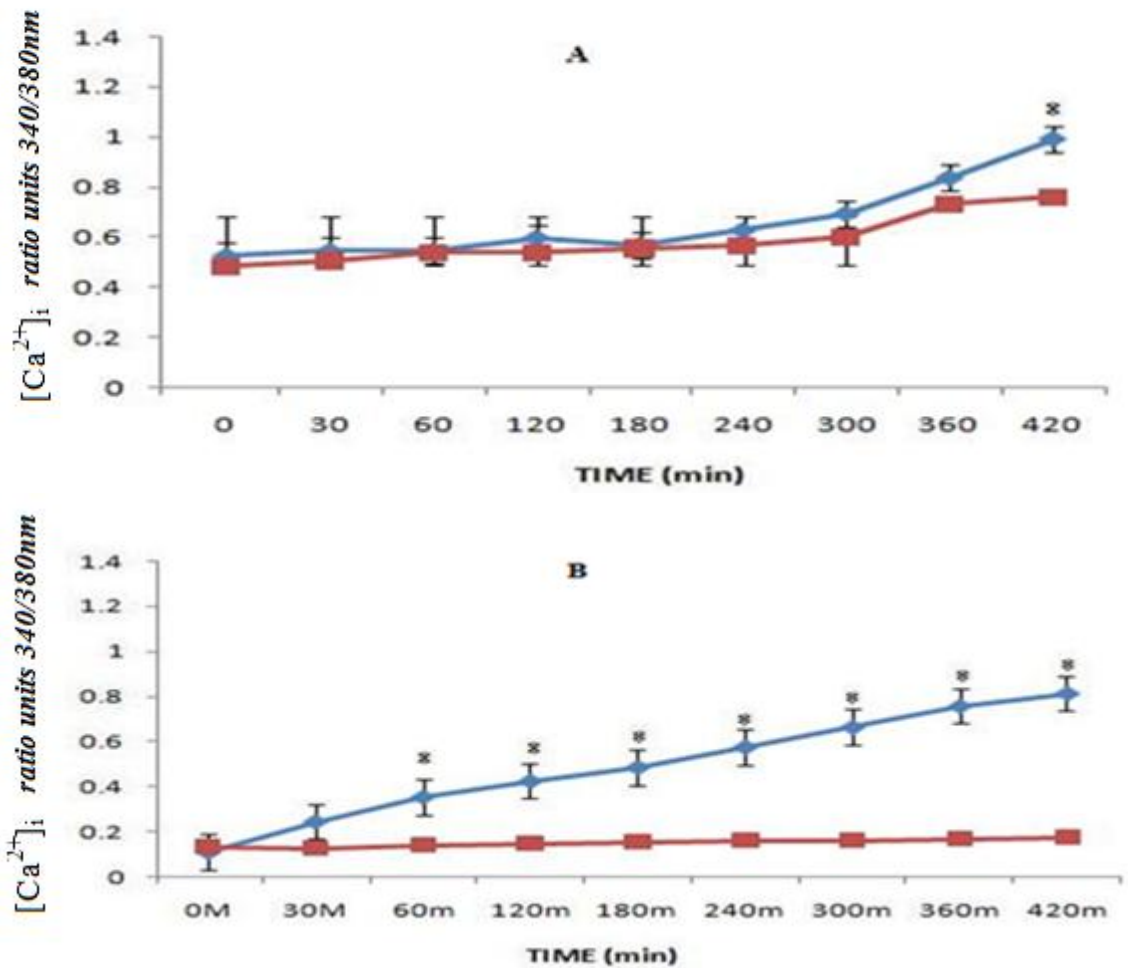
Figure 4.8: Time-course changes in $[Ca^{2+}]_i$ expressed as fluorescence ratio units (intensity) in Fura -2 AM loaded (A) U87- MG and (B) Sk Mel cell lines (solid diamonds) following incubation with 800 µg crude water-soluble extract of *M. charantia* for 420 min. The fluorescence intensity for untreated cells (solid squares) at different time points are also shown for comparison. Data are mean ± SD, n = 6; * $p < 0.05$ for treated compared to untreated cells in both A and B. Note the gradual time-course increases in $[Ca^{2+}]_i$ following treatment with crude water-soluble extract of *M. charantia* in Sk Mel cell line compared to control. Note also that there was only a small increase in $[Ca^{2+}]_i$ in U87-MG cell line following incubation with the crude water-soluble extract of *M. charantia* and this was only significant (* $p < 0.05$) compared to untreated control cells after 420 min of incubation.
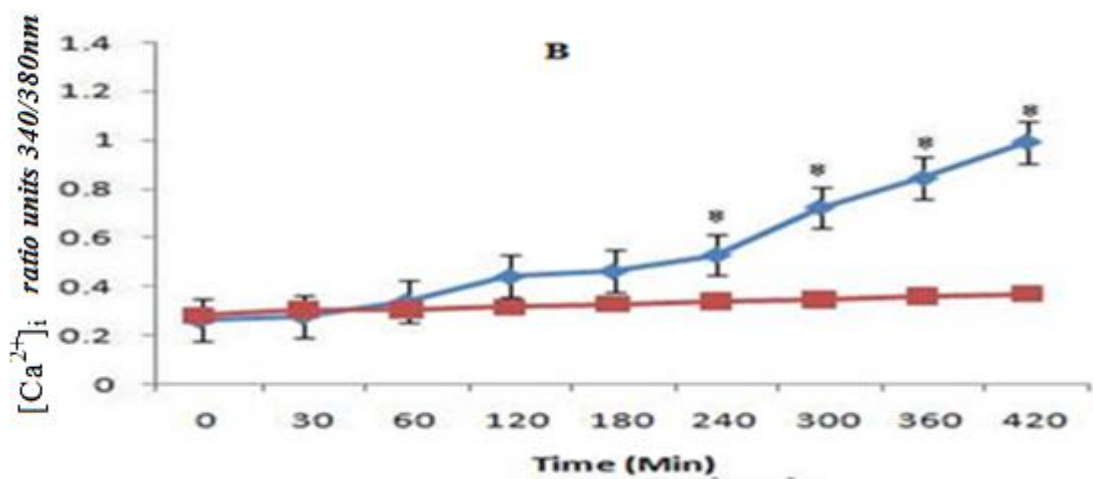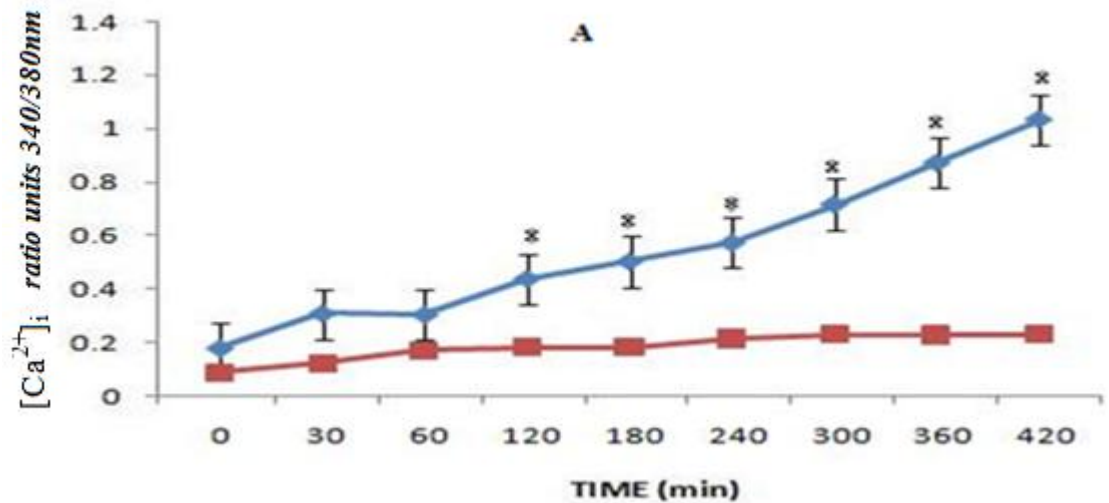
Figure 4.9: Time-course changes in $[Ca^{2+}]_i$ expressed as fluorescence ratio units (intensity) in Fura-2 AM loaded Corl-23 (A) and Weri Rb-1 (B) cell lines (solid diamonds) following incubation with 800 µg crude water-soluble extract of *M. charantia* for 420 min. The fluorescence intensity for untreated cells (solid squares) at different time points are also shown for comparison. Data are mean ± SD, n = 6; * p < 0.05 for treated compared to untreated cells in both A and B. Note the gradual time-course increases in $[Ca^{2+}]_i$ in both cell lines following treatment with crude water-soluble extract of *M. charantia* compared to untreated control cells.

Figure 4.10 shows the time-course changes in $[Ca^{2+}]_i$ in L6 muscle cell line (solid diamonds) following incubation with 800 µg of the crude water-soluble extract of *M. charantia*. The changes in $[Ca^{2+}]_i$ in untreated (control) L6 muscle cell line (solid squares) are also shown in figure 4.10 for comparison. The result shows that incubation of the L6 muscle cell line with the crude water-soluble extract of *M. charantia* can result in only a small increase in $[Ca^{2+}]_i$ reaching maximum after 240 min of incubation. However, this increase in $[Ca^{2+}]_i$ was more or less the same as untreated control cells. Thereafter, $[Ca^{2+}]_i$ in both treated and untreated L6 cells decreased gradually.

Figure 4.11 shows the crude water-soluble extract of *M. charantia* evoked maximal increases in $[Ca^{2+}]_i$ for the six different cancer cell lines as well as L6 skeletal muscle cell line after 420 min of incubation. Each value taken from figures 4.7- 4.10 at 420 min time point is expressed as the ratio units. The results show the basal $[Ca^{2+}]_i$ at 0 min, and increases in $[Ca^{2+}]_i$ in untreated and treated cells for a duration of 420 min. The results show that 800 µg crude water-soluble extract of *M. charantia* can evoke significant ($p < 0.05$) increases in $[Ca^{2+}]_i$ compared to control at 0 min as well as untreated cells over the same duration of incubation. In contrast, $[Ca^{2+}]_i$ in L6 skeletal muscle cell line remained more or less the same as the untreated cells.
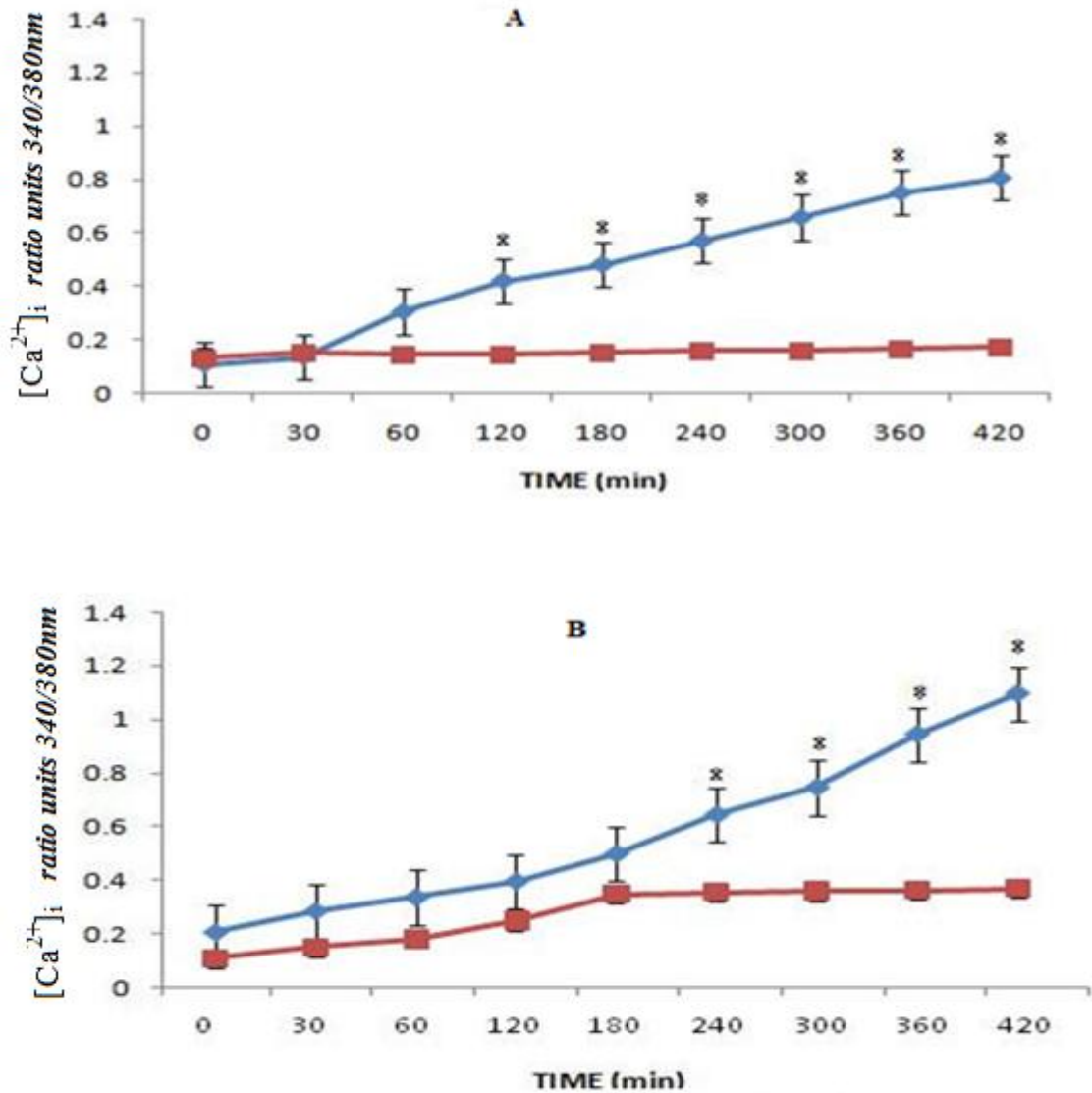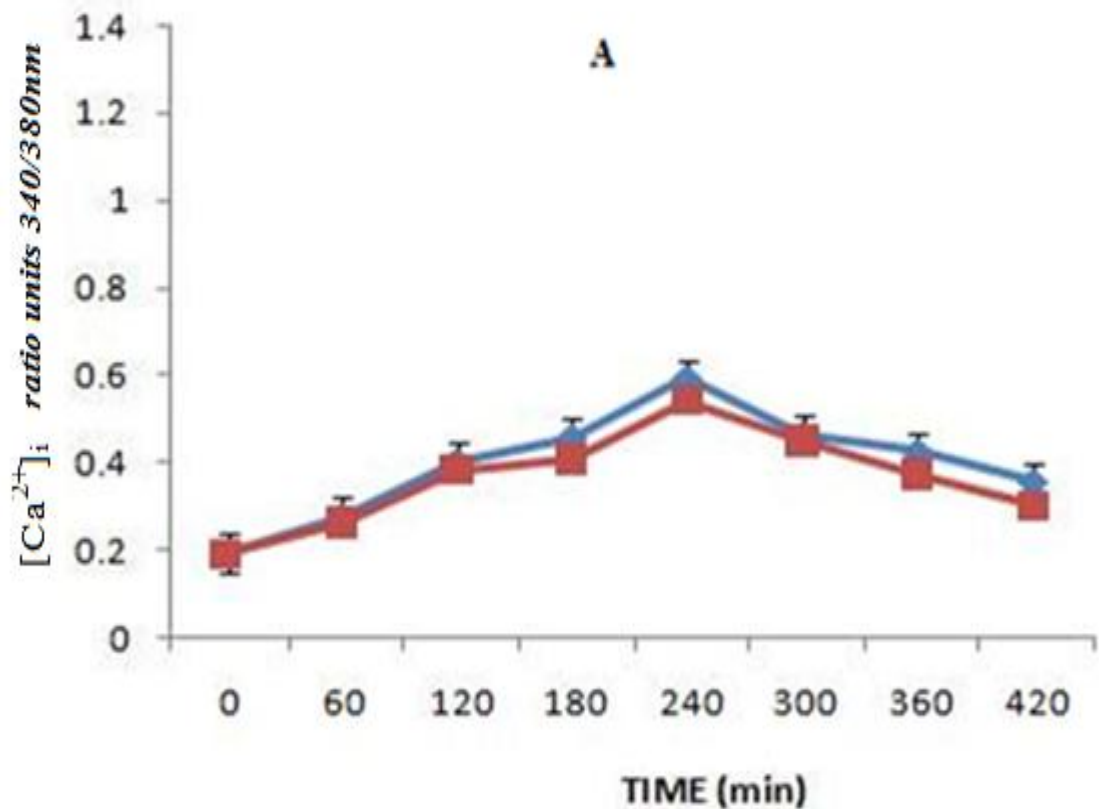
Figure 4.10: Time-course changes $[Ca^{2+}]_I$ expressed as fluorescence ratio units (intensity) in Fura-2 AM loaded healthy L6 muscle cell line (solid diamonds) following incubation with 800 µg crude water-soluble extract of *M. charantia* for 420 min. The fluorescence intensity for untreated cells (solid squares) at different time points are also shown for comparison. Data are mean ± SD, n = 6. Note that there are no significant (* $p > 0.05$) change in $[Ca^{2+}]_i$ comparing treated with untreated cells. However, the result shows a small increase in $[Ca^{2+}]_i$ in both test and control L6 cells for 120-240 min. Thereafter $[Ca^{2+}]_i$ decreased gradually in both test and control cells.
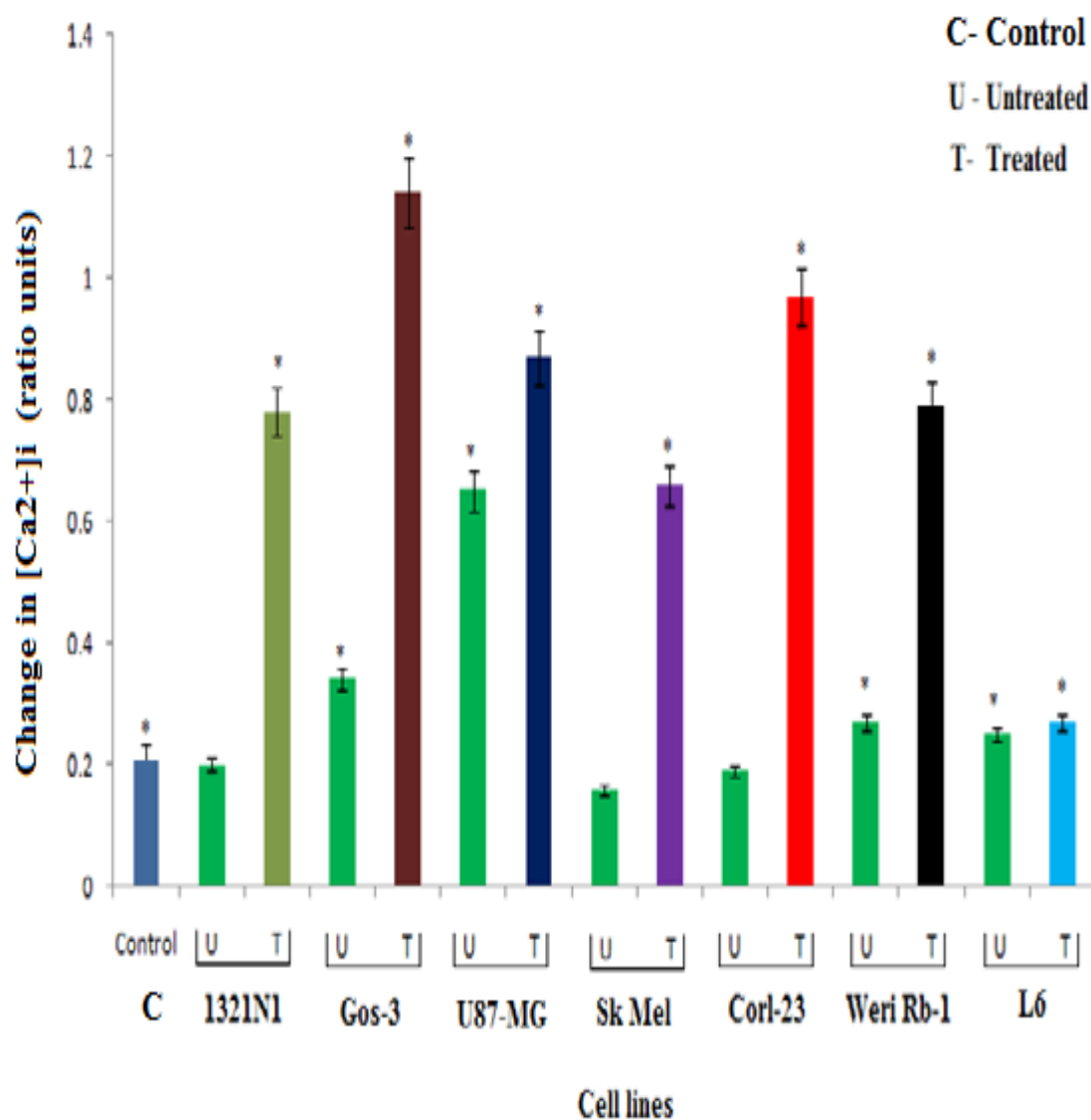
Figure 4.11: Bar charts showing maximal increases in $[Ca^{2+}]_i$ in basal control (C), untreated (U) cells and treated (T) cancer cell lines as well as L6 cell line following incubation with 800 µg crude water-soluble extract of *M. charantia* for 420 min. Data taken from (Figures 4.7- 4.10) and they are expressed as mean mean ± SD (ratio units), n = 6; (* $p < 0.01$) for test (T) compared to control (C) $[Ca^{2+}]_i$ at the start of the experiment (0 min) and also compared to untreated (U) cells at time point of 420 min. Note the large increases in $[Ca^{2+}]_i$ for 1321N1, Gos-3, Corl -23 and Weri Rb-1 cell lines.

4.2.4 Cellular mechanisms of action of α,β momorcharin on L6 and cancer cell lines

Since the crude water-soluble extract of *M. charantia* produced more or less similar effects on cell viability as α, β momorcharin, it was decided to investigate the mechanism(s) of action of α, β momorcharin on the same seven different cell lines for comparison.

4.2.5 Measurement of caspase-3 and 9 activities

Figure 4.12 shows caspase -3 activity in (A) the 6 cancer and L6 cell lines treated with 800 μM of the α, β momorcharin for 24 hours and (B) cell lines incubated alone with medium but without any α, β momorcharin for the same duration of 24 hrs. The results show that caspase-3 activity decreased significantly ($p < 0.05$) in both treated and untreated cell lines compared to the assay control caspase-3 activity. The decrease was much more pronounced in untreated cells compared to treated cells. Comparing cells treated with the α, β momorcharin with untreated (no α, β momorcharin) cells, the results show a significant ($p < 0.05$) increase in caspase -3 activity in the treated cells compared to untreated cells. It is also noteworthy, that caspase -3 activity increased slightly, but this was significant ($p < 0.05$) in L6 treated compared to untreated L6 muscle cells.
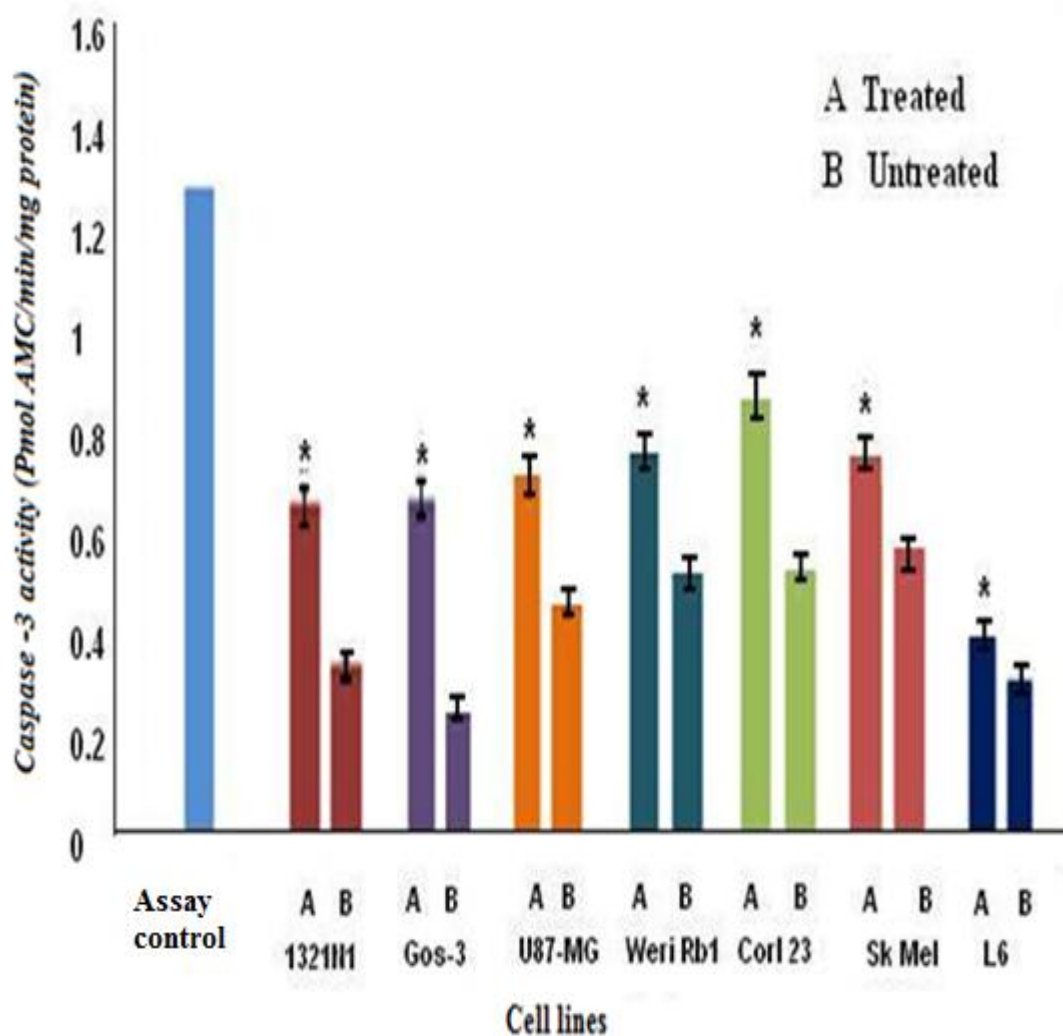
Figure 4.12: Bar charts showing caspase-3 activity in the six different cancer cell lines (1321N1, Gos-3, U87-MG, Sk Mel, Corl- 23, Weri Rb-1) and healthy L6 muscle cell line (A) treated with 800 μM of the α, β momorcharin for 24 hours and (B) control cancer and L6 cell lines incubated alone with the media without any α,β momorcharin for the same duration of 24 hrs. Assay kit control caspase-3 activity is shown in the figure for comparison. Data are mean ± SD, n = 4 different experiments in duplicate. Note that caspase-3 activity increased significantly (* $p < 0.05$) in treated cells compared to untreated cells.

Figure 4.13 shows the percentage difference (treated − untreated cell line for each) or increase in caspase -3 activity in all six different cancer cell lines (1321N1, Gos-3, U87-MG, Sk Mel, Corl- 23 and Weri Rb-1) and healthy L6 muscle cell line treated with 800 μM of the α, β momorcharin. Caspase -3 activity in each untreated cell line was taken as 100 %. Caspase-3 activity in each treated cell line was expressed as a percentage of untreated control and then substracted from the untreated control value. The results show that α, β momorcharin can evoke significant increases in caspase-3 activity in all cell line including L6. The effect of caspase-3 activity was much more pronounced in 1321N1 and Gos-3 cell line compared to the others.

Figure 4.14 shows caspase -9 activity in (A) the six cancer cell lines and L6 cell line treated with 800 μM of the α, β momorcharin for 24 hours and (B) the same cell lines incubated alone with media but without any α, β momorcharin for the same duration of 24 hrs. The results show that α, β momorcharin can elicit significant increases ($p < 0.05$) in caspase-9 activity in 1321N1, Gos-3, U87-MG, Corl -23 and Weri Rb-1 cancer cell lines compared to untreated cell line for each. There was no significant ($p > 0.05$) increase in caspase-9 activity in either Sk Mel and L6 healthy skeletal muscle cell line compared to their respective untreated cell line.

Figure 4.13: Bar charts showing the percentage difference (treated – untreated cell lines) or increase in caspase-3 activity in six different cancer cell lines (1321N1, Gos-3, U87-MG, Sk Mel, Corl- 23 and Weri Rb-1) and healthy L6 muscle cell line incubation with 800 μM α, β momorcharin for 24 hrs. The values for each untreated cell was taken as 100 %. Value from the respective treated cell was expressed as a percentage of the untreated control. This value was substracted from the respective untreated control to give the values plotted in the figure 4.13 from each cell line. Data are taken from Figure 4.12 and they are expresses as mean ± SD, n = 4 different experiments in duplicate. Note that caspase-3 activity activity increased significantly (* $p < 0.05$) in all cell lines including L6 compared to the untreated control value.
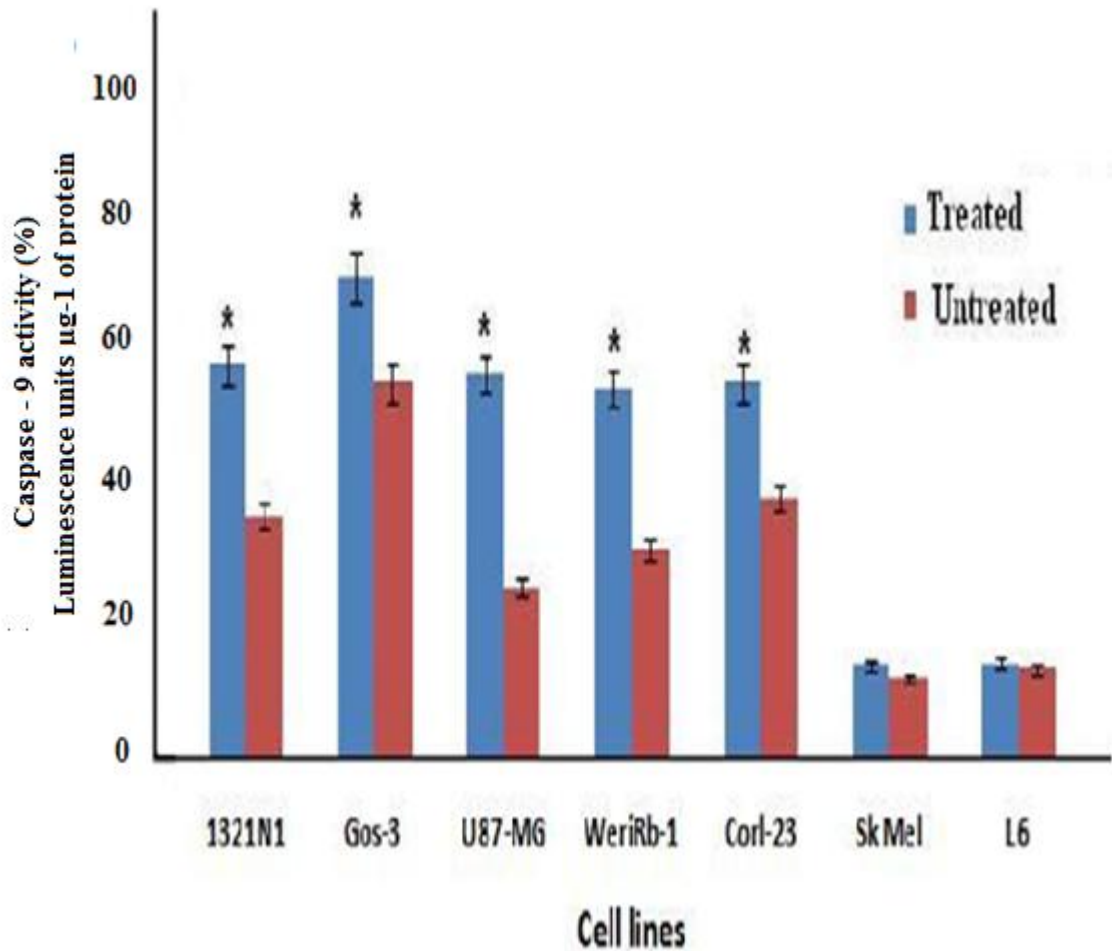
Figure 4.14: Bar charts showing caspase-9 activity in six different cancer cell lines (1321N1, Gos-3, U87- MG, Sk Mel-2, Corl- 23, Weri Rb-1) and healthy L6 muscle cell line following incubation with 800 µM of α, β momorcharin for 24 hrs. The caspase-9 activity for the untreated cell lines (no α, β momorcharin) is also shown in the figure for comparison. Data are mean ± SD, n = 4 different experiments in duplicate. Note that caspase-9 activity increased significantly (* $p < 0.05$) in treated compared to untreated cells for 1321N1, Gos-3, U87-MG, Corl-23 and Weri Rb-1 cell lines. There was no significant (* $p > 0.05$) change in caspase-9 activity in either Sk Mel or L6 cell line comparing to their respective control (untreated) cells.

Figure 4.15 shows the percentage difference (treated – untreated cell lines) or increase in caspase -9 activity in all six different cancer cell lines (1321N1, Gos-3, U87-MG, Sk Mel, Corl- 23 and Weri Rb-1) and healthy L6 muscle cell line treated with 800 μM of the α, β momorcharin. The value for each untreated cell line was taken as 100 % and the value for the respective treated cells was expressed as percentage of the untreated cells. Th treated value was substracted from the untreated value and the results plotted in figure 4.15 as increase in caspase-9 activity above untreated control. The results show that α, β momorcharincan evoke significant increases in caspase-9 activity in the 6 different cell lines. The results show that α, β momorcharin was much more effective in U87-MG compared to the other cells.

Figure 4.16 shows the activity of cytochrome-c in (A) the cancer cell lines treated with 800 μM of the α, β momorcharin for 24 hours and (B) the same cell lines incubated with media alone but without any α, β momorcharin for the same duration of 24 hrs. The control response of cytochrome-c is also shown in the figure for comparison. The results show that α, β momorcharin can evoke significant ($p < 0.05$) increases in cytochrome-c activity in all the cancer cell lines (1321N1, Gos-3, U87-MG, Sk Mel, Corl-23, Weri Rb-1 and L6) compared to either untreated cell lines or control cytochrome-c activity. In L6 skeletal muscle cell line, cytochrome-c activity increased significantly ($p < 0.05$) in treated (A) cells compared to untreated (B) cells but these values were less than the assay kit control cytochrome-c activity.
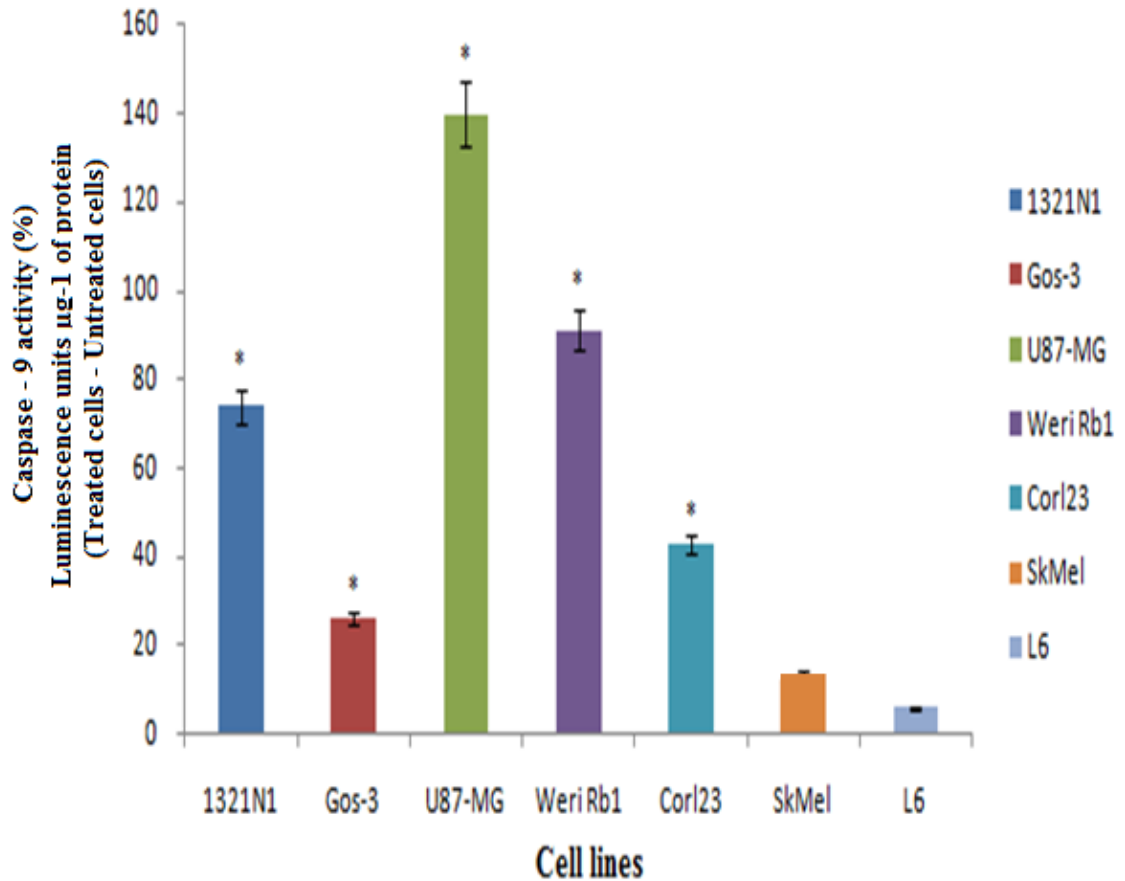
Figure 4.15: Bar charts showing the percentage difference (treated - untreated cell lines) or incerease in caspase-9 activity in six different cancer cell lines (1321N1, Gos-3, U87-MG, Sk Mel, Corl- 23 and Weri Rb-1) and healthy L6 muscle cell line incubation with 800 μM α, β momorcharin for 24 hrs. The value for each untreated control cell line was taken as 100 % and the value for the respective treated cells was expressed as a percentage of untreated control. The treated value was substracted from the untreated control value. Data are mean ± SD, n = 4 different experiments in duplicate. Note that caspase-9 activity increased significantly * $p < 0.05$ in treated compared to untreated cell line. Note that α, β momorcharin had little effect in L6 cell line.
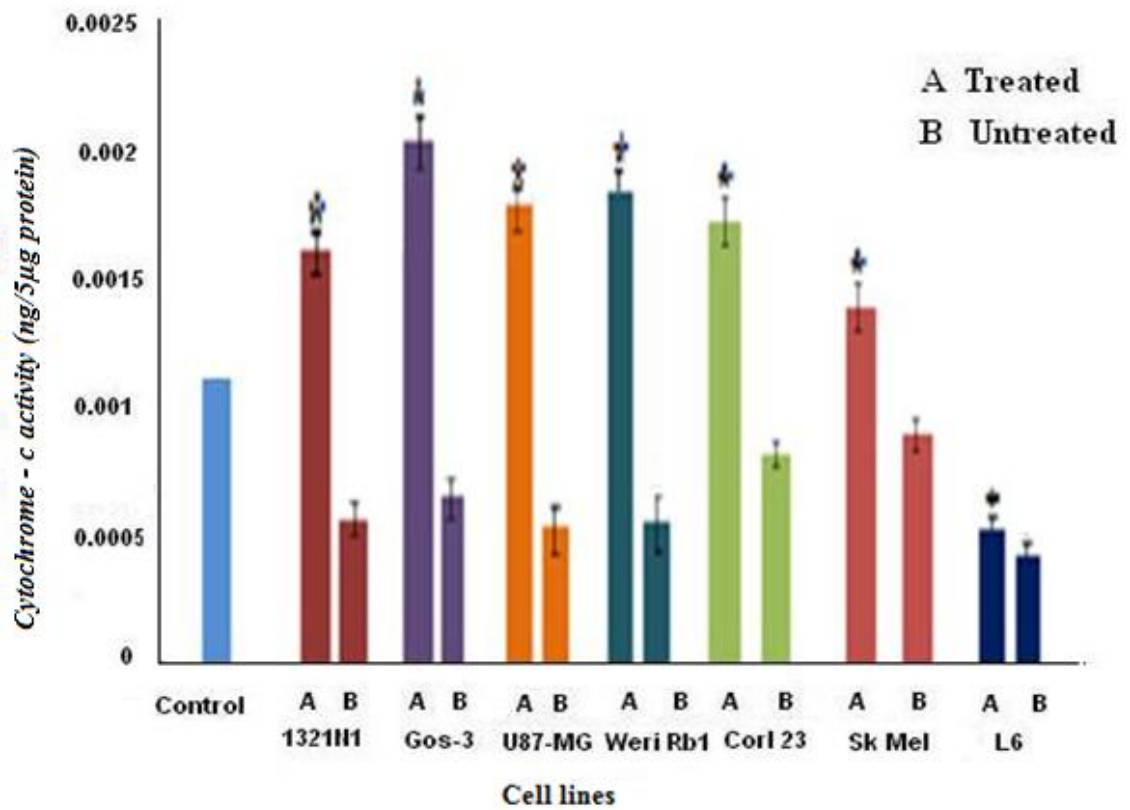
Figure 4.16: Bar charts showing cytochrome-c activity in (A) six different cancer cell lines (1321N1, Gos-3, U87-MG, Sk Mel-2, Corl- 23, Weri Rb-1) and healthy L6 muscle cell line following incubation with 800 μM α, β momorcharin for 24 hrs. The cytochrome-c activity (B) for the untreated (no α, β momorcharin) cell lines is also shown in figure for comparison. Similarly, the background assay kit control cytochrome-c activity in the absence of any cells is shown in the figure. Data are mean ± SD, n = 4 different experiments in duplicate. Note that cytochrome-c activity increased significantly (* $p < 0.05$) in all the treated cells compared to untreated cells. The results also show that cytochrome-c activity was maximal in Gos-3 cell line where as 1321N1, U87- MG, Sk Mel-2 and Weri Rb-1 contain more or less the same activity. However, cytochrome-c activity in L6 muscle cell line was the least compared to all the cancer cell lines.
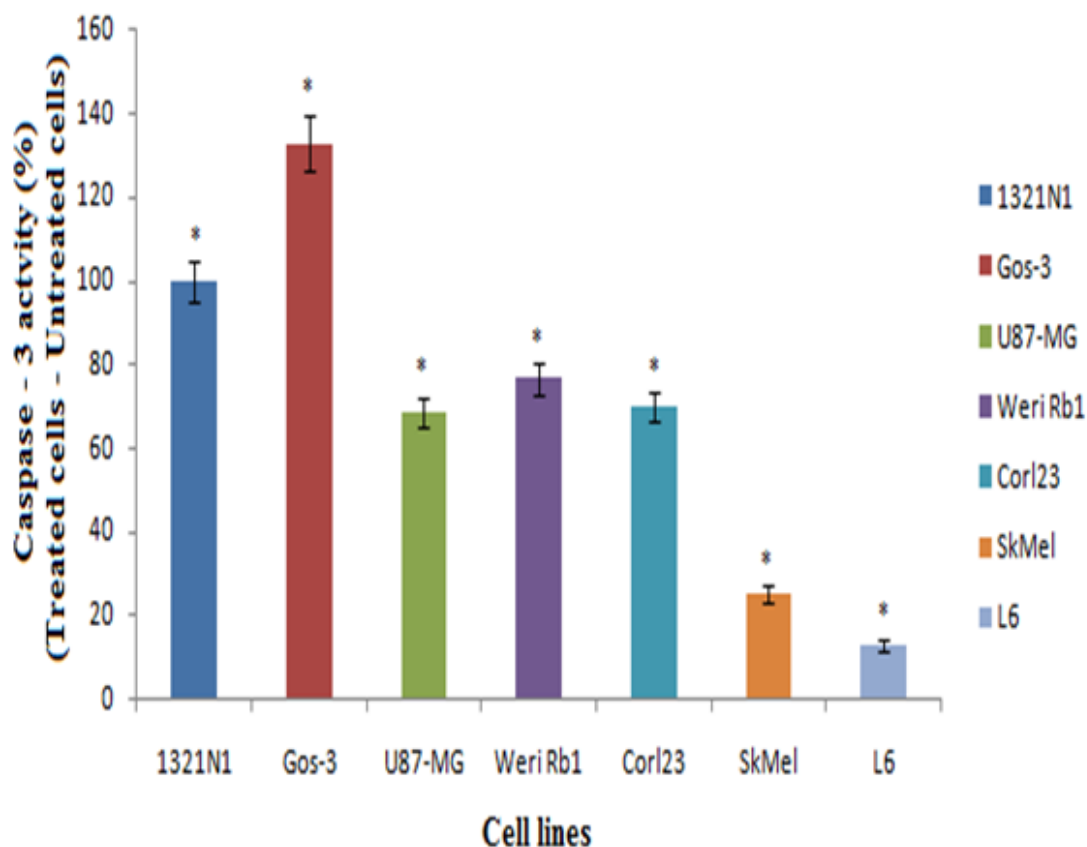
Figure 4.17 shows the percentage difference (treated – untreated cell lines) or increase in cytochrome-c activity in all six different cancer cell lines (1321N1, Gos-3, U87-MG, Sk Mel, Corl- 23 and Weri Rb-1) and healthy L6 muscle cell line treated with 800 μM of α, β momorcharin. The value for each untreated cell line was taken as 100 % and the value for the respected treated cell line was expressed as a percentage of the untreated cell line. The difference between treated and untreated for each cell line is plotted in figure 4.17. The results show that α, β momorcharin can evoke large and significant ($p < 0.05$) increases in cytochrome-c activity in 1321N1, Gos-3, Weri Rb-1 and Corl -23 cell line compared to the respective untreated cell lines. The results also show that α, β momorcharin had little effect on cytochrome – c activity in L6 skeletal muscle cell line.
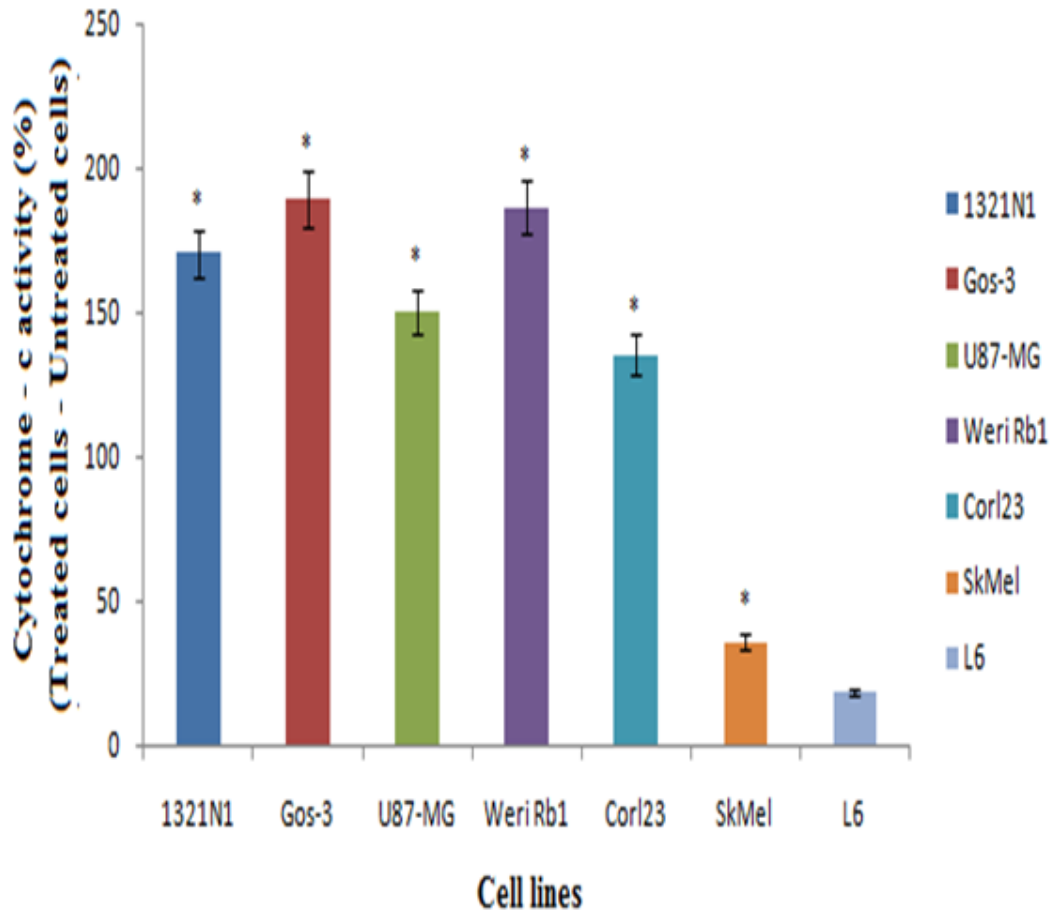
Figure 4.17: Bar charts showing the percentage difference (treated − untreated) or increase in cytochrome-c activity in six different cancer cell lines (1321N1, Gos-3, U87-MG, Sk Mel, Corl- 23 and Weri Rb-1) and healthy L6 muscle cell line following incubation with 800 µM α, β momorcharin for 24 hrs. All values are expressed as a percentage of the respective untreated control. The data were taken from figure 4.16 and they are expressed mean ± SD, n = 4; (* $p < 0.05$) for each cell line compared to their respective untreated cell line. Note that α, β momorcharin had little effect on cytochrome – c activity in L6 skeletal muscle cell line compared to large and significant effects in 1321N1, Gos-3,U87-MG, and Corl -23 cell lines.

4.2.6 Measurement of $[Ca^{2+}]_i$ in untreated and treated cells

Figure 4.18 shows the time-course changes in $[Ca^{2+}]_i$ expressed as fluorescence ratio units, in (A) 1321N1 and (B) Gos-3 cancer cell lines (solid diamonds) following incubation with 800 μM of α, β momorcharin for 420 min. The changes in $[Ca^{2+}]_i$ in untreated (control) 1321N1 and Gos-3 cancer cell lines (solid squares) are also shown in the figure for comparison. The results show that incubation of the two cancer cell lines with α, β momorcharin can result in significant ($p < 0.05$) time-dependent increases in $[Ca^{2+}]_i$ in both cancer cell lines compared to control (untreated) cells. Maximal increases in $[Ca^{2+}]_i$ was attained after 420 min of incubation. In control (untreated cell lines), $[Ca^{2+}]_i$ remained more or less stable in both cell lines after 420 min. The results also show that the increase in $[Ca^{2+}]_i$ in Gos-3 cell line was much more pronounced following incubation with α, β momorcharin compared to 1321N1 cell line.

Figure 4.19 shows the time-course changes $[Ca^{2+}]_i$ in expressed as fluorescence ratio units (A) U87- MG and (B) Sk Mel cancer cell lines following incubation with 800 μM of α, β momorcharin (solid diamonds). The changes in $[Ca^{2+}]_i$ in untreated (control) U87- MG and Sk Mel cancer cell lines are also shown in the figure for comparison (solid squares). The data show also that incubation of Sk Mel cell line with α, β momorcharin can result in a significant ($p < 0.05$) time-dependent increase in $[Ca^{2+}]_i$ compared to control (untreated) cells with maximal increases in $[Ca^{2+}]_i$ after 420 min of incubation. In control, $[Ca^{2+}]_i$ remained more or less stable after 420 min. In contrast, with untreated U87–MG cell line basal $[Ca^{2+}]_i$ remained more or less the same over 420 min. However, in U87-MG cell line treated with α, β momorcharin $[Ca^{2+}]_i$ decreased slightly for 0 - 420 min compared to untreated control but then increase gradually for 360 min to 420 min when it was significant ($p < 0.05$) compared to control untreated cells.
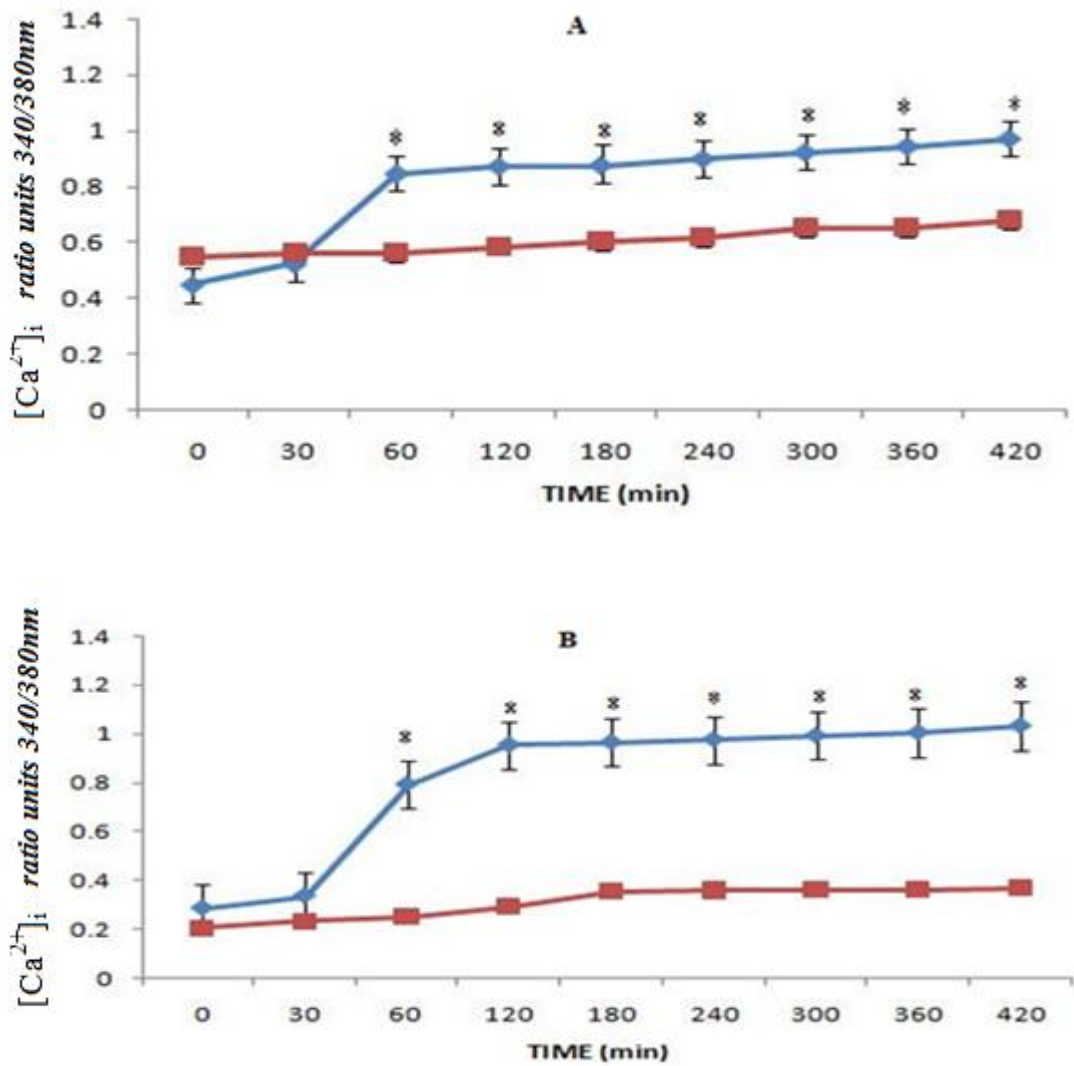
Figure 4.18: Time-course changes in $[Ca^{2+}]_i$ expressed as fluorescence ratio units (intensity) in Fura-2 AM loaded (A) 1321N1 and (B) Gos -3 cell lines following incubation with 800 μM α, β momorcharin for 420 min (solid diamonds). The fluorescence ratio units (intensity) for untreated cells at different time points are also shown for comparison (solid squares). Data are mean ± SD, n = 6; (* $p < 0.05$) for treated compared to untreated cells in both A and B for time points of 60, 120, 180, 240, 300, 360 and 420 min. Note the time-course increases in $[Ca^{2+}]_i$ following treatment with α, β momorcharin compared to untreated control. Note also the larger increase in $[Ca^{2+}]_i$ in Gos-3 cell line (B) treated with α, β momorcharin compared to 1321N1 cell line (A).
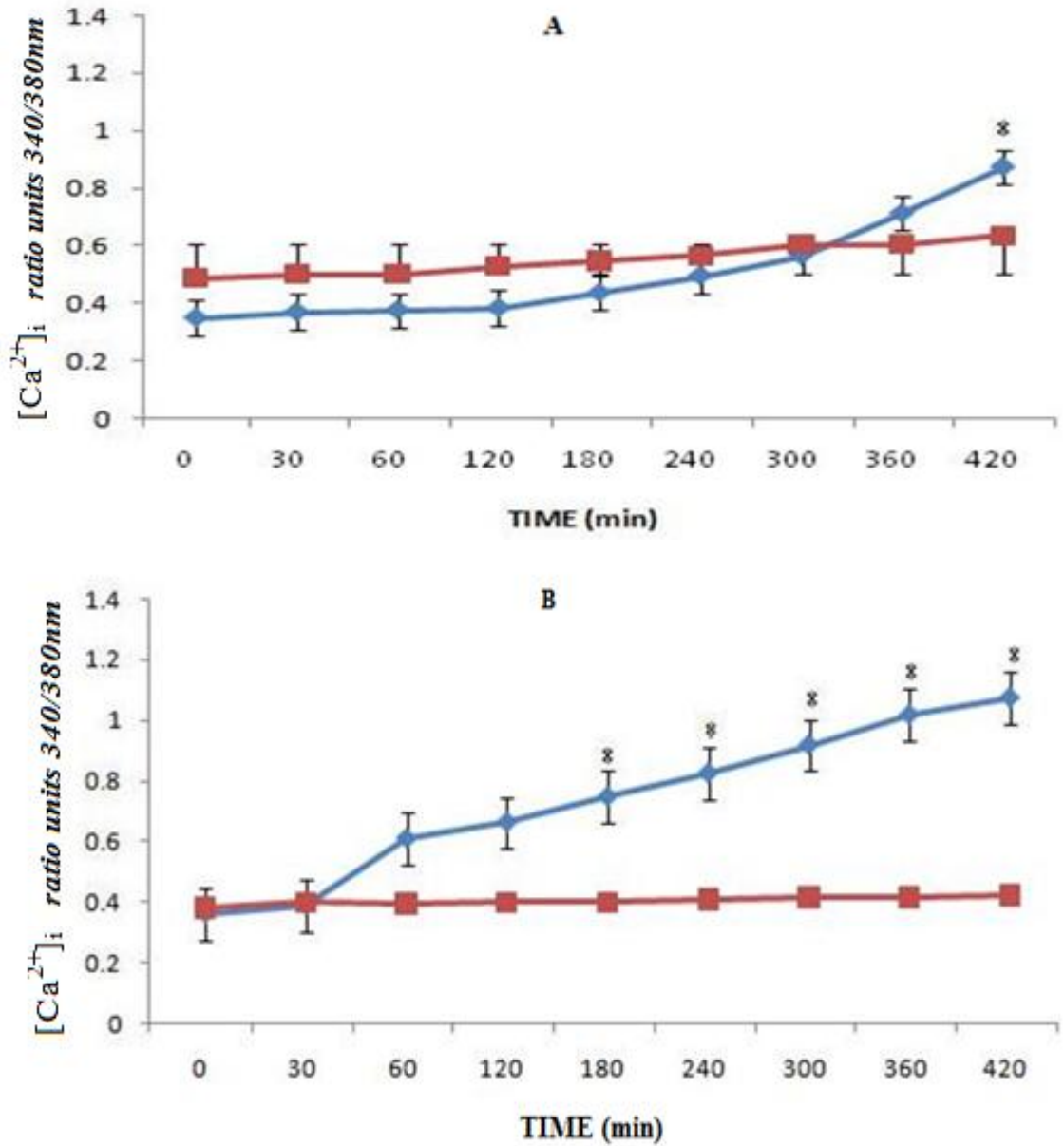
Figure 4.19: Time-course changes in $[Ca^{2+}]_i$ expressed as fluorescence ratio units (intensity) in Fura -2 AM loaded (A) U87-MG and (B) Sk Mel cell lines (solid diamonds) following incubation with 800 μM α, β momorcharin for 420 min. The fluorescence ratio units (intensity) for untreated cells (solid squares) at different time points are also shown for comparison. Data are mean ± SD, n = 6; (* p < 0.05) for treated compared to untreated cells in both A and B. Note the gradual time-course increases in $[Ca^{2+}]_i$ following treatment with α, β momorcharin compared to control (untreated cells).

Figure 4.20 shows the time-course changes in $[Ca^{2+}]_i$ expressed as fluorescence ratio units, in (A) Corl - 23 and (B) Weri Rb-1 cancer cell lines following incubation with 800 μM of the α, β momorcharin (solid diamonds). The changes in $[Ca^{2+}]_i$ in untreated (control) Corl-23, Weri Rb-1 cancer cell lines are also shown in figure for comparison (solid squares). The data show that incubation of the two cancer cell lines with α, β momorcharin can result in significant ($p < 0.05$) time-dependent increases in $[Ca^{2+}]_i$ in both cancer cell lines compared to control (untreated) cells with maximal increases in $[Ca^{2+}]_i$ at 420 min. In control, $[Ca^{2+}]_i$ remained more or less stable in both cell lines after 420 min of incubation.

Figure 4.21 shows the time-course changes in intracellular free calcium $[Ca^{2+}]_i$ expressed as fluorescence ratio units in L6 muscle cell line following incubation with 800 μM of the α, β momorcharin (solid diamonds). The changes in $[Ca^{2+}]_i$ in untreated (control) L6 muscle cell line are also shown in figure for comparison (solid square). The data show that incubation of the L6 muscle cell line with the α, β momorcharin can result in only a small increase in $[Ca^{2+}]_i$ reaching maximum after 420 min of incubation similarly to control (untreated) cells. Thereafter, $[Ca^{2+}]_i$ decreased gradually in both control and treated cells.
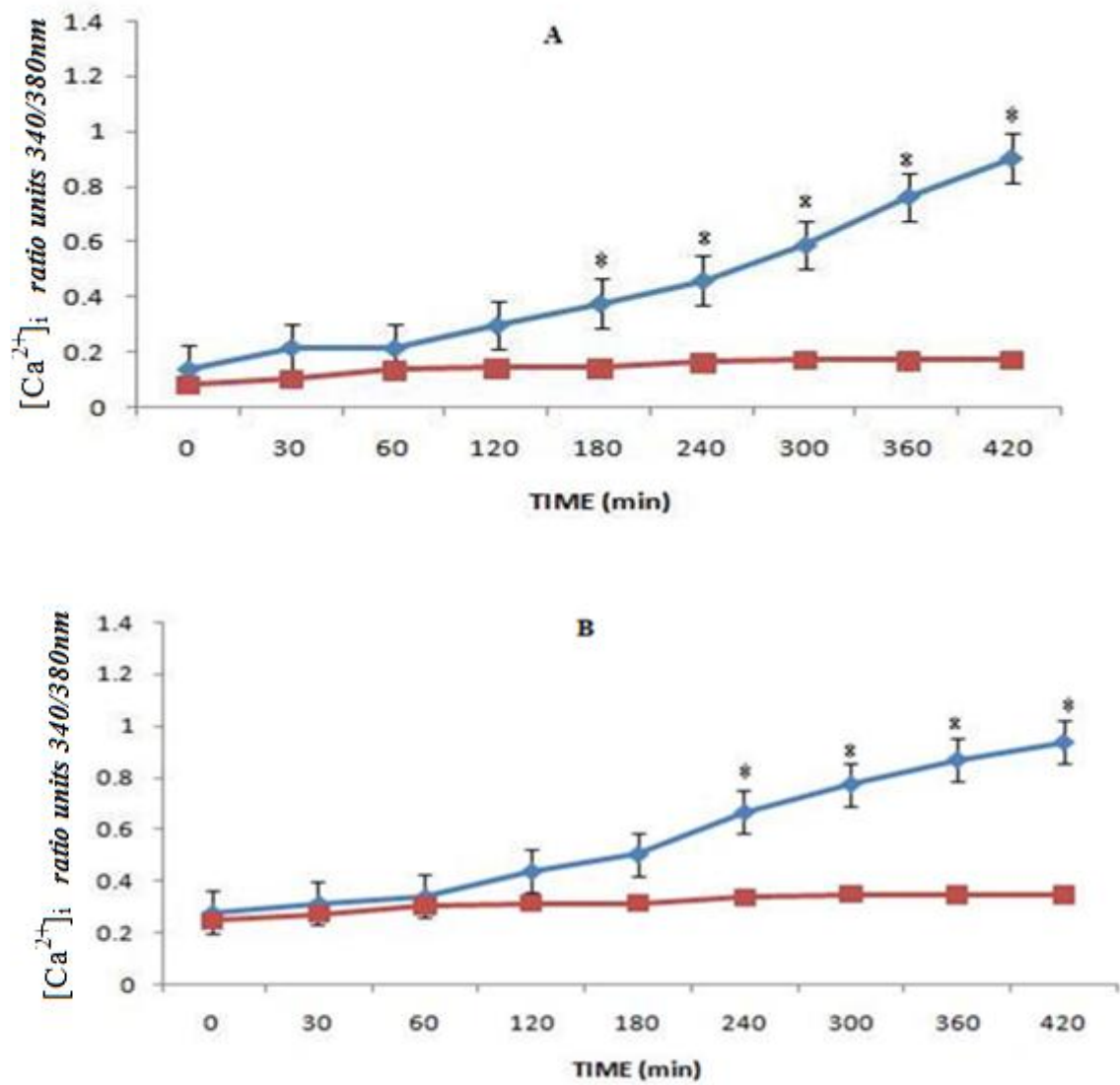
Figure 4.20: Time-course changes in $[Ca^{2+}]_i$ expressed as fluorescence ratio units (intensity) in Fura-2 AM loaded Corl-23 (A) and Weri Rb-1 (B) cell lines following incubation with 800 μM α, β momorcharin for 420 min (solid diamonds). The fluorescence ratio units (intensity) for untreated cells at different time points are also shown for comparison (solid squares). Data are mean ± SD, n = 6; (* p < 0.05) for treated compared to untreated cells in both A and B. Note the gradual time-course increases in $[Ca^{2+}]_i$ following treatment with α, β momorcharin compared to untreated control cells in which $[Ca^{2+}]_i$ remained at a steady-state basal level.
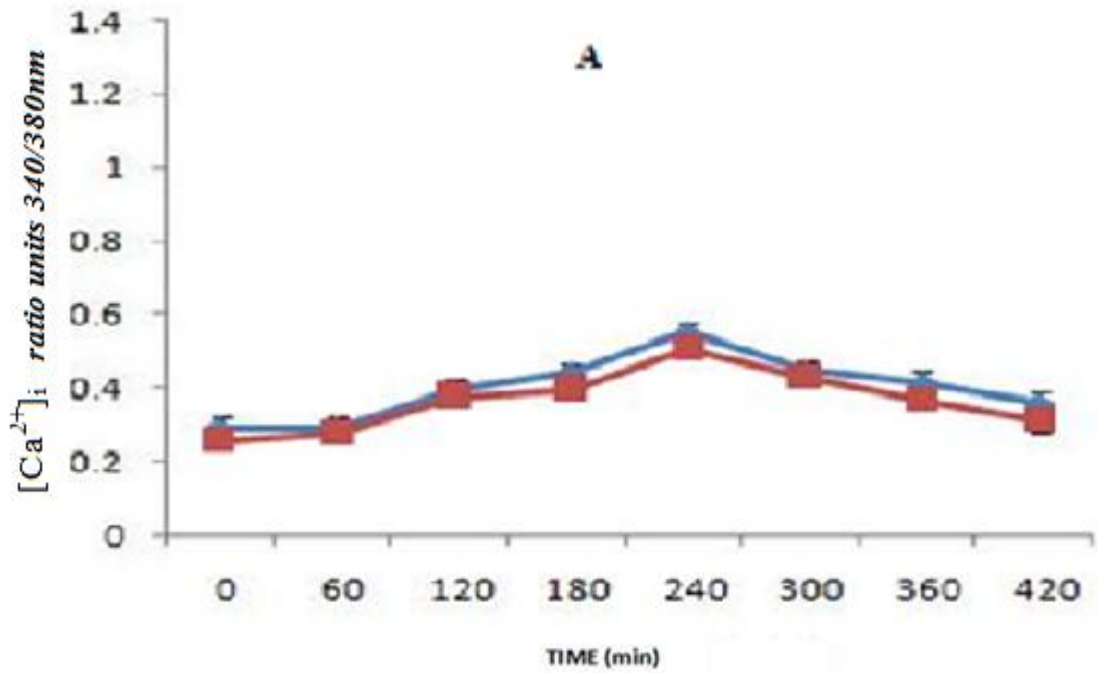
Figure 4.21: Time-course changes $[Ca^{2+}]_i$ expressed as fluorescence ratio units in Fura -2 AM loaded healthy L6 muscle cell line (solid diamonds) following incubation with 800 μM α, β momorcharin for 420 min. The fluorescence ratio units (intensity) for untreated cells (solid squares) at different time points are also shown for comparison. Data are mean ± SD, n = 6. Note that there was no significant (* p > 0.05) change in $[Ca^{2+}]_i$ comparing treated with untreated cells.

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Figure 4.22 shows the α, β momorcharin 800 μM evoked increases in $[Ca^{2+}]_i$ for the six different cancer cell lines as well as L6 skeletal muscle cell line after 420 min of stimulation. Each value taken from (Figures 4.18 - 4.21) is expressed as the maximal ratio units at 420 min time point. The results show the basal $[Ca^{2+}]_i$ at 0 min and maximal increases in $[Ca^{2+}]_i$ in untreated and treated cells at 420 min time point. The results show that 800 μM of the α, β momorcharin can evoke significant ($p < 0.05$) increases in $[Ca^{2+}]_i$ compared to control at 0 min as well as untreated cells after 420 min of incubation. In contrast, $[Ca^{2+}]_i$ in L6 skeletal muscle cell line remained more or less the same as the untreated cells.
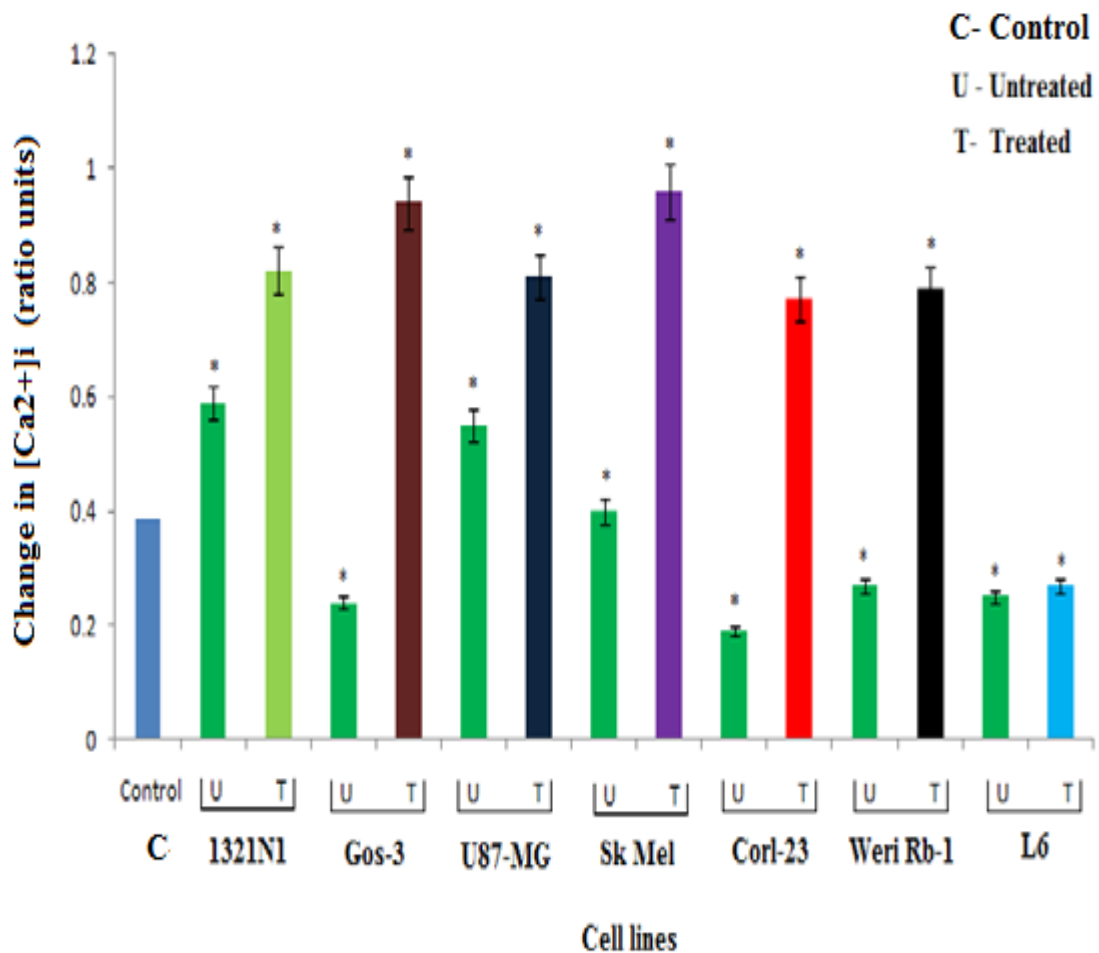
Figure 4.22: Bar charts showing the maximal increase (T) in $[Ca^{2+}]_i$ following incubation of the six cancer cell lines and L6 cell line with 800 µM of the α, β momorcharin for 420 min. Note that all the measurements were made at the 420 min time point. The results also show the increase in $[Ca^{2+}]_i$ in untreated (U) cells for the different cell lines for 420 min as well as the basal control (C) at time 0 min just before treatment with α, β momorcharin. Data were taken from figures 4.18 – 4.21 and they are expressed as mean ± SD, n = 6. Note the large increase in $[Ca^{2+}]_i$ following stimulation of each cancer cell line with α, β momorcharin for 420 min.

4.3 Discussion

The results presented in chapter 3 of this study have demonstrated significant anti-cancer effects of the crude water-soluble extract of *M. charantia* or the isolated and purified proteins, alpha, beta momorcharin of *M. charantia* on the six different cancer cell lines compared to untreated control. Anti-cancer drugs are believed to exert their 'killing' effects on cells via different cellular and sub-cellular mechanisms including damages to the mitochondria and microtubules, inhibition of kinases or by cellular calcium over-load (Tam *et al*, 1984 ; Tsao *et al*, 1990; Vikrant *et al*, 2001; Hajnoczky *et al*. 2006). This study employed two cellular pathways via which the crude water-soluble extract of *M. charantia* and alpha, beta momorcharin may exert their anti- cancer effects on cell death, namely, apoptosis and cellular calcium homeostasis.

 The results presented in this study have shown that either the crude water-soluble extract of *M. charantia* or the isolated and purified protein of *M. charantia,* namely alpha, beta momorcharin can elicit marked and significant changes in the activities of caspase-3 and caspase-9 and in the release of cytochrome-c in all the cell lines employed in this study compared to control untreated cell lines. In addition, both the crude water-soluble extract of *M. charantia* and alpha, beta momorcharin can elevate intracellular free calcium concentrations in all six-cancer cell lines compared to untreated control cells.

Apoptosis is programmed cell death and it is associated with damage of cell mitochondria in the body to elevate such intra-cellular mediators such as caspase-3 and caspase-9 and the release of cytochrome-c (Bernardi *et al*, 1994; Chandra *et al*. 2002). In previous studies, it was shown that anti-cancer drugs exert their lethality by inducing apoptosis in tumour cells *in vitro* and *in vivo* targeting both the mitochondrial and death receptor pathways (Gati *et al*.1991; Makin, 2002). There are two major apoptotic

pathways in mammalian cells namely the receptor (extrinsic) pathway and the mitochondrial (intrinsic) pathway (Hajnoczky *et al*. 2006). The receptor-mediated pathway is triggered by activation of cell death receptors followed by the activation of caspase-8, which in turn cleaves and activates downstream caspase-3 (Ashkenazi and Dixit, 1998). The mitochondrial pathway is initiated by cytochrome-c release from the mitochondria which promotes the activation of caspase-9 through activated caspase-9 which is responsible for the activation of cell death proteases (Ashkenazi and Dixit. 1998; Chandra *et al*. 2002).

In previous studies, it was shown that anti-cancer drugs exert their lethality by inducing apoptosis in tumour cells *in vitro* and *in vivo* targeting both the mitochondrial and death receptor pathways (Gati *et al*.1991; Makin. 2002). The activity of caspase-3 was determined using the caspase-3 activity kit. To evaluate the activity of caspase-3, A431 cells were treated with the 3.3 mM RIP or RIP-PEG conjugate, the cell lysates were respectively collected at 0, 12, 24, 36 h after exposure. The caspase activities were measured as fold of enzyme activity in comparison with control (Mengen L *et al*, 2009).

The results of the present study have shown that either the crude water-soluble extract of *M. charantia* or alpha, beta momorcharin can evoke significant and time-dependent increases in $[Ca^{2+}]_i$ in all the six cancer cell lines employed in this study compared to the L6 skeletal muscle cell line. Their effect was less pronounced in U87-MG cell line compared to others. The question where now arises is: What is the significance of elevated $[Ca^{2+}]_i$ in these cancer cell lines following treatment with either the crude water-soluble extract of *M. charantia* or alpha, beta momorcharin. Previous study have shown that sustained Ca^{2+} elevation can act as trigger for apoptosis or cell death (Hajnoczky *et al*. 2003). In addition, Ca^{2+} overloading in mitochondria can induce a cell

suicide programme by stimulating the release of apoptosis promoting factor like the release of cytochrome-c (Green *et al.* 1998; Wang and Ng 2001; Brooks *et al.* 2000; Hajnoczky *et al.* 2006). Cytosolic Ca^{2+} homeostasis in resting cells is achieved by balancing the leak of Ca^{2+} (entering from the outside of from the stores) by the constant removal of Ca^{2+} using pumps either on the plasma membrane or on the internal stores. These pumps ensure that cytoplasmic $[Ca^{2+}]_i$ remains low and that the stores are loaded with signal Ca^{2+}. In most cells, it is the internal stores (eg ER, SR, mitochondria) which provide most of the signal calcium (Streb *et al.* 1983). Furthermore, mitochondria have been found to play a pivotal role in Ca^{2+} signalling (Hajnoczky *et al.* 2003). The cellular free Ca^{2+} is an important physiological mediator and regulator in the stimulus-secreting coupling process in different epithelial cells (Yago *et al.*1999; Petersen. 1992), Many studies, have demonstrated mitochondrial Ca^{2+} overload as the link between complement deposition and the observed changes in mitochondrial physiology and the triggering of programmed cell death (PCD) (Irigoin *et al.* 2009). The mitochondrial Ca^{2+} overload is responsible for the increased $O(2)(*-)$ production (Jimenez and Hernandez-Cruz. 2001). The rates of mitochondrial membrane potential dissipation and mitochondrial Ca^{2+} uptake may determine cellular sensitivity to Ca^{2+} toxicity under pathological conditions, including ischemic injury (Gogvadze *et al.* 2001). It has also been suggested that only a subpopulation of mitochondria undergoes a permeability transition and releases apoptogens, whereas the remaining, undamaged mitochondria respire normally and produce ATP (Simpson *et al.* 2002; Kabir *et al.* 1999; Jimenez and Hernandez-Cruz. 2001).

Loss of Ca^{2+} homeostasis, often in the form of cytoplasmic increases, leads to cell injury. Depending upon the cell type and the intensity of Ca^{2+} toxicity, the ensuing pathology can be either reversible or irreversible (Gogvadze *et al.* 2004). Although multiple destructive

processes are activated by Ca^{2+}, lethal outcomes are determined largely by Ca^{2+} induced mitochondrial permeability transition (Johansson *et al*. 2003; Goldstein *et al*. 2000). This form of damage is primarily dependent upon mitochondrial Ca^{2+} accumulation, which is regulated by the mitochondrial membrane potential (Gogvadze *et al.* 2004). Together, the result presented in this study have demonstrated clearly that the anti-cancer effects of either the crude water-soluble extract of *M. charantia* or alpha, beta momorcharin are mediated via apoptosis and cellular Ca^{2+} overloading.

4.4 Conclusions

The results of this study have clearly demonstrated that either the crude water-soluble extract of *M. charantia* or the isolated and purified protein of *M. charantia,* namely alpha, beta momorcharin can evoke significant decreases in cancer cell viability (cell death) by exerting their anti-cancer effect on cells via damage of cell mitochondria body resulting in elevation in such cellular mediators as caspase-3 and caspase-9 and release of cytochrome-c and an elevation of intracellular free calcium concentration $[Ca^{2+}]_i$. A combination of all there factors may lead to Ca^{2+} overloading in the mitochondria resulting in cancer cell death. However, further experiments are required to investigate the sub- cellular mechanisms associated with cell death including the involvement of kinase and gene expressions for apoptotic mediators.

Chapter Five

General Discussion

5. General Disscusion

This study was divided into three main categories namely

1. Physiological investigation.

2. Morphological investigation and

3. Biochemical investigation

Emphasis in this discussion will now be focused on each investigation.

5.1 Physiological investigations

Firstly, the present study investigated the effect of the crude water-soluble and methanol soluble extracts of *M. charantia* and alpha, beta or alpha and beta momorcharins (proteins isolated and purified from *M. charantia*) and two commercially available anti-cancer drugs namely, vinblastine and temozolomide (TZM) on the viability (cell death) of six different cancer cell lines compared to healthy L6 skeletal muscle cell line. In some experiments, these agents were combined inorder to determine any possible potentiating or attenuating effect on the six different cancer cell lines (1321N1, Gos-3, U87-MG, Sk Mel, Corl -23, Weri Rb-1). The results have shown that the crude water-soluble extract of *M. charantia* can evoke significant time-course and dose-dependent decreases in cell viability of all six different cancer cell lines. In contrast, the crude water-soluble extract of *M. charantia* had no significant effect on the viability of the healthy L6 muscle cell line. Similar anti-cancer effects were obtained with alpha, beta momorcharin. In contrast, alpha or beta momorcharin or the crude methanol soluble extract of *M. charantia* had little or no effect on cell viability. The two commercially available anti-cancer drugs TMZ and vinblastine can evoke dose-dependent decreases in cell viability. Combining either TMZ or vinblastine with either the crude water-soluble

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extract of *M. charantia* or with alpha, beta momorcharin had only a small, but significant effect on cell viability compared to either alone. However, the decrease in cell viability during combined treatment of the different cancer cell lines was neither additive nor synergestic.

Until recently, very little was known about specific anti-cancer proteins from plants. Most previous studies have been undertaken using peptides as anticancer agents (Lolitkar and Rao. 1966; Yuwai *et al*. 1991). The anti-cancer property of *M. charantia* has been extensively studied in experimental animals and both beneficial and contradictory claims have been made regarding its effects (Bourinbaiar and Lee-Huang, 1996). The mechanism of action of *M. charantia* and its effects on the complications of cancer have not been well established.

In support of the current study, the beneficial anti-cancer effects of crude water-soluble extract of *M. charantia* and α, β momorcharin have been reported previously by several other investigators (Ganguly *et al*.2000; Jilka *et al*.1983; Singh *et al*.1998). A number of preliminary studies have shown that the crude water-soluble extracts of *M. charantia* posses anti-cancer activity against lymphoid, leukaemia, skin and prostatic cancers (Licastro *et al*.1980; Ng *et al*.1994; Sun *et al*. 2001). At present, the mechanism(s) involved in the crude water-soluble extract of *M. charantia* on cell death is not fully established. Several preliminary studies have been conducted using the crude water-soluble extracts of *M. charantia* and in all these studies; the chemical profile(s) of *M. charantia* was not documented. Few studies have demonstrated much significant biological activity of *M. charantia* compounds such as charantin, MAP 30, momordicin, alpha, beta momorcharin extracted from *M. charantia* (Grover *et al*. 2004). In a *in vivo* study, the crude water-soluble extract of *M. charantia* was shown to inhibit tumour formation in mice (Catherine *et al*. 1983).

At present, the mechanism(s) of action involved in either the crude water-soluble extract of *M. charantia* or alpha, beta momorcharin on cell death has not yet been established (Xiong *et al*. 2010). However, it has been reported that alpha, beta momorcharin is a type-1 ribosome inactivating protein (RIP), which has a number of biological activities including inhibition of tumour growth. On the alternativity, either the individual alpha or beta momocharin has excellent cytotoxicity to tumour cells (Xiong *et al*. 2010). Beta momorcharin, was about 15-fold more active than alpha momorcharin (Yeung *et al*.1988). In contrast, the present study has shown that either alpha momorcharin or beta momorcharin had no significant effect on cell viability. This was a rather surprising find compared to the results of other reported studies (Xiong *et al*. 2010; Yeung *et al*.1988).

5.2 Morphological investigations

The results of the physiological investigations have shown that the crude water-soluble extract of *M. charantia* can decrease the cell viability (cell death) in six different cancer cell lines. In the view of these observations, it was pertinent to examine the morphological basis of these physiological effects. The morphological investigations on the effects of the crude water-soluble extract of *M. charantia* and alpha, beta momorcharin were carried out in six different cancer cell lines. The results show that each glioma cell line seemed to lose their normal structure following incubation with either the crude water-soluble extract of *M. charantia* or with alpha, beta momorcharin. Moreover, each cell line aggregated forming clumps and in some cases, they seem to possess a donought-like shape of clumped cells following treatment for 24 hrs. In many cases, the glioma cells tend to lose their neuronal-like structures, decrease in cellular density, condensation, fragmentation and blebbing. These abnormal structural features are typical of either necrosis or apoptosis. The morphological studies have also shown

that the crude water-soluble extract of *M. charantia* or alpha, beta momorcharin had little or no effect on the cell viability of healthy L6 skeletal muscle cell line indicating that either of them does not have any adverse effect on control healthy cells. This interesting observation suggests that either the crude water-soluble extract of *M. charantia* or alpha, beta momorcharin can be used safely to treat cancer as well as with the combination of other commarcially avaliable anti-cancer drugs.. Both the crude water-soluble extract of *M. charantia* or with alpha, beta momorcharin can induce marked, morphological changes in the different cancer cell lines. They can also elicit significant decreases in cell viability, and it was necessary to investigate the cellular mechanism(s) by which they may evoke cell death.

5.3 Biochemical investigations

The results presented in this study have demonstrated that either the crude water-soluble extract of *M. charantia* or isolated and purified protein of *M. charantia,* namely alpha, beta momorcharin can elicit marked and significant changes in the activities of caspase-3 and caspase- 9 and release of cytochrome-c in all the cell lines employed in this study compared to control untreated cell lines. In addition, both the crude water-soluble extract of *M. charantia* and alpha, beta momorcharin can elevated intracellular free calcium concentration $[Ca^{2+}]_i$ in all six cancer cell lines compared to untreated control cells.

Experiments in this study examining Ca^{2+} homeostasis in the six different cancer cell lines (1321N1, Gos-3, U87-MG, Sk Mel, Corl -23, Weri Rb-1) treated with either crude water-soluble extract of *M. charantia* or alpha, beta momorcharin have demonstrated interesting findings. The results show gradual time-dependent and significant increases in the $[Ca^{2+}]_i$ following incubation with either crude water-soluble extract of

M. charantia or alpha, beta momorcharin compared to the untreated cells (control). This elevation in $[Ca^{2+}]_i$ following stimulation of either the crude water-soluble extract of *M. charantia* or alpha, beta momorcharin may be due to several cellular processes outlined in figure 5.2. These results are similar to previous studies employing pancreatic acinar cells during stress induced condition with H_2O_2 of deplete the mitochondria of $[Ca^{2+}]_i$ (Camello-Almaraz *et al*. 2002)

In previous studies, it was shown that anti-cancer drugs exert their lethality by inducing apoptosis in tumour cells *in vitro* and *in vivo* targeting both the mitochondrial and death receptor pathways (Gati *et al*.1991; Makin. 2002). The activity of caspase-3 was determined using the caspase-3 activity kit. To evaluate the activity of caspase-3, A431 cells were treated with the 3.3 mM RIP or RIP-PEG conjugate, the cell lysates were respectively collected at 0, 12, 24, 36 h after exposure. The caspase activities were measured as fold of enzyme activity in comparison with control (Mengen L *et al*, 2009).

The elevation in cytochrome-c and caspase-9 activation is consistent with evidences showing a direct interaction between mitochondria and intracellular calcium stores (see figure 5.1) (Rizzuto *et al*. 1998; Csordas *et al*.1999). There is structural and functional evidences suggesting the presence of specific and stable interactions between mitochondria and intracellular Ca^{2+} stores (e.g. mitochondrial and endoplasmic reticulum) which facilitate a rapid and nearly direct flux of Ca^{2+} from endoplasmic reticulum to mitochondria (Hajnoczky *et al*. 2006; Rizzuto *et al*. 1998; Camello-Almaraz *et al*. 2002; Filippini *et al*. 2003; Vay *et al*. 2007). These tight endoplasmic reticulum-mitochondria coupling may also serve to modulate Ca^{2+} release. In addition, it has been suggested that mitochondria colocalize in small sub-cellular regions where endoplasmic reticulum and mitochondria form close contact (Vay *et al*. 2007).
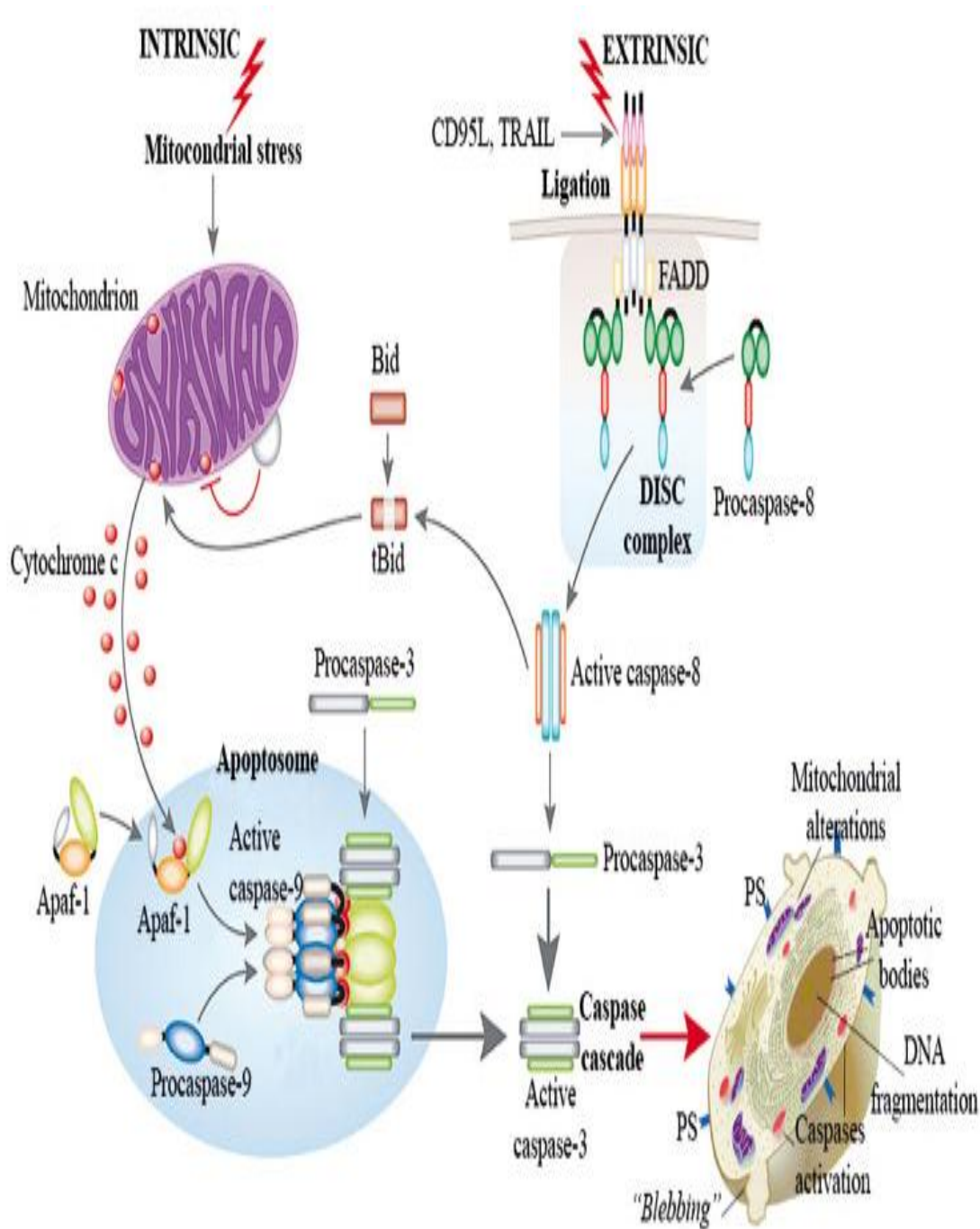
Figure 5.1: Over view of signal transduction pathways involved in apoptosis by

intrinsic and extrinsic pathways

(Adapted from Molecular cell biology. Harvey 5 edition, 2003)

Figure 5.1 illustrate both intrinsic and extrinsic pathways of apoptosis. Extrinsic

apoptosis signalling is mediated by the activation of so called "death receptors" which

are cell surface receptors that transmit apoptotic signals after ligation with specific ligands. Death receptors belong to the tumor necrosis factor receptor (TNFR) gene superfamily, including TNFR-1, Fas/CD95, and the TRAIL receptors DR-4 and DR-5 (Ashkenazi, 2002). All members of the TNFR family consist of cysteine rich extracellular subdomains which allow them to recognize their ligands with specificity, resulting in the trimerization and activation of the respective death receptor (Naismith and Sprang, 1998). Subsequent signalling is mediated by the cytoplasmic part of the death receptor, which contains a conserved sequence termed the death domain (DD). Adapter molecules like FADD or TRADD themselves possess their own DDs by which they are recruited to the DDs of the activated death receptor, thereby forming the so-called death inducing signalling complex (DISC). In addition to its DD, the adaptor FADD also contains a death effector domain (DED) which through homotypic DED-DED interaction sequesters procaspase-8 to the DISC. The local concentration of several procaspase-8 molecules at the DISC leads to their autocatalytic activation and release of active caspase-8. Active caspase-8 then processes downstream effector caspases which subsequently cleave specific substrates resulting in cell death. Cells harboring the capacity to induce such direct and mainly caspase-dependent apoptosis pathways (Scaffidi *et al*, 1998).

The link between the caspase signalling cascade and the mitochondria is provided by the Bcl-2 family member Bid. Bid is cleaved by caspase-8 and in its truncated form (tBID) translocates to the mitochondria where it acts in concert with the proapoptotic Bcl-2 family members Bax and Bak to induce the release of cytochrome c and other mitochondrial proapoptotic factors into the cytosol (Luo *et al*, 1998). Cytosolic cytochrome c is binding to monomeric Apaf-1 which then, in a dATP-dependent conformational change, oligomerizes to assemble the apoptosome, a complex of wheel-

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like structure with 7-fold symmetry, that triggers the activation of the initiator procaspase-9 (Acehan *et al*, 2002). Activated caspase-9 subsequently initiates a caspase cascade involving downstream effector caspases such as caspase-3, caspase-7, and caspase-6, ultimately resulting in cell death (Slee *et al*, 1999).

As described in Figure 5.1 he intrinsic pathway is triggered by cellular stress, specifically mitochondrial stress caused by factors such as DNA damage and heat shock (Adrain *et al*, 2002). Upon receiving the stress signal, the proapoptotic proteins in the cytoplasm, BAX and BID, bind to the outer membrane of the mitochondria to signal the release of the internal content. The signal of BAX and BID is not enough to trigger a full release. BAK, another proapoptotic protein that resides within the mitochondria, is also needed to fully promote the release of cytochrome c and the intra-membrane content from the mitochondria (Hague and Paraskeva, 2004). Following the release, cytochrome c forms a complex in the cytoplasm with adenosine triphosphate (ATP), an energy molecule, and Apaf-1, an enzyme. Following its formation, the complex will activate caspase-9, an initiator protein. In return, the activated caspase-9 works together with the complex of cytochrome c, ATP and Apaf-1 to form an apoptosome, which in turn activates caspase-3, the effector protein that initiates degradation. Besides the release of cytochrome c from the intra-membrane space, the intra-membrane content released also contains apoptosis inducing factor (AIF) to facilitate DNA fragmentation, and Smac/Diablo proteins to inhibit the inhibitor of apoptosis (IAP) (Hague and Paraskeva, 2004; Johnstone *et al*, 2002)
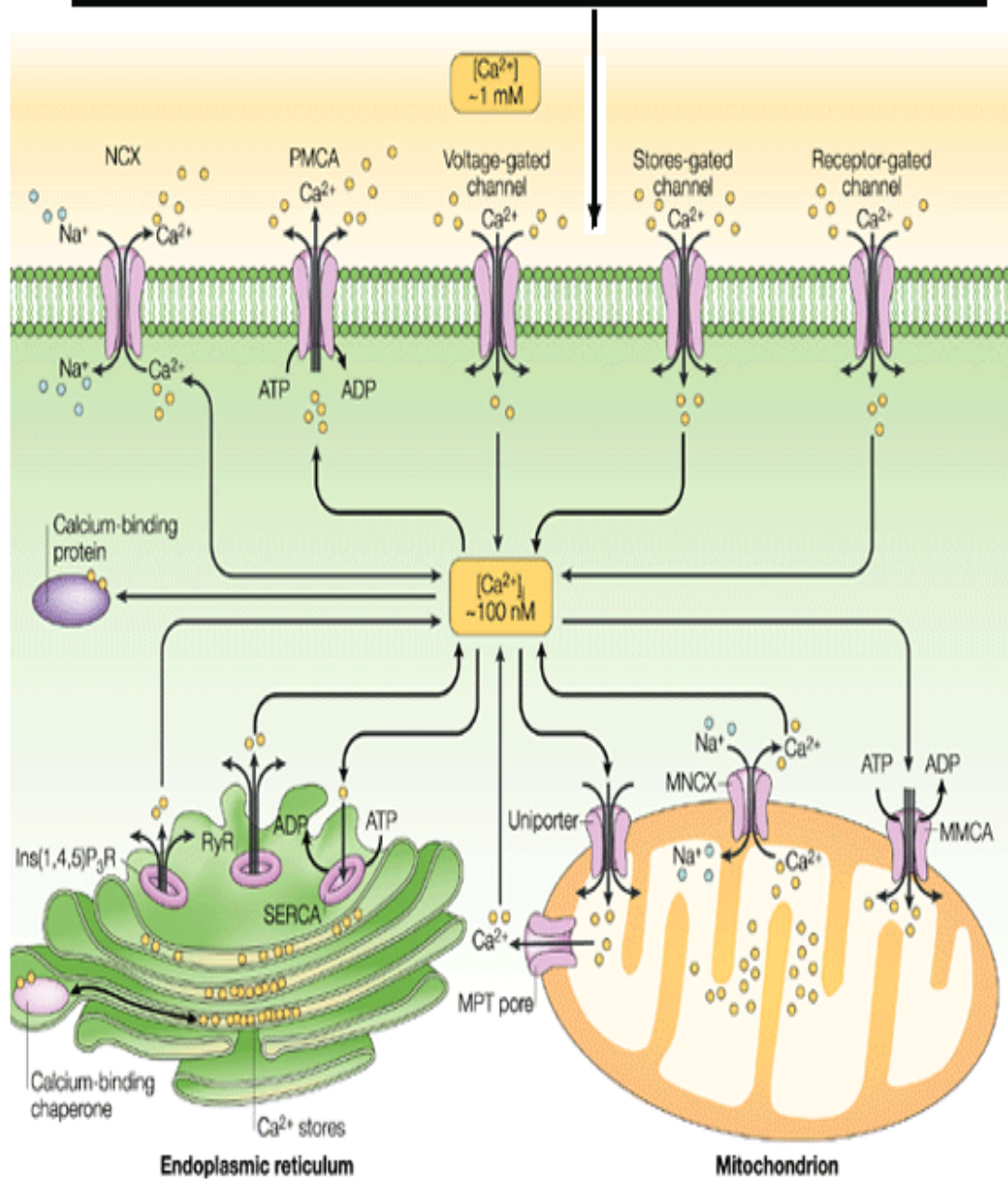
Figure 5.2: A modified schematic diagram showing the possible mechanism(s) whereby the crude water-soluble extract of *M. charantia* and alpha, beta momorcharin can exert their effect on cancer cell death by Ca^{2+} homeostasis pathway. (www.vanderbilt.edu/viibre/Projects/images/pfi).

Figure 5.2: shows the model of possible role of Ca^{2+} in cancer cells leading to cell death. Ca^{2+} is an universal signal transduction cation in cells modulating cell growth and differentiation and other physiological and biochemical process in cells (Johansson *et al.* 2003). The calcium levels outside cells are 10 000 times higher than free intracellular Ca^{2+}. However, free $[Ca^{2+}]_i$ is the physiologically active form of calcium (Clapham, 2007)). The level of free intracellular calcium ($[Ca^{2+}]_i$) is regulated and maintained as low (~100 nM) through the action of a number of binding proteins and ion exchange mechanisms. Each cell has a unique set of Ca^{2+} signals to control its function. Ca^{2+} signal transduction is based on rises in free cytosolic Ca^{2+} concentration. Ca^{2+} can flow from the extracellular space or be released from intracellular stores. The endoplasmic reticulum (ER) is a major site for sequestered Ca^{2+} ions. Recent studies indicate that the Golgi apparatus may also be a Ca^{2+} store in keratinocytes ((Bootman and Berridge, 1995; Clapham, 2007)). Ca^{2+} is accumulated into intracellular stores by means of Ca^{2+} pumps and released by inositol 1,4,5-trisphosphate (IP_3) via IP_3 receptors and by cyclic adenosine diphosphate ribose (cADPr) via ryanodine receptors (Berridge and Irvine. 1989). Extracellular Ca^{2+} enters the cell through various types of plasma-membrane Ca^{2+} channels. Soluble proteins, such as calmodulin, contribute to the buffering of cell Ca^{2+}, but membrane-intrinsic transporting proteins are more important. Ca^{2+} is transported across the plasma membrane (channel, pump, Na^+/Ca^{2+} exchanger) and across the membranes of organelles (Berridge and Irvine. 1989).

External signals arriving at the cell engage plasma membrane receptors to initiate cell-signalling pathways (see figure 5.2). One of the results is increased intracellular calcium concentration. On stimulation, this level can rise globally to in excess of 1 M. This increase can be generated from sources both within and outside the cell (Irigoin *et al.* 2009). The formation of IP_3 is the focal point for two major pathways, one initiated by a

family of G protein-linked receptors and the other by either receptors linked by tyrosine kinases directly or indirectly (Carafoli, 1994). These separate receptor mechanisms are coupled to energy-requiring transducing mechanisms which activate phospholipase C (PLC) to hydrolyse the lipid precursor phosphatidylinositol 4,5-biphosphate to generate both DAG and IP_3. The latter then binds to an IP_3 receptor (IP_3R) to mobilize stored calcium and to promote an influx of external calcium (Irigoin *et al*. 2009).

Extracellular Ca^{2+} enters the cell through plasma membrane Ca^{2+} channels and leaves the cell using Ca^{2+} pumps and Na^+/Ca^{2+} exchangers. Endoplasmic reticulum (ER) is a major site for sequestered Ca^{2+} ions. Ca^{2+} is accumulated in intracellular stores by means of Ca^{2+} pumps and released by inositol 1, 4, 5- trisphosphate (IP_3) via IP_3 receptors (IP_3R) and by cyclic adenosine diphosphate ribose (cADPr) via ryanodine receptors (RyR). Store-operated calcium channels (SOCs) open in response to depletion of the (ER) Ca^{2+} stores. Calcium influx factor (CIF) has postulated to mediate the signal from IP_3R to the plasma membrane store-operated calcium channels (SOCs) (Bootman and Lipp, 2001).

Figure 5.2 proposed the possible mechanism(s) whereby the crude water-soluble extract of *M. charantia* and alpha, beta momorcharin may exert their effects on cancer cell death. It is suggested that alpha, beta momorcharin (9.7 kDa), which is a large protein, can activate a membrane bound receptor on the cancer cells or possible penetration of cell due to osmosis. Once in the cell, the protein acts on the mitochondria to bring about apoptosis leading to the release of cytochrome-c and enhancing the activities of caspase-3 and caspase-9. Damage to the mitochondria can lead to the release of Ca^{2+} into the cytoplasm. It is also possible that the protein may also stimulate the influx of Ca^{2+} from the extra-cellular medium and its releases from the endoplasmic reticulum.

The rise in $[Ca^{2+}]_i$ coupled with the activities of caspase - 3 and caspase - 9 and release of cytochrome-c can lead to cellular Ca^{2+} overloading. These in turn can elicit cell death.

Another possible mechanism is that activation of the membrane bound receptor with either the crude water-soluble extract of *M. charantia* and alpha, beta momorcharin may lead to the generation of intracellular mediators. These in turn can stimulate cellular apoptosis and elevate $[Ca^{2+}]_i$. Together, they can induce cell death due to apoptosis and Ca^{2+} overload.

Alpha, beta momorcharin is a protein, which has been isolated from the fruit of *M. charantia*. Since the crude water-soluble extract of *M. charantia* can exert similar physiological, morphological and biological effect as the protein alpha, beta momorcharin, then it is possible to assume that the anti cancer effects of both are due to the same substance namely alpha, beta momorcharin. In addition to alpha, beta and alpha, beta momorcharin, it is worthwhile to examine the anticancer effects of the substances isolated from *M. charantia* including MAP-30, MRK29, alpha-momocharin, beta-momocharin and momordicin.

5.4 Conclusions

It can be concluded from this study that administration of either the crude water-soluble extract of *M. charantia* or isolated and purified protein of *M. charantia,* namely alpha, beta momorcharin can evoke significant decreases in cancer cell viability (an increase in cell death) without killing healthy cell line like L6 skeletal muscle cell line. These effects were both time and dose-dependent with maximal effect occurring after 24 hr at a dose of 800 µg. In contrast, either the crude methanol-soluble extract of *M. charantia* or alpha or beta momorcharin had little effect on cancer cell viability. Either

temozolomide or vinblastine, two commercially available anti-cancer drugs, can also elicit dose-dependent decreases in cancer cell viability. Anticancer drugs interfere with the growth of tumor cells, eventually causing their death. However, because these drugs are so powerful, they may also affect the growth of normal body cells, causing many side effects, some of which may be serious. Anticancer drugs are sometimes given together with other medicines. When using a combination of medicines either temozolomide or vinblastine with either the crude water-soluble extract of *M. charantia* or alpha, beta momorcharin had effect on the viability of each cell line compared to the effect of either alone. It is important to understand the advantages of using combination of temozolomide or vinblastine with either the crude water-soluble extract of *M. charantia* or alpha, beta momorcharin may have very less side effects when compare to individual anti-cancer drugs. It is concluded that *M. charantia* possesses anti-cancer properties since it can induce cell death.

The results of this study have also demonstrated that either the crude water-soluble extract of *M. charantia* or isolated and purified protein of *M. charantia,* namely alpha, beta momorcharin can increase the activities of caspase-3 and caspase - 9, the release of cytochrome-c and an elevation in intracellular free calcium concentrations. Together, the result suggest that either the crude water-soluble extract of *M. charantia* or alpha, beta momorcharin can exert their effect on cell death possible via an apoptotic mechanism involving Ca^{2+} overloading. Further experiments are required to determine the direct relationship between apoptosis and Ca^{2+} overload during cell death, measuring a number of kinases, which may be involved in the mechanism.

The results obtained from this study have enhanced our knowledge on the possible mechanism(s) of the anti-cancer effects of the crude water-soluble extract of *M. charantia* and alpha, beta momorcharin. However, further experiments are required to

investigate the sub-cellular mechanisms associated to cell death including the involvement of kinases and gene expressions for apoptotic mediators.

Scope of future work

The studies in this thesis open up many possibilities for further investigations.

Proposed goals of future research in to bitter gourd are:

Although *M. charantia* has been studied in non-randomized, controlled trials only, it is considered to be one of the most promising supplements with anti-cancer properties.

To better understand the mechanism of action in killing the cancer cells.

To test various *M. charantia* types from different environments and different growing sites for differences in their anti-cancer activity.

To perform long term and better randomized, controlled trials to assess the clinical efficacy and safety and the optimum dosage.

The reduction in size of the cancer cells has been due to necrosis or in fact apoptosis rather than just cellular atrophy. Since there are markers of these phenomena, one could examine these in order to gain knowledge about the nature of cellular atrophy.

It would be possible to do cancer cell based- cytokine with ELISA, RT-PCR, Western blotting measurements in order to examine which are associated with atrophy are induced.

To measure the protein kinases enzymes which are capable of transferring the γ phosphate group from ATP to serine, threonine or tyrosine residue in specific substrate proteins. Protein kinase are important targets for drug development

Chapter Six

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6. References

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Chapter Seven

Appendix

7. Appendix

Beneficial effects and cellular mechanism of action of *Momordica charantia (bitter gourd)* in the treatment of cancer

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Introduction

Prior to the availability of chemotherapeutic agents, dietary measures, including traditional medicines derived from plants, were the major forms of cancer treatment. A number of plants have been used previously to treat different cancers throughout the world. One such plant is *M.charantia* (Linn Family: Cucurbitaceae), whose fruit is known as Karela or bitter gourd. For a long time, several workers have studied the effects of this plant in cancer therapy. *M .charantia* has anti-carcinogenic properties and it can modulate xenobiotic metabolism and oxidative stress[1].

Aims

The main aim of this study is to extract, isolate, purify and characterize the active ingredient (s) of *M. charantia* and investigate its mode and cellular mechanism (s) of action as a potential chemotherapeutic agent in cancer therapy.
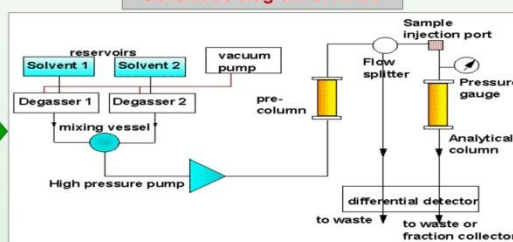
Methods

Soxhlet extraction

Dried *M.Charantia* powder (1 gram) was initially extracted with 400 ml with of methanol for 4 hrs. The methanol was then rota-evaporated.

↓

The sample residue was repeatedly (3 times) extracted with 30 ml of Methanol

↓

The extract was filtered with Whatmann filter paper no:4 and rota-evaporated to obtain a residue[1].

Schematic diagram of HPLC

Residue was analysed with C-18 inertsil ODS-3 column (5 μm, 4.6 mm × 259 mm id) and UV absorbance was monitored at 254 nm.

Mobile phase a. Trifluroacetic acid in acetonitrile.
b. Trifluroacetic acid in HPLC grade water.
c. Methanol HPLC grade.

Flow rate 1.0 ml/min, temperature was maintained 37°C, injection volume 10 μl [2]

Tissue culture

Cells like U87,1321NI and GOS-3 were cultured in 96 well plates

↓

Cells were then allowed to reach a confluence of 60-70%

↓

Different concentrations of isolated active ingredient (drug) are added to the cells

↓

Cells were then incubated for 24 hrs with and without ingredient

↓

Cells were then analysed by MTT and ATP assay for viability

Results
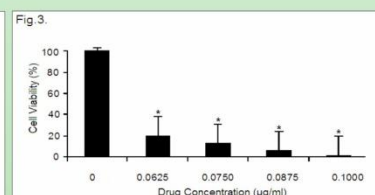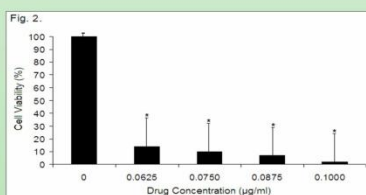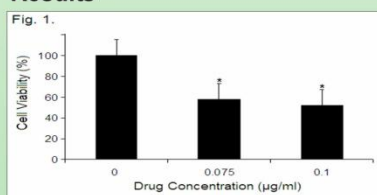
Glioma Cell lines viability for U87-MG (Fig. 1), GOS-3 (Fig. 2) and 1321N1 (Fig. 3) in the absence (control) and presence of different concentrations of *M. charantia* extract (ingredient). Data are ± SEM, n=5; * P< 0.001 for extract-treated cells compared to control.

These preliminary results have shown that *M. charantia* ingredient can evoke a significant (p<0.001) decrease in glioma cell viability for each cell line compared to untreated (control) cells. These effects were dose dependent.

Conclusion and Future Work

- A potent anticancer agent was extracted from *M.charantia* using methanol and tested on three different glioma cell lines.
- The extract (ingredient) evoked significant (p< 0.001) dose-dependent cytostatic effect on each cell line compared to untreated control.
- The next stage of the study is to determine the mechanism s) whereby *M. charantia* extract can induce cell death measuring cytosolic calcium, P53, caspase- 3 activity and cytochrome c.

References:

1. Ayala, R. S. and Luquede Castro, M. D (2001). Continuous subcritical water extraction as a useful tool for solation of edible essential oils. Food Chemistry **75**: 109-113.
2. Bourinbaiar AS, Lee-Huang S (1995). Potentiation of anti-HIV activity of anti-inflammatory drugs, dexamethasone and indomethacin, by MAP30, the antiviral agent from bitter melon. Biochem :**208(2)**:779-785.
3. Cunnick, J. E., et al (1990). "Induction of tumor cytotoxic immune cells using a protein from the bitter melon (Momordica charantia)." Cell Immunol. Apr **126(2)**: 278-289.

Effects of *Momordica charantia* fruit extract in the treatment of glioma cancer

G. Manoharan, R.W. Lea, T. J. Snape and J. Singh

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Introduction

Prior to the availability of chemotherapeutic agents, dietary measures, including traditional medicines derived from plants, were the major forms of cancer treatment. A number of plants have been used for the treatment of different cancers throughout the world[2]. One such plant is *Momordica charantia* (Linn Family: Cucurbitaceae), whose fruit is known as Karela or bitter gourd. For a long time, several workers have studied the effects of this plant extract in cancer therapy but the active ingredient and its mechanism of action are still unknown. *M charantia* has anti-carcinogenic properties and it can modulate xenobiotic metabolism and oxidative stress[1].

Aims

The main aim of this study is to extract, isolate, purify and characterize the active ingredient (s) of *M charantia* and investigate its mode and cellular mechanism (s) of action as a chemotherapeutic agent in cancer therapy.

Methods

Soxhlet extraction

1 gm of fine ground powder, sample extracted with Methanol for 4 hrs.

The sample residue was extracted repeatedly with 30 ml of Methanol.

The extract was filtered with Whatmann filter paper no:4

Evaporated under vacuum to remove the Methanol.

Schematic diagram of HPLC

Samples were analysed with C-18 inertsil ODS-3 column (5 μm, 4.6 mm × 259 mm id) and UV absorbance was monitored at 254 nm.

Mobile phase a. Trifluroacetic acid in acetonitrile.
b. Trifluroacetic acid in HPLC grade water.
c. Methanol HPLC grade.

Flow rate 1.0 ml/min, temperature was maintained 37°C, injection volume 10 μl.

Tissue culture

Cells like U87,1321NI and GOS-3 were cultured in 96 well plates

Cells were then allowed to reach a confluence of 60-70%

Different concentration of drug are added to the cells

Cells were then incubated for 24 hrs

Cells were then analysed by MTT assay

Results

Fig. 1.Time course effect of 0.0875 μg/ml *M. charantia* extract on three different glioma cell lines . Fig.2. Dose dependent effect of *M charantia* crude extract on viability of five different glioma cell lines. The effect of *M. charantia* on healthy L6 Muscle cell lines are also shown for comparison . Fig.3. Dose dependent effect of Alpha and Beta *Momocharin* extract on viability of five different glioma cell lines. Fig.4. Dose dependent effect of Temazolamide on three different cell lines. Data are mean± SEM, n=6 *, P < 0.05 for treated cells compared to untreated (control) cells.

Conclusion and Future Work

. The results have demonstrated that potent anticancer agent was extracted from *M Charanta* using Methanol and tested on three glioma cell lines.

. The crude extract and α and β Momocharin evoked significant (p<0.05) dose-dependent cytostatic effects on each cell lines compared to control.

. The next stage of the study is to determine the cellular and molecular mechanisms whereby *M Charantia* extract can induce cell death by measurement of cytosolic calcium, P53, caspase- 3 activity, cytochrome c.

References:

1.Ayala, R. S. and Luquede Castro, M. D (2001). Continuous subcritical water extraction as a useful tool for isolation of edible essential oils. Food Chemistry 75: 109-113.
2. Bourinbaiar AS, Lee-Huang S.(1995). Potentiation of anti-HIV activity of anti-inflammatory drugs, dexamethasone and indomethacin, by MAP30, the antiviral agent from bitter melon. Biochem :**208(2)**:779-785.
3. Cunnick, J. E., et al (1990). "Induction of tumor cytotoxic immune cells using a protein from the bitter melon (Momordica charantia)." Cell Immunol. Apr **126(2)**: 278-289.

Effects of *Momordica charantia* fruit extract with the combination of Temozolamide and Cisplatin in the treatment of glioma cancer

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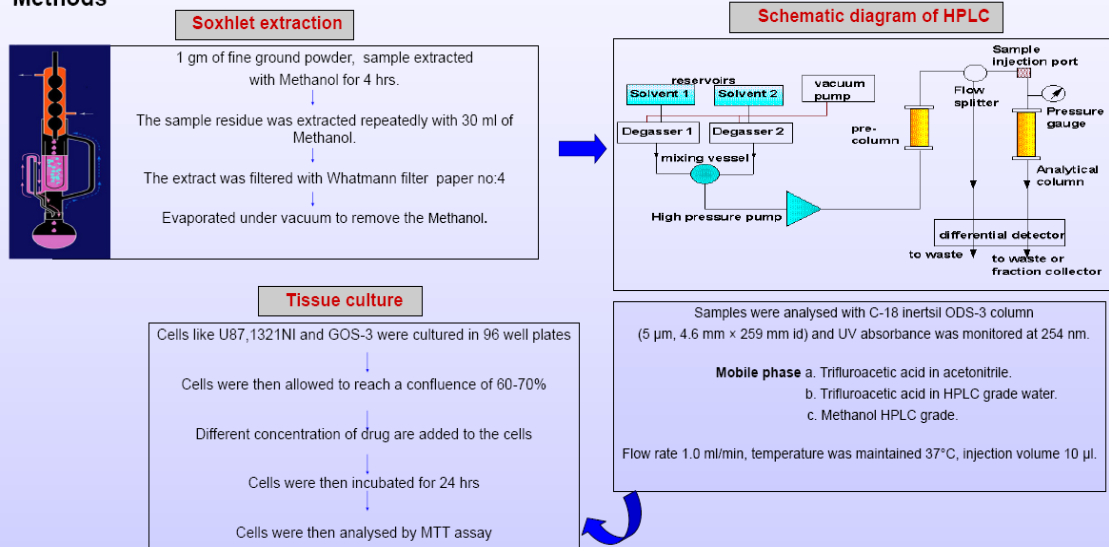
Introduction

Prior to the availability of chemotherapeutic agents, dietary measures, including traditional medicines derived from plants, were the major forms of cancer treatment. A number of plants have been used for the treatment of different cancers throughout the world[2]. One such plant is *Momordica charantia* (Linn Family: Cucurbitaceae), whose fruit is known as Karela or bitter gourd. Mainly, the fruit part of the bitter gourd is useful medicinally. It is made up of many different proteins and steroids that are chemically active. One of its chemicals displays cytotoxic activity and can inhibit guanylate cyclise, which is thought to be cause of psoriasis and leukemia. Another protein has been clinically shown to serve as anti-cancerous in animals. Alpha and Beta Momocharin has been tested as anti HIV and shows to stop virulence while the host cells were unaffected.

Aims

The main aim of this study is to extract, isolate, purify and characterize the active ingredient (s) of *M charantia* and investigate its mode and cellular mechanism (s) of action as a chemotherapeutic agent in cancer therapy.

Methods

Soxhlet extraction

1 gm of fine ground powder, sample extracted with Methanol for 4 hrs.

↓

The sample residue was extracted repeatedly with 30 ml of Methanol.

↓

The extract was filtered with Whatmann filter paper no:4

↓

Evaporated under vacuum to remove the Methanol.

↓

The samples were analysed with C-18 inertsil ODS–3 column, UV absorbance was monitored at 254nm

Tissue culture

Cells like U87,1321NI and GOS-3 were cultured in 96 well plates

↓

Cells were then allowed to reach a confluence of 60-70%

↓

Different concentration of drug are added to the cells

↓

Cells were then incubated for 24 hrs
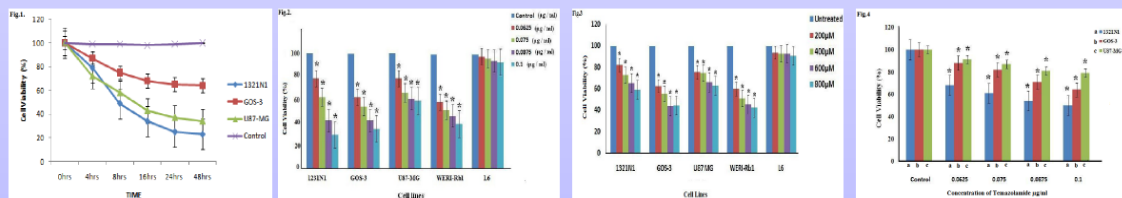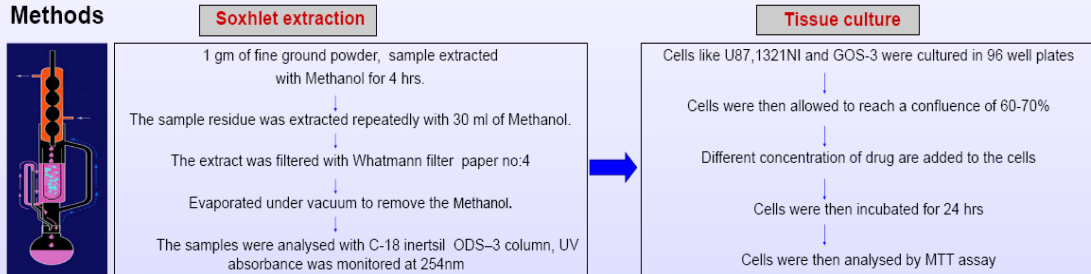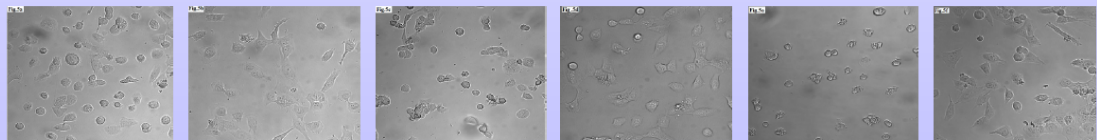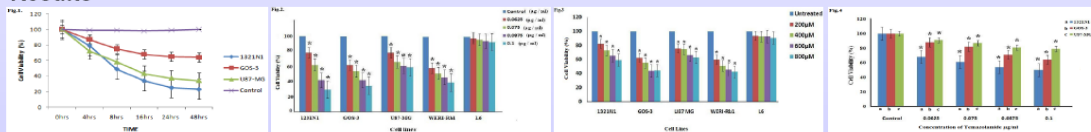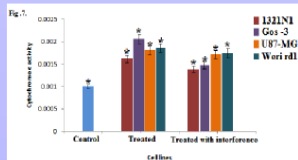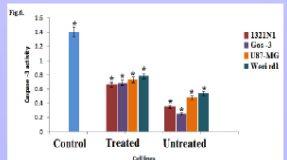
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Cells were then analysed by MTT assay

Results

Fig. 1.Time course effect of 0.0875 µg/ml *M. charantia* extract on three different glioma cell lines . Fig.2. Dose dependent effect of *M charantia* crude extract on viability of five different glioma cell lines. The effect of *M. charantia* on healthy L6 Muscle cell lines are also shown for comparison . Fig.3. Dose dependent effect of Alpha and Beta *Momocharin* extract on viability of five different glioma cell lines. Fig.4. Dose dependent effect of Temozolamide on three different cell lines. Data are mean± SEM, n=6 *, P < 0.05 for treated cells compared to untreated (control) cells.

Confocal image of 1321N1 cell line after 4 hrs of treating with Alpha Beta Momocharin (Fig 5a). Control of 1321N1 cell line (Fig 5b). Gos –3 cell line after 4 hrs of treating with Alpha Beta Momocharin (Fig 5c). Control of Gos –3 cell line (Fig 5d). U87-MG cell line after 4 hrs of treating with Alpha Beta Momocharin (Fig 5e). Control of U87-MG cell line (Fig 5f).

The study determines the mechanism where by *M Charantia* extract does not induce cell death by caspase –3 activity. The activity was measure by comparing with control by treated cells and untreated cells. (Fig.6.).

The study determines the mechanism where by *M Charantia* extract induce cell death by Cytochrome c activity. The activity was measure by comparing with control by treated cells and untreated cells. (Fig.7.).

Conclusion and Future Work

. The results have demonstrated that potent anticancer agent was extracted from *M Charanta* using Methanol and tested on three glioma cell lines. The crude extract and α and β Momocharin evoked significant (p<0.05) dose-dependent cytostatic effects on each cell lines compared to control. The next stage of the study is to determine the drug binging site and the receptors action and the Gene expression study.

References:

1.Ayala, R. S. and Luquede Castro, M. D (2001). Continuous subcritical water extraction as a useful tool for solation of edible essential oils. Food Chemistry 75: 109-113.

2. Bourinbaiar AS, Lee-Huang S.(1995). Potentiation of anti-HIV activity of anti-inflammatory drugs, dexamethasone and indomethacin, by MAP30, the antiviral agent from bitter melon. Biochem :208(2):779-785.

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Effects of *Momordica charantia* fruit extract with the combination of Temozolamide and Cisplatin in the treatment of glioma cancer

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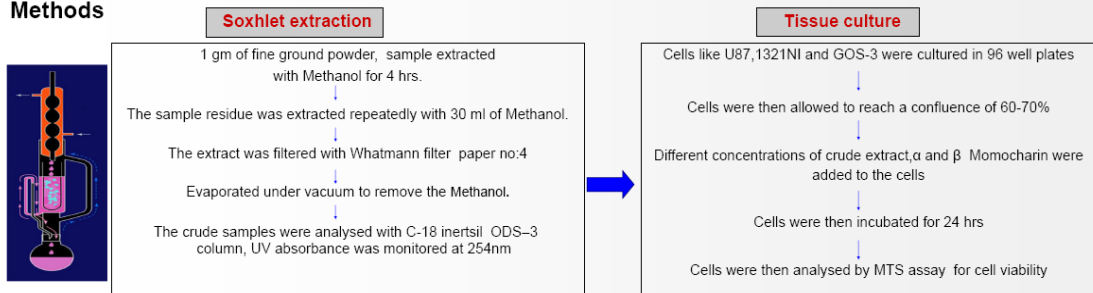
Introduction

Prior to the availability of chemotherapeutic agents, dietary measures, including traditional medicines derived from plants, were the major forms of cancer treatment. A number of plants have been used for the treatment of different cancers throughout the world[2]. One such plant is *Momordica charantia* (Linn Family: Cucurbitaceae), whose fruit is known as Karela or bitter gourd. Mainly, the fruit part of the bitter gourd is useful medicinally. It is composed of many different proteins and steroids that are chemically active[1]. One protein has been extracted and purified and clinically shown to posses anti-cancerous in animals. The compound is called α and β Momocharin and it can induce cell death in mouse skin carcinogenesis[3].

Aims

The aim of this study is to investigate the effect of crude water and methanol soluble extract of *M charantia* compared to α and β Momocharin either alone or in the combination with Cisplatin or with Temozolamide.
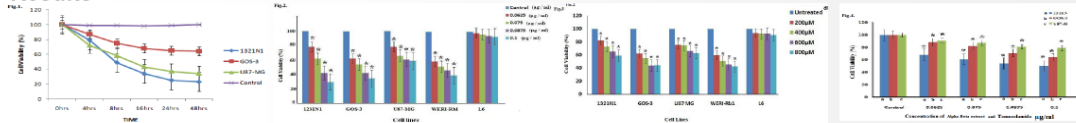
Methods

Soxhlet extraction

1 gm of fine ground powder, sample extracted with Methanol for 4 hrs.

↓

The sample residue was extracted repeatedly with 30 ml of Methanol.

↓

The extract was filtered with Whatmann filter paper no:4

↓

Evaporated under vacuum to remove the Methanol.

↓

The crude samples were analysed with C-18 inertsil ODS–3 column, UV absorbance was monitored at 254nm

Tissue culture

Cells like U87,1321NI and GOS-3 were cultured in 96 well plates

↓

Cells were then allowed to reach a confluence of 60-70%

↓

Different concentrations of crude extract,α and β Momocharin were added to the cells

↓

Cells were then incubated for 24 hrs

↓

Cells were then analysed by MTS assay for cell viability

Results

Fig. 1.Time course effect of 0.0875 µg/ml *M. Charantia* crude extract on three different glioma cell lines . Fig.2. Dose dependent effect of *M charantia* crude extract on viability of four different glioma cell lines. The effect of *M. charantia* on healthy L6 Muscle cell lines are also shown for comparison . Fig.3. Dose dependent effect of Alpha and Beta *Momocharin* extract on viability of four different glioma cell lines. Fig.4. Dose dependent effect of Temozolamide on four different cell lines. Data are mean± SEM, n=6 *, P < 0.05 for treated cells compared to untreated (control)
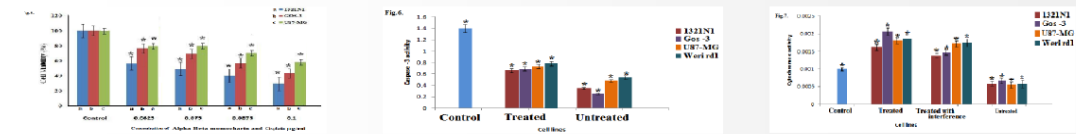

Fig.5. Dose dependent effect of Cisplatin on three different cell lines. Data are mean± SEM, n=6 *, P < 0.05 for treated cells compared to untreated. Fig.6. Measurement of Caspase –3 activity in untreated and *M Charantia* treated cells . Data are mean± SEM, n=6 *, P < 0.05 for treated cells compared to untreated (control) cells. Fig.7. Measurement of Cytochrome c activity in un-treated and *M Charantia* treated cells. Data are mean± SEM, n=6 *, P < 0.05 for treated cells compared to untreated (control) cells.
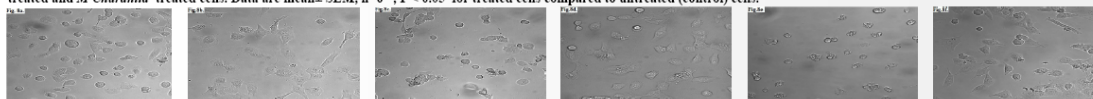

Fig .8a.Confocal image of 1321N1 cell line after 4 hrs of treating with Alpha Beta Momocharin. Fig. 8b. Control of 1321N1 cell line. Fig .8c. Gos –3 cell line after 4 hrs of treating with Alpha Beta Momocharin . Fig .8d.Control of Gos –3 cell line. Fig. 8e.U87-MG cell line after 4 hrs of treating with Alpha Beta Momocharin . Fig. 8f.Control of U87 -MG cell line .

Conclusion and Future Work

(A) The results have shown that either crude extract of *M charantia* , α and β Momocharin, Cisplatin and Temozolamide can evoke marked significant (p<0.05) dose-dependent anti-cancer effect on four different glioma compared to healthy L6 muscle cell line. (B) *M charantia* , α and β Momocharin seems to elicit its anti-cancer effect by inducing mainly, Cytochrome c activity compared to untreated cells. Future work will be based on cellular Ca^{2+} homeostasis.

References:

1. Ayala, R. S. and Luquede Castro, M. D (2001). Continuous subcritical water extraction as a useful tool for isolation of edible essential oils. Food Chemistry **75**: 109-113.
2. Bourinbaiar AS, Lee-Huang S.(1995). Potentiation of anti-HIV activity of anti-inflammatory drugs, dexamethasone and indomethacin, by MAP30, the antiviral agent from bitter melon. Biochem :**208(2)**:779-785.
3. Cunnick, J. E., et al (1990). "Induction of tumor cytotoxic immune cells using a protein from the bitter melon (Momordica charantia)." Cell Immunol. Apr **126(2)**: 278-289.

Cellular mechanism of action of α, β momorcharin on cell viability of different cancer cell lines.

G. Manoharan, R.W. Lea, T. J. Snape and J. Singh

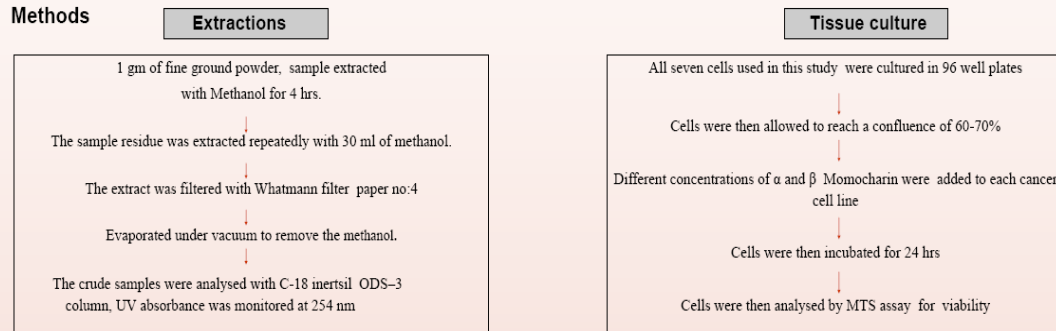School of Pharmacy and Biomedical Sciences, University of Central Lancashire, Preston, PR1 2HE

Introduction

Prior to the availability of chemotherapeutic agents, dietary measures, including traditional medicines derived from plants were the major forms of cancer treatment. A number of plants have been used for the treatment of different types of cancers throughout the world. One such plant is *M. charantia* (Linn Family: Cucurbitaceae), whose fruit is known as karela or bitter gourd [1]. Previous studies including these in our laboratory have demonstrated marked anti-cancer effects of *M. charantia* and α, β momocharin using several different cancer cell line[2,3]. However, the cellular and molecular mechanism(s) whereby *M. charantia* is exerting its anti-cancer effect is still unknown.

Aims

This study investigated the anti-cancer effects and the cellular mechanisms of action of different concentrations of α, β momocharin (200 μM - 800 μM) in 1321N1, Gos-3, U87-MG and Weri- Rd1 glioma cancer cell lines, human skin melanoma (Sk Mal-2) and large lung Carcinoma (Corl -23) compared to normal healthy L6 muscle cell line.

Methods

Extractions

1 gm of fine ground powder, sample extracted with Methanol for 4 hrs.

↓

The sample residue was extracted repeatedly with 30 ml of methanol.

↓

The extract was filtered with Whatmann filter paper no:4

↓

Evaporated under vacuum to remove the methanol.

↓

The crude samples were analysed with C-18 inertsil ODS–3 column, UV absorbance was monitored at 254 nm

Tissue culture

All seven cells used in this study were cultured in 96 well plates

↓

Cells were then allowed to reach a confluence of 60-70%

↓

Different concentrations of α and β Momocharin were added to each cancer cell line

↓

Cells were then incubated for 24 hrs

↓

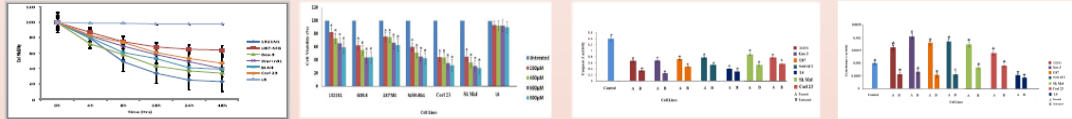Cells were then analysed by MTS assay for viability

Results

Fig. 1 : Time course effect of 800 μM α, β Momocharin extract on six different cancer cell lines. Fig.2: Dose dependent effect of α, β Momocharin extract on viability of the six different cancer cell lines compared to L-6 muscle cell line.. Fig.3: Measurement of Caspase –3 activity in untreated and α, β Momocharin-treated cells . Data are mean± SEM, n=6 *, P < 0.05 for treated cells compared to untreated (control) cells. Fig.4: Measurement of Cytochrome- c activity in untreated and α, β Momocharin- treated cells. Data are mean± SEM, n=6 *, P < 0.05 for treated cells compared to untreated (control) cells.

Calcium homeostatsis: Intracellular free calcium concentrations $[Ca^{2+}]_i$ in Fura 2- AM-loaded cancer cell lines in the absence and presence of 800 μM α, β Momocharin. The cells were loaded with 25 μM Fura 2- AM, and $[Ca^{2+}]_i$ measured at excitation wavelength of 485 nm and emission wavelength of 520 nm. Data are mean ± SEM, n=6. Note the time causes increasing in $[Ca^{2+}]_i$ following α, β Momocharin treatment.
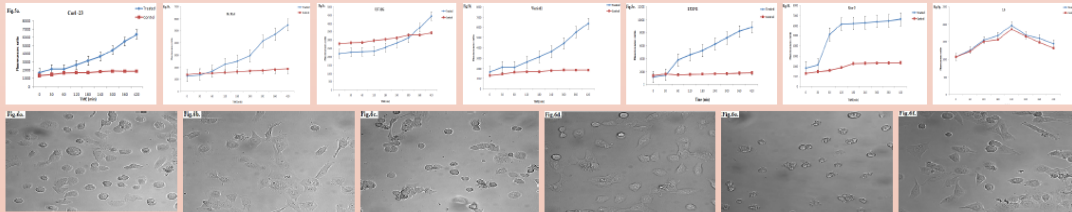

Fig .6: Confocal images of control and treated cell lines for 4 hrs with 800 μM α, β Momocharin. 6.a: Treated 1321N1 cell line . 6b: Untreated (control) of 1321N1 cell line. 6c: Treated Gos –3 cell line . 6d:Untreated (control) of Gos –3 cell line. 6e: Treated U87-MG cell line . 6f: Untreated (control) of U87-MG cell line. Note the decrease in cell viability following treatment with α, β Momocharin .

Conclusions and Future Work

The results have shown that α, β Momocharin, can evoke significant (p<0.05) dose-dependent anti-cancer effect on six different cancer cell lines compared to healthy L6 muscle cell line. The anti-cancer effects α, β Momocharin seems to be associated more with cellular $[Ca^{2+}]_i$ homeostatsis and Cytochrome- c activity, rather than cellular apoptosis. Future work will be based on Caspase – 9 activity and drug binding sites by fluorescence imaging.

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