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Screening of South African medicinal plant *Euphorbia tirucalli* for anticancer properties



Mpho Susan Choene

A Thesis submitted to the Faculty of Science under the school of Molecular and Cell Biology, University of the Witwatersrand, in partial fulfilment of the requirements for PhD.

Gauteng, Johannesburg, 2015

Declaration

I, Mpho Susan Choene (0412258x), am a student registered for the degree of Doctor of Philosophy (PhD) in the academic year 2015.

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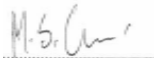
Signature Date: 20 May 2015

Abstract

Cancer is an enormous burden of disease, accounting for millions of deaths annually worldwide. Today, more people are dying from cancer than HIV/AIDS, malaria and tuberculosis combined. According to the American Cancer Society, it is expected that the global cancer burden will double by 2030 if preventative measures are not applied. Breast and gynaecological cancers remains a big scourge in developing countries, with breast cancer being the most common cancer and gynaecological cancers accounting for approximately 25% of all cancers in women in developing countries. Currently, the standard cancer treatments include surgery, radiation and chemotherapy. Adverse toxicities have been associated with these therapies and their effectiveness is also limited to drug resistance. The cost of treatment is another major burden. Limitations associated with these conventional cancer treatments have made discoveries of novel therapeutics which exhibit less toxicity and at a lowered cost of paramount importance. Medicinal plant extracts have recently attracted attention to modern medical science research with their non-lethal activity. Currently, up to 50% of the world drugs including chemotherapeutic drugs such as taxol and camptothecin are made from natural products or their derivatives. In this study we aimed to investigate the anti-cancer properties of the medicinal plant *Euphorbia tirucalli*. The crude extracts of *E. tirucalli* extracted using butanol; hexane and methanol solvents were screened for antiproliferative activity in breast (MCF-7 and MDA-MB231), ovarian (RMG-1) and cervical (SiHa) cancer cell lines. MTT assay and Real-Time Cell Analyzer (RTCA), xCELLigence were used to determine cytotoxicity of the extracts and calculate IC_{50}. From MTT and xCELLigence results, we observed that *E. tirucalli* extracts exhibited dose-dependent inhibition of cell proliferation with RMG-1 and MCF-7 cells being more sensitive than MDA-MB231 and SiHa cells to all three extracts for an unclear reason. The butanol extract appeared to exhibit

the most cytotoxicity with all cell lines reaching IC_{50} at low extract concentrations. Most therapies in anticancer treatment, such as chemotherapy, mainly induce cell death by causing either G_0/G_1 or G_2/M cell cycle arrest and then inducing an apoptotic pathway. Therefore, cell cycle arrest and the induction of apoptosis in cancer cells become the major indicators of anticancer effects. Cells were stained with propidium iodide dye to determine if cells were arrested at G_0/G_1 or G_2/M cell cycle stages while annexin V and PI staining were used to determine the type of cell death induced by the extracts. Cell cycle analyses revealed MCF-7, MDA-MB231 and SiHa cancer cells underwent arrest at G_0/G_1 following treatment with the plant extracts. Annexin V and PI staining revealed different proportions of apoptotic and necrotic populations. The extracts mainly induced apoptosis on MCF-7 and MDA-MB 231 cells, with the butanolic extracts inducing the most apoptosis. RMG-1 and SiHa cells had a high proportion of cells undergoing both late apoptosis and necrosis. The molecular mechanism of cell death induction was investigated using real time PCR and western blot. From the gene expression studies, p21 was observed to be over expressed in all cells following all treatments, in line with the observed cell cycle arrest at G_0/G_1. The extrinsic pathway of apoptosis was identified as the type of cell death induced with caspase 8 being overexpressed in MDA-MB 231 cells treated with butanol and hexane extracts. Upon further fractionation, flavonoids and especially isorhamnetin were identified as the active compounds in these extracts. Overall, the plant contains compounds that have some activity against cell proliferation and can be a promising tool to treat cancer cells. However, more work needs to be done to verify which compounds are mainly involved.

Keywords: apoptosis, cell cycle, x*Cell*igence, isorhamnetin, p21and *E. tirucalli*

Research outputs

Original publications

Publications forming part of PhD thesis

1. **Mpho S Choene** and Lesetja R Motadi (2015). CDK-interacting protein 1 overexpression as a predictive anti-cancer agent in breast cancer treated with *Euphorbia tirucalli* extracts. *J. Mol Bio*. (Accepted, *inpress*).

2. **Mpho S Choene** and Lesetja R Motadi (2015) Cell cycle and apoptosis induction in ovarian (RMG-1) and cervical cancer cell line (SiHa) via p21 activation by Flavonoids components isorhamnetin extract of *Euphorbia tirucalli*. Submitted.

3. **Mpho Choene,** Nonkululeko Mthembu, Zodwa Dlamini, Matlou Mokgotho, James Wachira and Lesetja Motadi (2012). Breast cancer: Small Molecules Targeting Apoptosis, a Prospective Approach to Safe Scientific Success. *Adv. Biol. Biotech*. 1: 833-844.

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2. Pontsho Moela, **Mpho S. Choene**, Lesetja R. Motadi (2014). Silencing RBBP6 (Retinoblastoma Binding Protein 6) sensitises breast cancer cells MCF7 to staurosporine and camptothecin-induced cell death. *Immunobiol*. 219: 593–601

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Poster presentations

1. South African Society for Biochemistry and Molecular Biology conference, Goudini Spa, Rawsonville. 2014, *Euphorbia mauritanica* extracts' anti-proliferative properties against breast cancer. **M.S. Choene** and L.R. Motadi.

Published conference output

1. **Mpho S. Choene** and Lesetja R. Motadi. 2014, Anti-proliferative properties of *Euphorbia mauritanica* medicinal plant against breast cancer. *Cancer Res*. 74: 3216. Appendix 1

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Abbreviations

Abbreviation	Description
3-GAP	3-geranyl-phloroacetophenone
3-PAP	3-prenyl-phloroacetophenone
AIDS	Acquired Immune Deficiency Syndrome
AIF	Apoptosis-Inducing Factor
Apaf-1	Apoptosis-protease-activating-factor 1
ApoG2	Apogossypolone
Bad	Bcl-2-associated death promoter
Bak-1	Bcl2-antagonist/killer 1
Bax	Bcl2-associated X protein
Bcl-2	B-cell lymphoma 2
BH	Bcl-2 homology domain
Bid	BH3 interacting-domain
Bik	Bcl-2-interacting killer
BIR	Baculovirus IAP repeat
BRCA 1	Breast cancer Gene 1
BRCA 2	Breast cancer Gene 2
Caspases	Cysteine-aspartic proteases
CARD	Caspase recruitment domain
CDKN-1	Cyclin-dependent kinase inhibitor 1
CHOP	CCAAT/enhancer-binding protein homologous protein
cIAP	Cellular IAP

dATP	Deoxyadenosine triphosphate
DCM	Dichloromethane
DD	Death domain
DED	Death effector domain
DISC	Death inducing signaling complex
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2, 2-diphenyl-1-picrylhydrazyl
DR	Death receptor
EGFR	Epidermal growth factor receptor
E. tirucalli	*Euphorbia tirucalli*
FADD	Fas-associated death domain
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HER2	Human epidermal growth factor receptor 2
HIV	Human Immunodeficiency Virus
HPLC	High Perfomance Liquid Chromatography
HPV	Human papillomavirus
IAP	Inhibitor of apoptosis
LEEP	Loop electrosurgical excision procedure
MDM 2	Murine double minute 2
MDMX	Murine double minute X

MeOH	Methanol
ML- IAP	IAP Melanoma IAP
MOMP	Mitochondrial outer membrane permeabilization
MTT	3-4,5-dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromide
NaCl	Sodium chloride
NAIP	Neuronal Apoptosis Inhibitory Protein
P53/Tp53	protein 53/tumour protein 53
Pen/strep	Penicillin/streptomycin
PCR	Polymerase chain reaction
PIG3	p53-inducible gene 3
RBBP6	Retinoblastoma binding protein 6
RING	Really Interesting New Gene
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RTCA	Real time cell analyser
RTK	Receptor tyrosine kinases
SD	Standard deviations
SDS	Sodium dodecyl sulphate
SMA	Spinal Muscular Atrophy
SPD	Styrylpyrone derivative
tBID	Truncated BH3 interacting-domain
TLC	Thin layer chromatography
TNF	Tumour necrosis factor
TRAIL	Tumour necrosis factor-related apoptosis-inducing ligand

UV	Ultraviolet
VEGF	Vascular endothelial growth factor
XIAP	X chromosome-linked IAP
XN	Xanthohumol
y-T3	YttriuM (III) Ion

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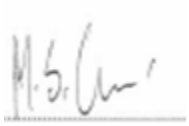
This letter is to confirm that the following manuscript "**Mpho Choene, Nonkululeko Mthembu, Zodwa Dlamini, Matlou Mokgotho, James Wachira and Lesetja Motadi (2012). Breast cancer: Small Molecules Targeting Apoptosis, a Prospective Approach to Safe Scientific Success. Adv. Biol. Biotech. 1:833-844.**" was prepared by me (Mpho S Choene). I have contributed 70% to the preparation of the paper which include: all the experiments and writing of the manuscript itself.



_____

Ms Mpho S Choene

CHAPTER 1 : INTRODUCTION

Part of this chapter has been published as follows: **Mpho Choene,** Nonkululeko Mthembu, Zodwa Dlamini, Matlou Mokgotho, James Wachira and Lesetja Motadi (2012). Breast cancer: Small Molecules Targeting Apoptosis, a Prospective Approach to Safe Scientific Success. *Adv. Biol. Biotech***.** 1:833-844. Appendix 2

1.1. Cancer

Cancer is an huge burden of disease globally. Tens of millions of people are diagnosed with cancer and more than half end up dying from it (Ma and Yu 2006). Today, more people are dying from cancer than HIV/AIDS, tuberculosis and malaria combined. According to the American Cancer Society, it is expected that the global cancer burden will double by 2030 growing to 21.4 million cases and 13.2 million deaths if preventative measures are not applied. Most of the incidence, morbidity and mortality of cancers occur in developing regions where access to medical and health resources is limited. The developing world is already burdened by cancers some of which are attributable to infectious diseases. In addition the incidence of cancer is further increased by the greater adoption of behaviours and lifestyles associated with urbanization such as smoking, physical inactivity and reproductive factors (Kanavos 2006). Breast and gynaecological cancers remain a big scourge in developing countries. Breast cancer is the most common cancer in females both in the developing and the developed world. Gynaecological cancers account for approximately 25% of all cancers in women in developing countries. It was reported that developing countries accounted for 77.7% of global estimates for new cases of the most common gynaecological cancers in 2009 (The Economist 2009). Currently, cervical cancer is the most common cancer, accounting for over 60% of the gynaecological cancer burden in developing

countries, with ovarian cancer being the second most common and the deadliest (Sankaranarayanan and Ferlay 2006).

1.1.1. Cervical cancer

Worldwide, cervical cancer is the third most common cancer in women ranking after breast and colorectal cancer accounting for almost 12% of all cancers in females. An estimated 528 000 new cases and 266 000 deaths were reported worldwide in 2012.

The world's cervical cancer burden lies heavily in the less developed countries including the Caribbean, Central and South America as well as southern, eastern and middle Africa. Eighty six percent of all cervical cancer cases and 88% of all deaths caused by cervical cancer have occurred in developing countries (Franco *et al.* 2001) (Globocan 2012). The cumulative rates for cervical cancer incidence and mortality were 1.9 and 1.1 in developing countries and 0.9 and 0.3 in developed countries (Arbyn *et al.* 2011). In South Africa, cervical cancer is the second most common cause of cancer related deaths, with over 3000 deaths per year for women, most likely due to the HIV pandemic (Hoque *et al.* 2013).

Epidemiological studies have shown an association between several factors and an increased risk of cervical cancer. The most consistent risk factor according to studies in the last 30 years has been sexual behaviour, including age at first sexual intercourse and the number of sexual partners, suggesting the existence of a sexually transmitted infectious agent (Plummer *et al.* 2012). In recent years, infection with certain strains of oncogenic human papillomavirus has been found to be the main casual factor of cervical cancer (Schiller and Lowy 2014).

Cervical cancer is a malignant tumour that starts in the cervical transformation zone. This zone is an area of metaplastic tissue between the glandular tissue of the endocervical canal and the squamous epithelium of the vagina. This zone is the most susceptible to cancer

growth even though the entire epithelium may be infected with HPV. Cervical carcinogenesis is a multi-step process that begins with HPV infection and requires establishment and persistence of the virus. In most people, the infection clears without medical intervention. If the viral infection persists, there is a higher risk of the development of high-grade precursor lesions which are likely to progress to cervical cancer if left untreated over a period of years (Bosch *et al*. 2002; Munoz *et al.* 2004). Persistence of the virus could be supported by the virus life cycle taking place away from dermal immune cells or lack of virus-induced cell lysis leading to no inflammatory response or altered immune response caused by the virus. This suggests that viral persistence depends on efficient negative cell cycle control allowing damaged genetic material to accumulate and immune evasion allowing the virus to go undetected (Gravitt 2011).

1.1.2. Ovarian cancer

The annual ovarian cancer incidence is estimated at 204 000 with 125 000 deaths globally. Ovarian cancer is the most lethal gynaecological malignancy with a fatality rate of over 70%. One of the main reasons for the high fatality rate is that most women are diagnosed with advanced disease (Jemal *et al*. 2008; Sankaranarayanan *et al.* 2006). Even though only about 20% of carcinomas are diagnosed whilst still localised to the ovaries, the five year survival of women with confined tumours is over 90%. The lifetime risk of developing the disease is 1 in 70 in developed countries (Cannistra 2004).

Most ovarian cancers are sporadic; however, hereditary predisposition accounts for about 10% of ovarian and breast cancer cases (Rauh-Hain *et al*. 2011). For this subset, a strong family history of breast and ovarian cancer is the most important risk factor. In genetically predisposed individuals, mutations in the BRCA genes account for 90% of the cancers.

BRCA 1 mutation carriers have a 40 to 50% chance of developing the disease whilst BRCA 2 mutation carriers have a 20 to 30% chance (Struewing *et al*. 1997).

The unknown pathogenesis and late stage of diagnosis contribute to the poor prognosis in ovarian cancer. It is now known that ovarian cancer is a heterogeneous disease composed of different types of tumours with different clinicopathologic features. Based on molecular genetic studies, the various types of ovarian cancers have been classified into two groups. The Type I tumours comprise low-grade serous, low-grade endometrioid, clear cell and mucinous carcinoma which are confined to the ovary on presentation. On the other hand Type II tumours are high-grade serous and high-grade endometrioid. Type II are the most aggressive and are usually present in advanced stages. These are usually present in 75% of epithelial ovarian carcinomas (Kurman and Shih 2010).

It has been well established that cancers arise as a result of accumulated genomic changes including gene amplifications, point mutations and deletions. Analysis of these genetic changes has led to the identification of cancer associated genes including DNA repair and tumour suppressor genes. Studies on genetic alterations in ovarian carcinomas found *TP*53 mutations to be present in over 80% of high-grade, serous carcinomas. *TP*53 mutations were found to be rare in low-grade serous carcinoma (Salani *et al*. 2008).

1.1.3. Breast cancer

Breast cancer is the most prevalent cancer in females, affecting as many as one in eight women in their lifetime. It can occur in both males and females, however, male breast cancer is rare. The estimated new cases for *in situ* breast cancers for 2015 are expected at about 60, 290 in the United Sates alone. The National Cancer Institute defines breast cancer as "Cancer that forms in tissues of breast, usually the ducts and the lobules". Deaths due to breast cancer

have been steadily declining in recent years; this most likely has to do with earlier detection and improved treatment.

1.2. Epidemiology

Developing countries seem to be heavily affected by the double burden of high infection-related cancers such as cervical cancer and an increasing incidence of cancers associated with a westernized lifestyle such as breast and ovarian cancer. The development of breast and ovarian malignancies is complex, involving numerous factors including environmental agents, genetic predisposition, reproductive and lifestyle factors (Kruk *et al*. 1999; Pieta *et al*. 2012; Struewing *et al*. 1997).

The majority of cancers can be attributed to lifestyle, diet, and environmental factors. Increased frequency of tobacco usage is seen as the most important risk factor in the development of cancer. Alcohol abuse is associated with an increased risk too, in addition, alcohol consumption was also found to enhance the carcinogenic effect of tobacco and other carcinogens, especially those of dietary origin (Pieta *et al*. 2012). Studies have linked physical activity to a lowered risk of developing breast and ovarian cancer; this may be due to its beneficial effect on the function of the immune system and hormonal regulation (Kruk *et al*. 1999). Furthermore, consumption of an insufficient amount of fruits and vegetables and being overweight have also been associated with an increased risk (Pieta *et al*. 2012).

Genetic and reproductive factors that are thought to contribute to cancer development include carriers of germline BRCA1 and BRCA2 mutations. BRCA mutations are believed to confer an increased risk of developing ovarian and breast cancer (Struewing *et al*. 1997). An initial germline mutation in one of these genes followed by a second mutation leading to the loss of function of the second allele is required for induction of tumouriginesis (Powell and Kachnic

2003). These genetic factors mostly show an autosomal dominant pattern of inheritance, with germline mutations rendering susceptibility to the following generation (Loubser *et al.* 2008; Ponzone and Baum 1998).

Nulliparity, early menarche and late menopause are associated with a higher risk of breast and ovarian carcinogenesis whilst parity, lactation and hysterectomy are associated with a decreased ovarian and breast cancer risk (Braem *et al.* 2010). The use of oral contraceptives reduces ovarian cancer risk by at least one-half, a benefit which increases with increasing duration of use and persists for at least 15 years after discontinuation. In contrast, oral contraceptives are believed to slightly increase the risk of breast cancer. However, the risk level goes back to normal ten years after discontinuing their use (Beaber *et al*. 2014; Braem *et al.* 2010; Hulka 1997).

Hormonal correlates of cervical cancer are few; it exhibits the epidemiologic characteristics of a sexually transmitted disease. Infection with certain strains of oncogenic human papillomavirus has been found to be the main casual factor of cervical cancer. Other sexually transmittable infections such as HIV and Chlamydia trachomatis are also thought to have increased the incidence of cervical cancer (Arbyn *et al.* 2011; Franco *et al*. 2001). With the prevalence of the HIV-AIDS epidemic in Africa, cervical cancer was defined as an AIDS-defining cancer by the U.S Center for Disease Control and Prevention (CDC 1993). In fact, a study carried out in Johannesburg, South Africa, found an an association between HIV infection and cervical cancer risk (Sitas *et al*. 2000).

1.3. Current treatments in cancer

Currently there are no effective treatments for persistent HPV infection but there are strategies to control HPV-associated lesions. The different types of treatment can be

classified into surgical and medical approaches; these are determined by the clinical tumour stage and cancer pathology at diagnosis (Table 1).

Treatment for abnormal cervical cells include conization, cryotherapy and loop electrosurgical excision procedure (LEEP). Conization is the removal of a cone-shaped piece of tissue from the cervix and cervical canal which is used for evaluation of any abnormalities. Cryotherapy employs the use of a cryoprobe set to below −22°C to freeze and destroy abnormal tissue whilst LEEP uses an electrical current passed through a thin wire loop for removal of abnormal tissue (Ma *et al*. 2012).

The treatment for malignant cervical tumours may include surgery, radiation and chemotherapy. For advanced cancers, combinational therapies may be administered. Surgical options for cervical cancer include either total or radical hysterectomy or radical trachelectomy. In total hysterectomy the uterus, including the cervix is removed. Radical hysterectomy on the other hand involves the removal of the uterus, cervix, parametrium, and a portion of the upper vagina. A different method called radical trachelectomy involves the removal of the cervix, surrounding tissue and lymph nodes, and the upper part of the vagina (Ma *et al.* 2012).

The treatment for ovarian cancer depends on the type and stage of the tumour at diagnosis. Therefore the following surgeries maybe performed: (i) bilateral salpingo-oophorectomy which is the removal of ovaries and fallopian tubes, (ii) total abdominal hysterectomy involves the removal of the womb and cervix and (iii) omentectomy which is the removal of the fatty layer that covers the bowel. The initial treatment recommended for women with ovarian cancer however, is surgical debulking/cytoreduction, regardless of disease stage. Cytoreduction is the removal of most if not all of the cancer tumour (Bristow *et al*. 2002;

Chang *et al*. 2012). Following cytoreduction, treatment with adjuvant systemic chemotherapy with taxanes and platinum drugs is recommended (Cristea *et al.* 2010).

For breast cancer, the surgical options include breast conserving surgery and mastectomy. Breast conserving surgery includes lumpectomy (partial mastectomy) which involves the removal of the diseased part of the breast and some surrounding normal tissue. Mastectomy involves removal of the entire breast and sometimes the surrounding tissue; this may include lymph nodes (Whelan *et al.* 1999).

Radiation therapy uses radiation such as x-rays to kill cancer cells. The two types of radiation therapy are external and internal radiation therapy. External radiation therapy uses a machine outside the body to kill cancer cells. Internal radiation therapy employs the use of radioactive substances which are placed near or directly into the cancer.

Chemotherapy involves the use of anticancer drugs to induce cancer cell death. Depending on the stage and type of cancer, chemotherapy treatment may either be localized or systemic. With systemic chemotherapy, drugs are either taken by mouth or injected into a vein, allowing the drugs to spread throughout the body. Currently the standard regime for chemotherapy for treatment of breast, ovarian and cervical cancer includes the following drugs: Cisplatin, Gemcitabine, Carboplatin, Paclitaxel and Topotecan. Depending on the cancer, these are either administered alone or in combination with other treatments such as radiation and surgery (Brave *et al.* 2006; Duenas-Gonzalez *et al.* 2011).

1.4. Apoptosis

Apoptosis, also known as programmed cell death, is an evolutionary conserved form of cell suicide. It is a fundamental process required for the development and health of multicellular organisms. Apoptotic cells undergo unique structural and morphological alterations which

can be distinguished from another type of cell death known as necrosis (Wyllie 1997). Apoptotic cells play an active role in their own cell death through activation of an internally coded suicide program, leading to a controlled regulated death. Structural features of apoptosis include condensation of nuclear chromatin, cell shrinkage, cleavage of chromosomal DNA and fragmentation of the nucleus. Phosphatidylserine residues which are normally embedded in the inner plasma membrane become translocated to the outer membrane and serve a signal to be rapidly taken up by phagocytes. In contrast, necrosis is an uncontrolled type of cell death which leads to lysis of cells, an inflammatory response and ultimately damage to surrounding cells. Cells undergoing necrosis swell, nuclear and cytosolic structures alter and the plasma membrane ruptures causing release of internal material into surrounding tissue (Thompson 1995; Wyllie 1997).

Apoptosis plays an important role throughout the life cycle of an organism. Its first crucial role is during the development of an embryo. It sculptures organs and allows for differentiation of fingers and toes. The failure of its regulation could lead to an observable phenotype characterized by various defects. In an average human adult, about 50 to 70 billion cells die every day to keep in balance with the numbers of new cells produced daily in self renewing tissues such as skin, bone marrow and gut. This homeostasis is an active process regulated through apoptosis (Reed 1999).

Apoptosis is a tightly regulated process which requires the coordinated activation and execution of various factors. Hence, malfunction of the apoptotic machinery has been implicated in pathogenesis of a numerous of clinical disorders. Increased apoptosis may lead to autoimmune diseases such as multiple sclerosis and rheumatoid arthritis, neurodegenerative disorders including Alzheimer's and Parkinson's diseases, and ischemic

injury for example, stroke. Diseases associated with decreased apoptosis include metabolic disorders, viral infections and various types of cancers (Fadeel *et al*. 1999).

Apoptosis has been a target of extensive research since the early 1990s. This is evidenced by the thousands of publications that are continuously being produced, making it difficult to keep track of newly identified proteins and molecules involved in its regulation. The decision of a cell to undergo apoptosis is dependent on the balance between positive signals (keep the cell alive) and negative signals (inducers of apoptosis). Multiple inducers of apoptosis have been identified of which some show selectivity to their targets, these include: DNA damage, tumour necrosis factor, ionizing radiation, viral infection and glucocorticoids. In response to these signals, a cascade of events is induced which will ultimately lead to cell death (Dan *et al*. 1999; Greenstein *et al.* 2002)

Apoptosis is mainly orchestrated by the activation of a cascade of aspartate specific cysteine proteases (caspases). There are two major pathways that lead to the activation of caspases: the first depends on the participation of the mitochondria (intrinsic pathway) while the second is triggered by the engagement of cell surface receptors (extrinsic pathway) (Chaudhary *et al*. 1997; Green and Kroemer 2004; Smith and Farrah 1994).

1.4.1. Intrinsic pathway

The mitochondrial pathway is activated in response to various types of intracellular stress such as DNA damage. In this pathway, activation of caspases is closely linked to permeabilization of the outer mitochondrial membrane. This permeabilization is regulated by signal inducing proapoptotic members of the Bcl-2 family (Green and Kroemer 2004). Upon disruption of the mitochondrial membrane, apoptogenic factors such as cytochrome c, Apoptosis-Inducing Factor (AIF) and Smac/Diablo which had been sequestered in the

mitochondrial intermembrane space become released into the cytoplasm (Schuler *et al*. 2000). Cytochrome c binds to apoptosis-protease-activating-factor 1 (Apaf-1). This aggregation requires the binding of dATP as a cofactor to form a large multi-subunit apoptosome complex. Once assembled, the apoptosome complex will recruit and activate initiator caspase-9 (Purring-Koch and McLendon 2000). Active caspase-9 in turn activates downstream effector caspases such as caspase-7 and caspase-3 which will lead to the execution phase of apoptosis (Li *et al.* 1997).

1.4.2. Extrinsic pathway

This pathway is mediated by activation of cell surface receptors belonging to the tumour necrosis factor (TNF) superfamily. Some members of this superfamily that have been well characterized include TNFR, DR4/5, CD-95 and the corresponding ligands being TNF, Trail and CD-95L, respectively (Smith and Farrah 1994; Chaudhary *et al*. 1997; Walczak and Krammer 2000). The binding of a ligand to the appropriate receptor induces receptor clustering, recruitment and activation of initiator caspases 8 and 10 and adapter protein Fas-associated death domain (FADD). This aggregation leads to the formation of the multi-protein complex known as the death inducing signaling complex (DISC) (Kim *et al.* 2000). Depending on the type of cell, caspase-8 will either directly activate caspase-3 or it can activate the mitochondrion pathway by truncating a proapoptotic member of the Bcl-2 family Bid into its active form tBid. The active tBid will thus trigger the mitochondrion pathway leading to apoptosis (Li *et al.* 1998).

1.5. Apoptosis and cancer

1.5.1. Small Molecules Targeting Extrinsic Pathway in Cancer

The observation that activation of the extrinsic pathway can amplify apoptotic signals initiated by cytotoxic drugs acting primarily through the induction of the mitochondrial

pathway has prompted the search for strategies capable of triggering the receptor-related cascade (Turner *et al.* 2005). This has led to the identification of the tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) and its death receptors DR4 and DR5 (Tutt et al 2002). Activation of DR4 and DR5, like that of Fas/APO, leads to activation of the initiator caspase, caspase-8, and its downstream targets (Erickson et al 2012). Interestingly, TRAIL seems to exert selective toxicity towards neoplastic cells, possibly because of the presence in normal cells of high levels of decoy receptors or the caspase-8 antagonist FLICE-inhibitory protein (Tutt *et al*. 2002). In this context, co-administration of TRAIL has been shown to increase the lethal effects of conventional cytotoxic drugs in several cancers (Hedau *et al*. 2004) (Figure 1). Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) induces programmed cell death preferentially in tumour cells. TRAIL holds enormous promise as a cancer therapeutic due to its highly selective apoptosis-inducing action on neoplastic versus normal cells. Moreover, a recently published Phase I clinical trial revealed that recombinant TRAIL administration is safe and well tolerated. In addition, dose escalation achieved peak TRAIL serum concentrations equivalent to those associated with preclinical antitumour efficacy (Volate *et al.* 2010). However, to exploit the opportunity to successfully treat cancers with TRAIL, the problems of TRAIL resistance in a variety of tumour cells must first be overcome (Kasibhatla *et al.* 2003). To address this setback, the use of several natural compounds has been evaluated. Bexarotene, which is a retinoid specifically selective for retinoid X receptors works by stopping the growth of cancer cells. In exploiting a combinational therapy with bexarotene, Ying *et al*. (2007) reported that the effect of bexarotene on TRAIL-mediated programmed cell death involved proximal events of the extrinsic pathway. However, downstream signals involved the intrinsic pathway in leukemia cancer cells (Figure 1). In another study Park et al. (2010) observed that both DR5 and CHOP

upregulation were required for γ-T3-induced apoptosis and DR5 was transcriptionally regulated by CHOP after γ-T3 treatment. Furthermore, apigenin induced extrinsic apoptosis pathway, up-regulating the levels of cleaved caspase-8, and inducing the cleavage of poly (ADP-ribose) polymerase in a different study. However, apigenin did not seem to induce apoptosis via the intrinsic mitochondrial apoptosis pathway as shown by lack of impact on levels of B-cell lymphoma 2 (Bcl-2) and Bcl- 2-associated X protein (Seo *et al*. 2010).

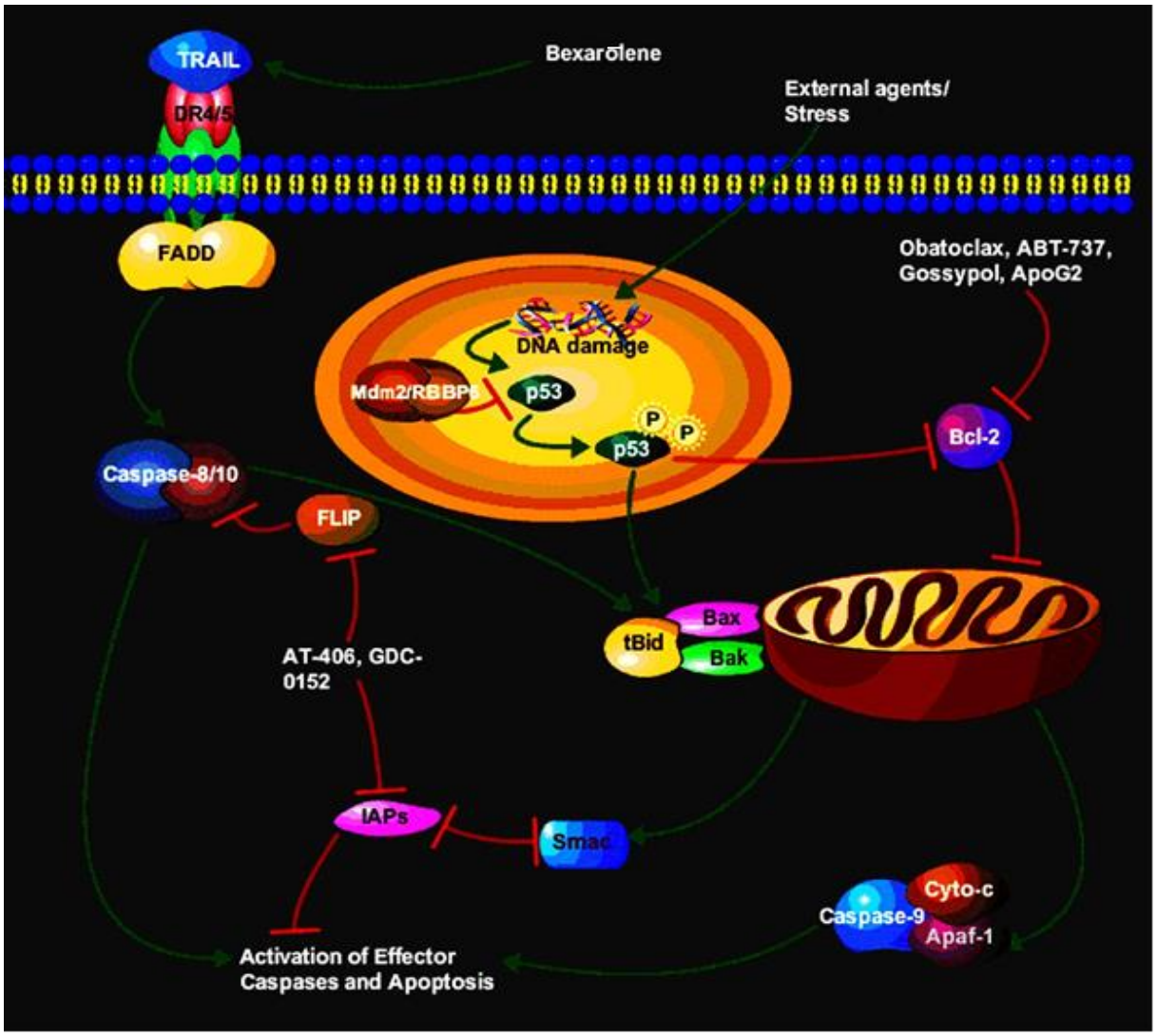


Figure 1 : Proposed steps of apoptosis induced by TRAIL ligand.

TRAIL ligands bind to and activate their receptors by inducing receptor trimerization. Activated receptors recruit adaptor molecules such as Fas-associating protein with death domain (FADD), which recruit procaspase 8 to the receptor complex, where it undergoes autocatalytic activation. Activated caspase 8 activates caspase 3 through two pathways; the complex one is that caspase 8 cleaves Bcl-2 interacting protein (Bid) and its COOH-terminal domain translocates to the mitochondria where it triggers cytochrome c release. The released cytochrome c binds to apoptosis protease activating factor-1 (Apaf-1) together with dATP and procaspase 9 and activates caspase 9. The cas-pase 9 cleaves procaspase 3 and activates caspase 3. In another pathway, p53 activation following external agents/stress results in the inhibition of pro-survival protein Bcl-2 and the activation of Bax that leads to activation of the mitochondria to release cyto-chrome c and caspases that end with apoptosis.

Moreover, apigenin reduced the tyrosine phosphorylation of HER2 (phospho-HER2 level) in MCF-7 HER2 cells, and upregulated the levels of p53, phospho-p53 and p21 in MCF-7 vec and MCF-7 HER2 cells (Seo *et al.* 2010). TRAIL receptor activating agents have been found to exhibit favourable *in vitro* and *in vivo* activity in the treatment of several malignancies, including breast and gynaecological cancers. Preclinical and early phase I studies have provided some support for the assumption that these novel agents are safe, with increased target specificity for malignant cells. When these targeted agents are combined with conventional chemotherapy drugs or radiotherapy, they appear to increase cell death over single agent modalities.

1.5.2. Small Molecules Targeting Intrinsic Pathway in Cancer

The mitochondrial pathway of apoptosis is the major mechanism of physiological cell death in vertebrates. In this pathway, proapoptotic members of the Bcl-2 family (contain only a BH3 domain) cause mitochondrial outer membrane permeabilization (MOMP), allowing the

release of cytochrome c, which interacts with Apaf-1 to trigger caspase activation and apoptosis. Activation of the intrinsic apoptotic pathway represents a major mechanism for breast cancer regression resulting from anti-estrogen therapy. The protein containing only BH3 domain is inducible by estrogen-starvation and anti-estrogen treatment and plays an important role in anti-estrogen induced apoptosis of breast cancer cells (Warr *et al*. 2008). In this section we present current targets of this pathway using small molecules of natural products. Recently, Jin *et al.* (2010), trying to exploit phytoestrogen to induce apoptosis in MCF-7, used daidzein which belongs to the isoflavone family, which is the most commonly ingested and most intensely studied type of phytoestrogen, often found in nuts, fruits, soybeans, and soy-based products. This study demonstrated that daidzein induces apoptosis through the generation of ROS that perturb mitochondrial function, leading to mitochondrial permeability, cytochrome c release, and caspase activation. They further reported the loss of the antiapoptotic mitochondrial Bcl-2 protein as partially responsible for the opening of the mitochondrial permeability transition pore (Jin *et al*. 2010).

A study by Kawiak *et al.* (2012) used plumbagin, which is a constituent of species of the plant genera *Drosera* and *Plumbago* that displays antineoplastic activity toward various cancers. They reported antiproliferative activity of plumbagin that was associated with apoptosis-mediated cell death, as revealed by caspase activation and an increase in the sub-G1 fraction of the cell cycle. Compound plumbagin increased the levels of the proapoptotic Bcl-2 family of proteins and decreased the level of the antiapoptotic Bcl-2 protein in SKBR3 and BT474 cells (Bender et al 2008). Thus, these findings indicated that plumbagin induces apoptosis in Her2-overexpressing breast cancers through the mitochondrial-mediated pathway and suggest its potential for further investigation for the treatment of Her2-overexpressing breast cancer (Figure 1). The mitochondrial pathway of apoptosis was further

shown to play a critical role in estradiol-induced apoptosis in long term estrogen-deprived breast cancer cells. Physiologic concentrations of estradiol could potentially be used to induce apoptosis and tumour regression in tumours that have developed resistance to aromatase inhibitors (Porter 2008). Plant-derived polyphenols inhibit cancer cell proliferation and induce apoptosis. Recently, prenylflavonoids and alkylphloroacetophenones have been reported for their *in vitro* antitumour activity (Satoh *et al.* 2001). Similarly, Cho *et al.* (2012) reported cytotoxic activity as a result of treating MCF -7 cell line with prenyl (3-PAP) and geranyl (3-GAP) derivatives of phloroacetophenone, and xanthohumol (XN), a prenylchalcone. In addition, proapoptotic Bax but not B-cell lymphoma 2 (Bcl-2) expression was increased by 3-GAP. In accordance with the Bax increase, 3-GAP induced mitochondrial cytochrome c release and activated caspase-9, an initiator of the caspase cascade. Currently there are several data that support or continue to show that small molecules, particularly the ones extracted from natural plants, present a supplementary and safer target in many cancers.

1.6. The Bcl-2 family

The Bcl-2 family of proteins are one of the primary regulators of the intrinsic pathway or the mitochondrial pathway. These proteins can be characterized into proapoptotic members: Bax, Bak, Bik, Bad and Bid, with the antiapoptotic members being Bcl-2 Mcl-1, Bcl-xl and Bcl-w. The proapoptotic members of this family work by promoting caspase activation and cell death. Some of them work by inactivating death inhibiting members of this family whilst others stimulate the release of cytochrome c from the mitochondria (Schimmer *et al*. 2008). The antiapoptotic members inhibit apoptosis by blocking release of cytochrome c from the mitochondria.

As our understanding of the field of apoptosis and its regulatory machinery evolves, it becomes important to identify and exploit new potential targets that restore proapoptotic

activities. The most promising recent strategies in the development of anticancer drugs involve targeting essential apoptotic genes and proteins directly to induce cell death. Members of the Bcl-2 family of proteins and IAPs have been shown to be very important regulators of caspases. Absence or impairment of caspase activation almost always leads to prevention of cell death. Novel strategies that will overcome caspase inhibition will be of great value in cancer therapeutics that aim to directly restore a tumour cell's apoptotic program. Hence, the development of small molecule agents that target IAPs and Bcl-2 appear to be an attractive therapeutic target.

The proapoptotic effectors such as Bax and Bak contain BH1, BH2, and BH3 domains, and a C-terminal transmembrane segment that selectively targets these proteins to the membranes of mitochondria and endoplasmic reticulum. The pro-survival (antiapoptotic) members Bcl-2, Mcl-1, Bcl-xl, Bcl-w contain a BH1, BH2, and BH3, but with the exception of Mcl-1, they also contain a BH4 domain. The last group, which are the proapoptotic members including Bid, Bad, Bik, Bim, Puma, and Noxa only have a BH3 domain (Fan *et al*. 2006) (Figure 2).

1.6.1. Regulation of apoptosis by the Bcl-2 Protein Family

The Bcl-2 family of proteins play a central role in the regulation of apoptosis. Whether it is the fate of a cell to undergo apoptosis is partly determined by the concentrations of pro- and anti- apoptotic members of the Bcl-2 family. Members of this family share sequence homology within conserved regions known as BCl-2 homology (BH) domains (Danial 2007). The proapoptotic effectors (BAX and BAK) and antiapoptotic (Bcl-2, Bcl-xl, Bcl-w and Mcl-1) members contain BH1, BH 2, and BH 3 domains. The domains are responsible for the formation of homodimers and heterodimers among members of the Bcl-2 family. In addition to the 3 domains, the antiapoptotic members contain a BH4 domain, with the exception of Mcl-1. This domain is involved in interactions with proteins outside of the Bcl-2 family. The

third group, the proapoptotic members (Bid, Bad, Bik, Bim, Puma, and Noxa) only have a BH3 domain (Wang *et al.* 1996). BH3 only proteins play a key role in promoting apoptosis. They sense apoptotic signals from a variety of insults, then translocate to the mitochondria. There they communicate with antiapoptotic and proapoptotic molecules of the BCl-2 family. This interplay eventually leads to activation of BAX and BAK in the mitochondria which will release apoptogenic factors such as cytochrome c resulting in apoptosis. The regulationof Bcl-2 family of protein is mainly controlled via the mitochondria. Bakwhich is located on the outer membrane of the mitochondria is prevented from forming a dimer with bax by bcl-2 xl which also resides on the surface of the mitochondria. Bax in the cytoplasm is also downregulated by bcl-2 through BH4 domain which prevents its translocation onto mitochondrial membrane. Following activation of p53, bcl-2 binds to p53 freeing bax that then translocates onto the mitochondrial membrane forming a dimer with bak that serves as a channel for cytochrome c release.

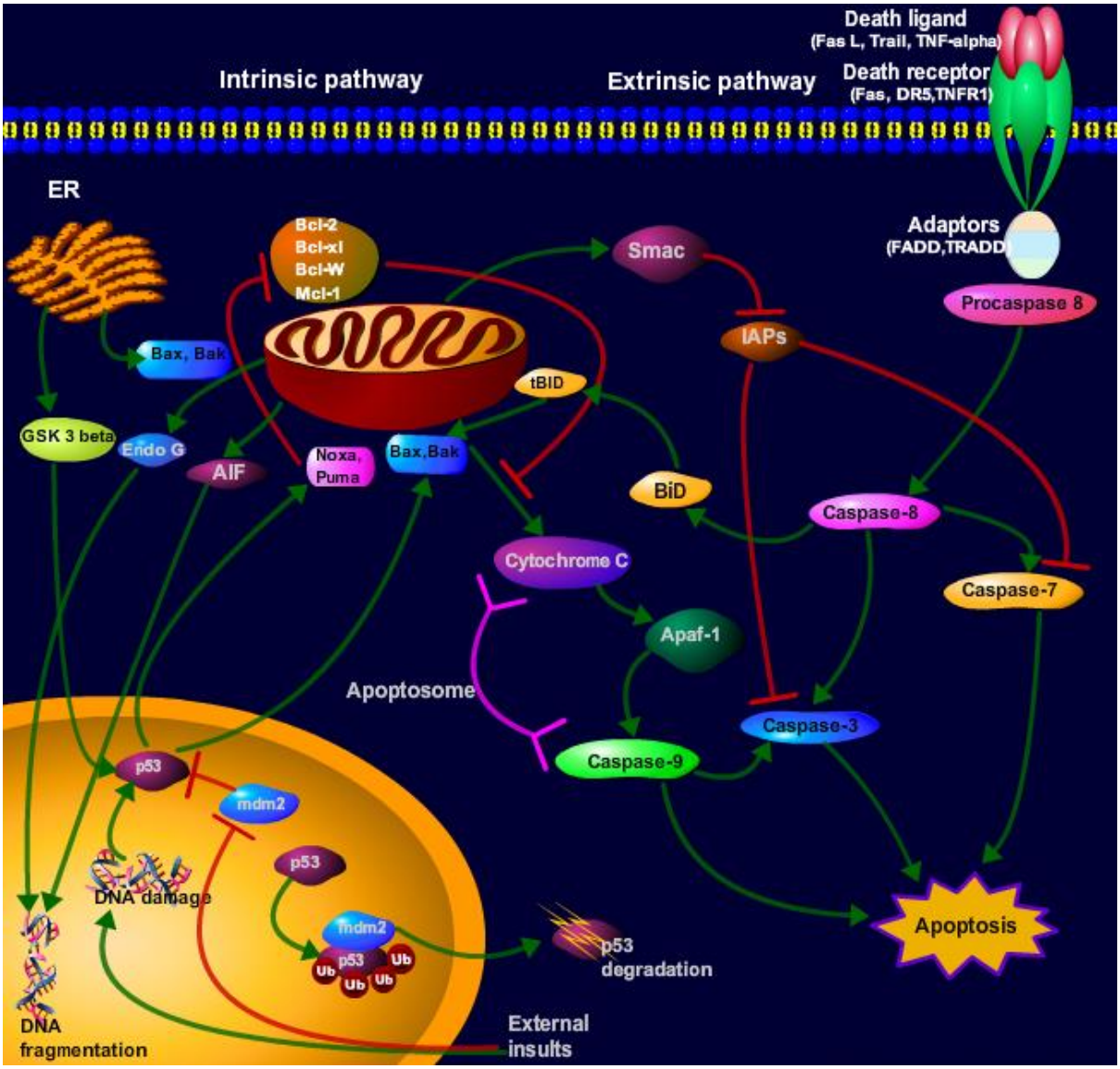


Figure 2 : A schematic representation of some major apoptotic signalling pathways.

Table 1 : Cancer current treatment strategies.

	Treatment	Target site	Limitations/Stage in research
1.	Chemotherapy	Whole body through blood system	Anemia and not specific
2.	Radiotherapy	High energy beam target individual cells	Skin problem/burn
3.	Hormonal therapy	Hormone replacement	Not all cancers are hormone
4.	**Targeted Therapies**	**Apoptosis**	**Mostly under improvement**
	a. Gossypol	Bcl-2 family	Still under clinical trials
	b. Apogossypolone	Bcl-2 family	Clinical trials
	c. Obatoclax	Bcl-2 inhibit pro-survival genes	Phase I
	***d. ABT*-737**	Bcl-2 and Bcl-xl	Clinical trials
	***e. AT*-406**	IAP molecule	Clinical trials
	***f. YM*155**	Survivin	Early clinical research
	***g. GDC*-0152**	cIAP1	Early clinical research
	h. I3C	BRCA1/2	Clinical trials
	i. Genistein	BRCA1/2	Clinical trials
	j. Tamoxifen	BRCA1/2 eostrogen	Clinical trials
	k. Iniparib	PARP	Clinical trials
	l. Cisplatin	Caspases	Inhibit IAP and activate caspases.
	m. Bexarotene	TRAIL	Preclinical research

A number of small molecules antagonists to pro-survival Bcl-2 have been developed and some are still undergoing clinical trials (Table 2). Some of these include:

1.6.1.1. Gossypol

Gossypol is a naturally occurring polyphenolic compound with BH3 mimetic property. It induces apoptosis in cells with high Bcl-2 expression. It was found to have a high affinity to multiple Bcl-2 family proteins. Thus it binds to and blocks the binding of antiapoptotic Bcl-2 to proapoptotic Bcl-2 family proteins (Powell *et al.* 2003). Gossypol has demonstrated anticancer activity against a wide range of malignancies including prostate cancer and human colorectal carcinoma (Alberts *et al.* 2002; Kishimoto *et al.* 2005). Gossypol is currently under clinical trials.

1.6.1.2. Apogossypolone (ApoG2)

Apogossypolone is a semi synthesized analogue of gossypol. It was created to reduce the toxicity of gossypol. Apogossypolone was designed by removing two aldehyde groups on polyphenolic rings of gossypol (King *et al.* 2001). It was due to these reactive two aldehyde groups that gossypol was thought to exhibit toxicity (Park *et al.* 2010). Similarly to gossypol, ApoG2 binds to Bcl-2 family proteins. In a study, ApoG2 was found to exhibit better stability and was also better tolerated by mice than gossypol. It showed significant inhibition to diffuse large-cell lymphoma, a type of non-Hodgkin's lymphoma, and was not toxic to normal peripheral blood lymphocytes (Peeters *et al*. 1994). Recently, a number of ApoG2 derivatives have been created such as Bl97D6. Bl97D6 inhibits binding of the BH3 peptide to the antiapoptotic Bcl-2 family proteins. It has been shown to be a potent inhibitor of human prostate cancer and human lung cancer (Imyanitov and Hanson 2004).

1.6.1.3. Obatoclax

Obatoclax is a small molecule novel drug designed to inhibit prosurvival Bcl-2 family proteins and thereby increase the cellular threshold for apoptosis in cancer cells. It acts as a BH3 mimetic and binds to prosurvival Bcl-2 proteins and neutralizes them (Cho *et al*.

20012). In a study, obatoclax showed significant cell viability reductions in human pancreatic cell lines (Cho *et al.* 20012). In phase I trials obatoclax exhibited antitumour activity in patients with refractory leukemia and myelodysplasia (Flygare *et al*. 2012).

1.6.1.4. ABT-737

ABT-737 is a small molecule inhibitor of the anti-apoptotic proteins Bcl- 2 and Bcl-xl but not Mcl-1. A study by Oltersdorf (2005) found that ABT-737 does not directly induce apoptosis but sensitizes cells to death signals, showing synergistic cytotoxicity with chemotherapeutic treatment and radiation. ABT-737 exhibits cytotoxicity against lymphoma, multiple myeloma and small-cell lung carcinoma lines (Herbst *et al.* 2010; Gartner *et al.* 2010).

1.7. Caspase in Cancer

Caspases are a class of cysteine-aspartyl proteases that are synthesized as inactive zymogens. They lie dormant in a healthy cell then due to cell death stimuli, they are proteolytically cleaved at two aspartate residues, generating mature active enzymes (Liggins *et al.* 2002; Huang *et al.* 2009). The generation of active caspases forms a cascade whereby initiator caspases interact with a specialized adapter molecule which results in its own activation and eventually activation of execution caspases which are responsible for the final pathway of apoptosis (Satoh *et al.* 2001, Liggins *et al.* 2002). Since all apoptotic pathways converge on caspases it renders them crucial effectors of apoptosis. Hence, it is very crucial that expression of caspases is tightly controlled. Deregulation of caspases has been implicated in a number of diseases. Over-expression has been linked to certain neurodegenerative diseases and ischemia-associated injury, whilst their suppression has been linked to a number of cancers.

1.7.1. Caspase-3 in Cancer

The role of caspase-3 in the response of cancer cells to chemotherapeutic drugs remains a controversial issue. The loss of caspase-3 expression as well as defaults in cytochrome c release from the mitochondria, which is required in most apoptotic pathways to activate caspase-3 through caspase-9 activation, are associated with multidrug resistance. Accordingly, expression of caspase-3 in the human MCF-7 breast tumour cell line (which is deficient for caspase-3) restores the apoptotic response to the topoisomerase II inhibitor, etoposide (Jin *et al.* 2010). Caspase-3 was also involved in breast cancer cell apoptosis upon exposure to anthracyclines and cisplatin (Erickson *et al.* 2012; Dean *et al.* 2007). Its role in tumour cell response to paclitaxel has been challenged (JunWei *et al*. 2011).

1.7.2. Initiator Caspases (8 and 9) in Cancer

The initiator caspases appear to display some specificity according to the type of apoptotic signal. Two main activation cascades for apoptosis induction have been described (Hougardy *et al*. 2005). Fas receptor-ligand interactions use caspase-8 activation to trigger the downstream executioner caspases. An alternative mitochondrial pathway, which is triggered by various anticancer agents, involves activation of caspase-9 upon recruitment to the mitochondria by cytochrome c and apoptosis protease activation factor-1(APAF-1) (Gartner *et al.* 2010). Mitomycin c which is one of the anticancer agents, led to an exclusive activation of caspase-8 but not of caspase-9 in comparison to various other drugs with differing mechanisms of action at concentrations formerly established as equitoxic in the NCI/ADR-RES cell line (Gartner *et al.* 2010). In contrast, Lee *et al*. (2003) using Styrylpyrone derivative (SPD) which is a pharmacologically active compound extracted from the plant *Goniothalamus* sp. of the Annonaceae family, have shown that procaspase-8 was not activated in MCF-7 cells but caspase-9 activation was detected in response to SPD treatment,

with the release of cytochrome c into the cytosol. In another study, prodigiosin which is a red pigment produced by *Serratia marcescens* with proapoptotic activity, was potently cytotoxic in both estrogen receptor positive (MCF-7) and negative (MDA-MB-231) breast cancer cell lines. It led to cytochrome c release, activation of caspases-9, -8 and -7 and cleavage of poly (ADPribose) polymerase protein typified the apoptotic event and caspase inhibition revealed that PG acts via the mitochondrial pathway (Zhou *et al.* 2012).

1.8. Inhibitors of apoptosis proteins (IAP)

The IAP's were discovered in baculoviruses. These proteins are known to function biochemically by suppressing the host's cell death response to a viral infection thereby allowing viral propagation in host insect cells (Gartner *et al.* 2010; Ambrosini *et al.* 1998). Several other cellular homologous IAPs were also identified in a range of species from *Drosophila* to vertebrates. Neuronal Apoptosis Inhibitory Protein (NAIP) was the first mammalian IAP to be identified during positional cloning in an effort to identify the causative gene for Spinal Muscular Atrophy (SMA) (Gartner *et al*. 2010). Other members of the mammalian inhibitor of apoptosis family of proteins, include the X-linked IAP (XIAP), cellular IAP 1 (cIAP1), cellular IAP II (cIAP2), melanoma IAP (ML- IAP), Survivin, Bruce, ILP-2 and Livin to name but a few. These are overexpressed in cancer cells thereby conferring cancer cell protection against a variety of proapoptotic stimuli by functionally regulating signal transduction pathways associated with malignancy (Balabhadrapathruni *et al.* 2000, Bodet *et al*. 2011).

Structurally the IAPs are similar, with one or more 70 - 80 amino acid baculovirus IAP repeat (BIR) domains with the core domain consisting of the variable sequence C(X)2C(X) 6W(X)3D(X)5H(X)6C with the X representing any amino acid (Herbst *et al*. 2010). A typical mammalian IAP family member contains a carboxy-terminal RING (Really Interesting New

Gene) zinc finger possessing a E3 ubiquitin ligase activity which directly regulates self-ubiquitination and protein degradation and the cellular IAP (cIAP 1 and cIAP 2) uniquely possesses the caspase recruitment domain (CARD) which mediates oligomerization with other CARD containing proteins. Although the mechanism of action is not known, the CARD in IAP may form protein-protein interactions with protease activating factor-1 (Apaf-1), and with some of the death domain (DD) or death effector domain (DED) containing proteins (Satoh *et al*. 2001) (Figure 2).

Table 2 : List of small molecule agents that target Bcl-2 and mode of action.

Small molecule	*Mechanism*	*Reference*
Gossypol	Binds to and blocks the binding of antiapoptotic Bcl-2 to pro-apoptotic Bcl-2 family proteins	Kitada, *et al*. 2003
Obatoclax	Acts as a BH3 mimetic and binds to prosurvival Bcl-2 proteins and neutralizes them	Huang, *et al.* 2009
Apogossypolone (ApoG2)	Binds to and blocks the binding of antiapoptotic Bcl-2 to pro-apoptotic Bcl-2 family proteins	Sun, *et al*. 2008
ABT-737	Inhibits the anti-apoptotic proteins Bcl-2 and Bcl-xl but not Mcl-1	Oltersdorf, *et al*. 2005

The fact that this family of proteins when overexpressed in human cells protects cells from stimuli that induce apoptosis provides the mechanism and way to explain treatment resistance in cancer cells (Bodet *et al.* 2011). One strategy to overcome this is to silence IAPs and this in turn can sensitize cells to apoptotic stimuli. Other efforts involve targeting the IAP proteins

by designing small molecules that can mimic the binding of the endogenous IAP antagonist second mitochondria-derived activator of caspases/direct IAP-binding protein, with low pI (Smac/ DIABLO) to a shallow groove on the surface of select IAP baculovirus repeat (BIR) domain, thereby inhibiting the inhibitor of apoptosis (IAP) (Gartner *et al.* 2010; Ambrosini *et al.* 1998).

1.8.1. IAP in Apoptosis and Cancer

Cancer cells thus tamper with the equilibrium of cell death and cell growth in order to survive. Apoptosis regulators such as IAPs play a fundamental role in oncogenesis because they suppress the intrinsic and extrinsic apoptosis pathway. This they do via the central mechanisms of IAP apoptotic suppression through direct caspase and pro-caspase inhibition (primarily caspase 3 and 7) in a cascade manner and modulation of and by the transcription factor NF-kappaB (Butler *et al*. 2000). In many cancers, IAP's are overexpressed or over activated; conferring cells an inability to die due to standard chemo resistance and radiation therapies. These in turn have a negative effect on normal healthy cells because DNA is damaged both chemically and physically (Gartner *et al*. 2010; Choudhuria *et al.* 2002). Novel yet effective and "safe" therapeutic targets are therefore required for the treatment of cancer. In the search for possible targeted markers that inhibit expression of IAPs, Smac/DIABLO was discovered in 2000, as a protein released from the mitochondria into the cytosol in response to apoptotic stimuli. It functions as an endogenous antagonist of several inhibitors of apoptosis proteins through direct binding. The interaction between Smac and IAPs involves the AVPI tetrapeptide binding motif on the N-terminus of Smac and a well-defined groove on the surface of these IAP proteins, providing an ideal site for the design of small-molecule Smac mimetics (Key *et al*. 2001; Kita *et al*. 2009). Potent and cell-permeable small-molecule Smac mimetics have provided powerful pharmacological tools for the study of the regulation

of apoptosis by IAP proteins. Several such compounds are now in early clinical trials as new anticancer agents. One focus has been on the generation of molecules that mimic the aminoterminus of mature Smac. AT-406, an orally-active, small molecule drug was designed to promote programmed cell death (apoptosis) in tumour cells by blocking the activity of IAPs thereby creating conditions in which apoptosis can proceed. AT-406 is therefore considered a multi-IAP antagonist (Cai *et al.* 2011).

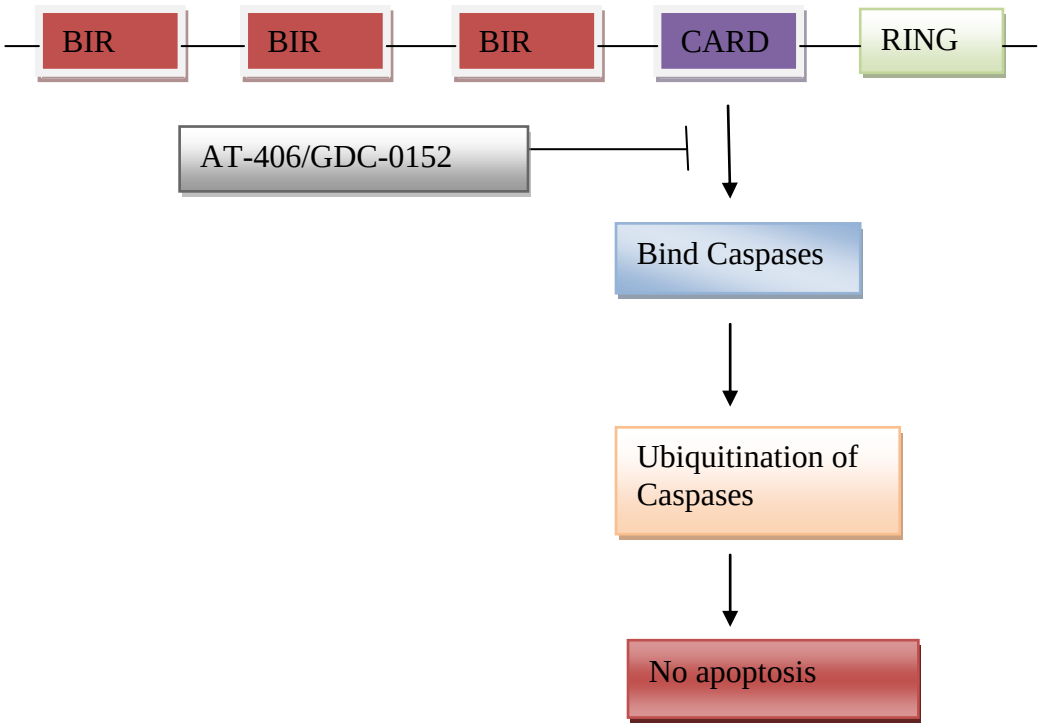


Figure 3 : Domain structure of IAP proteins.

Pro-survival proteins bear up to three domains BIR, CARD and RING. The BIR domain is necessary for IAP protein interaction with a number of proapoptotic factors, including invertebrate death inducers. Several small molecules have been identified that result in the inhibition of the binding of caspases to the CARD region on the IAP thereby resulting in activation of apoptosis.

IAPs are key components of the complex cascade of protein signaling that activates enzymes called caspases to initiate the breakdown of cancer cells (Kita *et al*. 2009; Cummings *et al*. 2002). AT-406 is thought to mimic the activity of Smac by binding to XIAP and preventing it from inhibiting caspase activation. Upon binding to cIAP1 and cIAP2, AT-406 induces rapid degradation of these proteins and promotes apoptosis through activation of the death-receptor complex and caspase-8 (Key *et al*. 2001). The other molecule, GDC-0152 has the best profile of these compounds: it binds to the XIAP BIR3 domain, the BIR domain of ML-IAP, and the BIR3 domains of cIAP1 and cIAP2. GDC-0152 achieves apoptosis induction by promoting degradation of cIAP1, inducing activation of caspase-3/7, and leads to decreased viability of breast cancer cells without affecting normal mammary epithelial cells (Kita *et al*. 2009). The second member of the IAP that has also been targeted is survivin. Survivin protein was found to be expressed in all of the most common human carcinomas but not in normal tissue. YM155, a small-molecule survivin suppressor resulted in suppression of survivin expression, caspase-3/7 activation, induction of spontaneous apoptosis, and growth inhibition. Similarly, valproic acid which is a broad-spectrum inhibitor of class I and II histone deacetylases and shows great anticancer activity in a variety of human cancers including breast cancer was found to significantly inhibit cell migration but not proliferation of human breast cancer MDA-MB-231 cells (Zhang *et al.* 2012). Ribonuclease expression was also found to promote apoptosis in human breast cells MDA-MB-231 by inhibiting the expression of Survivin and activating caspase-3 (Zhou *et al*. 2011). The novel findings of this review are that multiple IAPs are concomitantly expressed in breast cancers and in combination with clinically relevant IAP antagonists, promote apoptosis and reduce the cell turnover index of breast cancers.

1.9. The P53 Protein

P53, also known as guardian of the genome, plays a crucial role in maintaining genome integrity. It is one of the most studied tumour suppressors with a vital role in preventing cancer development. It has been proposed that it plays a role in cellular processes involved in DNA damage recognition and repair. Depending on the extent of DNA damage, p53 will either activate a cell cycle checkpoint, preventing the cells from advancing any further in the cycle until their DNA is repaired or if the damage is severe, p53 will cause the removal by apoptosis (Barnes and Camplejohn 1996).

P53 protein is normally present in low concentrations in normal, undamaged cells by virtue of its relatively short half life. This is mediated by Mdm2, an ubiquitin ligase which binds to p53 and decreases its stability through the ubiquitin-proteasome pathway, acting as its negative regulator (Fig 2 on page 19) (Momand *et al.* 1992). In response to DNA damage, p53 is rapidly activated by phosphorylation and no longer interacts with Mdm2. Phosphorylated p53 will act as a sequence specific DNA binding transcription factor which will activate or repress a number of target genes that mediate G1 or G2 cell cycle arrest, DNA repair, senescence, apoptosis or inhibition of metastasis (Harris and Levine 2005).

1.9.1. P53 in cancer

The p53 tumour suppressor gene prevents tumourigenesis in response to physiological and environmental stress. It plays a role in cell cycle progression, apoptosis and repair of DNA damage. It is involved in transcriptional regulation of proapoptotic genes associated with intrinsic and extrinsic pathways (Kawiak *et al.* 2012). Activated p53 increases the expression of p21 in DNA damaged cells and affects expression of p27 (Peeters *et al.* 1994). The upregulation of cyclin-dependent kinase inhibitors (CDKI), such as p21 and p27, is related to cell cycle arrest and contributes to downregulation of cyclin D1, cyclin E, cyclin-dependent

kinases (cdks) (von Haefen *et al*. 2003). The activation of p53 induces upregulation of proapoptotic Bax but not antiapoptotic Bcl-2. In addition, p53 mediated apoptosis involves the activation of Fas and the intrinsic mitochondrial pathway, which results in activation of caspase-8 and -9 (Figure 1 on page 14) (Altekruse *et al.* 2009). Thus, targeting p53 in cancer not only holds a promise for cancer eradication but also guarantees a safe elimination of those cells.

Many observations have implicated a p53 signaling pathway in the transcriptional activation of Bax in apoptosis as well as in the alteration of the Bcl-xL/Bax ratio, thereby indicating that the ratio between these pro and antiapoptotic proteins is one of the important factors deciding the fate of a cell (Hougardy *et al.* 2005; Gonzalez de Aguilar *et al.* 2000). Like many new plant extracts, the role of curcumin, a naturally occurring phytochemical responsible for the yellow colour of the commonly used spice turmeric, in regulating the balance between these pro and antiapoptotic factors was tested. The study revealed that curcumin induced apoptosis with an increase in p53 level in MCF-7 cell lines. Interestingly, the curcumin-induced increase in p53 expression precedes that of Bax thereby leading to the belief that p53 transactivates Bax expression (Hougardy *et al.* 2005). In the same study, the Bcl-xl levels remained almost unchanged thereby shifting the Bcl-xl/Bax ratio towards apoptosis. In recent times several small molecules have been identified, for example, Nutlin-3a and MI-219 that disrupt MDM2-p53 interaction resulting in inhibition of tumour growth. However they are less effective in cancer cells that express high levels of MDMX. Another molecule called benzofuroxan derivative, which is a MDMX inhibitor was found to activate p53, resulting in overexpression of proapoptotic genes (such as PUMA, BAX, and PIG3) in MCF-7. Importantly, this novel small-molecule p53 activator caused MCF-7 cells to undergo

apoptosis. It acted additively with Nutlin-3a to activate p53 and decrease the viability of cancer cells (Hunter *et al.* 2007).

1.10. Cell cycle and cancer

The cell cycle is a series of highly coordinated events that take place in a cell leading to its division, replication and eventually formation of two daughter cells. The cells grow at a steady pace during interphase. DNA synthesis only occurs at a portion of interphase. In eukaryotes, the cell cycle entails four distinct phases: The G_1 phase is the gap phase representing entry into the cell cycle, at this stage the cell is still growing but does not replicate. The G_1 is followed by the S-phase, which is where DNA replication takes place. Once DNA is synthesized, the G_2 phase follows, this is the second gap phase where cells grow rapidly and prepare to enter M phase. The M phase represents mitosis which is usually followed by cytokinesis or formation of two daughter cells (Caldon *et al.* 2006; Schwartz and Shah 2005).

Progression of cells through the cell cycle is an intricate process regulated by internal and extracellular signals which ensure that the various processes that take place during the different stages of the cell cycle occur in a coordinated manner. A catastrophic outcome would occur if cells proceeded to the next stage of the cell cycle before the previous step was properly completed. The different cellular processes including DNA replication, cell growth and mitosis require coordinated regulation and in eukaryotes, this is achieved by a series of cell cycle control checkpoints (Harashima *et al.* 2013).

The cell cycle control checkpoints ensure complete genomes are transmitted to daughter cells. The one major checkpoint arrests cells at G_2 in response to DNA damage or unreplicated DNA. G_2 arrest prevents cells from entering mitosis, it allows repair of double

stranded breaks. DNA damage can also arrest cells at G_1 checkpoint. G_1 arrest prevents cells from entering the S-phase, and allows damaged nucleotides to be repaired thereby fixing mutations in the genome.

At the heart of cell cycle control are a family of protein kinases called cyclin-dependent kinases (CDKs). They are controlled by an array of proteins and enzymes with the most important being cyclins. For the CDKs to be active, they need to be tightly bound to a cyclin, failure to bind to their respective cyclin renders them inactive. The negative regulation of CDKs is mediated by CDK inhibitors (Schwartz and Shah 2005). Depending on the stage of the cell cycle, , will be the determining factor for which particular CDK will be activated by its respective cyclin. In eukaryotes, there are three classes of cyclins, defined by the stage in the cell cycle where they bind their respective CDKs and their function. At G_1-phase, cyclin D is synthesized which forms complexes with Cdk4 and Cdk6; these commit the cell to DNA replication. Association between CDK2 and cyclin E drives the cell to the S-phase. At S-phase, cyclin A and cyclin E will interact with CDK2, driving the cell through the S-phase. Cyclin A-CDK1 and cyclin B-CDK1 complexes get the cells through the S-phase to mitosis transition (Schwartz and Shah 2005).

The most commonly mutated tumour suppressor associated with a number of cancers is p53. It functions in the G_1 checkpoint control to arrest cells with a damaged DNA and also contributes to G_2 arrest. Cells with mutated p53 gene do not arrest at G_1, allowing the damaged DNA to be replicated and passed on to daughter cells instead of being repaired. The inheritance of damaged DNA could lead to the accumulation of mutations leading to instability of the genome which could eventually lead to cancer.

p53 also functions as a transcription regulator of several genes, through which it can regulate events such as DNA synthesis. One of the genes whose transcription is regulated by p53 is p21(CIP1/WAF1). p21 is a potent cyclin-dependent kinase inhibitor which binds to and inhibits the activities of all mammalian Cdk-cyclin complexes. It thus functions as a regulator of cell cycle progression, causing arrest at G_1 and G_2 until DNA damage is repaired. If p53 is mutated, p21 will be unavailable to play its role as a stop signal for cell division (Agarwal *et al.* 1995).

1.11. New therapeutic agents

Breast and gynaecological cancers still remain a huge burden for women all over the world despite advances in treatment. Standard treatments usually involve surgery, chemotherapy and/or radiation either as single agents or in combination. Results, however, have been unsatisfactory due to patient relapse, development of drug resistance, adverse toxicity associated with treatments, or the high cost of treatment such as in HPV vaccines. Thus, there is a great need for the development of novel therapeutic interventions.

1.11.1. Anti angiogenetic agents

Angiogenesis is a complex process that plays an important role in the development of most types of cancer. The role of angiogenesis in cervical cancer seems related to HPV inhibition of p53 which is able to increase expression of vascular endothelial growth factor (VEGF) (Tomao *et al.* 2014). VEGF family proteins are the most potent proangiogenic factors and are associated with poor prognosis in many cancers. Activated VEGF promotes endothelial cell growth and migration. Blocking angiogenesis factors has been seen as a great potential for controlling cervical cancer growth. *Bevacizumab*, a recombinant humanized monoclonal antibody has been studied in a number of cancers including cervical cancer. *Bevacizumab* binds with high affinity and neutralises all isoforms of VEGF. Thus *bevacizumab* is able to

inhibit endothelial cell proliferation and vessel formation. It was the first angiogenic agent registered and is used extensively in the U.S.A. Bevacizumab has been successfully used in breast, ovarian, cervical, kidney, lung and brain cancers (Hurwitz *et al*. 2004; Sandler *et al.* 2006; Robert *et al.* 2011; Chinot 2011; Burger *et al*. 2011). An interplay between VEGF pathways and several growth factors, such as epidermal growth factor receptor (EGFR) and pathways involving receptor tyrosine kinases (RTKs) has also been implicated in tumouriginesis. Currently inhibitors of VEGF receptor tyrosine kinases such as *imatinib, pazopanib, sunitinib, sorafenib,* and *cediranib,* are at phase I and II clinical trials as treatment for cervical cancer. They are either used as single agents or in combination with cisplatin, carboplatin, paclitaxel or radiation (Vici *et al*. 2014).

1.11.2. Cancer vaccines

The recognition of HPV as the causative agent for cervical cancer created new research fronts in both the primary and secondary prevention of the disease. Subsequently, the development of an effective prophylactic vaccine against HPV was a major breakthrough as a primary preventative measure of cervical cancer (Franco *et al.* 2001).

Two preventative vaccines are currently approved in over 100 countries (Harper and Demars 2014). Gardasil and Cervarix are DNA-free virus-like particles assembled from recombinant HPV coat proteins. They are designed to elicit a strong humoral response with neutralizing antibodies. They have been shown to protect against the most prevalent oncogenic strains of HPV (HPV-16 and -18). These strains account for 70% of all cervical cancers Gardasil also protects against HPV-6 and -11, which are strains mostly associated with genital warts (Clifford *et al.* 2003). The high efficacy of the vaccines may dramatically decrease cervical cancer, preventing up to 70% of new cases in the future. Vaccination against HPV would offer a more efficient form of prevention especially in developing countries such as South

Africa where Pap screening programs have been largely ineffective. In South Africa, where cervical cancer is the leading cause of cancer-related death amongst women, the national Department of Health introduced the roll-out of the HPV vaccines targeting adolescents in 2014 (Richter *et al.* 2014).

A major limitation to these preventative vaccines is that they do not have a therapeutic effect. Once a person has an HPV infection, there is no way of eradicating the persistent infection before it develops pre-cancerous lesions. Currently large numbers of women already have established HPV infections which have not been treated mostly because they have not been detected and these are slowly progressing to malignancy phenotype. Even though mass vaccination with the HPV vaccines could be carried out worldwide, only in approximately 20 years will a significant reduction in cervical cancer incidence and prevalence be observed due to the slow development of the disease. Hence the importance of also developing therapeutic vaccines to control HPV-associated disease.

A promising avenue of clinical research in breast cancer is the identification of tumour-associated antigens that can be therapeutically targeted. In breast cancer, the most studied tumour-associated antigen is HER-2/neu (HER2). Breast cancers highly express Nelipepimut-S which is an immunogenic peptide from the HER2 protein. A peptide based vaccine NeuVax, has been developed to prevent or delay the recurrence of breast cancer in cancer survivors who are in remission following treatment with conventional therapies such as surgery, chemotherapy and radiation. NeuVax is the immunodominant nonapeptide derived from the extracellular domain of the HER2 protein, a well-established target for therapeutic intervention in breast carcinoma. NeuVax works by harnessing the patient's own immune system to seek out and attack cancer cells that express HER2/neu. Phase II clinical trials have

shown Neuvax to be safe and effective in stimulating HER2-specific immunity (Mittendorf *et al*. 2012).

Lynparza (olaparib), a new drug treatment for women with advanced ovarian cancer associated with defective BRCA genes, has recently been approved by the U.S. Food and Drug Administration. Lynparza is a poly ADP-ribose polymerase (PARP) inhibitor that blocks enzymes involved in repairing damaged DNA so as to preferentially kill cancer cells. It is the first PARP inhibitor to be approved for patients with platinum-sensitive relapsed BRCA-mutated ovarian cancer. Its approval was based on data from phase II clinical trials which evaluated its efficacy and safety and it was shown to significantly prolong progression free survival. Only mild to moderate symptoms including nausea, fatigue, vomiting and anaemia were associated with its use (Lederman *et al*. 2014).

1.11.3. Drugs derived from natural products

Since prehistoric times, in search of a cure against various ailments, humans have looked for drugs in nature. There is fossil evidence that suggests usage of plants as medicine dating back to the Middle Paleolithic age some 60,000 years ago (Solecki and Shanidar IV 1975). The struggle against illness led humans to seek medicine in roots, seeds, fruiting bodies, leaves, barks and other parts of plants. Developing countries have relied heavily on traditional medicine as a source of primary healthcare. However, in the 21st century, there has been a tremendous expansion in the use of traditional medicine including in those countries where modern conventional medicine predominates. The huge interest in medicinal plants may stem from various factors such as: (i) The toxicity associated with conventional medicine; natural products are perceived to be healthier, (ii) Plant based products are seen as eco-friendly and bio friendly and (iii) Development of multiple drug resistance, hence the need for new therapeutic agents (Gijtenbeek *et al*. 1999). This renewed interest in traditional medicine has

led to an increase in scientific studies that aim to explore some of the curative properties associated with medicinal plants.

Acknowledgement of the active elements of plants by contemporary science has allowed for their inclusion in pharmacotherapy as a source of modern drugs. In the current quest for plants as therapeutic agents, plants are either a) used as a whole or part as herbal remedy. For example, Echinacea and ginkgo biloba, b) used to isolate bioactive compounds that will be directly used as drugs e.g. taxol and vinblastine or c) used to produce bioactive compounds of known or novel structures as lead compounds for semisynthesis to compounds with higher activity or lower toxicity for e.g, teniposide and verapamil (Fabricant and Farnsworth 2001).

Medicinal plants have exhibited potent antibacterial, antifungal, antiviral and anticancer activities. Currently, up to 50% of the world's drugs are made from natural products or their derivatives (Gurib-Fakim 2006). Some of these drugs include penicillin, aspirin and quinine (Singh and Barrett 2006). Penicillin, one of the first and still a widely used antibiotic, was derived from *Penicillium* fungi. It is effective against bacterial infections caused by staphylococci and streptococci bacteria. Aspirin (Laich *et al.* 2002), extracted from white willow bark, is used as a blood thinner and to relieve inflammation, pain and fever (Jane and Botting 2003). Quinine on the other hand is used for its antimalarial, painkilling and anti-inflammatory properties and was derived from the bark of the cinchona tree (Iwu *et al.* 1999).

Thus far, a number of plant derived anticancer drugs have been approved by the Food and Drug Administration (FDA). Taxol, a diterpene alkaloid, was first discovered and isolated in the 1960s from the bark of a tree, *Taxus brevifolia.* It is now the first drug of choice in the treatment against metastatic breast and ovarian and lung cancer in most countries around the world (Weaver 2014). Topotecan is an alkaloid isolated from the leaves, bark and fruit of the

Camptotheca acuminata tree. It has been approved for the treatment of ovarian and lung cancers (Pizzolato & Saltz 2003). Etoposide and teniposide extracted from Podophyllum peltatum are used to treat leukemia, ovarian cancer, Hodgkin's disease, brain cancer and bladder tumours (Giri and Narasu 2000). Vinca alkaloids, including vinblastine, vincristine and vindesine, were discovered in the plant *Catharanthus roseus*. They are effective against many forms of leukemia, testicular, breast and ovarian cancer (Aniszewski 2007; Hait *et al.* 2007).

Plants have provided humans with important lifesaving pharmaceutical agents. A number of drugs derived from plants are now being used in the pharmaceutical industry whilst some are still undergoing clinical trials to test for their biological activity and safety. However, a huge proportion of higher plants have not been screened for biologically active compounds, hence it is imperative that in drug discovery plants remain at the forefront as an essential component in the search for new therapeutics.

1.12 Euphorbia tirucalli

South Africa has a huge flora biodiversity of which about 3689 plants are used as medicinal plants, of which 350 are commonly used and traded(Cherry 2005; Van Wyk *et al.* 2008). However, very few of these have been scientifically tested for safety and biological activity. The *Euphorbia* genus belongs to the Euphorbiaceae family. The Euphorbiaceae family has over 2000 species of which over 200 can be found in South Africa alone. The Euphorbiaceae family have a long history of usage as traditional medicine in Africa. The Euphorbia species has been widely used traditionally in the treatment of stomach, uterus, liver and kidney cancer (Sayed 1980).

Euphorbia tirucalli, also known as pencil plant, milkbush or kraalmelkbos, is a many-branched, succulent plant, which can grow up to ten metres long. This plant which can be found in Rwanda, Senegal, Angola, Ethiopia, Mauritius, Sudan, Kenya, Malawi, Tanzania, Uganda and Zanzibar originated from East Africa. It is abundant in the warmer regions of South Africa including Kwa Zulu Natal and Eastern Cape. *Euphorbia tirucalli* is able to grow under conditions in which most crops are unable to grow such as arid tropical areas with low rainfall, on poor eroded soils and at high altitudes (Van Damme 2001). *Euphorbia tirucalli* has traditional medicine use in many cultures.

In Malaysia, the root scrapings are taken for treatment of stomachache when mixed with coconut oil. In East Africa, the latex is used to treat hemorrhoids, snake bites, sexual impotence, epilepsy, toothache, warts, for extracting ecto-parasites, and cough among others (Van Damme 1989). In India, it is mostly used for spleen enlargement, for the treatment of, dyspepsia, bladder stones, asthma, dropsy, jaundice, colic, tumors, leprosy, biliousness and leucorrhoea. In small doses, the latex is used for, rheumatism, cough, toothaches, warts, earaches neuralgia, and scorpion bites (Kumar 1999). *Euphorbia tirucalli* is used against cancer, cancroids, epitheliomas, sarcomas, tumors, and warts in Brazil (Parekh *et al*. 2005; Schmelzer and Gurib-Fakim 2008).

Rationale

The aim of this study is to investigate the antitumour properties of the indigenous South African traditional plant *Euphorbia tirucalli* extracts on breast, ovarian and cervical cancer cell lines and to further identify secondary metabolites from crude plant extracts that show anticancer activity.

Study objectives

- To evaluate the anticancer activity of crude plant extracts of *Euphorbia tirucalli* against human breast, cervical and ovarian cancer cell lines.
- To determine if the plant extracts induce apoptosis in the cancer cell lines.
- To determine the effect the apoptotic inducing extracts have on expression levels of apoptotic genes
- To identify secondary metabolites from crude plant extracts that show anti-cancer activity.

CHAPTER 2 : MATERIALS AND METHODS

2.1. Materials

2.1.1. Plant collection

Fresh *Euphorbia tirucalli* plants were collected at the Limpopo Mankweng Township in February 2012. Identification of the bioactive compounds was conducted at the Biochemistry department, University of Limpopo and University of the Fort Hare. The anti-proliferative assays were performed in the department of Biochemistry at the University of the Witwatersrand, South Africa.

2.1.2. Plant storage

Due to fewer complications when working with dried plant material, stems were washed with water and cut into smaller parts. These were then evenly spread out and dried at room temperature under a stream of cold air for a week. When completely dried, the plants were ground to a fine powder using a pestle and mortar for maximum area coverage for the extraction process. Powdered plants were stored in properly sealed airtight glass jars in a dry and dark environment at room temperature until required.

2.1.3. Extraction procedure

All plant material was individually extracted by weighing 10 g of finely ground plant material and exhaustively extracting in 100 ml of the appropriate solvent (10:1 solvent to dry weight ratio) in 250 ml schott bottles. The mixture was shaken vigorously at high speed with a Labotec model 20.2 shaking machine for 24 hours. The mixture was then centrifuged at 5000 rpm for 5 minutes followed by filtering through Whatman No. 1 filter paper into pre-weighed labelled containers. The process was repeated twice and the resulting supernatant was combined. The extracts were dried under a stream of cold air at room temperature and the

quantity extracted per solvent was measured. The extraction solvents used were n-hexane, dichloromethane, chloroform, ethyl acetate, acetone, methanol and butanol (technical grade, Merck). The dried extracts were re-dissolved in dimethyl sulphoxide (DMSO) to give a stock concentration of 100 mg/ml. The quantified extracts were stored at -20°C until required.

2.2. Thin layer chromatography

Chemical constituents of the plants crude extracts were analysed by thin layer chromatography (TLC) (Merck Silica gel 60 F_{254}, 0.25 mm thick) using aluminium backed silica-gel coated plates (Sigma-Aldrich SA). TLC plates were loaded with 100 µg (10 µl of a 10 mg/ml extract in acetone) and were developed with the following mobile phases:

- Ethyl acetate/methanol/water = (EMW) 40:5,4:5 (v/v/v)

  polar/neutral
- Chloroform/ethyl acetate/formic acid = (CEF) 5:4:1 (v/v/v)

  intermediate polarity/ acidic
- Benzene/ethanol/ammonium hydroxide = (BEA) 90:10:1 (v/v/v)

  Non polar/basic

The chromatographs were developed in an enclosed tank saturated with the eluent vapour. Once dried the chromatographs were visualised under ultraviolet light at 254nm and 365 nm for compounds which are fluorescent. For detection and identification of separated chemical compounds not visible under UV light, vanillin spray reagent was used. Vanillin is composed of 0,1g vanillin, 28ml methanol and 1ml sulphuric acid. Once sprayed with vanillin, the plates were heated at 100°C for five minutes to allow optimal colour development.

2.3. Qualitative antioxidant activity of extracts

The qualitative antioxidant activity of the selected plants was tested using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma) method. Thin layer chromatographic plates were prepared as described (Section 2.4). To determine the antioxidant activity, the plates were sprayed with 0.2% DPPH in methanol and dried in the fumehood. A positive reaction for presence of antioxidants was detected as yellow bands against a purple background.

2.4. High Perfomance Liquid Chromatography

High Perfomance Liquid Chromatography (HPLC) is a technique used to separate compounds based on their polarity. It can be used for analyzing, purifying and quantifying compounds in a mixture. An Agilent 1260 binary pump (Agilent Technologies, Santa Clara, CA, USA) equipped with an injector and column coupled to a hybrid triple quadrupole mass spectrometer, TripleTOF 5600 (AB SCIEX, Concord, ON) was used. The experiment was carried out using Phenomenex C-18 silica (250 x 10mm, 5 micron) thermostated at 40°C as a stationary phase. The mobile phase started with 10% acetonitrile, 90% water containing 10 mM formic acid at a flow rate of 0.2 ml/min. The solvent ratio was changed through a linear gradient to 90% acetonitrile in 10% aqueous formic acid at 40 min. This ratio was maintained for 10 min after which the solvent ratio was changed back to the initial conditions.

2.5. Isolation and characterazation of Isorhamnetin

The subfraction 8 from the total extract was selected for purification based on the TLC profile. The subfraction had some spots on the TLC profile that were positive to vanillin-sulphuric acid and UV visible. The subfractions were subjected to silica gel packed chromatography. In brief, the chromatography was prepared as follows: silica gel was suspended in water and stirred gently until the formation of a slurry. This slurry was then poured carefully into previously cleaned, uprightly fitted; glass-wool sealed open glass

column (2.5cm inner diameter and 75cm length) till it is about three-fourths filled, with tapping the walls with a cushioned rod in order to avoid air-bubbles. 10g of methanol extract subfraction of the plant sample was subjected to silica packed column chromatography with 1% DCM: MeOH(1 1), 3% (1 1), 5% (1 1), 7% (1 1) and 10% solvent system. This was followed by DCM: MeOH: H_2O in a ratio 88:12:1 (1 1), 86:14:3 (1 1) and 85:15:3 (2 1) respectively. Several fractions were eluted depending on the visible changes in the colorful bands running out of the column and collected in dry glass bottles.

2.6. Cell lines and cell culture maintenance

2.6.1. Cell lines

In this study, the following cell lines were used to test for the cytotoxicity of *Euphorbia tirucali*: MCF-7 breast adenocarcinoma, MDA-MB-231 breast adenocarcinoma, SiHa cervical squamous cell carcinoma, RMG-1 ovarian clear cell carcinoma and MRC-5 normal lung fibroblast.

2.6.2. Cell culture expansion

MCF-7, MDA-MB-231, SiHa, RMG-1 and MRC-5 cell lines were routinely cultured in 25cm^2 tissue culture flasks with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (pen/strep). The media was stored at 4ºC and pre-warmed to 37ºC before use. The cells were grown up to 70-80% confluency before any experiment could be conducted on them. The cells were incubated at 37ºC, 95% humidity and 5% CO_2. The media, FBS and pen/strep were all purchased from Sigma.

2.6.3. Trypsinization

Confluent cells were washed twice with Phosphate Buffered Saline (PBS) and trypsinized (Trypsin-EDTA, Sigma) for 2-5 minutes. Supplemented media was used to deactivate the trypsin. Cells were either split into 2 flasks or frozen for future use in DMEM supplemented with 20% FBS and 10% DMSO.

2.7. Cytotoxicity by real time cell analysis

The *xCELLigence* system offers continual label-free and non-invasive analysis of cell proliferation, migration and invasion over time at a cell culture level. The RTCA measures changes in cellular behaviour using electrical impendance. The design of each well is such that 80% of the bottom is covered by microelectrodes. When a small voltage is applied to these wells impendance can be measured.

For this research, experiments were carried out using *xCELLigence* RTCA DP analyzer (Roche) which accommodates up to three E-plates (Roche Diagnostics GmbH). This system is composed of the following main components: impendance based real time cell analyzer (RTCA), RTCA control unit and disposable E-plate 16. The RTCA DP instrument was placed in a humidified incubator at 37°C and 5%CO_2. The gold microelectrodes were attached to the bottom to detect impendance changes expressed as Cell Index (CI) measurement. To each well, 100 µl of growth medium was added. After leaving the devices at room temperature for 30 minutes, the background impendance for each well was measured. Cells were harvested at an exponential growth phase using 0.05% trypsin-EDTA (Sigma) and counted using a haemocytometer. For each cell line, pilot experiments were conducted to determine cell numbers that allowed optimum growth for the entire incubation period. Ninety microlitres of the cell suspension was seeded into wells (40 000, 20 000, 10 000, 5 000, 2 500, 1 250 and 625 cells). For cytotoxicity assays, 90 µl cell suspension containing either

$1x10^4$ MCF-7, $2,5x10^4$ MDA-MB 231, SiHa, RMG-1 or MRC-5 cells was seeded into the wells. After leaving cells at room temperature for 30 minutes to allow for cell attachment, plates were transferred to the RTCA DP device and the CI was monitored by *xCELLigence* system. Twenty one hours after seeding cells, cells were treated over a period of 48 hours with different plant extracts at various concentrations (10, 30, 50 and 100 µg/ml dissolved in DMEM). Two negative control wells were included: Cells in DMEM and cells in DMSO dissolved in DMEM to a final concentration of 0,1%. Staurosporine at 0.1µM or taxol at 0.5µM or 2.5µM was added to positive control wells. Each concentration, including controls, was tested in duplicate within the same experiment. CI was monitored every 15 minutes and data was recorded by the supplied RTCA software.

2.8. Cytotoxicity by MTT assay

The MTT assay is a simple colorimetric assay used to measure cell cytotoxicity, proliferation or viability. This assay involves the ability of live cells to convert 3-4,5-dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromide (MTT) to produce a dark blue insoluble formazan. The resulting formazan crystals are dissolved in an organic solvent such as dimethyl sulfoxide (DMSO). The optical density of the solution can be measured on a multiwell spectrophotometer (Mosmann, 1983).

To determine the cytotoxicity of *E. tirucalli* extracts on the breast cancer cells, MTT assay was conducted. MCF-7 and MDA-MB 231 cells (5×10^3) were seeded into 96-well microtiter plates (F-Bottom, Greiner Bio-One) in volumes of 90 µl medium per well and then incubated overnight. Cells were treated with varying concentrations of *E. tirucalli* extracts (10, 30, 50 and 100 µg/ ml, w/v) for 24 hours. The MTT solution, prepared at 5 mg/ml in sterile PBS and filtered through a 0, 22 µm filter (Millipore, Bedford, MA), was added to each well at 10% v/v and incubated at 37ºC for 4 hours. To each well, 90 µl of DMSO was

added to ensure total solubility of formazan crystals and absorbance was recorded at 570 nm using the Multiskan^{TM} Go microplate reader. Each plate contained a blank, vehicle control, positive control, treated and untreated samples. Vehicle control cultures received DMSO (0,1%) alone. Cells treated with staurosporine at 0.1 µM served as a positive control.

Percentage Cell Viability was calculated as follows:

$$\%\ Cell\ viability = \frac{Absorbance\ of\ treated\ cells - Absorbamce\ of\ blank}{Absorbance\ of\ untreated\ cells - Absorbance\ of\ blank} \times 100$$

2.9. Annexin V/PI apoptosis assay

Cells undergoing apoptosis display certain typical morphological and physical alterations which can be measured using a flow cytometer. One of the major hallmarks of apoptosis is loss of membrane phospholipid asymmetry which leads to exposure of phosphatidylserine externally (Fadok *et al*. 1992). To quantify apoptotic cells, staining with fluorescein isothiocyanate (FITC) labelled Annexin V was conducted, Annexin-V detects the translocation of phosphatidylserine residues from the inner to the surface of the plasma membrane. To distinguish between apoptotic, viable and necrotic cell death, double staining with Annexin V-FITC and propidium iodide (PI) was used. PI detects necrotic cells with permeabilized plasma membrane. The test would thus discriminate PI positive (necrotic), FITC positive/ PI negative (early apoptotic). FITC positive/PI positive (late apoptotic) and FITC negative/PI negative (live) cells.

This technique was employed to determine the type of cell death *E. tirucalli* extracts were inducing on the cancer cell lines. Cells at 70-80% confluency in 25 cm^2 tissue culture flasks were treated with plant extracts at IC_{50} for 24 hours. Following treatment, both adherent and detached cells were collected and rinsed twice with cold PBS. The cell pellet was

resuspended in 500 µl of annexin binding buffer and incubated with 5 µl of Annexin V-FITC (Abcam) and 5 µl propidium iodide for 5 minutes. The cells were immediately analysed by flow cytometry using BD Accuri C6.

2.10. Cell cycle analysis by flow cytometry

This technique based on DNA content, can distinguish the proportion of cells that are in one of the three interphase cell cycle stages. Cells at G_2/M stage have about twice the DNA content of cells at G_0/G_1, whilst cells at S-phase have DNA content that lies between the two extremes. Propidium iodide (PI) is a red fluorescent dye which binds to DNA by intercalating between bases and stains both DNA and RNA. For specific DNA staining, PI is used in conjuction with ribonuclease RNAse for enzymatic removal of RNA. In this way, the more DNA cells have, the more dye they will uptake and hence fluoresce more brightly.

To determine if the plant extracts were inducing cell cycle arrest in the cancer cells, cell cycle analysis was performed using FxCycle™ PI/RNase Staining Solution (Life Technologies/ Thermo Fisher Scientific). Cells were treated with plant extracts at IC_{50} for 24 hours, followed by collection of both attached and detached cells. Pellet was rinsed twice with cold PBS and fixed in 70% ice-cold ethanol overnight at 20ºC. Fixed cells were then washed twice with PBS and DNA was stained with 500µl PI/RNase staining solution and incubated in the dark for 30 minutes. Flow cytometry analysis was carried out using BD Accuri C6.

2.11. Caspase 3/7 activity

Caspases are a family of cysteine proteases that play a key effector role in apoptosis. Initiator caspases cleave inactive preforms of effector caspases (including caspase 3 and -7) thereby activating them (Satoh *et al.* 2001, Liggins *et al.* 2002). Active effector caspases activate endonucleases and cleave substrates such as PARP, which then leads to the morphological

changes associated with apoptosis. The Caspase-Glo® 3/7 Assay is a luminescent assay that measures caspase-3 and -7 activities in purified enzyme preparations or cultures of adherent or suspension cells. The assay provides a proluminescent caspase-3/7 substrate, which contains the tetrapeptide sequence DEVD. This substrate is cleaved to release aminoluciferin, a substrate of luciferase used to generate a luminescent signal.

Caspase activity was assayed by measuring light intensity using Caspase-Glo® 3/7 (Promega) according to the manufacturer's protocol. Briefly, cells ($5x10^4$ cells/ well) were seeded on a white 96-well microplate and treated with plant extracts at IC_{50} for 24 hours. Caspase-Glo reagent was then added and incubated at room temperature for 30 min. The caspase activities were measured using the Glomax®-96 microplate luminometer. The assay was performed in triplicates.The results were presented as mean of Relative Light Units (RLU).

2.12. Total Ribonucleic Acid Extraction

Following 24 hour treatment of cells with the plant extracts, Total RNA was isolated using Quick RNA™ Miniprep kit (Zymoresearch) as per manufacturer's specifications. Briefly, cells were lysed directly in culture by removing liquid medium and adding 300 µl RNA lysis buffer directly to the cell monolayer. The lysate was transferred to a 1, 5 ml eppendorf tube and centrifuged at 10 000 x g for 1 minute to clear lysate. A Spin-Away Filter and collection tube were assembled as described in the manufacturer's manual. The resultant supernatant was transferred into the Spin-Away Filter-assemble and centrifuged at 10 000 x g for 1 minute. The resultant flow through was mixed with 100% ethanol at 1:1 ratio. A Zymo-Spin IIICG column and collection tube were assembled and the entire sample was transferred into the Zymo-Spin IIICG column-assembley followed by centrifugation at 10 000 x g for 30 seconds. The flow through was discarded and the Zymo-Spin IIICG column and collection

Tube were re-assembled. The High Zymo-Spin IIICG + collection tube were washed with 400 µl RNA Wash Buffer and centrifuged at 10 000 x g for 30 seconds, flow through was discarded. The Zymo-Spin IIICG + collection tube were incubated with a mixture of DNAse 1 and DNAse incubation buffer for 15 minutes followed by centrifugation at 10 000 x g for 30 seconds. The Zymo-Spin IIICG-assemble was washed with 400 µl RNA Prep Buffer and centrifuged 10 000 x g for 30 seconds. The flow through was discarded. The second wash was carried out with 700 µl RNA Wash Buffer and centrifuged 10 000 x g for 30 seconds. A third wash was performed with 400 µl RNA Wash Buffer and centrifuged at 10 000 x g for 2 minutes. The Zymo-Spin IIICG column was transferred into a sterile 1.5 ml micro centrifuge tube. Then 40 µl of DNase/RNase-Free water was added directly to the matrix of the column and centrifuged at 14 000 x g for 30 seconds for elution of total RNA. The total RNA was quantified using a nanodrop (NanoDrop technologies, USA), reading was taken at 260A. The A260/A280 ratio of ~2.0 was regarded as pure. The remaining RNA was stored at 70 ºC for future use.

2.12.1. Reverse Transcription

To conduct Real time PCR, mRNA was first transcribed to cDNA. cDNA was reverse transcribed using the ImProm-II Reverse Transcription System (Promega) according to the manufacturer's specifications. The kit uses the ability of reverse transcriptase to synthesize a new cDNA strand at the site determined by the sequence specific primers.

Reserve transcription was performed as per manufacturer's instructions. Briefly, 1µg of total RNA and 0.5 µg/reaction oligo $(dT)_{15}$ primers (Table 2.1) were mixed in sterile reaction tubes and heated to 70ºC for 5 minutes to allow for denaturation and primer annealing. These were immediately chilled in ice–water for 5 minutes. These were then mixed with the rest of the reverse transcription components as referred to in Table 4 and mixed briefly. The samples

were then incubated at 25ºC for 10 minutes for annealing followed by reverse transcription at 42ºC for 60 minutes. For inactivation of reverse transcriptase, samples were incubated at 70ºC for 15 minutes. The Multigene Gradient Thermal cycler system (Labnet International, Inc) was used for reverse transcription reaction.

Table 3 : Target RNA preparation for Reverse transcription

Total RNA	1 µg
Oligo $(dT)_{15}$	0.5 µg
Nuclease Free-water	5 µl
Final volume	5 µl

Table 4 : Cocktail for reverse transcription mix

Reagent	**Volume**	**Final concentration**
10X reaction buffer	4.0 µl	1X
25 mM Magnesium Chloride	5.0 µl	5mM
Deoxyribonucleotide mix	1.0 µl	0.5mM
RNase inhibitor	0.5 µl	20u
AMV reverse transcriptase	1.0 µl	1u
sdH_2O		To a final volume of 15µl
Total volume	**15 µl**	

2.12.2. Real time PCR using SYBR Green

There are three methods used for quantifying RNA, these include: Northern blot analysis and RNase protection assay and RT-PCR (reverse transcription-PCR). Compared to the other two, RT-PCR is the most sensitive technique for detecting mRNA levels since it can be used to amplify RNA from very small samples. Added advantages of RT-PCR include: The efficiency of the reaction can be precisely calculated, there is also no need to run the PCR product out on a gel after the reaction and it can be used to perform truly quantitative analysis of gene expression. Real time PCR is based on the principle of PCR where DNA is amplified in a tube using a thermophilic DNA polymerase. Real time PCR has the added advantage of being able to detect the amount of DNA formed after each cycle with fluorescent dyes. The intensity of the fluorescence emitted during RT-PCR correlates to the amount of DNA product formed. After a few RT-PCR cycles, fluorescence surpasses a threshold level (Cq) set above background fluorescence and starts to increase exponentially. The Cq is the metric used for analyzing qPCR results

SyBr green which is the simplest and least costly approach to detecting and quantitating RT-PCR products was used. It is a naturally fluorescing dye which can intercalate between double stranded DNA. When unbound, it exhibits low level fluorescence but emits brighter fluorescence upon binding to double stranded DNA. The disadvantage to using SyBr green is that it binds to and double stranded DNA, hence the importance of a melting curve analysis and running the PCR products on a gel to ensure there absence of non-specific products. For this study, the Thermo Scientific™ Luminaris™ qPCR Master Mix which is optimized for SYBR Green chemistry was used and the reaction was prepared is in table 5. Predesigned pimers- IDT PrimeTime® qPCR primers were used in this study. The primers are tested to ensure single product amplification of the correct size and high PCR efficiency. The reaction

contained the forward and reverse primer mixed in a single tube. Primers for the following genes were used in the study: p53, mdm-2, RBBP 6, caspase 3, caspase 8, bad, bax, bak-1, bcl-2 and CDKN-1. GAPDH was included as a reference gene. Real time PCR was run according to parameters indicated in table 6.

Table 5 : Cocktail for RT-PCR

Components	Volume
SYBR Green	5 µl
Forward and reverse primer mix	0.5 µl
Nuclease free water	3.5 µl
Template DNA	1 µl
Final Total Volume	10 µl

Table 6 : Real Time PCR parameters

Analysis	**Cycle**	**Segment**	**Temperature in °C**	**Hold time**
Enzyme activation	1	1	95	10 minutes
Quantification	40	Amplification Annealing Extension	95 55 72	15 seconds 30 seconds 30 seconds
None	1	Final extension	72	10 minutes
Melting	1	Melting curve Denaturation Annealing Extension	 95 65 97	 5 seconds 60 seconds Continuous
None	1	Cooling	40	15 seconds

2.12.3. Agarose gel electrophoresis

Agarose gel electrophoresis is the most effective way of separating nucleic acids of varying sizes (Lee *et al.* 2012). This technique separates DNA based on the size of the DNA molecule, voltage applied, agarose concentration and DNA conformation. It uses an electrical field to move the negatively charged DNA towards a positive electrode Ethidium bromide is usually the stain of choice since it intercalates between bases of DNA, allowing for visualization of DNA bands under UV light.

In this study, a 1% agarose gel electrophoresis prepared in 1x TBE and contained 3µl ethidium bromide (1 µg/ml) was used to separate DNA fragments according to their size and charge. A DNA ladder was included in the first lane as a guide to determine if the observed DNA bands in the sample lanes are actually DNA bands that were expected or not. The real time PCR products we run on the agarose gel to ensure only the specific product was amplified.

2.13. Western blot

Western blot is an immunoblotting technique used to detect specific proteins in a sample. It relies on the specificity of binding between a protein and an antibody raised against that protein. Proteins can be analysed from cells, tissue or even recombinant proteins (Kurien and Scofield 2006).

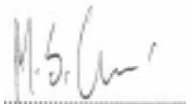
Western blot was employed in this study to determine if there were any changes in expression of proteins of apoptotic genes following treatment with the plant extracts. At 24 and 48 hours post treatment with plant extracts, cells were lysed using RIPA buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.1% SDS, 2 mM EDTA). Protein content was measured by the BCA assay and equal amounts were electrophoresed in SDS polyacrylamide gel and then

transferred onto nitrocellulose membranes. Membranes were subsequently immunoblotted with the anti-p53, anti-RBBP6, anti-Mdm-2, anti-BAX, anti-Bcl-2, anti-caspase-3, anti-caspase-9, anti-β-actin primary antibodies overnight then in horseradish peroxidase-conjugated secondary antibody for 1 hour. Protein bands were detected on the Chemidoc MP Imaging system following treatment with enhanced chemiluminescence reagent.

2.14. Statistical analysis

Experiments were either performed in duplicate or triplicate, data sets were expressed as means ± standard deviations (SD). Data were analysed using Student's t-test. Statistical significance was presented as ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

This letter is to confirm that the following manuscripts "**Mpho S Choene and Lesetja R Motadi. CDK-interacting protein 1 overexpression as a predictive anti-cancer agent in breast cancer treated with Euphorbia tirucalli extracts. Accepted JrMolBio 2015**" was prepared by me (Mpho S Choene) and independently send it to Journal of Molecular Biology. I have contributed 70% to the preparation of the paper which include: all the experiments and writing of the manuscript itself.



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Ms Mpho S Choene

CHAPTER 3 : CDK-INTERACTING PROTEIN 1 OVEREXPRESSION AS A PREDICTIVE ANTI-CANCER AGENT IN BREAST CANCER TREATED WITH *EUPHORBIA TIRUCALLI* EXTRACTS

This chapter has been accepted for publication in Journal of Molecular Biology. **Mpho S Choene** and Lesetja R Motadi. CDK-interacting protein 1 overexpression as a predictive anti-cancer agent in breast cancer treated with Euphorbia tirucalli extracts. Accepted *J.Mol.Bio* 2015. Appendix 3

Abstract

Africa, more specifically South Africa, has a remarkably rich flora biodiversity, comprising of about 8% (20 000) of the world's plant species. Of these, about 20% are used as medicine and, from these, about 350 are commonly used and traded medicinal plants (Gurib-Fakim 2006; Singh and Barrett 2006). The obstacle faced however, is that there is little or no knowledge on the molecular mechanisms of active compounds and the chemical profiles of some of these plants (Sayed 1980). In this study we look at the effects of *Euphorbia tirucalli* extracts in regulating cell death in breast cancer cell lines. In order to achieve this, *E. tirucalli* was collected in Limpopo, dried and crushed. The compounds were extracted using butanol, hexane and methanol at 1g per 10ml. Extracts were characterised using both TLC and LS-MS spectroscopy. Following the extract analysis, breast cancer cell lines (MCF-7 and MDA-MB 231) were treated with varying concentrations of the extracts for up to 48 hours. Cell viability, cell cycle and apoptosis were measured to assess the effects of the extracts. At IC_{50}, molecular analysis was performed to evaluate effect on apoptotic genes. Extracts were found to inhibit cell proliferation in a concentration and cell type-dependent manner. Cells were

also found to have been arrested at G_0/G_1 and in some cases apoptosis induced. In general most proapoptotic genes like Bax and cell cycle gene p21 were found to be significantly upregulated in cells treated with plant extracts. These results suggest that the three extracts might induce apoptosis or arrest cells at G_0/G_1 with the molecular mechanism that suggests activation of caspase-8 and Bax.

Keywords: apoptosis, cell cycle, xCelligence, breast cancer and *E. tirucalli*

3.1. Introduction

Plants of the Euphorbiaceae family have a long history of use as traditional medicine. In Africa and Fiji, a decoction or maceration of some Euphorbiaceae plants are used to treat asthma, cough, diabetes, cure ear-ache, bowel complaints and infertility (Singh 1986; Igoli *et al.* 2005). Some have been used for centuries as traditional medicine against cancers and tumours (Rizk 1987). In countries like Egypt, Euphorbia species are widely used in the treatment of stomach, uterus, liver and kidney cancer (Sayed 1980). The root of *E. tirucalli* is infused in boiling water and used to treat toothache. *Euphorbia tirucalli's* latex is used as treatment for warts (van Wyk *et al.* 2008). In recent years, the surge in pharmaceutical and phytotherapeutic research of medicinal plants has led to great discoveries. Today, up to 50% of the world's drugs are made from natural products or their derivatives (Gurib-Fakim 2006). Some of these drugs include aspirin, quinine and morphine amongst others (Singh and Barrett 2006). The recent surge in natural plants research has also led to production of drugs such as taxol, camptothecin and podofilox which are now being used for chemotherapy. These drugs were derived from parts of various plants (Itokawa *et al*. 2008; Pezzuto 1997). As researchers worldwide continue to explore plants for their anticancer properties, breast cancer should also be at the forefront of medicinal plant research, since it is now the leading cancer in females

(Siegel *et al*. 2012). In this study we explore the molecular mechanisms displayed by extracts of *E. tirucalli* in inducing cell death in breast cancer cell lines.

3.2. Results

3.2.1. Real-time monitoring of cell growth and IC50 concentration estimation

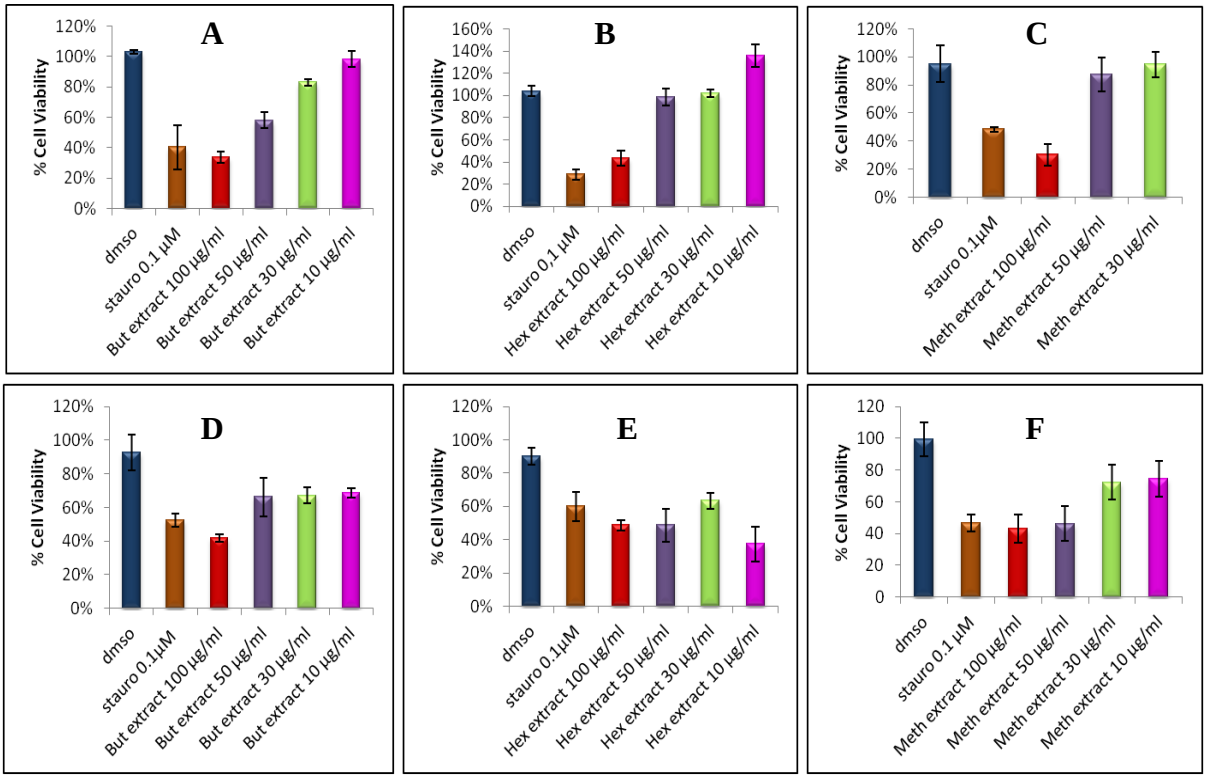


Figure 4 : Cell viability analysis using MTT assay.

(a-c) MCF-7 and (d-f) MDA-MB 231 cells were treated with varying concentrations of butanol (a & d), hexane (b & e) and methanol(c & f) extracts of *Euphorbia tirucalli* for 24 hours. IC_{50} was characterised as concentrations where 50% cell death was observed

MTT analysis of cell viability was analysed following treatment with different concentrations of Butanolic, Hexane and Methanolic extracts of *Euphorbia tirucalli* for 24 hours. The results indicated that inhibition of cell proliferation was concentration-dependent and cell line type-dependent. In MCF-7 the IC_{50} was estimated to be at about 60µg/ml in butanolic extract,

70µg/ml in hexane and 80µg/ml in methanolic extracts (Fig 4 a-c). The MDA-MB231 cell viability analysis indicated that the IC_{50} was observed at the following concentrations: about 40µg/ml in cells treated with butanolic extracts, 50µg/ml in cell treated with hexane and 100µg/ml in cells treated with methanolic extracts (Fig 4 d-f).

MTT assays are endpoint assays and can only detect cell viability/ cytotoxicity at a particular time point. Hence, a real-time proliferation assay (RTCA) would confer a better understanding of the kinetics of cell and plant extract interactions over an extended period of time. Experiments were carried out using *xCELLigence* RTCA DP analyzer (Roche) which offers continual label-free and non-invasive analysis of cell proliferation over time. Cell index (CI), were automatically calculated as live cells interact with electrodes in the E-plates.

As shown in Fig 5 d& g, a drastic decrease in CI following treatment with the butanol extract of *E. tirucalli* was observed, with the CI reaching 0 within a few hours for all concentrations tested in MDA-MB 231 (Fig. 5g). In MCF-7 (Fig 5d), at lower extract concentrations (10 µg/ml), the cells recover from the initial cytotoxic effects of extract and seem to re-proliferate. Butanol extract showed cytotoxicity at a concentration that was in a cell type-dependent manner with an IC_{50} of 15µg/ml and 30 µg/ml for MCF-7 and MDA-MB 231 cells respectively. On testing the growth profile of normal MRC-5 fibroblast, upon treatment, CI was decreased in butanol within 3 hours of treatment and later recovered quickly to normality (Fig 5a).

Addition of *E. tirucalli's* hexane extract showed a decline in CI in all cell lines (fig 5b, e &g). In MDA-MB 231 cells, IC_{50} was observed at 50µg/ml (Fig 5g) whereas in MCF-7 the suitable IC_{50} was observed at a mean 30 µg/ml (Fig. 5f). In comparison to butanol and hexane extract, methanolic extract showed different IC_{50} concentrations. In MDA-MB231 CI was

decreased at a 100µg/ml while in MCF-7 CI decreased at 50µg/ml (Fig 5h). In MRC-5 fibroblast CI was hardly sustained in its decreased level in all extracts which suggests that the three extracts might be decreasing cell proliferation in breast cancer only but not in normal cells.

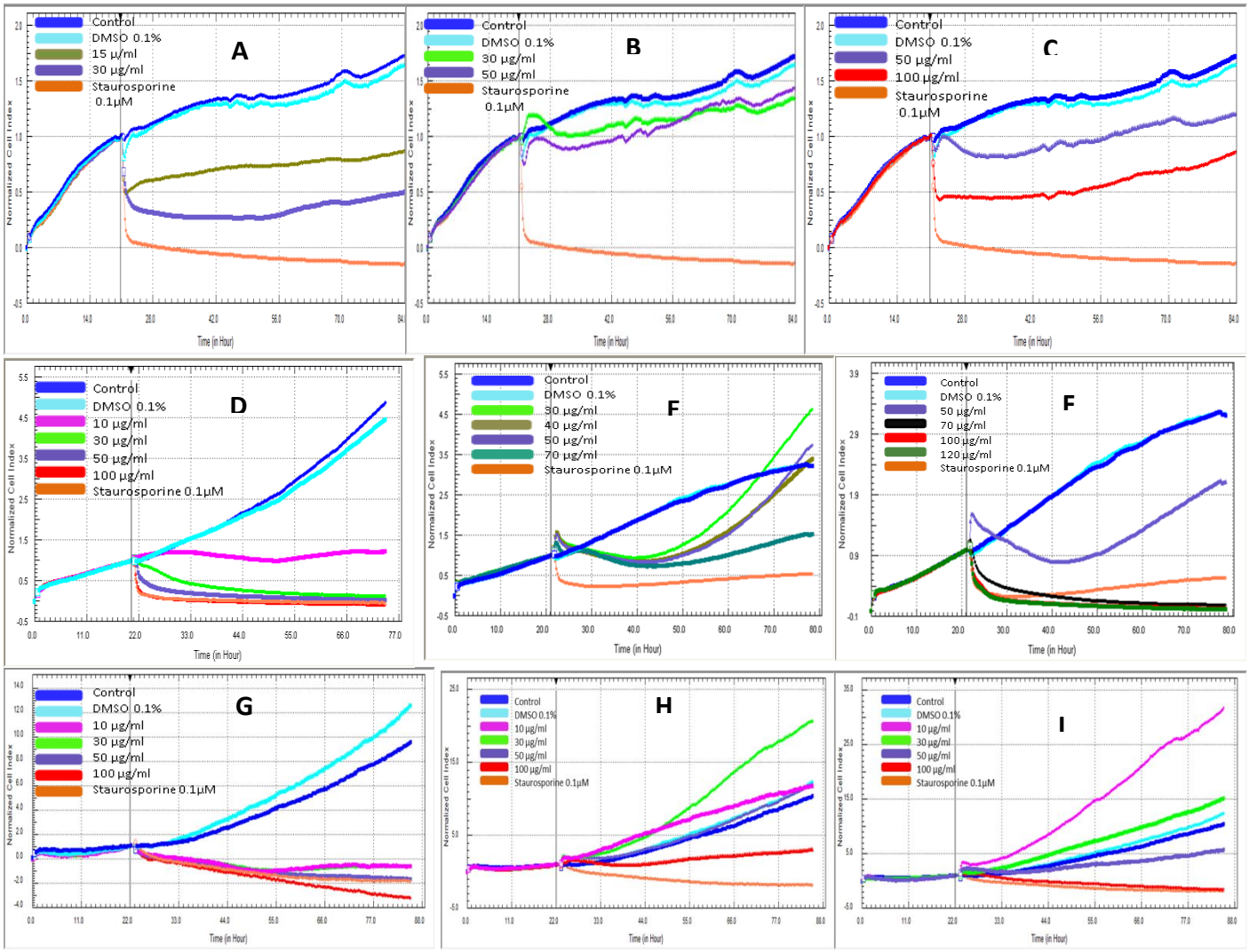


Figure 5 : Dynamic monitoring of *Euphorbia tirucalli* plant extract interaction with cells using RTCA analyser.

MRC-5(a-c), MCF-7(d-f) and MDA-MB 231 (g-i) cells were seeded for 21 hours, at which point, butanol (a, d & g), hexane (b, e & h) and methanol (c, f & i) extracts were added at

indicated concentrations. Cell growth was continuously monitored for over 48 hours. CI values were normalized to the point of compound addition indicated by the vertical black line

3.2.2. *Euphorbia tirucalli butanolic, hexane and methanolic extracts induces Apoptosis and Cell Cycle Arrest*

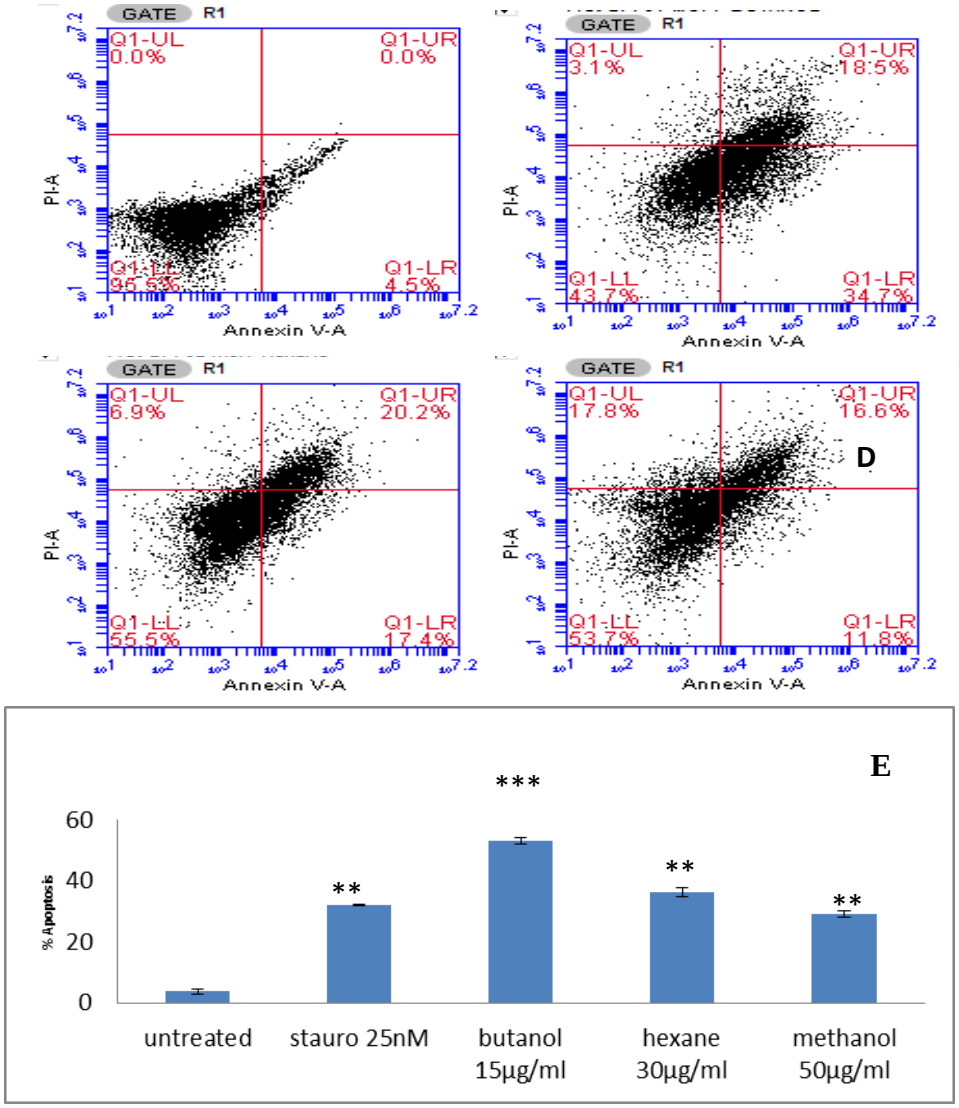


Figure 6 : MCF-7 apoptosis determination using Annexin V/PI staining.

Annexin V/PI stained MCF-7 cells following treatment with either **(b)** butanol, **(c)** hexane and **(d)** methanol extracts of *Euphorbia tirucalli* in comparison with untreated cells at 24 hours. Representative figures showing populations of viable (LL), early apoptotic (LR), late

apoptotic (UR) and necrotic (UL) cells. **(e)** Bar graphs showing percentages of apoptotic cells for each treatment sample at 24hours. Data were mean ± SD from two independent experiments (**P<0.01, ***P<0.001)

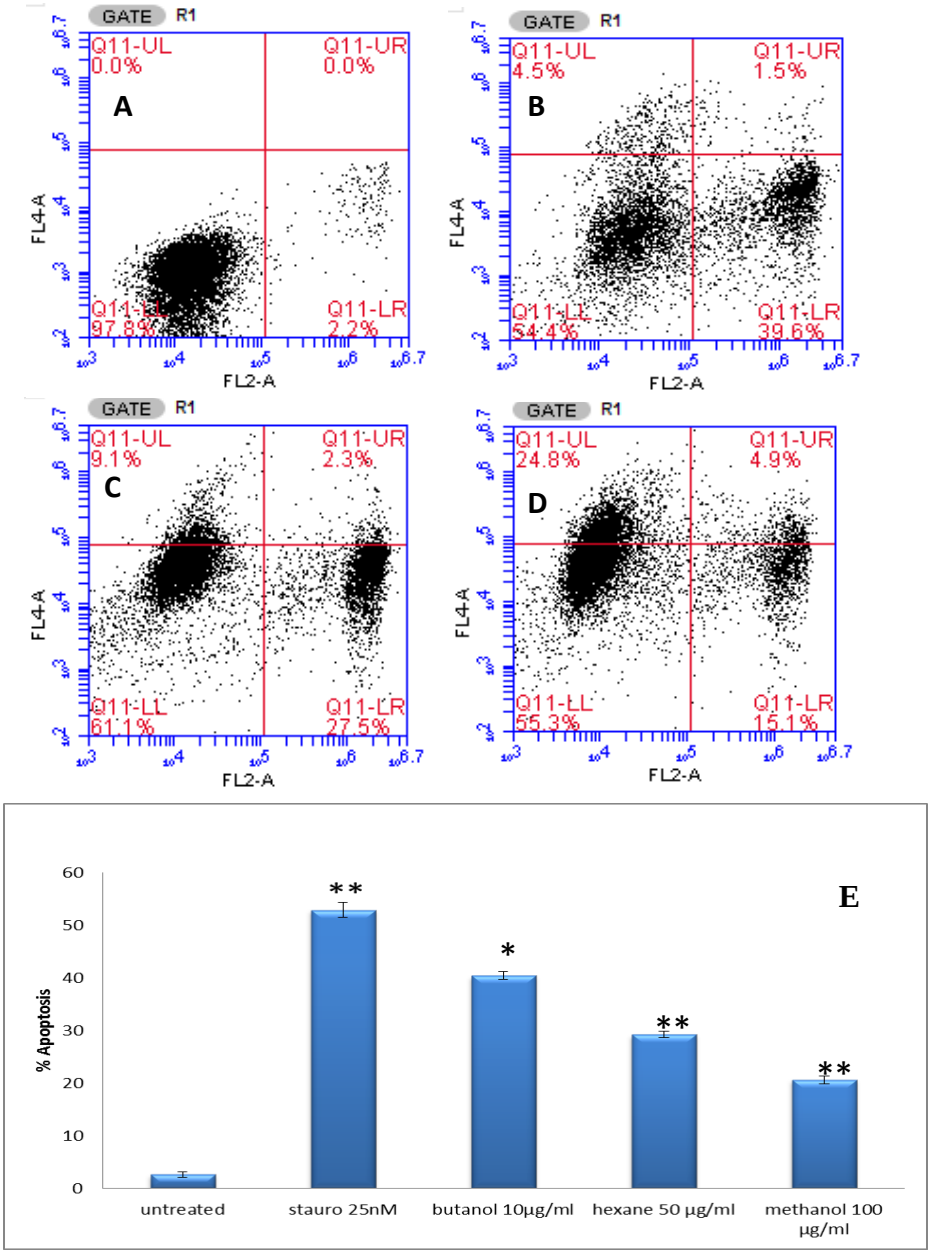


Figure 7 : MDA-MB 231 apoptosis determination using Annexin V/PI staining.

Annexin V/PI stained MDA-MB 231 cells following treatment with either **(B)** butanol, **(C)** hexane or **(D)** methanol extracts of *Euphorbia tirucalli* in comparison with control treatment

at 24 hours. Representative figures showing populations of viable (LL), early apoptotic (LR), late apoptotic (UR) and necrotic (UL) cells. **(E)** Bar graphs showing percentages of apoptotic cells for each treatment sample at 24 hours. Data were mean ± SD from two independent experiments (*P<0.05, **P<0.01).

To examine whether cells undergo apoptosis, untreated or extracts-treated MCF-7 and MDA-MB-231 breast cancer cells were stained with annexin V and PI. Flow cytometry analysis of stained cells can distinguish cells into four groups, namely viable (LL), early apoptotic (LR), late apoptotic (UR) and necrotic (UL) cells. As shown in Fig 6 & 7, exposure to different extracts of *Euphorbia tirucalli* at specific concentrations (butanolic 15µg/ml, hexane 30 µg/ml and methanolic 50µg/ml) resulted in higher population of apoptotic cells at 53% to 27.1% in MCF-7 cells (Fig. 6). In MDA-MB 231, the population of apoptotic cells in similar extracts but different concentrations (10 µg/ml, 50 µg/ml and 100 µg/ml) were higher at 41% to 20% (Fig. 7) compared to control (<3%). Overall there was less than 10% of population that showed necrotic signs.

Next we examined cell cycle distribution by staining treated breast cancer cells with propidium iodide and analyzed the percentages of G_0/G_1, S, G_2/M cell population using flow cytometry. MCF-7 cells treated with butanolic, hexane and methanolic extracts showed higher G_0/G_1 population (38%, 42% and 67%, respectively) as compared to the S-phase that indicates cell proliferation (Fig 8). MDA-MB-231 cells treated with hexane also showed higher G_0/G_1 population 37.8%. In addition, butanolic, hexane and methanolic treatment caused a concomitant decrease in the proportion of cells in the G_2/M phase of the cell cycle from control (10%) to below 5% in all MCF-7 treatments, and from control (8.2%) to less than 1% in treated MDA-MB-231 cells. Therefore, our data suggests that butanolic, hexane and methanolic extracts induced cell cycle arrest at the G_0/G_1 phase (Fig. 8).

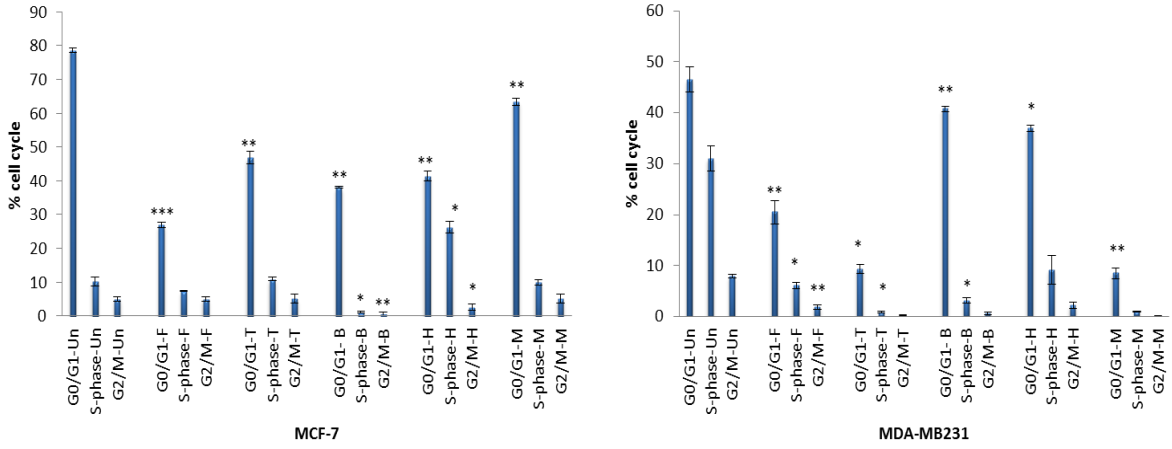


Figure 8 : Bar graphs representing cell cycle analysis of MCF 7 and MDA-MB231.

Cells were treated with butanolic, hexane and methanolic extracts of *Euphorbia tirucalli*. Data were mean ± SD from two independent experiments (*P<0.05, **P<0.01, ***P<0.001)

3.2.3. *Effects of extracts of Euphorbia tirucallion Caspase-3/7*

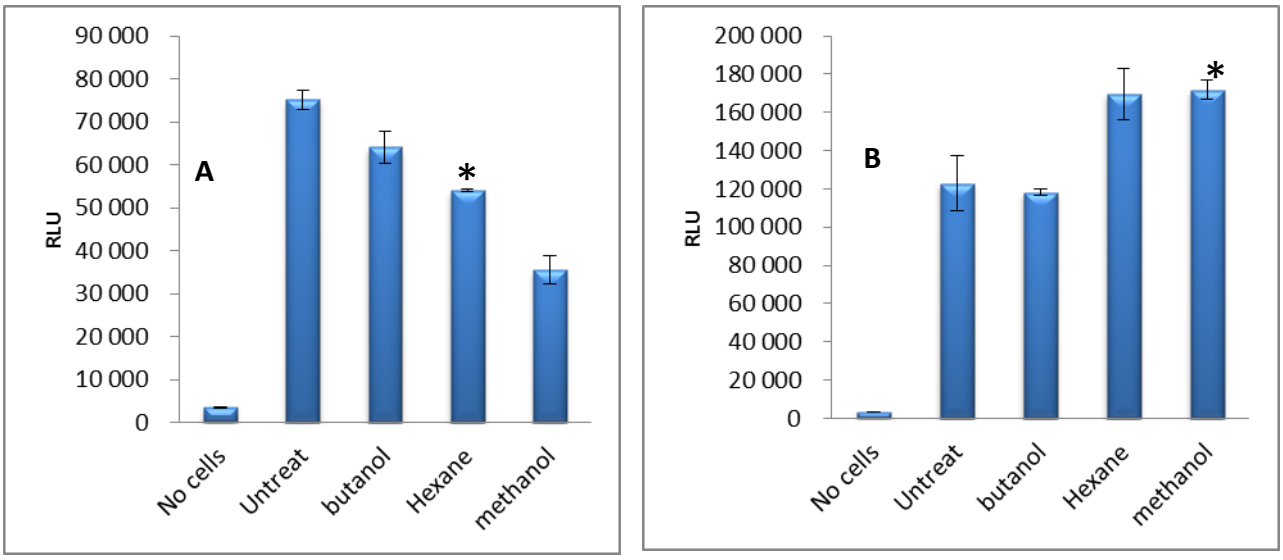


Figure 9 : Caspase 3/7 activity in MCF-7 and MDA-MB 231.

(A) MCF-7 and (B) MDA-MB 231 cells were treated with plant extracts' IC_{50} concentrations for 24 hours. Caspase 3/7 activities in treated and untreated cells were then determined by luminescence assay. Data were mean ± SD from three independent experiments (*P<0.05, **P<0.01)

Caspases are the hallmark of apoptosis and based on whether caspases are activated, apoptosis can be classified as caspase-dependent or caspase-independent. Depending on which caspases are activated, caspase-dependent pathways can further be divided into an extrinsic or intrinsic pathway. Both the intrinsic and extrinsic pathways involve activation of caspase-3/7 which is important for inducing downstream DNA cleavage molecules. To examine the molecular mechanism underlying the apoptosis process, we stained cells with aminoluciferin-labeled substrate of caspase and determined the caspase-3/7 activities by measuring the luminescence intensities after 24 hours of treatment. As shown in fig. 11, we observed a gradually increased caspase-3/7 activity in MDA-MB 231 cells treated with

hexane and methanolic extracts whereas that of butanolic remained unchanged as compared to the control. In MCF-7, there was significant reduction of caspase 3/7 activity in hexane treated cells (Fig. 9). Our data suggested that extracts of *Euphorbia tirucalli* induced activation of the extrinsic caspase pathway in MDA-MB 231 breast cancer cell lines.

3.2.4. Effects of Euphorbia tirucalli extracts on Anti-apoptotic, pro-apoptotic and cell cycle Molecules

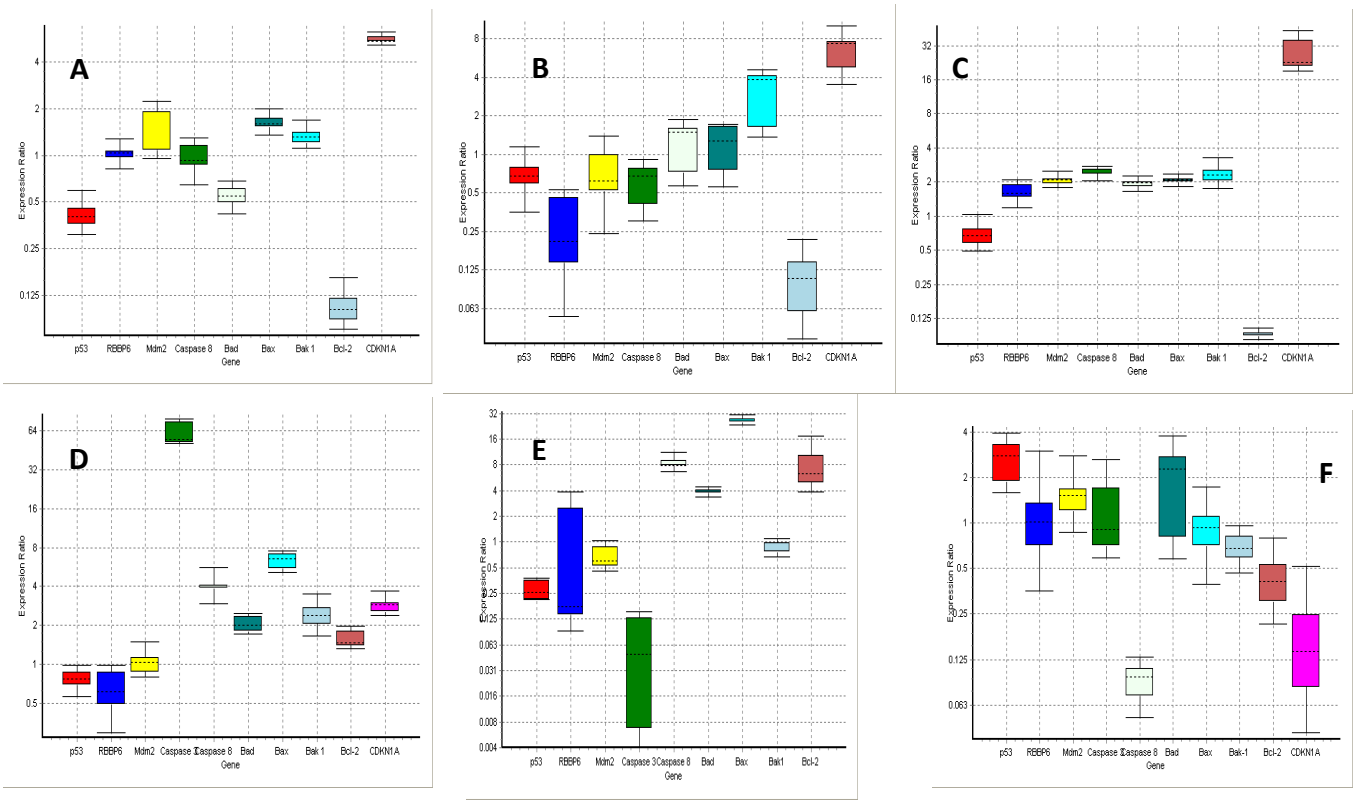


Figure 10 : Box plot representing Real Time PCR analysis using REST 2009 software.

(a-c) MCF-7 and (d-f) MDA-MB 231 gene expression analysis following treatment with (a & d) butanol, (b & e) hexane and (c & f) methanol extracts of *Euphorbia tirucalli* for 24 hours.

Cell survival is maintained by increased ratio of anti-apoptotic molecules to those of pro-apoptotic molecules. To examine if *Euphorbia tirucalli* extracts initiated apoptosis by affecting the cellular level of these molecules, we performed Real-Time PCR and western blot analysis using control to treated breast cancer cells. Our data showed that in MCF-7 treated with butanol, hexane and methanol extracts, Bcl-2 was down-regulated in sample groups in comparison to control groups by a mean factor of 0.105 (S.E. range is 0.083 - 0.129). In the same experiment both Bax and Bak were up-regulated in sample groups in comparison to control group by a mean factor of 1.619 -2.08 (S.E. range is 1.442 - 1.785) and 1.329-2.932 (S.E. range is 1.172 - 1.482) respectively. In MDA-MB 231 cells, the ratio of Bcl-2/Bax was reduced in comparison to control samples due to significant increase in the expression of Bax in sample group in comparison to control group by a mean factor of 6.352-26.518 folds. Bak was upregulated by a mean factor of 2.413 (S.E. range is 1.981 - 3.259) in hexane treated but remained statistically unchanged in butanol and methanol treated (Fig. 10). Bad analysis showed that it was only upregulated in MCF-7 treated with methanolic extracts whereas in MDA-MB 231 it was upregulated in butanolic extracts by a mean factor of 1.963 (S.E. range is 1.814 - 2.211) and 2.036 (S.E. range is 1.744 - 2.404) respectively.

Analysing the expression of both caspase 3 and 8, we observed that in MCF-7 both caspase 8 and 3 expression remained statistically unchanged in all the 3 extracts (Fig. 10: a, b & c). In MDA-MB231, both caspase 3 and 8 were up-regulated in sample groups in comparison to control groups in butanol treated cells by a mean factor of 59.866 (S.E. range is 50.807 - 76.344).and of 4.024 (S.E. range is 3.253 - 5.033) respectively. In hexane, caspase 8 was also up-regulated by a mean factor of 8.327 and that of caspase 3 remained unchanged. For methanol there was a down-regulation of caspase 8 and no change in caspase 3 expression (Fig. 10: d, e & f).

Next analysis was to examine the molecules that regulate cell cycle to see if there were any changes in their expression. p21 plays a crucial role in cell cycle as it inhibits cyclins thereby arresting cells. Analysis of CDKN1A (p21) revealed that it was up-regulated by all 3 extracts in MCF-7 in comparison to control group by a mean factor of 5.574 butanol, 6.396 hexane and 25.976 methanol. In MCF-7, p53 expression was down-regulated in butanol treated cell in comparison to control by a mean factor of 0.408 (S.E. range is 0.328 - 0.512) and in both hexane and methanol it statistically remained unchanged. In MDA-MB231, p53 was up-regulated in sample group in comparison to control group by a mean factor of 2.597 (S.E. range is 1.712 - 3.460) in methanol treated cells and it was down-regulated by a mean factor of 0.272 in hexane. The two p53 regulators, MDM2 and RBBP6 had different expression with MDM2 statistically not changed in all the 3 extracts in MCF-7. While RBBP6 was down-regulated in sample group in comparison to control group by a mean factor of 0.325 (S.E. range is 0.193 - 0.504) in hexane treated but was up-regulated in methanol treated by a mean factor of 1.649. In MDA-MB231 both MDM2 and RBBP6 were statistically unchanged in all treatments. Furthermore, RBBP6 was shown to have been spliced in all cancer as indicated by the figure 11.

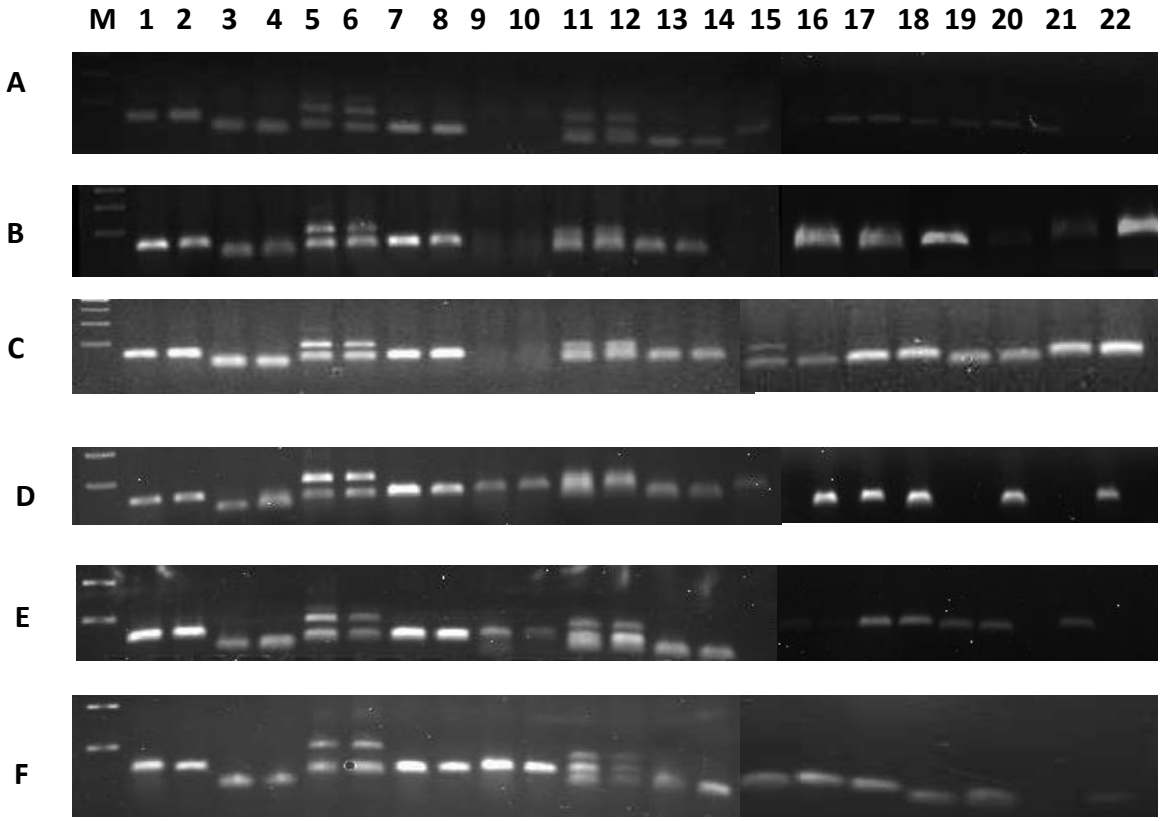


Figure 11 : Real time PCR products run on a 1% agarose gel electrophoresis.

(a-c) MCF-7 and (d-f) MDA-MB 231 cells treated with (a & d) butanol, (b & e) hexane and (c & f) methanol extracts of *E. tirucalli*. M=Marker, 1 = Untreated GAPDH, 2 = Treated GAPDH, 3 = p53 untreated, 4 = p53 treated, 5 = RBBP6 untreated, 6 = RBBP6 treated, 7 = Mdm-2 untreated, 8 = Mdm-2 treated, 9 = Caspase-3 untreated, 10 = Caspase-3 treated, 11 = Capsase-8 untreated,12 = Caspase-8 treated, 13 = Bad untreated, 14 = Bad treated, 15 = Bax untreated, 16 = Bax treated, 17 = Bak-1 untreated, 18 = Bak-1 treated, 19 = Bcl-2 untreated, 20 = Bcl-2 treated, 21 = P21 untreated, 22 = P21 treated

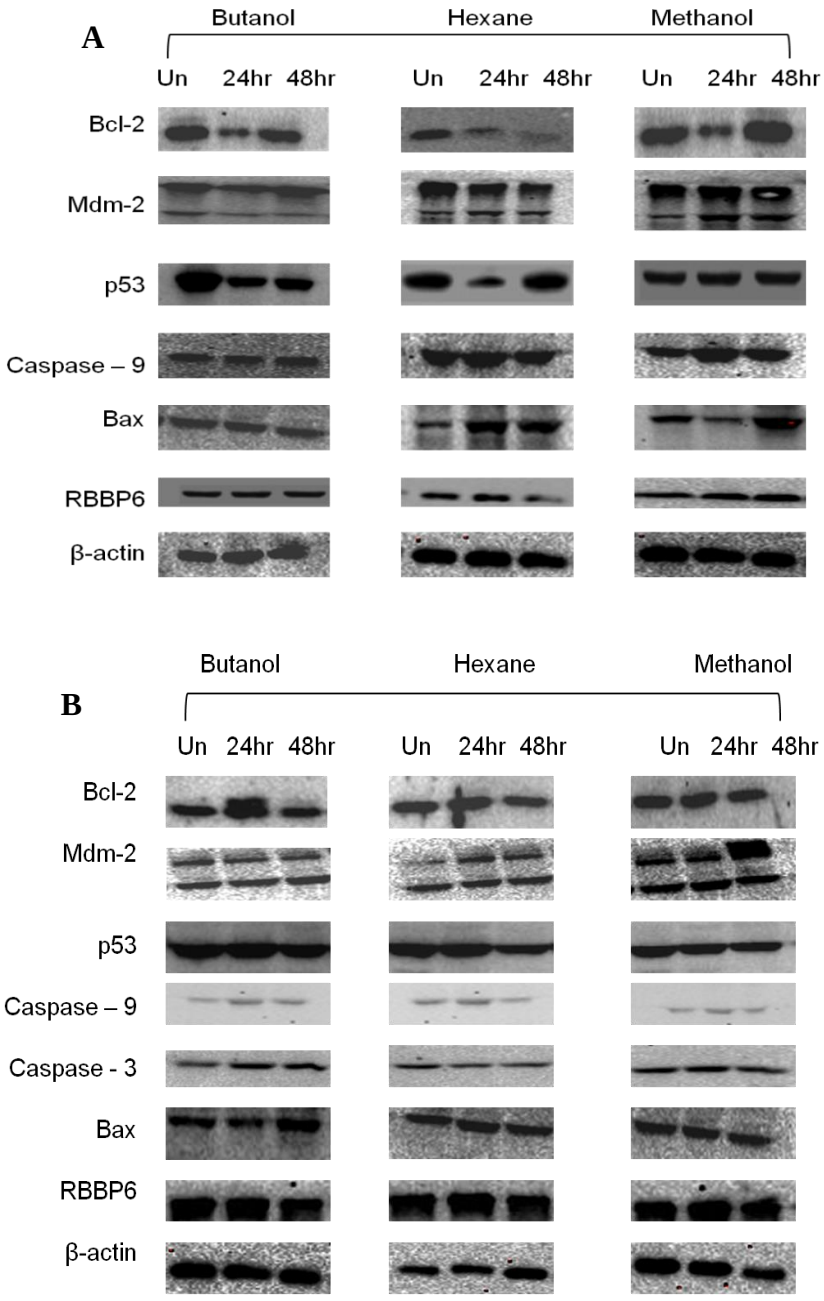


Figure 12 : Western blot analysis of protein expression

Protein expression was analysed in (a) MCF-7 and (b) MDA-MB 231 cells following treatment with butanol, hexane and methanol extracts of *E. tirucalli*. Un= Untreated samples. The results show a varying protein expression of different apoptotic genes 24 or 48 hours post treatment with the 3 plant extracts.

3.3. Discussion

In South Africa, between 1986 and 1992, cervical cancer was the leading cause of cancer amongst women, but recently breast cancer seems to have overtaken and is now the most common cancer in females and the development of novel therapeutic agents is urgently needed (Vorobiof *et al*. 2001; Siegel *et al.* 2011). Although several new therapeutics have been discovered, including chemotherapy, they remain ineffective if the disease was diagnosed in advanced stages or the side effects are unbearable to the users (Gehl *et al* 1996). In under developed and developing countries, natural compounds derived from plants have long been used to treat a number of human diseases. In this context, plant-derived compounds play an important role in the development of new anticancer agents against human breast cancer.

In this study, butanolic, hexane and methanolic extracts of *E.tirucalli* were tested for potential antiproliferative activity on MCF-7 and MDA-MB 231 human breast cancer cells. We showed that *E.tirucalli* effectively inhibited cell growth of MCF-7 and MDA-MB 231. Irrespective of the extracts, the antiproliferative activity was found to be concentration-dependent. It was also found that the antiproliferative activity of the extracts were different and specific towards the cell type. Overall, butanolic extract induced the maximum cancer cell death followed by hexane and methanolic extract. In all cases, antiproliferative activity was concentration-dependent and cell type-dependent. MTT is one of the oldest techniques that has been used to estimate the toxic concentration of compounds or new drugs. However, it lacks the accuracy to predict the exact concentration. In compensating for MTT, we employed xCELLigence assay to predict the accurate concentration that is potent and also to monitor real time analysis of cell proliferation following treatment with the three extracts in breast cancer cell lines and MRC 5 fibroblast that mimic normal cells.

xCELLigence technology measures impedance changes in a meshwork of interdigitated gold microelectrodes located at the well bottom (E-plate) or at the bottom side of a microporous membrane (Ke *et al.* 2010; Moniri *et al.* 2014). These changes are caused by the gradual increase of electrode surface occupation by proliferating cells during the course of time and thus can provide an index of cell viability (Meindl 2013). Staurosporin was chosen as it is widely used as a treatment for a variety of tumour types and previous reports on the use of staurosporin as a tool for cytotoxicity screening, including in breast cancer cell line, is documented (Moela *et al* 2014). From our studies we have observed that at 0.2 uM staurosporin was able to inhibit cell proliferation in all cell lines, this was predicted as in line with other reported results (Lui *et al* 2012). Profiling the different concentrations of plant extracts, it was evident that the type of cell proliferation associated with the extracts was cell type and concentration-dependent. Like in MTT and other reports, butanolic extracts showed toxicity in MCF-7 and MDA-MB231 at a much lower concentration, followed closely by extracts of hexane while methanol was the least toxic (Szic *et al.* 2014; Looi *et al.* 2013). Indeed, the xCELLigence device has been reported to generate compound-specific kinetic profiles on cultured cells. Hereby we demonstrate associations with the three extracts and hope to associate it with respective mechanisms of action. In fact, defects in the apoptotic pathway are thought to contribute to a number of human malignancies (Fulda and Debatin 2006; Fulda 2009). Thus, anticancer agents that induce apoptosis are one of the efficient strategies in cancer chemotherapy.

Since *E. tirucalli* extracts showed cell death and antiproliferative activity, with maximum cancer cell death induced at reasonable concentrations in both MCF-7 and MDA-MB 231 cells, we carried out a mechanism and characterization study of the type of induced cell death. Generally, anticancer agents have been reported to induce cell death by blocking cell

cycle progression and triggering tumour cell apoptosis (Shapiro and Harper 1999; Jansen *et al.* 2000). Therefore, cell cycle arrest and the induction of apoptosis in cancer cells become the major indicators of anticancer effects. The p53 protein is an essential molecule that arrests cells with damaged DNA in $G_1$and G_2, allowing time for DNA repair before the cell tries to enter the M phase (Kastan *et al.* 1991; Agarwal 1995). But if the cell is committed to division or the damage cannot be repaired, then p53 triggers a program cell death through the extrinsic and intrinsic apoptotic pathways. To elucidate the underlying mechanism of cell growth inhibition, cell cycle progression was then analysed for MCF-7 and MDA-MB231 cells after treatment with extracts of *E. tirucalli*. Flow cytometry analysis showed that treatment with the three extracts arrested the cell cycle at the G_0/G_1 phase and in most cases induced early and late apoptosis in both cell lines. Through Annexin and PI staining, flow cytometry showed that the number of dead MCF-7 and MDA-MB 231 was significantly increased in butanolic extracts in comparison to the other two extracts. These types of results were similar to those reported earlier in our laboratory: that methanolic extract exhibited apoptosis in MCF-7 (Choene and Motadi, 2012). Similarly, Mahassni and Al-Reemi (2012) reported that, in their study, extracts of Lepidium sativum seeds induced cell death in MCF-7. Moreover, all 3 extracts downregulated the expression of MDM2 and RBBP6 while upregulating the expression of p21 in MCF-7 and MDA-MB231 cells. Therefore, it is reasonable to speculate that the mechanisms by which *E. tirucalli* extracts alleviate cell growth may be partly via the induction of cell cycle arrest due to the upregulation of p21 that might then suppress cyclins. A similar mechanism was reported by Ujiki *et al* (2006) where they showed the effect of Luteolin on cell cycle and apoptosis in Hela cells. In another study by Krifa *et al* (2013) they also reported that aqueous gal extracts of *Limoniastrum*

guyonianum were able to induce apoptosis and arrest the cell cycle in human cervical cancer by upregulating p21 that resulted in suppression of Cyclin B1.

To understand the potential antitumour mechanisms of *E. tirucalli extracts*, the expression of pro and antiapoptotic genes was investigated in the present study. We found decreased expression of Bcl-2 mRNA and protein, but increased expression of Bax, Bad and caspase-8 mRNA or protein; therefore, the Bax: Bcl-2 ratio was elevated. In addition, the expression of MDM2 and RBBP6 mRNA and proteins was also downregulated with that of p21 highest in all cell lines and extracts. However, Bcl-2 mRNA and protein were significantly downregulated in MCF-7 cells treated with all three extracts compared with controls. In this study p53 was found to have either remained unchanged or decreased in some instances following treatment with the three plant extracts. The data presented in this study suggest that *E.tirucalli*-induced apoptosis in MDA-MB 231 cells is mediated by the death receptor and mitochondrial apoptotic pathways, as demonstrated by increased expression levels of caspase-8 and 3 after treatment.

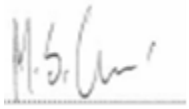
These pro and antiapoptotic proteins are the principal regulators of the intrinsic pathway of apoptosis (Williams and Smith 1993). Previous reports have shown that the ratio of Bax to Bcl-2 determines, in part, the susceptibility of cells to death signals (Raisova *et al* 2001). This was also evident in a study by Alshatwi *et al* (2012), where they found that methanolic extracts *Juglans regia* increased Bax protein levels in PC3 cells whereas the levels of Bak and Bcl-xL proteins were unchanged. In another study by Choi *et al.* (2011), Bcl-2 expression was significantly inhibited, whereas the expression of Bax, Tp53, and caspase-3 markedly increased in human prostate cancer cells treated with an extract of Green Husk of *Sugians regia*.

It is well established that Bcl-2 generally acts as a potent antiapoptosis gene by controlling several key steps in apoptosis signaling, including formation of ion channels in biological membranes that influence the permeability of intracellular membranes and cytochrome c release from mitochondria. The fact that, in this study Bcl-2 was downregulated but both Bax and Bad upregulated, suggested that the molecular mechanism that is associated with cell growth inhibition might be the one involving mitochondria activation and cytochrome c release.

Caspase −9 and −8 are upstream caspases that converge to caspase-3, a key effector molecule in the caspase-dependent cell apoptosis pathway that cleaves a number of cellular proteins including PARP, leading to DNA fragmentation and triggering apoptosis (Huppertz *et al.* 1999; Brauns *et al.* 2005). In this study, the caspase-3/7 activity was increased significantly in treated MDA-MB231 as compared to the control ($p<0.05$) which indicated enhanced caspase activation of apoptosis by methanolic treatment. In MCF-7 there was a decrease in caspase activity, however, that was not significant and which might have been due to the fact that caspase 3 has been reported to be lacking in MCF-7 (Devarajan *et al* 2002). The results obtained were supportive of Yaacob *et al. (*2010) who reported that an *S. crispus* extract induced apoptosis via caspase-3/7 activation in breast and prostate cancer. When caspase-8 was evaluated in both mRNA and protein following treatment with plant extracts, we have observed that it was upregulated in almost all treatmenst which suggested that the extrinsic pathway might be activated. Caspase inhibitor assay could be done to help in determining the involvement of various caspases in the apoptotic pathway. TLC analysis revealed the presence of unique phytochemicals in the *E. tirucalli* extracts suggesting that the differences and concentration of phytochemicals in the *E. tirucalli* extract was responsible for producing the maximum antiproliferative effect on the cancer cells. The results have shown that extracts

of *E. tirucalli* have a promising anticancer activity. Individual extracted compounds might be useful as anticancer treatment but they need further testing.

This letter is to confirm that the following manuscript "**Mpho S Choene and Lesetja R Motadi. Cell cycle and apoptosis induction in ovarian (RMG-1) and cervical cancer cell line (SiHa) via p21 activation by Flavonoids components isorhamnetin extract of *Euphorbia tirucalli*. Manuscript numbers: PHYMED-D-15-00177**" was prepared by me (Mpho S Choene). I have contributed 70% to the preparation of the paper which include: all the experiments and writing of the manuscript itself.



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Ms Mpho S Choene

CHAPTER 4 : CELL CYCLE AND APOPTOSIS INDUCTION IN OVARIAN (RMG-1) AND CERVICAL CANCER CELL LINE (SIHA) VIA P21 ACTIVATION BY FLAVONOIDS COMPONENTS ISORHAMNETIN EXTRACT OF *EUPHORBIA TIRUCALLI*

This chapter has been submitted for publication in Journal of Phytomedicine. **Mpho S Choene** and Lesetja R Motadi. Cell cycle and apoptosis induction in ovarian (RMG-1) and cervical cancer cell line (SiHa) via p21 activation by Flavonoids components isorhamnetin extract of *Euphorbia tirucalli*. Manuscript numbers: PHYMED-D-15-00177. Appendix 4

Abstract

Gynaecological cancers remain a huge burden to women in developing countries. Standard treatments usually involve surgery, chemotherapy and/or radiation either as single agents or in combination. Flavonoids have played a vital role in health because they help support detoxification of potentially tissue-damaging molecules and their intake has often, although not always, been associated with decreased risk of certain types of cancers, including lung and breast cancer. In this study we looked at the molecular analysis of the impact of Flavonoid components isorhamnetin extract from *Euphorbia tirucalli* on ovarian and cervical cancer. Euphorbia tirucalli plant material was individually extracted using butanol, hexane and methanol. Different compounds were identified and isolated using TLC, HPLC and Mass Spectrophotometer. Ovarian RMG-1 and cervical SiHa cells were treated with varying concentrations of isolated flavonoid isorhamnetin extracts and monitored for over 48 hours. Cell viability was analysed using MTT assay and x*CELL*igence. Cell cycle and apoptosis were measured by flow cytometry. Real time PCR and western blot was performed to evaluate the effect of the extracts on apoptotic genes. The treated ovarian cells were found to

be arrested at G_0/G_1 cell cycle arrest. Apoptosis was induced in most of the treatments on both cell lines. The cell cycle gene p21 was found to be significantly upregulated in both cell lines for all treatments. The proapoptotic Bax was found to be upregulated in most treatments. Isorhamnetin inhibited cell proliferation in a cell type and concentration-dependent manner. Cell cycle arrest was accompanied by upregulation of p21.

Keywords: apoptosis, cell cycle, *Euphorbia tirucalli,* isorhamnetin and p21

4.1. Introduction

Cancer is a leading cause of morbidity and mortality worldwide, with approximately 14 million new cases and 8.2 million cancer related deaths reported in 2012. Africa, Asia, Central and South America account for over 60% of the world's total cancer cases and 70% of the world's cancer deaths (Siegel et al. 2012; World Cancer report 2014). Currently, limitations to these conventional treatments such as adverse toxicity and the high cost of treatment have made it important to develop novel therapeutic agents which exhibit less toxicity and at a lowered cost. These types of agents have commonly been found in medicinal plants. Flavonoids are a class of secondary metabolites in plants chemically, they have the general structure of a 15-carbon skeleton, which consists of two phenyl rings and a heterocyclic ring. Flavonoids are best known for their antioxidant and anti-inflammatory health benefits as well as the support of the reduced risk of cancers such as lung and breast. However, it is important to note that the amount of flavonoids required to provide the above health benefits is not certain. Some of the activities attributed to flavonoids include: anti-allergic, anticancer, antioxidant, anti-inflammatory and antiviral. The flavonoid, Quercetin, is known for its ability to relieve hay fever, eczema, sinusitis and asthma while isorhamnetin, which is present in many plants and is also a metabolite of quercetin, is known to reduce the risk of cancer. There are a few studies which demonstrate similar activity for isorhamnetin

and its glycosides (Ma et al. 2007). In this study we looked at the molecular impact of isorhamnetin isolated from Euphorbia tirucalli on ovarian and cervical cancer cell

4.2. Results

4.2.1. Real-time monitoring of cell growth and IC50 concentration estimation

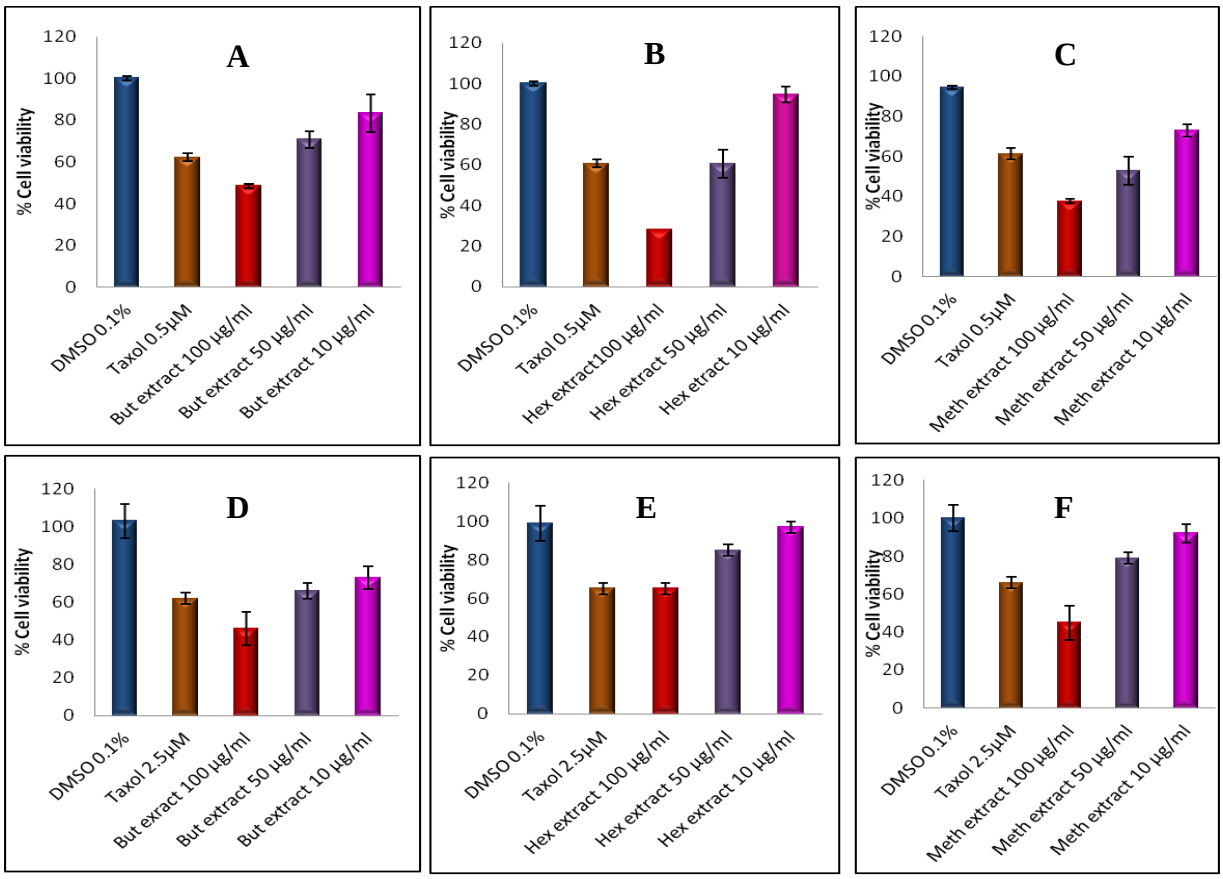


Figure 13 : Cell viability analysis using MTT assay.

(a-c) RGM-1 and **(d-f)** SiHa cells were treated with varying concentrations of **(a & d)** butanol, **(b & e)** hexane and **(c & f)** methanol isorhamnetin extract of *Euphorbia tirucalli* for 24 hours. Taxol at the indicated concentration was included as a positive control.

Cell viability was analysed by MTT following treatment with different concentrations of isorhamnetin extracts for 24 hours. The results indicated that inhibition of cell proliferation was concentration-dependent and cell line type-dependent (Fig 13). Since MTT assays are

endpoint, we confirmed cell growth inhibition by the use of real-time proliferation assay (RTCA). Cell index (CI) was automatically calculated as live cells interact with electrodes attached to the bottom of the E-plates. RGM-1 and SiHa cells were treated with isorhamnetin extract at different concentrations and cell proliferation was monitored for over 30 hours. The IC_{50} at 24 hours post treatment was estimated (Appendix 5). In RMG-1, the IC_{50} was estimated at 10µg/ml in butanolic extract, 30µg/ml in hexane and 30µg/ml in methanolic extracts. In SiHa cells, IC_{50} was estimated at 15µg/ml in butanolic extract, 60µg/ml in hexane and 100µg/ml in methanolic extracts. Futhermore, cells were treated with isorhamnetin of *E. tirucalli* extracts at concentrations that induced 50% cell death (IC_{50}), and cell proliferation was monitored for over 60 hours (Fig 14).

In RMG-1 cells, treatment with hexane and methanol extracts caused a rapid decline in CI, whilst treatment with butanol extract caused a slower decline. In SiHa cells, a rapid decline in CI could also be observed upon treatment with hexane and methanol extracts. A slower decline in CI was observed in cells treated with butanolic extract.

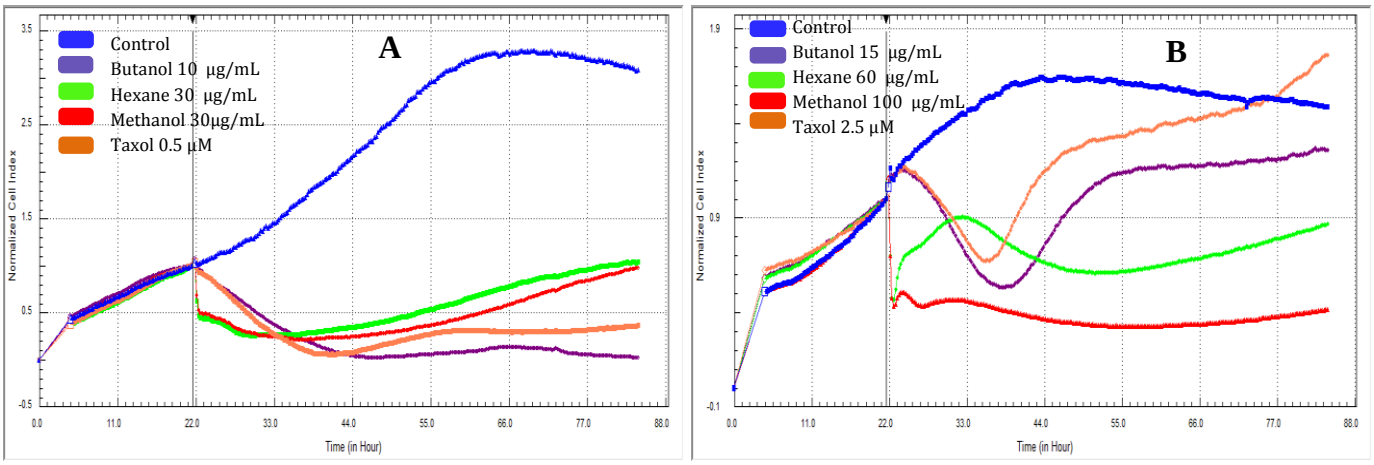
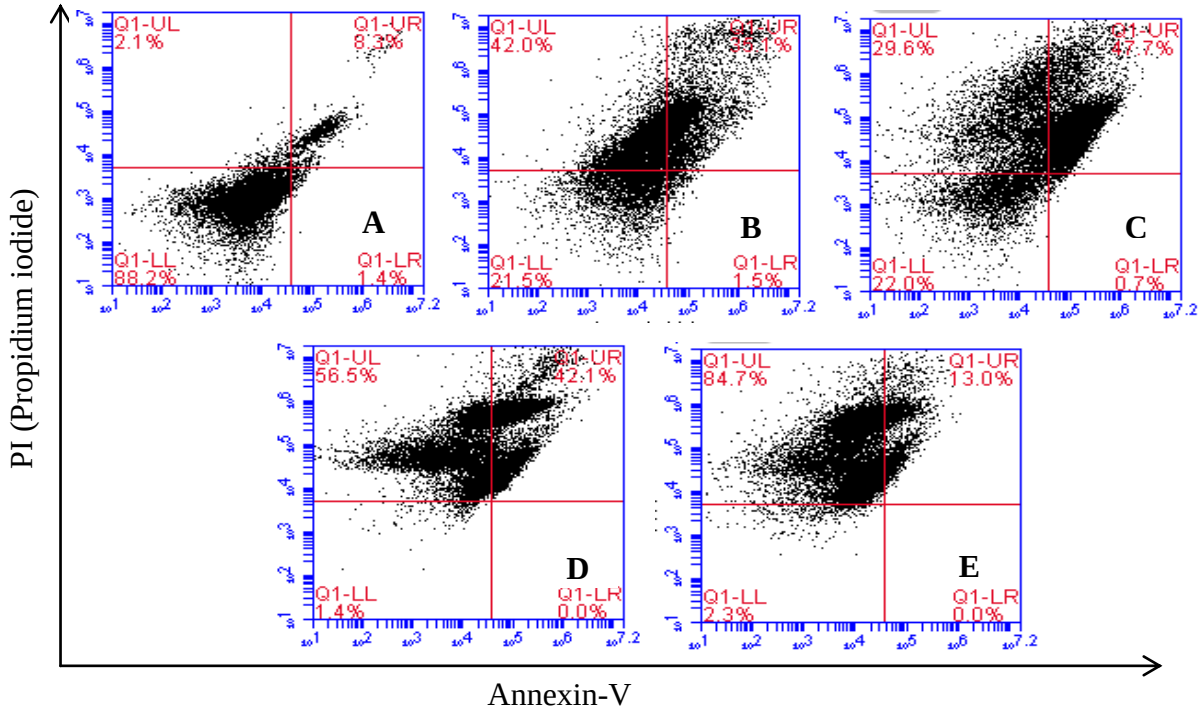


Figure 14 : Dynamic monitoring of isorhamnetin plant extract interaction with using RTCA analyser

(a) RGM-1 and **(b)** SiHa cells were seeded for 22 hours, at which point, butanol, hexane and methanol extracts at indicated IC_{50} were added. Cell growth was monitored for over 60 hours. Taxol at the indicated concentration was included as a positive control. CI values were normalized to the point of compound addition indicated by the vertical black line.

4.2.2. Euphorbia tirucalli butanolic, hexane and methanolic isorhamnetin extracts induces Apoptosis and Cell Cycle Arrest



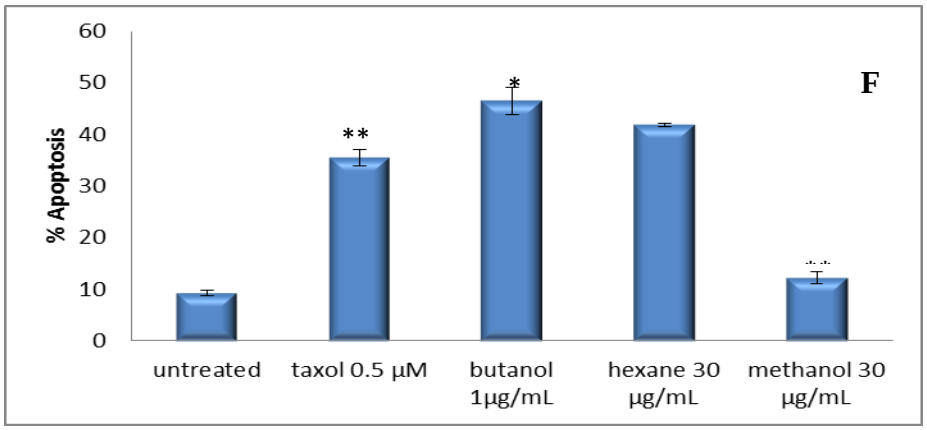
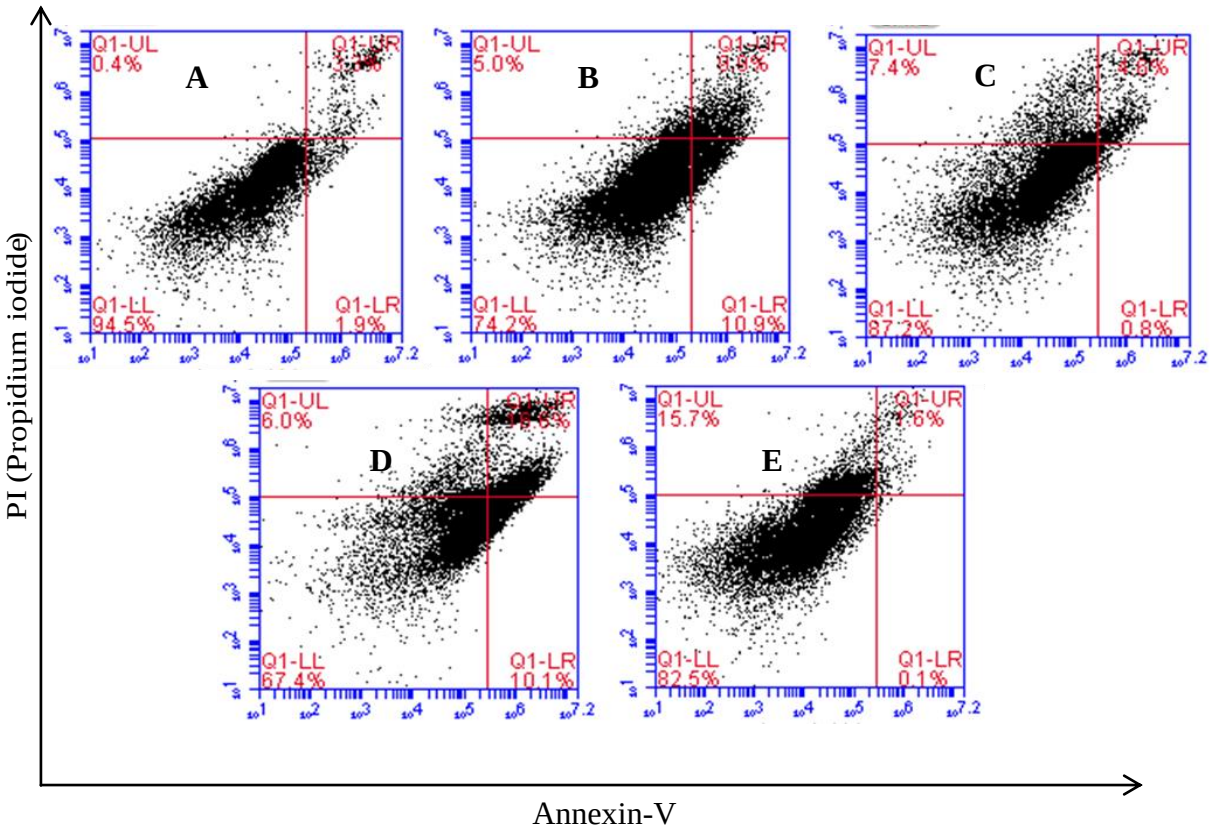


Figure 15 : RGM-1 cells apoptosis determination using Annexin V/PI staining.

Annexin V/PI stained RGM-1 cells following treatment with either **(c)** butanol, **(d)** hexane and **(e)** methanol isorhamnetin extracts of *Euphorbia tirucalli* in comparison with untreated cells at 24 hours. **(b)** Taxol was included as a positive control. Representative figures showing populations of viable (LL), early apoptotic (LR), late apoptotic (UR) and necrotic

(UL) cells. (f) Bar graphs showing percentages of apoptotic cells for each treatment sample at 24hours. Data were mean ± SD from two independent experiments (*P<0.05, **P<0.01)



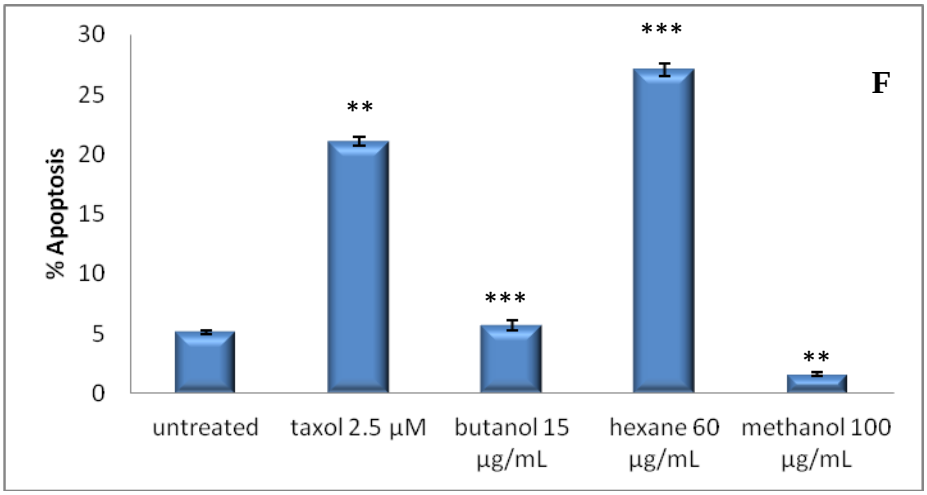


Figure 16 : SiHa cells apoptosis determination using Annexin V/PI staining.

Annexin V/PI stained SiHa cells following treatment with either **(c)** butanol, **(d)** hexane and **(e)** methanol isorhamnetin extracts of *Euphorbia tirucalli* in comparison with untreated cells

at 24 hours. **(b)** Taxol was included as a positive control. Representative figures showing populations of viable (LL), early apoptotic (LR), late apoptotic (UR) and necrotic (UL) cells. **(f)** Bar graphs showing percentages of apoptotic cells for each treatment sample at 24hours. Data were mean ± SD from two independent experiments (**P<0.01, ***P<0.001)

To determine if the observed cell death was due to apoptosis, flow cytometry was employed. RMG-1 and SiHa cells were treated with isorhamnetin for 24 hours then stained with annexin V and PI. In RMG-1 cells, the butanolic extract seemed to induce the most apoptosis compared to the other two extracts at 48.4% (Fig 15c), followed by the hexane with an apoptotic population of 42.1% (Fig 17d). The methanolic extracts seemed to induce minimal apoptosis at 13%, with a majority of the cells undergoing necrosis (Fig 15e).

As shown in 18, in SiHa cell lines, hexane extracts induced the highest population of apoptotic cells at 27% (Fig 16c), as compared to the butanolic (5.7%)(Fig 16d) and methanolic extracts (1.6%)(Fig 16e).

Next we examined cell cycle distribution by staining treated ovarian and cervical cells with PI and analyzed the percentages of G_0/G_1, S, G_2/M cell population using flow cytometry. An increase in the population undergoing cell death could be observed in RMG-1 cells treated with isorhamnetin.

A higher G_0/G_1 population, was observed in SiHa cells treated with isorhamnetin at 29,8%, 68,2% and 64,2% respectively as compared to the control cells. Cells treated with the butanolic extract also showed an increase in the G_2/M cell population at 14.7% compared to the untreated cells (Fig 17).

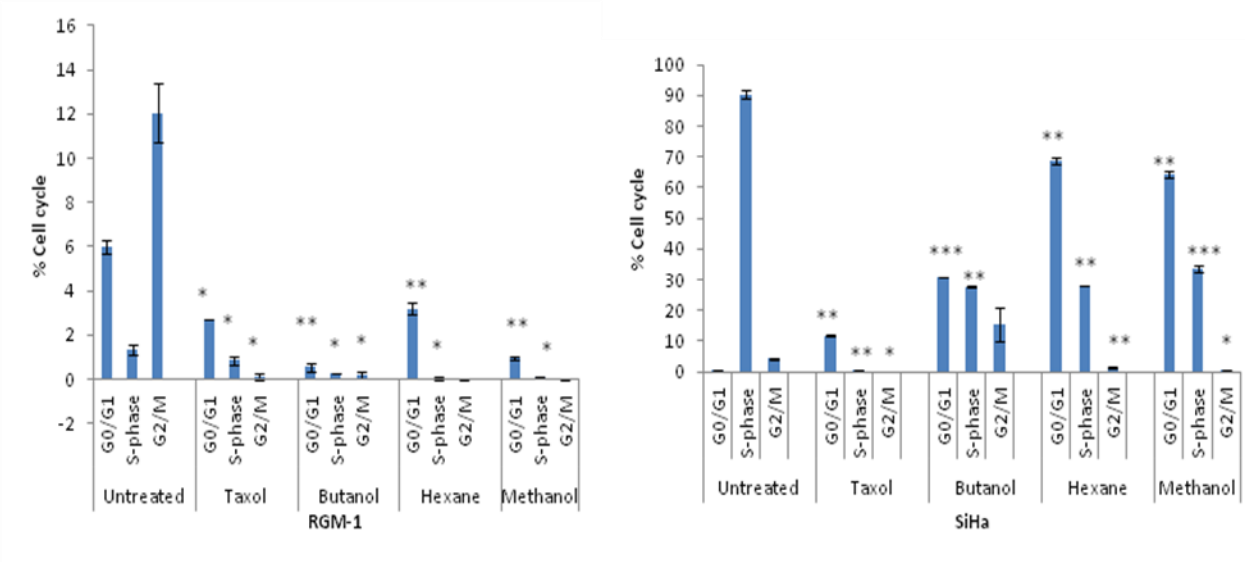


Figure 17 : Bar graphs representing cell cycle analysis of RMG-1 and SiHa cells

Cells were treated with butanolic, hexane and methanolic isorhamnetin extracts of *Euphorbia tirucalli*. Data were mean ± SD from two independent experiments (*P<0.05, **P<0.01, ***P<0.001)

4.2.3. Effects of Euphorbia tirucalli extracts on Anti-apoptotic, pro-apoptotic and cell cycle Molecules

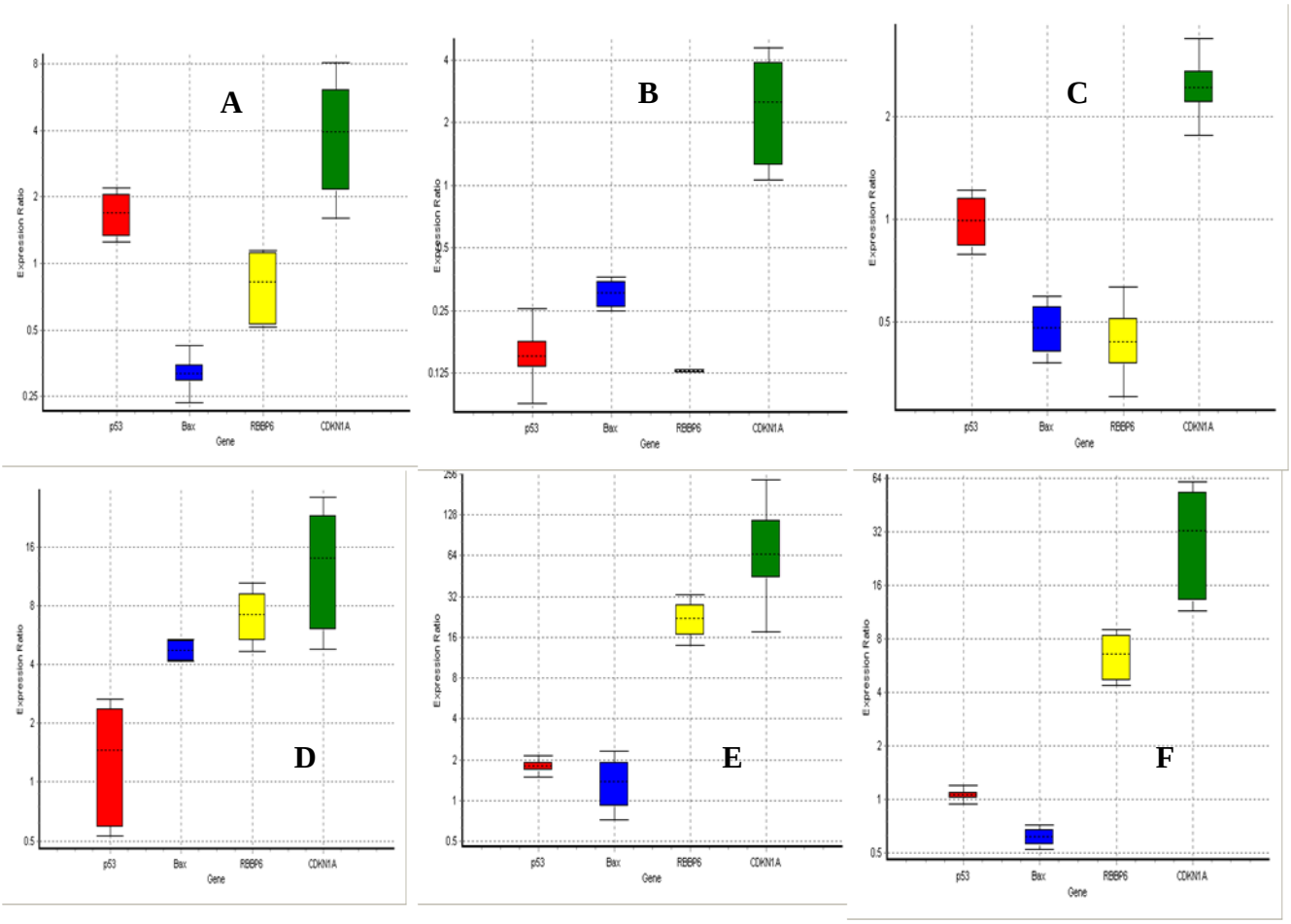


Figure 18 : Box plots representing Real Time PCR analysis using REST 2009 software.

(a-c) RMG-1 and (d-f) SiHa gene expression analysis following treatment with (a & d) butanol, (b & e) hexane and (c & f) methanol isorhamnetin extracts of *Euphorbia tirucalli* for 24 hours.

Real-Time PCR data showed that Bax was statistically down-regulated upon treating RMG-1 cells with isorhamnetin. Western blot analysis however, showed an increase in the butanol and methanol extracts treated samples. In SiHa cells, Bax was significantly upregulated in cells treated with butanol extracts by a mean factor of 4.736 in comparison to the control group but remained statistically unchanged in hexane and methanol treated. No significant

changes were observed in the expression of Bcl-2. Analysing caspase 3 and -9, we observed that in RMG-1 both caspase 9 and 3 expression remained statistically unchanged while in SiHa cells, a slight increase in caspase 9 expression was observed in butanol and methanol extracts.

Next analysis was to examine the molecules that regulate cell cycle to see if there were any changes in their expression. p53 functions as tumour suppressor and a transcription regulator of several genes. One of the genes it regulates is p21, also known as CDKN1A. p21 plays a crucial role in cell cycle arrest. Analysis of p21 revealed that it was up-regulated by the butanol, hexane and methanol isorhamnetin extracts by a mean factor of 3.584, 2.214 and 2.438 respectively in RGM-1 cells. In SiHa cells, up-regulation was observed in cells treated with all extracts at a mean factor of 11.746, 21.448 and 26.276 respectively.

In RMG-1 cells, p53 was up-regulated by a mean factor of 1.653 in butanol treated and down-regulated by a mean factor of 0.151 in hexane but remained statistically unchanged in methanol treated. In SiHa cells, p53 was statistically unchanged in butanol and methanol treated but up-regulated by a mean factor of 1.789 in hexane treated.

The expression of two p53 regulators, RBBP6 and mdm-2 was also investigated. In RGM-1 cells, RBBP6 was statistically unchanged in butanol extracts, but was down-regulated by a mean factor of 0.130 in hexane and 0.436 in methanol treated cells. In SiHa cells, RBBP6 was significantly up-regulated in butanol, hexane and methanol with mean factors of 7.035, 21.448 and 6.258 respectively. Western blot analysis showed a slight decrease in mdm-2 expression at 48 hours post treatment with the three extracts in RMG-1 cells. A slight increase in mdm-2 could be observed for butanol and hexane treated SiHa cells.

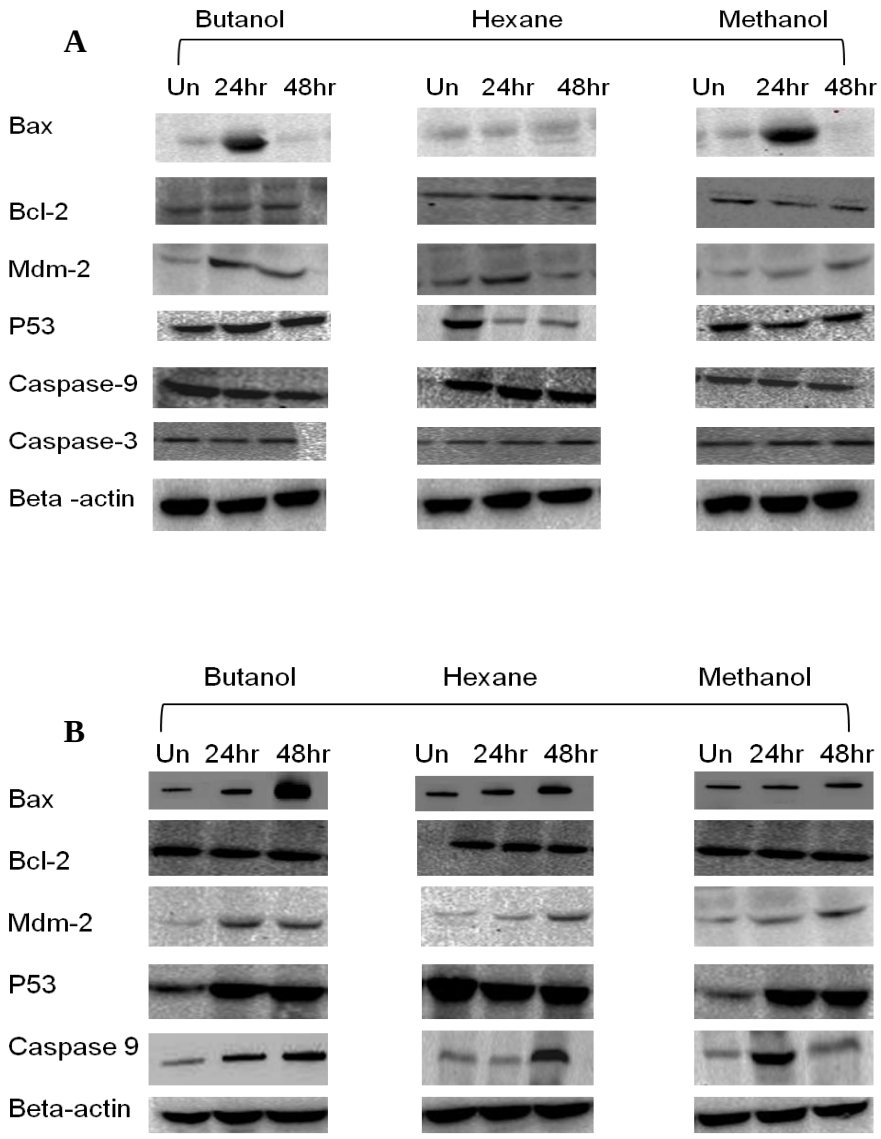


Figure 19 : Western blot analysis of protein expression in (a) RMG-1 and (b) SiHa cells following treatment with isorhamnetin extracts of *E. tirucalli*.

Un= Untreated samples. The results show a varying protein expression of different apoptotic genes 24 or 48 hours post treatment with the 3 plant extracts.

4.3. Discussion

The gynaecologic cancer burden is huge in developing countries accounting for 25% of all new cancers among women (Ferlay *et al* 2008). Of these, cervical cancer is the most common cancer of the female reproductive tract, whilst ovarian cancer is the most lethal of the gynaecological cancers (Sherris *et al* 2001; Jemal *et al* 2008). Plants have long been a source of important lifesaving pharmaceutical agents in developing countries. A number of drugs derived from plants are currently being used in the pharmaceutical industry (Veeresham 2012). One of the most important secondary metabolites of plants that were seen to have positive effect on health are flavonoids, which are antioxidants that play a role in reducing cancer development. Isorhamnetin is an O-methylated flavonol, a type of flavonoid. It can be found in many plants including *Euphorbia tirucalli* and is also a metabolite of quercetin (Li *et al* 2014).

In this study, we evaluated the anticancer activity of the Isorhamnetin extracts on RMG-1 and SiHa cancer cells. Isorhamnetin inhibited cell proliferation, decreased cell viability and induced cell death in a dose-dependent and cell type specific manner. A shortcoming of the use of endpoint cytotoxicity assays such as MTT is that they are invasive. Another is their inability to identify the appropriate time point for conducting any given assay. To address these issues, a Real-Time Cell Analyzer (RTCA) - xCELLigence System, which is a non-invasive and label-free way to continuously monitor cellular behaviour, was employed.

The xCELLigence system was used to predict the accurate concentration that is potent and also to monitor real time analysis of cell proliferation following treatment with Isorhamnetin extracted using 3 different solvents. The potency exhibited on the cells was dependent on the solvent type used for extraction. As shown from the results presented in Figure 16, the butanol extract exhibited the most potent activity on both RMG-1 and SiHa cell lines

(IC_{50}=10 µg/ml and IC_{50}= 15µg/ml) as compared to the hexane and methanolic extracts. According to the American National Cancer Institute (NCI), the criteria for cytotoxic activity of a crude extract is an IC_{50} of less than 30 µg/ml (IC_{50}<30 µg/ml) (Itharat *et al.* 2004). Using this criterion, the Isorhamnetin butanol extract could be considered as of promising anticancer potential. Both the hexane and methanol extracts showed an IC_{50}=30 µg/ml on RGM-1 cells, however, an IC_{50}=60 µg/ml and IC_{50}=100 µg/ml respectively was observed on SiHa cells. RGM-1 cells showed a higher sensitivity to taxol since 0,5 µM resulted in ~ 40% cell death whereas SiHa required five times that concentration (2,5µM) to induce the same amount of cell death which were similar to other studies (Rowinsky and Donehower 1995; Lee *et al.* 2005).

Following determination of the $IC_{50,}$ we then treated the cells with the appropriate IC_{50} concentration for all the three different solvents and the behaviour of the cells was monitored over time. It was interesting to observe that the extract decreased cell viability; however, the cells eventually started to recover from the initial cytotoxic effects of the extract and seemed to re-proliferate. Since the extracts seemed to induce cytotoxicity on the two cells, it was then important to determine if this cell death was as a result of apoptosis or necrosis.

Apoptosis is a tightly regulated process that plays a key role in maintaining tissue homeostasis (Kerr *et al.* 1972; Renehan *et al.* 2001. Discoveries of chemotherapeutic agents that can induce and recover the apoptotic response in tumour cells would be an efficient strategy. Isorhamnetin extracts showed antiproliferative activity on both cervical and ovarian cancer cell lines. We then aimed to investigate the type of cell death these extracts were inducing. Most therapies in anticancer treatment, such as chemotherapy, mainly induce cell death by causing either G_0/G_1 or G_2/M cell cycle arrest and then inducing an apoptotic pathway (Morgan 2003). Therefore, cell cycle arrest and the induction of apoptosis in cancer

cells become the major indicators of anticancer effects. Annexin V and PI staining were used to determine the type of cell death induced by the extracts. Flow cytometric analysis of the two cell lines revealed different proportions of apoptotic and necrotic cells. The butanolic extract seemed to induce the most apoptosis in RMG-1 cells. With the hexane extract, an almost equal population underwent apoptosis as those undergoing necrosis. Methanolic extracts seemed to induce necrosis in both RGM-1 and SiHa cells. The hexane extract seemed to induce a higher population of cells that undergo apoptosis in SiHa than the other two solvents. Lee and colleagues' *in vitro* study showed that isorhamnetin induced apoptosis, which is mediated by mitochondria-dependent caspase activation.

Cell cycle analyses using flow cytometer revealed that the majority of RGM-1 cells underwent cell death upon treatment with isorhamnetin. However, they arrested cell cycle at the G0/G1 phase in SiHa cells. In another study Jamillo *et al.* (2010) observed that isorhamnetin was able to increase the number of cells in G_2/M phase which correlated with our study. To test the mechanism behind the G_0/G_1 phase arrest observed in SiHa cells treated with isorhamnetin, we analysed p21 expression. We found an upregulation of p21 in treated RMG-1 and an even drastically higher upregulation in SiHa treated cells.

A complex cascade of cell signaling interactions is involved in cell cycle regulation and induction of the apoptotic pathway. One of these important regulators is p53 and, in fact, over 50% of cancers have been found to have a mutated dysfunctional p53 (Abbas and Dutta 2009; Wang and Sun 2010). We observed upregulated p53 in RMG-1 cells treated with butanolic extract and methanolic extract in SiHa cells. Mdm-2 and RBBP6 are negative regulators of p53 which were downregulated in RMG-1 cells following treatment with isorhamnetin.

The Bcl-2 family encompasses pro and antiapoptotic proteins that are primary regulators of the intrinsic or the mitochondrial pathway. The proapoptotic members of this family such as Bax, induce the release of cytochrome c and other proapoptotic factors, promoting caspase activation and eventually cell death (Schimmer *et al.* 2008). Real-Time PCR data showed that Bax was statistically downregulated upon treating RMG-1 cells with isorhamnetin. Western blot analysis however, showed a significant increase in Bax in the butanolic and methanolic extract-treated samples. In SiHa cells, Bax was significantly upregulated in cells treated with the butanolic extract and western blot analysis showed an increase in Bax in both butanolic and hexane treatments. No significant changes were observed in the expression of the antiapoptotic Bcl-2. In a similar type of study, myricetin-induced apoptosis correlated with the modulation of Bcl-2 family proteins and activation of the caspase-3 in human bladder cancer ((Hollams *et al.* 2002; Raghavan and Bohjanen 2004; Sun *et al.* 2012). The results we observed in our study show that the expression of both caspases remained unchanged in RGM-1 cells treated with isorhamnetin. However, a slight increase in caspase-9 could be observed in SiHa cells.

CHAPTER 5 : PHYTOCHEMICAL ANALYSIS OF *E. TIRUCALLI* EXTRACTS

5.1. Introduction

This chapter covers the phytochemical analysis of extracts of *E. tirucalli* following extraction with three different solvents i.e. methanol, butanol and hexane. We show the different fractions identified and secondary metabolite that were positive in our extracts.

5.2. Results

5.2.1. Identification of compounds from Euphorbia tirucalli using TLC and identification of secondary metabolites

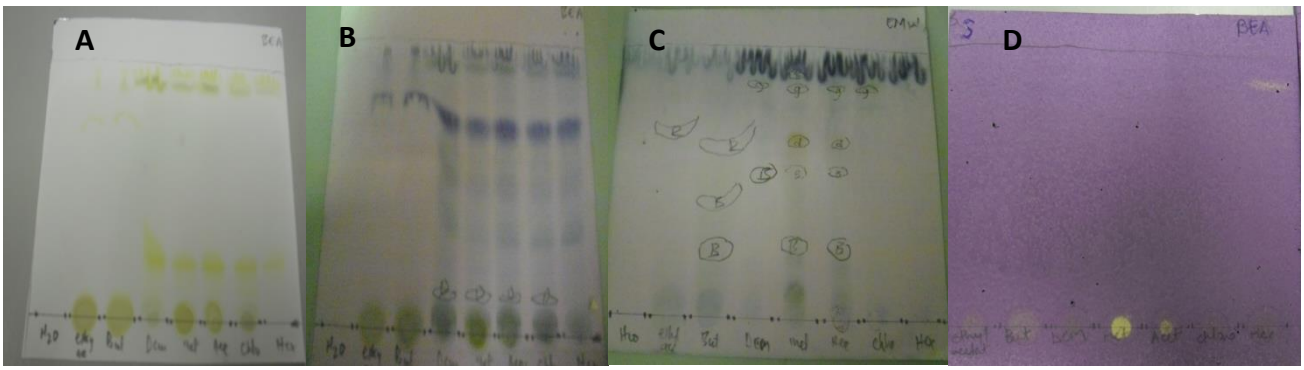


Figure 20 : Thin layer chromatographic separation of butanolic, hexane and methanolic fraction from *E. tirucalli.*

Chemical analysis of the butanolic, hexane and methanolic fractions showed the presence of tannins, flavonoids, phenols, antioxidants and terpenes (Table 7, fig. 20). The LC-MS analysis has shown the presence of a variety of compounds in the *E. tirucalli* extracts which have been shown to possess anticancer properties by previous research (Table 8).

Table 7 : Secondary metabolites tests of *E. tirucalli*

Secondary metabolite	**Presence**
Tannins	Positive
Terpenes	Positive
Steroids	Negative
Cardiac glycosides	Negative
Flavonoids	Positive
Saponins	Negative
Phenols	Positive

5.2.2. HPLC- MS analysis

For analysis of phytochemicals present in *Euphorbia tirucalli*, HPLC analysis (HPLC-MS) coupled to mass spectroscopy was used. The advantage to HPLC-MS is its ability to analyse compounds that exhibit high polarity or have a high molecular mass or are thermally labile. This would ensure that a broad spectra of peaks from various molecules, each with a unique mass-to-charge ratio (m/z), would be detected from the sample (Figure 21) (Zhu *et al*., 2013). The figures below (are profiles of compounds identified from LC-MS of the methanol, butanol and hexane extracts of *E. tirucalli*. The mass of these compounds were identified by determining the charge that the quadrupoles were at upon molecular impact and the compound's retention time (RT). The list of compounds found in the extracts was then tabulated below (Table 8).

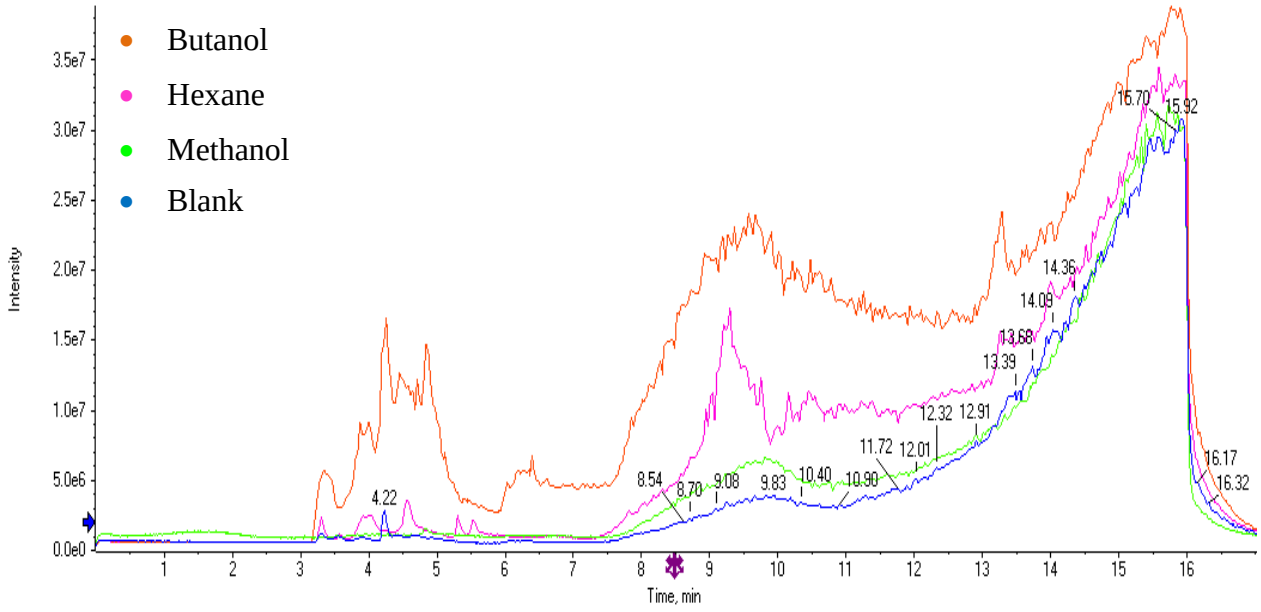
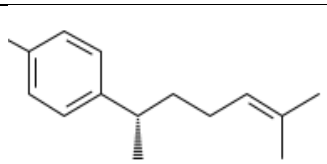
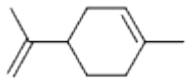
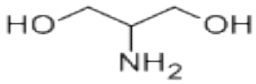
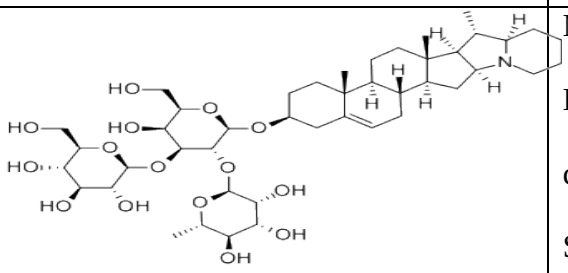
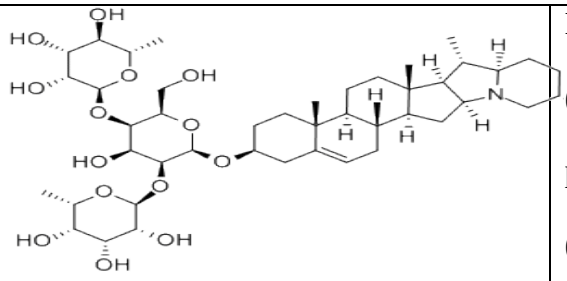
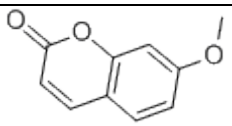
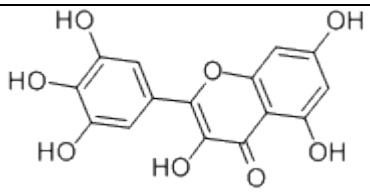
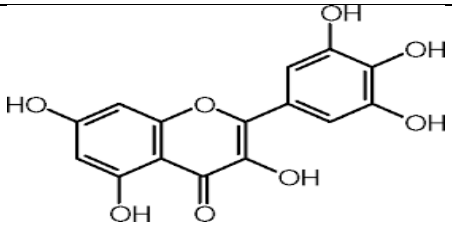
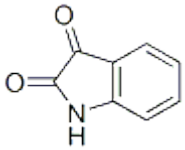
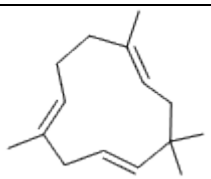
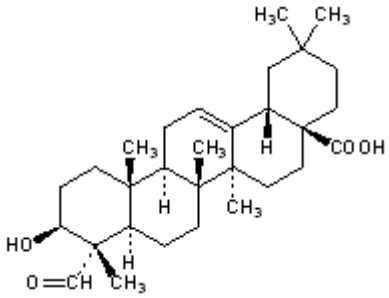
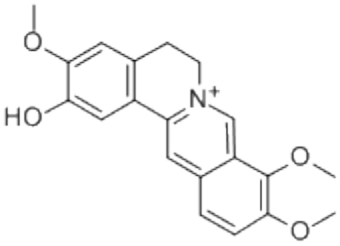
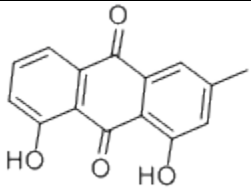
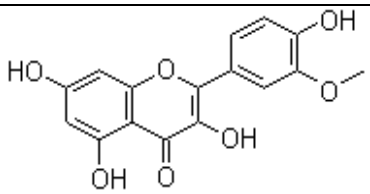


Figure 21 : HPLC-MS chromatogram of *E. tirucalli* butanol, hexane and methanol extract

Table 8 : Phytochemicals identified in *Euphorbia tirucalli* extracts

Compound	Structure	Solvent and cancer type for antiproliferative effect	Reference
Alpha-Curcumene		Methanol, hexane, Ovarian cancer	Shin and Lee 2013
d-limonene		Methanol, butanol, mammary carcinomas	Crowell and Gould 1994
Serinol		Methanol, human neuroblastoma, glioma, medulloblastoma, adenocarcinoma	Bieberich *et al*. 2002
Alpha-solanine		Methanol, butanol, Human pancreatic cancer cell lines, SW1990 and Panc-1	Sun *et al*. 2014

Alpha-chaconine		Hexane, prostate (LNCaP) and prostate cancer-3 (PC-3).	Reddivari *et al.* 2010
Alpha-pinene		Hexane, metastatic melanoma.	Matsuo *et al.* 2011
Herniarin		Hexane, ovarian (CH1), lung (A549) and melanoma (SK-MEL-28)	Valiahdi *et al.* 2013
Linamarin		Butanol, MCF-7, HT-29 and HL60 cells	Idibie *et al.* 2007
Myricetin		Butanol, HCT-15 human colon cancer cells	Kim *et al.* 2014
Isatin		Butanol, K562 cell line	Aboul-Fadl *et al.* 2012

Alpha-humulene		Methanol, breast cancer MCF-7, colon cancer HCT-116	el Hadri *et al.* 2010
Gypsogenin		Methanol, Saos-2 and MCF-7	Emirdağ-Öztürka *et al.* 2014
Columbamine		human osteosarcoma U2OS	Bao *et al.* 2012
Chrysophanol		Butanol, human breast and colon adenocarcinoma	Yuenyongsawad *et al.* 2014
Isorhamnetin		Butanol, human hepatocellular carcinoma	Teng *et al.* 2006

5.2.2.1. Specific compounds

This section covers the selected individual compounds identified with LC-MS.

Compound 1: herniarin

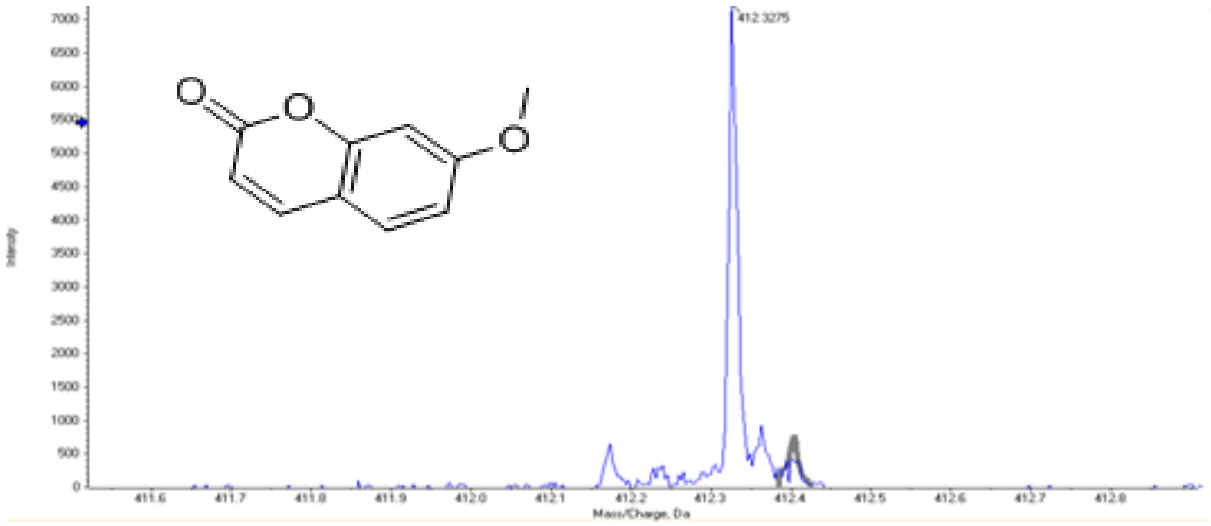


Figure 22 : LC-MS chromatographic profiles herniarin. Compound structure displaying highlighted peaks at 412.32 m/z

Herniarin (C30H50) is one of the compounds that was identified in the hexane extract of *E. tirucalli*. The compound has a mass spectrum found at 15.631 min (Figure 13A), with peaks highlighted at 412.32 m/z for the protiated compound (M+H+) and 410.39 and 411.39 for its isotopic species (M+1 and M+2 respectively) respectively.

Compound 2: Isatin

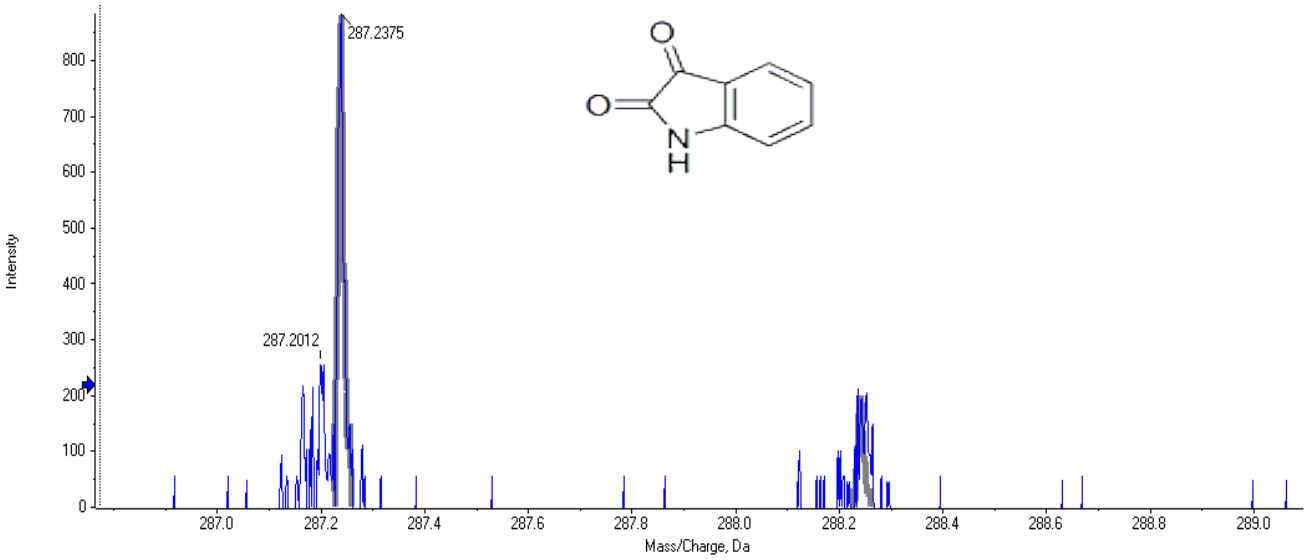


Figure 23 : LC-MS chromatographic profiles Isatin. Compound structure displaying highlighted peaks at 287.20 and 287.23 m/z

Isatin (C20H30O1) is one of the compounds that were identified in the butanol extract of *E. tirucalli*. The compound has a mass spectrum found at 13.383 min, with peaks highlighted at 286.2375 m/z for the protiated compound (M+H+) and 287.2375 for its isotopic species (M+1).

Compound 3: Alpha-solanine

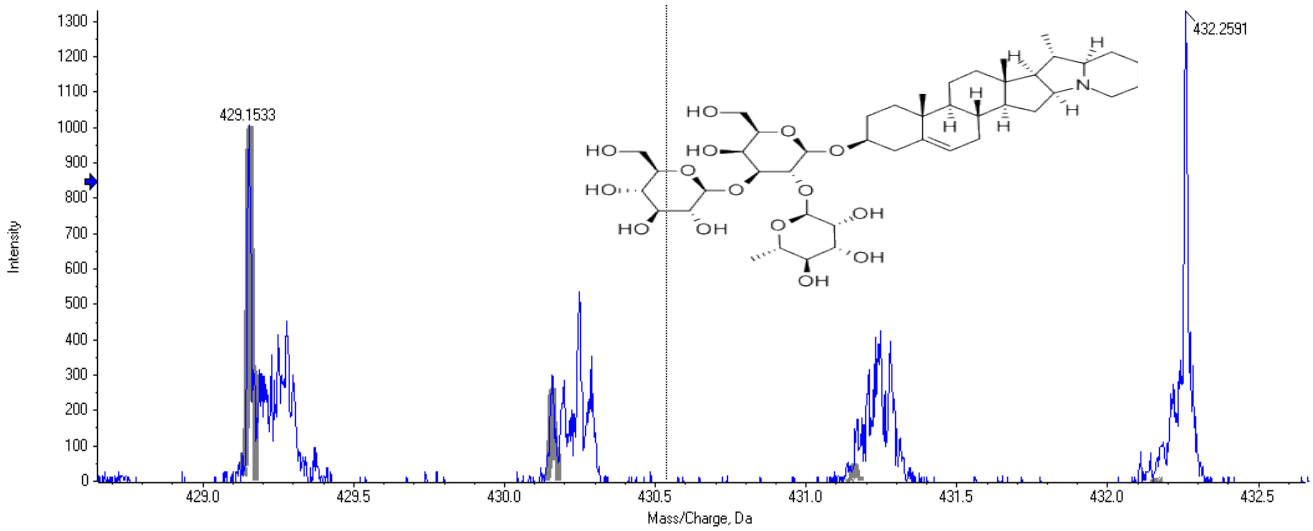


Figure 24 : LC-MS chromatographic profiles Alpha-solanine. Compound structure displaying highlighted peaks at 429.15 and 432.25 m/z

Alpha-solanine (C23H24O8) is one of the compounds that was identified in the hexane extract of *E. tirucalli*. The compound has a mass spectrum found at 11.58 min, with peaks highlighted at 428.14 m/z for the protiated compound (M+H+) and 429.1543 (M+1).

Compound 4: linamarin

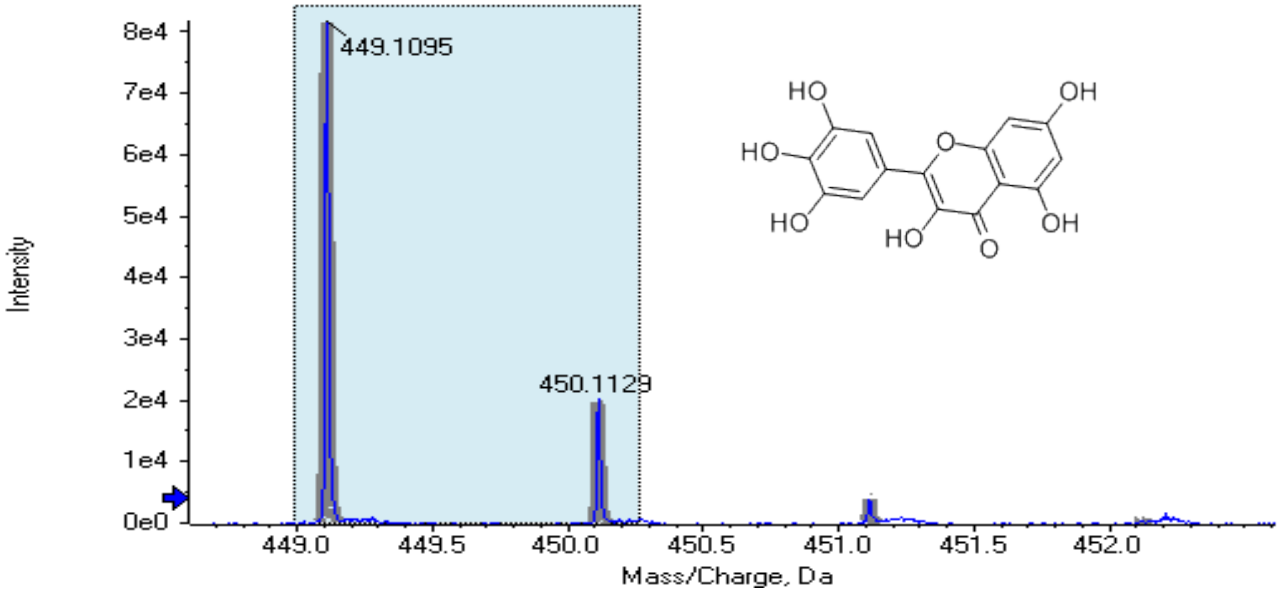


Figure 25 : LC-MS chromatographic profiles linamarin. Compound structure displaying highlighted peaks at 449.10 and 450.11 m/z

Linamarin (C21H20O11) is one of the compounds that were identified in the butanol extract of *E. tirucalli*. The compound has a mass spectrum found at 10.09 min, with peaks highlighted at 448.1078 m/z for the protiated compound (M+H+) and 429.1543 and 450.15 for its isotopic species (M+1 and M+2 respectively) respectively.

Compound 5: α-curcumene

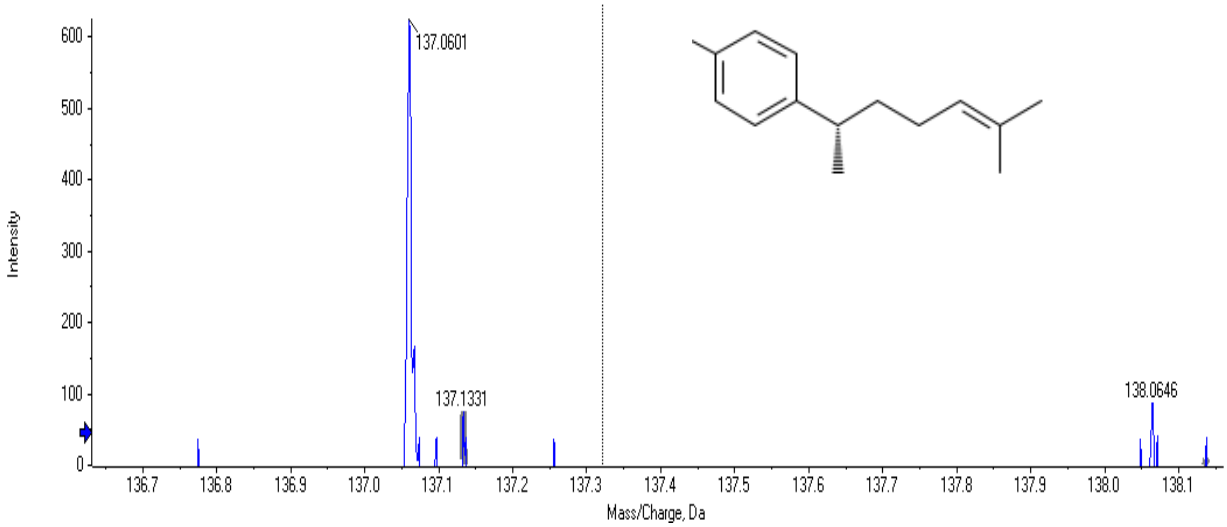


Figure 26 : LC-MS chromatographic profiles α-curcumene. Compound structure displaying highlighted peaks at 137.13 and 137.06 m/z

Alpha curcumene (C10H16) is one of the compounds that were identified in methanol and hexane extracts of *E. tirucalli*. The compound has a mass spectrum found at 14.7 min, with peaks highlighted at 136.13 m/z for the protiated compound (M+H+) and 137.13 for its isotopic species (M+1).

5.3. Discussion

The LC-MS is the most effective form of data analysis as it ensures that a wide range of compound identification is achieved. These include polar, non-polar, thermoliable and semi-volatile compound identification (Korfmacher, 2005). Compound identification was achieved through non-target screening of the extracts of *E. tirucalli*. Compound confidence was determined by mass accuracy, retention time and isotope pattern, this is performed by matching the results with those found in the ABSciex library. Retention time (RT) is the amount of time a compound spends in the column (Korfmacher, 2005). It is the sum of the time it takes a compound to move from the column to detector. A compound's RT will vary

depending on the pressure used- as it affects flow rate of the solvent), the nature of the stationary phase (both the solvent and particle size), the exact composition of the solvent and the temperature of the column (Vipul *et al*. 2005). Most of the metabolic compounds have similar characteristics and size and tended to be identified at similar retention time.

The TLC was used to identify different fragments of compounds and secondary metabolites also indicated different types which showed presence of flavonoids which are mainly associated with anticancer activity (Fig 24). In this study, we were interested in identifying compounds that were able to induce apoptosis or cell cycle arrest in cancer cells. As observed in the results section. Several compounds were identified that have a history of being used in anticancer studies such as α-curcumene, linamarin, columbamine, serinol and gypsogenin. These compounds were tested in variety of cancer cells extracted from different plants. In all cases they were found to inhibit cell death in these cells.

5.4. Conclusion

This chapter has shown that compounds present in *E. tirucalli* are mainly flavonoids and terpenes that were previously associated with anticancer activity.

CHAPTER 6 : GENERAL DISCUSSION AND CONCLUSION

Cancer is a major public health problem worldwide which exacts an enormous economic, emotional, and physical cost from society. Millions of people are diagnosed with cancer annually and, according to the American Cancer Society, it is expected that the global cancer burden will double by 2030 if preventative measures are not applied. Advances have been made in cancer treatments but these conventional therapies cause adverse side effects and merely extend a person's lifespan by just a couple of years. Hence, it becomes important to identify and exploit novel potential anticancer therapeutics. Medicinal plants have recently attracted the attention of the scientific and pharmaceutical communities and a number of publications have documented their therapeutic potential. Attention has been drawn to their anticancer potential due to the limitations that have been associated with the current cancer treatment including their adverse toxicities, high cost and patient relapse. The advantage of using medicinal plants as a cancer treatment is their relatively non-toxic nature. The search for anticancer agents from plants started in the 1950s with the discovery of vinca alkaloids isolated from the plant *Catharanthus roseus* (Hait *et al*. 2007; Ngan *et al.* 2000). This resulted in further plant research programs that led to the discovery of many novel chemotypes with a range of cytotoxic activities.

Medicinal plant research involves identification of secondary metabolites produced by plants, and these have wide ranging chemical, physical and biological activities. In our analysis of the plant *E. tirucalli*, using the following techniques TLC, HPLC and Mass Spectroscopy, it was found to constitute a source for a variety of flavonoids, alkaloids, saponins and terpenes (table 4.1) In several studies similar to ours, a number of these plant secondary metabolites extracted from different plants have been used successfully as anticancer agents (Aboul-Fadl *et al.* 2012; Kim *et al.* 2014). The terpenes are one of the largest and most diverse groups of

secondary metabolites from plants. Limonoids, which are a group of triterpenes present in our plant extracts, have previously been used successfully in inhibiting cell proliferation in human tumour cell lines using *in vitro* bioassays (Ahn 1994). Most of the earliest isolated pure compounds with biological activity were alkaloids. Alkaloids, isolated from medicinal plants have shown potent antiproliferative properties against a variety of cancers. Alkaloids such as camptothecin and vinblastine have successfully been developed into anticancer drugs (Jian *et al*. 2012). Columbamine, an alkaloid we identified in *E. tirucalli*, was previously isolated from the herb *Coptis chinensis* and was found to inhibit proliferation and neovascularization of metastatic osteosarcoma cells (Bao *et al*. 2012).

Phenolics and polyphenols which include phenols and flavonoids are another group of secondary metabolites. The flavonoids myricetin and isorhamnetin were found to be present in *E. tirucalli*. These flavonoids have previously demonstrated potent anticancer activities against bladder and colorectal cancers (Sun *et al*. 2012; Saud *et al*. 2013). Saponins are a group of naturally occurring glycosides, and about 150 natural saponins have been found to exhibit significant anticancer properties (Man *et al*. 2010). The saponin, gypsogenin which we identified in *E. tirucalli*, has previously demonstrated an inhibitory effect on human cancer cell lines (Emirdağ-Öztürk *et al*. 2014). Even though all this work has been performed on single secondary metabolites and they continued to show a positive response towards cancer cells *in vitro*, they continue to fail as we progress to *in vivo* analysis due to the fact that these groups of compounds work as antagonists to minimise the cytotoxicity of each other. We, therefore, opted to work with a cocktail of these secondary metabolites to see if they can offer any hope in the treatment of cancer cells.

6.1. Cytotoxicity assay

The MTT assay is a simple colorimetric assay used to measure cell cytotoxicity, proliferation and viability. This assay is based on the conversion MTT into formazan crystals by mitochondrial dehydrogenase activities in living cells. The total mitochondrial activity is related to the number of live cells. The resulting formazan, which is insoluble in water, requires the addition of an organic solvent such as DMSO to solubilize the crystals before absorbance can then be measured. This assay was used to test the antiproliferative properties of butanolic, hexane and methanolic extracts of *Euphorbia tirucalli* against breast, cervical and ovarian cancer cells lines. The results suggest that *E. tirucalli* extracts exhibited cytotoxicity in a cell type and concentration-dependent manner for all three extracts. Butanol extracts exhibited the most cytotoxicity on MCF-7 and MDA-MB 231 breast cancer cells, compared with SiHa and RMG-1 with IC_{50} values of 60, 40, 80 and 100 µg/ml respectively. MDA-MB231 cells showed the highest sensitivity to hexane extracts, with an IC_{50} of 50 µg/ml, followed by RMG-1 cells with an IC_{50} of 60 µg/ml. RMG-1 cells were the most sensitive to methanol extracts with an IC_{50} of 50 µg/ml as compared to SiHa, MCF-7 and MDA-MB 231 cells with IC_{50} of 90, 80 and 100 µg/ml respectively.

There are several limitations to the use of endpoint assays such as MTT for cytotoxicity assays: (i) It is labor intensive, requiring a labeling step (ii) If the extracts being tested interfere with mitochondrial function, false positives may be obtained (iii) they provide limited information of the dynamics of drug interaction with the cells (iv) Since MTT is absorbance based, cells tend to interfere with the method of detection and can greatly obscure the data. For these reasons, MTT should only be used as a preliminary method for screening cytotoxicity.

To validate the observed results, cell growth inhibition was confirmed by the use of real-time proliferation assay (RTCA) which offers non-invasive analysis of cell proliferation over time. As cells interact with electronic biosensors at the bottom of the plates, a cell-electrode impedance response is generated that correlates with the number of viable cells. The advantages to this system include non-invasiveness to cells, no labelling and, unlike endpoint assays, it allows real time measurement of cell viability. Furthermore, real-time monitoring allows calculation of time-dependent IC_{50} values when testing cytotoxic extracts.

Experiments were carried out using xCELLigence RTCA DP analyzer (Roche). For the determination of $IC_{50,}$ cells were treated with the extracts for 24 hours. It was observed that IC_{50} for almost all treatments was much less than that estimated by the MTT assay. This suggested that the *E. tirucalli* extracts inhibited cell proliferation at much lower concentrations than previously suggested by MTT. From the RTCA data we can deduce that not only did *E. tirucalli* extracts induce cytotoxicity in a cell type but also in a concentration-dependent manner. However, the advantage with the RTCA was that at each time point we were able to analyse the IC_{50} even though the general outcome indicated that the extracts were able to inhibit cell proliferation in a concentration-dependent manner. The RTCA data suggests that the butanolic extracts exhibited the most potency on all cell lines with IC_{50} values of 10, 15, 15 and 30 µg/ml for RMG-1, SiHa, MCF-7 and MDA-MB231 cells respectively. The methanol extracts appeared to be the least cytotoxic with IC_{50} values of 30, 100, 50 and 100 RMG-1, SiHa, MCF-7 and MDA-MB231 respectively. RMG-1 and MCF-7 cells showed the most sensitivity to hexane extracts with IC_{50} of 30 µg/ml for both cell lines as compared to MDA-MB 231 and SiHa cells at an IC_{50} of 50 and 60 µg/ml respectively.

Our findings are in line with a study by Eljabarin *et al*. (2014), who tested the anticancer properties of the methanolic extracts of *E. tirucalli* on EMT-6 mouse breast cancer cells. It

was found that the extracts drastically reduced viability of the cells at low concentrations. Cell proliferation was inhibited in a concentration-dependent manner. A different study investigated the antitumour properties of euphol, a tetracyclic triterpene alcohol which is the main constituent of *E. tirucalli*. Their results were in line with our findings: euphol demonstrated potent antitumour activity on a wide range of cancers, including breast, prostate, lung, pancreas and cervical cancer in a dose and time-dependent manner (Reis *et al.* 2013). The MTT and RTCA results showed that *E. tirucalli* exhibited antiproliferative properties on different cancer cell lines. However, it was important to determine the mechanism by which the observed cell death is induced.

6.2. Cell cycle analysis by flow cytometry

To investigate the functional mechanism of *E. tirucalli* in inhibiting cell proliferation, its effect on the regulation of the cell cycle in cancer cells was determined. Cell cycle analysis is used to distinguish the proportion of cells that are in one of the three interphase cell cycle stages. There are three distinct stages within interphase, called G1, S, and G2 phases. During G1 cells make proteins needed for DNA replication. If conditions are right, cells commit to DNA synthesis at S phase, at this point cells will have more DNA than cells at G1. A G2 phase follows where cells continue to grow and synthesize a variety of proteins; cells at G2 will have about twice the DNA content of cells at G1. DNA content in cells can be readily quantitated by flow cytometry of cells stained with propidium iodide, a fluorescent DNA intercalating dye. The premise with propidium iodide dye is that it is stoichiometric, this means it binds in proportion to the amount of DNA present in the cell. In this way, cells at G2 phase will take up proportionally more dye and fluoresce twice as brightly as cells in G1 which can then be measured by flow cytometry.

Cancer cells were treated with extracts at an IC_{50} and then fixed with 70% ethanol 24 hours following treatment. Cells were then stained with propidium iodide and analysed using a flow cytometer. In breast cancer cells, cell cycle arrest was observed at G0/G1 in both MCF-7 and MDA-MB231 cells following treatment with plant extracts. Furthermore, S-phase showed a lower cell population which is an indication that the cells were unable to divide. Cell cycle analyses revealed that the majority of RGM-1 cells underwent cell death upon treatment with all three extracts. However, they arrested cell cycle at the G0/G1 phase in SiHa cells.

Results are in line with a study by Wang *et al.* (2013), who examined the anticancer effect of euphol, a tetracyclic triterpene alcohol from *E. tirucalli* against T47D human breast cancer cells. They observed cell cycle G1 arrest and proliferation inhibition of T47D cells upon treating cells with euphol. We showed that extracts of *E. tirucalli* induced cell cycle arrest in the cancer cells. Deregulation of the cell cycle has been implicated in carcinogenesis. Thus, cell cycle has emerged as one of the attractive therapeutic targets in the treatment of cancer. Currently a majority of the chemotherapeutic agents cause cell cycle arrest either at G0/G1 or the G2/M stage

6.3. Annexin V/PI apoptosis assay

Apoptosis is an evolutionary conserved form of cell suicide, which is fundamental for the development, health and in maintenance of tissue homeostasis in multicellular organisms. Defects in apoptosis regulation have been implicated in a number of malignancies. This process is characterized by specific morphologic features, including cleavage of DNA and condensation of the cytoplasm and nucleus. One of the major hallmarks of apoptosis is loss of membrane phospholipid asymmetry which leads to exposure of phosphatidylserine residues externally (Fadok *et al*. 1992). Annexin V, a Ca2+-dependent phospholipid-binding protein, has high affinity for these residues. Fluorescein isothiocyanate (FITC) labelled Annexin-V

can be used to detect the phosphatidylserine residues translocated to the surface of the plasma membrane. Propidium iodide (PI) detects necrotic cells with permeabilized plasma membrane. Double staining with Annexin V and PI enables quantification of apoptotic, viable and necrotic cells. The test would thus discriminate PI positive (necrotic), FITC positive/ PI negative (early apoptotic), FITC positive/PI positive (late apoptotic) and FITC negative/PI negative (live) cells.

To determine whether the observed cell death was as a result of apoptosis, cells treated with *E. tirucalli* extracts for 24 hours at an IC_{50} were double stained with annexin and PI and analysed using flow cytometry. The data acquired showed that in the untreated samples, the majority of cells were viable and non-apoptotic. In contrast, when cells were treated with *E. tirucalli* extracts, a lower proportion of cells remained viable. There was an increase in early apoptotic cell populations in MDA-MB 231 cells treated with all three extracts, however, an increase in the necrotic population was also observed in those treated with methanolic extracts. An increase in early and late apoptotic populations was observed for MCF-7 cells treated with all three extracts. In RMG-1 and SiHa cells treated with all three extracts, an increase in late apoptotic and necrotic populations could be observed. These results are in line with a study by Lin *et al.* (2012) who investigated the anticancer effects of euphol against human gastric cancer cells. Euphol treatment was found to significantly induce apoptosis in the CS12 AGS, and MKN45 gastric cancer cell lines.

A limitation to this assay includes its inability to distinguish cells that have died as a result of the necrotic pathway versus those that have undergone apoptotic death. Since cells which are considered viable, with intact membranes, will obviously exclude PI and Annexin V, cells that are in early apoptosis will be Annexin V positive and PI negative. Membranes of cells that are in late apoptosis or cells that are already dead are permeable to both Annexin V and

PI. Hence it is difficult to determine if cells that are already dead died via the apoptotic or necrotic pathways. It would thus be beneficial to measure apoptosis over time and track them as cells move from Annexin V and PI negative, to Annexin V positive and PI negative up until the final stages of where they stain Annexin V and PI positive. In this way, one could get a better insight as to how cell death occurred as opposed to one single observation which does to give a clear picture.

To examine the mechanisms underlying the observed antiproliferative effects of *E. tirucalli* we went on to investigate the expression of genes involved in the regulation of apoptosis.

6.4. Caspase 3/7 activity

Apoptosis is a complex process which requires an intricate balance between a number of regulatory genes. Regulation of apoptosis is mainly orchestrated by the activation of caspases, which are a family of cysteine proteases that can be classified into initiator and effector caspases. Activation of effector caspase-3/7 plays a central role in inducing important downstream proteins associated with execution of apoptosis.

To examine the molecular mechanism underlying the apoptosis process, we stained cancer cells with aminoluciferin-labeled substrate of caspase and determined the caspase-3/7 activities by measuring the luminescence intensities after 24 hours of treatment. From the data, we observed a gradual increased caspase-3/7 activity in MDA-MB 231 cells treated with hexane and methanolic extracts whereas those treated with butanolic extracts remained unchanged as compared to the control. In contrast, there was a significant reduction of caspase 3/7 activity in hexane treated MCF-7 cells. It is important to note that MCF-7 cells are one of the breast cancers which have a deletion in the caspase-3 gene, making them

highly resistant to chemotherapeutics. Caspase-3 is an important effector caspase that plays a role in the execution-phase of apoptosis.

Our data suggest that apoptosis induced by extracts of *Euphorbia tirucalli* was caspase-3 dependent in MDA-MB231 cells. However, this was not the case in MCF-7 cells where apoptosis induction by the extracts does not correlate with the activation of caspase-3 but may activate different apoptotic pathways and other effector caspases.

6.5. Prediction of molecular mechanism

We showed that extracts of *E. tirucalli* induced cell cycle arrest and apoptosis in the cancer cells. However, the molecular mechanism underlying cell cycle arrest and apoptosis were still not clear at this stage. Real-time PCR and western blot were used to gain further insight into the mechanisms underlying the observed antiproliferative effects. Real-time PCR is the most sensitive technique for detecting mRNA levels even from very small samples. An advantage to real time PCR is that the efficiency of the reaction can be precisely calculated, PCR product do not need to be run out on a gel after the reaction and it can be used to perform truly quantitative analysis of gene expression. Real-time PCR is able to detect the amount of DNA formed after each cycle with fluorescent dyes. The intensity of the fluorescence emitted during RT-PCR correlates to the amount of DNA product formed. In this study, to investigate expression of genes between cells treated with extracts of *E. tirucalli* and untreated cells, we used SyBr green, which is a naturally fluorescing dye which can intercalate between double stranded DNA. When unbound, it exhibits low level fluorescence but emits brighter fluorescence upon binding to double stranded DNA.

In gene expression studies it is important to study not only the expression of a gene at RNA level but also at a protein level. Western blot is an immunoblotting technique used to detect

specific proteins in a sample: it relies on the specificity of binding between a protein and an antibody raised against that protein. To identify proteins involved in cell cycle arrest and apoptosis, proteins extracted from treated and untreated cells were separated on a SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked to prevent unspecific binding and stained with specific antibodies. Bands were then detected on a Chemidoc imaging system upon developing with a chemiluminescence reagent.

To test the mechanism behind the G0/G1 phase arrest observed in treated cancer cells, p21 expression was analysed. P21 is a potent cyclin-dependent kinase inhibitor that is usually expressed in the G0/G1 phase of the cell cycle. From our results, it was observed that the three extracts upregulated the expression of p21 in all the cell lines. A similar mechanism was reported by Wang et al 2014, where they showed the effect of euphol on cell cycle in human breast cancer cell lines. This upregulation of p21 is noteworthy as the development of some cancers has been associated with a defective p21.

A complex cascade of cell signaling interactions is involved in cell cycle regulation and induction of the apoptotic pathway. One of these important regulators is p53. p53 is a tumour suppressor which is essential for cell cycle arrest at G1and G2 and apoptosis regulation (Kastan *et al*. 1991; Agarwal 1995). The importance of p53 as a tumour suppressor is evidenced in its high frequency of mutation in human cancers. Approximately 50% of human cancers have been found to be mutated in p53 (Potten and Wilson 2004). From our results, we observed an upregulation of p53 in MDA-MB231 and SiHa cells treated with methanol extracts of *E. tirucalli.* Butanol extracts upregulated p53 in RGM-1 cells. We also investigated the expression of two p53 negative regulators: mdm2 and RBBP6. RBBP6 was downregulated in butanol extract treated MCF-7 cells and in RGM-1 cells treated with

hexane and methanol extracts. The three extracts resulted in decreased mdm-2 expression in RMG-1 cells but remained statistically unchanged in other cell lines.

The Bcl-2 family encompasses pro and antiapoptotic proteins that are primary regulators of the intrinsic pathway. The proapoptotic members of this family such as Bax and Bak 1 induce release of cytochrome c and other proapoptotic factors, promoting caspase activation and eventually cell death (Schimmer *et al* 2008). In MCF-7 cells treated with *E. tirucalli* extracts, Bcl-2 was downregulated whilst Bax and Bak 1 were upregulated. In MDA-MB 231 cells, the ratio of Bcl-2/Bax was reduced in comparison to control samples due to the significant increase in the expression of Bax. In RMG-1 and SiHa cells, Bax was significantly upregulated whilst Bcl-2 remained unchanged. Interestingly, a study by Ma *et al*. (2007) investigated the anticancer properties of isorhamnetin on human esophageal squamous carcinoma cell line Eca-109 and observed similar results. Isorhamnetin is a flavonoid which we also found to be present in our plant *E. tirucalli*. On treating the Eca-109 cells with isorhamnetin, they observed an increased expression of Bax and a decreased expression of Bcl-2, resulting in an increased ratio of BAX to BCL-2. Similarly, Lin *et al.* (2012) observed an upregulated BAX expression and downregulated Bcl-2 protein expression following treatment of gastric cancer cells with euphol. The mitochondrial pathway of apoptosis is the major mechanism of physiological cell death in vertebrates and its activation represents a major mechanism for cancer regression. The importance of regulation of the Bcl-2 family in cancer is evidenced by the number of small molecule antagonists to pro-survival Bcl-2 that have been developed. Some of these are still undergoing clinical trials. Drugs such as Obatoclax and Gossypol work by blocking the binding of antiapoptotic Bcl-2 to proapoptotic Bcl-2 family proteins thereby inducing apoptosis in cells (Powell *et al.* 2003; Cho *et al.* 2012). These drugs exhibited antitumour activity in patients with refractory leukemia,

myelodysplasia, prostate cancer and colorectal carcinoma (Ko *et al.* 2007; Schimmer *et al.* 2008; Volate *et al.* 2010).

Caspases are a family of cysteine proteases that are involved in mediating cell death signaling. The caspase−9 and 8 are upstream caspases that converge to caspase-3, a key effector molecule in the caspase-dependent cell apoptosis pathway that cleaves a number of cellular proteins including PARP, leading to DNA fragmentation and triggering apoptosis. Following treatment with *E. tirucalli* extracts, expressions of caspase-9, 8 and 3 were determined. In MDA-MB231, both caspase-3 and 8 were upregulated in butanol treated cells whilst caspase-8 was also upregulated in hexane treated cells which suggested activation of the extrinsic pathway. However, in MCF-7 caspase-8 and 9 expression remained statistically unchanged. Analysing caspase-3 and 9 in RMG-1, we observed that their expression remained statistically unchanged while in SiHa cells, a slight increase in caspase-9 expression was observed in butanolic and methanolic extracts.

6.6. Conclusion

Apoptosis is essential to proper tissue and organ development, and defects in this pathway are known to be involved in cancer development. Any strategy to a successful cancer therapy, must involve the induction of apoptosis in these affected cells so that they can naturally commit themselves to apoptosis. Inducers of apoptosis are considered good agents in anticancer therapeutics. Medicinal plant extracts have recently attracted attention to modern medical science research with their non-lethal activity. In this study, crude extracts of *E. tirucalli* extracted using butanol, hexane and methanol solvents were screened for antiproliferative activity in breast (MCF-7 and MDA-MB231), ovarian (RMG-1) and cervical (SiHa) cancer cell lines. In this study, we observed that *E. tirucalli* extracts were able to inhibit cancer cell proliferation in a concentration and cell type-dependent manner.

In trying to classify the type of cell death or the molecular mechanism associated with such inhibition, cell cycle arrest at G_0 was observed as the mode of inhibition resulting from treated cells with this compound with p21 being over expressed in all cells and all treatments. The extrinsic pathway of apoptosis was identified as the type of cell death induced with caspase-8 being overexpressed in most treatment. In trying to identify the type of compounds that might be responsible for this cell death, flavonoids, especially isorhamnetin, was identified as the main compound responsible the type of cell death.

In conclusion, this study has suggested that extracts of *E. tirucalli* inhibit cancer cell proliferation by increasing the expression of p21 that leads to cell cycle arrest and the induction of apoptosis through the extrinsic pathway.

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Appendix 1



Cancer Research

The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

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Abstract 3216: Anti-proliferative properties of Euphorbia mauritanica medicinal plant against breast cancer

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Abstract

Over the decades, the increased interest in the use of natural resources in pharmaceutical and phytotherapeutic research has led to manufacture of drugs (including anti-cancer drugs) derived from natural sources With our increased understanding of the molecular mechanisms underlying cancer progression and cell death, it is therefore necessary to test if there are any natural products that can be used to act as antitumor agents. As breast cancer continues to be the leading cause of cancer in females, we aimed to exploit the use of South African traditional medicine, Euphorbia mauritanica as a tool for anti breast cancer therapy. Euphorbia mauritanica was tested for anti-proliferative properties against MCF-7 and Cama-1 breast cancer cell lines. The following solvents were used to extract active compounds from E. mauritanica: n-hexane, dichloromethane, chloroform, ethyl acetate, acetone, methanol and butanol. A real time cell analyzer (Xcelligence) was used to monitor cell growth over a period of 48 hours following treatment of cells with the extracts. Butanolic extracts showed the highest level of cytotoxicity on both MCF-7 and Cama-1 cell lines with IC50 of 15 and 30 µg/ml respectively. These were followed by hexanolic extracts with IC50 of 30 and 50 µg/ml and methanolic extracts with IC50 of 50 and 100 µg/ml. Flow cytometry using annexin V was further employed to determine if cell death was as a result of apoptosis and the data confirmed that this was most likely the case. On investigating whether the observed apoptosis was via p53 mediated pathway, se 9, caspase3, RBBP6 and mdm2 showed that

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Appendix 2

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Breast cancer: Small molecules targeting apoptosis, a prospective approach to safe scientific success

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ABSTRACT

Breast carcinoma represents the second leading cause of cancer death in developed countries amongst women. Current cytotoxic chemotherapy plays an important role in the management of patients with hormone-insensitive or metastatic breast carcinoma, although most of them ultimately develop recurrences. Therefore, there is a need for novel targets and treatment strategies in patients with advanced breast carcinoma that is refractory to conventional chemotherapy. This paper summarizes current knowledge on breast cancer targets and molecular mechanisms that follows apoptosis induction.

Keywords: Breast Cancer; Apoptosis; p53; Natural Products

1. INTRODUCTION

Breast cancer is a malignant tumour that starts in the breast tissue; it usually starts in the ducts or in lobules. It can occur in both males and females; however, male breast cancer is rare. It is the second leading cancer in the world following lung cancer, with an estimated 1.15 million cases a year [1]. The American Cancer Society estimated 230,480 cases were diagnosed in women for 2011 in the USA alone, resulting in 39,970 deaths [2]. Breast cancer is primarily a postmenopausal women's disease, with the American National Cancer Institute's Cancer Review estimating the average age of women at risk to be 61 years of age. Approximately 0 case have being reported for women below 20, but the figures are gradually increasing with every decade [3].

1.1. Epidemiology

The lifetime risk of an individual developing breast cancer cannot be attributed to one factor alone. A number of variables play a role in the development of breast malignancies. Even though it is fairly difficult to pinpoint one cause as the main risk factor for breast cancer, studies have shown there are certain genetic, reproductive and lifestyle factors that play a major role in its induction and progression. One of these factors is age, with studies finding that the incidence rates double with every 10 years up until menopause, where the rates start to dramatically decline [4]. Exposure to radiation and use of oral contraceptives may be associated with induction of breast cancer. Very small fraction of breast cancers could be attributed to preventable lifestyle behaviour such as obesity, alcohol consumption, lack of physical activity and long term use of hormone replacement therapy. In several studies conducted to determine the role these factors have on breast cancer incidence, the data appears to be inconsistent, hence it is hard to establish the severity these factors play in breast cancer induction that is if they even do at all [5,6]. In developed countries, incidence rates are higher relative to developing countries. However, incidence rates are steadily increasing in developing countries [7]. This increase may be attributed to the adoption of a more westernized lifestyle which encompasses a more sedentary lifestyle, delayed childbearing, high alcohol use and a diet high in fat [8,9].

In a lot of breast malignancies, hormones play a major role. It is known that estrogen starts breast tissue proliferation, and studies have now shown that the longer a woman is exposed to estrogen, the greater the risk of developing breast cancer. Hence, women with an early age at menarche (<12 years), were found to be 2.2 times more likely to develop breast cancer in the future than those with late menarche. In a different study, women who started menopause late *i.e.* at the age of 55, were found to have 44% higher breast cancer incidence rates than women who started menopause at 45. This may be due to a 10-year increase in the amount of estrogen the

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women were exposed to. Exposure to excessive estrogen plays a role in hyperestrogenia. Hyperestrogenia could trigger breast cancer in two ways: Either by breast epithelium tumour promotion or by DNA damage which could be caused by estrogen metabolites directly [10,11].

Another important factor which may increase susceptibility to breast cancer is inheritance of susceptibility genes, it has been estimated that 7% - 10% of breast cancers arise due to inherited mutated genes. The major predisposing factors to breast cancer are breast susceptibility genes, known as BRCA1 and BRCA2, which are important for early onset of breast cancer [11,12]. An initial germline mutation in one of these genes followed by a second mutation leading to the loss of function of the second allele is required for induction of tumourigenesis [13]. These genetic factors mostly show an autosomal dominant pattern of inheritance, with germline mutations rendering susceptibility to the following generation [14,15].

1.2. BRCA in Breast Cancer

Defects in DNA repair results in increased genomic instability, which can lead to malignant transformation. Additionally, defects in DNA repair renders cells sensitive to DNA damaging agents. BRCA1 and BRCA2 are important DNA repair proteins that are required for effective repair of DNA double-strand breaks by homologous re combination and DNA replication fidelity [16]. This they do by interacting with a recombinational repair protein *Rad*51, thereby maintaining genomic integrity [17,18]. In accordance with their role in DNA repair; a cell with a mutant BRCA gene will not be able to have a functional protein product thereby leading to defects in DNA repair mechanisms. Inherited mutations in BRCA1 and BRCA2 increase the risk of developing various cancers, especially breast and ovarian cancers. Tumours that develop in patients with inherited BRCA1/2 mutations are generally believed to be BRCA1/2 deficient. Cancer cells with BRCA1/2 deficiency are defective in DNA repair by homologous recombination and sensitive to interstrand DNA crosslinking (ICL) agents. The BRCA genes are thought to be tumour suppressors since the wild type allele is often lost in tumours of heterozygous people [19]. Though BRCA mutations have been implicated in a lot of breast cancer cases, there are still breast cancers which occur due to deregulation of other genes. A substantial proportion of susceptibility to breast cancer can be attributed to mutations in cell cycle regulation and apoptosis genes.

BRCA and Small Molecule Targets

Defects in DNA repair render cells sensitive to DNA damaging agents. BRCA1 and BRCA2 are important DNA repair proteins, hence, cancer cells with BRCA1/2 deficiency are defective in DNA repair. Several management strategies have been suggested for reduction of the cancer risk in individuals who bear the BRCA1 or BRCA2 mutations. In recent studies, small molecules have been targeted to induce DNA repair. Indole-3-carbinol (I3C) and genistein are naturally occurring chemicals derived from cruciferous vegetables and soy, respectively, with potential cancer prevention activity for hormone-responsive tumours [20]. Fan *et al.* showed that I3C induced BRCA1 expression and that both I3C and BRCA1 inhibit oestrogen (E2)-stimulated oestrogen receptor (ER-alpha) activity in human breast cancer cells. They further reported that a combination of both I3C and genistein induced the expression of both BRCA1 and BRCA2 genes in breast cancer cells. In another study, tamoxifen, a selective oestrogen receptor regulator, was reported to have reduced the risk of developing breast cancer among women with inherited BRCA1 or BRCA2 mutations [19].

The first human phase I study reported that a single agent therapy with olaparib, an oral selective inhibitor of PARP1, has promising antitumour activity in advanced solid tumours with either BRCA1 or BRCA2 mutations. A very recent multi-centre proof-of-concept phase II trial further demonstrated a positive result with olaparib single agent therapy in women with both advanced breast cancer and BRCA1 or BRCA2 mutations [16]. Iniparib another PARP inhibitor similar to olaparib, has been reported in a phase I study to improve the clinical benefits and survival of patients with metastatic triple-negative breast cancer, which shares clinical and pathological features with hereditary BRCA1-related breast cancer [21]. Increasing knowledge of the roles of BRCA1 and BRCA2 genes in DNA repair and the relations between their mutations and cancer predisposition suggests the therapeutic potential of strategies targeting the BRCA1 and BRCA2 defects.

2. APOPTOSIS AND BREAST CANCER

Apoptosis is a series of highly coordinated and complex processes, which multicellular organisms use to ensure elimination of defective and damaged cells [20]. Defective apoptosis has been implicated in development and progression of malignant phenotypes. Apoptosis is controlled by a number of proteins which require various triggers to be activated resulting in a cascade of signaling modules which eventually lead to cell death [22]. Based on the triggering stimulus, there are at least two main pathways that can activate apoptosis [23]. The first is the intrinsic pathway or the mitochondrial pathway. Apoptotic signals that trigger activation of this pathway will lead to a cascade of events that will result in the release of cytochrome *c* sequestered in the mitochondrial intermembrane space [24]. The second pathway is the extrin-

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sic pathway, which is triggered through receptors of the tumour necrosis factor (TNF) superfamily. Both these pathways converge to a common pathway which leads to activation of a proteolytic cascade of caspases (**Figure 1**).

2.1. Small Molecules Targeting Extrinsic Pathway in Breast Cancer

The observation that activation of the extrinsic pathway can amplify apoptotic signals initiated by cytotoxic drugs acting primarily through the induction of mitochondrial pathway has prompted the search for strategies capable of triggering the receptor-related cascade [25]. This has led to the identification of the tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) and its death receptors DR4 and DR5 [26]. Activation of DR4 and DR5, like that of Fas/APO, leads to activation of the initiator caspase,

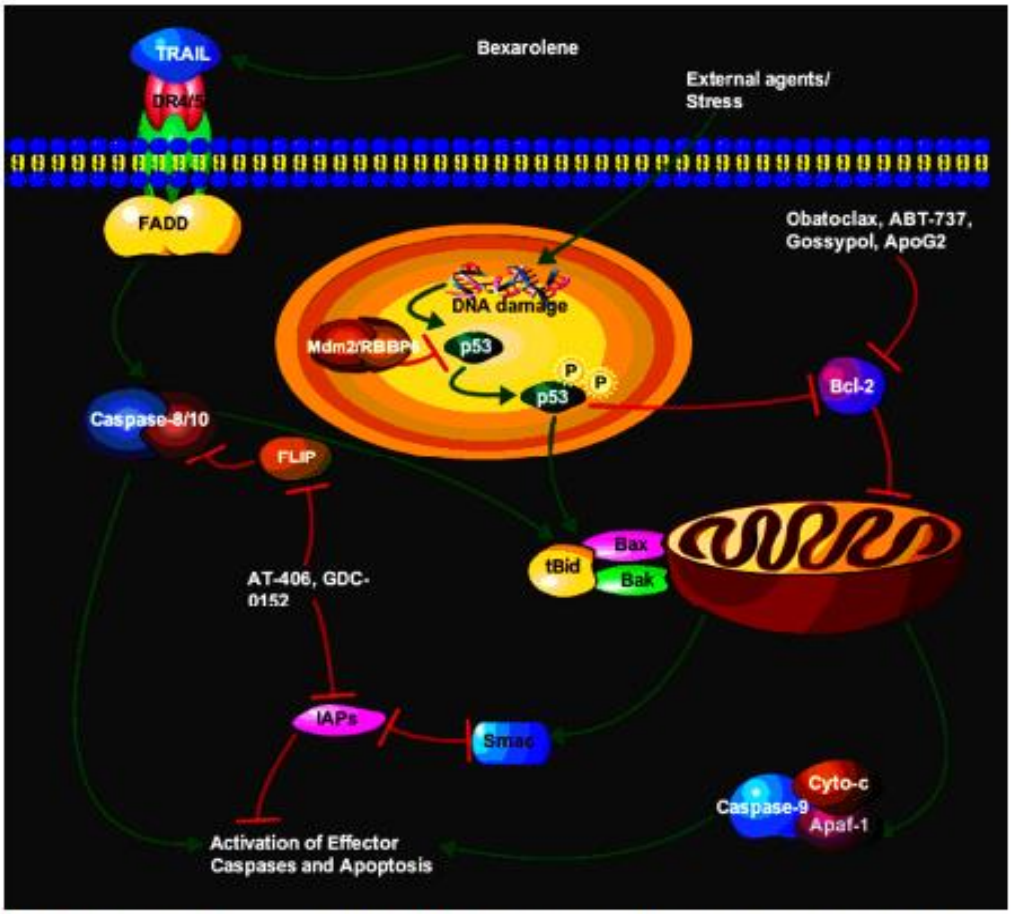

Figure 1. Proposed steps of apoptosis induced by TRAIL ligand. TRAIL ligands bind to and activate their receptors by inducing receptor trimerization. Activated receptors recruit adaptor molecules such as Fas-associating protein with death domain (FADD), which recruit procaspase 8 to the receptor complex, where it undergoes autocatalytic activation. Activated caspase 8 activates caspase 3 through two pathways; the complex one is that caspase 8 cleaves Bcl-2 interacting protein (Bid) and its COOH-terminal domain translocates to the mitochondria where it triggers cytochrome *c* release. The released cytochrome *c* binds to apoptosis protease activating factor-1 (Apaf-1) together with dATP and procaspase 9 and activates caspase 9. The caspase 9 cleaves procaspase 3 and activates caspase 3. In another pathway, p53 activation following external agents/stress results in the inhibition of pro-survival protein Bcl-2 and the activation of Bax that leads to activation of the mitochondria to release cytochrome *c* and caspases that end with apoptosis.

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caspase-8, and its downstream targets [27]. Interestingly, TRAIL seems to exert selective toxicity towards neoplastic cells, possibly because of the presence in normal cells of high levels of decoy receptors or the caspase-8 antagonist FLICE-inhibitory protein [26]. In this context, coadministration of TRAIL has been shown to increase the lethal effects of conventional cytotoxic drugs in several cancers [28] (**Figure 1**).

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induces programmed cell death preferentially in tumour cells. TRAIL holds enormous promise as a cancer therapeutic due to its highly selective apoptosis-inducing action on neoplastic versus normal cells Moreover, a recently published Phase I clinical trial revealed that recombinant TRAIL administration is safe and well tolerated, and that dose escalation achieved peak TRAIL serum concentrations equivalent to those associated with preclinical antitumor efficacy [29]. However, to exploit the opportunity to successfully treat cancers with TRAIL, the problems of TRAIL resistance in a variety of tumor cells must first be overcome [30]. In trying to address this setback, several natural compounds have been evaluated as possible solutions. Bexarotene, which is a retinoid specifically selective for retinoid X receptors works by stopping the growth of cancer cells. In exploiting a combinational therapy with bexarotene, Ying *et al.* (2007) reported that the effect of bexarotene on TRAIL-mediated programmed cell death involved proximal events of the extrinsic pathway; however, downstream signals involved the intrinsic pathway in leukemia cancer cells (**Figure 1**). In another study by Park *et al.*, they observed that both DR5 and CHOP upregulation were required for γ-T3-induced apoptosis and DR5 was transcriptionally regulated by CHOP after γ-T3 treatment. In another study, apigenin induced extrinsic apoptosis pathway, up-regulating the levels of cleaved caspase-8, and inducing the cleavage of poly (ADP-ribose) polymerase. However, apigenin did not induce apoptosis via intrinsic mitochondrial apoptosis pathway since this compound did not decrease mitochondrial membrane potential maintaining red fluorescence and did not affect the levels of B-cell lymphoma 2 (Bcl-2) and Bcl-2-associated X protein [31]. Moreover, apigenin reduced the tyrosine phosphorylation of HER2 (phospho-HER2 level) in MCF-7 HER2 cells, and up-regulated the levels of p53, phospho-p53 and p21 in MCF-7 vec and MCF-7 HER2 cells [31]. TRAIL receptor activating agents have been found to exhibit favourable *in vitro* and *in vivo* activity in treatment of several malignancies, including breast and gynaecological cancers. Preclinical and early phase I studies have provided some support to the assumption that these novel agents are safe, with increased target specificity for malignant cells. When these targeted agents are combined with conventional chemotherapy drugs or radiotherapy, they appear to increase cell death over single agent modalities.

2.2. Small Molecules Targeting Intrinsic Pathway in Breast Cancer

The mitochondrial pathway of apoptosis is the major mechanism of physiological cell death in vertebrates. In this pathway, proapoptotic members of the Bcl-2 family (contain only a BH3 domain) cause mitochondrial outer membrane permeabilization (MOMP), allowing the release of cytochrome *c*, which interacts with Apaf-1 to trigger caspase activation and apoptosis. Activation of the intrinsic apoptotic pathway represents a major mechanism for breast cancer regression resulting from anti-estrogen therapy. The BH3-only protein is inducible by estrogen-starvation and anti-estrogen treatment and plays an important role in anti-estrogen induced apoptosis of breast cancer cells [32]. In this section we present current targets of this pathway using small molecules of natural products. Recently, Jin *et al.* (2010) trying to exploit phytoestrogen to induce apoptosis in MCF-7, they used Daidzein which belongs to the isoflavone family, which is the most commonly ingested and most intensely studied type of phytoestrogen, often found in nuts, fruits, soybeans, and soy-based products. In their studies Jin *et al.* (2010) demonstrated that daidzein induces apoptosis through the generation of ROS that perturb mitochondrial function, leading to mitochondrial permeability, cytochrome *c* release, and caspase activation. They further reported the loss of the antiapoptotic mitochondrial Bcl-2 protein as partially responsible for the opening of the mitochondrial permeability transition pore.

In another study using Plumbagin a constituent of species of the plant genera *Drosera* and *Plumbago* which displays antineoplastic activity toward various cancers, Kawiak *et al.* (2012) reported antiproliferative activity of Plumbagin that was associated with apoptosis-mediated cell death, as revealed by caspase activation and an increase in the sub-G1 fraction of the cell cycle. Compound Plumbagin increased the levels of the proapoptotic Bcl-2 family of proteins and decreased the level of the antiapoptotic Bcl-2 protein in SKBR3 and BT474 cells [33]. Thus, these findings indicated that Plumbagin induces apoptosis in Her2-overexpressing breast cancers through the mitochondrial-mediated pathway and suggest its potential for further investigation for the treatment of Her2-overexpressing breast cancer (**Figure 1**). The mitochondrial pathway of apoptosis was further shown to play a critical role in estradiol-induced apoptosis in long-term estrogen-deprived breast cancer cells. Physiologic concentrations of estradiol could potentially be used to induce apoptosis and tumour regression in tumours that have developed resistance to aromatase inhibitors [34]. Plant-derived polyphenols inhibit cancer

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cell proliferation and induce apoptosis. Recently, prenyl-flavonoids and alkyl-phloroacetophenones have been reported for their *in vitro* antitumor activity [35]. In the same study Cho *et al.* (2012) reported cytotoxic activity as a result of treating MCF-7 cell line with prenyl (3-PAP) and geranyl (3-GAP) derivatives of phloroacetophenone, and xanthohumol (XN), a prenyl-chalcone. In addition, pro-apoptotic Bax but not B-cell lymphoma 2 (Bcl-2) expression was increased by 3-GAP. In accordance with the Bax increase, 3-GAP induced mitochondrial cytochrome *c* release and activated caspase-9, an initiator of the caspase cascade. Currently there are several data that support or continue to show that small molecules particularly the ones extracted from natural plants present a supplementary and safer target in many cancers.

3. THE BCL-2 FAMILY (TABLE 1)

The Bcl-2 family of proteins are one of the primary regulators of the intrinsic pathway or the mitochondrial pathway. These proteins can be characterized into proapoptotic members: Bax, Bak, Bik, Bad and Bid, with the anti-apoptotic members being Bcl-2 Mcl-1, Bcl-xl and Bcl-w. The proapoptotic members of this family work by promoting caspase activation and cell death. Some of them work by inactivating death inhibiting members of this family whilst others stimulate the release of cytochrome *c* from the mitochondria [36]. The anti-apoptotic members inhibit apoptosis by blocking release of cytochrome *c* from the mitochondria.

As our understanding of the field of apoptosis and its regulatory machinery evolves, it becomes important to identify and exploit new potential targets that restore pro-apoptotic activities. The most promising recent strategies in the development of anti-cancer drugs involve targeting essential apoptotic genes and proteins directly to induce cell death. Members of the Bcl-2 family of proteins and IAPs have been shown to be very important regulators of caspases. Absence or impairment of caspase activation almost always leads to prevention of cell death. Novel strategies that will overcome caspase inhibition will be of great value in cancer therapeutics that aim to directly restore a tumour cells apoptotic program. Hence, the development of small molecule agents that target IAPs and Bcl-2 appear to be attractive therapeutic target.

The proapoptotic effectors such as Bax and Bak contain BH1, BH2, and BH3, and a C-terminal transmembrane segment that selectively targets these proteins to the membranes of mitochondria and endoplasmic reticulum. The pro-survival (antiapoptotic) members Bcl-2, Mcl-1, Bcl-xl, Bcl-w contain a BH1, BH2, and BH3, but with the exception of Mcl-1, they also contain a BH4 domain. The last group, which are the proapoptotic members including Bid, Bad, Bik, Bim, Puma, and Noxa only have a BH3 domain [37] (**Figure 1**).

A number of small molecules antagonists to pro-

Table 1. Breast cancer current treatment strategies.

Treatment	Target site	Limitations/Stage in research
1. Chemotherapy	Whole body through blood system	Anemia and not specific
2. Radiotherapy	High energy beam target individual cells	Skin problem/burn
3. Hormonal therapy	Hormone replacement	Not all cancer are due to hormonal
4. **Targeted Therapies**	**Apoptosis**	**Mostly under improvement**
a. *Gossypol*	Bcl-2 family	Still under clinical trials
b. *Apogossypolone*	Bcl-2 family	Clinical trials
c. *Obatoclax*	Bcl-2 inhibit pro-survival genes	Phase I
d. *ABT-737*	Bcl-2 and Bcl-xl	Clinical trials
e. *AT-406*	IAP molecule	Clinical trials
f. *YM155*	Survivine	Early clinical research
g. *GDC-0152*	cIAP1	Early clinical research
h. *I3C*	BRCA1/2	Clinical trials
i. *Genistein*	BRCA1/2	Clinical trials
j. *Tamoxifen*	BRCA1/2 oestrogen	Clinical trials
k. *Iniparib*	PARP	Clinical trials
l. *Cisplatin*	Caspases	Inhibit IAP and activate caspases.
m. *Bexarotene*	TRAIL	Preclinical research

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survival Bcl-2 have been developed and some are still undergoing clinical trials. Some of these include:

3.1. Gossypol

Gossypol is a naturally occurring polyphenolic compound with BH3 mimetic property. It induces apoptosis in cells with high Bcl-2 expression. It was found to have a high affinity to multiple Bcl-2 family proteins, and thus it binds to and blocks the binding of antiapoptotic Bcl-2 to pro-apoptotic Bcl-2 family proteins [38]. Gossypol has demonstrated anticancer activity against a wide range of malignancies including prostate cancer and human colorectal carcinoma [39,40]. Gossypol is currently under clinical trials.

3.2. Apogossypolone (ApoG2)

Apogossypolone is a semi synthesized analogue of gossypol. It was created to reduce the toxicity of gossypol. Apogossypolone was designed by removing two aldehyde groups on poly-phenolic rings of gossypol [41]. It was due to these reactive two aldehyde groups that gossypol was thought to exhibit toxicity [20]. Similarly to gossypol, ApoG2 binds to Bcl-2 family proteins. In a study, ApoG2 was found to exhibit better stability and was also better tolerated by mice than gossypol. It showed significant inhibition to diffuse large-cell lymphoma, a type of non-Hodgkin's lymphoma, and was not toxic to normal peripheral blood lymphocytes [42]. Recently, a number of ApoG2 derivatives have been created such as Bl97D6. Bl97D6 inhibits binding of the BH3 peptide to the anti-apoptotic Bcl-2 family proteins. It has been shown to be a potent inhibitor of human prostate cancer and human lung cancer [43].

3.3. Obatoclax

Obatoclax is a small molecule novel drug designed to inhibit prosurvival Bcl-2 family proteins and thereby increase cellular threshold for apoptosis in cancer cells. It acts as a BH3 mimetic and binds to prosurvival Bcl-2 proteins and neutralizes them [44]. In a study, obatoclax showed significant cell viability reductions in human pancreatic cell lines [44]. In phase I trials obatoclax exhibited antitumour activity in patients with refractory leukemia and myelodysplasia [45].

3.4. ABT-737

ABT-737 is a small molecule inhibitor of the anti-apoptotic proteins Bcl-2 and Bcl-xl but not Mcl-1. A study by Oltersdorf (2005) found that ABT-737 does not directly induce apoptosis but sensitizes cells to death signals, showing synergistic cytotoxicity with chemotherapeutic treatment and radiation. ABT-737 exhibits cytotoxicity against lymphoma, multiple myeloma and small-cell lung carcinoma lines [46,47].

4. CASPASE IN BREAST CANCER

Caspases are a class of cysteine-aspartyl proteases that are synthesized as inactive zymogens. They lie dormant in a healthy cell then due to cell death stimuli, they are proteolytically cleaved at two aspartate residues, generating mature active enzymes [48,49]. The generation of active caspases forms a cascade whereby initiator caspases interact with a specialized adapter molecule which results in its own activation and eventually activation of execution caspases which are responsible for the final pathway of apoptosis [35,48]. Since all apoptotic pathways converge on caspases it renders them crucial effectors of apoptosis. Hence, it is very crucial that expression of caspases is tightly controlled. Deregulation of caspases has been implicated in a number of diseases. Overexpression has been linked to certain neurodegenerative disease and ischemia-associated injury, whilst their suppression has been linked to a number of cancers.

4.1. Caspase 3 in Breast Cancer

The role of caspase-3 in the response of breast cancer cells to chemotherapeutic drugs remains a controversial issue. The loss of caspase-3 expression as well as defaults in cytochrome *c* release from the mitochondria, which is required in most apoptotic pathways to activate caspase-3 through caspase-9 activation, are associated with multidrug resistance. Accordingly, expression of caspase-3 in the human MCF-7 breast tumor cell line (which is deficient for caspase-3) restores the apoptotic response to the topoisomerase II inhibitor, etoposide [50]. Caspase-3 was also involved in breast cancer cell apoptosis upon exposure to anthracyclines and cisplatin [27,51]. Its role in tumor cell response to paclitaxel has been challenged [11].

4.2. Initiator Caspases (8 and 9) in Breast Cancer

The initiator caspases appear to display some specificity according to the type of apoptotic signal. Two main activation cascades for apoptosis induction have been described [52]. Fas receptor-ligand interactions use caspase-8 activation to trigger the downstream executioner caspases. An alternative mitochondrial pathway, which is triggered by various anticancer agents, involves activation of caspase-9 upon recruitment to the mitochondria by cytochrome *c* and apoptosis protease activation factor-1(APAF-1) [47]. Mitomycin *c* which is one of the anticancer agents, led to an exclusive activation of caspase-8 but not of caspase-9 in comparison to various other drugs with differing mechanisms of action at con-

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centrations formerly established as equitoxic in the NCI/ADR-RES cell line [47]. In a contrary study by Lee at al using Styrylpyrone derivative (SPD) which is a pharmacologically active compound extracted from the plant *Goniothalamus sp.* of the Annonaceae family have shown that procaspase-8 was not activated in MCF-7 cells but caspase-9 activation was detected in response to SPD treatment, with the release of cytochrome *c* into the cytosol. In another study, prodigiosin which is a red pigment produced by Serratia marcescens with pro-apoptotic activity was potently cytotoxic in both estrogen receptor positive (MCF-7) and negative (MDA-MB-231) breast cancer cell lines. It lead to cytochrome *c* release, activation of caspases-9, -8 and -7 and cleavage of poly (ADPribose) polymerase protein typified the apoptotic event and caspase inhibition revealed that PG acts via the mitochondrial pathway [53].

5. P53 IN BREAST CANCER

The p53 tumor suppressor gene prevents tumorigenesis in response to physiological and environmental stress and plays a role in cell cycle progression, apoptosis and repair of DNA damage. p53 has be involved in transcriptional regulation of pro-apoptotic genes associated with intrinsic and extrinsic pathways [54]. The activation of the p53 pathway leads either to cell cycle arrest or to apoptosis. Activated p53 increases the expression of p21 in DNA damaged cells and affects expression of p27 [42]. The up-regulation of cyclin dependent kinase inhibitors (CDKI), such as p21 and p27, is related to cell cycle arrest and contributes to down-regulation of cyclin D1, cyclin E, cyclin-dependent kinases (cdks) [55]. The activation of p53 induces up-regulation of pro-apoptotic Bax but not anti-apoptotic Bcl-2. Also, p53-mediated apoptosis involves the activation of Fas and the intrinsic mitochondrial pathway, which results in activation of caspase-8 and -9 (**Figure 1**) [56]. So, targeting p53 in cancer not only holds a promise to cancer eradication but also guarantees a safe elimination of those cells.

Many observations have implicated p53 signaling pathway in the transcriptional activation of Bax in apoptosis, but also in the alteration of the Bcl-xL/Bax ratio, thereby indicating that the ratio between these pro- and anti-apoptotic proteins is one of the important factors deciding the fate of a cell [52,57]. Like many new plant extracts, the role of curcumin a naturally occurring phytochemical responsible for the yellow colour of the commonly used spice turmeric in regulating the balance between these pro- and anti-apoptotic factors was tested and it revealed that curcumin induced apoptosis with an increase in p53 level in MCF-7 cell lines. Interestingly, the curcumin-induced increase in p53 expression precedes that of Bax thereby leading to believe that p53 transactivates Bax expression [52]. In the same study, the Bcl-xl levels remained almost unchanged thereby shifting the Bcl-xl/Bax ratio towards apoptosis. In recent times several small molecules have been identified e.g., Nutlin-3a and MI-219 that disrupt MDM2-p53 interaction resulting in inhibition of tumor growth, however they are less effective in cancer cells that express high levels of MDMX. Another molecule called benzofuroxan derivative following treatments of MCF-7 cells with this small-molecule MDMX inhibitor activated p53, resulting in elevated expression of proapoptotic genes (e.g., PUMA, BAX, and PIG3). Importantly, this novel small-molecule p53 activator caused MCF-7 cells to undergo apoptosis, and acted additively with Nutlin-3a to activate p53 and decrease the viability of cancer cells [58]. **Table 2** shows other small molecules that are involved in p53-induced apoptosis.

6. INHIBITORS OF APOPTOSIS PROTEINS (IAP)

The IAP's were discovered in baculoviruses and these proteins are known to biochemically function by suppressing the host's cell death response to a viral infection thereby allowing viral propagation in host insect cells [47,59]. Several other cellular homologous IAP's were also identified in a range of species such as *Drosophila* to vertebrates. Neuronal Apoptosis Inhibitory Protein (NAIP) was the first mammalian IAP to be identified during positional cloning in an effort to identify the causative gene for Spinal Muscular Atrophy (SMA) [47]. Other members of mammalian inhibitor of apoptosis family of proteins, include the X chromosome-linked IAP (XIAP), cellular IAP 1 (cIAP1), cellular IAP II (cIAP2), melanoma IAP (ML-IAP), Survivin, Bruce, ILP-2 and Livin to name but a few. These are overexpressed in cancer cells thereby conferring cancer cell protection against a variety of proapoptotic stimuli by

Table 2. List of small molecule agents that target Bcl-2 and mode of action.

Small molecule	*Mechanism*	*Reference*
Gossypol	Binds to and blocks the binding of antiapoptotic Bcl-2 to pro-apoptotic Bcl-2 family proteins	Kitada, *et al.* 2003
Obatoclax	Acts as a BH3 mimetic and binds to prosurvival Bcl-2 proteins and neutralizes them	Huang, *et al.* 2009
Apogossypolone (ApoG2)	Binds to and blocks the binding of antiapoptotic Bcl-2 to pro-apoptotic Bcl-2 family proteins	Sun, *et al.* 2008
ABT-737	Inhibits the anti-apoptotic proteins Bcl-2 and Bcl-xl but not Mcl-1	Oltersdorf, *et al.* 2005

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functionally regulating signal transduction pathways associated with malignancy [60,61].

Structurally, the IAPs are similar with one or more 70 - 80 amino acid baculovirus IAP repeat (BIR) domains with the core domain consiting of the variable sequence $C(X)_2C(X)_6W(X)_3D(X)_3H(X)_6C$ with the X representing any amino acid [46]. A typical mammalian IAP family member contains a carboxy-terminal RING (Really Interesting New Gene) zinc finger possessing a E3 ubiquitin ligase activity which directly regulates self-ubiquitination and protein degradation and the cellular IAP (cIAP 1 and cIAP 2) uniquely possesses the caspase recruitment domain (CARD) which mediates oligomerization with other CARD containing proteins. Although the mechanism of action is not known, the CARD in IAP may form protein-protein interactions with protease activating factor-1 (Apaf-1), and with some of the death domain (DD) or death effector domain (DED) containing proteins [35] (**Figure 2**).

The fact that this family of proteins, when over-expressed in human cells protects cells from stimuli that induces apoptosis provides the mechanism and way to explain treatment resistance in cancer cells [61]. One strategy to overcome this is to silence IAPs and this in turn can sensitize cells to apoptotic stimuli. Other efforts involve targeting the IAP proteins by designing small molecules that can mimic the binding of the endogenous IAP antagonist second mitochondria-derived activator of caspases/direct IAP-binding protein, with low pI (Smac/DIABLO) to a shallow groove on the surface of select IAP baculovirus repeat (BIR) domain, thereby inhibiting the inhibitor of apoptosis (IAP) [47,59].

IAP in Apoptosis and Cancer

Cancer cells thus temper with the equilibrium of cell death and cell growth in order to survive. Apoptosis regulators such as IAPs play a fundamental role in oncogenesis because they suppress the intrinsic and extrinsic apoptosis pathway. This they do via the central mechanisms of IAP apoptotic suppression through direct caspase and pro-caspase inhibition (primarily caspase 3 and 7) in a cascade manner and modulation of and by the transcription factor NF-kappaB [62]. In many cancers IAP's are over-expressed or over-activated, conferring cells to no longer be able to die in resistance to standard chemo and radiation therapies which in turn have a negative effect on normal healthy cells because DNA is damaged chemically and physically [47,63]. Novel yet effective and "safe" therapeutic targets are therefore required for the treatment of cancer.

In search for possible targeted markers that inhibit expression of IAPs, Smac/DIABLO was discovered in 2000, as a protein released from the mitochondria into the

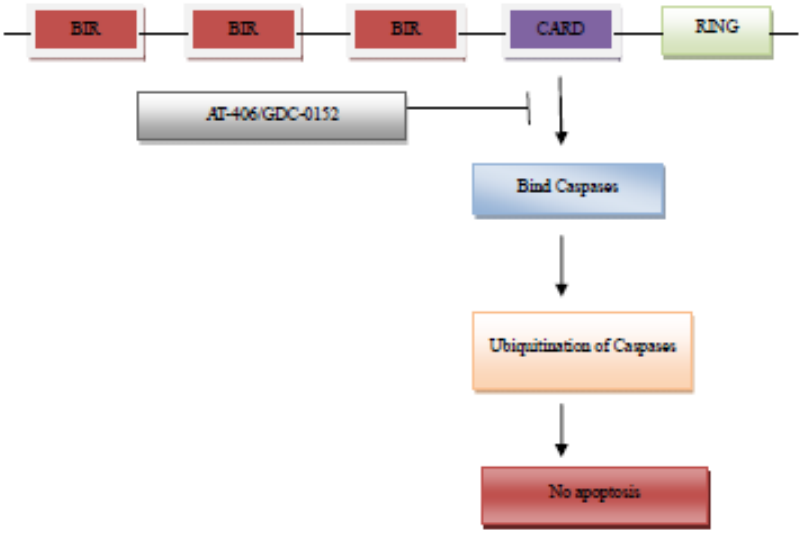

Figure 2. Domain structure of IAP proteins. Pro-survival proteins bear up to three domains BIR, CARD and RING. The IAP family mediated caspase-ubiquitination pathway that then results in the pro-survival of the cell and its escape to cell death by apoptosis. The BIR domain is necessary for IAP protein interaction with a number of proapoptotic factors, including invertebrate death inducers. Several small molecules have been identified that result in the inhibition of the binding of caspases to the CARD region on the IAP thereby resulting in activation of apoptosis.

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cytosol in response to apoptotic stimuli. It functions as an endogenous antagonist of several inhibitors of apoptosis proteins through direct binding. The interaction between Smac and IAPs involves the AVPI tetrapeptide binding motif on the N-terminus of Smac and a well-defined groove on the surface of these IAP proteins, providing an ideal site for the design of small-molecule Smac mimetics [13,64]. Potent and cell-permeable small-molecule Smac mimetics have provided powerful pharmacological tools for study of the regulation of apoptosis by IAP proteins, and several such compounds are now in early clinical trials as new anticancer agents. One focus has been on the generation of molecules that mimic the aminoterminus of mature Smac. AT-406 which is an orally-active, small molecule drug was designed to promote programmed cell death (apoptosis) in tumour cells by blocking the activity of IAPs thereby creating conditions in which apoptosis can proceed. AT-406 is considered a multi-IAP antagonist.

IAPs are key components of the complex cascade of protein signaling that activates enzymes called caspases to initiate the breakdown of cancer cells [64,65]. AT-406 is thought to mimic the activity of Smac by binding to XIAP and preventing it from inhibiting caspase activation. Upon binding to cIAP1 and cIAP2, AT-406 induces rapid degradation of these proteins and promotes apoptosis through activation of the death-receptor complex and caspase 8 [13] The other molecule, GDC-0152 has the best profile of these compounds; it binds to the XIAP BIR3 domain, the BIR domain of ML-IAP, and the BIR3 domains of cIAP1 and cIAP2. GDC-0152 achieves apoptosis induction by promoting degradation of cIAP1, inducing activation of caspase-3/7, and leads to decreased viability of breast cancer cells without affecting normal mammary epithelial cells [64]. The second member of the IAP that has also been targeted is survivin. Survivin protein was found to be expressed in all the most common human carcinomas but not in normal tissue. YM155, a small-molecule survivin suppressor resulted in suppression of survivin expression, caspase-3/7 activation, induction of spontaneous apoptosis, and growth inhibition. In another study by Zhang *et al.* (2012), valproic acid which is a broad-spectrum inhibitor of class I and II histone deacetylases and shows great anticancer activity in a variety of human cancers including breast cancer was found to significantly inhibit cell migration but not proliferation of human breast cancer MDA-MB-231 cells. Ribonuclease expression was also found to promote apoptosis in human breast cells MDA-MB-231 by inhibiting the expression of Survivin and activating caspase-3 [66]. The novel findings of this review is that multiple IAPs are concomitantly expressed in breast cancers, and that, in combination with clinically relevant IAP antagonists promote apoptosis and reduce the cell turnover index of breast cancers.

7. FUTURE FOCUS ON BREAST CANCER

It is now recognized that the mechanism of action of chemotherapeutic drugs often involves the induction of cancer cell apoptosis, and that apoptosis resistance is a major contributing factor in chemotherapeutic drug resistance. Therefore, restoring apoptosis signalling in cancer cells with targeted therapeutics has enormous potential to improve the outcome of cancer chemotherapy by reversing a major mechanism of drug resistance. In this review, we assessed the outlook for improving the outcome of cancer therapy by targeting different apoptotic genes using small molecules and exploring the possibility of using plant extracts as a target to induce apotosis. Novel modalities of cancer therapies that improves the efficacy of apoptosis as well as chemotherapeutic drugs and lessen the toxicity of these agents by targeting specific genes would be an improvement to the current status of available drugs. Moreover, upregulation of the pro-apoptotic signaling proteins or suppression of specific anti-survival signaling pathways by agents directed at increasing pro-apoptotic proteins may acutely induce chemosensitization of resistant cancer cells.

8. CONCLUSION

Although the mechanism that used to triggers apoptosis in breast cancer using small molecules is still unclear, the identified mechanism of cell death provides a convenient window for further defining the upstream events and elucidating the mechanisms. Finally, the fact that the proapoptotic action are independent of p53 further emphasizes that small molecule analogues are potentially useful to the development of new therapeutics that induce apoptosis in breast cancer.

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Appendix 3

CDK-interacting protein 1 overexpression as a predictive anti-cancer agent in breast cancer treated with *Euphorbia tirucalli* extracts

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Abstract

Medicinal plant extracts have recently attracted attention to modern medical science research with their non-lethal activity. Currently, up to 50% of the world drugs including chemotherapeutic drugs such as taxol and camptothecin are derived from natural products. *Euphorbia tirucalli* has a long history of usage as traditional medicine in Africa and has been widely used in the treatment of different cancers. In this study, we explore the medicinal properties of *Euphorbia tirucalli* extracts in breast cancer development. To achieve this, *E. tirucalli* was dried, crushed and compounds extracted using butanol, hexane and methanol at 1g per 10ml. Extracts were characterised using both TLC and LC-MS spectroscopy. Breast cancer cell lines (MCF-7 and MDA-MB 231) were treated with varying concentrations of the extracts for up to 48 hours. Cell viability assay, cell cycle, apoptosis and gene expression were analysed. Extracts were found to contain different types of secondary metabolites mainly terpenes and flavonoids. In cells, extracts were found to inhibit cell proliferation in a concentration and cell type dependent manner. Analysis of the cause of antiproliferation revealed that most cells were found to be arrested at G0/G1 phase with p21 overexpressed. In general most pro-apoptotic genes like Bax and caspase 8 were found to be significantly up-regulated in cells treated with plant extracts. These results suggest that the extracts might induce cell cycle arrest at G0/G1 with p21 attributing to these molecular mechanism.

Keywords: apoptosis, cell cycle, xCelligence, breast cancer and *E. tirucalli*

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1. Introduction

Plants of the Euphorbiaceae family have a long history of use as traditional medicine. In Africa and Fiji, a decoction or maceration of some Euphorbiaceae plants are used to treat asthma, cough, diabetes, cure ear-ache, bowel complaints and infertility [1, 2]. Some have been used for centuries as traditional medicine against cancers and tumours [3]. In countries like Egypt, *Euphorbia* species are widely used in the treatment of stomach, uterus, liver and kidney cancer [4]. The root of *E. tirucalli* is infused in boiling water and used to treat toothache. *Euphorbia tirucalli's* latex is used as treatment for warts [5]. In recent years, the surge in pharmaceutical and phytotherapeutic research of medicinal plants has led to great discoveries. Today, up to 50% of the world drugs are made from natural products or their derivatives [6]. Some of these drugs include aspirin, quinine and morphine amongst others [7]. The recent surge in natural plants research has also led to production of drugs such as taxol, camptothecin and podofilox which are now being used for chemotherapy. These drugs were derived from parts of various plants [8, 9]. As researchers worldwide continue to explore plants for their anti-cancer properties, breast cancer should also be at the forefront of medicinal plant research, since it is now the leading cause of cancer in females [10]. In this study we explore the molecular mechanisms played by extracts of *E. tirucalli* in inducing cell death in breast cancer cell lines.

2. Methods

2.1 Plant materials

Euphorbia tirucalli plants were collected in Limpopo Mankweng Township in February 2012. The plant material was authenticated by Dr Egen from University of Limpopo Herberium

2.2 Preparation of Euphorbia tirucalli extract

Collected fresh *Euphorbia tirucalli* stems were washed with water and cut into smaller parts. These were then evenly spread out and dried at room temperature under a stream of cold air for a week. When completely dried, the plants were ground to a fine powder using a pestle and mortar for maximum area coverage for the extraction process. All plant material was individually extracted by weighing 10 g of finely ground plant material and exhaustively extracting in 100 ml of the appropriate solvent (10:1 solvent to dry weight ratio) in 250 ml schott bottles. The mixture was shaken vigorously at high speed with a Labotec model 20.2 shaking machine for 24 hours. The mixture was centrifuged at 5000 rpm for 5 minutes then filtered through Whatman No. 1 filter paper into pre-weighed labelled containers. The extracts were dried under a stream of cold air at room temperature and the percentage yield was obtained. The extraction solvents used were n-hexane, dichloromethane, chloroform, ethyl acetate, acetone, methanol and butanol (technical grade, Merck). Extracts to be used for biological studies were re-dissolved in dimethyl sulphoxide (DMSO) to give a stock concentration of 100 mg/ml. The quantified extracts were stored at -20°C until required.

2.3 Cell culture

MCF-7, MDA-MB 231 and MRC-5 were obtained from ATCC. All cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 1% penicillin/streptomycin. All cells were maintained in a humidified incubator at 37ºC with 5% CO_2. All cell lines were subconfluently grown and passaged by treating them with 0.05% trypsin-EDTA.

2.4 MTT assay

To determine the cytotoxicity of *E. tirucalli* plant extracts on the breast cancer cells, MTT assay was conducted. MCF-7 and MDA-MB 231 cells (5 x 10^3) were seeded into 96-well microtiter plates in 90 µl culture medium and then incubated overnight. Cells were treated with varying concentrations *E. tirucalli* extracts (dissolved in dimethyl sulfoxide, DMSO) for 24 hours. The MTT solution, prepared at 5 mg/ml in sterile PBS, was added to each well at 10% v/v and incubated at 37ºC for 4 hours. DMSO was added to ensure total solubility of formazan crystals and absorbance was recorded at 570nm using the Multiskan™ Go microplate reader. Each plate contained a blank, vehicle control, positive control, treated and untreated samples. Vehicle control cultures received DMSO (0,1%) alone. Cells treated with staurosporine at 0.1µM served as a positive control.

2.5 Real time cell growth assay

In vitro cell proliferation was measured using x*CELL*igence Real Time Cellular Analysis (RTCA) system. Briefly, the background impendance was measured following addition of 100 µl of growth medium to the 16X E-plates. Cell suspension containing 1x10^4 MCF-7, 5x10^3 MDA-MB 231 or 2x10^4 MRC-5 cells was seeded into the wells and attachment and proliferation were monitored using the RTCA system. Upon reaching logarithmic phase, cells were treated with 10 µl *E. tirucalli* extracts at various concentrations (µg/ml) and continuously monitored for over 48 hours. Vehicle control cultures received DMSO (0, 1%) alone. Cells treated with staurosporine at 0.1µM served as a positive control. Impendance changes expressed as cell index (CI) were automatically calculated as live cells interact with electrodes in the E-plates, correlating with viability or cytotoxicity of cells over time.

2.6 Annexin V/PI apoptosis assay

This technique was employed to determine the type of cell death *E. tirucalli* extracts were inducing. Annexin-V detects the translocation of phosphatidylserine from the inner to the surface of the plasma membrane, which is characteristic of apoptotic cells, whereas PI detects necrotic cells with permeabilized plasma membrane. Cells were treated with plant extracts at IC_{50} for 24 hours. Following treatment, both adherent and detached cells were collected and rinsed twice with cold PBS. The cell pellet was resuspended in 500 µl of annexin binding buffer and incubated with 5 µl of Annexin V-FITC and 5 µl propidium iodide for 5 minutes. The cells were immediately analysed by flow cytometry

2.7 Cell cycle analysis by flow cytometry

Cell cycle analysis was performed by propidium iodode (PI) based measurements of cell DNA content using flow cytometry. Cells were treated with plant extracts at IC_{50} for 24 hours, followed by collection of both attached and detached cells. Pellet was rinsed twice with cold PBS and fixed in 70% ice-cold ethanol overnight at 20ºC. Fixed cells were then washed twice with PBS and DNA was stained with 500µl PI/RNase staining solution and incubated in the dark for 30 minutes. Flow cytometry analysis was carried out using BD Accuri C6.

2.8 Caspase 3/7 activity

Caspase activity was assayed by measuring light intensity using Caspase-Glo® 3/7 (Promega) according to the manufacturer's protocol. Briefly, cells ($5x10^4$ cells/ well) were seeded on a white 96-well microplate and treated with plant extracts at IC_{50} for 24 hours. Caspase-Glo reagent was then added and incubated at room temperature for 30 min. The caspase activities were measured using the Glomax®-96 microplate luminometer. The assay was performed in triplicates and the results were presented as mean of Relative Light Units (RLU)

2.9 Real time PCR

Cells were treated with plant extracts at IC_{50} for 24 hours. The total RNA from treated and untreated samples was isolated using the Zymoresearch Quick RNA™ Miniprep. The RNA concentrations were measured using the NanoDrop and normalised for cDNA synthesis. Total RNA was reverse transcribed using the ImProm-II Reverse Transcription System (Promega) according to manufacturer's specifications. Real time PCR was carried out in a Lightcycler 480 using Luminaris Color HiGreen qPCR Mastermix with specific primers. Amplification products obtained by PCR were electrophoretically separated on a 1% Agarose gel, stained with ethidium bromide and visualized on the Chemidoc MP Imaging system. The data was analysed using REST 2009 software.

2.10 Western blot

At 24 and 48 hours post treatment with plant extracts, cells were lysed using RIPA buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.1% SDS, 2 mM EDTA). Protein content was measured by the BCA assay and equal amounts were electrophoresed in SDS polyacrylamide gel and then transferred onto nitrocellulose membranes. Membranes were subsequently immunoblotted with the anti-p53, anti-RBBP6, anti-Mdm-2, anti-BAX, anti-

Bcl-2, anti-caspase-3, anti-caspase-9, anti-β-actin primary antibodies overnight then in horseradish peroxidase-conjugated secondary antibody for 1 hour. Protein bands were detected on the Chemidoc MP Imaging system following treatment with enhanced chemiluminescence reagent.

2.11 Phytochemistry analysis

Chemical constituents of the plants crude extracts were analysed by thin layer chromatography (TLC) (Merck Silica gel 60 F_{254}, 0.25 mm thick) using aluminium backed silica-gel coated plates (Sigma-Aldrich SA). TLC plates were loaded with 100 µg (10 µl of a 10 mg/ml extract in acetone) and were developed with the following eluent systems: Ethyl acetate/methanol/water = (EMW) 40:5,4:5 (v/v/v) polar/neutral and Benzene/ethanol/ammonium hydroxide = (BEA) 90:10:1 (v/v/v) Non polar/basic. For detection and identification of separated chemical compounds not visible under UV light, vanillin spray reagent was used. Vanillin is composed of 0,1g vanillin, 28ml methanol and 1ml sulphuric acid. The qualitative antioxidant activity of the selected plants was tested using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma) method.

2.12 Statistical analysis

Experiments were either performed in duplicate or triplicate, data sets were expressed as means ± standard deviations (SD). Data were analysed using Student's t-test. Statistical significance was presented as ***P < 0.001, **P < 0.01, *P < 0.05.

3. Results

3.1 Real-time monitoring of cell growth and IC50 concentration estimation.

Cell viability was analysed using the MTT assay following treatment with different concentrations of butanolic, hexane and methanolic extracts of *Euphorbia tirucalli* after 24 hours. The results show that inhibition of cancer cell proliferation was concentration and cell line type depending again on the solvent type. In MCF-7 the IC_{50} was estimated to be 60µg/ml in butanolic extract, 70µg/ml in hexane and 80µg/ml in methanolic extracts (Fig1 a-

c). In MDA-MB231, IC_{50} was estimated as 40µg/ml in butanolic, 50µg/ml in hexane and 100µg/ml in methanolic extracts (Fig 1 d-f).

MTT assays are endpoint assays and can only detect cell viability/ cytotoxicity at a particular time point. Hence, we employed a real-time proliferation assay (RTCA). Experiments were carried out using *xCELLigence* RTCA DP analyzer (Roche) which offers continual label-free and non-invasive analysis of cell proliferation over time. Cell index (CI), were automatically calculated as live cells interact with electrodes in the E-plates. As shown in Fig 2 d& g, a drastic decrease in CI following treatment with the butanol extract was observed in MDA-MB 231. In MCF-7 (Fig 2d), at lower extract concentrations (10 µg/ml), the cells recover from the initial cytotoxic effects. Butanolic extract inhibited cell proliferation in a cell type dependent manner with IC_{50} of 15µg/ml and 30µg/ml for MCF-7 and MDA-MB 231 cells respectively. In MRC5 normal fibroblast at similar concentrations, there was no statistical change in the CI in both treated and untreated cells (Fig 2a). When cells were treated with hexane extracts, CI decreased in all cell lines (fig 2b, e & g) with MDA-MB 231's IC_{50} observed at 50µg/ml (Fig 2g) and MCF-7 at a mean of 30µg/ml (Fig. 2f).

3.2 Euphorbia tirucalli butanolic, hexane and methanolic extracts induces Apoptosis and Cell Cycle Arrest

Analysing cell cycle distribution using propidium iodide staining, MCF-7 cells treated with butanolic, hexane and methanolic extracts showed higher G0/G1 population at 38%, 42% and 67%, respectively (Fig 3). While MDA-MB-231 treated with hexane and methanolic also showed higher G0/G1 population at 37.8% and 42.5%. In addition, butanolic, hexane and methanolic extracts resulted in a decrease in the proportion of cells in the S-phase from 10% in untreated to 5% in all MCF-7 treatments cells and from 8.2% in untreated to less than 1% in treated cells. These data suggests that treatment with butanolic, hexane and methanolic extracts induced cell cycle arrest at the G0/G1 phase (Fig. 3& 4).

Following the observation that the extracts were able to inhibit cell proliferation and induce cell cycle arrest, apoptosis was examined in MCF-7 and MDA-MB-231 breast cancer cells lines to check if the effect was due to cell death by apoptosis. Flow cytometry analysis of stained cells can distinguish cells into four groups, namely viable (LL), early apoptotic (LR), late apoptotic (UR) and necrotic (UL) cells. As shown in Fig 6 & 7, exposure to different

extracts of *Euphorbia tirucalli* at specific concentrations (butanolic 15µg/ml, hexane 30µg/ml and methanolic 50µg/ml) resulted in higher population of apoptotic cells in MCF-7 cells ranging between 53% to 27.1% depending on the solvent type (Figure 6). In MDA-MB 231, the population of apoptotic cells in butanolic 10 µg/ml, hexane 50 µg/ml and methanolic 100 µg/ml extracts were higher at 41% to 20% (Fig. 7) as compared to untreated cells <3%. Overall there was less than 10% of population that was characterised as necrotic.

(i) 3.3 Effects of extracts of Euphorbia tirucallion Caspase-3/7

Caspases are the hallmark of apoptosis and based on whether caspases are activated, apoptosis can be classified as caspase-dependent or caspase-independent. Depending on which caspases are activated, Caspase-dependent pathway can further be divided into extrinsic or intrinsic pathway. Both the intrinsic and extrinsic pathway involves activation of caspase-3/7 which is important for inducing downstream DNA cleavage molecules. To examine the molecular mechanism underlying apoptotic process, we stained cells with aminoluciferin-labeled substrate of caspase and determined the caspase-3/7 activities by measuring the luminescence intensities after 24 hours of treatment. As shown in fig. 8, we observed a gradual increase in caspase-3/7 activity in MDA-MB 231 treated with hexane and methanolic extracts whereas in butanolic extract caspase3/7 remained unchanged. In MCF-7, there was significant reduction of caspase 3/7 activity in hexane treated cell (Fig. 8). Our data suggest that extracts of *Euphorbia tirucalli* induces activation of caspases 3/7 activity in MDA-MB 231 but not in MCF-7 breast cancer cells.

(ii) 3.4 Effects of Euphorbia tirucalli extracts on Anti-apoptotic, pro-apoptotic and cell cycle Molecules

Cell survival is maintained by increased ration of anti-apoptotic molecules to those of pro-apoptotic molecules. To examine if *Euphorbia tirucalli* extracts induce apoptosis by activating apoptotic genes, we performed Real-Time PCR and western blot analysis. Our data showed that in MCF-7 treated with butanol, hexane and methanol extracts, Bcl-2 was down-regulated in treated cells by a mean factor of 0.105 (S.E. range is 0.083 - 0.129). While that of Bax and Bak were up-regulated by a mean factor of 1.619 -2.08 (S.E. range is 1.442 -

1.785) and 1.329-2.932 (S.E. range is 1.172 - 1.482) respectively as compared to the untreated cells. In MDA-MB 231 cells, the ratio of Bcl-2/Bax was reduced due to a significant increase in the expression of Bax in treated cell by a mean factor of 6.352-26.518 folds. Bak was upregulated by a mean factor of 2.413 (S.E. range is 1.981 - 3.259) in hexane treated but remained statistically unchanged in butanolic and methanolic treated cells (Fig. 8). Bad analysis showed that it was only upregulated in MCF-7 treated with methanolic extracts whereas in MDA-MB 231 it was upregulated in butanolic extracts by a mean factor of 1.963 (S.E. range is 1.814 - 2.211) and 2.036 (S.E. range is 1.744 - 2.404) respectively.

Analysing the expression of both caspase 3 and 8, we observed that in MCF-7 both caspase 8 and 3 expression remained statistically unchanged (Fig. 8: a, b & c). While in MDA-MB231, caspase 3 and 8 were up-regulated in butanolic treated cells by a mean factor of 59.866 (S.E. range is 50.807 - 76.344) and of 4.024 (S.E. range is 3.253 - 5.033) respectively as compared to untreated cells. In hexane, caspase 8 was also up-regulated by a mean factor of 8.327 while that of caspase 3 remained unchanged. For methanolic extracts, there was a down-regulation of caspase 8 and no change in caspase 3 expression (Fig. 8: d, e & f).

p21 plays a crucial role in cell cycle as it inhibits cyclins thereby arresting cells. Analysis of CDKN1A (p21) revealed that it was up-regulated in MCF-7 following treatment with the plants extracts in comparison to untreated cells by a mean factor of 5.574 in butanol, 6.396 in hexane and 25.976 in methanol. The same was true in MDA-MB 231 which also showed overexpression of p21 in treated cells. p53 analysis in MCF-7, showed a down-regulation in butanolic extracts by a mean factor of 0.408 (S.E. range is 0.328 - 0.512) whereas in hexane and methanolic extracts remained statistically unchanged. In MDA-MB231, p53 was up-regulated by a mean factor of 2.597 (S.E. range is 1.712 - 3.460) in methanolic treated cells and it was down-regulated by a mean factor of 0.272 in hexane treated cells. While the two p53 regulators, i.e. MDM2 and RBBP6 had differential expression, with MDM2 statistically unchanged in MCF-7 treated cells. While RBBP6 was down-regulated in hexane treated MCF-7 cells by a mean factor of 0.325 (S.E. range is 0.193 - 0.504) but up-regulated in methanolic treated cells by a mean factor of 1.649. In MDA-MB231 both MDM2 and RBBP6 were statistically unchanged in all treatments.

3.5 Identification of compounds from Euphorbia tirucalli using TLC and LC-MS

Chemical analysis of the butanolic, hexane and methanolic fractions showed the presence of tannins, flavonoids, phenols, antioxidants and terpenes (Table 1, fig. 11). And LC-MS analysis has shown presence of a variety of compounds extracted from E. *tirucalli* with most being previously identified and studied in other cancers. However, these compounds were never isolated and identified in this particular plant (Table 2).

4. Discussion

In South Africa, between 1986 and 1992, cervical cancer was the most leading cause of cancer amongst women, but recently breast cancer seems to have overtaken and is now the most common cancer in females and development of novel therapeutic agents are urgently needed [11, 10]. Although several new therapeutics have been discovered including chemotherapy, they remain ineffective if the disease was diagnosed in advanced stages or the side effects are unbearable to the users [12]. In under developed and developing countries, natural compounds derived from plants have long been used to treat a number of human diseases. In this context, plant-derived compounds play an important role in the development of new anti-cancer agents against human breast cancer.

In this study, butanolic, hexane and methanolic extracts of *E. tirucalli* were tested for potential antiproliferative activity on MCF-7 and MDA-MB 231 human breast cancer cells. We showed that *E. tirucalli* effectively inhibited cell growth of MCF-7 and MDA-MB 231. Irrespective of the extracts, the antiproliferative activity was found to be concentration dependent. It was also found that the antiproliferative activity of the extracts were different and specific towards the cell type. Overall, butanolic extract induced the maximum cancer cell death followed by hexane and methanolic extract. In all cases, antiproliferative activity was concentration dependent and cell type dependent. Our findings are in line with a study by Aljabarin *et al* [13], who tested the anti-cancer properties of the methanolic extracts of *E. tirucalli* on EMT-6 mouse breast cancer cells, whereby they also observed cell proliferation inhibition in a concentration dependent manner. Studies on other Euphorbia species also reported similar anticancer activities that were dependent on cell type or concentration [14, 15, 16, 17]. Further studies by Amirghofran *et al*. [18] found extracts of *Euphorbia cheiradenia* to inhibit cell proliferation in leukemic cancer lines (K562 and Jurkat) in a dose dependent manner.

MTT is one of the oldest techniques that have been used to estimate the toxic concentration of compounds or new drugs. However, it lacks the accuracy to predict the exact concentration, in compensating for MTT, we employed xCELLigence assay to predict the accurate concentration that is potent and also monitor real time analysis of cell proliferation following treatment with the 3 extracts in breast cancer cell lines and MRC 5 fibroblast that mimic normal cells. *xCELLigence* technology measures impedance changes in a meshwork of interdigitated gold microelectrodes located at the well bottom (E-plate) or at the bottom side of a microporous membrane [19, 20]. These changes are caused by the gradual increase of electrode surface occupation by proliferating cells during the course of time and thus can provide an index of cell viability [21]. Staurosporin was chosen as it is widely used as a treatment for a variety of tumour types and previous reports on the use of staurosporin as a tool for cytotoxicity screening including in breast cancer cell line is documented [22]. From our studies we have observed that at 0.2 uM staurosporin was able to inhibit cell proliferation in all cell lines, this as predicted in line with other reported results [23]. Profiling the different concentrations of plant extracts, it was visible that the type of cell proliferation associated with the extracts was cell type and concentration dependent. Like in MTT and other reports, butanolic extracts showed toxicity in MCF-7 and MDA-MB231 at a much lower concentration, followed closely by extracts of hexane and methanol was the least [24, 25]. Indeed, the xCELLigence device has been reported to generate compound-specific kinetic profiles on cultured cells, hereby we demonstrate associations with the three extracts and hope to associate it with respective mechanisms of action. In fact, defects in apoptotic pathway are thought to contribute to a number of human malignancies [26, 27]. Thus, anti-cancer agents that induce apoptosis are one of the efficient strategies in cancer chemotherapy.

Since, *E. tirucalli* extracts showed cell death and antiproliferative activity, with maximum cancer cell death induced at reasonable concentrations in both MCF-7 and MDA-MB 231 cells; we carried out mechanism and characterization study of the type of induced cell death. Generally, anticancer agents have been reported to induce cell death by blocking cell cycle progression and triggering tumour cell apoptosis [28, 29]. Therefore, cell cycle arrest and the induction of apoptosis in cancer cells become the major indicators of anticancer effects. The p53 protein is an essential molecule that arrests cells with damaged DNA in G1and G2, allowing time for DNA repair before the cell tries to enter the M phase [30, 31]. But if the

cell is committed to division or the damage cannot be repaired, then p53 triggers a program cell death through the extrinsic and intrinsic apoptotic pathways. To elucidate the underlying mechanism of cell growth inhibition, cell cycle progression was then analysed for MCF-7 and MDA-MB231 cells after treatment with extracts of *E. tirucalli*. Flow cytometry analysis showed that treatment with the three extracts arrested the cell cycle at the G0/G1 phase and in most cases induced early and late apoptosis in both cell lines. Through Annexin and PI staining, flow cytometry showed that the number of dead MCF-7 and MDA-MB 231 was significantly increased in butanolic extracts in comparison to the other two extracts. These types of results were similar to those reported earlier in our laboratory that methanolic extract exhibited apoptosis in MCF-7 [32]. Similarly in a study by Mahassni and Al-Reemi [33] they reported that extracts of Lepidium sativum seeds induced cell death in MCF-7. In another study by Hsieh *et al* [34] using the *Euphorbia formosana* Root Extract in leukaemia cells they found them to induce apoptosis and cell cycle. Moreover, all 3 extracts downregulated the expression of MDM2 and RBBP6 while upregulating the expression of p21 in MCF-7 and MDA-MB231 cells. Therefore, it is reasonable to speculate that the mechanisms by which *E. tirucalli* extracts alleviates cell growth may be partly via the induction of cell cycle arrest due to the upregulation of p21 that might then suppress cyclins. A similar mechanism was reported by Ujiki *et al* [35] where they showed that the effect of Luteolin on cell cycle and apoptosis in Hela cells. In another study by Krifa *et al* [36] the also reported that aqueous gal extracts of *Limoniastrum guyonianum* was able to induce apoptosis and arrest cell cycle in human cervical cancer by upregulating p21 that resulted in suppression of Cyclin B1.

To understand the potential antitumour mechanisms of *E. tirucalli* extracts, the expression of pro- and antiapoptotic genes was investigated in the present study. We found decreased expression of Bcl-2 mRNA and protein, but increased expression of Bax, Bad and caspase 8 mRNA or protein; therefore, the Bax:Bcl-2 ratio was elevated. In addition, the expression of MDM2 and RBBP6 mRNA and proteins was also downregulated with that of p21 highest in all cell lines and extracts. However, Bcl-2 mRNA and protein were significantly downregulated in MCF-7 cells treated with all three extracts compared with controls. In this study p53 was found to have either remained unchanged or decreased in some instances following treatment with the three plant extracts. In a study by Amirghofran *et al* [18] using Leukaemia cell lines they also found that extracts of Euphorbia cheiradenia were able to

increase Bax/Bcl2 ratio and caspase 3 activation. The data presented in this study suggest that *E. tirucalli* -induced apoptosis is mediated by the death receptor and mitochondrial apoptotic pathways, as demonstrated by increased expression levels of caspase-8 and 3 after treatment.

These pro- and antiapoptotic proteins are the principal regulators of the intrinsic pathway of apoptosis [37]. Previous reports have shown that the ratio of Bax to Bcl-2 determines, in part, the susceptibility of cells to death signals [38]. This was also evident in a study by Alshatwi *et al* [39], where they found that methanolic extracts J*uglans regia* increased Bax protein levels in PC3 cells whereas the levels of Bak and Bcl-xL proteins were unchanged. In another study by Alshatwi *et al* [39], Bcl-2 expression was significantly inhibited, whereas the expression of Bax, Tp53, and caspase-3 markedly increased in human prostate cancer cell treated with extract of Green Husk of *Sugians regia*. It is well established that Bcl-2 generally acts as a potent anti-apoptosis gene by controlling several key steps in apoptosis signaling, including formation of ion channels in biological membranes that influence permeability of intracellular membranes and cytochrome c release from mitochondria. The fact that, in this study Bcl-2 was downregulated but both Bax and Bad upregulated suggested that the molecular mechanism that is associated with cell growth inhibition might be the one involving mitochondria activation and cytochrome c release.

The caspase −9 and −8 are upstream caspases that converge to caspase-3, a key effector molecule in the caspase-dependent cell apoptosis pathway that cleaves a number of cellular proteins including PARP, leading to DNA fragmentation and triggering apoptosis [40, 41]. In this study, the caspase-3/7 activity was increased significantly in treated MDA-MB231 as compared to control ($p<0.05$) which indicated enhanced caspase activation of apoptosis by methanolic treatment. In MCF-7 there was a decrease in caspase activity however that was not significant which might have been due to the fact that caspase 3 has been reported to be lacking in MCF7 [42]. Results obtained were supportive to Yaacob *et al.*[43] that reported *S. crispus* extract induced apoptosis via caspase-3/7 activation in breast and prostate cancer. When caspase 8 was evaluated in both mRNA and Protein following treatment with plant extracts, we have observed that it was upregulated in almost all treatment which suggested that extrinsic pathway might be activated. Caspase inhibitor assay could be done to help in determining involvement of various caspases in apoptotic pathway.

TLC and LC-MS analysis revealed the presence of unique phytochemicals in the *E. tirucalli* extracts suggesting that the differences and concentration of phytochemicals in the *E. tirucalli* extract was responsible for producing the maximum antiproliferative effect on the cancer cells (Table 1 & 2). A number of the phytochemicals identified in *E. tirucalli* have previously been isolated from other sources and tested against different cancers [44, 45]. Emirdağ-Öztürka *et al*. [46] tested the anti-cancer activities of the compound gypsogenin against two cancer cell lines: Saos-2 and MCF-7 and found it to exhibit antiproliferative activities against the cancer cell lines. A different study investigated the antiproliferative properties of alpha-chaconine in lymph-node carcinoma of the prostate (LNCaP) and prostate cancer-3 (PC-3), and it was found to inhibit cell proliferation [47]. Idibie *et al.* [48] showed that linamarin was able to exhibit cytotoxic effects on the cells (Table 2) against cancer cell lines: MCF-7, HT-29 and HL60. Most of the compound we identified to be present in *E. tirucalli* have also been previously reported to exhibit anticancer properties. It is possible that the same compounds were accountable for the anti-proliferative activity we observed in breast cancer cells we tested. In future, it would be interesting to isolate and to test these compounds individually or as cocktail in the breast cancer cells to determine if they can induce cell death without on their own without being toxic.

5. Conclusion

The results have shown that extracts of *E. tirucalli* have a promising anticancer activity. However individual extracted compound might be useful as anticancer treatment but they need further testing.

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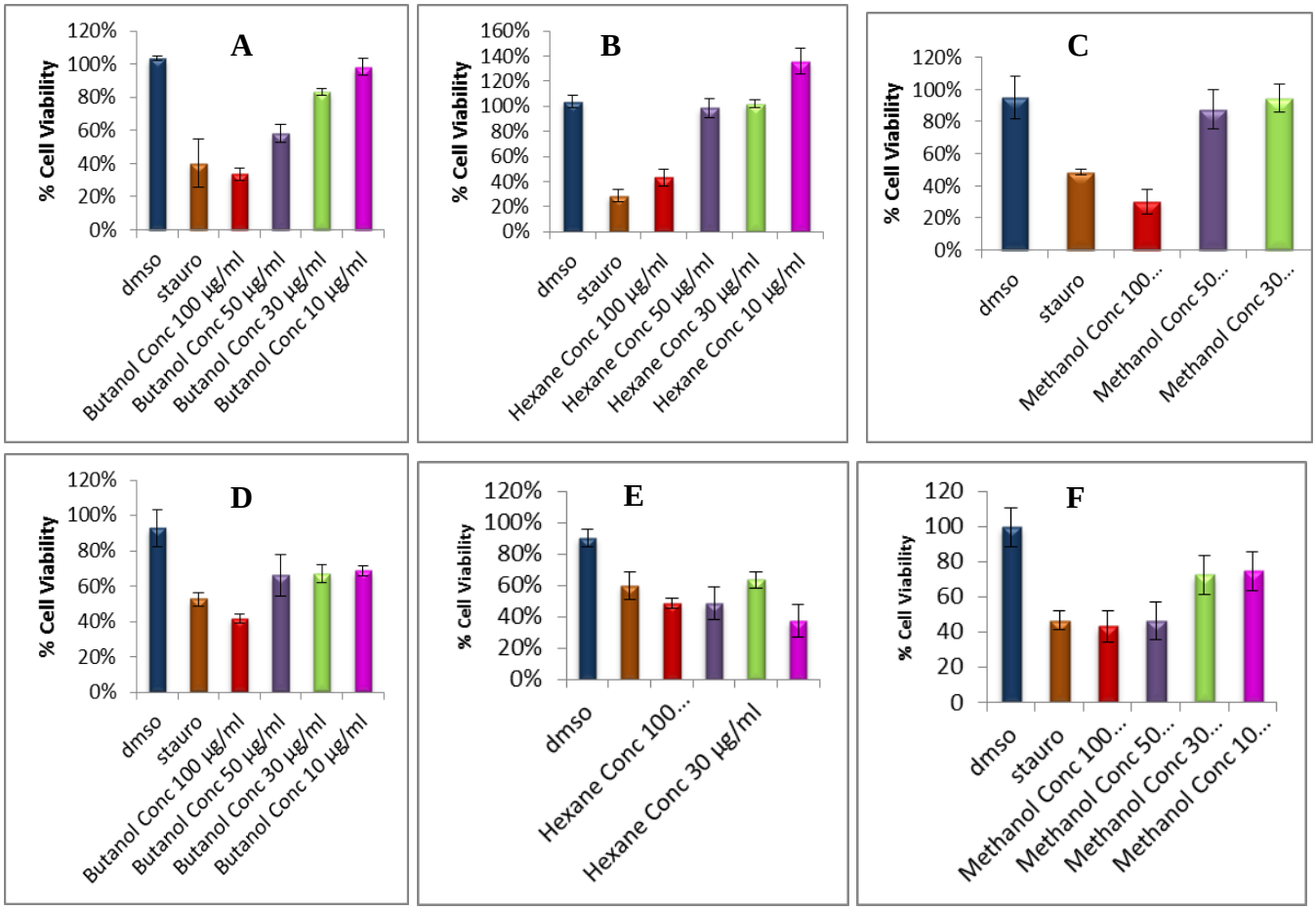


Fig. 1 Cell viability analysis using MTT assay. (a-c) MCF-7 and (d-f) MDA-MB 231 cells were treated with varying concentrations of butanol (a & d), hexane (b & e) and methanol(c & f) extracts of *Euphorbia tirucalli* for 24 hours. IC50 was characterised as concentrations where 50% cell death was observed.

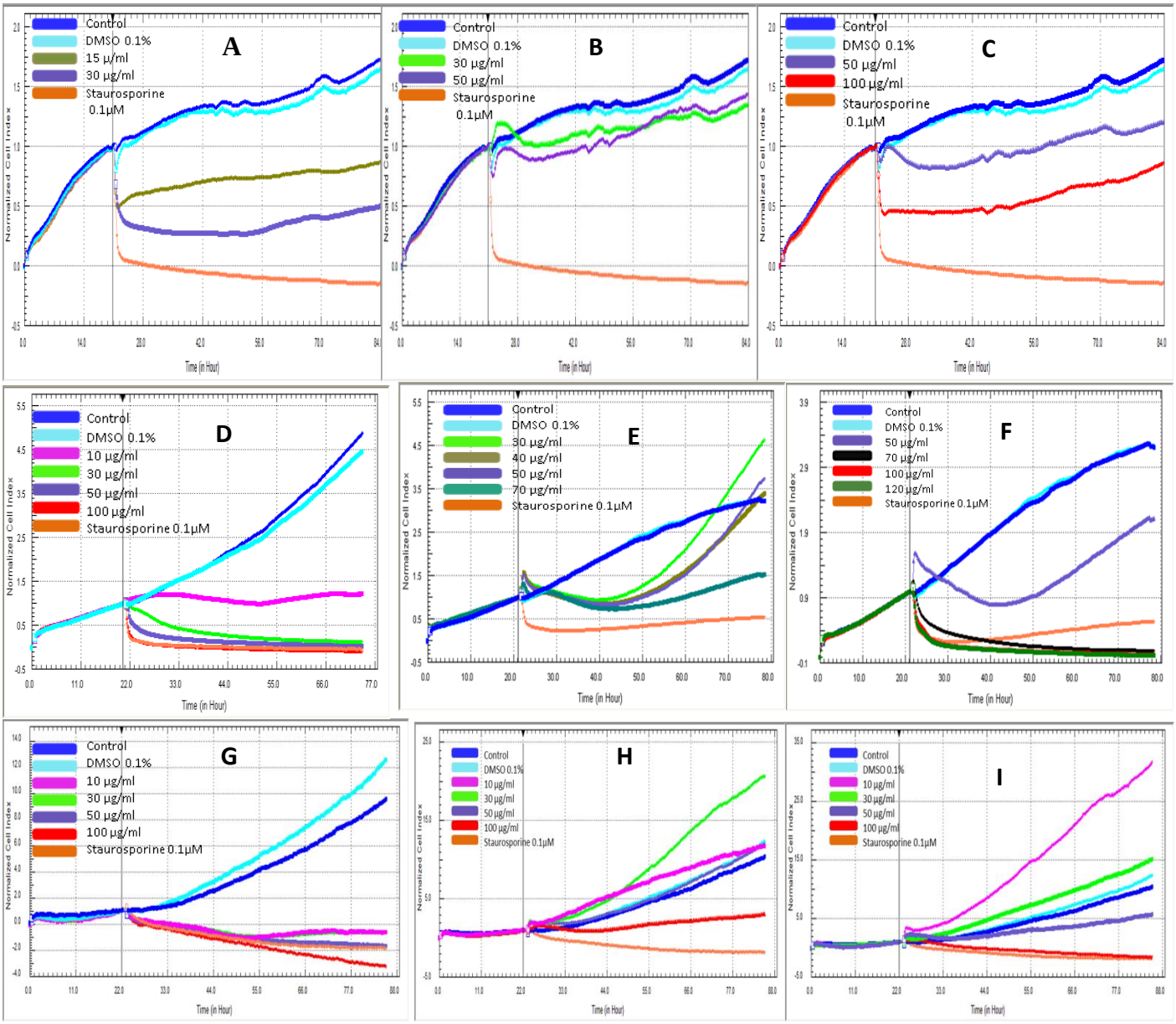


Fig. 2 Dynamic monitoring of *Euphorbia tirucalli* plant extract interaction with MRC-5(a-c), MCF-7(d-f) and MDA-MB 231 (g-i) cells using RTCA analyser. Cells were seeded for 21 hours, at which point, butanol (a, d & g), hexane (b, e & h) and methanol (c, f & i) extracts

were added at indicated concentrations. Cell growth was continuously monitored for over 48 hours. CI values were normalized to the point of compound addition indicated by the vertical black line

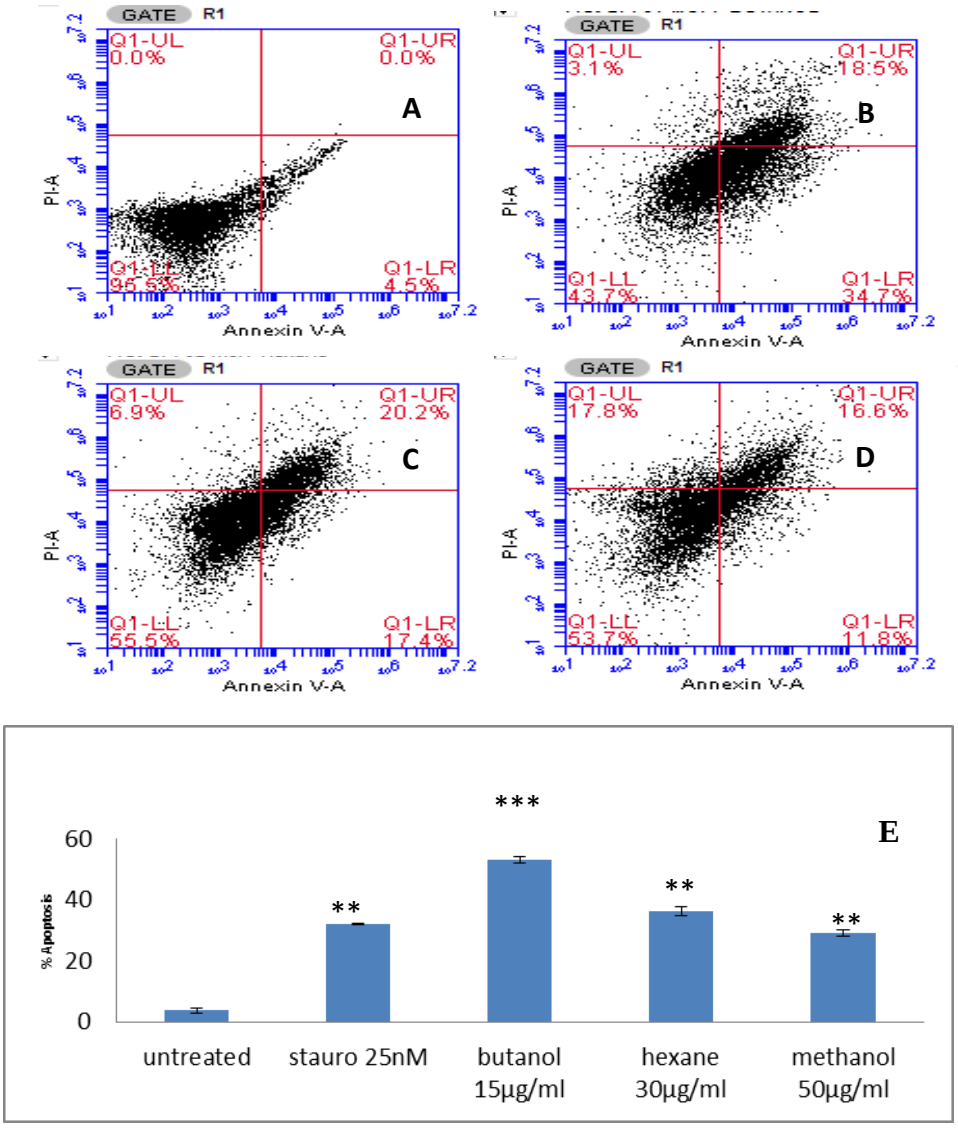
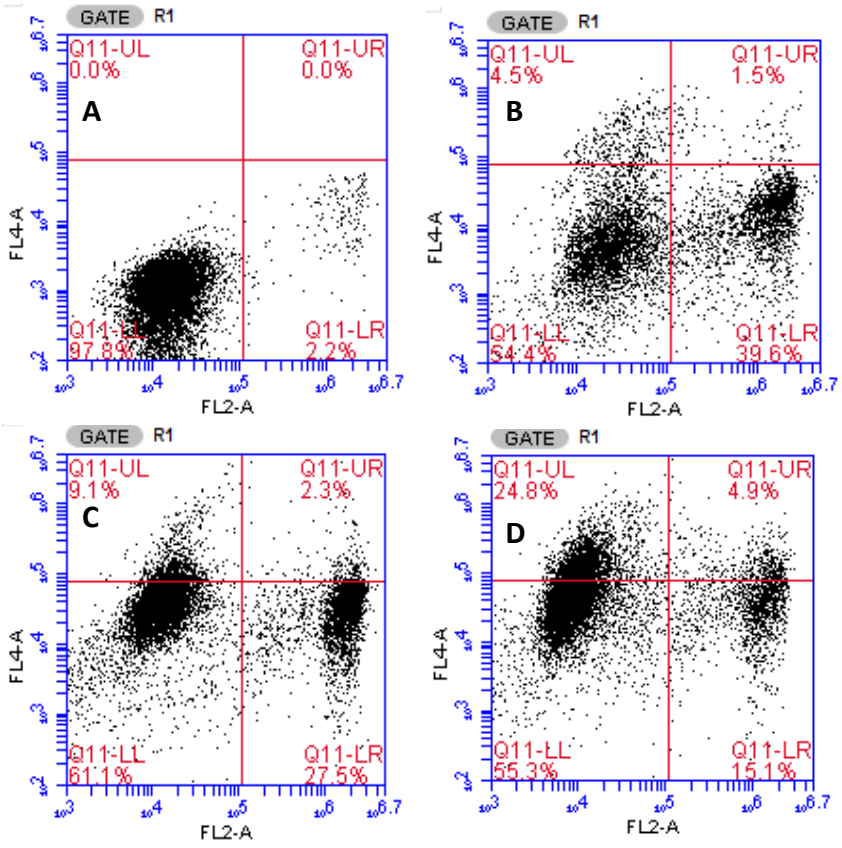


Fig. 3 MCF-7 apoptosis determination using Annexin V/PI staining. Annexin V/PI stained MCF-7 cells following treatment with either **(b)** butanol, **(c)** hexane and **(d)** methanol extracts of *Euphorbia tirucalli* in comparison with untreated cells at 24 hours. Representative figures showing populations of viable (LL), early apoptotic (LR), late apoptotic (UR) and

necrotic (UL) cells. **(e)** Bar graphs showing percentages of apoptotic cells for each treatment sample at 24hours. Data were mean ± SD from two independent experiments (**P<0.01, ***P<0.001)



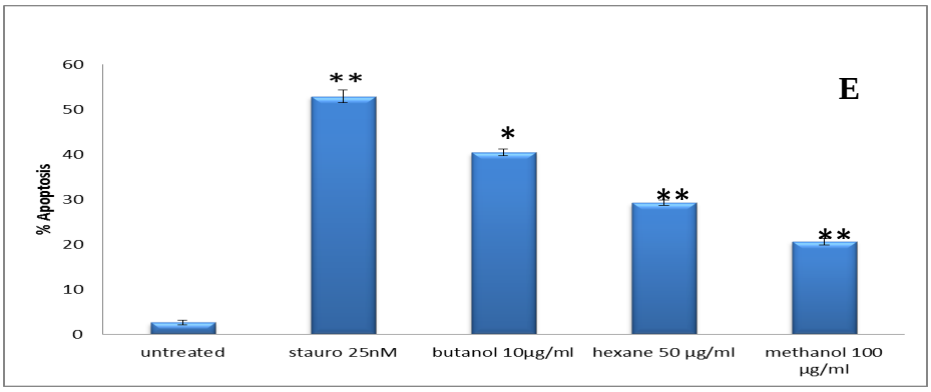


Fig. 4 MDA-MB 231 apoptosis determination using Annexin V/PI staining. Annexin V/PI stained MDA-MB 231 cells following treatment with either **(B)** butanol, **(C)** hexane or **(D)** methanol extracts of *Euphorbia tirucalli* in comparison with control treatment at 24 hours. Representative figures showing populations of viable (LL), early apoptotic (LR), late apoptotic (UR) and necrotic (UL) cells. **(E)** Bar graphs showing percentages of apoptotic cells for each treatment sample at 24hours. Data were mean ± SD from two independent experiments (*P<0.05, **P<0.01)

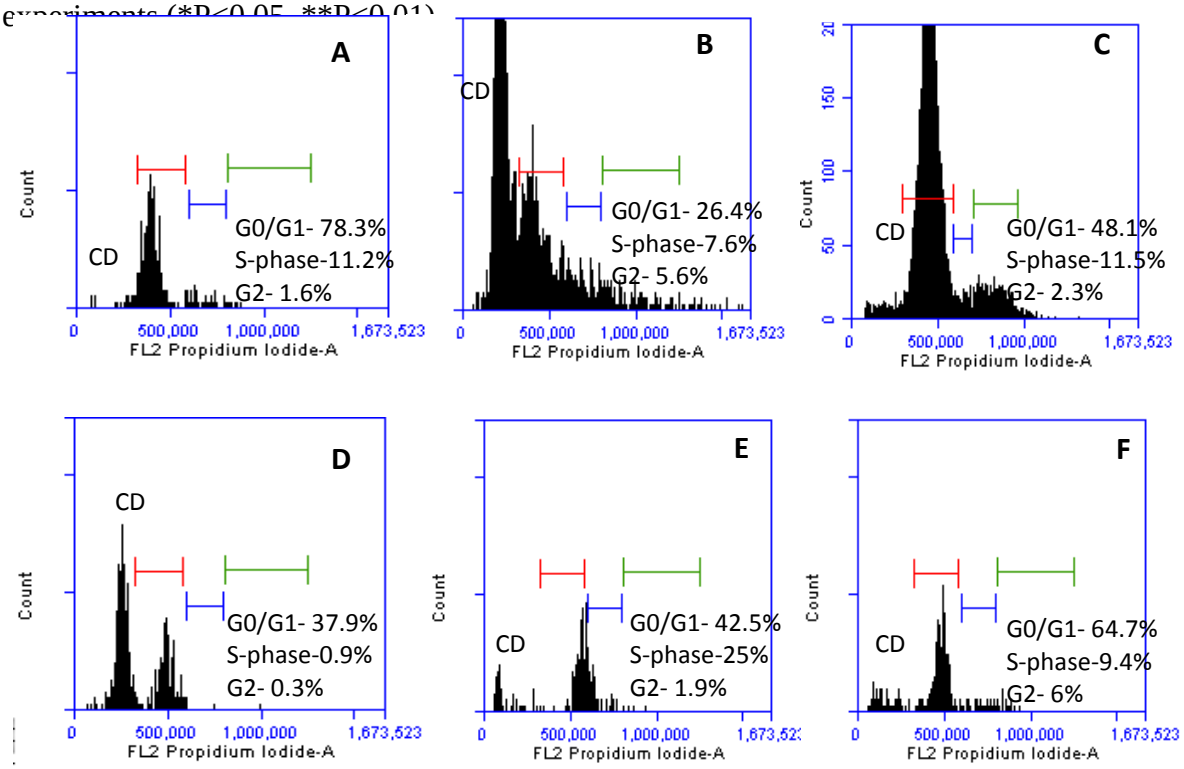


Fig. 5 Effects of *Euphorbia tirucalli* extracts on cancer cell cycle progression. MCF-7 cell cultures received (a) no treatment, (b) flash frozen cells, (c) treated with taxol, (d) treated with butanolic, (e) hexane and (f) methanolic extracts of *E. tirucalli*. CD represents cell death

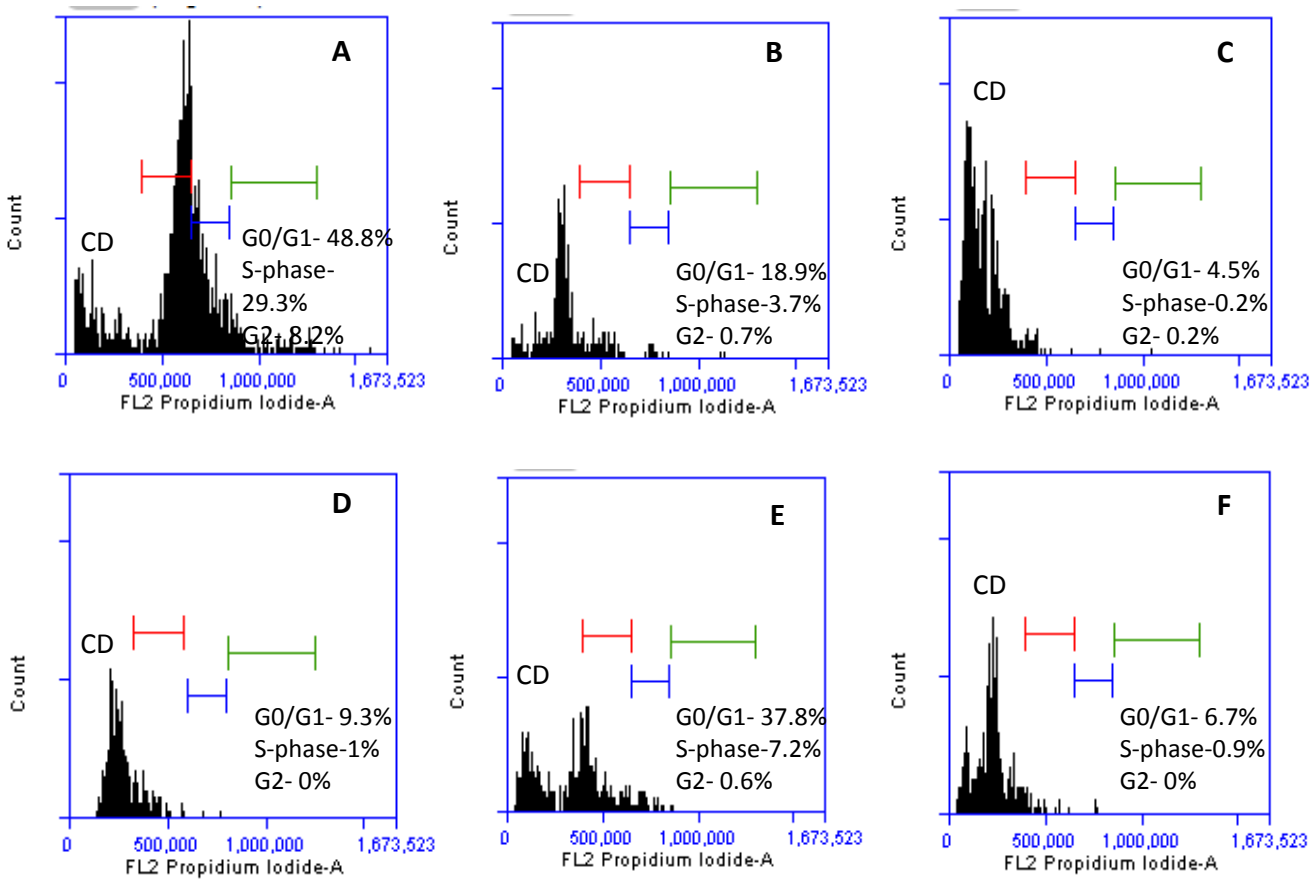


Fig. 6 Effects of extracts of *Euphorbia tirucalli* on cancer cell cycle progression. MDA-MB 231 cell cultures received (a) no treatment, (b) flash frozen cells, (c) treated with taxol, (d) treated with butanolic, (e) hexane and (f) methanolic extracts of *E. tirucalli*. CD represents cell death

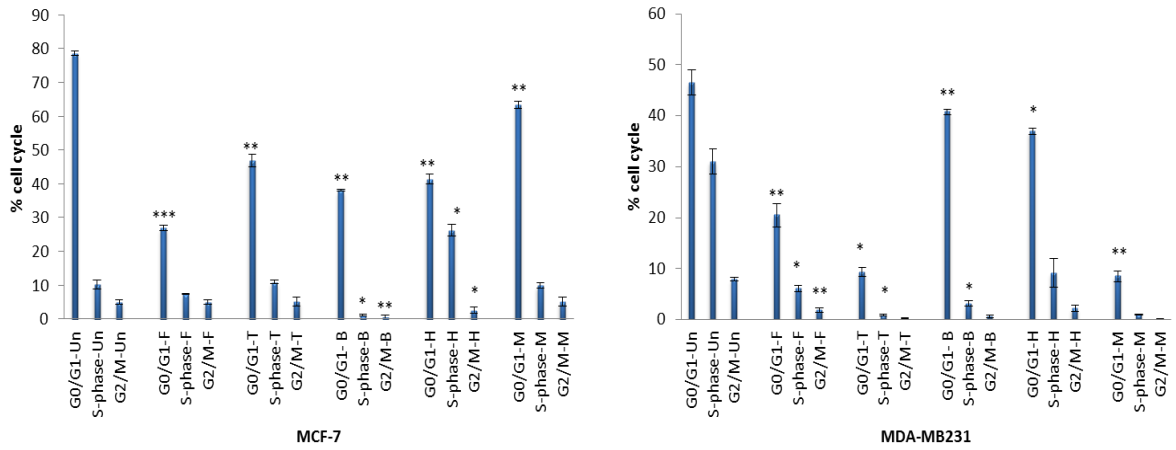


Fig. 7 Bar graphs representing cell cycle analysis of MCF 7 and MDA-MB231 treated with butanolic, hexane and methanolic extracts of *Euphorbia tirucalli*. Data were mean ± SD from two independent experiments (*P<0.05, **P<0.01, ***P<0.001)

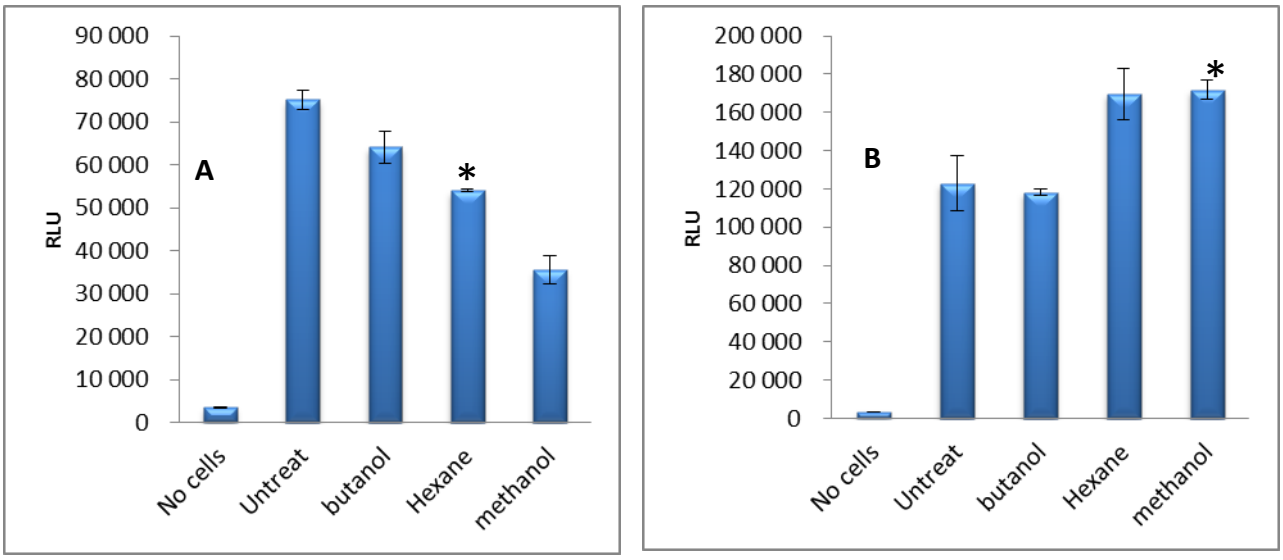


Fig. 8 Caspase 3/7 activity in MCF-7 and MDA-MB 231. (A) MCF-7 and (B) MDA-MB 231 cells were treated with plant extracts' IC_{50} concentrations for 24 hours. Caspase 3/7 activities in treated and untreated cells were then determined by luminescence assay. Data were mean ± SD from three independent experiments (*P<0.05, **P<0.01)

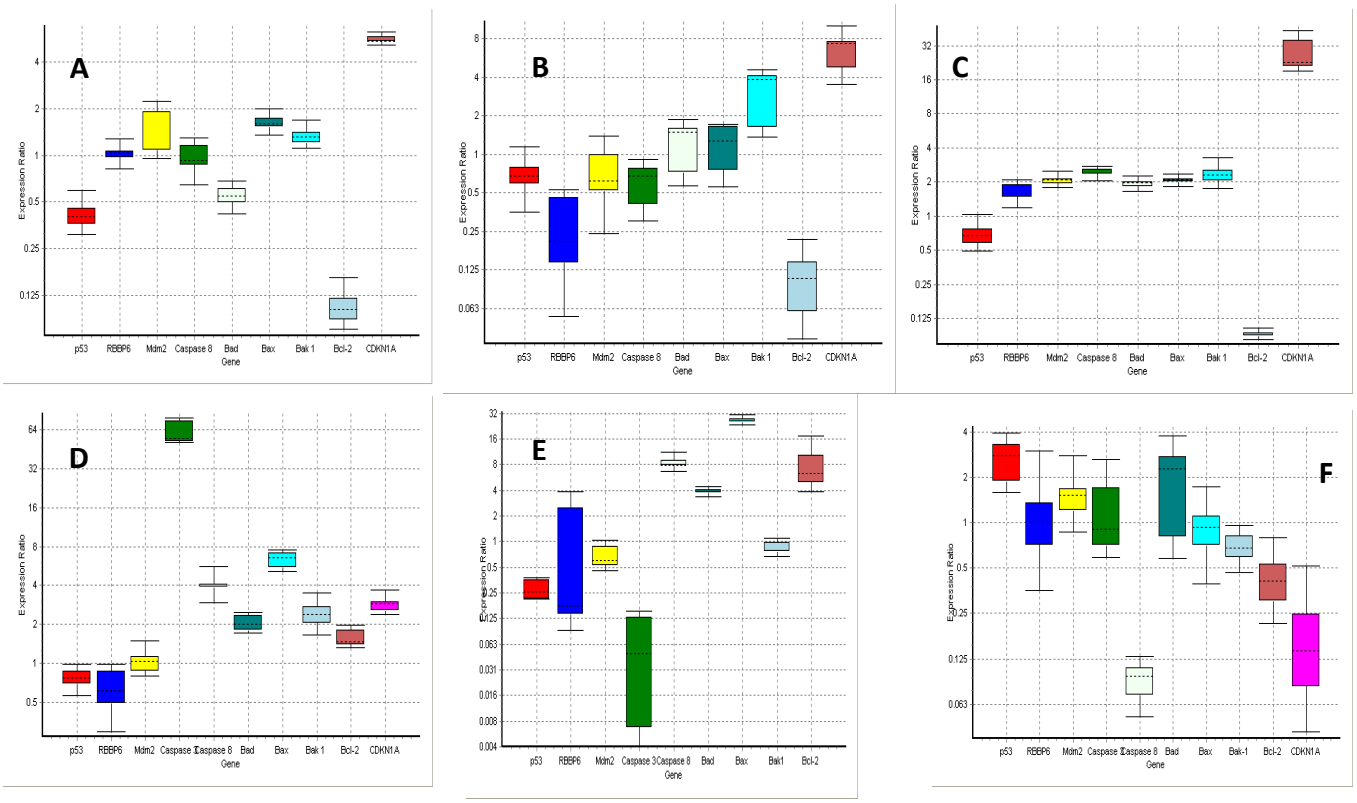


Fig. 9 Box plot representing Real Time PCR analysis using REST 2009 software. (a-c) MCF-7 and (d-f) MDA-MB 231 gene expression analysis following treatment with (a & d) butanol, (b & e) hexane and (c & f) methanol extracts of *Euphorbia tirucalli* for 24 hours.

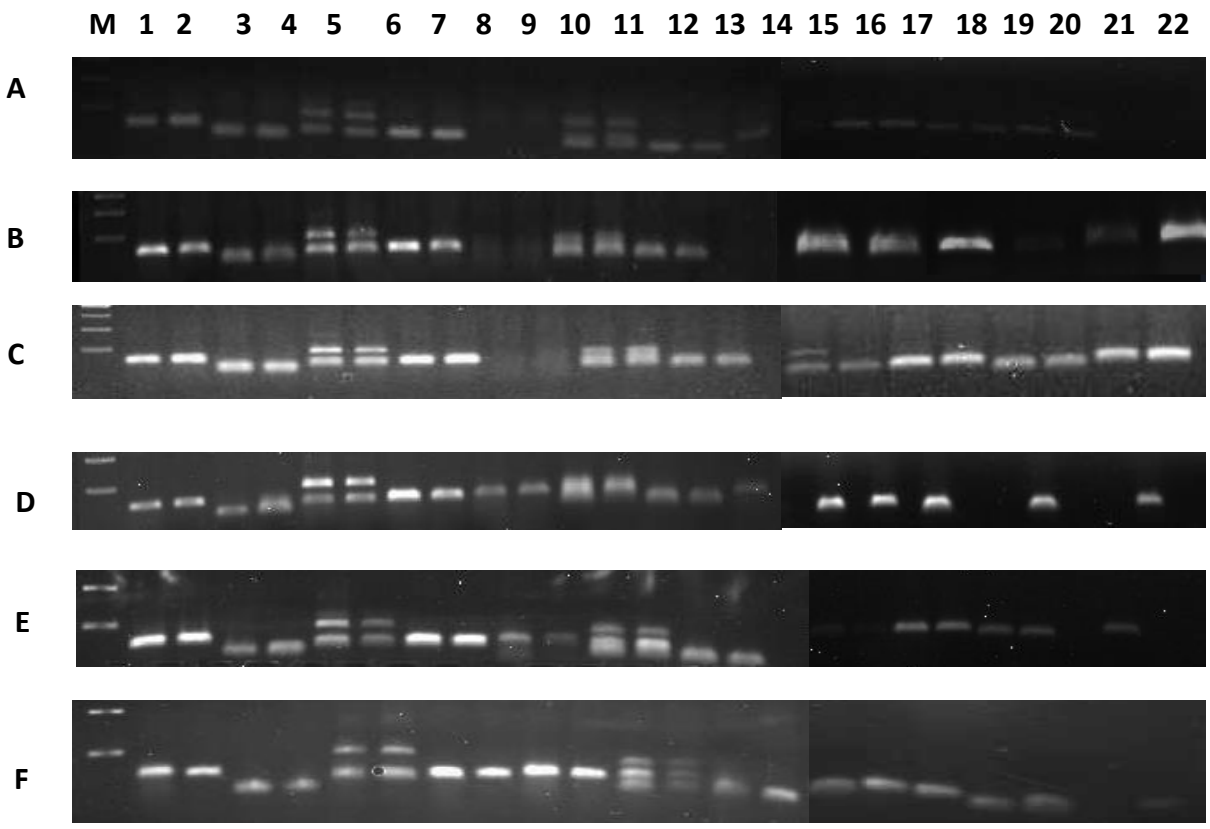


Fig.10 Real time PCR products run on a 1% agarose gel electrophoresis. (a-c) MCF-7 and (d-f) MDA-MB 231 cells treated with (a & d) butanol, (b & e) hexane and (c & f) methanol extracts of *E. tirucalli*. M=Marker, 1 = Untreated GAPDH, 2 = Treated GAPDH, 3 = p53 untreated, 4 = p53 treated, 5 = RBBP6 untreated, 6 = RBBP6 treated, 7 = Mdm-2 untreated, 8 = Mdm-2 treated, 9 = Caspase-3 untreated, 10 = Caspase-3 treated, 11 = Capsase-8 untreated,12 = Caspase-8 treated, 13 = Bad untreated, 14 = Bad treated, 15 = Bax untreated, 16 = Bax treated, 17 = Bak-1 untreated, 18 = Bak-1 treated, 19 = Bcl-2 untreated, 20 = Bcl-2 treated, 21 = P21 untreated, 22 = P21 treated

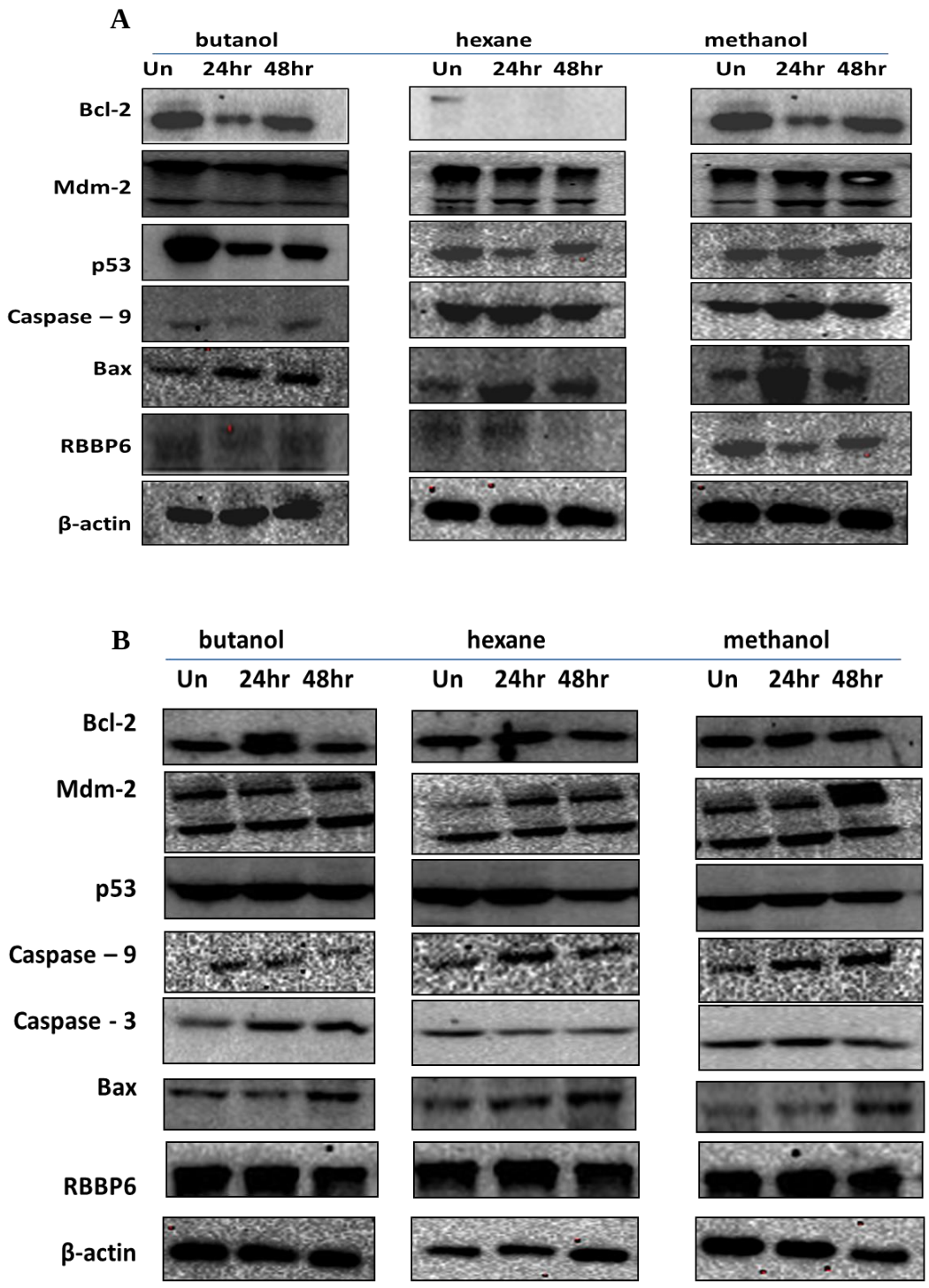


Fig. 11 Western blot analysis of protein expression in (a) MCF-7 and (b) MDA-MB 231 cells following treatment with butanol, hexane and methanol extracts of *E. tirucalli*. Un= Untreated samples. The results show a varying protein expression of different apoptotic genes 24 or 48 hours post treatment with the 3 plant extracts after.

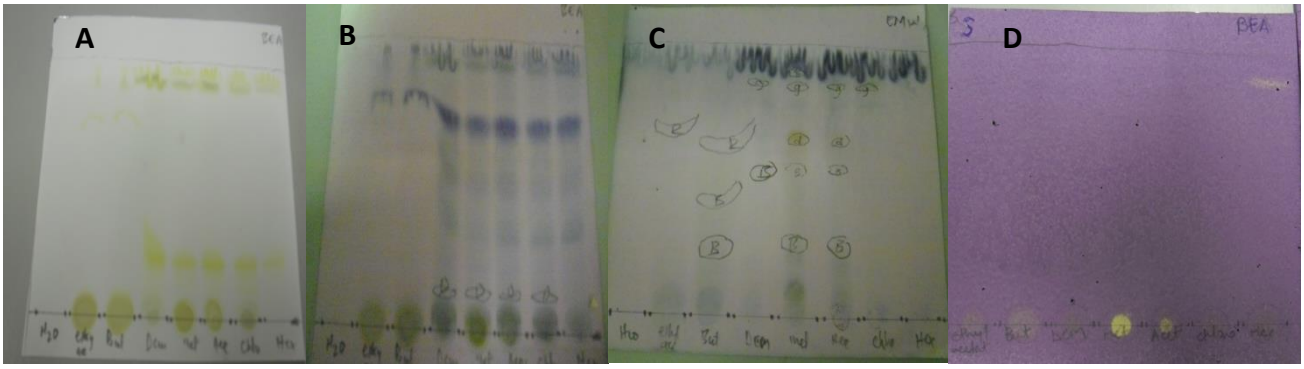
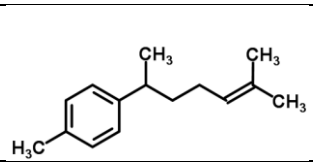
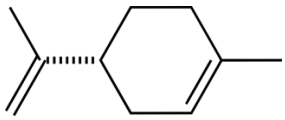
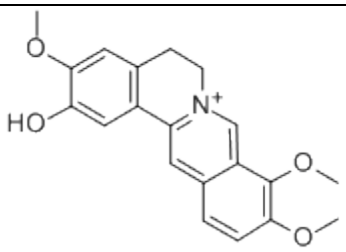
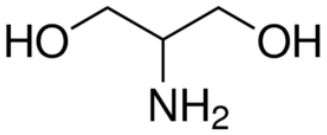
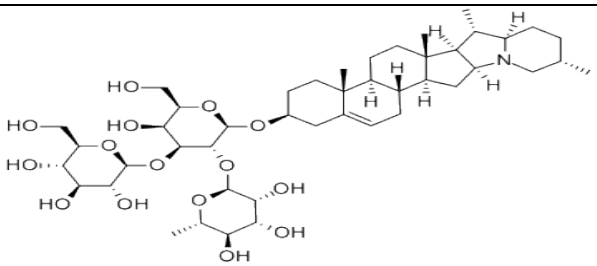
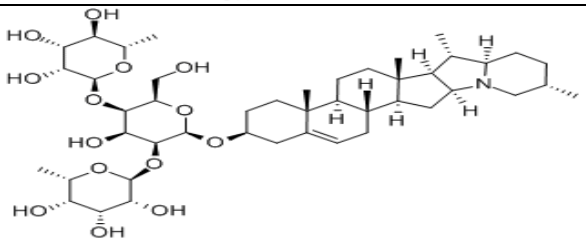


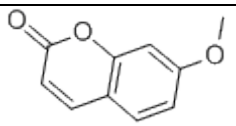
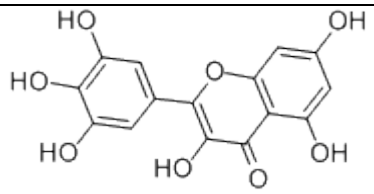
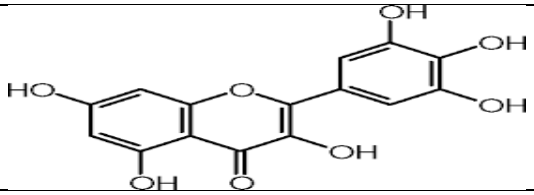
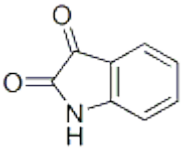
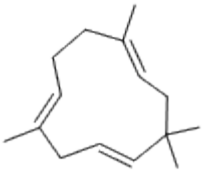
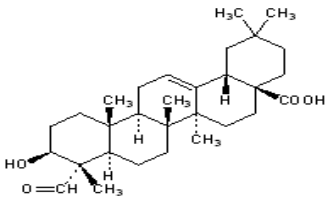
Fig. 12 Thin layer chromatographic separation of butanolic, hexane and methanolic fraction from *E. tirucalli* the arrow indicates the terpenes and antioxidant compounds.

Table 1: Secondary metabolites tests of *E. tirucalli*

Secondary metabolite	**Presence**
Tannins	Positive
Terpenes	Positive
Steroids	Negative
Cardiac glycosides	Negative
Flavonoids	Positive
Saponins	Negative
Phenols	Positive

Table 2: LC- MS representation of terpene compounds identified from *E. tirucalli* extracts.

Compound	**Structure**	**Solvent and cancer type for antiproliferative**	**Reference**
Alpha-curcumene		Methanol, hexane Ovarian cancer,	[49]
d-limonene		Methanol, butanol	[50]
Columbamine		Methanol, human osteosarcoma U2OS	[51]
Serinol		Methanol, human neuroblastoma, glioma, medulloblastoma, and adenocarcinoma	[52]
Alpha- solanine		Methanol, butanol,	[53]
Alpha-chaconine		Hexane, antiproliferative properties in lymph-node carcinoma of the prostate (LNCaP) andprostate cancer-3 (PC-3) .	[47]

Alpha-pinene		Hexane, induce apoptosis in metastatic melanoma.	[55]
Herniarin		Hexane, cytotoxic against ovarian (CH1), lung (A549) and melanoma (SK-MEL-28) cell lines.	[56]
linamarin		Butanol. Cytotoxic effects on MCF-7, HT-29 and HL60 cells	[48]
myricetin		Butanol, anticancer effects of myricetin on HCT-15 human colon cancer cells	[45]
isatin		Butanol, antiproliferative activity against tumourigenic K562 cell line	[44]
Alpha-humulene		Methanol	[57]
gypsogenin		Methanol. antiproliferative activity against two cancer cell lines (Saos-2 and MCF-7),	[46]

Appendix 4

Elsevier Editorial System(tm) for Phytomedicine
Manuscript Draft

Manuscript Number: PHYMED-D-15-00177

Title: Cell cycle and apoptosis induction in ovarian (RMG-1) and cervical cancer cell line (SiHa) via p21 activation by Flavonoids components isorhamnetin extract of Euphorbia tirucalli.

Article Type: Original Article

Section/Category: Cancer

Keywords: Apoptosis, p21 and cell cycle

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First Author: Mpho S Choene, M.Sc

Order of Authors: Mpho S Choene, M.Sc; lesetja Raymond Motadi, PhD

Abstract: Gynaecological cancers remain a huge burden to women in developing countries. Standard treatments usually involve surgery, chemotherapy and/or radiation either as single agents or in combination. Flavonoids have played a vital role in health because they also help support detoxification of potentially tissue-damaging molecules, their intake has often, although not always, been associated with decreased risk of certain types of cancers, including lung and breast cancer. In this study we looked at the molecular analysis of the impact of Flavonoids components isorhamnetin extract from Euphorbia tirucalli on ovarian and cervical cancer. Euphorbia tirucalli plant material was individually extracted using butanol, hexane and methanol. Different compounds were identified and isolated using TLC, HPLC and Mass Spectrophotometer. Ovarian RMG-1 and cervical SiHa cells were treated with varying concentrations of isolated flavonoid component isorhamnetin extracts of E. tirucalli and monitored for over 48 hours. Cell viability was analysed using MTT assay and xCELLigence RTCA (real time cell analyzer). Cell cycle and apoptosis were measured by flow cytometry to assess the effects of the extracts. Molecular analysis using real time PCR and western blot was performed to evaluate effect of the extracts on apoptotic genes at IC50. The extracts inhibited cell viability in a cell type and concentration dependent manner. The treated ovarian cells were found to be arrested at G0/G1 cell cycle arrest. Apoptosis was induced in most the treatments on both cell lines. The cell cycle gene p21 was found to be significantly up-regulated in both cell lines for all treatments. The pro-apoptotic Bax was found to be upregulated in most treatments Flavonoid component isorhamnetin inhibited cell proliferation in a cell type and concentration dependent manner; it arrested cell cycle by upregulating p21.

Cell cycle and apoptosis induction in ovarian (RMG-1) and cervical cancer cell line (SiHa) via p21 activation by Flavonoids components isorhamnetin extract of *Euphorbia tirucalli*.

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Abstract

Gynaecological cancers remain a huge burden to women in developing countries. Standard treatments usually involve surgery, chemotherapy and/or radiation either as single agents or in combination. Flavonoids have played a vital role in health because they also help support detoxification of potentially tissue-damaging molecules, their intake has often, although not always, been associated with decreased risk of certain types of cancers, including lung and breast cancer. In this study we looked at the molecular analysis of the impact of Flavonoids components isorhamnetin extract from *Euphorbia tirucalli* on ovarian and cervical cancer. *Euphorbia tirucalli* plant material was individually extracted using butanol, hexane and methanol. Different compounds were identified and isolated using TLC, HPLC and Mass Spectrophotometer. Ovarian RMG-1 and cervical SiHa cells were treated with varying concentrations of isolated flavonoid component isorhamnetin extracts of *E. tirucalli* and monitored for over 48 hours. Cell viability was analysed using MTT assay and x*CELL*igence RTCA (real time cell analyzer). Cell cycle and apoptosis were measured by flow cytometry to assess the effects of the extracts. Molecular analysis using real time PCR and western blot was performed to evaluate effect of the extracts on apoptotic genes at $IC_{50.}$ The extracts inhibited cell viability in a cell type and concentration dependent manner. The treated ovarian cells were found to be arrested at G0/G1 cell cycle arrest. Apoptosis was induced in most the treatments on both cell lines. The cell cycle gene p21 was found to be significantly up-regulated in both cell lines for all treatments. The pro-apoptotic Bax was found to be upregulated in most treatmentsFlavonoid component isorhamnetin inhibited cell proliferation in a cell type and concentration dependent manner; it arrested cell cycle by upregulating p21.

Keywords: isorhamnetin, cell cycle, apoptosis, p21 and *Euphorbia tirucalli*

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1. Introduction

Cancer is a leading cause of morbidity and mortality worldwide, with approximately 14 million new cases and 8.2 million cancer related deaths reported in 2012. Africa, Asia, Central and South America account for over 60% of the world's total cancer cases and 70% of the world's cancer deaths [1;2]. Currently, limitations to these conventional treatments such as adverse toxicity and high cost of treatment have made it important to develop novel therapeutics agents which exhibit less toxicity and at a lowered cost. These types of agents have been popular in medicinal plants. Flavonoids are a class of plant secondary metabolites. Chemically, they have the general structure of a 15-carbon skeleton, which consists of two phenyl rings and heterocyclic ring. Flavonoids are best known for their antioxidant and anti-inflammatory health benefits as well as the support of the reduced risk of cancer such as lung and breast. However, it is important to note that the amount of flavonoids required to provide the above health benefits is not certain. Some of the activities attributed to flavonoids include: anti-allergic, anti-cancer, antioxidant, anti-inflammatory and anti-viral. The flavonoids quercetin is known for its ability to relieve hay fever, eszema, sinusitis and asthma. While isorhamnetin which is present in many plants and is also a metabolite of quercetin is known to reduce the risk of cancer. There are a few studies which demonstrate similar activity for the isorhamnetin and its glycosides [3]. In this study we looked at the

molecular impact of isorhamnetin isolated from *Euphorbia tirucalli* on ovarian and cervical cancer cells.

2. Methods

2.1 Plant materials

Euphorbia tirucalli plants were collected in Limpopo Mankweng Township in February 2012. The plant material was authenticated by Dr Egen from University of Limpopo Herberium

2.2 Preparation of Euphorbia tirucalli extract

Collected fresh *Euphorbia tirucalli* stems were washed with water and cut into smaller parts. These were then evenly spread out and dried at room temperature under a stream of cold air for a week. When completely dried, the plants were ground to a fine powder using a pestle and mortar for maximum area coverage for the extraction process. All plant material was individually extracted by weighing 10 g of finely ground plant material and exhaustively extracting in 100 ml of the appropriate solvent (10:1 solvent to dry weight ratio) in 250 ml schott bottles. The mixture was shaken vigorously at high speed with a Labotec model 20.2 shaking machine for 24 hours. The mixture was centrifuged at 5000 rpm for 5 minutes then filtered through Whatman No. 1 filter paper into pre-weighed labelled containers. The extracts were dried under a stream of cold air at room temperature and the percentage yield was obtained. The extraction solvents used were n-hexane, methanol and butanol (technical grade, Merck). Different compounds were identified and isolated using TLC, HPLC and Mass Spectrophotometer. The isolated extracts to be used for biological studies were re-dissolved in dimethyl sulphoxide (DMSO) to give a stock concentration of 100 mg/ml. The quantified extract was stored at -20°C until required.

2.3 Cell culture

RMG-1 and SiHa were obtained from ATCC. All cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 1% penicillin/streptomycin. All cells were maintained in a humidified incubator at 37ºC with 5% CO2. All cell lines were subconfluently grown and passaged by treating them with 0.05% trypsin-EDTA.

2.4 MTT assay

To determine the cytotoxicity of isorhamnetin extract of *E. tirucalli* on the ovarian and cervical cancer cells, MTT assay was conducted. RMG-1 and SiHa cells (5×10^3) were seeded into 96-well microtiter plates in 90 µl culture medium and then incubated overnight. Cells were treated with varying concentrations of isorhamnetin (dissolved in dimethyl sulfoxide, DMSO) for 24 hours. The MTT solution, prepared at 5 mg/ml in sterile PBS, was added to each well at 10% v/v and incubated at 37ºC for 4 hours. DMSO was added to ensure total solubility of formazan crystals and absorbance was recorded at 570nm using the MultiskanTM Go microplate reader. Each plate contained a blank, vehicle control, positive control, treated and untreated samples. Vehicle control cultures received DMSO (0,1%) alone. Cells treated with taxol served as a positive control.

2.5 Real time cell growth assay

In vitro cell proliferation was measured using xCELLigence Real Time Cellular Analysis (RTCA) system. Briefly, the background impendance was measured following addition of 100 µl of growth medium to the 16X E-plates. Cell suspension containing $3x10^4$ RMG-1 or $7x10^4$ SiHa cells was seeded into the wells and attachment and proliferation were monitored using the RTCA system. Upon reaching logarithmic phase, cells were treated with 10 µl isorhamnetin at various concentrations (µg/ml) and continuously monitored for over 48 hours. Vehicle control cultures received DMSO (0, 1%) alone. Cells treated with taxol served as a positive control. Impendance changes expressed as cell index (CI) were automatically calculated as live cells interact with electrodes in the E-plates, correlating with viability or cytotoxicity of cells over time.

2.6 Annexin V/PI apoptosis assay

This technique was employed to determine the type of cell death isorhamnetin was inducing. Annexin-V detects the translocation of phosphatidylserine from the inner to the surface of the plasma membrane, which is characteristic of apoptotic cells, whereas PI detects necrotic cells with permeabilized plasma membrane. Cells were treated with plant extracts at IC_{50} for 24 hours. Following treatment, both adherent and detached cells were collected and rinsed twice with cold PBS. The cell pellet was resuspended in 500 µl of annexin binding buffer and incubated with 5 µl of Annexin V-FITC and 5 µl propidium iodide for 5 minutes. The cells were immediately analysed by flow cytometry

2.7 Cell cycle analysis by flow cytometry

Cell cycle analysis was performed by propidium iodode (PI) based measurements of cell DNA content using flow cytometry. Cells were treated with plant extracts at IC_{50} for 24 hours, followed by collection of both attached and detached cells. Pellet was rinsed twice with cold PBS and fixed in 70% ice-cold ethanol overnight at 20ºC. Fixed cells were then washed twice with PBS and DNA was stained with 500µl PI/RNase staining solution and incubated in the dark for 30 minutes. Flow cytometry analysis was carried out using BD Accuri C6.

2.8 Real time PCR

Cells were treated with isorhamnetin at IC_{50} for 24 hours. The total RNA from treated and untreated samples was isolated using the Zymoresearch Quick RNA^{TM} Miniprep. The RNA concentrations were measured using the NanoDrop and normalised for cDNA synthesis. Total RNA was reverse transcribed using the ImProm-II Reverse Transcription System (Promega) according to manufacturer's specifications. Real time PCR was carried out in a Lightcycler 480 using Luminaris Color HiGreen qPCR Mastermix with specific primers. Amplification products obtained by PCR were electrophoretically separated on a 1% Agarose gel, stained with ethidium bromide and visualized on the Chemidoc MP Imaging system. The data was analysed using REST 2009 software.

2.9 Western blot

At 24 and 48 hours post treatment with isorhamnetin, cells were lysed using RIPA buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.1% SDS, 2 mM EDTA). Protein content was measured by the BCA assay and equal amounts were electrophoresed in SDS polyacrylamide gel and then transferred onto nitrocellulose membranes. Membranes were subsequently immunoblotted with the anti-p53, anti-RBBP6, anti-Mdm-2, anti-BAX, anti-Bcl-2, anti-caspase-3, anti-caspase-9, anti-β-actin primary antibodies overnight then in horseradish peroxidase-conjugated secondary antibody for 1 hour. Protein bands were detected on the Chemidoc MP Imaging system following treatment with enhanced chemiluminescence reagent.

2.10 Statistical analysis

Experiments were either performed in duplicate or triplicate, data sets were expressed as means ± standard deviations (SD). Data were analysed using Student's t-test. Statistical significance was presented as ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

3. Results

3.1 Real-time monitoring of cell growth and IC50 concentration estimation.

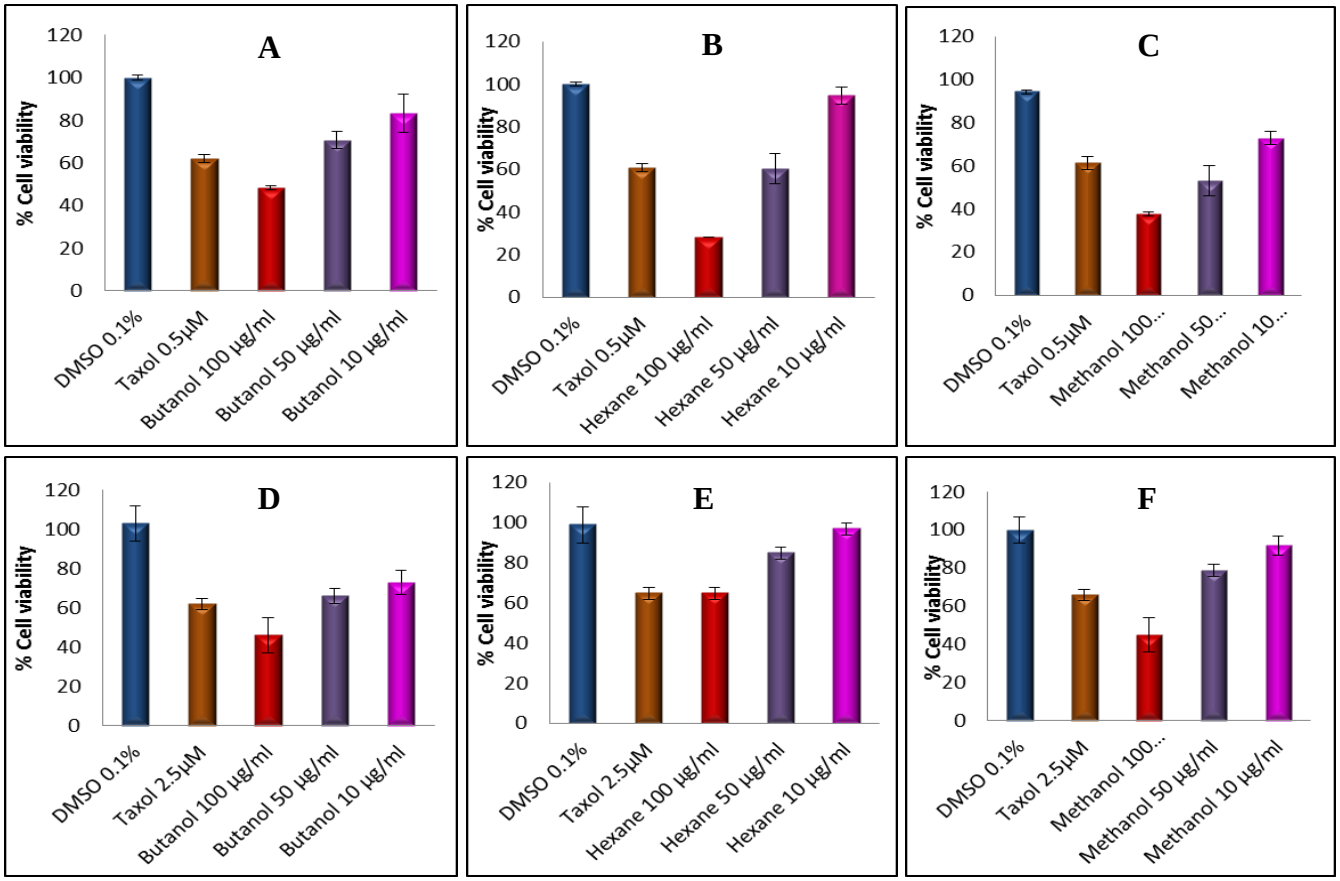


Fig. 1 Cell viability analysis using MTT assay. **(a-c)** RGM-1 and **(d-f)** SiHa cells were treated with varying concentrations of **(a & d)** butanol, **(b & e)** hexane and **(c & f)** methanol isorhamnetin extract of *Euphorbia tirucalli* for 24 hours. Taxol at the indicated concentration was included as a positive control.

Cell viability was analysed by MTT following treatment with different concentrations of butanolic, hexane and methanolic isorhamnetin extracts of *Euphorbia tirucalli* for 24 hours. The results indicated that inhibition of cell proliferation was concentration dependent and cell line type dependent. In RMG-1, the IC_{50} was estimated at 100µg/ml in butanolic extract, 60µg/ml in hexane and 50µg/ml in methanolic extract (Fig1 a-c). In SiHa cells, IC_{50} was

estimated at 80µg/ml in butanolic extract, >100µg/ml in hexane and 90µg/ml in methanolic extracts.

Since MTT assays are endpoint, we confirmed cell growth inhibition by the use of real-time proliferation assay (RTCA) which offers non-invasive analysis of cell proliferation over time. Experiments were carried out using xCELLigence RTCA DP analyzer (Roche). Cell index (CI), were automatically calculated as live cells interact with electrodes attached to the bottom of the E-plates.

RGM-1 and SiHa cells were treated with isorhamnetin extract at different concentrations and cell proliferation was monitored for over 30 hours. The IC_{50} at 24 hours post treatment with plant extract was estimated. In RMG-1, the IC_{50} was estimated at 10µg/ml in butanolic extract, 30µg/ml in hexane and 30µg/ml in methanolic extracts. In SiHa cells, IC_{50} was estimated at 15µg/ml in butanolic extract, 60µg/ml in hexane and 100µg/ml in methanolic extracts. Futhermore, cells were treated with isorhamnetin of *E. tirucalli* extracts at concentrations that induced 50% cell death (IC_{50}), and cell proliferation was monitored for over 60 hours (Fig 2).

In RMG-1 cells, treatment with hexane and methanol extracts caused a rapid decline in CI, whilst butanol extract caused a slower decline in CI. Cells treated with hexane and methanol extracts recover from the initial cytotoxic effects of the extract and seem to re-proliferate (Fig 2a). In SiHa cells, a rapid decline in CI could also be observed upon treatment with hexane and methanol extracts. A slower decline in CI was observed on treating with the butanol extract. Cells treated with all three extracts seem to recover and re-proliferate, with butanol extracts recovering quickly to normality (Fig 2b).

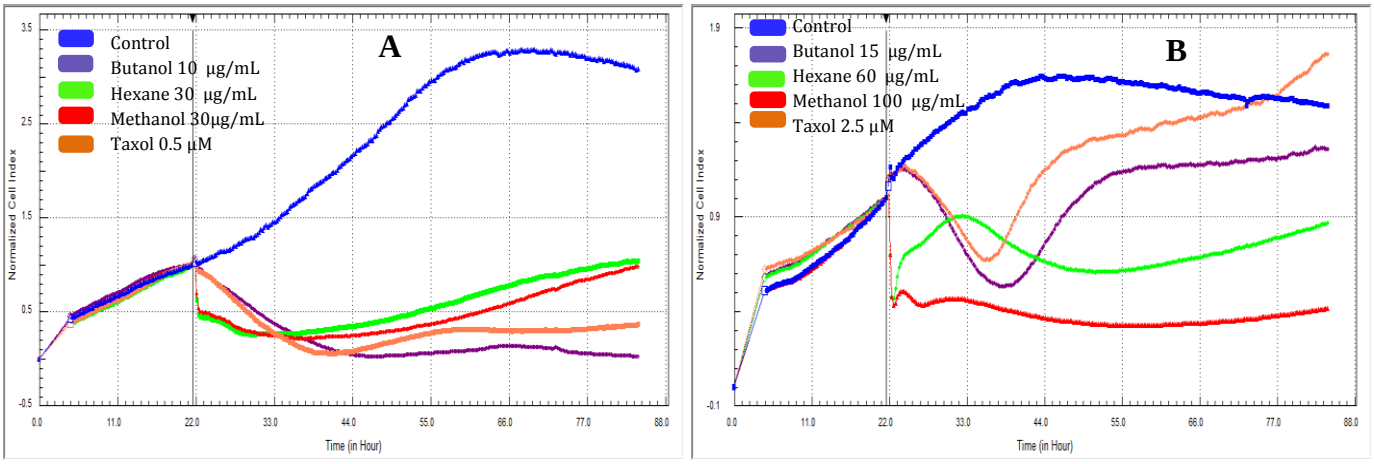
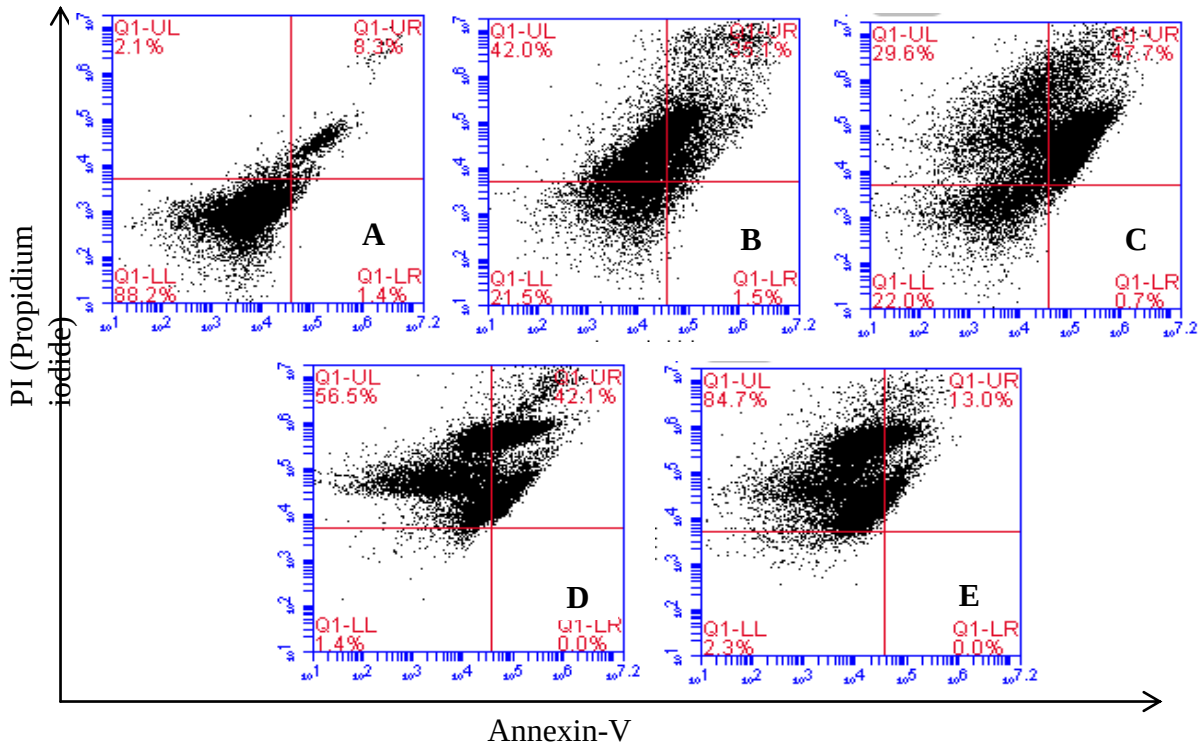


Fig. 2 Dynamic monitoring of isorhamnetin plant extract interaction with **(a)** RGM-1 and **(b)** SiHa cells using RTCA analyser. Cells were seeded for 22 hours, at which point, butanol, hexane and methanol extracts at indicated IC_{50} were added. Cell growth was monitored for over 60 hours. Taxol at the indicated concentration was included as a positive control. CI values were normalized to the point of compound addition indicated by the vertical black line.

3.2 Euphorbia tirucalli butanolic, hexane and methanolic isorhamnetin extracts induces Apoptosis and Cell Cycle Arrest



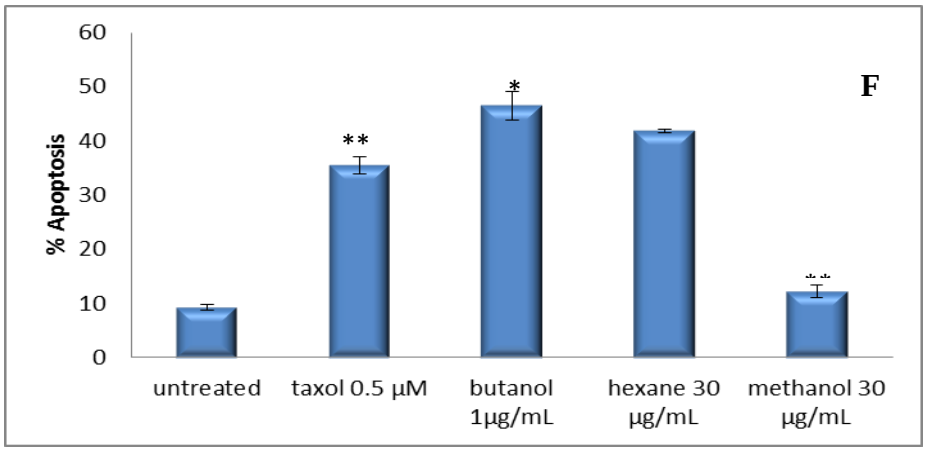
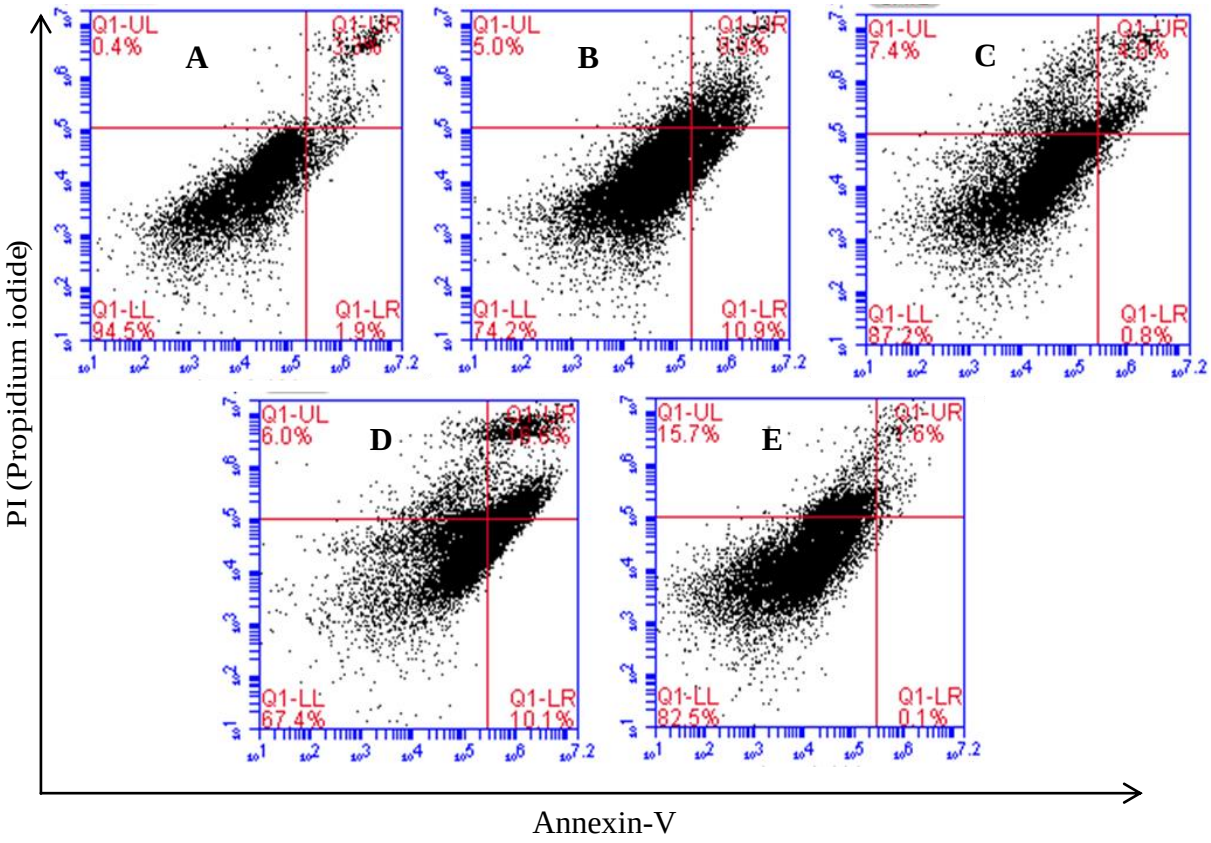


Fig. 3 RGM-1 cells apoptosis determination using Annexin V/PI staining. Annexin V/PI stained RGM-1 cells following treatment with either **(c)** butanol, **(d)** hexane and **(e)** methanol isorhamnetin extracts of *Euphorbia tirucalli* in comparison with untreated cells at 24 hours. **(b)** Taxol was included as a positive control. Representative figures showing populations of viable (LL), early apoptotic (LR), late apoptotic (UR) and necrotic (UL) cells. (f) Bar graphs showing percentages of apoptotic cells for each treatment sample at 24hours. Data were mean ± SD from two independent experiments (*P<0.05, **P<0.01)



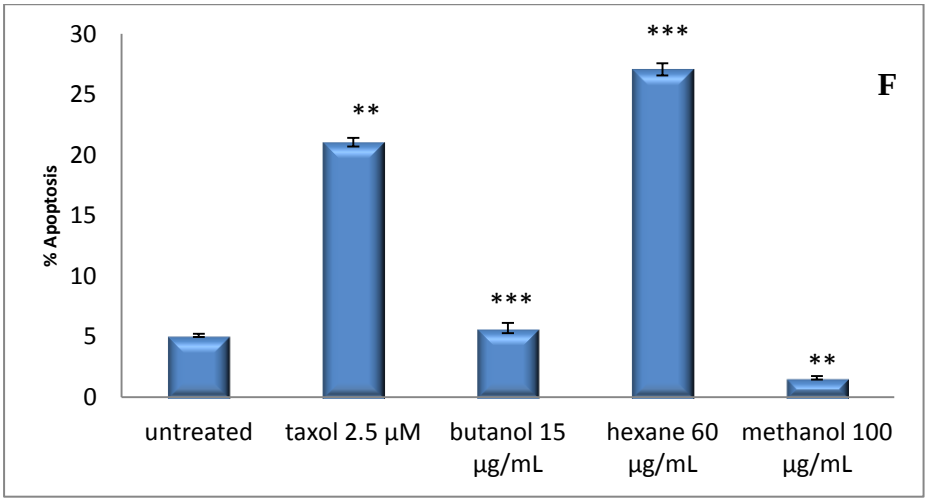


Fig. 4 SiHa cells apoptosis determination using Annexin V/PI staining. Annexin V/PI stained SiHa cells following treatment with either **(c)** butanol, **(d)** hexane and **(e)** methanol isorhamnetin extracts of *Euphorbia tirucalli* in comparison with untreated cells at 24 hours. **(b)** Taxol was included as a positive control. Representative figures showing populations of viable (LL), early apoptotic (LR), late apoptotic (UR) and necrotic (UL) cells. **(f)** Bar graphs showing percentages of apoptotic cells for each treatment sample at 24hours. Data were mean ± SD from two independent experiments (**P<0.01, ***P<0.001)

To determine if the observed cell death was due apoptosis flow cytometry was employed. Untreated and RMG-1 and SiHa cancer cells treated with isorhamnetin extract, at IC_{50} for 24 hours were stained with annexin V and PI. Flow cytometry analysis of stained cells can distinguish cells into four groups, namely viable (LL), early apoptotic (LR), late apoptotic (UR) and necrotic (UL) cells. In RMG-1 cells, the butanolic extract seemed to induce the most apoptosis compared to the other two extracts at 48.4% (Fig 3c), followed by the hexane extracts with an apoptotic population of 42.1% (Fig 3d). The methanolic extracts seemed to induce minimal apoptosis at 13%, with a marjority of the cells undergoing necrosis (Fig 3e).

As shown in Fig 4, in SiHa cell lines, hexane isorhamnetin extracts of *E. tirucalli* induced the highest population of apoptotic cells at 27% (Fig 4c), as compared to the butanolic (5.7%)(Fig 4d) and methanolic extracts (1.6%)(Fig 4e).

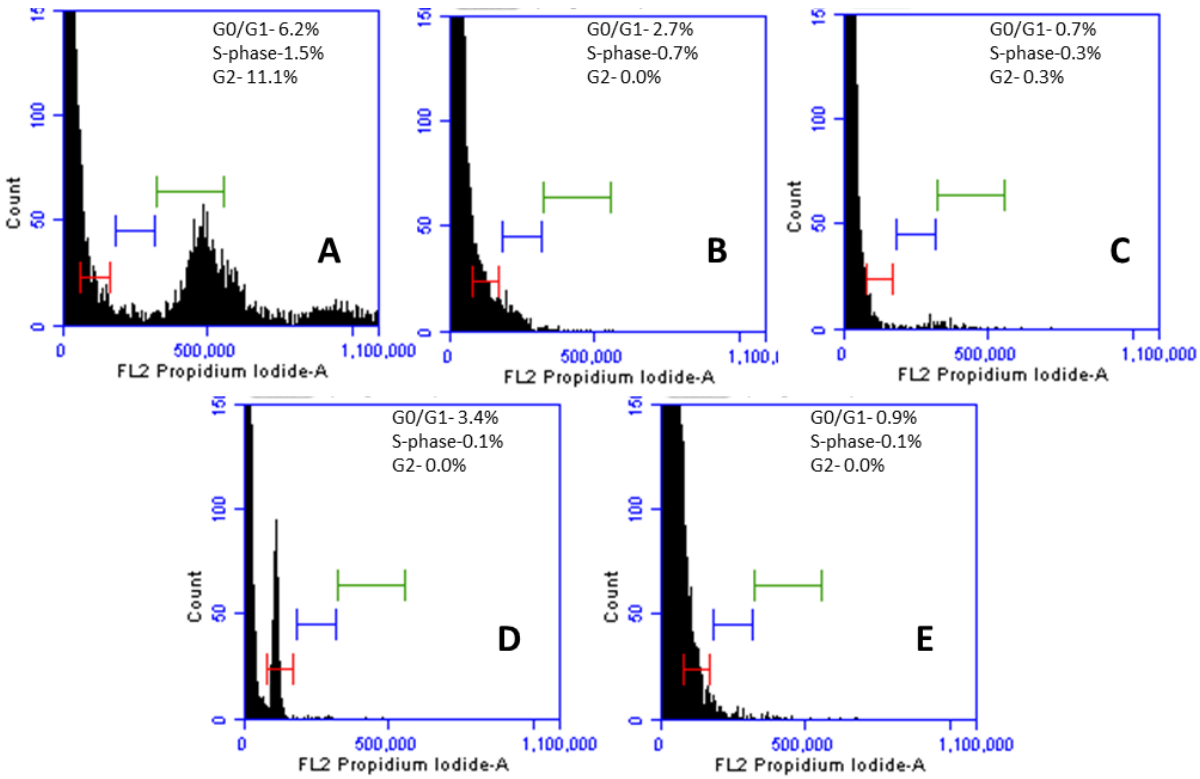


Fig. 5 Effects of isorhamnetin extracts on cancer cell cycle progression. RMG-1 cell cultures received (a) no treatment, (b) treated with taxol, (c) treated with butanolic, (d) hexane and (e) methanolic isorhamnetin extracts of *E. tirucalli*.

Next we examined cell cycle distribution by staining treated ovarian and cervical cancer cells with propidium iodide and analyzed the percentages of G0/G1, S, G2/M cell population using flow cytometry. An increase in the population undergoing cell death could be observed for RMG-1 cells treated with butanolic, hexane and methanolic isorhamnetin extracts of *E. tirucalli*

A higher G0/G1 population, could be observed for SiHa cells treated with butanolic, hexane and methanolic extracts with an increase of 29,8%, 68,2% and 64,2% respectively as compared to the control cells. Cells treated with the butanolic extract also showed an increase in the G2/M cell population at an increase of 14.7% compared to the untreated.

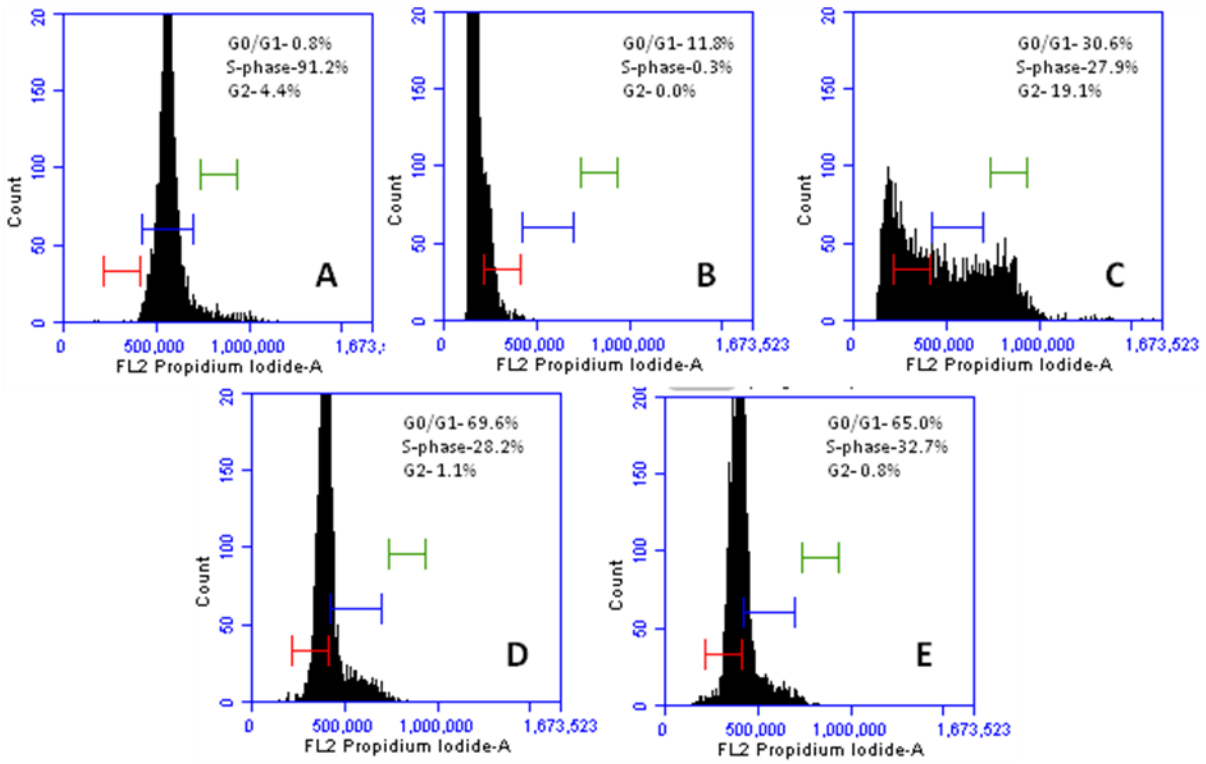


Fig. 6 Effects of isorhamnetin extracts on cancer cell cycle progression. SiHa cell cultures received (a) no treatment, (b) treated with taxol, (c) treated with butanolic, (d) hexane and (e) methanolic isorhamnetin extracts of *E. tirucalli*.

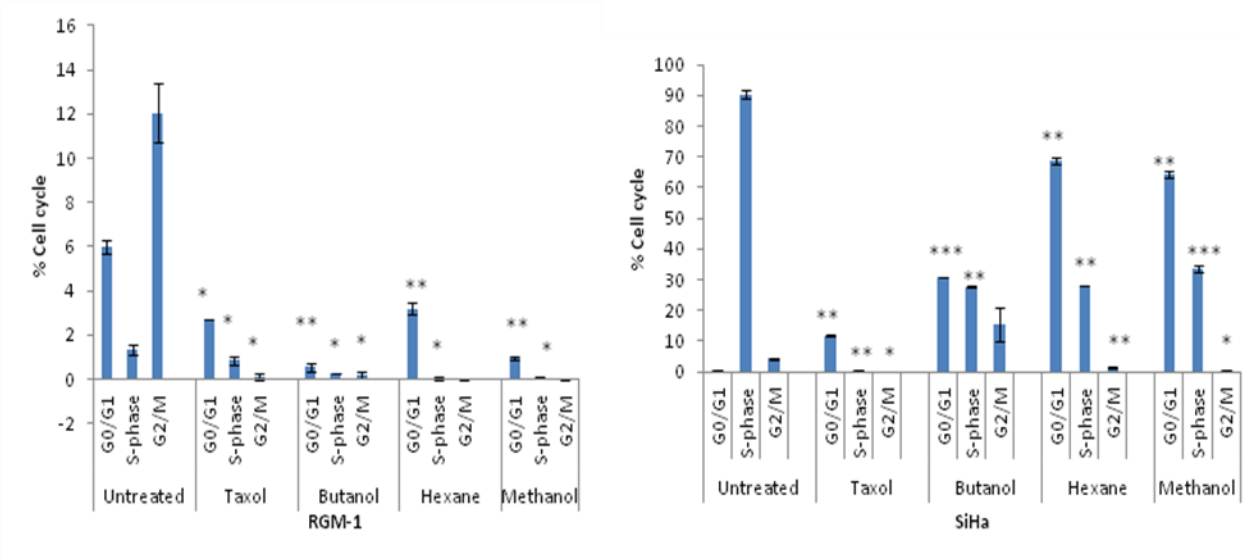


Fig. 7 Bar graphs representing cell cycle analysis of RMG-1 and SiHa cells treated with butanolic, hexane and methanolic isorhamnetin extracts of *Euphorbia tirucalli*. Data were mean ± SD from two independent experiments (*P<0.05, **P<0.01, ***P<0.001)

3.3 Effects of Euphorbia tirucalli extracts on Anti-apoptotic, pro-apoptotic and cell cycle Molecules

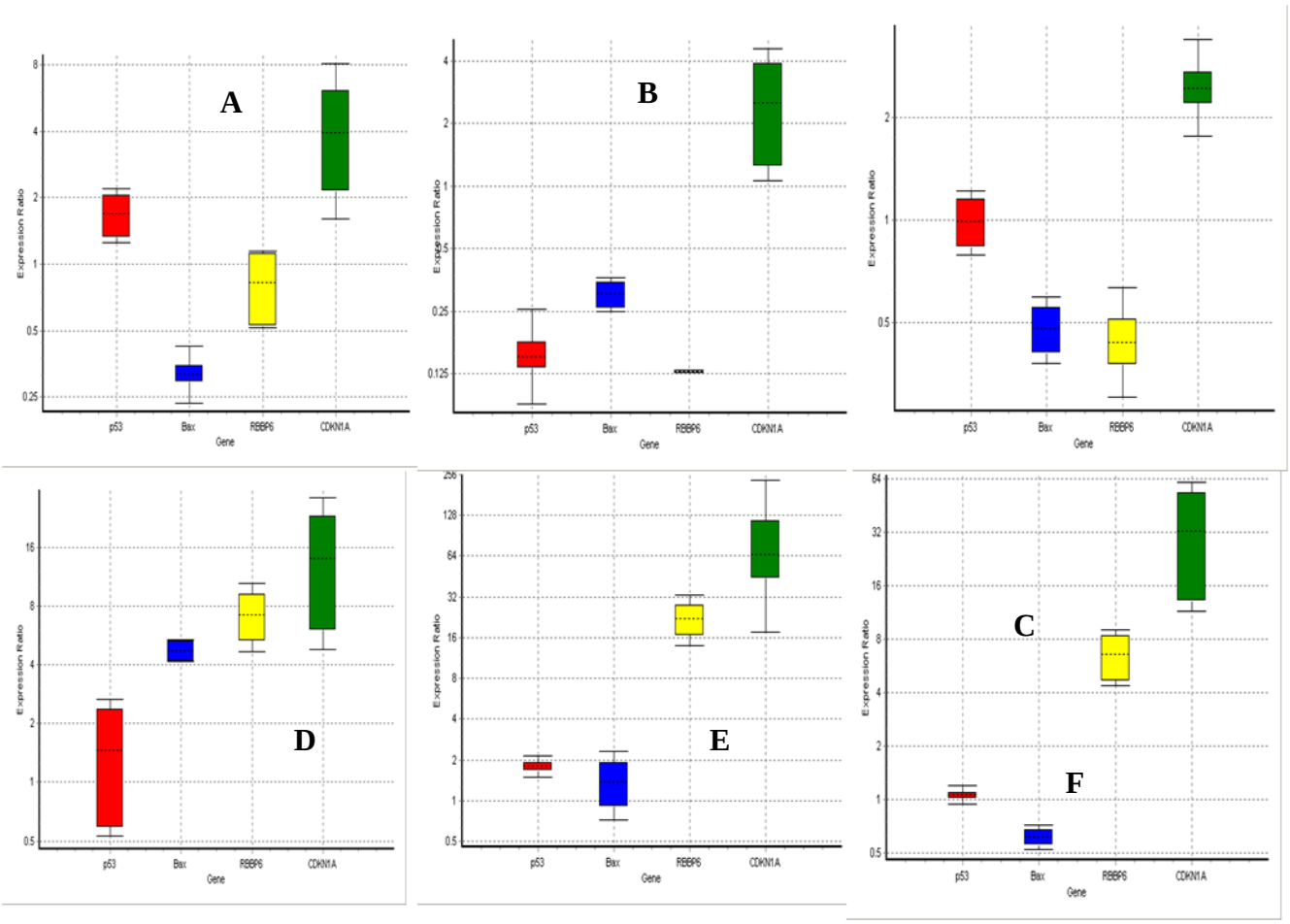


Fig. 8 Box plots representing Real Time PCR analysis using REST 2009 software. (a-c) RMG-1 and (d-f) SiHa gene expression analysis following treatment with (a & d) butanol, (b & e) hexane and (c & f) methanol isorhamnetin extracts of *Euphorbia tirucalli* for 24 hours.

To examine if isorhamnetin extracts initiated apoptosis by affecting the cellular level of these molecules, we performed Real-Time PCR and western blot analysis using control and treated. Real-Time PCR data showed that Bax was statistically down-regulated upon treating RMG-1 cells with butanolic, hexane and methanolic extracts. Western blot analysis however, showed an increase in the butanol and methanol extracts treated samples 24 hours following treatment. In SiHa cells, Bax was significantly upregulated in cells treated with butanol

extracts by a mean factor of 4.736 (S.E. range is 4.169 - 5.380) in comparison to the control group but remained statistically unchanged in hexane and methanol treated. No significant changes were observed in the expression of Bcl-2 on both cells lines in response to all treatments.

Analysing the expression of both caspase 3 and -9, we observed that in RMG-1 both caspase 9 and 3 expression remained statistically unchanged in all the 3 extracts, in SiHa cells, a slight increase in caspase 9 expression following treatment with butanol and methanol extracts could be observed.

Next analysis was to examine the molecules that regulate cell cycle to see if there were any changes in their expression. p53, functions as tumour suppressor and a transcription regulator of several genes. One of the genes it regulates is p21, also known as CDKN1A. p21 plays a crucial role in cell cycle arrest. Analysis of p21 revealed that it was up-regulated by the butanol, hexane and methanol isorhamnetin extracts by a mean factor of 3.584 (S.E. range is 1.932 - 6.872), 2.214 (S.E. range is 1.185 - 4.185) and 2.438 (S.E. range is 2.039 - 2.976) respectively in RGM-1 cells. In SiHa cells, an even more drastic up-regulation was observed in cells treated with all extracts. Up-regulation by a mean factor of 11.746 (S.E. range is 5.548 - 25.365), 21.448 (S.E. range is 15.540 - 29.985) and 26.276 (S.E. range is 12.362 - 56.199) was observed for the butanol, hexane and methanol extracts respectively.

In RMG-1 cells, p53 was up-regulated by a mean factor of 1.653 (S.E. range is 1.289 - 2.122) in butanol treated and it was down-regulated by a mean factor of 0.151 in hexane and remained statistically unchanged in methanol treated. In SiHa cells, p53 was statistically unchanged in butanol and methanol treated cells but was up-regulated by a mean factor of 1.789 (S.E. range is 1.584 - 2.029) in hexane treatments.

The expression of two p53 regulators, RBBP6 and mdm-2 was also investigated. In RGM-1 cells, RBBP6 was statistically unchanged in butanol extracts, but was down-regulated by a mean factor of 0.130 in hexane and 0.436 in methanol treated extracts in comparison to control group. In SiHa cells, RBBP6 was significantly up-regulated by all three extracts with mean factors of 7.035, 21.448 and 6.258 respectively for butanol, hexane and methanol extracts. Western blot analysis showed a slight decrease in mdm-2 expression at 48 hours

post treatment with the three extracts in RMG-1 cells. A slight increase in mdm-2 could be observed for butanol and hexane treated SiHa cells.

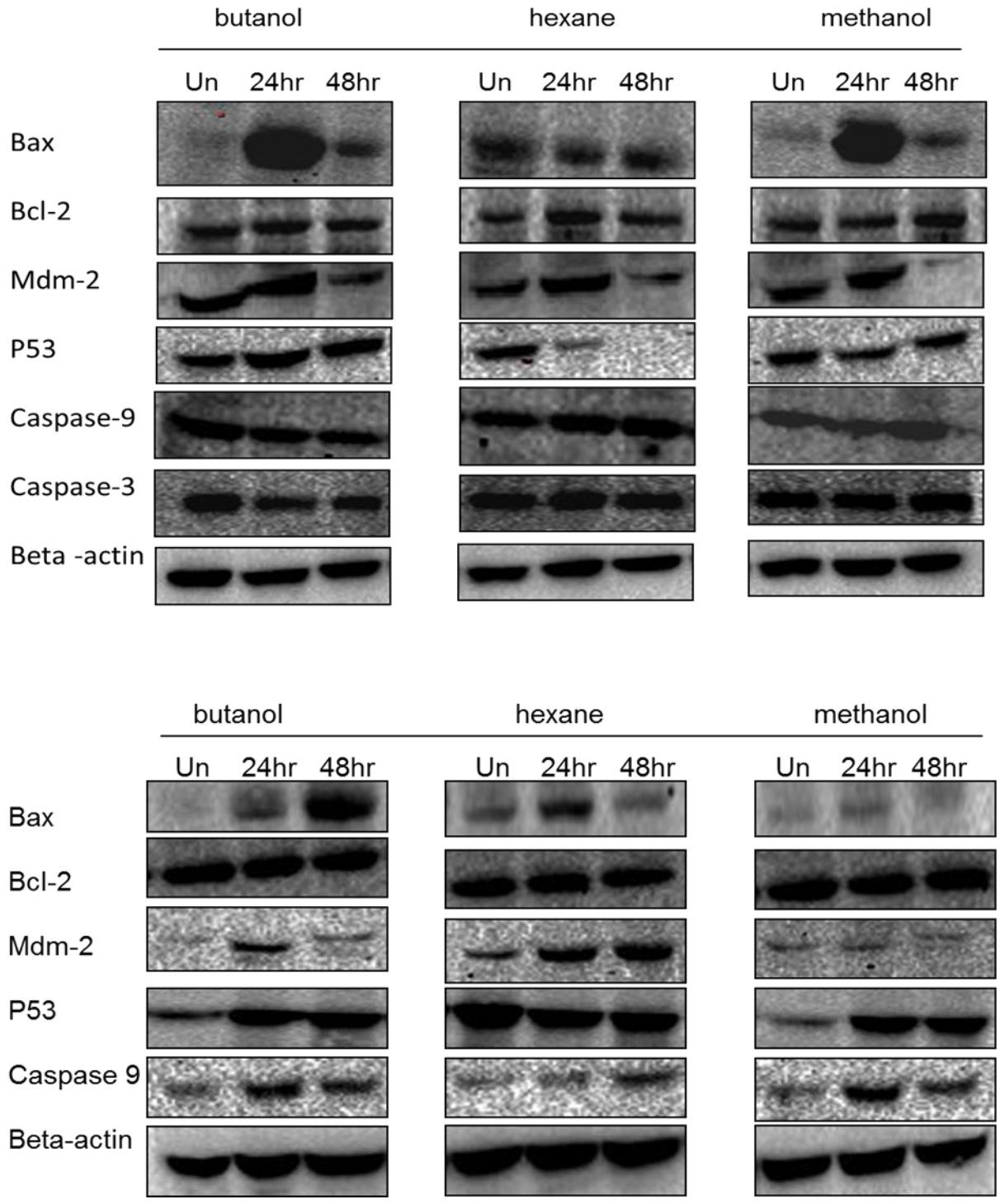


Fig. 9 Western blot analysis of protein expression in (a) RMG-1 and (b) SiHa cells following treatment with butanol, hexane and methanol isorhamnetin extracts of *E. tirucalli*. Un= Untreated samples. The results show a varying protein expression of different apoptotic genes 24 or 48 hours post treatment with the 3 plant extracts.

4. Discussion

The gynaecologic cancer burden is huge in developing countries accounting for 25% of all new cancers among women (Ferlay *et al* 2008). Of these, cervical cancer is the most common cancer of the female reproductive tract, whilst ovarian cancer is the most lethal of the gynaecologic cancers (Sherris *et al* 2001; Jemal *et al* 2008). Major strides have been made in developing effective therapeutics including chemotherapy, but results haven't always been satisfactory. Plants have long been a source of important lifesaving pharmaceutical agents in developing countries. A number of drugs derived from plants are currently being used in the pharmaceutical industry (Veeresham 2012). Thus, it is important that plants remain at the forefront in drug discovery as an essential component in new therapeutics, including chemotherapy. One of the most important secondary metabolites of plants that were seen to have positive effect on health are flavonoids, which are antioxidants that play a role in reducing cancer development. Isorhamnetin is an O-methylated flavonol, a type of flavonoid. It can be found in many plants including *Euphorbia tirucalli* and is also a metabolite of quercetin (Li *et al* 2014).

In this study, we evaluated the anticancer activity of the butanolic, hexane and methanolic Isorhamnetin extracts of *Euphorbia tirucalli* against ovarian RMG-1 and cervical SiHa cancer cells. Isorhamnetin inhibited cell proliferation, decreased cell viability and induced cell death in a dose dependent and cell type specific manner. A shortcoming to the use of endpoint cytotoxicity assays such as MTT is that they are invasive and their inability to identify the appropriate time point for conducting any given assay. To address this issue, a Real-Time Cell Analyzer (RTCA) - xCELLigence System, which is a non-invasive and label-free way to continuously monitor cellular behaviour, was employed.

The xCELLigence system was used to predict the accurate concentration that is potent and also monitor real time analysis of cell proliferation following treatment with Isorhamnetin

extracted using 3 different solvents. The potency exhibited on the cells was dependent of the solvent type used for extraction. As shown from the results presented in Figure 2, the butanol extract exhibited the most potent activity on both RMG-1 and SiHa cell lines (IC_{50}=10 µg/ml and IC_{50}= 15µg/ml) as compared to the hexane and methanolic extracts. According to the American National Cancer Institute (NCI), the criteria for cytotoxic activity of a crude extract is an IC_{50} of less than 30 µg/ml (IC_{50}<30 µg/ml) (Itharat *et al* 2004). Using this criterion, the Isorhamnetin butanol extract could be considered as of promising anticancer potential. Both the hexane and methanol extracts showed an IC_{50}=30 µg/ml on RGM-1 cells, however, an IC_{50}=60 µg/ml and IC_{50}=100 µg/ml respectively was observed on SiHa cells. Taxol was used in our study as positive control for the reason that it is widely used in the treatment of cancer and is it a plant extracted compound. It has been used for cytotoxicity screening in ovarian and cervical cancer, and was found to elevate apoptosis-related, immune response-related and cell cycle check point-related proteins (Rowinsky and Donehower 1995; Lee *et al* 2005). RGM-1 cells showed a higher sensitivity to taxol since 0,5 µM resulted in~40% cell death whereas SiHa required five times that concentration (2,5µM) to induce the same amount of cell death.

Following determination of the $IC_{50,}$ we then treated the cells with the appropriate IC_{50} concentration for all the three different solvents and the behaviour of the cells was monitored over time. It was interesting to observe that the extract decreased cell viability; however, the cells eventually started to recover from the initial cytotoxic effects of the extract and seemed to re-proliferate. Since the extracts seemed to induce ctytotoxicity on the two extracts, it was then important to determine if this cell death was as a result of apoptosis or necrosis.

Apoptosis is a tightly regulated process that plays a key role in maintaning tissue homeostasis (Renehan *et al* 2001). An imbalance or inactivation of important apoptotic pathways has been implicated in a number of cancers (Kerr *et al* 1972). Hence, discoveries of chemotherapeutic agents that can induce and recover the apoptotic response in tumour cells would be an efficient strategy in anticancer treatments. Isorhamnetin extracts of *E. tirucalli* showed anti-proliferative activity on both cervical and ovarian cancer cell lines, we then aimed to investigate the type of cell death these extracts were inducing. Most therapies in anticancer treatment, such as chemotherapy mainly induce cell death by causing either G0/G1 or G2/M cell cycle arrest and then inducing an apoptotic pathway (Morgan 2003). Therefore, cell cycle

arrest and the induction of apoptosis in cancer cells become the major indicators of anticancer effects. Annexin V and PI staining were used to determine the type of cell death induced by the extracts. Flow cytometric analysis of the two cell lines revealed different proportions of apoptotic and necrotic cells. Isorhamnetin butanolic extract seemed to induce the most apoptosis in RMG-1 cells. With the hexane extract, an almost equal population underwent apoptosis as those undergoing necrosis. Methanolic extracts seemed to induce necrosis in both RGM-1 and SiHa cells. Hexane extract seemed to induce a higher population of cells that undergo apoptosis in SiHa than the other two solvents. Lee and colleague *in-vitro* study showed that isorhamnetin induced apoptosis, which is mediated by mitochondria-dependent caspase activation.

Cell cycle analyses using flow cytometer revealed that majority of RGM-1 cells underwent cell death upon treatment with isorhamnetin, however, they arrested cell cycle at the G0/G1 phase in SiHa cells. In another study by Jamillo *et al* (2010), they observed that isorhamnetin was able to increase the number of cells in G2/M phase which correlated with our study. To test the mechanism behind the G0/G1 phase arrest observed in SiHa cells treated with isorhamnetin, we analysed p21 expression. P21 is a potent cyclin dependent kinase inhibitor that is usually expressed in the G0/G1 phase of the cell cycle (Abbas and Dutta 2009). We found an up-regulation of p21 in treated RMG-1 and an even drastically higher up-regulation in SiHa treated cells.

A complex cascade of cell signaling interactions is involved in cell cycle regulation and induction of the apoptotic pathway. One of these important regulators is p53, in fact, over 50% of cancers have been found to have a mutated dysfunctional p53 (Wang and Sun 2010). With p53 playing a crucial role in cell cycle arrest and apoptosis induction, we aimed to investigate if the extracts could upregulate its expression. We observed that p53 was up-regulated in RMG-1 cells treated with butanol extract and in SiHa cells treated with methanol extract. Mdm-2 which is a negative regulator of p53 was downregulated in RMG-1 cells following treatment with isorhamnetin. RBBP 6, which is another negative regulator of p53, was also down regulated in RGM-1 cells treated with hexane and methanol extracts.

The Bcl-2 family encompasses pro- and antiapoptotic proteins that are primary regulators of the intrinsic or the mitochondrial pathway. The proapoptotic members of this family such as Bax, induce release of cytochrome c and other pro-apoptotic factors, promoting caspase activation and eventually cell death (Schimmer *et al* 2008). Real-Time PCR data showed that Bax was statistically down-regulated upon treating RMG-1 cells with Isorhamnetin. Western blot analysis however, showed a significant increase in Bax in the butanolic and methanolic extracts treated samples after 24 hours. Real-time PCR assays measure RNA expression levels at a certain time point. Studies have revealed that various mRNAs have different half-lives, which are related to the physiological role of that mRNA. For example, housekeeping genes are known to have a long half-life, whereas proteins involved in cell cycle and development have a much limited half-life (Hollams *et al* 2002). Post-transcriptional regulation of gene expression includes mRNA processing, splicing and translation (Raghavan and Bohjanen 2004). Hence, it is possible that 24 hours post treatment, Bax mRNA had long undergone post-tanscriptional processing which included translation of the protein that was observed to have been up-regulated on western blot protein analysis. In SiHa cells, Real-Time PCR data showed Bax was significantly upregulated in cells treated with butanolic Isorhamnetin extracts and western blot analysis showed an increase in Bax in both butanolic and hexane treatments. No significant changes were observed in the expression of the anti-apoptotic Bcl-2 on both cells lines in response to all treatments. In similar type of study, myricetin-induced apoptosis correlated with the modulation of Bcl-2 family proteins and activation of the caspase-3 in human bladder cancer (Sun *et al* 2012). The results we observed in our study shows that the expression of both caspases remained unchanged in RGM-1 cells treated with isorhamnetin, however, a slight increase in caspase 9 could be observed upon treatment with isorhamnetin in SiHa cells extracted with butanol and methanol. These results are the first to show the isolated isorhamnetin from *E. tirucalli* to be able to induce cell death in a p21 dependent manner.

5. Conclusion

Isorhamnetin extract of *E. tirucalli* was able to increase the expression of p21 that lead to cell cycle arrest and the induction of apoptosis through the extrinsic pathway.

Acknowlegments

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Appendix 5

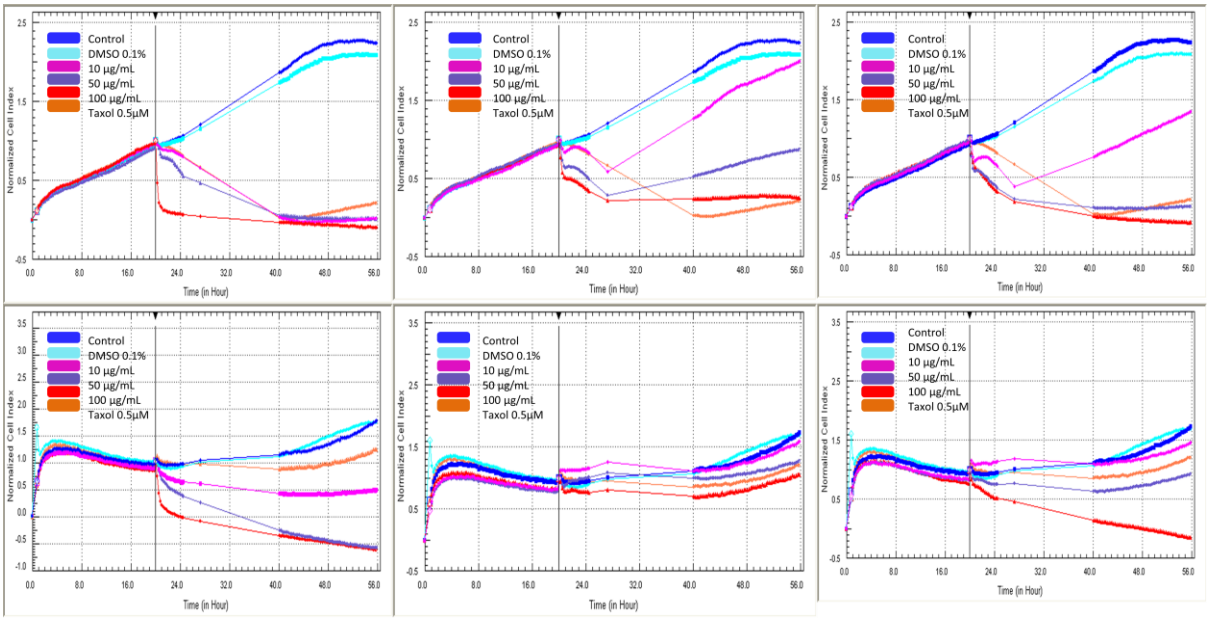

Figure A: Dynamic monitoring of *Euphorbia tirucalli* plant extracts interaction with cells using RTCA analyser.

RMG-1(a-c) and SiHa (d-f) cells were seeded for 21 hours, at which point, butanol (a & d), hexane (b, & e) and methanol (c, & f) extracts were added at indicated concentrations. Cell growth was continuously monitored for over 48 hours. CI values were normalized to the point of compound addition indicated by the vertical black line