An investigation of the effects of selected Chinese herbal remedies on cancer cells *in vitro*

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This thesis is being submitted in partial fulfilment of the requirements of Kingston University for the award of PhD

January 2007

Abstract

Chinese herbal remedies (CHRs) are commonly prescribed for the treatment of cancer, however their use is often based on the belief systems of traditional Chinese medicine (TCM), and there is relatively little information regarding their efficacy or biological action. Thus, the aim of this investigation was to select a range of CHRs with some suggestion of tumour modulatory activity, and to devise and implement a strategy with which to investigate their direct toxicity to cancer cells, in an attempt to elucidate their potential efficacy and mode of action.

The CHRs *Oldenlandia diffusa* (OD), Long Dan Xie Gan Wan (LD), *Polygonum multiflorum* (PM) and *Polyporus umbellatus* (PU) were selected for this investigation, and their direct cytotoxic potential against cancer cells examined using an *in vitro* cell-based system. Initially, water and ethanol extracts of each CHR were made and their toxicity evaluated against a range of cancer cell lines (HL60, HT29, HCT-8, HeLa and CHO). The results of this study suggested that water extracts of OD, LD and PM, but not PU, were significantly toxic to a range of cancer cell types. Further investigation (using the HL60 and HT29 cell lines) suggested that OD and LD induced apoptosis in cancer cell lines *in vitro* through activation of the intrinsic pro-apoptotic signalling pathway (characterised by activation of caspase 9), possibly through the induction of genotoxic damage, and that this activity was related to the combined actions of a number of cytotoxic compounds, and not to a single constituent. Furthermore, OD and LD were found to be less toxic to cultured primary blood lymphocytes (PBLs), thus further suggesting that there may be some scientific basis for their use in the treatment of cancer. Further investigation into the cytotoxic action of water extracts of PM revealed that it was inducing necrosis in cancer cell lines and not apoptosis, thus suggesting that PM does not possess anticancer activity.

Overall, the results of this investigation suggest that OD and LD may be a source of novel chemotherapeutic agents, and that there may be some scientific basis for their traditional use in the treatment of cancer.

Acknowledgements

I would like to thank my supervisors, Lucy Jones, James Barker and particularly Elizabeth Opara for their help and guidance during this project. In addition, I'm grateful to Gurm Sappal, Michael Tsang, Jayne Reeves, Nico Forraz, Keith Jones and Amin Mirza for their assistance in the lab.

I'd also like to thank Polly Hayes, Louise Plows, Simon Gould, Hiren Patel, Bridgette Manship, Audrey Lacchini, Christianne Micallef, Sachin Dhanani, sporty Jenny and (especially) old Jenny for their moral support during my time at Kingston Uni ☺

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List of Abbreviations

ALS	Alkali labile site
APCI	Atmospheric Pressure Chemical Ionization
APS	Ammonium Persulphate
B[a]P	Benzo[a]pyrene
CAM	Complementary and alternative medicine
CHR	Chinese herbal remedy
CS	Capillary electrophoresis
DES	Diethylstilbestrol
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DSB	Double strand break
ECL	Enzymatic chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EtBr	Ethidium bromide
FACS	Fluorescence activated cell sorter (flow cytometer)
FBS	Foetal bovine serum
FGF	Fibroblast growth factor
G1	Gap 1 (cell cycle stage)
G2	Gap 2 (cell cycle stage)
GC	Gas chromatography
GC-MS	Gas chromatography-Mass spectrometry
HPLC	High performance liquid chromatography
HRP	Horse radish peroxidase
IAP	Inhibitor or apoptosis
LAK	Lymphocyte activated killer
LC	Liquid chromatography
LC-MS	Liquid chromatography-Mass spectrometry
LD	Long Dan Xie Gan Wan
LMP	Low melting point agarose
M	Mitosis
MeOH	Methanol

MTT	Mitochondrial reduction of tetrazolium salts (assay)
MWT	Molecular weight
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NEAA	Non-essential amino acid
NK	Natural killer
NMP	Normal melting point agarose
OD	*Oldenlandia diffusa*
PBL	Primary blood lymphocyte
PBS	Phosphate buffered saline
PHA	Phytohemagglutinin
PI	Propidium iodide
PM	*Polygonum multiflorum*
PS	Phosphatidylserine
PSA	Prostate specific antigen
PU	*Polyporus umbellatus*
RNA	Ribonucleic acid
S	Synthesis (cell cycle stage)
SDS	Sodium dodecyl sulphate
SM	Secondary (plant) metabolite
SSB	Single strand break
TBS	Tris buffered saline
TBS/T	Tris buffered saline/Tween 20
TCM	Traditional Chinese medicine
TEM	Transmission electron microscope
TLC	Thin layer chromatography
TNFR	Tumour necrosis factor receptor
TUNEL	TdT-mediated dUTP Nick-End Labelling (assay)
UA	Ursolic acid
WHO	World Health Organization

Chapter 1. Introduction

1.2. An introduction to Traditional Chinese Medicine (TCM)

Traditional Chinese medicine (TCM) is one of the oldest and most widely used medical systems in the world, and is today practised in over 140 countries by more than 300,000 practitioners. Traditional Chinese Medicine (TCM) encompasses the use of acupuncture, massage, exercise and Chinese herbal remedies (CHRs) for the treatment of disease and the maintenance of general health (Scheld, 1999).

Chinese herbal remedies (CHRs), which range from the prescription of a single herb to remedies containing more than 20 separate ingredients, are central to the ancient practice of TCM, and their use in Western society is becoming increasingly common as the popularity of all forms of complementary and alternative medicine (CAM) continues to grow (WHO, 2003). Yet despite their popularity and worldwide use, much of the information regarding their efficacy is anecdotal, and there are growing concerns regarding the potential safety of these remedies when used alone or in combination with Western treatments (Ernst, 2002). As such, there is a growing need to examine the safety and efficacy of these remedies.

1.2. The foundation and history of TCM

1.2.1. The theories of yin-yang and The Five Elements

The foundations of TCM are based on the theories of yin-yang and The Five Elements. The yin-yang theory was originally formed to explain the relationships and patterns that occurred in nature, later on it was used to understand the complex relationships that occurred in the human body. The *Yellow Emperor's Classic of Medicine* (*Huang Di Nei Jing*), written around 300BC, set down the theoretical foundations of TCM that are still used today. The yin-yang theory proposes everything has an opposing yin and yang aspect; these are mutually controlled and inhibited by each other, which results in a state of dynamic balance. In the human body, the activity (yang) of our body is nourished by the physical form (yin), while the physical form is created and maintained through the activity of the body. If the dynamic balance that these opposing forces create is disturbed, people become ill. TCM seeks to restore this balance and cure the patient. All factors that cause yin and

yang disharmony are related to the "pernicious evils" (outside evils that cause disease). The Five Elements Theory expands on the yin-yang theory, and both are often used simultaneously. The Five Elements theory proposes everything is made of five basic elements: earth, water, fire, wood and metal. An imbalance between these elements within the body can result in illness (Shen-nong Limited n.d). In addition to the theories of yin and yang and The Five Elements it is also believed the free flow of Qi (the most basic material from which the body is formed) and blood (essential in maintaining normal function) are essential in the maintenance of health, and that their disruption can result in illness. When an individual becomes ill, the disharmony that has led to their illness is diagnosed by a TCM practitioner through examination of their pulse, mouth, tongue, throat, eyes and disease profile. After diagnosis, a patient will be prescribed a treatment that will restore the balance within their body, thereby curing the disease (Tse, 2003).

The plants, fungi and herbs used in TCM to make CHRs are ascribed specific yin-yang and elemental properties, and are prescribed by TCM practitioners to restore the imbalances in the body that cause illness. The CHRs used in the treatment of disease can range from preparations of single herbs to remedies containing up to 20 separate ingredients. There are thousands of CHRs described in Chinese literature, most commonly containing 4-12 separate ingredients. The efficacy of CHRs is traditionally attributed to the combination of ingredients within that remedy, with individual ingredients used to treat different symptoms of the same disease or to counteract the unwanted side-effects of other herbs within that CHR preparation. For multi-component herbal preparations each ingredient is typically ranked into one of four separate groups depending on its role within that CHR. The "imperial herb" within a remedy is the main ingredient, and is responsible for the main action of that CHR. The "ministerial herbs" within a preparation promote the action of the imperial herb, "assistant herbs" reduce the side-effects of the imperial herb, and "servant herbs" coordinate the actions of the other herbs. The rank of individual herbs varies between preparations and the condition for which that remedy is prescribed (Lee, 2000).

1.2.2. The history of TCM (300BC - 19th Century)

Many of the CHRs prescribed by TCM practitioners today have been used in the treatment of specific diseases for thousands of years. The first TCM pharmacopoeia to be published was the *Divine Husbandman's Classic of Materia Medica* (*Shen Nong Bencao Jing*), written around 300BC. It contains 252 medicines of plant origin, 67 from animals and 46 minerals. Each of the medicines in the book is placed into one of three categories. The "superior category" contains 120 medicines considered non-toxic and containing invigorating effects that preserve vitality and prolong life. The second category contains 120 "average medicines" used to prevent illness and restore vitality. The third category lists 125 "inferior medicines" that were considered toxic and should only be used to treat certain ailments. *Divine Husbandman's Classic of Materia Medica* is still used today by Chinese physicians because of the detailed information it contains (The American Academy of Acupuncture and Oriental Medicine n.d.). Other notable texts include Sun Simiao's (581-682AD) *Prescriptions for Emergencies worth a Thousand Gold* (*Beiji Qianjin Yaofang*), published in 652AD. It is in 30 volumes and contains 4,500 medicinal formulas and detailed information about acupuncture, nutrition, massage and exercise, and is traditionally hailed as the first encyclopaedia of clinical practise. Sun Simiao wrote a second book towards the end of his life called *A Supplement to the Essential Prescriptions Worth a Thousand Gold* (*Qianjin Yifang*), which is also in 30 volumes. It is believed that the world's first official pharmacopoeia was published in China during the Tang Dynasty (618-907AD). It was compiled between 657-659AD by Su Jing and around 20 others and entitled *Newly Revised Materia Medica* (*Xinxiu Bencaro*). It lists 844 herbs and contains illustrations based on genuine herbal specimens. It was not until 1498AD that the first official Western Pharmacopoeia was published in Florence (*Nuovo Receptario*), and the more famous *Pharmacopoeia of Nuremburg* between 1542-1546AD (New Chinese Medic n.d.), illustrating that Western medicine is a much more recent concept than TCM.

1.2.3. Modern TCM

The theories and practices of TCM remained unchallenged in China until the latter part of the 19th Century. However, during the Quing Dynasty (1644-1911AD) Western influence arrived, and with it Christian missionaries and doctors. The response to Western medicine varied greatly. Some TCM practitioners denied its

value, some tried to merge the two by stressing the benefits of each while others argued TCM was unscientific and should be banned. In 1911 Sun Yat-sen became the first leader of the Provisional Chinese Republic. Raised with a Western education, Sun Yat-sen was sceptical of the theories and practices of TCM and his views were shared by other high-ranking members of government.

During the first half of the 20th Century epidemics were rampant in China, with cholera, plague, smallpox and famine resulting in the government emphasising the importance of public hygiene and the adoption of Western medicine. Traditional Chinese medicine (TCM) became a symbol of "backward China", and resulted in public health officials drawing up the proposal "A Case for the Abolishment of the Old Medicine to Thoroughly Eliminate Public Health Obstacles". Written by Yu Ai and Wang Qizang, it stated important Chinese medical principles (yin and yang and The Five Elements) were fraudulent, and therefore the practise of Chinese medicine should be restricted, and the establishment of new Chinese medicine schools banned. Although the proposal passed the first legislative session on February 26th 1929 it was never implemented because of the heavy protests it generated by TCM practitioners and members of the general public.

In 1942, the outlawed communist Mao Zedong, residing in the Yan'an area of China, expressed similar views to those in power at the time, instructing his Red army to uproot all "Shamanic beliefs and superstitions" and establish model public health villages. He accused TCM practitioners of being no better than "circus entertainers, snake oil salesmen and street hawkers". Mao Zedong became the Leader of the Peoples Republic of China on October 1st, 1949.

Between 1953 and 1959 Mao Zedong revised his stance against TCM. Russia was perceived as a potential threat and Mao wanted to diminish China's growing reliance on the Soviets (including their purchase of medical equipment and pharmaceutical drugs) and create a form of communism that could put him and China on the world stage. He wanted to create a society that was self-reliant and patriotic; TCM promoted this. In 1954 Mao introduced the "Chinese-Western medicine integration" programme, for which Mao announced a search for "2,000 first rate Western medicine physicians who are to assist in the evolvement of Chinese medicine". The

2,000 doctors enlisted for this programme attended "seminars for the study of Chinese medicine by Western medicine physicians on leave" over a period of 1-2 years. Of those who entered this programme, only 10% passed, possibly due to their fundamental lack of belief in TCM, having previously obtained Western doctorates.

In 1966, Mao found himself locked in a power struggle within the communist party, and to remove his political opponents he unleashed The Cultural Revolution (1966-76). Mao directed popular anger against other members of the party leadership and in doing so became supreme commander of the nation and army. During the Cultural Revolution ideological cleansing began with attacks by Red Guards on so-called "intellectuals" to remove "bourgeois" influences. Another goal of The Cultural Revolution was to eradicate feudalist influences, and artefacts, temples and churches were demolished and books were publicly burned. During this time, much of the physical heritage of TCM was destroyed and its traditional belief systems lost public favour. Between 1966 and 1971 no new students were allowed into any academic institution and hundreds of thousands of existing students were forced to leave their studies. In 1972 "Colleges for Workers, Peasants and Soldiers" opened all over China, providing 3 year vocational programs for various studies including medicine, with doctors receiving rudimentary training in Eastern and Western modalities. It was not until the end of the Cultural Revolution in 1976 and the death of Mao that academics, physicians and TCM practitioners could safely return into society (Fruehauf, 1999).

In China today, TCM is used by the state to promote Chinese culture, and TCM and Western medicine are practised side by side. At present there are almost 3,000 dedicated TCM hospitals in China, and almost 40% of all healthcare services are provided by TCM practitioners. Furthermore, over 95% of the Western medical hospitals in China contain TCM wards and outpatient departments (Scheld, 1999). In addition to its growing popularity in China, TCM is also gaining popularity throughout the Western world.

1.3. The growing popularity of TCM in Western society

The World Health Organization (WHO) describes traditional medicine as

> "Health practises, approaches, knowledge and beliefs incorporating plant, animal and mineral based medicines, spiritual therapies, manual techniques and exercise, applied singularly or in combination to treat, diagnose and prevent illnesses or maintain well-being." (WHO, 2003)

In industrialised countries, adaptations of these traditional medical practises are termed "Complementary" or "Alternative" medicines (CAM), and include TCM. The uses of all forms of CAM are rapidly spreading in industrialized countries. In Europe, North America and other industrialized regions more than 50% of the population have used some form of CAM at least once. In Germany, 90% of the population have used a natural remedy at least once. According to the USA Commission of Alternative and Complementary medicines, 158 million adults in the US spent £9.8 billion on traditional remedies in 2000. In the United Kingdom, annual expenditure on traditional medicines is £132 million, while the global market for traditional medicines currently stands at over £34.5 billion and is continually growing (WHO, 2003).

The increasing popularity of all forms of CAM, including TCM, has been attributed to a range of factors. One of the most important is the media, with the internet, magazines, radio and television programmes informing people of potentially beneficial alternative therapies available for a range of diseases and chronic conditions. The media also presents Western medicine as "artificial" in a climate where the benefits of "natural" and "organic" products are constantly stressed, encouraging the use of CAM and herbal remedies in particular (Vuckovic & Nichter, 1997). The prevalence of CAM use in Western society varies between social groupings. In general, social status, age, sex and general health dictate the probability that an individual will use some form of CAM, with most studies indicating people who use CAM are typically better educated, of higher socioeconomic status and more likely to be young and female (Cassileth & Vickers, 2005).

Complementary and alternative medicines (CAM) are used in conjunction with Western medicine, as an alternative to Western treatment or as dietary supplements. Those individuals that most frequently use CAM as an alternative to Western medicine suffer from chronic conditions; the most common complaints presented to CAM practitioners are musculoskeletal problems, allergies, arthritis and stress related ailments. These individuals typically use CAM because Western medicine has been unable to effectively treat their conditions (Kelner & Wellman, 1997). Furthermore, an individual's choice to use some form of CAM is not necessarily based on clinical validation by Western science or reports of potential benefit by Western scientists. For example, in a survey of 500 peri- and postmenopausal women 79% used botanical dietary supplements, with only 3% obtaining information regarding the efficacy of those supplements from healthcare professionals before using them (Mahady *et al*, 2003).

The use of CAM in conjunction with traditional Western treatment is especially common amongst cancer patients. In a survey of cancer patients in a hospital in Florida (US), 80% of patients reported the use of some form of CAM, with 54% using herbal products (Bernstein & Grasso, 2001). Amongst cancer patients CAM user rates are at their highest in patients undergoing chemotherapy (Hyodo *et al*, 2005).

1.4. Dangers associated with the use of CHRs

As a result of the growing popularity of CHRs and other herbal products in Western society two key issues regarding the safety of these remedies have appeared. The first involves the current lack of regulation regarding the sale of these remedies (which include the correct identification of herbs and monitoring levels of adulterants), and the second key issue regards the potential interaction of herbal remedies with Western treatments.

1.4.1. Regulation, incorrect identification and adulteration

An example of the potential danger associated with the incorrect identification of herbs is the case of 100 young Belgian women who followed a slimming regimen containing a number of Chinese herbs, one of which was subsequently found to

contain high concentrations of aristolochic acid. In four of these patients multifocal transitional cell carcinomas were observed (Cosyns *et al,* 1998). The herb inadvertently placed into the pills was *Aristolochia fangchi*, a herb known to have nephrotoxic and carcinogenic effects (Nortier *et al,* 2000). Approximately 200 cases of nephrotoxicity related to the ingestion of *Aristolochia* species in CHR preparations have now been reported worldwide (Arlt *et al*, 2002). Other commonly used herbs have also been found to contain components with dangerous effects. *Aristolochia manshuriensis* Kom has been shown to induce acute renal failure in rats, and has also been proven to be oncogenic (Qui *et al*, 2000). *Coptis Chinensis*, a herb traditionally prescribed to combat inflammation and liver disease, has been investigated and experiments have suggested it increases the resistance of tumour cells to anti-tumour drugs. Its major constituent, berberine, has been shown to up-regulate expression of the multidrug-resistant transporter gene pgp-170 in a number of different cell lines (Lin *et al*, 1999a), reducing cell sensitivity to the cancer chemotherapeutic drug Paclitaxel (Lin *et al*, 1999b).

Studies have shown a significant proportion of Chinese herbs imported into Europe contain high levels of toxins, but due to a lack of quality control protocols these products are freely sold. Lead, mercury, cadmium, arsenic, copper and thallium have all been found in Chinese remedies. Analysis of 317 batches of dried Chinese herbs delivered to a hospital of Chinese medicine in Germany found 3.5% of their samples contained heavy metal contamination above legal limits. Lack of regulation can also lead to the sale of herbs containing high levels of pesticides, herbicides, microorganisms and mycotoxins. Contamination is most likely a result of herbs being grown in contaminated soil or their inadequate processing and storage, however not all contamination can be regarded as accidental, as some heavy metals are added intentionally to some prescriptions for their supposed medicinal attributes (Ernst 2002).

The lack of regulation concerning herbal remedies can also result in the sale of medicines that have been subject to deliberate adulteration: caffeine, paracetamol, diazepam and heavy metals have all been added to CHRs to make them more effective (Ernst 2002). One of the most recent examples of deliberate adulteration of a CHR is the case of PC-SPES. Produced by the company BotanicLabs and

introduced into the US in 1996, PC-SPES was marketed as a dietary supplement for "prostate health" (PC standing for prostate cancer, and *spes* Latin for "hope"). This remedy rapidly became known throughout the prostate cancer community as patients began speaking out about its effectiveness through the internet and support groups (White, 2002). Various studies assessing the potential effectiveness of PC-SPES on prostate cancer patients gave promising results. In one trial, the effects of PC-SPES on androgen dependent and independent cancer patients were assessed using serial serum prostate specific antigen (PSA) level measurements. Of 33 patients with androgen-dependent prostate cancer treated with PC-SPES, all experienced a PSA decline of $\geq$ 80%, and no patient developed PSA progression. Of 37 patients with androgen-independent prostate cancer, 19 (54%) experienced a PSA decline of $\geq$50%. However, common side-effects amongst patients treated with PC-SPES were similar to those treated with oestrogen therapy, and included loss of libido and potency, gynaecomastia and gynecodynia (Small *et al*, 2000). Allegations that PC-SPES contained the synthetic oestrogen diethylstilbestrol (DES) appeared on e-mail list severs of prostate cancer patients in 2001. Incidences of PC-SPES causing abnormalities in clotting times and severe bleeding amongst some patients were also published (Lock *et al*, 2001). Sovak *et al* (2002) analyzed lots of PC-SPES manufactured between 1996 and mid 2001 and found those manufactured between 1996 and 1999 contained the synthetic compounds indomethacin (a non-steroidal anti-inflammatory drug) and DES, with a gradual decline in the levels of these adulterants after 1999. In addition, lots produced from July 1998 were also found to contain another adulterant, the anticoagulant warfarin. As a result, BotanicLabs went out of business in on June 1, 2002, and PC-SPES is no longer available.

1.4.2. Herb-Western drug interaction

The potential for herb-drug interactions amongst individuals who use Western medicine in conjunction with CHRs and other herbal products are also becoming an increasing concern as their prevalence of use continues to grow. People often neglect to tell their GP they are taking alternative medicines when being prescribed Western treatments. Around half of people who use alternative medicines do not see the need to inform their GP. This leads to the possibility of herb-drug interactions (Ernst 2002). Studies in various cancer centres have shown CAM use can range

from 32% to 83% in patients who deal with radiation oncologists, but patients are not asked if they use CAM when their histories are taken (Micke, 2003).

Providing accurate and clinically relevant advice to patients regarding the possibility of herb-drug interactions is a challenge for both doctors and alternative medicine practitioners. Clinical articles relating to case reports of possible interactions between herbal medicines and cardiovascular drugs (published between January 1996 and February 2003) highlight the problems concerning the clinical impact of herb-drug interactions. Compiled using a Medline search, 43 case reports and 8 clinical trials relating to direct herb-drug interactions were uncovered (Izzo *et al*, 2005). The cardiovascular drug most commonly involved was warfarin, an anticoagulant that antagonizes the cofactor function of vitamin K. Boldo, curbicin, fenugreek, garlic, danshen, devil's claw, don quai, ginko and lyceum all caused over-anticoagulation (each herb contains anticoagulant compounds that provided an additive anticoagulant effect when combined with warfarin). Ginseng, green tea and soy all decreased the anticoagulant effect of warfarin (due to the high levels of vitamin K they contain). Another cardiovascular drug identified as interacting with CHRs was digoxin, a cardiac glycoside that originates from foxglove and increases contractility of heart muscle and is used in the treatment of heart failure. Guar gum was found to reduce the effectiveness of digoxin by reducing its absorption. Another example is aspirin, which is used in the prophylactic treatment of transient cerebral ischemia, to reduce the incidence of recurrent myocardial infarction (MI) and decrease mortality in post-MI patients. Spontaneous hyphema (bleeding in the eye) has been observed when associated with ginkgo, and increased bioavailability when associated with tamarind.

When used in combination with Western medicine, herbal remedies have been shown to modulate the effects of drugs through alterations in their absorption, metabolism and excretion. Interactions may also be synergistic or additive, or may directly antagonize the effect of a drug (Izzo *et al*, 2005). Another growing concern is that people who first go to TCM practitioners with a problem may be given a treatment that masks the effects of the disease, but does not cure it. This masking lengthens the time between a patient becoming ill and being given proven Western treatment.

In addition to patient safety issues regarding TCM, there are also fears the growing herbal market may pose a danger to biodiversity through over harvesting of raw products, possibly leading to the destruction of natural habitats (WHO, 2003). Another concern regarding the increased use of CHRs is that 13% of TCM products are reportedly derived from animals. Their use is often based on belief rather than scientific fact. Tiger bone and rhino horn are important examples as they are endangered species illegally hunted to supply the growing trade in TCM. Their use in medicine is listed as one of the four main reasons tiger populations are threatened with extinction. It is also a concern that those animal products used in CHRs could potentially transmit diseases to humans (Still, 2003).

These concerns regarding the safety of CHRs, which include accidental and deliberate adulteration and the potential dangers associated with their use alone and in combination with Western treatments are becoming increasingly important as the popularity of TCM in Western society continues to grow.

To address these important issues in Europe, international EU regulations regarding quality control and identification of herbs are currently being constructed (The Herb Society, n.d.), and investigations into herb-Western drug interactions are becoming increasingly common and in the future will be used to inform patients of the potential dangers associated with using CHRs and other medicinal remedies in combination with Western treatments (Fugh-Berman, 2000).

In addition to analysing the potential dangers associated with CHRs, investigations that attempt to provide a scientific basis for their use are also becoming increasingly common as the popularity of TCM continues to grow. However, there is, at present, little in the way of a standard approach to investigate CHRs and consistent and definitive data concerning their biological actions.

1.5. Investigations into the biological actions of CHRs and difficulties associated with their study

Chinese herbal remedies (CHRs) are traditionally developed using the belief systems of TCM, and their efficacy is based on patient observation. In contrast, Western scientists base the efficacy of medicines on clinically defined factors, and explain the actions of drugs and other remedies through interactions of individual compounds with specific cellular targets.

Due to the fact that the compounds within CHRs, and their biological actions remain largely undefined, investigations into the pharmacological activities of CHRs requires a multidisciplinary approach, which may include the field of ethnopharmacology for investigations into the traditional uses of selected herbs and herbal remedies, botany for correct identification of herbal products, biological science for analysis of the effects of herbal products in mammalian systems and analytical chemistry for identification of the compounds within these remedies (Rates, 2001).

1.5.1. Approaches to investigate the biological actions of CHRs

There are two approaches to examining the biological actions of CHRs. One is to examine the action of a CHR in its entirety, and the other is to examine the biological actions of compounds found within CHRs in isolation. To examine the action of a CHR in its entirety is to analyse the action of a remedy that has been developed by TCM practitioners using their belief systems, with the efficacy and development of that remedy based on patient observation. Obtaining information regarding the disease for which that CHR is prescribed, how that remedy is prepared and whether it is prescribed through oral or non-oral administration provides a basis for its analysis (Rates, 2001). If a CHR is found to exert a biological action, investigations can then be conducted to determine if a single or multiple compounds are responsible for the observed biological action of that remedy. This can lead to the isolation of pure active principals (where a single compound is found to be responsible for the action of that remedy), active fractions (where a herbal fraction is found to exert the action of a CHR in its entirety) or to the development of improved formulations (Lee, 2000).

The other common research method used to study CHRs is to first isolate the compounds within a remedy and then test the biological actions of these isolated compounds in a range of biological settings. However, these pharmacological studies do not take into consideration the traditional belief systems of TCM, in which the multiple actions of compounds within a remedy are believed to be responsible for the efficacy of that remedy. Individual compounds can interact in a number of ways, for example through mutual accentuation (where compounds intensify the effects of one another), enhancement (where one compound increases the effectiveness of another), or through counteraction (where one compound opposes the effectiveness of another) (Yuan & Lin, 2000), therefore it is possible the observed actions of individual compounds will be different from the biological actions of CHRs in their entirety.

1.5.2. Identification and standardization of CHRs

The question of whether to study CHR constituents in isolation or to consider the CHR as a whole is one of the many difficulties associated with the study of CHRs, and the correct identification of herbs and standardization of CHR remedies are two other hurdles to overcome in the study of these CHRs. The correct identification of herbs and CHRs is essential to prevent investigations into the actions of the wrong CHRs, and the publication of inaccurate data. Common identification procedures for CHR constituents may include microscopic analysis (to examine morphological characteristics) and chromatographic fingerprint profiling (using thin layer chromatography (TLC), high performance liquid chromatography (HPLC), liquid chromatography (LC), gas chromatography (GC), gas chromatography-mass spectrometry GC-MS, liquid chromatography-mass spectrometry (LC-MS), LC/MS/MS and capillary electrophoresis (CE)) (Lee, 2000).

There are also difficulties in CHR research associated with nomenclature, with herbs having multiple names and spellings. For example, in English, liquorice is also spelt licorice, and has the botanical names *Glycyrrhiza uralensis* and *Glycyrrhiza galbra*, and the pharmaceutical name Radix glycyrrhizae (Tse 2003). Therefore when reviewing literature regarding the medicinal effects of this remedy searches have to be performed for each of its common names. If this is not done, a comprehensive literature review regarding the known effects of this remedy cannot be made.

Lack of standardization with regard to CHR formulations is a key problem in the study of multi-component remedies: CHRs can be made from up to 20 separate herbs, and CHRs with common names can be made up differently by different practitioners. In some instances the proportions of each herb may vary, while in others entirely different herbs may be used to make a remedy with the same name. Furthermore, many CHRs are tailored to individual patient's symptoms, with specific herbs added to a standard preparation after consultation with a TCM practitioner. This lack of standardization makes accurate comparative analysis between different research groups studying the same CHRs very difficult. In addition to difficulties associated with identification and standardization, the activities of the herbs, plants and fungi used in CHRs may vary depending on where they were grown, climate condition, type of soil and their time of harvest (Yuan & Lin, 2000), again making comparative analysis very difficult.

Accidental and deliberate adulteration can also pose a problem in CHR research, making remedies look like they exert a specific biological effect when they have none (for example the effect of PC-SPES on prostate cancer, described above), or the masking of a genuine biological action through the presence of an adulterant. Furthermore, the actions of many CHRs are investigated in multiple biological environments, and if one effect is found to be a result of adulteration the other effects of that CHR have to be re-examined. For example, the finding that deliberate adulterants in PC-SPES were responsible for its reported action against prostate cancers cast doubts on the observed biological action of PC-SPES in other biological contexts. Research published by Sadava *et al* (2002) showed PC-SPES to be cytotoxic and pro-apoptotic to both drug sensitive (H69) and drug resistant (H69V) small cell lung carcinoma (SCLC) cell lines. Upon subsequent investigation, these actions of PC-SPES were found to be independent of the adulterants associated with their effectiveness regarding prostate cancer (Sadava & Winesburg, 2005). Therefore some of the positive effects of PC-SPES may be genuine, but all research must be re-examined to determine whether all of the effects of PC-SPES are a result of the adulterants or constituents of PC-SPES.

Despite the difficulties associated with the study of medicinal remedies, CHRs are potentially a rich source of diverse therapeutic agents and formulations that may be useful in the treatment of a range of diseases. Of particular interest are those remedies with suggested cancer chemotherapeutic activity, with studies suggesting CHRs may be a rich source of novel cancer chemotherapeutic agents (Mann, 2002), and that CHR prescriptions themselves may be of chemotherapeutic value while exhibiting few of the adverse side-effects associated with modern chemotherapeutic drugs (Izuka *et al*, 2000).

1.6. Anticancer Activities of CHRs

Cancer, the uncontrolled proliferation of a mutated cell within the body, is one of the greatest killers in Western society, and more than 270,000 people in the UK alone are diagnosed with cancer each year (Office for National Statistics, 2005).

1.6.1. Carcinogenesis

Cancers develop through the accumulation of mutations within a normal cell that allow it to grow uncontrollably and evade programmed cell death (apoptosis). The vast majority of mutations that give rise to cancer are not inherited, but arise spontaneously as a consequence of chemical damage to DNA resulting in the altered function of crucial genes. DNA damage and mutations do occur naturally within cells, however it is estimated that ~70% of cancers in Western populations are attributed to Western lifestyles, with the use of tobacco accounting for ~30% of those cancers, and other leading factors including poor diet and un-protected exposure to sunlight (Turner *et al*, 2000).

Chemical damage to DNA itself is not a mutagenic event: DNA replication and cell division are necessary to convert chemical damage to an inheritable change in DNA, which is known as a mutation. Multiple mutations within a number of genes that control cell growth and death are required within a cell to make it cancerous. As the likelihood of a single cell simultaneously acquiring multiple mutations is small, it is believed a sequential process of mutation acquisition is responsible for the development of cancer. In this model a cell with a single mutation clonally expands until the population rises to millions - in this population a second mutation is more likely to occur. This process of clonal expansion is repeated, with each generation

becoming progressively better adapted to an independent life. Clinically, this process is observed as disease progression, which is characterised by an increased growth rate, acquisition of the ability to invade neighbouring tissue, and after the application of chemotherapeutic agents, drug resistance.

For a cancer cell to develop and proliferate effectively it is believed that their response to 5 major control pathways must be augmented. These include:

1. Development of independence to growth stimulatory signals
2. Development of a refractory state to growth inhibitory signals
3. Development of resistance to apoptosis
4. Development of an infinite proliferative capacity
5. Development of angiogenic potential

While normal cells can only proliferate in response to external, typically locally produced growth factors (such as epidermal growth factor (EGF) and fibroblast growth factor (FGF)), cancer cells have the ability to activate their own proliferative pathways through a variety of mechanisms, including the inappropriate synthesis of growth factors or growth factor receptors, activation of downstream signal transduction pathways and the inappropriate activation of nuclear transcription factors.

Cancer cells must also become refractory to a range of growth inhibitory signals. For example, virtually all cells in the body communicate with their neighbours through small pores called connexons, or gap junctions, and these are used to transmit growth inhibitory signals. However, it has been found that the expression of gap junctions and their function is frequently down-regulated in cancer cells.

Apoptosis, or programmed cell death, is central to normal tissue homeostasis, the removal of virally infected cells and the destruction of mutated cells that may become cancerous. During apoptosis cellular organelles and chromatin are rapidly degraded and the cell broken down into apoptotic bodies before being taken up by phagocytic cells and surrounding tissues (without stimulating a localized inflammatory immune response). Apoptosis can be triggered through a variety of

external signalling pathways (including death receptor activation and the loss of local contact signals within tissues), and internal signalling pathways (activated in response to the detection of cellular or genomic damage). For a cancer cell to become successful it must evade apoptotic induction.

In normal cells, division can only occur a limited number of times, after which they enter a state of "replicative senescence"; however cancer cells can proliferate indefinitely. The life span of a normal cell is limited by the progressive erosion of the ends of chromosomes, called telomeres: during each cell division these become shorter as small RNA primers are used to initiate DNA synthesis and these regions are not replicated. In stem cells the enzyme telomerase is used to maintain telomere length, and in many cancer cells this enzyme is re-activated, thus allowing them to proliferate indefinitely (Bertram, 2001).

1.6.2. Conventional cancer therapies

As cancerous cells are essentially normal cells that are dividing in an uncontrolled manner, the challenge of cancer therapy is to find a way to kill those cancerous cells without damaging normal cells and tissues. One common means of treatment is the physical removal of tumours through surgery. Although successful, surgical removal is not always possible, and depends on the type and location of the tumour. Other key therapies used to treat cancer include radiotherapy and chemotherapy. Both rely on one of the key features of cancer cells that separate them from the majority of cells within the body: their rapid rate of cell division.

Radiotherapy uses precisely targeted high-energy rays to damage those cancerous cells that make up a tumour and induces apoptosis in them. Although effective, radiotherapy is only used in the treatment of solid tumours. In contrast, chemotherapeutic agents are injected into the bloodstream and attack cancerous cells throughout the body. Chemotherapeutic drugs are essentially toxic chemicals that kill dividing cells. As many of the cells and tissues within the body do not constantly proliferate they are not affected. However, those normal cells within the body that do divide rapidly (including bone marrow cells, hair follicle cells and immune cells) are typically affected by chemotherapeutic agents, leading to adverse side effects for the patient (Verweij & Jonge, 2000).

1.6.3. Investigating the anticancer potential of CHRs

The search for novel chemotherapeutic agents to treat cancer is very common, and includes the development of more effective analogues of existing drugs with increased activity and/or reduced toxicity to non-cancerous cells and tissues, creating novel combinations of existing drugs to enhance activity and reduce toxicity, and investigations to discover novel classes of agents with chemotherapeutic potential (Cancer Research UK, n.d.). As many CHRs are already used for the treatment of cancer, they are an attractive target for the discovery of novel chemotherapeutic agents.

Based on approaches used to study CHRs (see Section 1.5), investigations into the chemotherapeutic potential of CHRs may include examining the effects of individual compounds within those remedies in a range of biological settings, or examining the anticancer potential of those CHRs in their entirety. While examination of individual compounds may lead to the isolation and characterization of novel chemotherapeutic compounds, the efficacy of a CHR is traditionally attributed to that remedy in its entirety, with individual compounds acting in combination in a synergistic or additive way against a single, or multiple biological targets (Yuan & Lin, 2000). Thus, to examine a CHR in its entirety is to examine a traditional therapy that has been developed by TCM practitioners using their traditional belief system and patient observation in response to it (the remedy) (Rates, 2001). Furthermore, examination of the anticancer potential of a CHR in its entirety could be quite fruitful as it may reveal a range of biological activities, with further investigation resulting in the isolation of active fractions or the characterization of individual compounds that exert a chemotherapeutic effect (Lee, 2000). However, this approach has a number of already identified drawbacks including a lack of standardization which could arise from different growing locations and harvest times, specific patient needs, differences in preparation, and adulteration (Yuan & Lin 2000, Ernst 2002). These drawbacks could ultimately lead to the generation of inconsistent and possibly misleading data. In addition, as CHRs can contain up to 20 separate ingredients, which can potentially contain hundreds of individual compounds, they, as a result, could interact in a number of different ways, with multiple cellular targets (Lee, 2000). Thus, investigations into the chemotherapeutic potential of CHRs requires a multidisciplinary approach which includes the

determination of the biological actions of those remedies, and chemical analysis to elucidate those constituents/compounds responsible for the biological action of that remedy.

Despite the difficulties associated with the study of CHRs, studies have shown that some CHRs can elicit various anticancer activities, which include enhancement of immune function against tumours (Chen *et al*, 2006), sensitisation of tumours to common therapeutic drugs and traditional therapies (Frese *et al*, 2003), direct toxicity to cancer cells (Lee *et al*, 2002) or a combination of the above. In addition, many of those therapies that have been shown to elicit a direct anticancer action have been found to exhibit few of the adverse side effects associated with modern chemotherapeutic drugs (Izuka et al, 2003), thus highlighting their potential use in the treatment of cancer in the future. However, for many CHRs commonly used to treat cancer much of the information regarding their anti-cancer activity is anecdotal. In addition, some investigations into the anti-cancer activity of popular CHRs have generated conflicting data. Therefore, to fully understand the potential efficacy and also safety of these CHRs a more thorough understanding of their mode of action is required.

1.7. Aim of study

Chinese herbal remedies (CHRs) are commonly prescribed for the treatment of cancer, however their use is often based on the traditional belief systems of TCM rather than any scientific proof of their efficacy or biological action. Thus, the aim of this investigation was to select a range of CHRs with some suggestion of tumour modulatory activity and to investigate their direct toxicity to cancer cells in an attempt to elucidate their mode of action. Furthermore, due to the inherent difficulties associated with studying the biological actions of CHRs, which include a lack of standardization of CHR prescriptions, adulteration, the incorrect identification of herbs and the potential of CHRs exerting multiple biological activities, part of this project also focused on devising a strategy with which to investigate the biological actions of these remedies in a manner that addressed these issues. As such, a multidisciplinary approach was chosen, which involved examining the known biological actions and traditional uses of CHRs, identified through a systematic search of the literature and consultation with a TCM

practitioner, investigation into the various biological actions of these remedies, and chemical analysis of these remedies to determine whether a single, or multiple constituents/compounds were responsible for the observed biological actions of those remedies.

The first objective of this investigation was the selection of CHRs for investigation: With more than 5,000 CHRs traditionally used for the treatment of a variety of diseases, it was essential to select CHRs that had been shown to possess some form of anticancer action. Thus, CHR selection was based on the traditional uses of those remedies and existing research suggesting that they may exhibit a chemotherapeutic potential.

The second objective of this investigation was to then assess the direct cytotoxic potential of the selected CHRs against cancer cells using an *in vitro* cell based system. An *in vitro* model was employed as it is a commonly used, effective way of characterizing the cytotoxicity of chemicals in a controlled environment against a variety of specific cell types, in this case a range of cancer cell lines, without the need for animal testing (Tuschl & Schwab, 2004). Furthermore, as CHRs are typically boiled in water and then drunk as a tea, the cytotoxic actions of water extracts of each CHR were examined to determine whether traditional preparations of these remedies exhibit a cytotoxic potential against a range of cancer cells. The effect of ethanol extracts was also investigated to determine whether smaller, volatile compounds within the CHRs possess anticancer activity.

The third objective of this investigation was to then characterize the chemotherapeutic potential of those CHR extracts found to exert a direct cytotoxic action against the cancer cells. To assess the chemotherapeutic potential of these extracts, their ability to induce apoptosis in cancer cells was examined.

The fourth objective of this investigation was to further characterize any apoptosis-inducing activity of the CHR extracts. This was achieved by examining those pathways involved in triggering apoptosis in those cancer cell lines exposed to the CHR extracts, and examining the potential intracellular targets of those CHR extracts that led to apoptotic induction.

The fifth objective of this investigation was to then determine the effect of each cytotoxic CHR extract on non-cancer cells so as to determine the viability of these CHR extracts as potential chemotherapeutic agents, and to investigate their safety via their impact on 'normal' cells *in vitro*.

The final objective of this investigation was to assess the role of individual constituents/compounds in the observed actions of the selected CHRs. To address this issue, an activity-guided fractionation study was performed, in which CHR extracts were split into fractions and the cytotoxic potential of each examined. Those extracts found to exert a significant toxic action were then subject to chemical analysis, in order to determine whether a single, or multiple compounds were found to be responsible for the observed actions of those CHR extracts studied in this investigation.

Chapter 2. Selection of Chinese Herbal Remedies

2.1. Introduction

More than 5,000 CHRs are used in TCM for the treatment of various forms of disease, including a range of cancers, however the scientific basis for this use is unclear. Furthermore, the use of CHRs in Western society is becoming increasingly common as the popularity of all forms of CAM continues to grow (Ernst, 2002), however this use is often based on anecdotal evidence of their efficacy rather than scientific proof or clinical validation.

Anecdotal reports of CHRs exerting a beneficial anticancer action are primarily spread through the Internet (Vuckovic & Nichter, 1997), and while some websites provide unbiased information regarding the potential benefits and dangers associated with the use of CHRs, much of the available information regarding the efficacy of these remedies is either misleading or unsubstantiated, as illustrated in the advert for the anticancer CHR "Tumour Shrinker", shown below.

> "There is a cure which your doctor or drug company didn't know or didn't want you know it
>
> *Herbal Doctor Remedies* are formulated according to Traditional Chinese Herbal medicines, scientifically prepared, purified, concentrated, clinically tested, 100% all natural, no side effects, very powerful and with remarkable effectiveness, and are recommended by Chinese herbal Doctor for over thousands years."
>
> **Tumor Shrinker #325**
> ***Composition:*** Laminaria, Phellodendron, Anemarrhena, Platycodon, Sparganum, Zedoaria, Forsythia, Coptis, Licorice, Scute, Paonia, Pueraia, Bupleurum, TangKuei, Gentiana, Cimicifuga, Tricosanthes, Sargasum. $35.00/90 Caps
>
> ***Indication:*** This is the most powerful formula to shrink tumor or lumps, fibrocystic diseases,unknown tumors, for lymphadenitis in the neck, scrofula ,thyroma, breast cancer, and goiter by disperses Qi stagnancy , dispel heat-dampness. . $35.00/90 Caps.
>
> **www.herb-doc.com**

Thus, due to the fact that TCM bases the efficacy of CHRs on a series of belief systems that are not linked to traditional Western scientific values, and the fact that many of the supposed anticancer activities of many CHRs are unsubstantiated

anecdotal reports of their effectiveness, the first challenge in this project was to identify appropriate CHRs for analysis in this investigation. To achieve this goal, CHRs were primarily selected for this study on the basis of a review of scientific articles published by research groups that had examined the anticancer potential of various CHRs. Furthermore, as the aim of this investigation was to analyse the direct cytotoxic potential of a range of CHRs against cancer cells, their selection for this investigation was primarily based on preliminary studies that had shown those CHRs to exhibit some form of direct cytotoxic action against cancer cells, or had been shown to elicit some form of *in vivo* anticancer activity whose mechanism of action was unknown, but could potentially be related to a direct cytotoxic action.

Once the review of peer-reviewed literature had been completed, the traditional uses of those remedies were then also examined in order to see if those CHRs were traditionally prescribed for the treatment of cancer, or if there was any available anecdotal evidence of their efficacy. Thus, in addition to the above literature search, a TCM practitioner was also consulted to provide further information with regards to remedies that may potentially elicit an anticancer action and may be worth investigating.

2.2. Materials and Methods: Selection of CHRs

The selection of CHRs for use in this investigation began with a search of peer reviewed literature (published between 1990-2003) using PubMed and a range of broad search terms such as "alternative therapies and cancer" and "Chinese herbal remedies and cancer" to compile a list of CHRs that had been found exhibited some form of cancer modulatory effect.

These search criteria highlighted a broad selection of articles describing the anticancer potential of a range of CHRs. However, these broad search terms did not distinguish between CHRs that had been found to exhibit a direct cytotoxic action against cancer cells, an indirect anticancer potential through immune-enhancing activity or anticancer activities that involved reducing the side-effects of traditional Western therapies.

Thus, from the above search, CHRs were selected that had been found to exhibit *in vitro* cytotoxicity against cancer cells or an *in vivo* anticancer action whose direct cytotoxic potential had not been assessed, then all articles relating to these specific CHRs were compiled by performing PubMed searches for each of those CHRs to obtain all known information regarding their anticancer potential. Furthermore, to ensure a comprehensive literature review of each of these CHRs, searches were performed using all known names and spellings for each of these remedies (as CHRs and individual herbs can posses a variety of names and spellings) (Tse, 2003).

After the peer-reviewed literature search, the traditional uses of these remedies, anecdotal evidence of their efficacy, and the traditional beliefs surrounding their use were investigated using the Internet and a range of search engines (Google, Yahoo, AskJeeves) to provide more information regarding their anticancer potential. In addition to these literature reviews, a TCM practitioner was also consulted, who gave suggestions of potential remedies that may exhibit an anticancer potential but had not previously been investigated in a biological setting.

Once compiled, the above information was then used to select CHRs for analysis in this investigation, with the final criteria for the inclusion of CHRs in this

investigation based on preliminary findings that those CHRs may elicit a direct action against cancer cells, however their cytotoxic potential against a range of cancer cell lines had not yet been thoroughly investigated.

2.3. Results: Selected CHRs

Those CHRs selected for this study were *Oldenlandia diffusa* (OD), Long Dan Xie Gan Wan (LD), *Polygonum multiflorum* (PM) and *Polyporus umbellatus* (PU). The traditional uses of these remedies, the research relating to their known cytotoxic action (published between1990 and 2003) and the rationale for their selection is summarized below.

2.3.1. *Oldenlandia diffusa* (OD)

Oldenlandia diffusa (OD) is also known by the alternate Latin botanical name *Hedyotis diffusa*, the pharmaceutical name *Herba hedyotis diffusae* and the Pin Yin name Bai hua she she cao, and is a member of the Rubiaceae family. It is found mainly in the south-eastern provinces of China, and grows at low altitudes in moist fields. *Oldenlandia diffusa* (OD) is collected in the summer and autumn of each year and either used fresh or dried in sunlight for use later in the year. The whole plant is used medicinally and raw material is characterized by stems with small dried leaves. *Oldenlandia diffusa* (OD) is a popular folk medicine that was first described in 1949 in "Herbal Records of Guanxi" (Dharmananda, 2005).

Traditionally, OD is described as having bitter, sweet and cold properties, and is used to treat fire toxicity, for clearing heat, treating abscesses, toxic sores, ulcerations, swellings, snake bites, tumours, bronchitis, tonsillitis, haemorrhoids, burning urinary tract infections, damp heat jaundice and promoting urination. *Oldenlandia diffusa*'s (OD's) medical properties are described as anti-bacterial, anti-inflammatory, anti-tumour, anti-viral and immune enhancing. *Oldenlandia diffusa* (OD) is specifically prescribed for the treatment of lung, liver and rectal tumours. *Oldenlandia diffusa* (OD) can be taken orally (in the form of a tea) or topically. Oral dosage can range from between 10-60g of raw material per day (Chu, 2003).

From the literature search, a number of groups were found that have researched the direct anti-cancer action of OD. Sadava *et al* (2002) investigated the effect of water extracts OD on a small cell lung cancer cell line (H69), a multi-drug resistant small cell lung cancer cell line (H69VP) and a normal lung epithelium cell line (BEAS-2). IC50 values showing the concentration of OD needed to inhibit the growth of each cell line by 50% over 4 days revealed OD to be equally toxic to the H69 and H69VP

cell lines, while having a significantly reduced effect on the normal BEAS-2 cell line at the same concentration. Apoptosis was observed in both the H69 and H69VP cell lines, with gene arrays showing a high increase in expression of the pro-apoptotic genes MdM2, Bax, p21 and caspases 6, 9 and 10, and a small increase in expression of Bcl-$_{XL}$, Bad, Trial, Traf 5 and Nik in the H69 cell line after exposure to OD.

In a separate study, purified MeOH extracts of OD were found to significantly inhibit the proliferation of the cultured tumour cell lines A549 (lung), SK-OV-3 (ovary), SK-MEL-2 (skin), XF498 (brain), HCT-15 (colon), SNU-1 (stomach, L1210 (murine leukaemia) and B15-F_0 (murine melanoma). Furthermore, cytotoxicity guided fractionation of those MeOH extracts led to the isolation of ursolic acid (UA) as an active cytotoxic principle (Kim *et al*, 1998).

In addition to the *in vitro* cytotoxic action of OD, two separate studies have shown oral administration of OD can significantly inhibit the growth of transplanted tumours in mice (Yoshida *et al*, 1997), (Wong *et al*, 1996).

2.3.2. Long Dan Xie Gan Wan

Long Dan Xie Gan Wan (LD) was primarily selected for this study because it was recommended by Professor Ma, an active TCM practitioner. Long Dan Xie Gan Wan (LD) is traditionally prescribed when a patient is diagnosed as having excess liver heat, excess gall bladder heat and damp heat accumulation. It is used for the treatment of a range of conditions, including gall stones, gall bladder diseases, hepatitis, herpes, shingles, cystitis, hyperthyroidism, migraines and jaundice. Long Dan Xie Gan Wan (LD) was first listed in the "Analytic Collection of Medicine Formulas" (Yi Fang Ji Jie) in 1682AD. As with many CHRs, LD can be made from a variety of different ingredients, or the same ingredients used in varying proportions, depending on where it is made. The traditional ingredients typically used to make LD are *Radix Scutellariae* (Huang Qin), *Fructvs Gardeniae* (Zhi zi), *Radix glycyrrhizae* (Gan cao), *Aristolochia manshuriensis* (Mu Tong), *Radix rehmanniae* (Di huang), *Radix Gentianae* (Long dan), *Radix angelicae sinensis* (Dang gui), *Semen Plantaginis* (Che qian zi), *Radix Bupleuri* (Cai hu) and *Rhizoma alismatis* (Ze xie) (RxList, 2004). However, those LD remedies containing

Aristolochia manshuriensis (Mu Tong) are no longer available as *Aristolochia* species contain the toxic and carcinogenic aristolochic acids (Cosyns *et al*, 1998).

The LD remedy selected for this investigation was produced by Shanghai Chinese Herbs Works (Shanghai, China). In this formulation *Aristolochia manshuriensis* (Mu Tong) has been replaced by *Medulla tetrapanacis* (Tong cao), while all the all other herbs within the formula are found in the original recipe described above. The recommended dosage of LD is 8 pills taken 3 times daily (RxList, 2004). The constituents and relative proportions of each herb in the LD remedy used in this study are described below.

Table 2.3.2.1. The constituents of Long Dan Zie Gan Wan

Herb	Relative quantity
Radix gentianiae (Long Dan)	14.29%
Radix bupleuri (Cai hu)	14.29%
Radix scutellariae (Huang qin)	7.1%
Fructvs gardeniae (Huang qin)	7.1%
Rhizoma alismatis (Ze xie)	14.29%
Semen plantaginis (Che qian zi)	14.29%
Radix angelicae sinensis (Dang gui)	7.1%
Radix rehmanniae (Di huang)	14.29%
Radix glycyrrhizae (Gan cao)	7.1%
Medulla tetrapanacis (Tong cao)	7.1%

Long Dan Xie Gan Wan (LD) is not traditionally prescribed in the treatment of cancer, and there is no research regarding the effects of LD in any biological context. However, there are herbs within LD that are prescribed for the treatment of cancer, (*Radix bupleuri*, *Radix scutellariae* and *Rhizoma alismatis*) and the anticancer activities of a number of the individual herbs within LD have been assessed, and are described below.

Radix bupleuri (botanical name Bupleurum scorzoneraefollium wild.) is traditionally described as having bitter, pungent and slightly cold properties. Its functions are to release the exterior, clear heat and pacify the liver, and it is used in the treatment of fever, irregular menstruation and diarrhoea. *Radix bupleuri* was first described in Shen Nong Bencao Jing. Acetone extracts of *Radix bupleuri* have been shown to be toxic to A549 human lung cancer cells and induce apoptosis *in vitro*, while being significantly less toxic to WI38 human normal lung fibroblasts. It is believed apoptosis was induced through inhibition of the enzyme telomerase (Cheng *et al*, 2003).

The anticancer activities of water extracts of *Radix bupleuri* have also been studied, and it has been found that i.p. injection of *Radix bupleuri* can significantly inhibit the growth of WEHI-164 tumour cells that have been implanted into BALB/c mice (Kok *et al*, 1995).

Radix scutellariae is the root of *Scutellaria baicalensis*, and is traditionally used to remove damp heat, quench fire and counteract toxicity. It is prescribed in the treatment of nausea, vomiting, dysentery, jaundice, fever, spitting blood and threatened abortion (Chang *et al*, 2007). The major flavonoid constituents of *Radix scutellariae* are baicalin, baicalein and wogonin, each of which have been shown to inhibit the proliferation of the bladder cancer cell lines EJ-1, KU-1 and MBT-2 in a dose-dependent manner (Ikemoto *et al*, 2000). Baicalin has also been shown to inhibit the growth of a range of prostate cancer cell lines and to induce apoptosis in those cell lines (Chan *et al*, 2000). Oral administration of *Radix scutellariae* has been found to inhibit the growth of MBT-2 tumour cells implanted in C3/HeN mice (Ikemoto *et al*, 2000).

Rhizoma alismatis is used to transform dampness and promote water metabolism. It is traditionally prescribed to treat turbid urine, diarrhoea, retention of phlegm and other fluids (Fong *et al*, 2007). Three triterpenes cytotoxic to cancer cells have been isolated from methanol extracts of *Rhizoma alismatis* through activity-guided fractionation, these are alisol B 23-acetate, alisol C 23-acetate and alisol A 24-acetate (Lee *et al*, 2001).

3.2.3. Polygonum multiflorum

Many species of *Polygonum* are valued for their medicinal properties, and include *Polygonum chinensis*, *Polygonum persicaria*, *Polygonum viscosum*, *Polygonum hydropiper* and *Polygonum multiflorum* (PM). The genus *Polygonum* is a source of a wide range of phenolic compounds, flavonoids, anthraquinones, stilbenes and tannins. *Polygonum multilflorum* (PM) is a medicinal vine plant in TCM that is commonly prescribed to the elderly to restore vitality. It is used by TCM practitioners throughout the world, and is widely regarded as one of the most important of all CHRs (Lin *et al*, 2003).

Polygonum multiflorum (PM) has not previously been examined for its potential anticancer activities *in vitro*, however a range of compounds within PM have been shown to exert antimutagenic and anticancer activities. In an *in vitro* test system, PM fractions were tested for potential antimutagenic action in Salmonella typhimurium TA98 incubated with benzo[a]pyrene (B[a]P). The ethyl acetate soluble fraction of the CHR exhibited substantial dose-dependent antimutagenic activity. The major constituents of the extract were tannins, of which epigallocatechin, epigallocatechin gallate, epicatechin gallate and tannic acid all significantly inhibited mutagenicity. To test the effects of PM as a whole *in vivo*, F344/DuCrj male rats were subcutaneously injected with B[a]P, then received water extracts of PM for 50 weeks. PM was found to significantly reduce tumour incidence (Horikawa *et al*, 1994). In a separate study, epigallocatechin gallate and epigallocatechin were found to suppress the growth of the human colon carcinoma LoVo cell line in a dose-dependent manner, and furthermore to induce apoptosis in that cell line. In addition, epicatechin gallate was found to induce G1 arrest in the LoVo cell line (Xiaohua *et al*, 2000).

Anthraquinone-containing extracts of the root of PM have been found to enhance hepatic mitochondrial glutathione antioxidant status in mice, providing protection against hepatotoxicity induced by carbon tetrachloride (CCl 4)-intoxication. The active constituent is believed to be the anthraquinone emodin (Chiu *et al*, 2002). Emodin has been shown to prevent proliferation of the cancer cell lines K562, Calu-1, Vero, HeLa and Wish (Kuo *et al*, 1997). In addition, emodin has been found to

induce apoptosis in cervical cancer cell lines through activation of the intrinsic apoptotic cascade (Srinivas *et al*, 2003).

3.2.4 *Polyporus umbellatus* (PU)

Polyporus umbellatus (PU) (traditionally known as Chuling or Zhuling) is a medicinal mushroom described as having a sweet, bland taste and neutral nature. It is traditionally used to promote urination and leach out damp, and is prescribed in the treatment of difficult urination, bladder cancer and diarrhoea (Dharmananda, 2002).

The literature search revealed an *in vivo* animal study in which the oral administration of PU to mice with implanted tumours was found to inhibit tumour growth and increase the lifespan of those mice by 71.6% compared to a control group. In addition, the combined administration of PU and mitomycin C (a bioreductive alkylating agent used for chemotherapy for gastric and pancreatic cancer) increased the lifespan of tumour-bearing mice by 119.9% (You *et al*, 1994).

Furthermore, two separate studies identified by the literature search have examined the prophylactic effect of PU administration on the postoperative recurrence of bladder cancer in patients in China. The first study looked at the postoperative recurrence of bladder cancer over an average of 70.8 months after surgery, and found tumour recurrence was reduced from 65.1% in the control group to 33.3% in the PU group, with no evidence of any adverse side effects (Yang *et al*, 1994). The second study followed a group of postoperative bladder cancer patients for an average of 7.6 years after their surgery, and found the incidence of cancer recurrence decreased from 64.7% in a control group to 34.9% in the PU group. Furthermore, the prophylactic treatment of PU was found to be more successful than prophylactic treatment using mitomycin C, which had a recurrence rate of 41.7% (Yang *et al*, 1999).

2.4. Discussion

Four CHRs were selected for this investigation on the basis of a literature review and consultation with a TCM practitioner. Those CHRs selected for this investigation were *Oldenlandia diffusa* (OD), Long Dan Xie Gan Wan (LD), *Polygonum multiflorum* (PM) and *Polyporus umbellatus* (PU).

Oldenlandia diffusa (OD) was selected for this study because it is commonly prescribed for the treatment of lung, liver and rectal cancers (Chu, 2003) and yet there was relatively little information regarding its potential mode of action (at the time of this literature search to select CHRs for analysis). A single study had focused on the cytotoxic potential of water extracts of OD, revealing water extracts of OD to be significantly more toxic to lung cancer cells than normal lung epithelial cells (Sadava *et al*, 2002), thus providing some scientific basis for its use in the treatment of lung cancer. While this study suggested OD may exhibit an anticancer action against lung cancers, the cytotoxic action of water extracts of OD against other cancer cell types had not been assessed. In another study, MeOH extracts of OD were found to inhibit the growth of a number of cancer cell types *in vitro*, again suggesting OD may posses a cytotoxic action against cancer cells (Kim *et al*, 1998). However, this study did not investigate the type of cell death induced by MeOH extracts of OD on those cell lines, or compare the toxicity of those extracts to their effect on normal cell types. Furthermore, while oral administration of OD has been found to inhibit the growth of transplanted tumours in mice, suggesting OD may posses a genuine anticancer activity (Yoshida *et al*, 1997), (Wong *et al*, 1996), these studies did not asses the role of direct cytotoxicity to cancer cells in the observed anticancer action of OD. Thus, while OD had been shown to exhibit anticancer *in vitro* and *in vivo*, there was a lack of information regarding the basis for its toxicity, or if its action was cancer cell-specific, therefore it was selected for this investigation.

Long Dan Xie Gan Wan (LD), a commonly used herbal preparation containing 10 separate ingredients, was initially considered for inclusion in this study after it was recommended by a TCM practitioner (Professor Ma). While the activity of LD in its entirety has not been previously investigated (in any biological context), a range of *in vitro* and *in vivo* studies (described above) have revealed some of its herbal

constituents do exhibit a range of direct cytotoxic activities against cancer cells, thus suggesting LD may itself posses some anticancer activity. Therefore LD was selected for this investigation.

Polygonum multiflorum (PM) is a widely used CHR that is commonly prescribed to the elderly for its invigorating effects (Lin *et al*, 2003). While a number of studies have found PM contains compounds that exhibit an anticancer activity when tested in isolation (Xiaohua *et al*, 2000), (Kuo *et al*, 1997), there is relatively little information regarding the anticancer action of PM when used in its entirety, therefore PM was selected for this investigation.

Polyporus umbellatus (PU) is traditionally prescribed for the treatment of bladder cancer (Dharmananda, 2002), and the prophylactic use of PU has been found to significantly reduce the postoperative recurrence of bladder cancer in clinical trials (Yang *et al*, 1994&1999). In addition, *in vivo* studies have shown PU significantly inhibits the growth of transplanted tumours in mice (You *et al*, 1994). Yet despite the reported *in vivo* anticancer potential of PU, the cytotoxic potential of PU against cancer cells has not been previously reported, hence its inclusion in this study.

In summary, OD, LD, PM and PU are commonly used CHRS that have previously been found to elicit an anticancer action in their entirety, or to possess constituents that have been shown to elicit a cytotoxic action against cancer cells. However, the cancer cell-specific cytotoxic action of these CHRs in their entirety have not been investigated in detail. As such, OD, LD PM and PU were selected for this investigation.

Chapter 3. Growth Inhibition of cancer cell lines *in vitro* by selected CHRs

3.1. Introduction

Chinese herbal remedies (CHRs) are recognized as a potential source of novel chemotherapeutic compounds, and as such investigations into their anticancer action are becoming increasingly common.

3.1.1. Cytotoxicity Screens

The first stage in examining the cytotoxic potential of CHRs against cancer cells typically involves creating crude aqueous or polar extracts of these remedies and conducting a preliminary cytotoxicity screen against cancer cells. Aqueous extracts are examined as CHRs are traditionally boiled in water and ingested in the form of a tea, while polar extracts are examined to look for evidence of small, polar compounds within those CHRs that may exert an anticancer action. Cytotoxicity screens may involve the use of an *in vivo* model in which cytotoxicity is assessed using tumour bearing animals, or an *in vitro* model in which the cytotoxic potential of those extracts are tested directly on cancer cell lines (Davila *et al*, 1998). Today, *in vitro* methods are often preferred for cytotoxicity screens, and this is for a number of reasons, which include

- *In vitro* screening can be conducted on a large scale against a range of cancer cell types in a more cost-effective manner than *in vivo* screening
- Mechanisms of toxicity can be studied with greater reproducibility and in greater detail *in vitro*, as *in vivo* models are complicated by structural and functional heterogeneity that do not allow for mechanisms to be clearly defined or reproducibly examined (Davila *et al*, 1998).
- *In vitro* screening is more popular with the general public as it does not involve animal testing

The cytotoxic potential of CHR extracts against cancer cell lines can be assessed *in vitro* by examining one of a number of characteristics associated with toxicity, which include inhibition of cell proliferation (which can be characterized by measuring protein and DNA synthesis using radioactive labelling assays), decreased metabolic activity (which can be assessed using the mitochondrial reduction of tetrazolium

salts into insoluble dye (MTT) assay) and decreased cell viability (which can be determined by measuring cell numbers and using vital dyes such as trypan blue) (NIH, August 2001).

If crude CHR extracts are found to exert a growth inhibitory or toxic action against cancer cells based on these preliminary studies, they are then subject to further investigation, which may involve bioactivity guided-fractionation studies to elucidate the active principles within those crude extracts, or further characterization of the cytotoxic action of those remedies in their entirety (Balunas & Kinghorn, 2005).

3.1.2 Difficulties associated with screening CHRs for cytotoxic potential

There are a number of difficulties associated with examining the cytotoxic potential of CHRs. For example, soil contamination, variability depending on where herbs were grown, at what time they were harvested and which parts of the herb were used may influence the results obtained in a study (Ernst, 2002) (Yuan & Lin, 2002). Furthermore, deliberate adulteration of CHR preparations and the incorrect identification of herbs (Ernst, 2002) (Seki *et al*, 2005) may lead to the publication of incorrect data. As such, these issues must be taken into consideration when designing experiments to examine the cytotoxic potential of CHRs.

3.1.3 The cytotoxic potential of OD, LD, PM and PU against cancer cells

Previous studies, highlighted in the initial literature review (of papers published between 1990-2003), suggested that the CHRs *Oldenlandia diffusa* (OD), Long Dan Xie Gan Wan (LD), *Polygonum multiflorum* (PM) and *Polyporus umbellatus* (PU) could potentially elicit a direct cytotoxic action against cancer cells.

Oldenlandia diffusa (OD) is traditionally prescribed for the treatment of lung, liver and rectal cancers (Chu, 2003), and a previous *in vitro* study had shown water extracts of OD could elicit a cytotoxic action against the H69 lung cancer cell line (Sadava *et al*, 2002). However, the cytotoxic potential of water extracts of OD against other cancer cell types had not been examined. Furthermore, while another study had found that MeOH extracts of OD could exert a cytotoxic action against a range of other cancer cell types, including A549 (lung), SK-OV-3 (ovary), SK-MEL-

2 (skin), XF498 (brain), HCT-15 (colon), SNU-1 (stomach, L1210 (murine leukaemia) and B15-F_0 (murine melanoma) cell lines (Kim *et al*, 1998), it did not characterize the mode of cell death induced by MeOH extracts of OD.

Long Dan Xie Gan Wan (LD) is a CHR preparation made of 10 separate herbs, and a number of these, including *Radix bupleuri* (Cheng *et al*, 2003), *Radix scutellariae* (Ikemoto *et al*, 2000) and *Rhizoma alismatis* (Lee *et al*, 2001), had previously been shown to elicit a cytotoxic action against cancer cells in isolation *in vitro*. However, the anticancer action of this CHR in its entirety had not been examined.

Polygonum multiflorum (PM) had previously been found to reduce tumour incidence in rats subcutaneously injected with B[a]P (Horikawa *et al*, 1996) and to contain the cytotoxic compounds epigallocatechin gallate, epigallocatechin and emodin (Xiaohua *et al*, 2000) (Kuo *et al*, 1997). However, the cytotoxic potential of this CHR in its entirety against cancer cells had not been examined.

Polyporus umbellatus (PU) is traditionally prescribed for the treatment of bladder cancer (Dharmananda, 2002), and had been shown to significantly inhibit the postoperative recurrence of bladder cancer in humans (Yang *et al*, 1994) (Yang *et al*, 1999), and to increase the lifespan of sarcoma 180 tumour-bearing mice (You *et al*, 1994). However, no *in vitro* studies had been conducted to characterize the role of direct cytotoxicity to cancer cells in the observed anticancer action of PU.

Thus, as the initial literature review revealed that the cytotoxic potential of OD, LD, PM and PU had not previously been examined in detail against cancer cells, despite studies suggesting they may possess chemotherapeutic potential, the aim of this study was to conduct a preliminary screen to examine the direct cytotoxic action of OD, LD, PM and PU against a range of cancer cell types.

3.1.4. Devising a strategy with which to examine the mode of cell death induced by OD, LD, PM and PU in cancer cells

Preliminary cytotoxicity screening of CHRs typically begins with the creation of aqueous or polar extracts of those remedies, followed by investigation into their direct cytotoxic potential against cancer cells using one of a number of markers for

toxicity. Thus, for this investigation, water and ethanol extracts of each CHR were made and their cytotoxic potential examined against cancer cells. Water extracts were examined because CHRs are traditionally boiled in water and those remedies ingested in the form of a tea. Ethanol extracts were examined to look for evidence of smaller, polar compounds within those CHRs exerting an anticancer action.

For this study, the direct cytotoxic potential of the selected CHRs against cancer cells was examined using an *in vitro* test system. The advantages of this test system over *in vivo* analysis include the fact that cytotoxic activity can be investigated in isolation without any immune-enhancing activity against cancer cells contributing to observed toxicity, and that experiments can be conducted in a reproducible fashion without *in vivo* structural and functional heterogeneity augmenting results (Davila *et al*, 1998). In order to examine the spectrum and specificity of activity of these CHRs, a range of cancer cell lines were used, and included a leukaemic cell line (HL60), gut associated cancer cells (HT29 and HCT-8), cervical cancer cells (HeLa) and a non-human cancer cell line (CHO).

A range of morphological characteristics can be examined as markers of toxicity, including inhibition of cell proliferation, which can be determined by measuring incorporation of ^{3}H labelled thymidine, decreased metabolic activity, which can be measured using the MTT assay, and a decrease in whole cell numbers, which can be determined using a haemocytometer and a vital dye such as trypan blue. For this study, cytotoxicity was determined by counting viable cell numbers after exposure to the CHRs using the trypan blue exclusion assay. The ^{3}H labelled thymidine incorporation assay was not used because this assay cannot differentiate between growth arrest and cell death, and the MTT assay was not selected because this is a colorimetric assay and the CHR preparations were darkly coloured and would have affected results. Finally, to address a number of the potential difficulties associated with CHR research, which include the correct identification of herbs, deliberate adulteration of CHR preparations and batch variability augmenting results, single batches of each CHR were purchased from a single reputable source and used throughout this investigation.

3.2. Materials and Methods

3.2.1. Preparation of CHRs

Oldenlandia diffusa (OD), Long Dan Xie Gan Wan (LD), *Polygonum multiflorum* (PM) and *Polyporus umbellatus* (PU) were obtained locally from the same TCM practitioner (London, UK). The CHRs OD, PM and PU were obtained in their raw state. To make water extracts (infusions) of OD, PM and PU, 3g of each CHR were crushed and then placed in distilled water (30ml) and boiled for 1 hour under reflux. The resultant solution was centrifuged for 20 minutes at room temperature at 3000rpm and syringed through a 0.45μm sterile filter (Nalgene, Hereford, UK). For ethanol extracts (tinctures), 3g of OD, PM and PU were placed in ethanol (30ml) and boiled under reflux for 1 hour, after which time 20ml was rotary evaporated and the resultant solid re-suspended in distilled water then centrifuged and sterile filtered as described above. The CHR LD was obtained in a pre-processed tablet form and was therefore not boiled, instead 3g were crushed then incubated in water or ethanol (30ml) at 37°C for 1 hour, after which time the water extract was centrifuged and sterile filtered as described above. Ethanol extracts were also prepared as described above.

3.2.2. Cell Culture

HL60 (human promyelotic leukaemia), HT29 (human colon adenocarcinoma), HCT-8 (ileocecal carcinoma), HeLa (human cervix epitheloid carcinoma) and CHO (Chinese hamster ovary) cancer cell lines were obtained from The European Collection of Cell Cultures (ECACC, Sailsbury, UK). The HL60, CHO and HeLa cell lines were grown in RPMI 1640 medium (Gibco, Paisley, UK) containing 10% foetal bovine serum (FBS) (Gibco, Paisley, UK). The HCT-8 cell line was also grown in RPMI 1640 media containing 10% FBS, supplemented with 5mM L-glutamine (Sigma, Poole, UK) and 2mM non-essential amino acids (NEAAs) (Gibco, Paisley, UK). The HT29 cell line was grown in minimum essential medium (MEM) (Gibco, Paisley, UK) containing 10% FBS, 2mM L-glutamine and 2mM NEAAs. All cell lines were incubated at 37°C in an atmosphere containing 5% CO_2. Cell numbers were determined using the trypan blue exclusion assay (Gibco, Paisley, UK). Cell lines were passaged when concentrations reached 10^6 cells/ml

and diluted to 10^5 cells/ml, thereby maintaining a stock solution containing cells at concentrations ranging from 10^5 to 10^6 cells/ml.

3.2.3. Growth Inhibition Study

In order to determine the cytotoxic potential of the selected CHRs against the cancer cell lines selected for this study in a reproducible and consistent manner, the National Institutes of Health (NIH, August 2001) recommendations for *in vitro* cytotoxicity test protocols were incorporated into this experiment. These recommendations include:

1. Never use cells after thawing from frozen stock (allow 1-2 pasages)
2. Use cell lines that divide rapidly with doubling times of less than 30 hours under standard culture conditions
3. Initial seeding should be done at a density that allows rapid growth through the exposure period
4. Use only cells in the exponential stages of growth
5. Chemical exposure period should be at least one cell cycle, i.e. 24-72 hours
6. Complete a concentration response using a progression factor that yields graded effects between no effect and total toxicity

For the growth inhibition study, three separate solutions of both the water and ethanol extracts of each CHR were made, and the growth inhibitory actions of each of these extracts examined at a final concentration of 1:10, 1:100 and 1:1000 on each of the cell lines over a period of 72 hours. Growth curves were carried out in 24-multiwell plates. Three identical plates were made for each of the experiments at 0hrs, and then one of these was counted and discarded after 24, 48 and 72 hours. Cell viability was measured by determining whole cell numbers using a haemocytometer, and viable cells were excluded by using the vital dye trypan blue (Gibco, Paisley, UK).

3.2.4. Data presentation and statistical analysis

Data for the growth inhibition study are expressed as mean ± standard deviation from the mean. The mean values of each concentration at each time point were compared to control values using ANOVA.

3.3. Results

3.3.1. *Oldenlandia diffusa* (OD)

The growth inhibition study showed that the water extract of OD generally exerted a toxic effect on all cell lines at 1:10 (Figure 3.3.1). It exerted its greatest toxic effect at 1:10 on the HL60, HeLa and CHO cell lines, killing all cells within the first 24 hours of exposure. At 1:10 it also exerted a statistically significant (p=0.05) growth inhibitory effect on the HT29 cell line at all time points, and after 48 and 72 hours on the HCT-8 cell line. At 1:100 and 1:1000, the water extract of OD caused statistically significant (p=0.05) growth inhibition of the HL60 cell line at all time points. The cell line least sensitive to the effects of the water extract of OD appeared to be the HT29 cell line, with no statistically significant growth inhibitory effect at 1:100 or 1:1000.

The ethanol extract of OD had a far less pronounced growth inhibitory effect on each cell line compared to the water extract of OD (Figure 3.3.2). At 1:10, the ethanol extract exerted a significant (p=0.05) growth inhibitory effect when compared to control cell growth on the HL60 cell lines after 48 and 72 hours, and at 48 hours on the HT29 and CHO cell lines. However, a 1:10 ethanol extract of OD had no effect on the HeLa or HCT-8 cell lines. The ethanol extract of OD had no significant growth inhibitory effect at 1:100 or 1:1000 on any cell line, with the exception of the HeLa cell line after 72 hours at 1:1000.

3.3.2. Long Dan Xie Gan Wan (LD)

The growth inhibition study showed the water extract of LD generally exerted a toxic effect at 1:10 on all cell lines after 48 and 72 hours of incubation (Figure 3.3.3). The water extract of LD exerted its greatest toxic effect on the CHO and HL60 cell lines, killing all CHO's at 1:10 within the first 24 hours of exposure, and all HL60's after 72 hours of exposure. At 1:10 the water extract of LD also exerted a significant (p=0.05) growth inhibitory effect on the HCT-8 and HeLa cell lines after 48 and 72 hours of exposure, and in the HT29 cell line after 72 hours. The effect of the water extract of LD at 1:100 and 1:1000 was greatly reduced when compared the effects of LD at 1:10, only causing significant growth inhibition after 72 hours in the HL60 and CHO cell lines (at both 1:100 and 1:1000).

The toxic effect of the ethanol extract of LD was far less than that of the water extract on the CHO and HL60 cell lines at 1:10: viable cells were still present after 72 hours of exposure. However, significant (p=0.05) growth inhibition was observed in all cell lines exposed to LD at 1:10 for 72 hours (Figure 3.3.4).

3.3.3. *Polygonum multiflorum* (PM)

The growth inhibition study showed the water extract of PM generally exerted a toxic effect at high concentrations, and a growth inhibitory effect at lower concentrations on all cell lines studied (Figure 3.3.5). At 1:10, the water extract of PM killed all HL60, HCT-8 and CHO cells within the first 24 hours of exposure. Significant toxicity (p=0.05) was also observed on the HT29 and HeLa cell lines after exposure to a 1:10 preparation of PM for 24, 48 and 72 hours. Significant growth inhibition (p=0.05) was also observed at 1:100 and 1:1000 at various time points on each cell line.

In contrast to the water extract of PM, the ethanol extract exhibited very little statistically significant toxic or growth inhibitory activity on any of the cells lines used in this investigation (Figure 3.3.6). No significant growth inhibition was observed at 1:100 or 1:1000 at any time point on any cell line. At 1:10, growth inhibition was observed on the HL60 and CHO cell lines after 72 hours, and after 48 hours on the HeLa cell line.

3.3.4. *Polyporus umbellatus* (PU)

The growth inhibition study showed the water extract of PU to exert a significant (p=0.05) growth inhibitory effect on the HL60 cell line after 72 hours of exposure at 1:10, but no effect on any other cell line at any concentration (Figure 3.3.7). The ethanol extract of PU had no effect on the CHO, HeLa or HCT-8 cell lines at any concentration or time point (Figure 3.3.8). Significant growth inhibition (p=0.05) was observed on the HL60 cell line after 48 hours at 1:10 1:100 and 1:1000, and after 72 hours at 1:10. An inhibitory effect was also observed on the HeLa cell line at 1:10 and 1:1000 after 48 hours.

Figure 3.3.1 Growth inhibitory effects of water extracts of OD

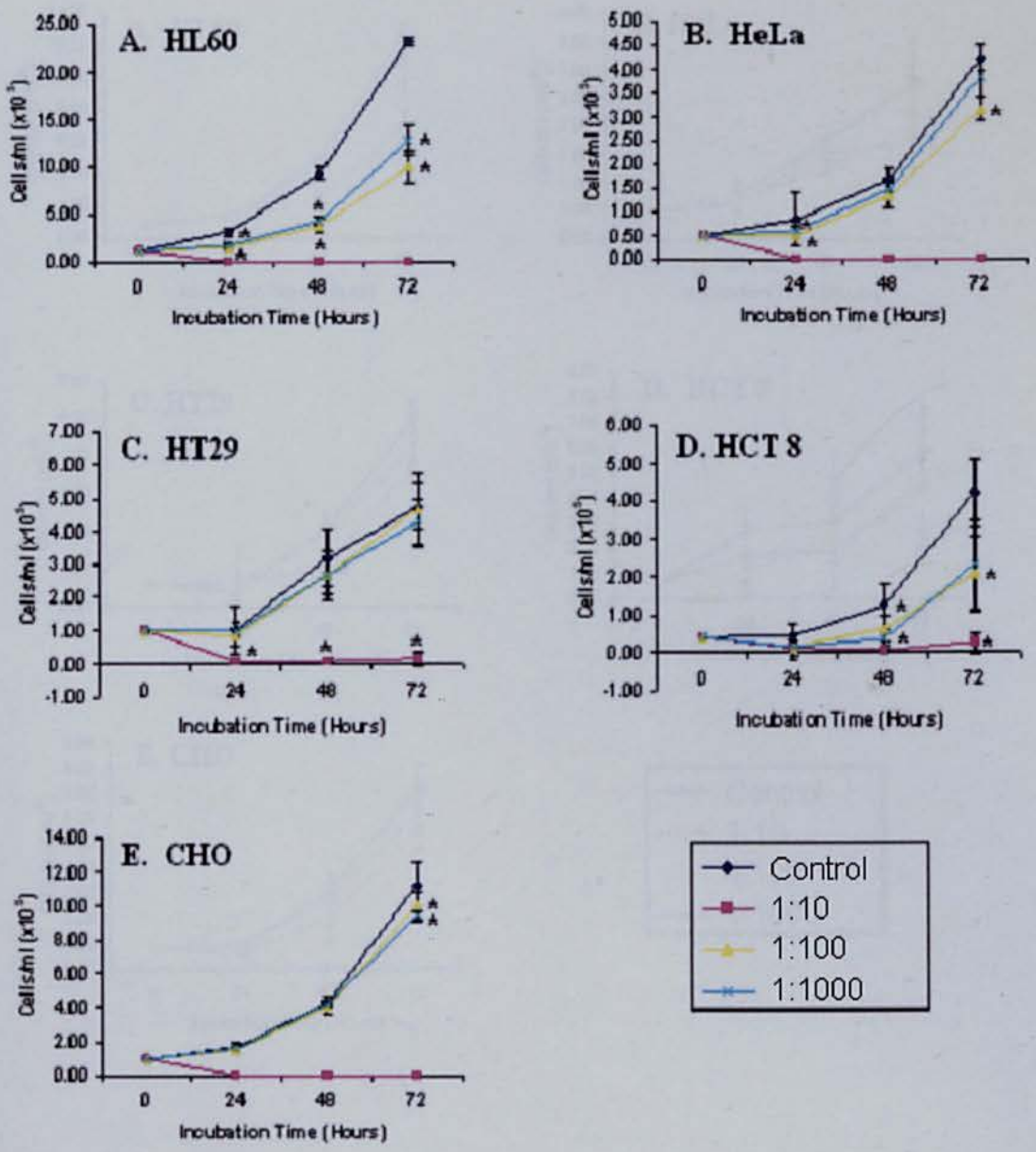


Figure 3.3.1 Incubation of water extracts of OD at 1:10, 1:100 and 1:1000 on the HL60, HeLa, HT29, HCT-8 and CHO cell lines for 24, 48 and 72 hours compared to normal (control) cell growth, ± standard deviation from the mean, *denotes statistical significance (p=0.05).

Figure 3.3.2 Growth inhibitory effects of ethanol extracts of OD

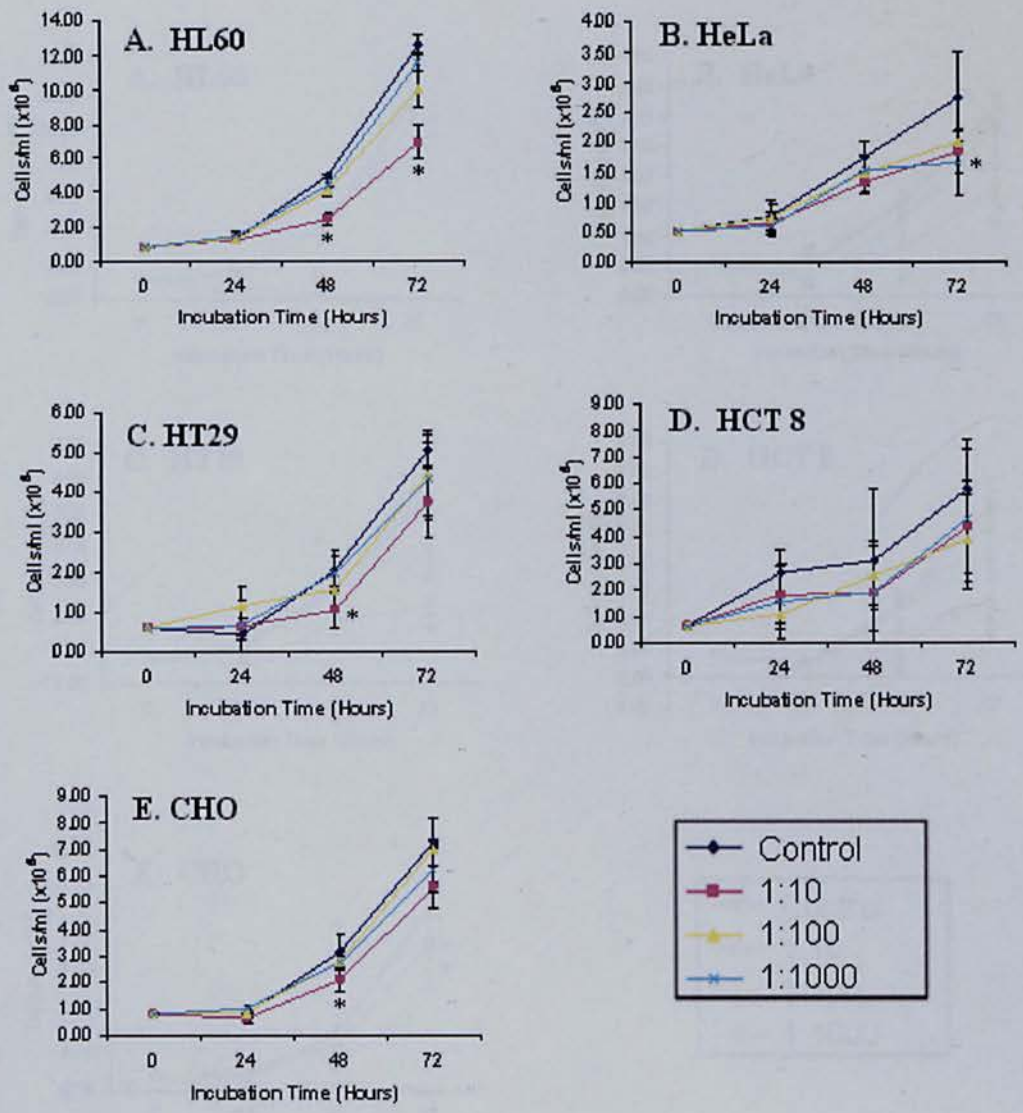


Figure 3.3.2 Incubation of ethanol extracts of OD at 1:10, 1:100 and 1:1000 on the HL60, HeLa, HT29, HCT-8 and CHO cell lines for 24, 48 and 72 hours compared to normal (control) cell growth, ± standard deviation from the mean. *denotes statistical significance (p=0.05).

Figure 3.3.3 Growth inhibitory effects of water extracts of LD

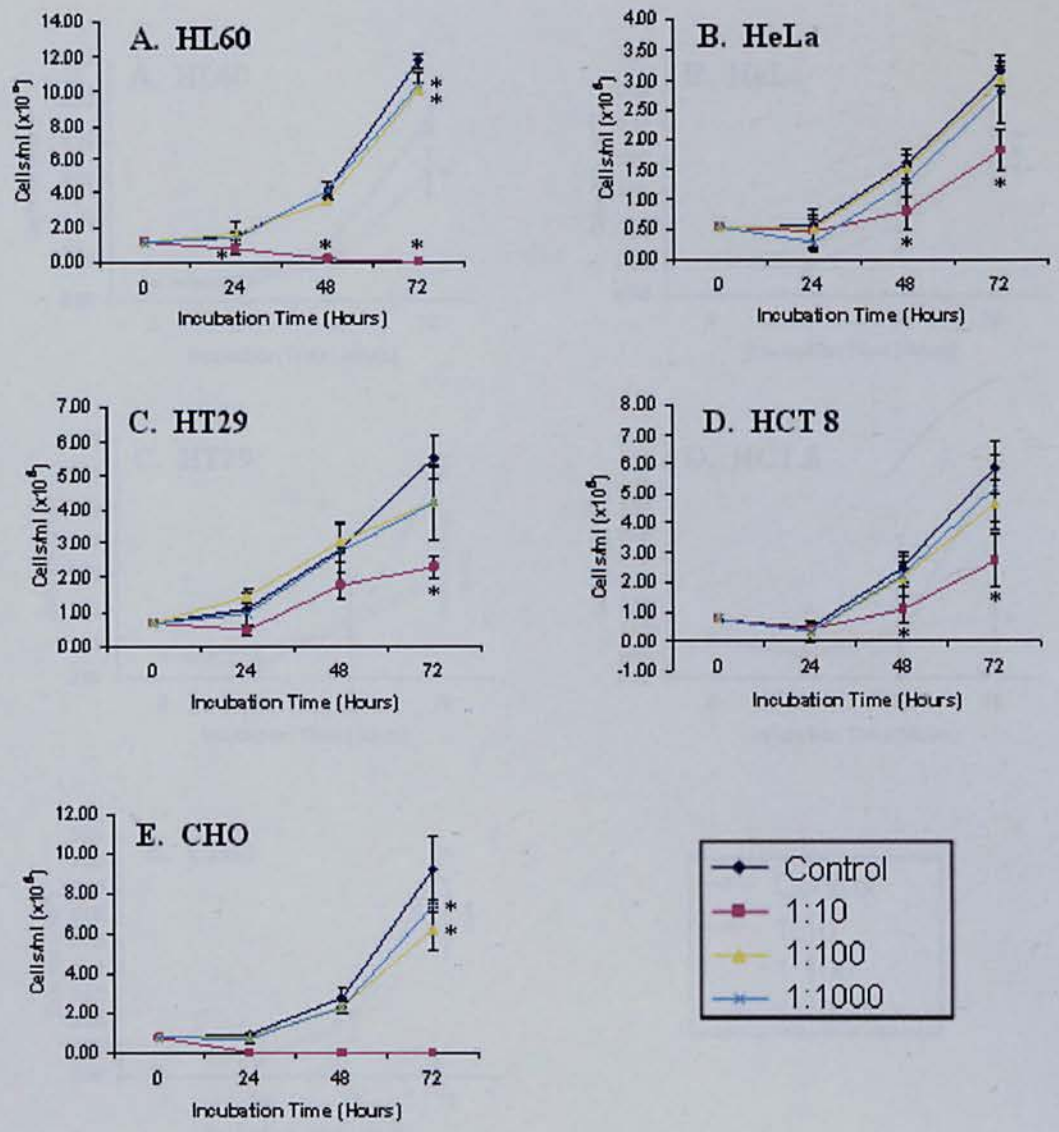


Figure 3.3.3 Incubation of water extracts of LD at 1:10, 1:100 and 1:1000 on the HL60, HeLa, HT29, HCT-8 and CHO cell lines for 24, 48 and 72 hours compared to normal (control) cell growth, ± standard deviation from the mean, *denotes statistical significance (p=0.05).

Figure 3.3.4 Growth inhibitory effects of ethanol extracts of LD

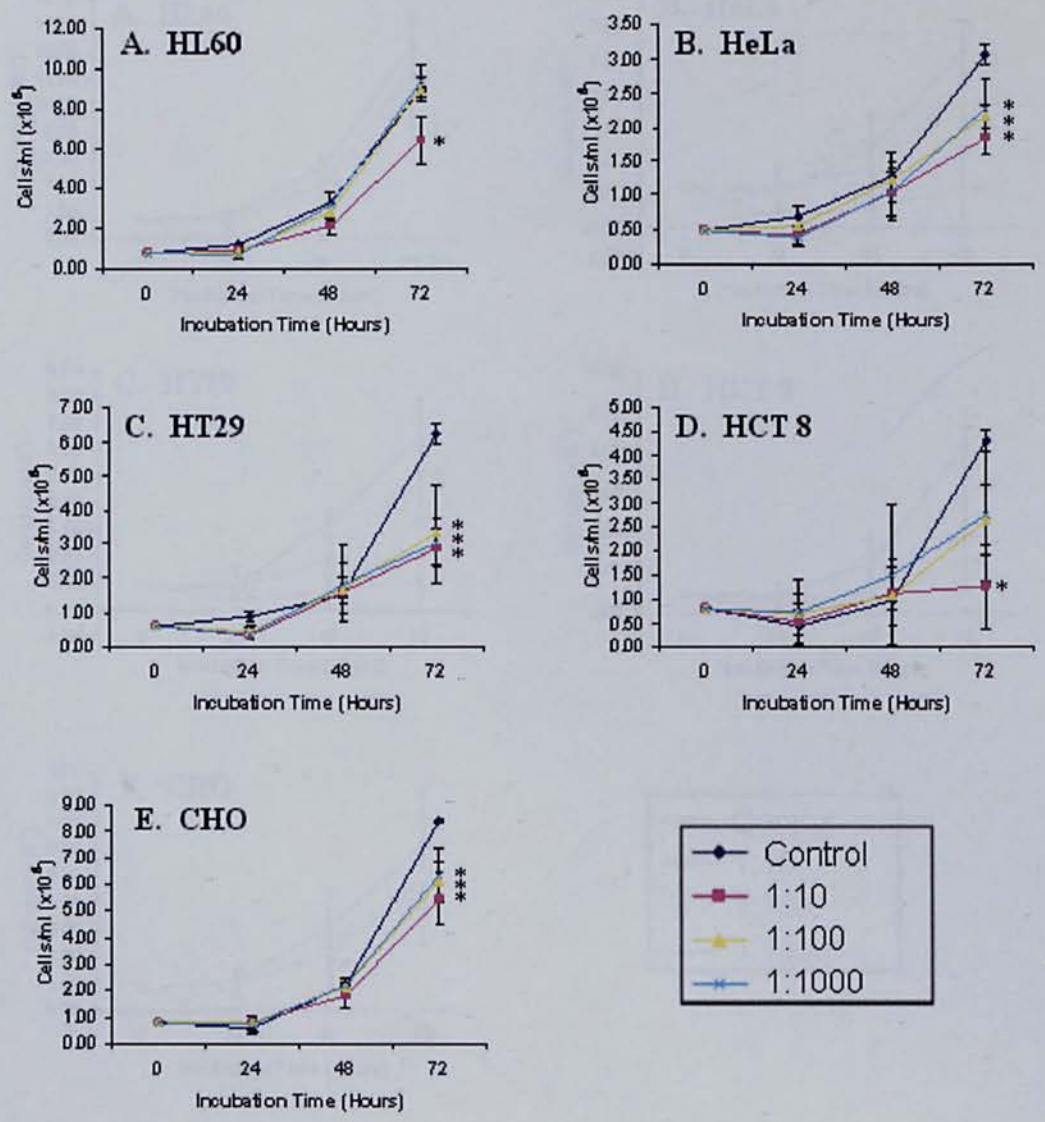


Figure 3.3.4 Incubation of ethanol extracts of LD at 1:10, 1:100 and 1:1000 on the HL60, HeLa, HT29, HCT-8 and CHO cell lines for 24, 48 and 72 hours compared to normal (control) cell growth, ± standard deviation from the mean, *denotes statistical significance (p=0.05).

Figure 3.3.5 Growth inhibitory effects of water extracts of PM

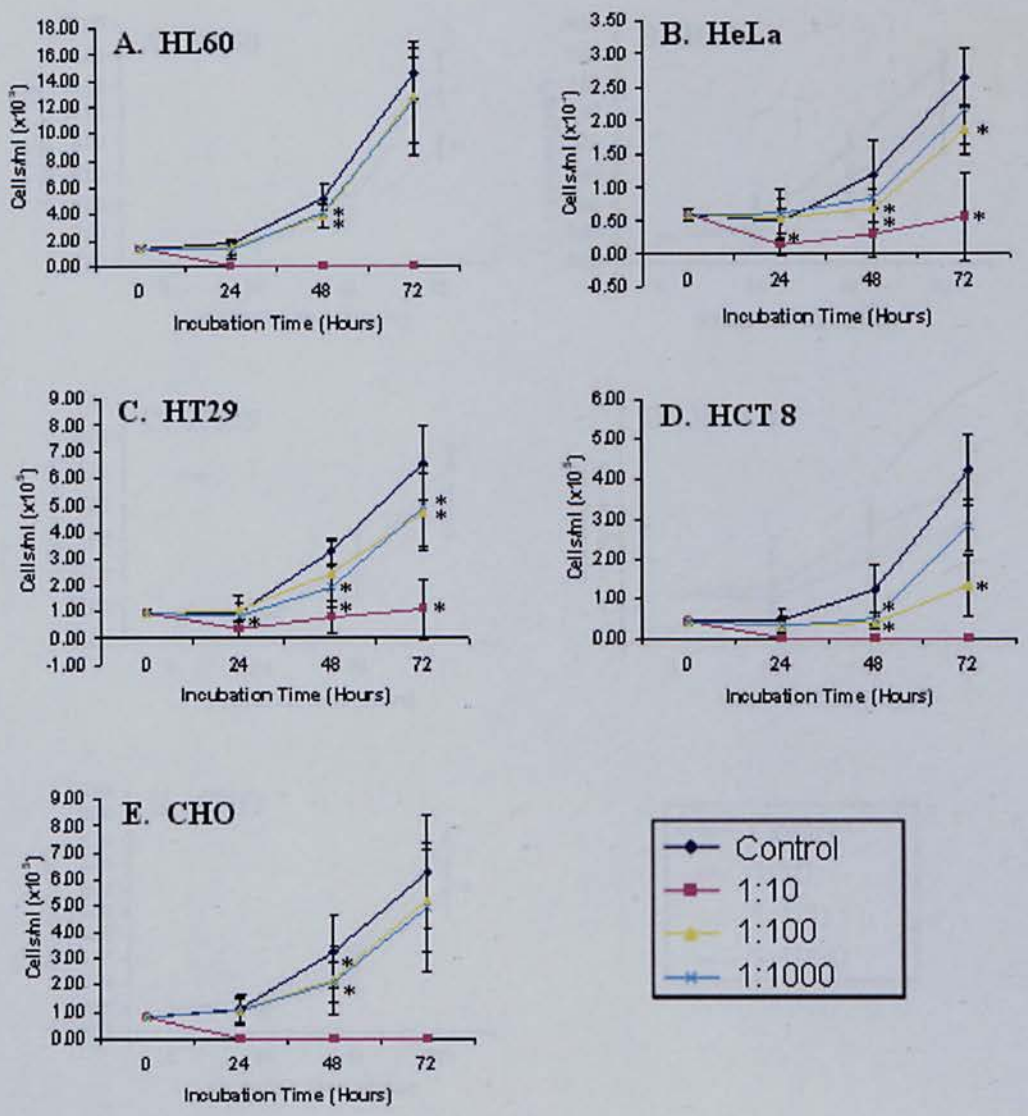


Figure 3.3.5 Incubation of water extracts of PM at 1:10, 1:100 and 1:1000 on the HL60, HeLa, HT29, HCT-8 and CHO cell lines for 24, 48 and 72 hours compared to normal (control) cell growth, ± standard deviation from the mean, *denotes statistical significance (p=0.05).

Figure 3.3.6 Growth inhibitory effects of ethanol extracts of PM

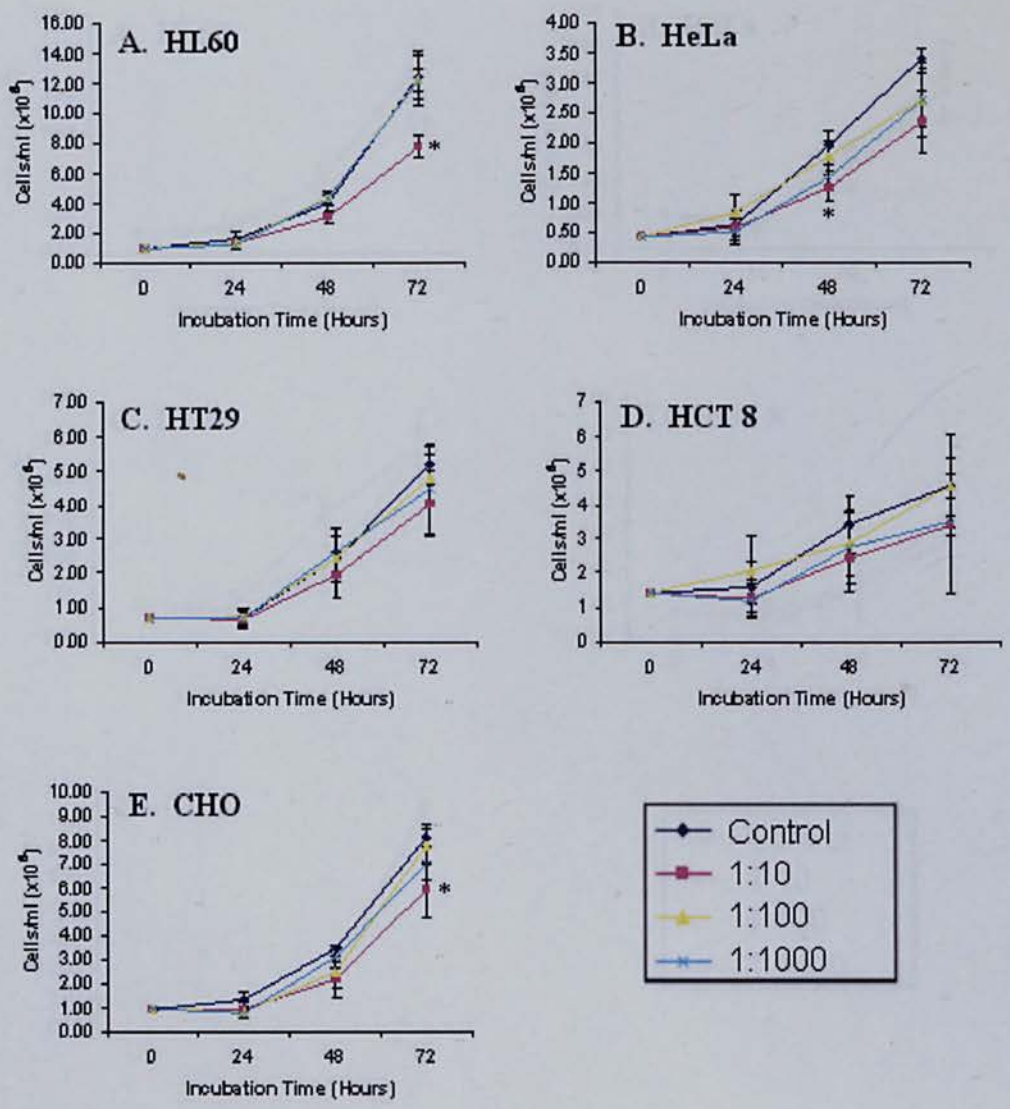


Figure 3.3.6 Incubation of ethanol extracts of PM at 1:10, 1:100 and 1:1000 on the HL60, HeLa, HT29, HCT-8 and CHO cell lines for 24, 48 and 72 hours compared to normal (control) cell growth, ± standard deviation from the mean, *denotes statistical significance (p=0.05).

Figure 3.3.7 Growth inhibitory effects of water extracts of PU

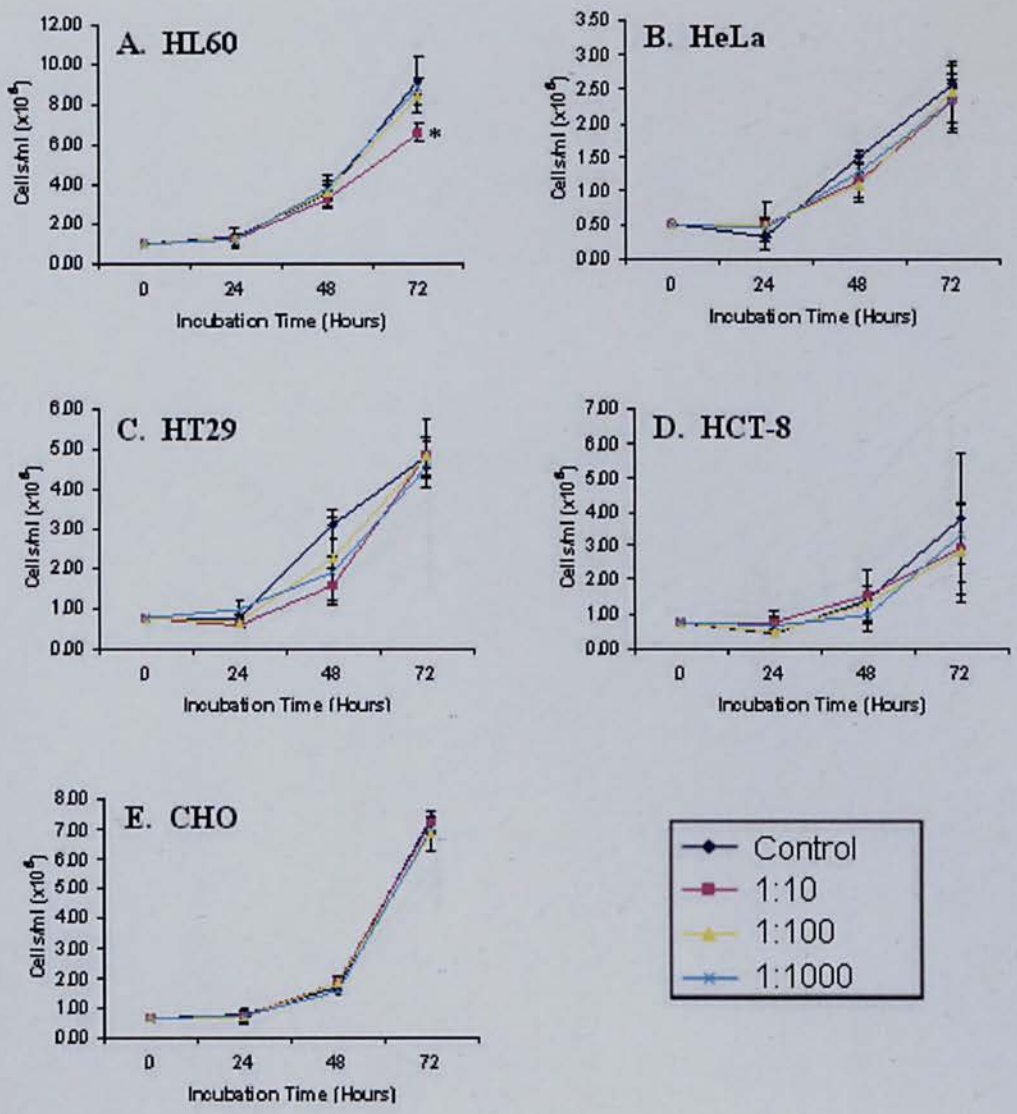


Figure 3.3.7 Incubation of water extracts of PU at 1:10, 1:100 and 1:1000 on the HL60, HeLa, HT29, HCT-8 and CHO cell lines for 24, 48 and 72 hours compared to normal (control) cell growth, ± standard deviation from the mean, *denotes statistical significance (p=0.05).

Figure 3.3.8 Growth inhibitory effects of ethanol extracts of PU

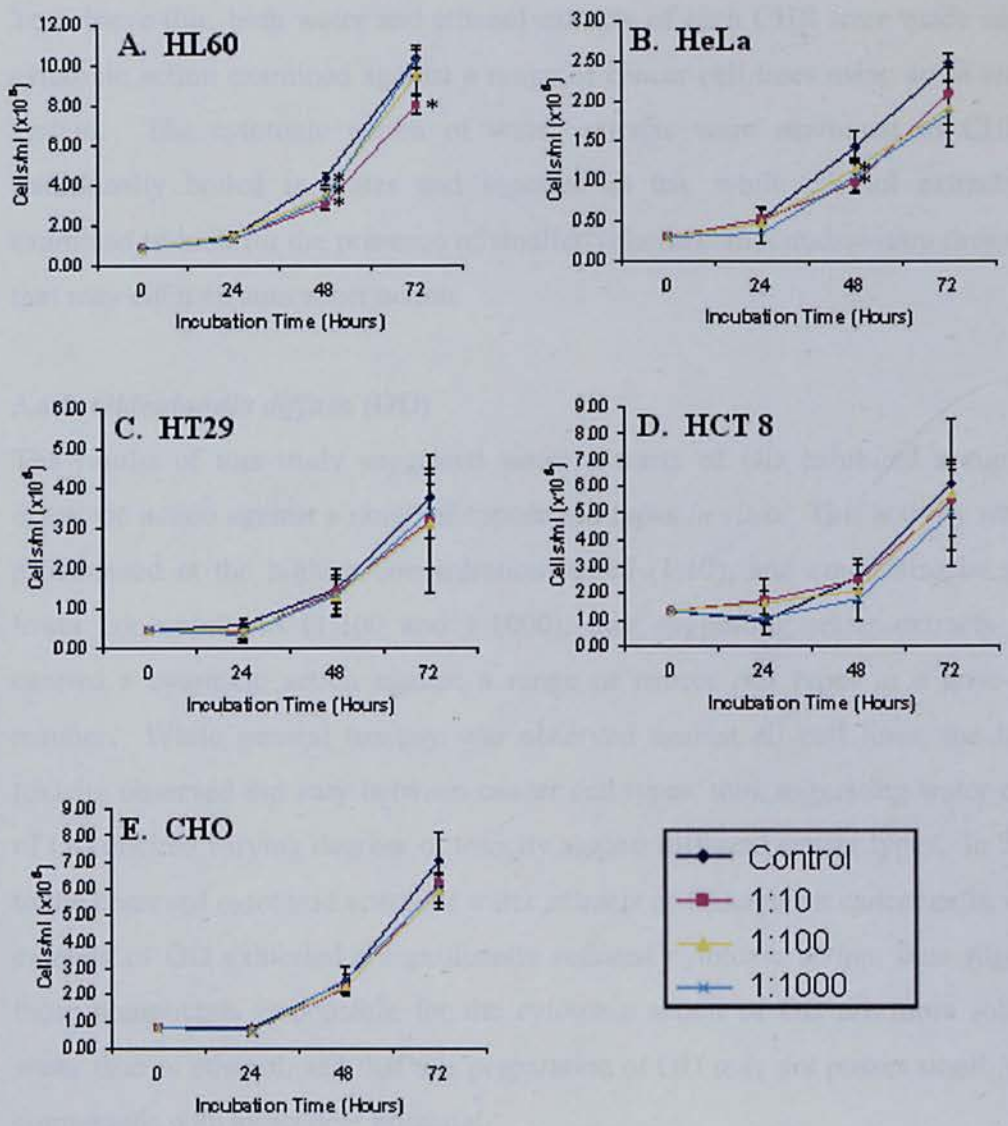


Figure 3.3.8 Incubation of ethanol extracts of PU at 1:10, 1:100 and 1:1000 on the HL60, HeLa, HT29, HCT-8 and CHO cell lines for 24, 48 and 72 hours compared to normal (control) cell growth, ± standard deviation from the mean, *denotes statistical significance (p=0.05).

3.4. Discussion

The aim of this investigation was to perform a preliminary study to determine the cytotoxic potential of the CHRs OD, LD, PM and PU against a range of cancer cells. To achieve this, both water and ethanol extracts of each CHR were made and their cytotoxic action examined against a range of cancer cell lines using an *in vitro* test system. The cytotoxic action of water extracts were examined as CHRs are traditionally boiled in water and ingested as tea, while ethanol extracts were examined to look for the presence of smaller volatile compounds within those CHRs that may elicit an anticancer action.

3.4.1. *Oldenlandia diffusa* (OD)

The results of this study suggested water extracts of OD exhibited a significant cytotoxic action against a range of cancer cell types *in vitro*. This activity was most pronounced at the highest concentration tested (1:10), and could also be seen at lower concentrations (1:100 and 1:1000), thus suggesting water extracts of OD exerted a cytotoxic action against a range of cancer cell types in a dose-related manner. While general toxicity was observed against all cell lines, the level of toxicity observed did vary between cancer cell types, thus suggesting water extracts of OD elicited varying degrees of toxicity against different cancer types. In contrast to the observed cytotoxic action of water extracts of OD against cancer cells, ethanol extracts of OD exhibited a significantly reduced cytotoxic action, thus suggesting those compounds responsible for the cytotoxic action of OD are more soluble in water than in ethanol, and that this preparation of OD may not posses small, volatile compounds with anticancer potential.

The cytotoxic action of water extracts of OD observed in this study are consistent with the studies highlighted in the initial literature search (of papers published between 1990-2003) that showed extracts of OD could exhibit anticancer activity *in vitro* (Sadava *et al*, 2002) (Kim *et al*, 1998), and are also consistent with studies published after 2003 which have shown extracts of OD elicit a cytotoxic action against a range of cancer cell types *in vitro*, including breast cancer cell lines (MCF-7 and MDA-MB-453), lung epithelial carcinoma (A5-49), human cervix carcinoma (C-33A), murine melanoma (B16-F10), prostate cancer cell lines (Tsu-Pt1, Du-145 and Ln-Cap) (Gupta *et al*, 2004), and the HL60 cell line (Yadav & Lee, 2006).

3.4.2. Long Dan Xie Gan Wan (LD)

The results of this investigation suggested that water extracts of LD exerted a significant growth inhibitory/toxic effect against all the cell lines studied here at 1:10, and a limited growth inhibitory effect on each cell line at lower concentrations. (1:100 and 1:1000) However, the toxicity of LD did appear to vary between cancer cell types, suggesting LD may exhibit more toxicity to some cancer cell types than others. The growth inhibitory effect of the ethanol extract of LD was significantly reduced when compared to the effect of the water extract, suggesting those compounds in LD that exert an anticancer action are more soluble in water than in ethanol, and that LD does not contain smaller, volatile compounds with anticancer potential.

Long Dan Xie Gan Wan (LD) is not commonly used in the treatment and management of cancer, and the anticancer activity of LD has not been investigated. However, the literature review of scientific articles published between 1990-2003 revealed some of its constituents (*Radix bupleuri*, *Radix scutellariae* and *Rhizoma alismatis*) had been shown to express anticancer activity (Cheng *et al*, 2003), (Kok *et al*, 1995), (Ikemoto *et al*, 2000), (Chan *et al*, 2000), (Lee *et al*, 2001). As such, it is possible that the action of LD observed here may have been related to the combined actions of these CHRs.

Furthermore, subsequent studies have further examined the anticancer potential of the herbal constituents of LD, and these may have also contributed to the observed cytotoxic action of LD seen here: a triterpene derivative, saikosaponin D has been isolated from *Radix bupleuri* and has been found to inhibit the growth of A549 lung cancer cells dose dependently *in vitro*, and to induce apoptosis (Hsu *et al*, 2004). Furthermore, acetone extracts of *Radix bupleuri* have been found to induce cell cycle arrest at the G2/M checkpoint in A549 cells *in vitro*, and daily i.p. administration of *Radix bupleuri* to mice bearing A549 cells resulted in growth suppression and regression of the cancer cells with no effect on mouse body weight. It is believed saikosaponins were not responsible for these actions as saikosaponins are non-polar compounds and would not be present in the acetone extract of *Radix bupleuri* (Cheng *et al*, 2005). In addition, baicalein, also isolated from *Radix bupleuri* has

been shown to inhibit the growth of human lung carcinoma CH27 cells through arrest in S phase of the cell cycle and through induction of apoptosis (Lee *et al*, 2005).

The growth inhibitory effects of 11 flavonoids isolated from *Radix scutellariae* on the HL60 cell line revealed baicalin, wogonin, 2',3',5,7-tetrahydroxy flavone and viscidulin III all exhibited a strong growth inhibitory effect, while 2',5,6',7-tetrahydroxy flavone, 2',5,6',7-tetrahydroxy flavanone and scullcapflavone II exhibited weaker inhibitory effects (Sonoda *et al*, 2004).

The anticancer action of one of the major components found within *Fructvs gardeniae*, the glycoside geniposide, has also been studied. As the isolation of glycosides is difficult due to their high polarity, the anticancer activity of the acetylated product of geniposide ($(Ac)_5GP$) has been examined (Peng *et al*, 2004), and has been found to be cytotoxic to rat C6 glioma cells *in vitro*, inducing apoptosis and cell cycle arrest.

3.4.3. *Polygonum multiflorum* (PM)

In this study, water extracts of PM were found to be significantly toxic to a range of cancer cell lines at 1:10, with a reduced yet still significant effect at 1:100 and 1:1000 on different cell lines at various time points, suggesting PM exerts a dose and time dependent growth inhibitory/toxic effect on a range of cancer cell types *in vitro*. Again, this effect was significantly less for ethanol extracts of PM, suggesting the cytotoxic compounds in PM are water and not ethanol soluble.

The cytotoxic action of PM against cancer cells observed in this study may be related to the actions of those cytotoxic compounds highlighted as constituents of PM in the initial literature review, which include epigallocatechin gallate, epigallocatechin and emodin (Xiaohua *et al*, 2000) (Kuo *et al*, 1997). Furthermore, a subsequent study has found that catechin, another constituent of PM, exhibits moderate cytotoxic activity to HSC-2 human carcinoma fibroblasts while being less toxic to normal HGF-2 fibroblasts (Babich *et al*, 2005). Thus, this compound may also play a role in the cytotoxic action of PM.

3.4.4. *Polyporus umbellatus* (PU)

In this study, water extracts of PU were found to exert very little cytotoxic effect on any cell line at any concentration. Ethanol extracts also exhibited very little effect at any concentration. Thus, these results suggest that neither water nor ethanol extracts of PU are directly toxic to the cancer cell lines.

Polyporus umbellatus (PU) was selected for this investigation because it is traditionally prescribed for the treatment of bladder cancer (Dharmananda, 2002), had been found to significantly inhibit the postoperative recurrence of bladder cancer in patients (Yang *et al*, 1994), (Yang *et al*, 1997), and to inhibit the growth of sarcoma 180 fibroblast tumour cells transplanted into mice (You *et al*, 1994), and yet the direct cytotoxic action of PU against cancer cells had not previously been reported.

Based on the results of this study, the previously reported anticancer action of PU *in vivo* may not have been related to PU exerting a directly cytotoxic action against cancer cells. Instead, its anticancer action may have been related to it exerting immune-enhancing activity against cancer cells, a theory which my be supported by previous *in vivo* histopathological studies that have shown PU to enhance lymphocyte infiltration into implanted sarcoma 180 tumours in mice (You *et al*, 1994).

Although the specific immune-enhancing action of PU against cancer cells has not previously been reported, a number of studies have focused upon the immunomodulatory potential of CHRs against cancer cells, and have found CHRs can enhance immune function against cancer cells through a variety of mechanisms. For example, the medicinal mushroom *Phellinus linteus*, traditionally used in North-east Asia for the treatment of various forms of cancer, contains acidic polysaccharides that can stimulate the tumoricidal activities of peritoneal macrophages and also activate T-cell mediated immunity against malignant cells *in vivo* (Kim *et al*, 2004). Other examples of CHRs exerting immune-enhancing activity against tumours include *Cordyceps Sinensis*, which is able to increase MHC expression in tumour cells (Chiu *et al,* 1998), *Radix Bupleuri*, which can induce the

cytotoxic activity of macrophages, NK and LAK cells (Kok *et al,* 1995), and the herbs *Astragalus membranaceus* and *Ligustrum lucidum*, which can reverse the inhibitory effects of some tumour cells on macrophage function (Rittenhouse *et al,* 1991). Thus, it is possible the anticancer action of PU observed in *in vivo* studies may be related to the augmentation of immune function against cancer cells.

3.4.5. Conclusions

Overall, the results of this preliminary study suggest that OD, LD and PM contain water soluble compounds that generally elicit a dose-related toxic action against a range of cancer cell types *in vitro*, and that there are no small volatile compounds within these CHRs that elicit a significant anticancer action.

The cytotoxic action of water extracts of OD observed in this study is consistent with previous studies that have examined the cytotoxic action of OD on a range of other cancer cell types. Thus, this study further suggests that OD elicits a cytotoxic action against a wide variety of cancer cell types, and that its anticancer activity may not be limited to the cancers for which it is traditionally prescribed. The cytotoxic potential of LD and PM against cancer cells have not previously been examined, therefore this is the first study to suggest that LD and PM may exhibit a cytotoxic action against cancer cells in their entirety *in vitro*. However, the observed cytotoxicity of LD and PM are consistent with previous studies that suggest the constituents of these CHRs may possess an *in vitro* anticancer action.

While these studies suggest that water extracts of OD, LD and PM elicit a dose-related cytotoxic action against cancer cells *in vitro*, they do not provide information regarding the mode of cell death induced by those CHRs (these results cannot be used to differentiate between apoptotic induction or necrotic cell death), or provide information regarding the apparent growth inhibitory action of these CHRs at lower concentrations (these results cannot be used to differentiate between growth arrest or lower levels of cell death). Furthermore, the results of this preliminary study do not provide information regarding whether the cytotoxic action of these CHRs are related to the action of individual compounds within those remedies, or the actions of those CHRs in their entirety.

Finally, the results of this study suggest that neither water nor ethanol extracts of PU exhibit a significant cytotoxic action against cancer cells *in vitro*. This may suggest that the previously reported anticancer action of PU observed *in vivo* may be related to some form of immune-enhancing activity against cancer cells and not direct cytotoxicity, however an investigation into the immune-enhancing potential of PU would be required to address this possibility.

Chapter 4. Characterization of the mode of cell death induced by water extracts of *Oldenlandia diffusa*, Long Dan Xie Gan Wan and *Polygonum multiflorum*

4.1. Introduction

In elucidating the mode of action of cytotoxic test compounds against cancer cells using an *in vitro* test system, growth inhibition data can only give an indication that such compounds are toxic to the cell lines used in that investigation, and cannot be used to determine the mode of cell death induced by those compounds, which is essential in elucidating and therefore understanding their mode of biological action. Thus, although the results of the growth inhibition study revealed water extracts of OD, LD and PM exhibited a dose-related toxic action against a variety of cancer cell types *in vitro*, including a leukaemic cell line (HL60), gut associated cancer cells (HT29 and HCT-8), cervical cancer cells (HeLa) and a non-human cancer cell line (CHO), these data did not provide information regarding the mode of cell death induced by those CHRs.

4.1.1. Apoptosis versus necrosis

There are two distinct forms of cell death: apoptosis and necrosis. Apoptosis, also known as programmed cell death, is an active process that facilitates the removal of unwanted or damaged cells without eliciting an immune response or damaging surrounding cells and tissues. Apoptosis can be triggered through external signalling (including the withdrawal of pro-survival signals or the receipt of pro-apoptotic signals), or internally through the detection of cellular damage or viral infection. During apoptosis cells exhibit a range of unique morphological characteristics, which include loss of membrane asymmetry and attachment, condensation of cytoplasm and the nucleus, internucleosomal cleavage of DNA, and ultimately formation of apoptotic bodies which can be eliminated by phagocytic cells and surrounding tissues without eliciting a localized inflammatory immune response (Turner *et al*, 2000).

In contrast to apoptosis, necrosis is a passive, uncontrolled form of cell death caused by gross cellular injury. Necrosis is characterized by cell swelling and mitochondrial damage which leads to a rapid depletion of energy levels, the

breakdown of homeostatic control, cell membrane lysis and the release of cytoplasmic contents which triggers local inflammation (Turner *et al*, 2000).

4.1.2. Examining the mode of cell death induced by OD, LD and PM

While the results of the growth inhibition study showed water extracts of OD, LD and PM were cytotoxic to a range of cancer cell types, they did not address the issue of whether their cytotoxic action resulted in the induction of apoptosis or necrosis in those cell lines, and this information is essential in understanding their mode of biological action.

The mode of cell death induced by water extracts of OD, LD and PM in their entirety against a range of cancer cell types had not previously been reported at the time of this investigation. However, a previous study highlighted in the initial literature review (of papers published between 1990-2003) had focused on the apoptosis inducing activity of water extracts of OD against lung cancer cells: in a study by Sadava *et al* (2002), *Oldenlandia diffusa* (OD), which is traditionally prescribed for the treatment of lung, liver and rectal cancers, was found to induce apoptosis in both drug-sensitive (H69) and multi-drug resistant (H69VP) small cell lung cancer cell lines *in vitro*. However, as this study only focused on determining the mode of cell death induced by OD in lung cancer cells, it did not address the question of whether the apoptotic action of OD was specific to the cancers for which it is traditionally prescribed, or whether OD induces apoptosis in a range of cancer cell types.

In addition, the initial literature review also revealed studies that had focused on the mode of cell death induced by the cytotoxic constituents of LD in isolation: acetone extracts of *Radix bupleuri* (one of the constituents of LD) had been found to induce apoptosis in the A549 human lung cancer cell line (Chang *et al*, 2003), and baicalin, a compound found in *Radix scutellariae* (another LD constituent), had been shown to induce apoptosis in a range of prostate cancer cell lines (Chan *et al*, 2000), thus suggesting LD may contain pro-apoptotic compounds. However, the mode of cell death induced in cancer cells by LD in its entirety had not been reported.

The literature review also revealed that epigallocatechin gallate, epigallocatechin and emodin, which are constituents of PM, had all been shown to induce apoptosis in various cancer cell lines when examined in isolation (Xiaohua *et al*, 2000) (Srinivas *et al*, 2003). However, while these studies suggest PM contains compounds that induce apoptosis in isolation in a range of cancer cell lines, the mode of cell death induced by PM in its entirety had not previously been examined.

Thus, in order to further characterize the cytotoxic action of water extracts of OD, LD and PM against cancer cell lines *in vitro*, the aim of this study was to devise and implement a strategy with which to examine the mode of cell death induced by water extracts of OD, LD and PM in cancer cells.

4.1.3. Devising a strategy with which to examine the mode of cell death induced by OD, LD and PM

A range of experimental techniques can be used to examine the morphological characteristics unique to apoptotic cell death, and as such can be used to differentiate between apoptotic induction and necrotic cell death in populations of cells exposed to cytotoxic test compounds. These techniques include light microscopy, which can be used to examine membrane blebbing and nuclear fragmentation (characteristics unique to apoptosis), transmission electron microscopy (TEM), which can be used to study apoptotic characteristics at the sub-cellular level, and flow cytometry (or fluorescence activated cell sorting (FACS)), which can be used to examine a range of morphological characteristics associated with apoptosis, including genomic fragmentation and loss of membrane asymmetry (Heerde *et al*, 2000). The most commonly used of these assays are the Annexin V, propidium iodide (PI) staining and TUNEL (TdT-mediated dUTP Nick-End Labelling) assays, and these are often used in combination to confirm apoptotic induction in cell populations exposed to cytotoxic test compounds (Tuschl & Schwab, 2004).

The Annexin V assay examines one of the early morphological characteristics unique to apoptotic cell death, which is the loss of membrane asymmetry before loss of membrane integrity. In the Annexin V assay, the translocation of phosphatidylserine (PS) residues from the internal to the outer face of the plasma

membrane before loss of membrane integrity is used as a marker of apoptotic induction (Vermes *et al*, 1995). In contrast to the Annexin V assay, which examines one of the early morphological characteristics of apoptosis, the PI staining and TUNEL assays are both used to examine one of the latter morphological characteristics of apoptosis, which is genomic fragmentation. The PI staining assay looks for reduced DNA content within permeabilised cells as a marker of genomic fragmentation (Otsuki *et al*, 2003), while the TUNEL assay looks for evidence of 3′ hydroxyl-termini DNA strand breaks within fixed cells as a marker of endonuclease-mediated genomic fragmentation (Otsuki *et al*, 2003).

While light microscopy and TEM are an effective means of characterising apoptotic induction, they are not favoured in cytotoxicity screening as they are relatively time-consuming techniques and rely on the qualitative scoring of comparatively few cells within a test population. In contrast, FACS analysis provides a reliable method of analyzing a high number of cells within a short period of time (10,000 cells are typically scored at a flow rate of 300-1,000 cells per second). Furthermore, a range of well-characterized techniques, including the Annexin V, PI staining and TUNEL assays, can be used to examine the various morphological characteristics of apoptosis. Thus, for this investigation, the mode of cell death induced by water extracts of OD, LD and PM in cancer cells *in vitro* was investigated by performing the Annexin V, PI staining and TUNEL assays using FACS analysis.

As the growth inhibition study revealed the cytotoxic actions of these CHRs were most pronounced at 1:10, with significant cell death occurring within 24 hours of initial exposure, the mode of cell death induced by these CHRs were examined at a concentration of 1:10 within the first 24 hours of exposure. In addition, two cell lines were selected to examine the mode of cell death induced by water extracts of OD, LD and PM: these were the HL60 and HT29 cell lines. Two cell lines were used in this study in order to determine whether these CHRs induced the same form of cell death in different cancer cell types, as varying degrees of toxicity were observed between different cancer cells lines in the growth inhibition study and this may have been related to these CHRs inducing different types of cell death in different cancer cell lines. The HL60 cell line was primarily chosen for further

study because OD, LD and PM exerted their greatest toxic effect on this cell line at 1:10. The HT29 cell line was selected because water extracts of OD, LD and PM exerted a significant growth inhibitory effect on this cell line at 1:10, and it is gut associated; each of the CHRs are ingested orally and could potentially come into direct contact with gut-associated tumours.

4.2. Materials and Methods

4.2.1. Cell culture & CHR preparation

Cell culture and CHR preparation were performed as described in Chapter 3.

4.2.2. The Annexin V assay

The Annexin V assay was performed using the BD Pharmingen™ Annexin V-FITC Apoptosis Detection Kit (Becton Dickenson, Oxford, UK). HL60 and HT29 cell lines were exposed to OD, LD and PM for 6,12 16 and 24 hours, then washed twice with cold PBS and re-suspended in binding buffer (10mM Hepes/NaOH (pH 7.4), 140mM NaCl, 2.5mM $CaCl_2$) at a concentration of 10^6 cells/ml. Of this, 100µl was transferred to a 1.5ml microfuge tube and 5µl of Annexin V-FITC (buffered in 50mM Tris, pH 8.0, 80mM NaCl, 1mM EDTA, 0.09% w/v sodium azide and 0.2% BSA) and 5µl PI (Sigma, Dorset, UK) (50µg/ml in 1x PBS) added. This was gently vortexed and left to incubate for 15 minutes before analysis by flow cytometry (using a BD FacsCalibur Flow Cytometer running CellQuest Pro (Becton Dickenson, Oxford, UK), see appendix A1).

The fluorochrome FITC was analyzed using the FL1 detector, while PI was detected using FL2. Before sample data was acquired the detectors were calibrated for analysis (see appendix A2). Data were collected in the form of dot plots, then represented as density plots. Cells in the bottom left quadrant of the density plots are viable (Annexin V-FITC and PI negative), cells in the lower right quadrant are apoptotic (Annexin V-FITC positive and PI negative), and cells in the upper right quadrant are late stage apoptotic/necrotic (Annexin V-FITC and PI positive).

4.2.3. PI staining for sub-G1 peaks

HL60 and HT29 cell lines at a starting concentration of 10^5 cells/ml were made and OD, LD and PM added for 4, 8 and 24 hours. Following incubation, cells were centrifuged for 5 minutes at 800rpm and the resultant pellet re-suspended in 1ml ice-cold 70% ethanol and left on ice for 30 minutes. Samples were then centrifuged at 800rpm for 5 minutes, the supernatant was then removed, the cell pellet washed twice in ice cold PBS and re-suspended in PBS (1ml). To remove double stranded RNA, 1 unit of DNAse free RNAse A (Promega, Hertfordshire, UK) was added to

the cell suspension, and incubated for 30 minutes at 37°C. Finally, PI (100μl) was added to the cell suspension, and cells were stored on ice and protected from light until analysis.

Flow cytometry was used to analyse samples. For apoptotic analysis 10,000 of all events detected by the flow cytometer were counted and saved. In order to generate accurate histograms gating was performed to separate single cells that had passed through the flow cell from 2 (doublets) or more cells that had passed through the flow cell at the same time, which are scored as a single event by the flow cytometer (see appendix A3).

4.2.4. TUNEL assay

The TUNEL assay was performed using the Promega DeadEnd™ Fluoremetric TUNEL system (Promega, Southampton, UK). Flasks were seeded with HL60 and HT29 cells at a starting concentration of 10^5 cells/ml. After exposure to OD, LD and PM for 4, 8 and 24 hours cells ($2x10^6$) were spun down at 800rpm for 5 minutes and the supernatant removed. Cells were washed once with 4°C PBS and then re-suspended in PBS (0.5ml) and fixed by adding 1% methanol-free formaldehyde (5ml) and incubating on ice for 20 minutes. Cells were then centrifuged at 300g for 10 minutes at 4°C, then the supernatant was removed and the cells re-suspended and washed in PBS. Following this, cells were centrifuged for 10 minutes at 300g and then re-suspended in PBS (0.5ml). To the cell suspension 70% ice-cold ethanol (5ml) was added and this was kept at -20°C overnight. This suspension was then centrifuged at 300g for 10 minutes, the supernatant was removed and cells were re-suspended in PBS (1ml) and transferred to a microcentrifuge tube. The cells were then centrifuged at 300g for 10 minutes, then the supernatant was removed and the cells re-suspended in 80μl equilibration buffer (containing 200mM potassium cacodylate, 25mM Tris-HCL, 0.2mM DTT, 0.25mg/ml BSA and 2.5mM cobalt chloride) and incubated at room temperature for 5 minutes. Cells were then centrifuged at 300g for 10 minutes, then the supernatant was removed and the cells were re-suspended in 50μl TdT incubation buffer (45μl equilibration buffer, 5μl nucleotide mix (containing 50μM fluorescin-12-dUTP, 100μM dATP, 10mM tris-HCL (pH 7.6) and 1mM EDTA) and 1μl TdT enzyme (500u)) and incubated for 60

minutes at 37°C. This reaction was terminated by adding 1ml of 20mM EDTA and vortexing gently. The solution was then centrifuged at 300g for 10 minutes, after which the supernatant was removed and cells re-suspended in 1ml of 0.1% Triton® X-100 solution in PBS containing 5mg/ml BSA. This solution was then centrifuged for 10 minutes at 300g for 10 minutes, after which the supernatant was removed and the cell pellet re-suspended in 0.5ml of propidium iodide (Sigma, Poole, UK) solution (5μg/ml in PBS) containing 250μg of DNase-free RNase A (Promega, Southampton, UK), and incubated at room temperature in the dark for 30 minutes.

Flow cytometric analysis was carried out using a BD FACSCalibur flow cytometer. TUNEL data are represented as density plots showing DNA content on the x axis (using FL2) and the relative number of DNA strand breaks on the y-axis (using FL2).

4.3. Results

4.3.1. *Oldenlandia diffusa* (OD)

The Annexin V assay revealed apoptotic induction in both HL60 and HT29 cell lines exposed to OD at 1:10. For unchallenged HL60 cells 85.5% of cells were viable (lower left quadrant), 6.4% apoptotic (lower right quadrant) and 7.4% non-viable (upper right quadrant) (Figure 4.3.1.1 A). After a 6 hour incubation (Figure B) the apoptotic population rose to 12.4%. After 12 hours of exposure to OD (Figure C) the number of viable cells decreased to 63% with a concomitant increase in both apoptotic and non-viable cell populations. After 16 hours of exposure (Figure D) the number of viable cells fell to 52.8%, 29% of cells were in the early stages of apoptotic induction and 18.1% were non-viable. In the HT29 cell line apoptosis could not be detected after 12 or 16 hours (Figures F and G), however after 24 hours there was a distinct apoptotic population (26.3%) and the number of non-viable cells rose to 18.3%.

PI staining of both HL60 and HT29 cell lines exposed to the water extract of OD revealed apoptotic induction in the HL60 cell line after 4 hours, and after 24 hours in the HT29 cell line. For the HL60 4 hour control, 1.3% of cells within the population were apoptotic (Figure 4.3.1.2 A). HL60 cells exposed to OD for 4 hours had 15.5% of cells apoptosing (Figure B). After 8 hours the apoptotic population rose from 1.51% in the control (Figure C) to 46.28% (Figure D). After 4 and 8 hours of exposure to OD there was no evidence of an apoptotic population in the HT29 cell line (Figures E-H). After 24 hours of exposure the apoptotic population had risen from 2.12% in the control (Figure I) to 80.98% (Figure J).

The results from the TUNEL assay indicated apoptotic induction in the HL60 cell line after 4 hours, and after 24 hours in the HT29 cell line when exposed to the water extract of OD at 1:10. The HL60 and HT29 controls had a distinct population of viable cells (Figures 4.3.1.3 A and C respectively), with some apoptotic cells above this region, and some apoptotic cells with a reduced complement of DNA. HL60 cells exposed to OD for 4 hours had a distinct region of cells containing an increased number of double strand breaks which is indicative of apoptosis (Figure B). It also shows an increased number of cells with a reduced DNA content, which is also

indicative of apoptosis. In contrast, no distinct region of apoptotic cells could be observed in HT29 cells exposed to a 1:10 concentration of OD for 4 hours (Figures C and D). However, HT29 cells exposed to OD for 24 hours had a distinct region of cells above those found in the control (Figure F), which is indicative of apoptosis.

4.3.2. Long Dan Xie Gan Wan (LD)

The Annexin V assay revealed apoptotic induction in both HL60 and HT29 cell lines exposed to a 1:10 concentration of LD. Figure 4.3.2.1 A shows control values for an unchallenged HL60 population, with 85.5% of cells viable and 6.4% of the cell population undergoing apoptosis. After a 6 hour exposure to LD the population of actively apoptosing cells had risen to 14.7% (Figure B), after 12 hours to 26.4% (Figure C), and after 16 hours to 39.4% (Figure D). In the HT29 control (Figure E) the apoptotic population was 4.5%. There was a slight increase in apoptosing cells after 12 and 16 hours (Figures F and G), however it was not until 24 hours after initial exposure (Figure 1H) that a distinct apoptotic population was observed, accounting for 18.2% of the cell population.

Propidium iodide (PI) staining of the HL60 cell line exposed to a 1:10 water extract of LD for 4 and 8 hours revealed apoptotic induction, and after 24 hours in the HT29 cell line. For the HL60 4 hour control, 1.3% of cells within the population were apoptotic (Figure 4.3.2.2 A). HL60 cells exposed to LD for 4 hours had 30.2% of cells apoptosing (Figure B). After 8 hours the apoptotic population had risen to 56.5% (Figure D). There was no evidence of apoptotic induction in the HT29 cell line after 4 and 8 hours of exposure (Figures E-H). The HT29 24 hour control contained an apoptotic population of 2.12% (Figure I), for HT29 cells exposed to LD for 24 hours 68.91% of cells were apoptotic (Figure J).

The results from the TUNEL assay indicate apoptotic induction after 4 hours of exposure to the water extract of LD in the HL60 cell line, and after 24 hours in the HT29 cell line, with no evidence of apoptotic induction in the HT29 cell line after 4 hours of exposure. The HL60 and HT29 controls (Figures 4.3.2.3 A, C and E) show normal populations of cells spread between channels 200 and 400 on the x-axis. The distinct cell population above that of the viable cells (as seen in the controls) for the 4 hour-exposed HL60 and 24 hour exposed HT29 cell lines (Figures B and F,

respectively) are indicative of apoptosis. These regions contain cells with an increased proportion of double-strand breaks compared to controls, which is indicative of cells undergoing apoptosis.

4.3.3. Polygonum multiflorum (PM)

Results from the Annexin V assay indicate a time dependent increase in both apoptotic and dead cells in both HL60 and HT29 cell lines exposed to a 1:10 extract of PM (Figure 4.3.3.1). Figure A shows a normal HL60 cell population, with a viable cell population of 85.5%, and an apoptotic population of 6.4%. After 6 hours of exposure the apoptotic population has risen to 10% (Figure B), and after 12 hours it has increased to 38.1% (Figure C). After 16 hours, 89.2% of cells are non-viable, with 9.9% of the population apoptosing and only 0.9% of the population viable (Figure D). A similar trend can be observed when HT29 cells are exposed to PM over a period of 24 hours, with the proportion of viable cells decreasing over time, and a concomitant increase in both apoptotic and non-viable cells. After 12 and 16 hours the apoptotic population rises from 4.5% in the control to 14.2% after 12 hours (Figure F) and 13.2% after 16 hours (Figure G). After 24 hours 65.6% of the cell population are non-viable (Figure H). However, the results do not show the presence of a typically distinct apoptotic population in the lower right quadrant, as seen with cells incubated with OD and LD.

PI staining shows apoptotic induction in the HL60 cell line at 4 hours, and in the HT29 cell line after 24 hours of exposure to PM at 1:10 (Figure 4.3.3.2). Figure A shows the HL60 4 hour control, with viable cells in region M1 (93% of events), and apoptotic cells in region M2 (containing 1.3% of events scored). Figure B shows the effect of a 4 hour exposure of HL60 cells to PM, with cells in the apoptotic sub-G1 region rising to 22.04%. Figures E-H show PM has no effect on HT29 cells after 4 and 8 hours of exposure. Figure I shows the HT29 24 hour control, with 2.7% of cells in the sub-G1 region, and Figure J shows the effect of a 24 hour exposure of HT29's to PM; in this histogram 29.05% of cells are in the apoptotic sub-G1 region. However, the sub-G1 region does not contain a sub-G1 peak typically seen in a cell population containing apoptotic cells.

TUNEL assay results indicated apoptotic induction in the HL60 cell line after 4 hours but not the HT29 cell line, however apoptotic induction was observed after 24 hours of exposure in the HT29 cell line. The HL60 and HT29 controls (Figures 4.3.3.3 A, C and E respectively) showed normal populations of cells spread between channels 200 and 400 on the x-axis. The distinct population above the area of viable cells in the density plot of HL60 cells exposed to PM for 4 hours is indicative of apoptotic cells (Figure B). Figure E, exposure of HT29 cells for 24 hours shows movement of the entire cell population from a viable to non-viable, apoptotic cell population.

4.3.1. Examining the mode of cell death induced by water extracts of OD in the HL60 and HT29 cell lines

Figure 4.3.1.1 Annexin V assay showing HL60 and HT29 cells exposed to water extracts of OD

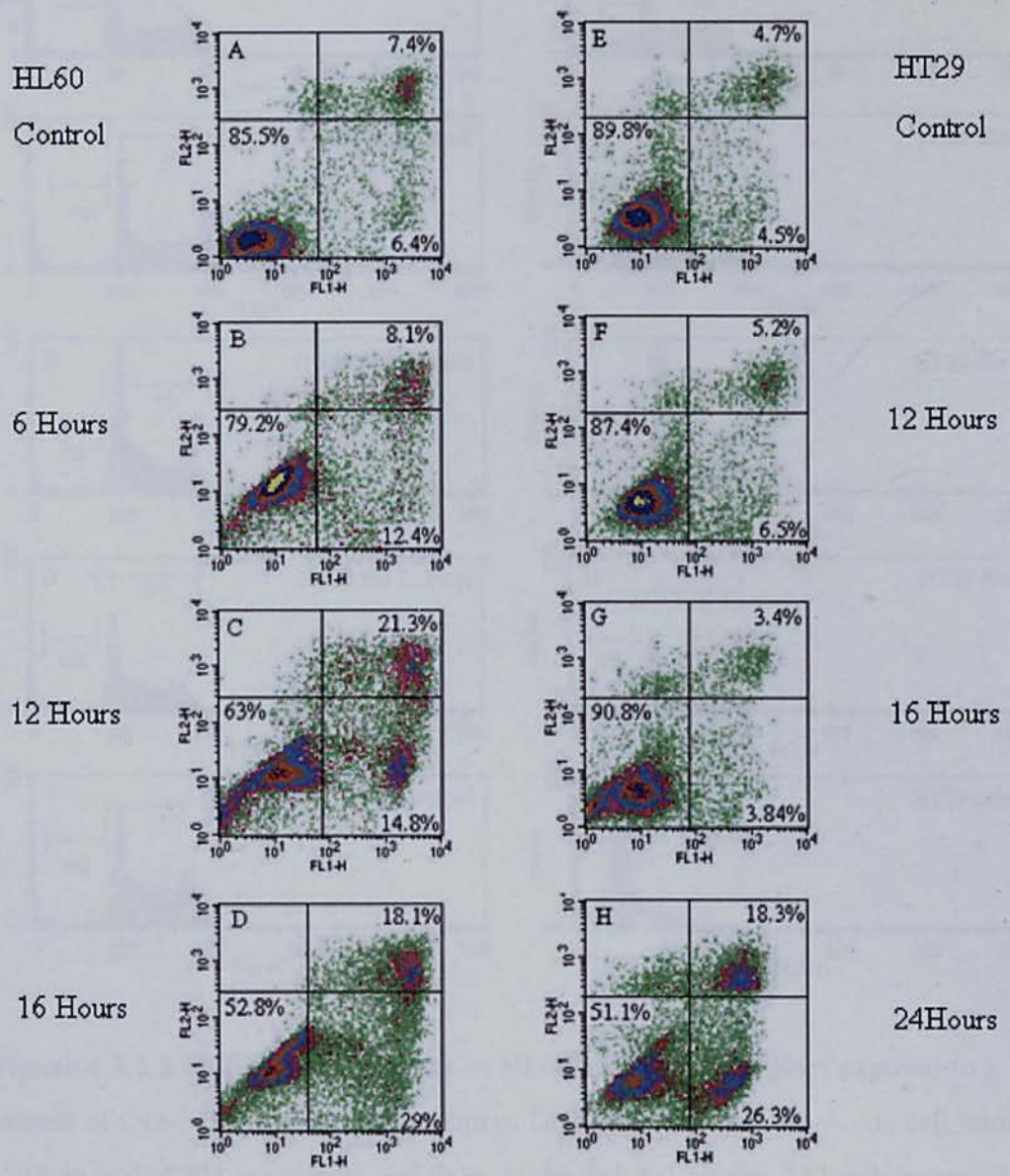


Figure 4.3.1.1 Annexin V staining of HL60 and HT29 cell lines exposed to the water extract of OD. The x-axis shows Annexin V-FITC binding and the y-axis staining of the vital dye propidium iodide. Cells in the lower left quadrant are viable, cells in the lower right are apoptotic and those in the upper right are non-viable dead/late stage apoptotic cells.

Figure 4.3.1.2 Propidium iodide staining for apoptotic sub-G1 regions in HL60 and HT29 cell lines exposed to water extracts of OD

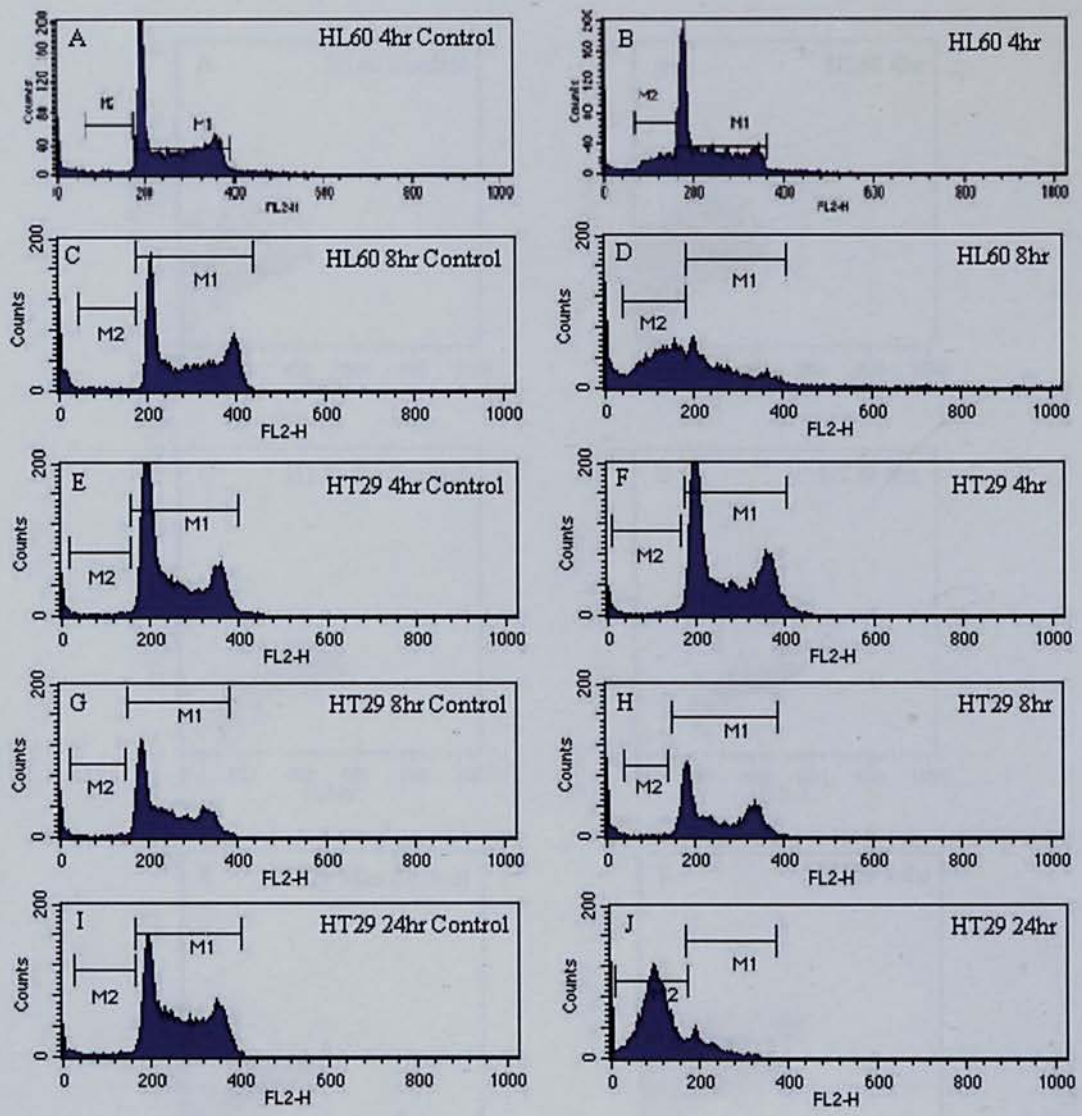


Figure 4.3.1.2 Cell cycle histograms of HL60 and HT29 cell lines exposed to a 1:10 extract of OD. The x-axis shows relative DNA content, and the y-axis cell number. Cells in region M1 are viable, and those in the Sub-G1 region, M2, are apoptotic. A: HL60 4 hour control. B: HL60 cells exposed to OD for 4 hours. C: HL60 8 hour control. D: HL60 cells exposed to OD for 8 hours. E: HT29 4 hour control. F: HT29 cells exposed to OD for 4 Hours. G: HT29 8 hour control. H: HT29 cells exposed to OD for 8 hours. I: HT29 24 hour control. J: HT29 cells exposed to OD for 24 hours.

Figure 4.3.1.3 TUNEL analysis of HL60 and HT29 cell lines exposed to water extracts of OD for 4 hours and 24 hours.

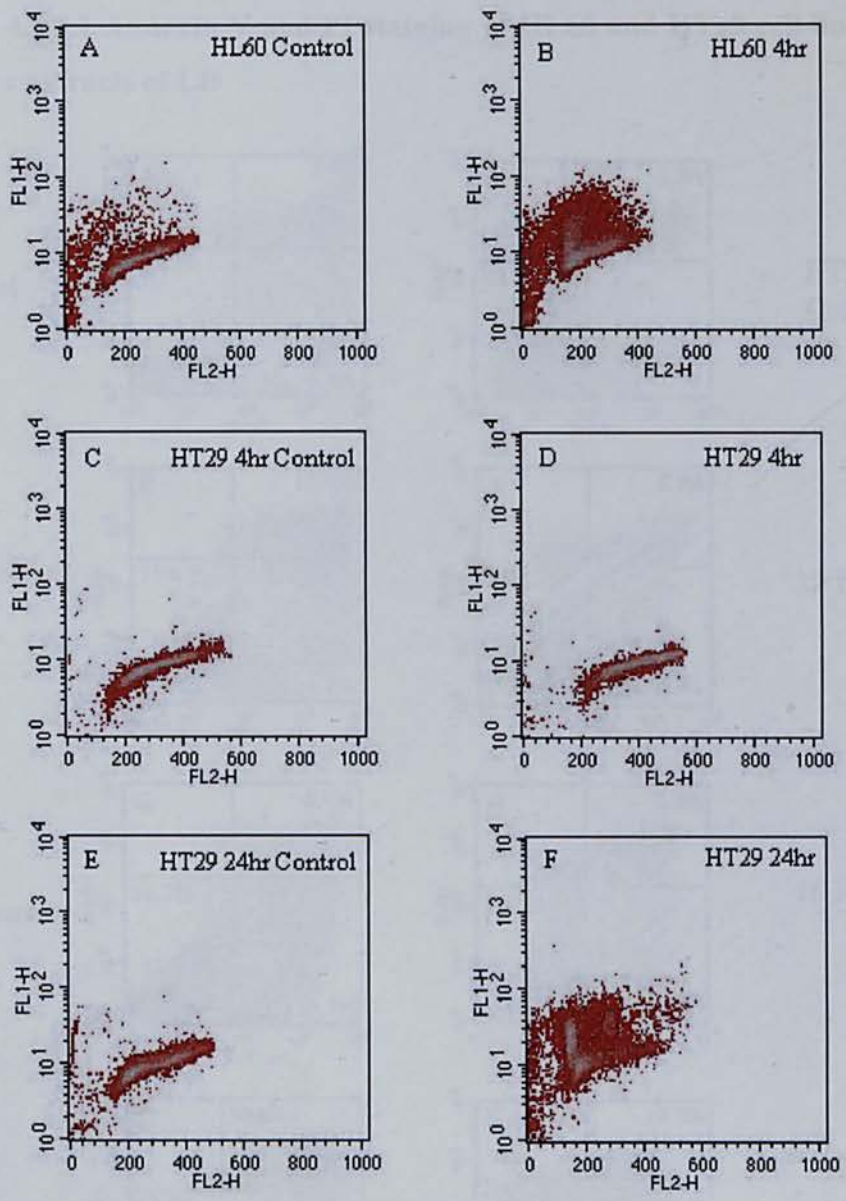


Figure 4.3.1.3 TUNEL analysis of the HL60 and HT29 cell lines exposed to a 1:10 extract of OD. The x-axis shows relative DNA content, and the y-axis the number of double strand breaks within cells. Figure A: the HL60 control and B: effect of a 4 hour incubation of HL60s with OD. Figure C: the HT29 4 hour control and D: the effect of a 4 hour exposure to OD. Figure E: the HT29 24 hour control and F: the effect of a 24 hour exposure to OD.

4.3.2. Examining the mode of cell death induced by water extracts of LD in the HL60 and HT29 cell lines

Figure 4.3.2.1 Annexin V and PI staining of HL60 and HT29 cell lines exposed to water extracts of LD

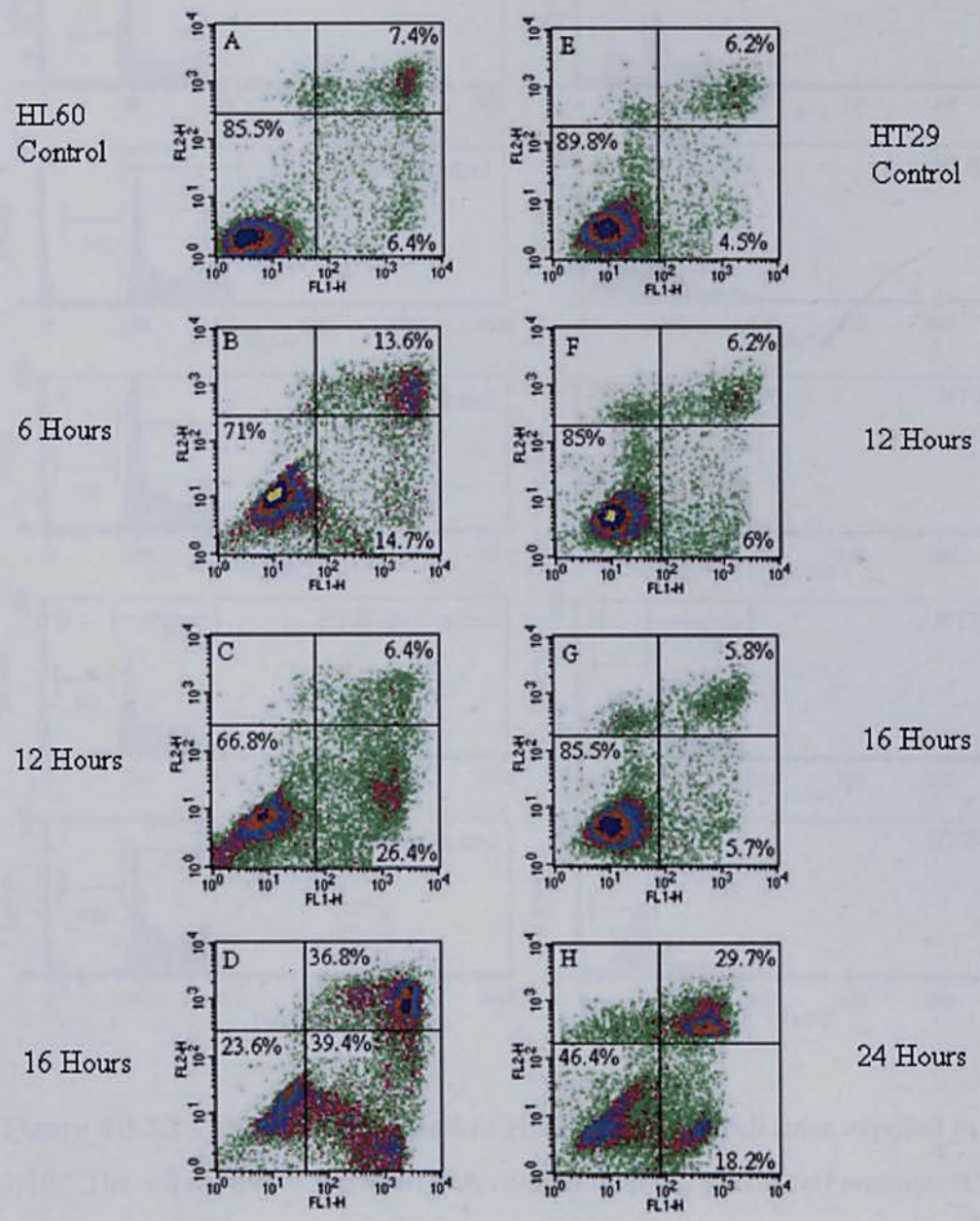


Figure 4.3.2.1 Annexin V staining of HL60 and HT29 cell lines exposed to the water extract of LD at 1:10. The x-axis shows Annexin V-FITC binding and the y-axis staining of the vital dye propidium iodide. Cells in the lower left quadrant are viable, cells in the lower right are apoptotic and those in the upper right are non-viable dead/late stage apoptotic cells.

4.3.2.2 Propidium iodide staining for apoptotic sub-G1 regions in HL60 and HT29 cell lines exposed to water extracts of LD

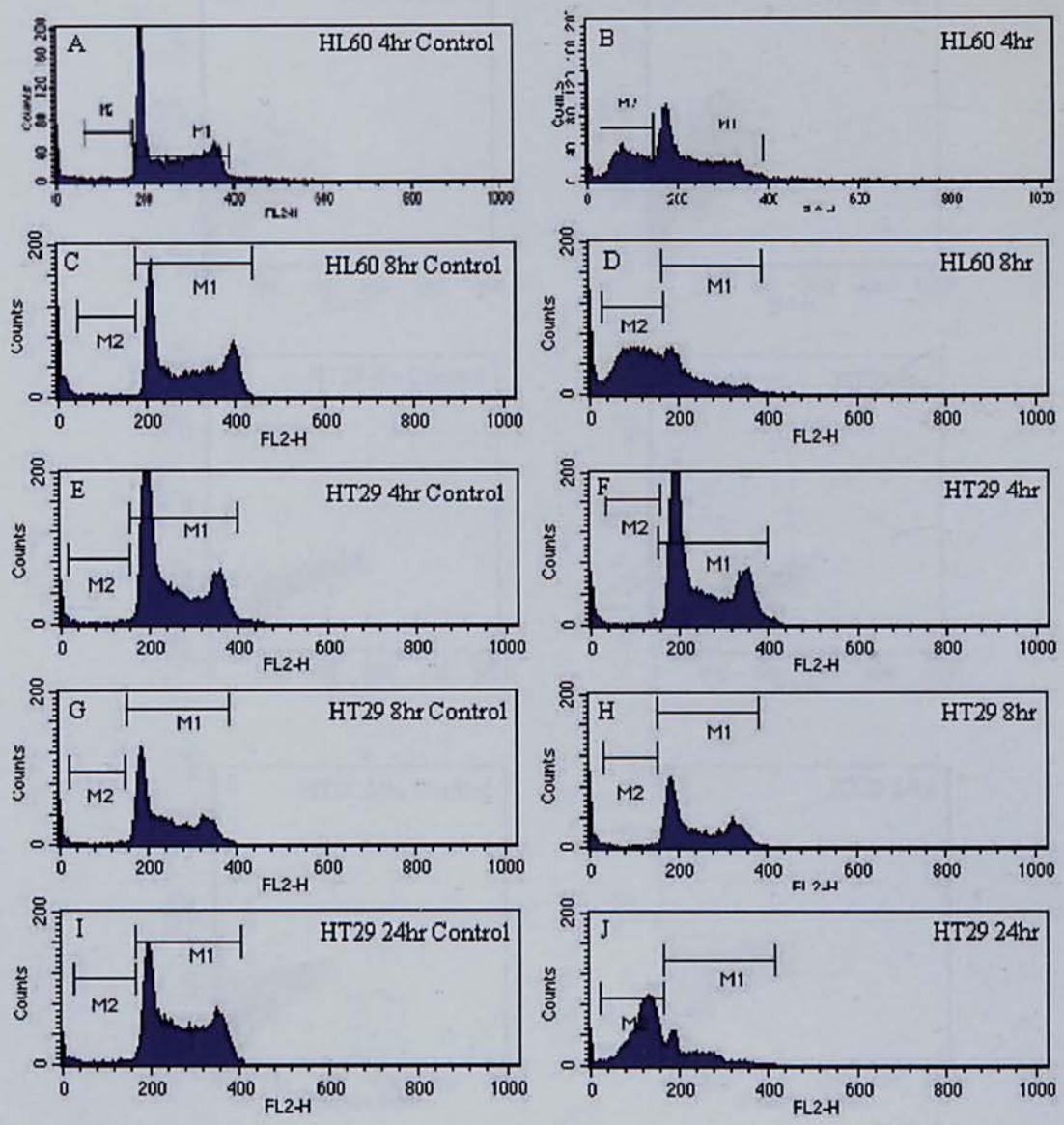


Figure 4.3.2.2 Cell cycle histograms of HL60 and HT29 cell lines exposed to LD at 1:10. The x-axis shows relative DNA content, and the y-axis cell number. Cells in region M1 are viable, and those in the Sub-G1 region, M2, are apoptotic. A: HL60 4 hour control. B: HL60 cells exposed to LD for 4 hours. C: HL60 8 hour control. D: HL60 cells exposed to LD for 8 hours. E: HT29 4 hour control. F: HT29 cells exposed to LD for 4 Hours. G: HT29 8 hour control. H: HT29 cells exposed to LD for 8 hours. I: HT29 24 hour control. J: HT29 cells exposed to LD for 24 hours.

Figure 4.3.2.3 TUNEL analysis of HL60 and HT29 cell lines exposed to water extracts of LD for 4 hours and 24 hours.

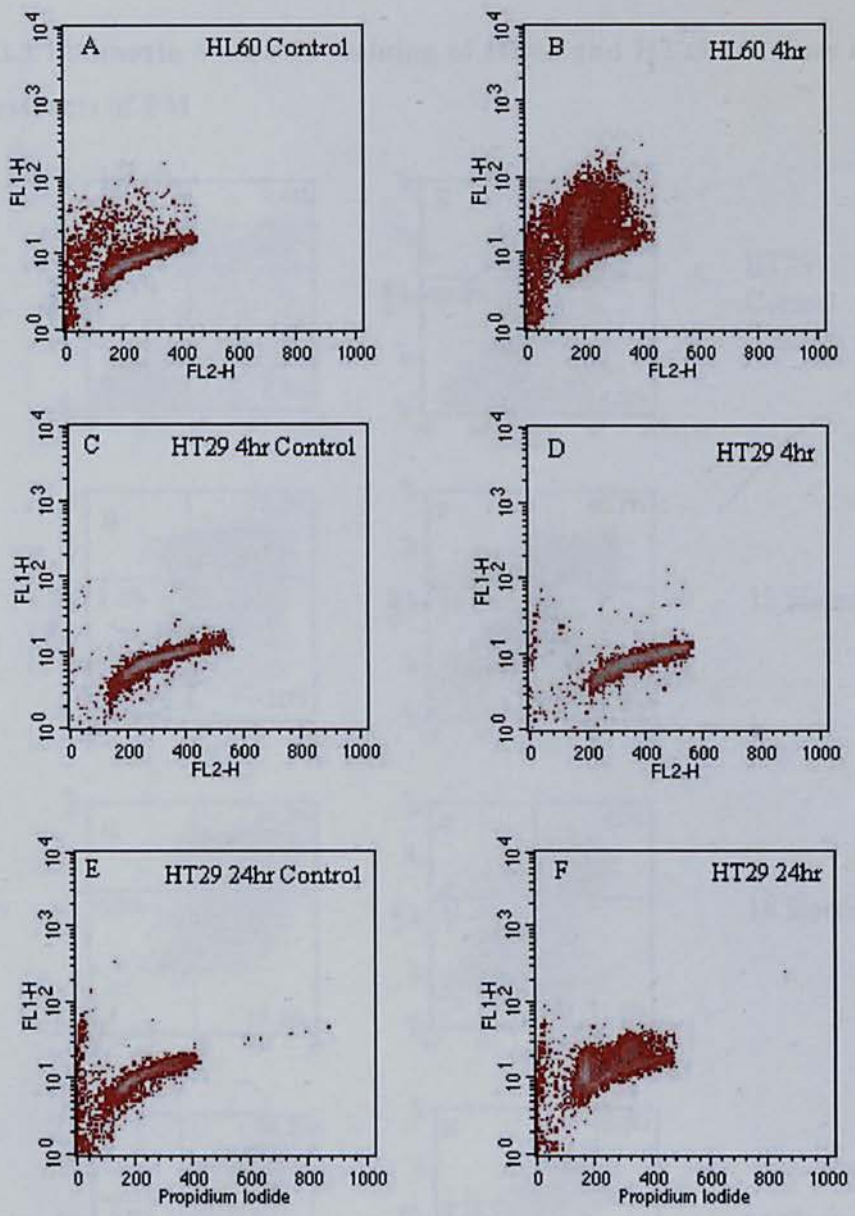


Figure 4.3.2.3 TUNEL analysis of the HL60 and HT29 cell lines exposed to LD at 1:10. The x-axis shows relative DNA content, and the y-axis the number of double strand breaks within cells. Figure A: the HL60 control and B: effect of a 4 hour incubation of HL60s with LD. Figure C: the HT29 4 hour control and D: the effect of a 4 hour exposure to LD. Figure E: the HT29 24 hour control and F: the effect of a 24 hour exposure to LD.

4.3.3. Examining the mode of cell death induced by water extracts of PM in the HL60 and HT29 cell lines

Figure 4.3.3.1 Annexin V and PI staining of HL60 and HT29 cell lines exposed to water extracts of PM

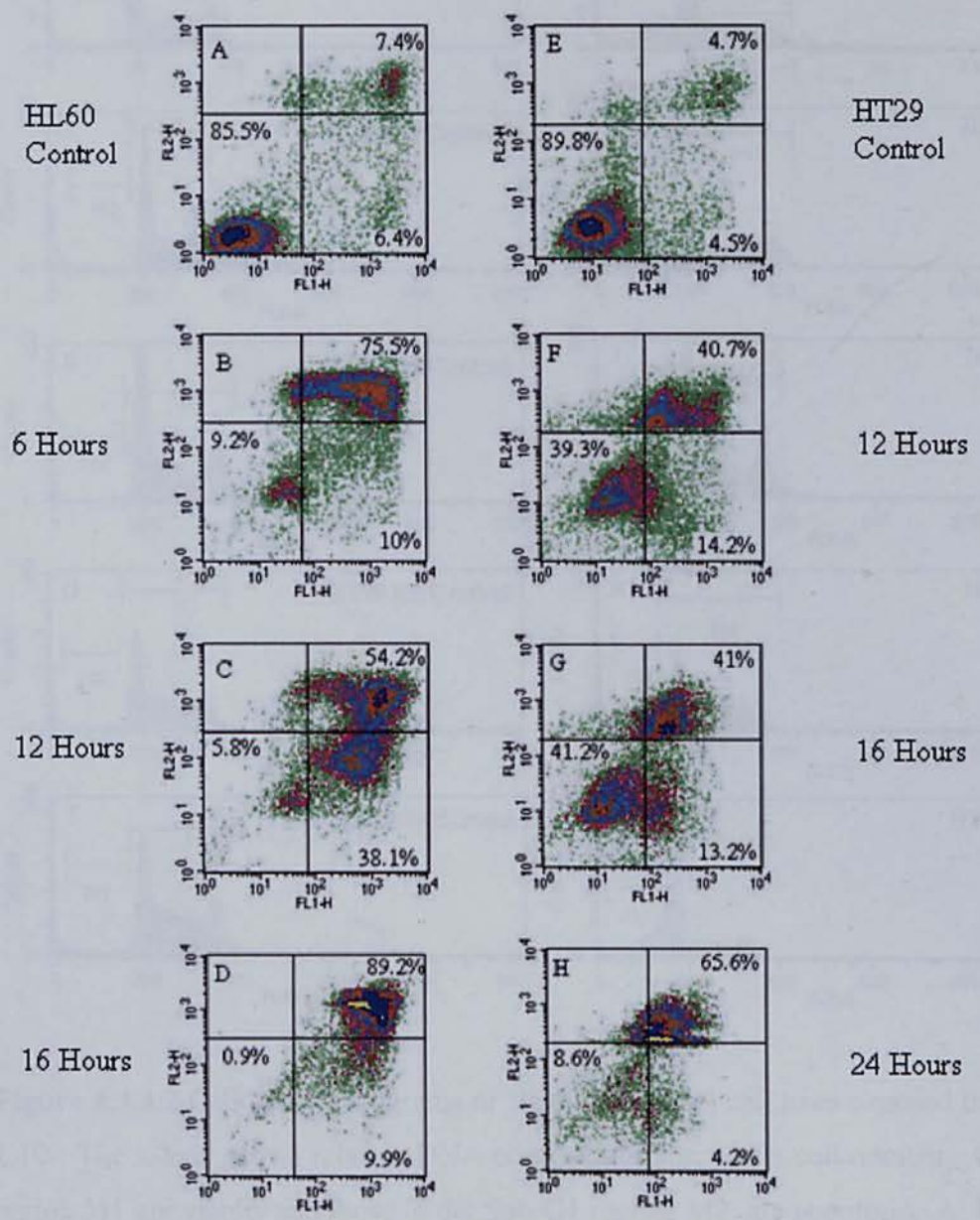


Figure 4.3.3.1 Annexin V staining of HL60 and HT29 cell lines exposed to PM at 1:10. The x-axis shows Annexin V-FITC binding and the y-axis staining of the vital dye propidium iodide. Cells in the lower left quadrant are viable, cells in the lower right are apoptotic and those in the upper right are non-viable dead/late stage apoptotic cells.

Figure 4.3.3.2 Propidium iodide staining for apoptotic sub-G1 regions in HL60 and HT29 cell lines exposed to water extracts of PM

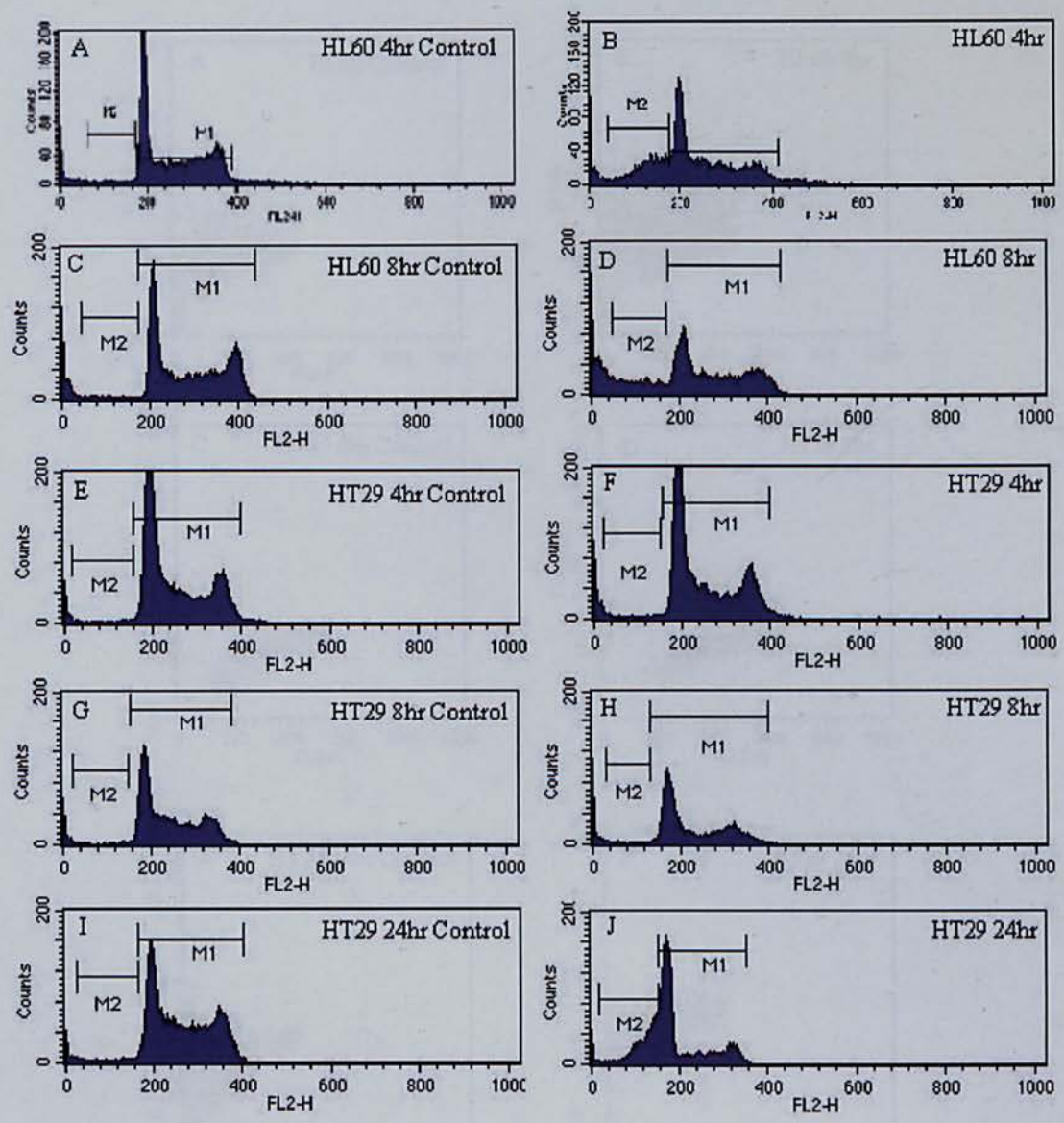


Figure 4.3.3.2 Cell cycle histograms of HL60 and HT29 cell lines exposed to PM at 1:10. The x-axis shows relative DNA content, and the y-axis cell number. Cells in region M1 are viable, and those in the Sub-G1 region, M2, are apoptotic. A: HL60 4 hour control. B: HL60 cells exposed to PM for 4 hours. C: HL60 8 hour control. D: HL60 cells exposed to PM for 8 hours. E: HT29 4 hour control. F: HT29 cells exposed to PM for 4 Hours. G: HT29 8 hour control. H: HT29 cells exposed to PM for 8 hours. I: HT29 24 hour control. J: HT29 cells exposed to PM for 24 hours.

Figure 4.3.3.3 TUNEL analysis of HL60 and HT29 cell lines exposed to water extracts of PM for 4 hours and 24 hours, respectively.

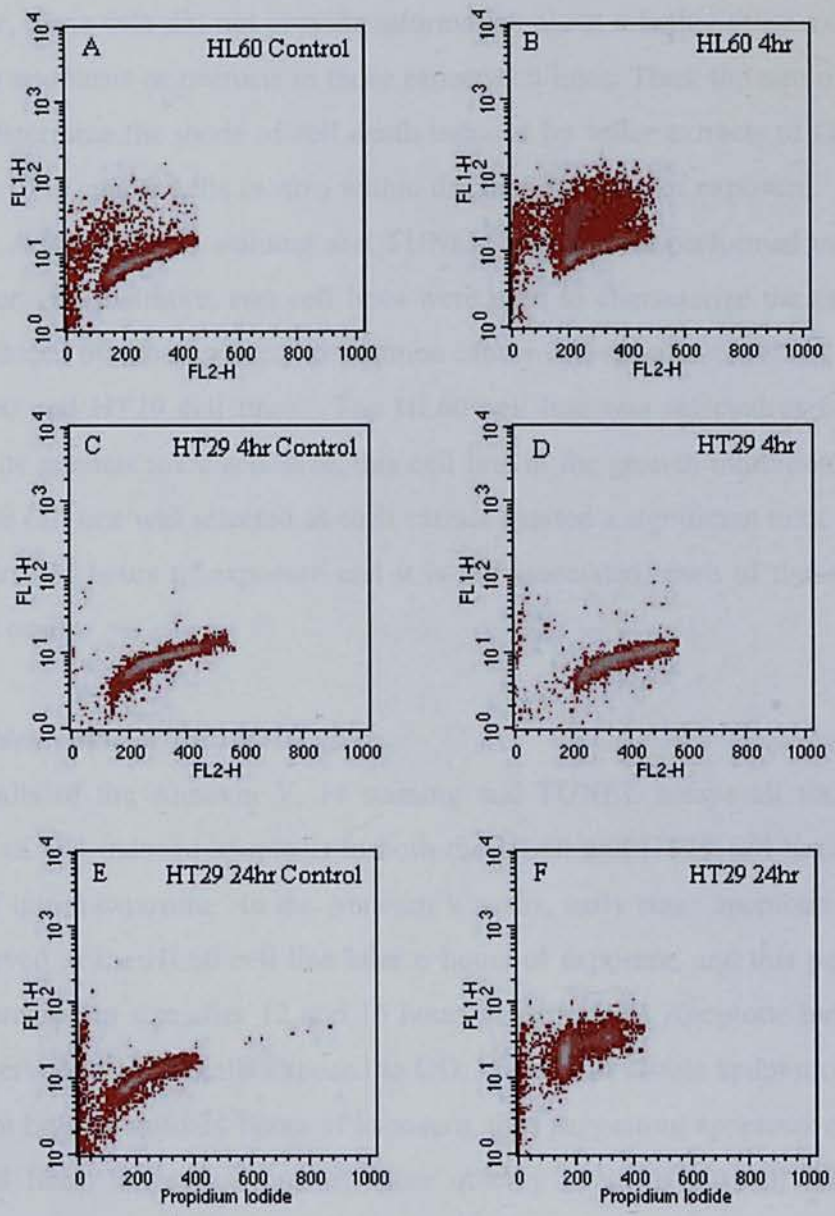


Figure 4.3.3.3 TUNEL analysis of the HL60 and HT29 cell lines exposed to PM at 1:10. The x-axis shows relative DNA content, and the y-axis the number of double strand breaks within cells. Figure A: the HL60 control and B: effect of a 4 hour incubation of HL60s with PM. Figure C: the HT29 4 hour control and D: the effect of a 4 hour exposure to PM. Figure E: the HT29 24 hour control and F: the effect of a 24 hour exposure to PM.

4.4. Discussion

Results from the growth inhibition study showed water extracts of OD, LD and PM were highly toxic to a range of cancer cell lines at 1:10 after 24 hours of exposure. However, these data did not provide information about whether those extracts were inducing apoptosis or necrosis in those cancer cell lines. Thus, the aim of this study was to determine the mode of cell death induced by water extracts of OD, LD and PM at 1:10 in cancer cells *in vitro* within the first 24 hours of exposure. To achieve this, the Annexin V, PI staining and TUNEL assays were performed using a flow cytometer. Furthermore, two cell lines were used to characterize the mode of cell death induced by those extracts to examine cancer cell-specific activities; these were the HL60 and HT29 cell lines. The HL60 cell line was selected as each extract exerted its greatest toxic action on this cell line in the growth inhibition study, and the HT29 cell line was selected as each extract exerted a significant toxic effect on it in the first 24 hours of exposure and it is gut associated: each of these CHRs are ingested orally.

4.4.1 *Oldenlandia diffusa* (OD)

The results of the Annexin V, PI staining and TUNEL assays all showed water extracts of OD induced apoptosis in both the HL60 and HT29 cell lines within 24 hours of initial exposure. In the Annexin V assay, early stage apoptotic cells could be observed in the HL60 cell line after 6 hours of exposure, and this population of cells increased in size after 12 and 16 hours of exposure. Apoptotic induction was also observed in HT29 cells exposed to OD, however a visible apoptotic population could not be seen until 24 hours of exposure, thus suggesting apoptosis occurring in both cell lines, however occurring more quickly in the HL60 cell line. Further investigation using the PI staining and TUNEL assays also revealed apoptotic induction in both the HL60 and HT29 cell lines after exposure to water extracts of OD. Again, apoptotic induction was observed in the HL60 cell line before the HT29 cell line, suggesting OD elicits a more rapid apoptosis-inducing action in the HL60 cell line than the HT29 cell line.

The results of this study are consistent with the growth inhibition study, in which water extracts of OD were found to be toxic to both the HL60 and HT29 cell lines at 1:10. Furthermore, the results of this study suggest the observed cytotoxic action of

OD in the growth inhibition study occurred through apoptotic induction in the HL60 and HT29 cell lines. That apoptotic induction occurs more quickly in the HL60 cell line than the HT29 cell line is also consistent with the growth inhibition study in that all HL60 cells were killed in the first 24 hours of exposure to water extracts of OD at 1:10 in the growth inhibition study, while some HT29 cells remained viable after 24 hours of exposure.

Furthermore, these data are also consistent with the previous study published by Sadava *et al* (2002) which showed water extracts of OD could induce apoptosis in both small cell lung cancer (H69) and multi-drug resistant small cell lung cancer (H69VP) cell lines, and with subsequent studies that have shown extracts of OD can induce apoptosis in a range of other cancer cell lines *in vitro*, including breast cancer cell lines (MCF-7 and MDA-MB-453), lung epithelial carcinoma (A5-49), human cervix carcinoma (C-33A), murine melanoma (B16-F10), prostate cancer cell lines (Tsu-Pt1, Du-145 and Ln-Cap) (Gupta *et al*, 2004) and the HL60 cell line (Yadav & Lee, 2006). Taken together, this and other studies suggest the apoptosis-inducing activity of water extracts of OD are not limited to a single cancer cell type, which may suggest the cytotoxic action of water extracts of OD observed against the other cancer cell lines used in the initial screen (HeLa, CHO and HCT-8) may be related to apoptotic induction, and that the observed anticancer action of OD *in vivo* (Yoshida *et al*, 1997), (Wong *et al*, 1996) may be related to a direct cytotoxic action against cancer cells.

4.4.2 Long Dan Xie Gan Wan (LD)

The Annexin V, PI staining and TUNEL assays also revealed apoptotic induction in HL60 and HT29 cell lines exposed to water extracts of LD at 1:10. As with OD, the Annexin V assay revealed water extracts of LD began to induce apoptosis in the HL60 cell line after 6 hours of initial exposure, and the number of dead and apoptotic cells increased in size after 12 and 16 hours. Apoptosis was also observed in the HT29 cell line, however a distinct apoptotic population could not be seen until cells had been exposed for 24 hours. Apoptotic induction was also seen in both the HL60 and HT29 cell lines exposed to LD using the PI staining and TUNEL assays, and again apoptotic induction was observed in the HL60 cell line before the HT29 cell line.

The results of this study are consistent with the growth inhibition study, in which water extracts of LD were found to be significantly cytotoxic to both the HL60 and HT29 cell lines at 1:10. The results of this study are also consistent with the growth inhibition study in that water extracts of LD were found to exert a greater cytotoxic action against the HL60 cell line than the HT29 cell line in the growth inhibition study, and in this study were found to induce apoptosis more quickly in the HL60 cell line than the HT29 cell line. Furthermore, the results of this study suggest the observed cytotoxic action of LD against the HL60 and HT29 cell lines in the growth inhibition study occurred as a result of apoptotic induction by LD, and as LD was found to induce apoptosis in both the HL60 and HT29 cell lines, it is also possible the observed cytotoxic action of LD against the HeLa, CHO and HCT-8 cell lines in the growth inhibition study also occurred through apoptotic induction.

The apoptosis-inducing activity of LD against cancer cells *in vitro* has not previously been reported. However, previous studies have revealed that a number of the herbal constituents of LD and the compounds within them can induce apoptosis in cancer cells: A triterpene derivative, saikosaponin D, isolated from *Radix bupleuri* (one of the constituents of LD), has been found to induce apoptosis in A549 lung cancer cells (Hsu *et al*, 2004). Furthermore, acetone extracts of *Radix bupleuri* have been found to induce apoptosis in A549 cells, and it is believed saikosaponins were not responsible for these actions, as saikosaponins are non-polar compounds and would not be present in acetone extracts of *Radix bupleuri* (Cheng *et al*, 2005). Baicalin, isolated from *Radix scutellariae* has been shown to inhibit the growth of a range of prostate cancer cell lines and to induce apoptosis in those cell lines (Chan *et al*, 2000), and has also been shown to inhibit the growth of human lung carcinoma CH27 cells through induction of apoptosis (Lee *et al*, 2005). One of the major components found within *Fructvs gardeniae* is the glycoside geniposide. As the isolation of glycosides is difficult due to their high polarity, the anticancer activity of the acetylated product of geniposide ($(Ac)_5GP$) has been examined (Peng *et al*, 2004). $(Ac)_5GP$ has been found cytotoxic to rat C6 glioma cells *in vitro*, inducing apoptosis (Chang *et al*, 2002). Finally, a triterpenoid, alisol B acetate, from *Rhizoma alismatis*, has been found to exert dose-dependent cytotoxic activity against the

human hormone-resistant prostate cancer cell line PC-3 and to induce apoptosis (Huang *et al*, 2005).

Thus, the apoptosis-inducing activity of LD observed in this study may be related to the combined actions of those compounds in LD that have previously been shown to exhibit anticancer activity. However, while these studies show that the constituents of LD possess apoptosis-inducing activity, it is not known whether these compounds are present in the water extracts of LD used in this study, nor is it clear whether these compounds would be found in sufficient levels in this preparation of LD to individually elicit an apoptosis-inducing activity. Thus, although it is possible that these compounds may be present within this preparation of LD, their role in the observed action of LD in this study remains to be elucidated.

4.4.3 *Polygonum multiflorum* (PM)

While the results of the Annexin V, PI staining and TUNEL assays all showed water extracts of PM to induce cell death in both the HL60 and HT29 cell lines at 1:10 within 24 hours of exposure, the mode of cell death induced by PM could not clearly be defined based on the results of this study: the Annexin V assay showed movement of HL60 cells from the lower left quadrant (containing viable cells) to the upper right quadrant (representing dead cells) after 6 hours of exposure, with no clearly defined cell population in the lower right quadrant (representing early stage apoptotic cells), thus suggesting cells dying through necrosis. This trend could also be observed in HT29 cells exposed to water extracts of PM at 1:10, although more slowly, as this trend became apparent after 12, 16 and 24 hours of exposure. For the PI staining assay, cell death was observed after 4 hours in the HL60 cell line and after 24 hours in the HT29 cell line, characterized by an increase in the number of cells in the sub-G1 region of cell cycle histograms. However, those sub-G1 regions were not distinct sub-G1 peaks characteristic of apoptosis, as they were connected to the G1 peak. As such, the results of this assay suggest water extracts of PM are inducing cell death in both cell lines, however the mode of cell death did not generate sub-G1 peaks typically associated with apoptotic induction. In contrast to the Annexin V and PI staining assays, which suggested water extracts of PM did not kill HL60 and HT29 cells through apoptotic induction at 1:10, the results of the TUNEL assay showed clearly defined apoptotic populations in HL60 and HT29 cells exposed to

water extracts of PM at 1:10, after 4 hours in the HL60 cell line and after 24 hours in the HT29 cell line.

Overall, these results are consistent with the findings of the growth inhibition study, in that they clearly show water extracts of PM are significantly toxic to both the HL60 and the HT29 cell line, and furthermore that they induce cell death more quickly in the HL60 cell line than the HT29 cell line. However, these data do not clearly define the mode of cell death induced by PM, as the Annexin V and PI staining assays suggest cells are dying through necrosis, while the TUNEL assay shows distinct apoptotic populations of cells after exposure to PM.

The contradictory results generated by these assays may be explained through the cytotoxic action of PM against the HL60 and HT29 cell lines and the markers for apoptotic induction examined here: the PI staining assay looks for evidence of genomic fragmentation as a marker of apoptotic cells death and the TUNEL assay for evidence of the generation of 3′OH DNA strand breaks, while the Annexin V assay looks at changes in cell surface characteristics as a marker of apoptotic induction. Thus, if PM were to induce its cytotoxic action through generation of genomic damage, resulting in necrotic cell death, this may be seen using the PI staining and TUNEL assays, however would not be seen using the Annexin V assay. This may also explain why a sub-G1 peak not characteristic of apoptosis was seen in the PI staining assay. Furthermore, these results also highlight the importance of examining the mode of cell death using multiple techniques.

While the cytotoxic action of PM in its entirety has not previously been reported, a number of the compounds within it have been shown to induce apoptosis in cancer cells in separate studies: epigallocatechin gallate and epigallocatechin have been found to induce apoptosis in the human colon carcinoma LoVo cell line (Xiaohua *et al*, 2000), while emodin has been found to induce apoptosis in cervical cancer cell lines (Srinivas *et al*, 2003) and in the A549 cell line (Su *et al*, 2005). However, apoptotic induction was not observed in HL60 and HT29 cell lines exposed to whole water extracts of PM at 1:10. This may be explained through the possibility that there are other compounds within PM that induce necrosis yet have not previously been reported, or that the levels of those apoptosis-inducing compounds within this

preparation were so high that cells died through necrosis instead of apoptotic induction: studies have shown that high levels of pro-apoptotic compounds can induce so much damage that apoptotic machinery cannot be triggered, and cells instead die through necrosis (Lennon *et al*, 1991). However, further investigation would be required to confirm this.

4.4.4. Comparing the observed toxicities of OD, LD and PM against the HL60 and HT29 cell lines

In addition to showing that OD and LD induce apoptosis in the HL60 and HT29 cell lines while PM induces necrosis, the results of this study also show each CHR to be more toxic to the HL60 cell line than the HT29 cell line, with cell death occurring more quickly in HL60 cell populations than HT29 cell populations exposed to a 1:10 concentration of each CHR. That OD, LD and PM have a more pronounced effect on the HL60 cell line than the HT29 cell line is consistent with a previous study that has shown HL60 cells to be more sensitive to a range of toxic stimuli than the HT29 cell line (Shao *et al*, 1997). These results also suggest that each CHR exerts the same effect on both cell lines, although at a different rate, suggesting the toxicity of each CHR may not be cancer cell-type specific, and that each may induce the same form of cell death in various cancer cell types.

4.4.5. Conclusions

The Annexin V, PI staining and TUNEL assays, which each examine distinct morphological characteristics associated with apoptosis, consistently showed apoptotic induction in both HL60 and HT29 cell populations exposed to water extracts of OD and LD, thus suggesting this experimental approach was successful in elucidating the mode of cell death induced by OD and LD in the HL60 and HT29 cell lines.

However, this methodological approach was less successful in elucidating the mode of cell death induced by PM in the HL60 and HT29 cell lines, as the Annexin V, PI staining and TUNEL assays gave a series of contradictory results, with the TUNEL assay suggesting apoptotic induction and the Annexin V and PI staining assays suggesting necrosis. While genomic fragmentation may account for these disparate results, further investigation would be required to confirm this. In addition, further

investigation would also be required to confirm the mode of cell death induced by PM in the HL60 and HT29 cell lines.

The contradictory results of this study with regards to PM highlight the importance of examining multiple morphological characteristics associated with apoptosis to confirm the mode of cell death induced by cytotoxic compounds, thus justifying the use of the Annexin V, PI staining and TUNEL assays for this investigation. However, the limitations of studies that examine the morphological characteristics of apoptosis are that they cannot be further used to characterize how apoptosis was induced by those test compounds, which is essential in further elucidating their mechanism of action.

Chapter 5. The role of the intrinsic and extrinsic apoptotic signalling cascades in cell death mediated by *Oldenlandia diffusa*, Long Dan Xie Gan Wan and *Polygonum multiflorum*

5.1. Introduction

The ability of cytotoxic test compounds to induce apoptosis in cancer cell lines *in vitro* can be determined by examining the morphological characteristics unique to apoptosis in cancer cells exposed to those compounds. However, these morphological data cannot be used to determine which intracellular pro-apoptotic signalling cascade is activated in response to the cytotoxic actions of those test compounds, and this information is essential in elucidating their mode of biological action. Thus, although the results of the Annexin V, PI staining and TUNEL assays suggested OD and LD were inducing apoptosis in the HL60 and HT29 cell lines, they did not provide information regarding which pro-apoptotic signalling cascade was activated in response to their cytotoxic activity, and therefore could not be used to further characterize their mode of biological action.

5.1.1. The apoptotic signalling cascade

Apoptosis is a caspase-dependent process. The caspases (cysteine-dependent aspartate specific proteases) are a family of cysteine proteases that are present within the cytoplasm as tightly controlled procaspases, and upon activation are cleaved to form active caspases. Unlike many proteases, caspases are highly specific and only cleave select target proteins at the C-terminal end of aspartic acid residues. Caspases are organised into a signalling cascade with initiator caspases responsible for activating effector caspases. Initiator caspases (caspases 2, 8, 9 and 10) contain long prodomains that interact with their specific activators, while effector caspases (caspases 3, 6 and 7) contain shorter prodomains and are activated through proteolytic cleavage by upstream caspases.

There are two primary means of inducing caspase-dependent apoptosis, one involving stimulation of cell surface death receptors and the other perturbation of the mitochondria. The cell surface, or extrinsic signalling cascade is characterized by activation of caspase 8, while the mitochondrial, or intrinsic signalling cascade is

characterised by activation of caspase 9 (Schimmer *et al*, 2001). The intrinsic and extrinsic signalling cascades are distinct pro-apoptotic pathways.

5.1.2. Death Receptor-mediated apoptosis

Death receptors are primarily used by the immune system to control cell numbers, in particular limiting the number of mature lymphocytes within the body, and are also used by cytotoxic T lymphocytes to induce apoptosis in virus infected cells. The death receptors are a subset of the tumour necrosis factor receptor (TNFR) superfamily, and include Fas (CD 95), TNFR1, TRAIL receptors 1 and 2 and Death Receptor-3 (DR3). Each of the death receptors differ in ligand specificity, activating binding partners and downstream effectors (Schimmer *et al*, 2001). The relay of extracellular apoptotic signals through binding of specific ligands by death receptors ultimately results in the cleavage of procaspase 8 into its active form and the induction of apoptosis (Cain *et al*, 2002).

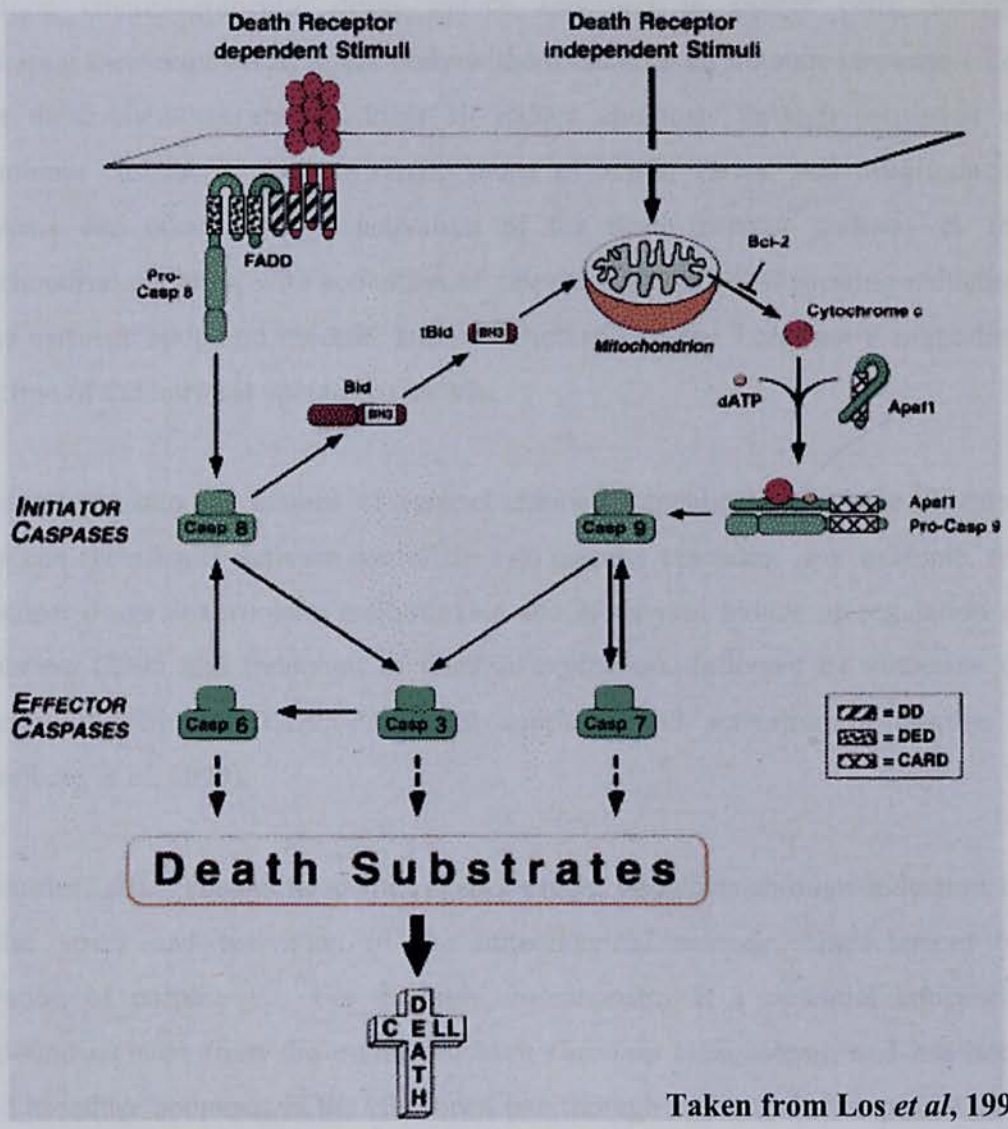
5.1.3. The Mitochondrial apoptotic pathway

Mitochondrial disruption is typically the end-point for a multitude of diverse proapoptotic signals that result in the activation of caspase 9. Cellular stresses such as ionizing radiation, chemotherapy, heat shock and cytokine withdrawal all ultimately lead to mitochondrial membrane disruption. The pathways that connect these activators to the mitochondria are often complex and poorly understood. They are complex because a given activator, such as chemotherapy, can up-regulate multiple intermediates that impact the mitochondria through multiple effectors (Schimmer *et al*, 2001).

5.1.4. Executioner caspase 3

Caspase 3 is the central executioner protease in the caspase signalling cascade, and is directly activated by caspases 8 and 9 (Figure 5.1.4.1). Its active form is a heterodimer containing 17kD and 12kD subunits derived from the 32kD proenzyme. Activated caspase 3 cleaves other caspases and the cytoplasmic and nuclear substrates responsible for dismantling the cell into apoptotic bodies (Turner *et al*, 2002).

Figure 5.1.4.1 Summary diagram showing the extrinsic and intrinsic caspase cascades



Taken from Los *et al*, 1999

Figure 5.1.4.1 The pathway on the left summarizes the extrinsic apoptotic cascade, with ligation of death receptors ultimately leading to activation of initiator caspase 8 followed by activation of executioner caspase 3. The pathway on the right summarizes the intrinsic, or mitochondrial caspase cascade, characterized by activation of initiator caspase 9 followed by activation of executioner caspase 3. Cross-talk between these pathways is also summarized.

5.1.5. The role of the intrinsic and extrinsic caspase cascades in cell death mediated by cytotoxic compounds

Common chemotherapeutic drugs induce apoptosis in cancer cells, thereby facilitating their removal from the body without eliciting an immune response. Yet while these chemotherapeutic drugs all induce apoptosis through activation of executioner caspase 3, their cytotoxic mode of action varies, and drug-induced apoptosis can occur through activation of the death-receptor pathway or the mitochondrial pathway, with activation of caspase 8 and not 9 suggesting induction of the extrinsic apoptotic cascade, and induction of caspase 9 and not 8 suggesting induction of the intrinsic apoptotic cascade.

Investigations into the actions of various chemotherapeutic agents have revealed drugs can specifically activate one of the two caspase cascades. For example, the anticancer drugs doxorubicin, methotrexate and bleomycin induce up-regulation of membrane CD95 and induction of CD95L expression, followed by autocrine or paracrine induction of CD95-dependent apoptosis and activation of caspase 8 (Weselberg *et al*, 1999).

In contrast, other chemotherapeutic agents trigger apoptosis through induction of cellular stress and activation of the mitochondrial cascade, characterized by activation of caspase 9. For example, α-mangostin is a potential anticancer compound isolated from the traditional herb *Garcinia mangostana*, and has been found to induce apoptosis in the HL60 cell line through activation of caspase 9 with no evidence of caspase 8 activation, thus suggesting induction of some form of cellular damage is responsible for its apoptosis-inducing action (Matsumoto *et al*, 2004). The flavonoid myricetin has also been shown to activate caspase 9 and not 8 in HL60 cells (Ko *et al*, 2005), and the antifungal agent econazole has also been shown to activate caspase 9 and not 8 in the COLO 205 cell line (Ho *et al*, 2005).

5.1.6. Examining the role of the intrinsic and extrinsic caspase cascades in cell death mediated by water extracts of OD, LD and PM

As discussed above, cytotoxic compounds can induce apoptosis through activation of the extrinsic caspase cascade, characterised by activation of caspase 8, or the intrinsic caspase cascade, characterised by activation of caspase 9, and as these

pathways are activated in response to distinct apoptotic stimuli, determining which pathway is activated by cytotoxic test compounds provides information regarding their mode of biological action. Thus, to further characterize the apoptosis-inducing action of water extracts of OD and LD against cancer cells, their ability to activate caspases 8 and 9 in cancer cells was investigated.

The apoptotic pathways activated in response to the cytotoxic actions of LD or its constituents had not previously been reported at the time of the initial literature review (of papers published between 1990-2003). However, the initial literature review did reveal that water extracts of OD had previously been shown to induce apoptosis in the H69 small cell lung cancer cell line through activation of caspase 9, thus suggesting a role for the intrinsic caspase cascade in the apoptosis-inducing activity of OD against this lung cancer cell line (Sadava *et al*, 2002). However, this study only focused on a single cancer cell type, and the role of the intrinsic and extrinsic apoptotic pathways in cell death mediated by OD in other cancer cell types had not previously been reported.

In order to examine the activation status of caspases 8 and 9 in cancer cells exposed to water extracts of OD and LD, Western blotting was used, as it is a technique that can specifically differentiate between activated caspases and inactive procaspases within cells exposed to cytotoxic test compounds. Furthermore, the HL60 cell line was used to examine the ability of OD and LD to induce apoptosis in cancer cells. The HL60 cell line was selected as OD and LD were found to be highly toxic to the HL60 cell line in this investigation. Furthermore, water extracts of OD and LD were used at 1:10 in this study because of their observed apoptosis-inducing activity in the Annexin V, PI staining and TUNEL assays, and as the morphological characteristics of apoptosis could be observed after 4 hours, caspase activation was examined after 0-8 hours of exposure. In addition to examining caspase 8 and 9 activation, activation of executioner caspase 3 was also studied to confirm the role of the caspase cascade in cell-death mediated by OD and LD.

In addition, as classical apoptosis is known as a caspase-dependent process, the role of caspase activation in PM-mediated cell death was also examined in the HL60 cell line. This was done to confirm the results of the Annexin V and PI staining assays,

and to provide further evidence that PM induces necrosis in cancer cells and not apoptosis.

5.2. Materials and Methods

5.2.1. Cell culture & CHR preparation

Cell culture and CHR preparation were performed as described in Chapter 3.

5.2.2. Investigation of caspase activation using Western blotting

Initially, protein extracts were prepared using the Blue Loading Buffer Pack (62.5mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% glycerol, 0.01% (w/v) bromophenol blue and 41.7mM DTT) (Cell Signaling, Hertfordshire). The amount of protein required to produce a clear signal was determined by creating extracts at various protein concentrations and probing for β-actin (Cell Signaling, Hertfordshire). Extracts were made at concentrations of $3x10^5$, $5x10^5$, $7.5x10^5$ and $1x10^6$ cells/12μl loading buffer. As a result of this experiment, extracts to examine caspase 3 activation using the Blue Loading Buffer Pack were prepared at $3x10^5$ cells/12μl, with 12μl run in each well.

In later experiments, Chaps extraction buffer (Cell Signaling, Hertfordhire) was used instead of the Blue Loading Buffer Pack due to the limited caspase signal it generated. Flasks were seeded at $1x10^5$ cells/ml and CHR added for 0-8 hours. After incubation, cells were washed then re-suspended in Chaps cell extract buffer (containing 50mM Pipes/HCl (pH 6.5), 2mM EDTA, 0.1% Chaps, 20μg/ml Leupeptin, 10μg/ml Pepstatin A, 10μg/ml Aprotinin and 5mM DTT), and frozen until use. Protein from $5x10^5$ cells was run in each lane.

Electrophoresis was performed using a Bio-Rad mini protean III electrophoresis tank. For initial experiments a 10% separating gel was used, which contained 1.43ml water, 1.875ml Tris (pH 8.8) (0.2M Tris base, 1.36M NaCl, adjusted to pH 8.8 with HCl), 1.5ml 30% Acrylamide (Sigma, Dorset), 25μl 20% SDS (Sigma, Dorset), 6.3μl TEMED (Sigma, Dorset), and 150μl 10% APS (Sigma, Dorset). For later experiments a 12% separating gel was made, which contained 930μl water, 1.875ml Tris (pH 8.8), 2ml 30% Acrylamide, 25μl 20% SDS, 6.3μl TEMED, and 150μl 10% APS. The stacking gel contained 1.64ml water, 313μl Tris (pH6.8), 450μl 30% Acrylamide, 12.5μl 20% SDS, 6.3μl TEMED and 75μl 10% APS. For each gel, 10μl of biotinylated molecular weight marker (Cell Signaling,

Hertfordshire) was added to lane 1. Each gel was run at 159 volts for approximately 50 minutes, until the dye-front reached the bottom of the gel.

After electrophoresis, protein was transferred from the gel onto a 0.45μm nitrocellulose membrane (Bio-Rad, Hertfordshire) using a Bio-Rad Trans-Blot® SD semi-dry transfer cell (Bio-Rad, Hertfordshire) and transfer buffer (25mM Tris, pH 8.3, 192mM glycine, with 20% MeOH and 0.5% SDS). Proteins were transferred at 15v/300ma for 30 minutes.

After transfer, the membrane was incubated in 5ml blocking buffer (1x TBS/T and 5% w/v non-fat dry milk) for 1 hour, then incubated overnight at 4°C with gentle agitation in primary antibody dilution buffer (1 TBS/T and 5% milk) and antibody at 1:1000 dilution. The antibodies used in this study bound to the active forms of caspases 3, 8 and 9, and β-actin antibody was used as a control for relative protein content in each lane (Cell Signaling, Hertfordshire, UK). The caspase 3 polyclonal antibody bound to the 17kDa and 19kDa subunits of active caspase 3. The caspase 8 polyclonal antibody bound to the 22kDa large subunit of active caspase 8. The caspase 9 polyclonal antibody bound to the 47kDa inactive pro-caspase, and the 35 and 37kDa active fragments. The β-actin monoclonal antibody bound to the 45kDa β-actin protein.

After overnight incubation the membrane was washed then incubated in HRP-conjugated anti-rabbit secondary antibody (1:2000) and HRP-conjugated anti-biotin antibody (1:1000) (Cell Signallin, Hertfordshire, UK), and protein visualized using LumiGLO® and ECL Hyperfilm™ (Amersham Biosciences, Buckinghamshire).

Results were scanned onto a computer using an HP Scanjet 5300C scanner at a resolution of 300dpi and edited using Microsoft Office Picture Manager.

5.3. Results

To determine the necessary protein levels needed to analyze caspase activation, protein samples from HL60 cells were made and lanes containing protein from $3x10^5$, $5x10^5$, $7.5x10^5$ and $1x10^6$ cells run and β-actin protein levels detected by ECL (Figure 5.3.1). A 10% separating gel was used. Results showed a maximum of $3x10^5$ cells could be used before the signal became distorted. The gel was also used to determine the amount of biotinylated ladder needed to provide a comparable signal to that of the protein being analyzed. As a result of this experiment protein extracted from $3x10^5$ cells were subsequently used in each lane. A colorimetric protein assay was not performed as the Blue loading buffer pack initially used contained blue pigment.

Subsequent blots to look for caspase 3 activation by OD were made by extracting protein using the Blue Loading Buffer Pack and running protein from $3x10^5$ cells in each lane. Results (Figure 5.3.2) showed a weak signal was generated, and the 17kD and 19kD fragments had not resolved in the 10% separating gel. As a result a 12% gel was used in subsequent experiments, which was able to resolve the separate bands (Figure 5.3.3). However, the signal generated was still very weak when compared to control protein detection (β-actin staining). As a result of the weak signal, Chaps cell extract buffer was used instead of the Blue Loading Buffer Pack, which was able to generate a much stronger caspase signal. Chaps cell extract buffer was used for the experiments described below.

5.3.1. The effect of OD on activation of caspases 3, 8 and 9

Results showed caspases 3 and 9 were activated after 2 hours of exposure (Figure 5.3.4). Caspase 3 could be detected until 6 hours after initial exposure, while the active fragments of caspase 9 could only be detected after 2 and 4 hours of initial exposure to OD. Furthermore, the overall levels of procaspase 9 (47kD) decreased over time. For caspase 8, non-specific binding appeared around 22kD in the control and at all time points. It is unlikely that the band seen was caspase 8, as there was no evidence of caspase 3 activation at 0hrs (in the control), thus it did not appear caspase 8 was activated in response to OD. β-actin was used as a control for total protein content within each lane, and showed equal amounts of protein were present within each lane.

5.3.2. The effect of LD on activation of caspases 3, 8 and 9

Results show activation of caspase 3 after 2, 4, 6 and 8 hours of exposure to 1:10 extracts of LD (Figure 5.3.5). Caspase 9 could also be detected after 2, 4 and 6 hours of initial exposure, with levels of procaspase 9 decreasing over time. Again, for caspase 8 non-specific binding appeared around the 22kD marker, and no evidence of caspase 8 activation could be seen. β-actin staining reveals equal protein levels within each well.

5.3.3. The effect of PM on activation of caspases 3, 8 and 9

There was no evidence of activation of caspases 3, 8 or 9 in HL60 cells exposed to PM at 1:10 for 0-8 hours (Figure 5.3.6). There was a decrease in levels of procaspase 9 over time, however there was also a decrease in total protein levels as shown by the β-actin positive control. There was also a decrease in the level of non-specific binding observed at 22kD (the site of caspase 8), however, this was likely due to decreased protein levels as shown by β-actin staining.

Figure 5.3.1 Determining necessary protein concentration for caspase analysis in HL60 cells exposed to CHRs and characterisation of biotinylated ladder

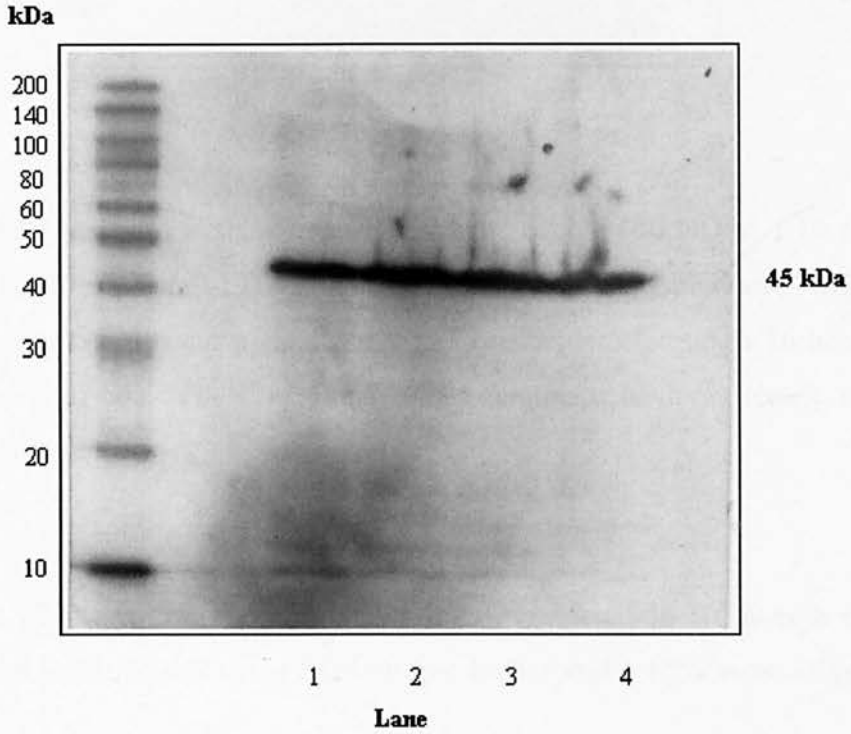


Figure 5.3.1 Western blot showing protein extracted from $3x10^5$ (lane 1), $5x10^5$ (lane 2), $7.5x10^5$ (lane 3) and $1x10^6$ (lane 4) HL60 cells using the Blue Loading Buffer Pack. Results show optimum results were generated with the addition of $3x10^5$ cells in a lane, as smearing occurs at higher protein concentrations. The far left lane shows the biotinylated ladder used in this study, which contains 10 molecular weight markers ranging from 10-200kDa.

Figure 5.3.2 Time course showing caspase 3 activation in HL60 cells exposed to OD using a 10% separating gel and protein extracted using the Blue Loading Buffer Pack

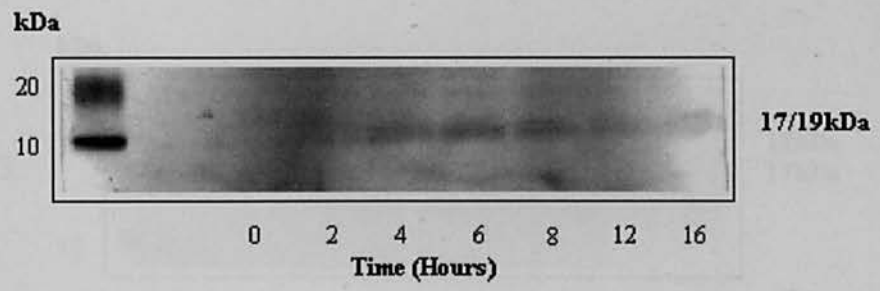


Figure 5.3.2 Caspase 3 activation in HL60 cells exposed to OD at 1:10 for 0-16 hours. The 17kDa and 19kDa bands represent cleaved, activated caspase 3. Activation appears to occur after 4 hours and can be detected up to 16 hours after initial addition of OD. The 17kDa and 19kDa fragments have not clearly resolved due to the 10% gel.

Figure 5.3.3 Time course showing activation of caspase 3 in HL60 cells exposed to OD and β actin control using SDS sample buffer and a 12% separating gel

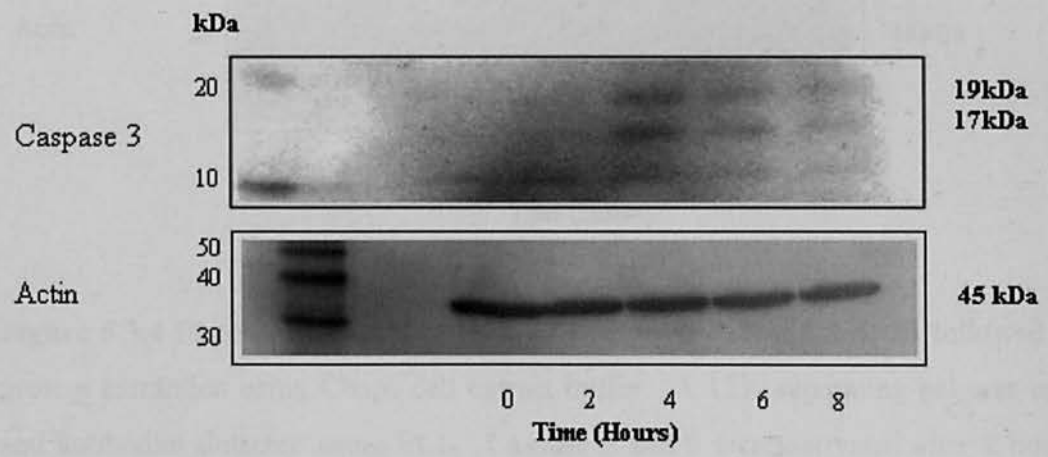


Figure 5.3.3 Caspase 3 activation in HL60 cells exposed to OD at 1:10 for 0-8 hours. The 17kDa and 19kDa bands represent cleaved, activated capase 3. Caspase 3 appears to be activated after 4 hours. A 12% separating gel was used, which has resolved the 17kDa and 19kDa fragments. The β actin control shows equal amounts of protein have been loaded into each lane.

Figure 5.3.4 Time course showing activation of caspases 3 and 9 but not 8 in the HL60 cell line exposed to OD using Chaps cell extract buffer

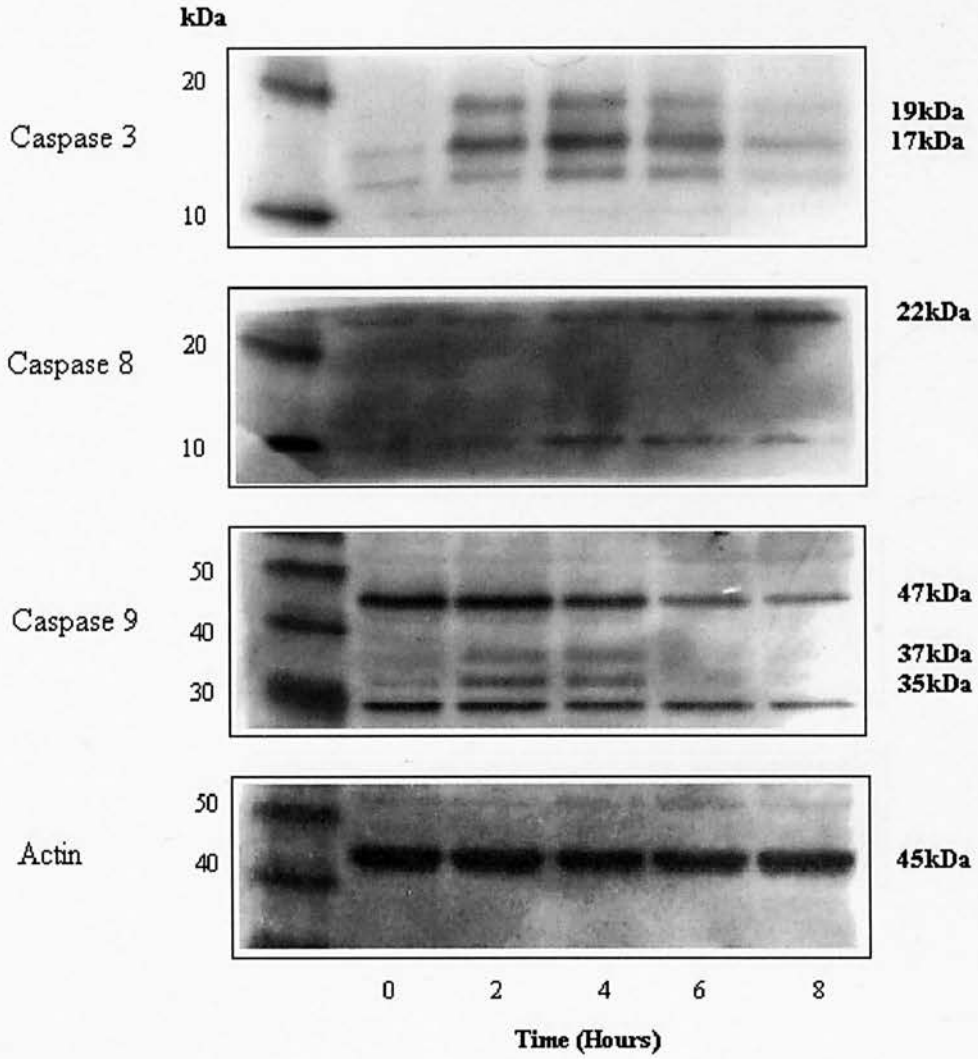


Figure 5.3.4 Exposure of HL60 cells to OD for 0, 2, 4, 6 and 8 hours followed by protein extraction using Chaps cell extract buffer. A 12% separating gel was used and antibodies detected using ECL. Caspase 3 and 9 were activated after 2 hours, which is indicative of apoptotic induction. Caspase 8 was not activated, however there appears to be non-specific binding around the site of activated caspase 8. β actin was used as a control to examine the protein content within each lane.

Figure 5.3.5 Time course showing activation of caspases 3 and 9 but not 8 in the HL60 cell line exposed to LD using Chaps cell extract buffer

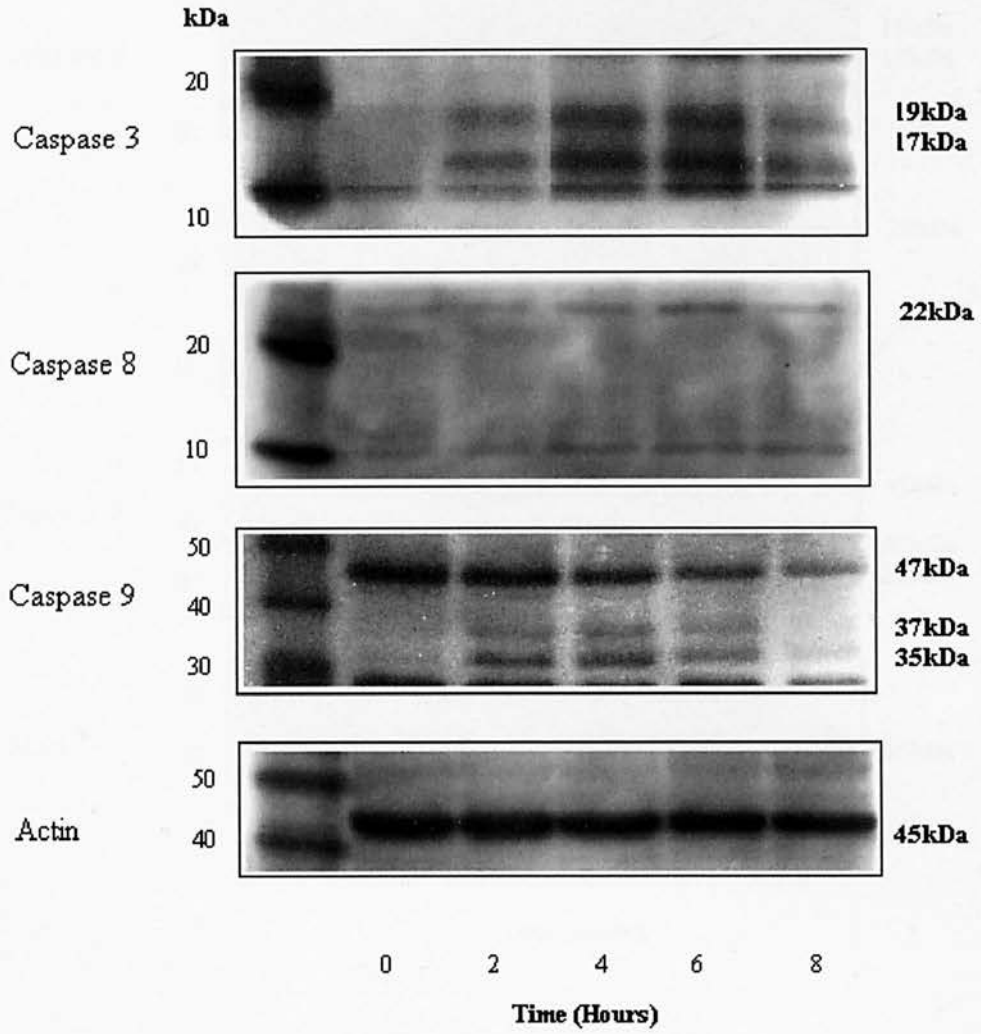


Figure 5.3.5 Exposure of HL60 cells to LD for 0, 2, 4, 6 and 8 hours followed by protein extraction using Chaps cell extract buffer. A 12% separating gel was used and antibodies detected using ECL. Caspase 3 and 9 were activated after 2 hours, which is indicative of apoptotic induction. Caspase 8 was not activated, however there appears to be non-specific binding around the site of activated caspase 8. β actin was used as a control to examine the protein content within each lane.

Figure 5.3.6 Time course examining caspase activation in HL60 cells exposed to PM using Chaps cell extract buffer

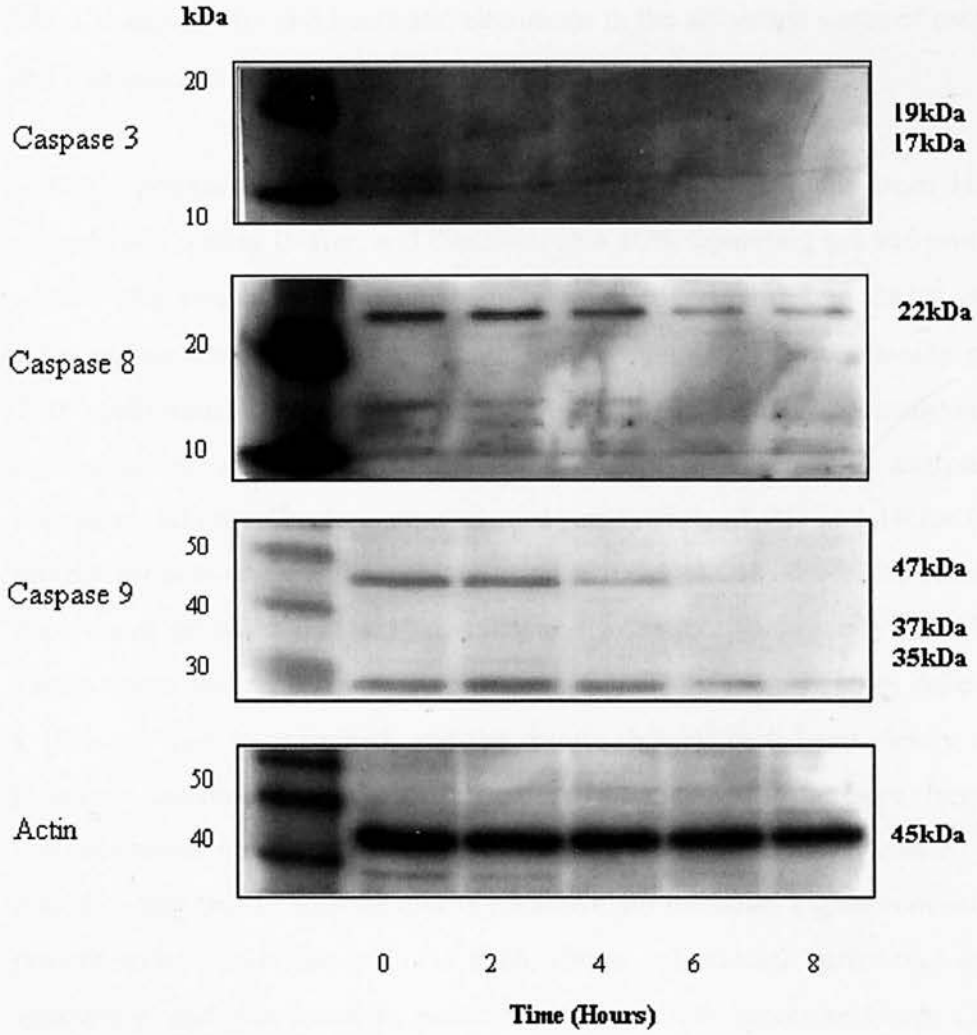


Figure 5.3.6 Exposure of HL60 cells to PM for 0, 2, 4, 6 and 8 hours followed by protein extraction using Chaps cell extract buffer. A 12% separating gel was used and antibodies detected using ECL. There was no evidence of caspase activation. β actin was used as a control to examine the protein content within each lane.

5.4. Discussion

To determine the role of the intrinsic and extrinsic apoptotic cascades in cell death mediated by OD, LD and PM, HL60 cells were exposed to 1:10 water extracts of OD, LD and PM for 0-8 hours and alterations in the activation states of caspases 3, 8 and 9 investigated using Western blotting.

Initially, protein extracts of varying concentrations were made from HL60 cells using Blue Loading Buffer, and these run on a 10% separating gel and probed for β-actin. The results of this initial study revealed protein from $3x10^5$ cells were sufficient to generate a strong signal. Furthermore, samples containing more than $3x10^5$ cells generated distorted bands so were not appropriate for analysis. Having determined an appropriate protein concentration with which to analyse caspase activation, HL60 cells were exposed to water extracts of OD at 1:10 for 0-8 hours, protein extracts made and separated using a 10% gel. However, the results of this experiment revealed that a 10% gel was insufficient to separate the 17kDa and 19kDa bands that represent activated caspase 3. Thus, these samples were re-run on a 12% gel then re-examined, and the results showed each band clearly separated. However, the results generated showed a weak signal. Furthermore, larger protein volumes could not be added to address this issue using samples created using the Blue Loading Buffer because results became distorted when higher concentrations of protein were run on the gel. As such, Chaps cell extract buffer was used as an alternative, and was found to generate clearer results, therefore Chaps cell extract buffer was used for all subsequent studies.

5.4.1. *Oldenlandia diffusa* (OD)

The results of this study showed 1:10 water extracts of OD activated caspases 3 and 9 in the HL60 cell line after 2 hours of exposure while having no effect on caspase 8. As such, the results of this study suggest that water extracts of OD induced apoptosis in the HL60 cell line through activation of the intrinsic apoptotic pathway, which is typically activated in response to the detection of cellular stress.

The intrinsic pathway requires disruption of the mitochondrial membrane and the release of the mitochondrial proteins cytochrome c, Smac/DIABLO and HtrA2 into the cytoplasm to enable activation of caspase 9. Cytochrome c, which normally

shuttles electrons between protein complexes of the respiratory chain, binds to Apaf-1 (apoptotic protease activating factor 1) when released into the cytosol, and in the presence of ATP recruits and activates procaspase 9 to form a complex called the apoptosome, while Smac/DIABLO and HtrA2 antagonize the actions of inhibitors of apoptosis (IAPs) such as XIAP, which would otherwise block activation of caspase 9 (Martinou & Green, 2001).

Mitochondrial outer membrane permeabilization is regulated by interactions between members of the Bcl-2 protein family, which are divided into two main groups according to whether they promote or inhibit apoptosis. Pro-apoptotic members are further divided according to whether they contain multiple BH domains or only BH3 (Bcl 2 family members have up to four Bcl 2 homology (BH) domains, which corresponds to α-helical segments):

Anti-apoptotic Bcl-2 family members

Bcl-2, Bcl-$_{XL}$, Bcl-w, Mcl-1, A1, Boo/Diva

Pro-apoptotic Bcl-2 family members

Multidomain: Bax, Bak, Bok

BH3-only: Bid, Bad, Bim, BMF, Bik, Hrk/DP5, Blk, Nip3, BNip3/Nix, Puma, Noxa

(Kuwana & Newmeyer, 2003)

BH3 only proteins are essential initiators of apoptosis in response to cellular stress. It is currently believed that, in general, varying apoptotic stimuli are mediated through the activation of one or more BH3-only proteins, which then go on to interact with anti-apoptotic Bcl-2 family members or multi-domain pro-apoptotic family members, and this ultimately results in mitochondrial membrane disruption through the pore-forming abilities of activated Bax and Bak (Willis & Adams, 2005).

As Bcl-2 family members are central regulators of apoptosis, it is possible that OD may be inducing apoptosis in HL60 cells through the induction of some form of intracellular damage that is relayed to the mitochondria by Bcl 2 family members,

which ultimately culminates in activation of caspase 9. Furthermore, due to the lack of caspase 8 activation observed here, these results suggest the apoptosis-inducing activity of OD is not related to the ability of OD to augment death receptor activity, an ability which has previously been reported in other apoptosis-inducing compounds (Weselberg *et al*, 1999).

The results of this study are consistent with the findings of the Annexin V, PI staining and TUNEL assays of this investigation, in that the morphological characteristics associated with apoptosis were detected in HL60 cells exposed to OD using these assays, and caspase activation was observed here: apoptosis is a caspase-dependent process. Furthermore, caspase activation is known to precede the morphological characteristics of apoptosis, including endonuclease activation and PS exposure on the plasma membrane (Kroemer, 2002), and in this study caspase activation was observed after two hours, while the morphological characteristics of apoptosis could not be seen in HL60 cells exposed to OD until they had been exposed for 4 hours.

The results of this study are also consistent with the study by Sadava *et al* (2002), in which water extracts of OD were found to induce apoptosis in the H69 lung cancer cell line through activation of caspase 9, and with the study by Yadav & Lee (2006), which showed caspase activation in HL60 cells exposed to 70% EtOH extracts of OD. Taken together, these results suggest that activation of the intrinsic apoptotic pathway by water extracts of OD is not limited to a single cancer cell type, or to the cancers for which OD is prescribed (which include lung cancer). Thus, it is possible that the apoptosis-inducing activity of OD observed on the HT29 cell line in the previous chapter may also be related to its ability to activate the intrinsic caspase cascade, and that the observed cytotoxic action of OD against those cell lines used in the growth inhibition may also be related to its ability to activate the intrinsic apoptotic pathway.

5.4.2. Long Dan Xie Gan Wan (LD)

Activation of caspases 3 and 9, but not 8, was also observed in HL60 cells exposed to 1:10 water extracts of LD after 2 hours of incubation. Again, this would suggest apoptosis was not initiated in those cells through an activity related to augmentation

of death receptor activity, such as up-regulation of CD95-Fas receptors or production of CD95 ligand, and that the apoptosis-inducing activity of LD may instead be related to the induction of some form of intracellular damage that triggers the intrinsic caspase cascade, possibly as a consequence of signalling from pro-apoptotic Bcl-2 family members, as discussed above (Borner, 2003).

Caspase activation by LD in the HL60 cell line is consistent with the results of the Annexin V, PI staining and TUNEL assays, as they all show LD to be inducing apoptosis in the HL60 cell line. As with OD, caspase activation by LD could be seen after two hours of exposure, while the morphological characteristics of apoptosis could be observed after 4 hours of exposure, and these results are consistent as caspase activation precedes the morphological characteristics associated with apoptosis.

While the apoptosis-inducing activity of LD in its entirety has not previously been reported, the apoptosis-inducing activity of some of the constituents of LD and the compounds within them have been examined since the initial literature review, and have been shown to induce apoptosis through both signalling pathways. Furthermore, some of these constituents have been shown to induce apoptosis through activation of both signalling cascades: while the intrinsic and extrinsic apoptotic pathways are considered distinct signalling pathways, cross-talk can occur. For example, the Bcl-2 family member Bid can be cleaved by caspase 8 to form tBid, which can translocate to the mitochondria and cause release of cytochrome c, leading to activation of caspase 9 (Huang *et al*, 2004). Furthermore, Fas receptors can activate the MAP3 kinase ASK1, which launches a phosphorylation cascade that culminates in the activation of JNK. Active JNK can phosphorylate substrates such as c-Jun and p53, and induce apoptosis through a number of processes including activation of Bcl-2 family proteins, which activate the mitochondrial cascade. TNFRI and II can also activate the MAP/JNK pathway (as above) by binding to the adaptor proteins TRADD and TRAF (Schimmer *et al*, 2001). In addition to activation of the mitochondrial caspase cascade by death receptors, activation of the mitochondrial cascade can lead to activation of caspase 8, typically through activation of caspase 3 and 6 by caspase 9, a process that has been observed in B-lymphoma cells (Weider *et al*, 2001).

One of the herbal constituents of LD is *Radix bupleuri*, and a triterpene derivative, saikosaponin D, has been isolated from *Radix bupleuri* and its effect on the A549 lung cancer cell line investigated. Saikosaponin D has been found to induce apoptosis in A549 cells through up-regulation of Fas/Apo1 and membrane and soluble Fas Lignad, which leads to activation of caspase 8 and up-regulation of Bax (Hsu *et al*, 2004). Furthermore, acetone extracts of *Radix bupleuri* have been found to induce apoptosis in A549 cells through activation of caspases 3 and 9. It is believed saikosaponins were not responsible for these actions as saikosaponins are non-polar compounds and would not be present in the acetone extract of *Radix bupleuri* (Cheng *et al*, 2005).

Another constituent of LD is the herb *Fructvs gardeniae*, and one of the major components found within *Fructvs gardeniae* is the glycoside geniposide. As the isolation of glycosides is difficult due to their high polarity, the anticancer activity of the acetylated product of geniposide ($(Ac)_5GP$) has been examined (Peng *et al*, 2004). $(Ac)_5GP$ has been found cytotoxic to rat C6 glioma cells *in vitro*, inducing apoptosis and cell cycle arrest at G_0/G_1 by increasing levels of c-Myc, p53 and pro-apoptotic Bax while decreasing levels of anti-apoptotic Bcl-2 (Chang *et al*, 2002). However further study revealed that despite increased levels of p53 and Bax there was no evidence of caspase 9 activation or apoptosis occurring through the mitochondrial pathway. Instead, translocation and activation of PKCδ (Peng *et al*, 2004) was found to lead to apoptosis via activation of the JNK/FasL/Fas/caspase8/caspase3 signalling cascade (Peng *et al*, 2005).

Finally, a triterpenoid, alisol B acetate, from *Rhizoma alismatis* (another constituent of LD), has been found to exert dose-dependent cytotoxic activity against the human hormone-resistant prostate cancer cell line PC-3. Alisol B acetate was found to activate initiator caspases 8 and 9 and effector caspase 3, and up-regulate expression of the proapoptotic Bcl 2 family member Bax (Huang *et al*, 2005).

However, while these previous studies have shown the constituents of LD contain a range of compounds that induce apoptosis through both the intrinsic and extrinsic caspase cascades in isolation, the results of this study suggest that, in its entirety, the

LD preparation used in this study induces apoptosis solely through the intrinsic cascade. This may be due to the possibility that those compounds that induce apoptosis through the extrinsic cascade may be present in low levels or absent within this preparation of LD (LD is made from a combination of 10 herbal ingredients), or because HL60 cells are known to be relatively resistant to Fas-mediated apoptosis, which would suggest those compounds may not exert a pro-apoptotic activity against this cell line (O'Gorman *et al*, 2001).

5.4.3. *Polygonum multiflorum* (PM)

The growth inhibition study clearly showed water extracts of PM were highly toxic to a range of cancer cell lines at 1:10. Further study into the mode of cell death induced by PM was conducted using the Annexin V, PI staining and TUNEL assays, and while the Annexin V and PI staining assays suggested PM was inducing necrosis, TUNEL data suggested apoptosis was being induced. Thus, to further investigate the mode of cell death induced by PM, and to determine the role of the caspases in cell death mediated by PM, the ability of PM to activate caspases 3, 8 and 9 was examined.

The results of this study showed no evidence of caspase activation, and as apoptosis is considered a caspase-dependent process, these data further support the findings of the Annexin V and PI staining assays, and suggest that PM triggers cell death in the HL60 cell line through necrosis. The observed decrease in total protein levels over time, as seen in the β-actin loading control and in pro-caspase 9 is likely a result of cell death occurring through necrosis over time. Based on these data, it is also possible that PM induces necrosis in the HT29 cell line, and the other cancer cell lines used in the growth inhibition study.

While the mode of cell death induced by extracts of PM in their entirety have not previously been reported, some of the constituents of PM have been shown to induce apoptosis in cancer cell lines when examined in isolation: epigallocatechin gallate and epigallocatechin have been found to induce apoptosis in the human colon carcinoma LoVo cell line (Xiaohua *et al*, 2000), and emodin has been shown to prevent proliferation of the cancer cell lines K562, Calu-1, Vero, HeLa and Wish (Kuo *et al*, 1997). In addition, emodin has been found to induce apoptosis in

cervical cancer cell lines through activation of the intrinsic apoptotic cascade (Srinivas *et al*, 2003), and in A549 cells emodin has been found to activate caspase 9 through induction of oxidative injury (Su *et al*, 2005), thus supporting the role of the intrinsic cascade.

Thus, these data suggest that although the epigallocatechin gallate, epigallocatechin and emodin can induce apoptosis in isolation, PM in its entirety induces necrosis. This may be due to the large number of cytotoxic compounds within PM that act in a combinatorial fashion to kill cells before they have time to apoptose (Lennon *et al*, 1991), however further investigation would be required to determine the necrosis-inducing activity of PM.

5.4.4. Conclusions

The aim of this study was to determine the role of the intrinsic and extrinsic apoptotic signalling cascades in cell death mediated by OD, LD and PM in cancer cells. As such, HL60 cells were exposed to 1:10 concentrations of OD, LD and PM for 0-8 hours, and caspase 3, 8 and 9 activation examined using Western blotting.

The results of this study revealed caspase 3 and 9 activation in HL60 cells after 2 hours of exposure to OD and LD, suggesting the intrinsic caspase cascade was activated in response to the cytotoxic actions of these CHRs. Furthermore, there was no evidence of caspase activation by PM, which would suggest cell death occurring through necrosis. The results of this study are consistent with the results of the previous chapter, in which OD and LD were found to induce apoptosis in the HL60 cell line and PM necrosis, and provide further information regarding the cytotoxic actions of OD, LD and PM against cancer cells.

Chapter 6. Alterations in the cell cycle characteristics of cancer cell lines by *Oldenlandia diffusa*, Long Dan Xie Gan Wan and *Polygonum multiflorum*

6.1. Introduction

Growth inhibition studies can provide information regarding the dose-related toxicity of test compounds against cancer cell lines *in vitro*, however these studies cannot be used to characterize the role of cell cycle arrest in the observed growth inhibitory actions of cytotoxic test compounds. Thus, while the results of the growth inhibition study showed *Oldenlandia diffusa* (OD), Long Dan Xie Gan Wan (LD) and *Polygonum multiflorum* (PM) were significantly toxic to a range of cancer cell lines at 1:10 and exerted a growth inhibitory action at 1:100 and 1:1000, (compared to control cell growth), they could not be used to determine whether this growth inhibitory action occurred as a result of dose dependent toxicity leading to cell death (via apoptosis or necrosis), or as a result of cell cycle arrest.

6.1.1. The cell cycle and cell cycle arrest

The eukaryotic cell cycle is an ordered and highly regulated process that consists of DNA replication followed by cell division. The cell cycle is comprised of four distinct stages: G1 (Gap 1), S (Synthesis), G2 (Gap 2) and M (Mitosis). G1 is the longest phase of the cell cycle, during which cells prepare for replication. S-phase is the synthesis phase of the cell cycle, where DNA is replicated and a complete copy of each chromosome made. G2 is a short gap phase, which occurs after S phase. The final stage of the cell cycle is M, and during this stage new chromosomes are segregated equally between two daughter cells before division (Turner *et al*, 2000).

To ensure the integrity of a cell and its genetic material during division a cell must successfully progress through three cell cycle checkpoints, known as G1, S phase and G2/M. These checkpoints contain a series of sensors that detect various forms of cellular damage, a signal transduction cascade that directs the sensors output, and effectors that can either inhibit cell cycle progression to allow a cell time to repair that damage before it proceeds through the cell cycle, or induce apoptosis if that damage is irreparable (Lukas *et al*, 2004).

6.1.2. Alterations in cell cycle characteristic by natural products and chemotherapeutic agents

Many cytotoxic compounds, including common cancer chemotherapeutic drugs and naturally occurring cytotoxic compounds, are regarded as cell cycle agents as they selectively kill actively dividing cells (which include cancer cells) while having no effect on non-dividing cells. These agents have the ability to arrest cells at specific stages of the cell cycle (depending on their mode of action) through the activation of cell cycle checkpoints, and to induce apoptosis in these cells (Fischer *et al*, 2004). Cell cycle agents can be divided into generic groups depending on which cell cycle checkpoint(s) they trigger, and are summarized below.

6.1.2.1. G1/S and S phase arrest agents

G1/S and S phase arrest agents can be subdivided into three distinct groups depending on their mode of action. These include compounds that directly affect DNA synthesis, for example chemotherapeutic anthracyclins, which interfere with enzymes involved in DNA replication, compounds that affect the nucleotide pool, for example purine and pyrimidine analogues, which integrate into DNA during S phase and block cell cycle progression, and finally DNA damaging agents such as plant alkylating agents, which act directly on DNA, causing cross-linking of DNA strands, abnormal base-pairing and DNA strand breaks (American Cancer Society, n.d.).

6.1.2.2. G1 and G2-phase arrest agents

G1 and G2-phase arrest agents include DNA damaging agents and inhibitors of protein synthesis and degradation. Clinically used synthetic DNA damaging agents in cancer therapy include the nitrogen mustards, while natural DNA damaging agents in clinical use include alkylating agents such as mitomycin c, and the antibiotic bleomycin (Hung *et al*, 1996).

6.1.2.3. G2 phase-specific arrest agents

Topoisomerase inhibitors are G2 phase-specific arrest agents. Topoisomerase I and II are enzymes that maintain the topology of DNA, and their inhibition interferes with both transcription and the replication of DNA by upsetting DNA supercoiling. Examples of topoisomerase II inhibitiors include etoposide and tenopiside from the Mayapple tree (Hung *et al*, 1996).

6.1.2.4. M-phase-specific arrest agents

Mitotic phase-specific arrest agents primarily act by interfering with cytoskeletal organization, predominantly affecting the microtubules required for spindle formation and chromosome segregation, although some agents interfere with actin, which is required for cytokinesis. Examples of compounds that prevent microtubule function during mitosis include the chemotherapeutic drugs paclitaxel (first isolated from the Pacific yew tree) and vinblastine (first isolated from the Madagascar periwinkle), which disrupt microtubule function, and monastrol, which blocks microtubule motor protein function (Fischer *et al*, 2004).

6.1.3. Alterations in cell cycle characteristics by OD, LD and PM

In the initial growth inhibition study OD, LD and PM were found to be toxic to a range of cancer cell lines at a range of dilutions, generally killing those cell lines at 1:10 while exerting an apparent growth inhibitory action at lower concentrations (compared to normal cell growth). Yet while these studies showed OD, LD and PM exerted a growth inhibitory action at lower concentrations, they did not address the issue of whether this occurred as a result of cell cycle arrest, as has been observed with other cytotoxic compounds (discussed above), or as a result of dose-related cell death. Thus, the aim of this study was to examine the role of cell cycle arrest in the observed cytotoxic actions of OD, LD and PM against cancer cells at lower concentrations. Furthermore, as cell cycle checkpoints can trigger apoptosis in response to large levels of intracellular damage, the role of checkpoint signalling in cell death mediated by OD, LD and PM at 1:10 was also investigated.

The ability of OD, LD and PM in their entirety to augment cell cycle characteristics had not been reported at the time of this study. However, a previous study had shown epicatechin gallate, a cytotoxic constituent of PM, could induce G1 arrest in the LoVo cell line (Xiaohua *et al*, 2000), thus suggesting the preparation of PM used in this study may contain a compound that can induce cell cycle arrest in cancer cells.

To examine the role of cell cycle arrest in the cytotoxic actions of OD, LD and PM the HL60 and HT29 cell lines were used. Two cell lines were used to examine potential cancer cell-type specific activity. The HL60 cell line was used because

each CHR generally exerted their greatest growth inhibitory action against this cell line in the growth inhibition study, and the HT29 cell line was used because each CHR also exerted a significant cytotoxic action against this cell line (although to a lesser extent), and because it is gut associated.

To look for evidence of cell cycle arrest in HL60 and HT29 cells exposed to OD, LD and PM, these cell lines were exposed to each CHR at a concentration of 1:50 and 1:100 for 24, 48 and 72 hours and their cell cycle characteristics compared to those of unchallenged cell populations. These two concentrations were used over a period of 72 hours to look for evidence of those CHRs exerting a time and dose-related growth inhibitory action without inducing an extensive cytotoxic action. The effects of OD, LD and PM at 1:50 and 1:100 were examined over a period of 72 hours to allow cell cycle progression in the presence of the selected CHRs before analysis.

To examine the role of cell cycle arrest in the previously observed growth inhibitory actions of OD, LD and PM, propidium iodide (PI) staining and FACS analysis were performed. Propidium iodide staining and FACS analysis were used as PI binds stochiometrically to double stranded DNA, thus cells in each stage of the cell cycle can be defined according to their relative DNA content. After acquisition of data, the relative percentage of cells in each stage of the cell cycle were calculated using the freeware software programme Cyclred, and compared to the relative percentage of cells in each stage of the cell cycle in control (unchallenged) cell populations. In addition to examining alterations in cell cycle characteristics, PI staining can also be used to look for evidence of cell death, characterized by sub-G1 regions. Thus, the role of cell death in the growth inhibitory actions of OD, LD and PM at 1:50 and 1:100 were also examined.

Finally, to examine the role of cell cycle checkpoint signalling (at G1, S or G2/M) in death mediated by OD, LD and PM at 1:10 in the HL60 and HT29 cell lines, histograms of viable and apoptotic populations were generated and overlaid using data from the TUNEL assay to determine whether cells were dying at a specific stage of the cell cycle, which would suggest specific cell cycle checkpoint activation

may have been responsible for the apoptosis-inducing actions of OD, LD and PM at 1:10.

6.2. Materials and Methods

6.2.1. Cell culture & CHR preparation

Cell culture and CHR preparation were performed as described in Chapter 3.

6.2.2. Cell cycle analysis using PI staining

HL60 and HT29 cell lines were seeded at a starting concentration of 10^5 cells/ml and OD, LD and PM added at 1:10, 1:50 and 1:100 for 24, 48 and 72 hours. Following incubation, cells were centrifuged for 5 minutes at 800rpm and the resultant pellet re-suspended in 1ml ice-cold 70% ethanol and left on ice for 30 minutes. Samples were then centrifuged at 800rpm for 5 minutes, the supernatant was then removed, the cell pellet washed twice in ice cold PBS and re-suspended in PBS (1ml). To remove double stranded RNA, 1 unit of DNAse free RNAse A was added to the cell suspension, and incubated for 30 minutes at 37°C. Finally, PI (100µl) was added to the cell suspension, and cells were stored on ice and protected from light until analysis. For cell cycle analysis 15,000 of all events were saved and the percentage of cells in each stage of the cell cycle calculated using the freeware Cyclred.

Figure 6.2.3.1 Data output from Cyclred

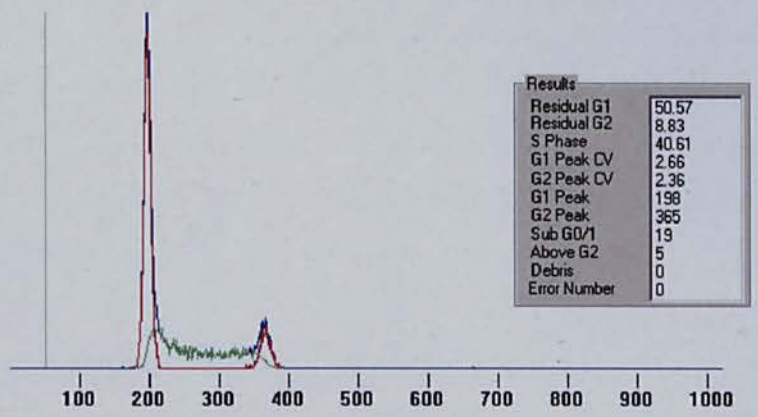


Figure 6.2.3.1 Cell cycle histogram analysis using Cyclred. The red region above channel 200 represents cells in G1. The green line represents cells in S phase of the cell cycle. The red region between channels 350 and 400 represent cells in G2 phase of the cell cycle. The results window shows the relative percentage of cells in each stage of the cell cycle.

6.2.3. TUNEL assay

TUNEL data were collected as described in Chapter 4. Analysis of the cell cycle stage in which apoptosis was being induced are expressed as overlaid histogram plots showing all cells, viable cells and apoptotic cells, with data gated from TUNEL density plots and FL2-Area versus FL2-Width plots, as described in the appendix (section A4).

6.3. Results

6.3.1. Alterations in HL60 and HT29 cell cycle characteristics by OD

Analysis of alterations in the cell cycle characteristics of HL60 cells exposed to OD at 1:50 and 1:100 using the freeware Cyclred did not generate a series of consistent time or dose-related results. However, visual inspection of the histograms generated showed dose and time dependent apoptotic induction (Figure 6.3.1). After 24 hours HL60 cells exposed to both 1:50 and 1:100 concentrations of OD showed normal cell cycle histograms, with low levels of apoptosing cells (Figures A and D). After 48 hours the number of viable cells was decreased, and the sub-G1 region grew accordingly, with the effect being more pronounced at 1:50 than 1:100 (Figures B and E respectively). After 72 hours at 1:50 most cells were apoptotic or dead (Figure C). At 1:100 after 72 hours there was still a visible cell cycle histogram, but it was smaller than at 24 or 48 hours, with a large apoptotic sub-G1 region (Figure F).

Cell cycle analysis of HT29 cells exposed to OD at 1:50 and 1:100 for 24, 48 and 72 hours did generate some consistent results (represented as bar charts, Figure 6.3.2), with the most notable effect being observed after 24 hours of incubation (Figure A). For this incubation time there was a dose dependent decrease in cells in G1, with a concomitant increase in cells in both S and G2 phases of the cell cycle. After 48 hours there was still a dose dependent decrease in cells in G1 phase of the cell cycle and a relative increase in cells in S and G2 compared to controls (Figure B). However, after 72 hours this pattern was no longer apparent (Figure C).

Overlaid histograms of TUNEL data showing viable and apoptotic HL60 and HT29 cells exposed to water extracts of OD at 1:10 for 4 and 24 hours respectively revealed apoptosis occurring in cells irrespective of their position in the cell cycle (Figure 6.3.3).

6.3.2. Alterations in cell cycle characteristics by LD

When analysed using Cyclred, cell cycle histograms of HL60 cells exposed to LD at 1:50 and 1:100 for 24, 48 and 72 hours did not generate a consistent set of time and dose-related data. However, visual inspection of the cell cycle histograms generated

showed dose and time dependent apoptotic induction (Figure 6.3.4). Figures A, B and C show the effects of LD at 1:50 after 24, 48 and 72 hours, respectively. At all time points there were consistent levels of apoptosing cells and over time a decrease in the relative numbers of viable cells. Figures D, E and F show the effect of LD at 1:100 at 24, 48 and 72 hours. At all time points there were low levels of apoptosing cells.

Cell cycle analysis of HT29 cells exposed to 1:50 and 1:100 concentrations of LD for 24, 48 and 72 hours did generate some consistent results (represented as bar charts) (Figure 6.3.5). Results showed a relative decrease in cells in G1 phase of the cycle compared to the control, with a concomitant increase in cells in S phase at all time points, and a small increase in the relative number of cells in G2 phase of the cell cycle.

TUNEL data examining the cell cycle stage in which HL60 and HT29 cells were dying after exposure to LD at 1:10 for 4 and 24 hours, respectively, revealed apoptosis occurring in all cell cycle stages (Figure 6.3.6).

6.3.3. Alterations in cell cycle characteristics by PM

Analysis of cell cycle histograms of HL60 cells exposed to PM at 1:50 and 1:100 for 24, 48 and 72 hours did not generate a consistent set of time or dose-related results. However, cell death was shown at both concentrations, with a greater effect observed at 1:50 compared to 1:100 (Figure 6.3.7). Figures A, B and C show the effect of a 1:50 extract of the CHR after 24, 48 and 72 hours. After a 24 hour exposure at 1:50, there are few viable cells within the cell cycle (between channels 200 and 400 on the x-axis), and after 48 and 72 hours of exposure no viable cells are visible. At 24 hours there was a distinct cell cycle histogram with cells clearly visible in G1, S and G2 phases of the cell cycle, and a defined sub-G1 region. After 48 hours the number of viable cells decreased, and after 72 hours the majority of events scored were in the sub-G1 region. Although there was an increase in the relative number of cells in the sub-G1 region, a characteristic sub-G1 peak was not visible.

Cell cycle analysis of HT29 cells exposed to 1:50 and 1:100 extracts of PM for 24, 48 and 72 hours using Cyclred generated consistent time and dose-dependent results (Figure 6.3.8): there was a relative decrease in cells in G1 phase of the cycle compared to the control, with a concomitant increase in cells in both S and G2 phases of the cell cycle. This effect was most obvious after 24 hours (Figure A), and could also be seen after 48 hours of incubation. After 72 hours, the relative numbers of cells in each stage of the cycle were similar to those of the controls.

Overlaid histograms of viable and apoptotic HL60 and HT29 cell populations exposed to PM at 1:10 for 4 and 24 hours, respectively, revealed cells dying in a cell cycle-independent manner (Figure 6.3.9).

Figure 6.3.1 Cell cycle analysis of the HL60 cell line exposed to 1:50 and 1:100 extracts of OD for 24, 48 and 72 hours

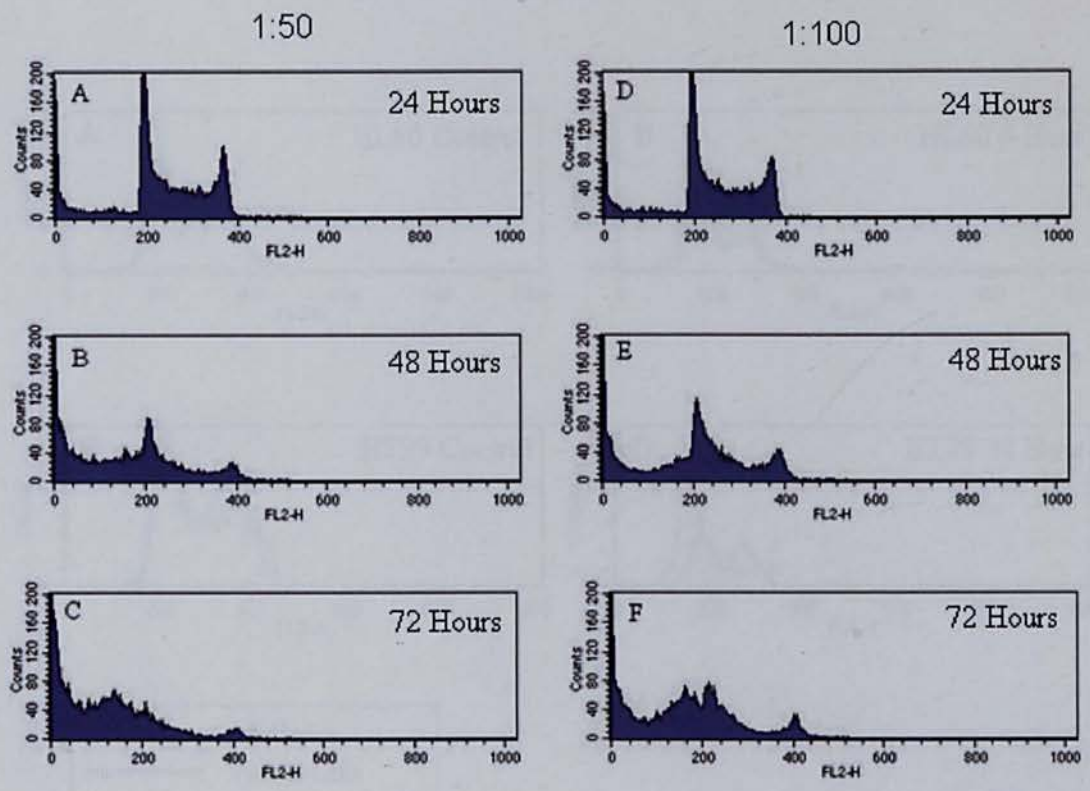


Figure 6.3.1 Cell cycle histograms of HL60 cells exposed to OD at 1:50 and 1:100. The peak in channel 200 shows cells in G1, and the peak in 400 cells in G2 of the cell cycle. The sub-G1 region represents apoptotic cells. Figures 6A-C show the effects of a 1:50 extract on cell cycle characteristics after 24, 48 and 72 hours, respectively. Figures 6D-F show the effects of a 1:100 extract after 24, 48 and 72 hours, respectively.

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Figure 6.3.3 Overlaid histograms from TUNEL assay data showing where in the cell cycle HL60 and HT29 cells apoptosed when exposed to a 1:10 extract of OD for 4 hours and 24 hours, respectively

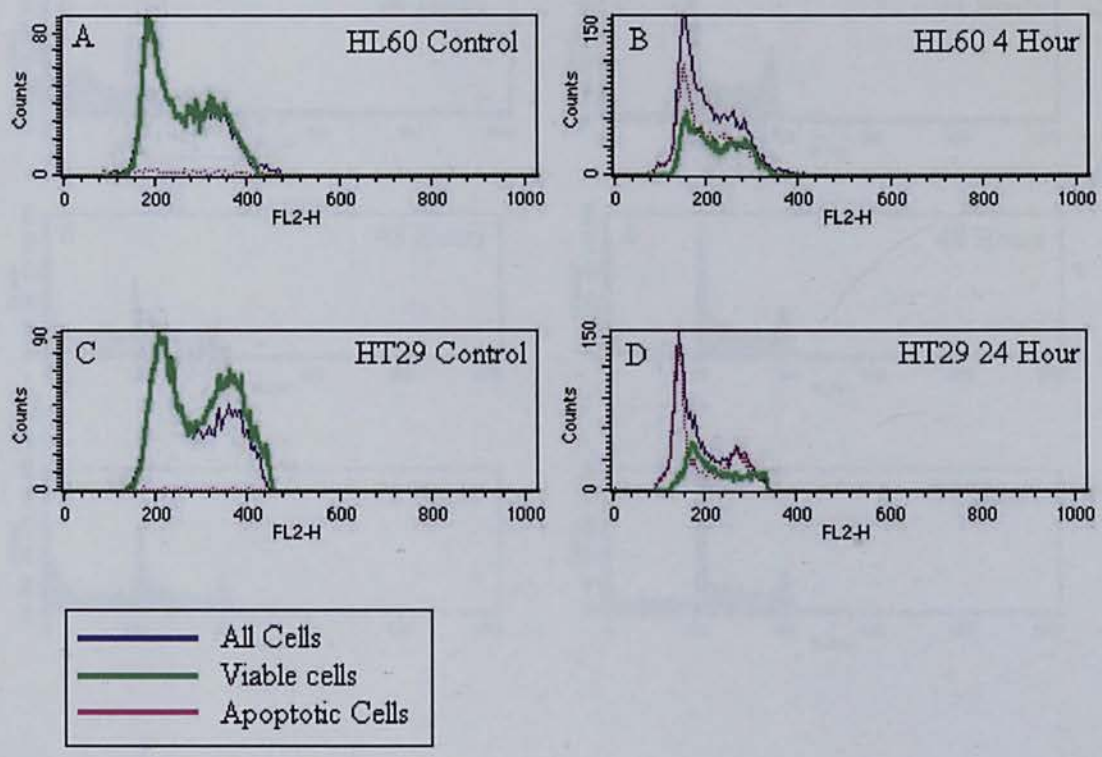


Figure 6.3.3 Overlaid cell cycle histograms generated using gated TUNEL assay data. The x axis shows the relative DNA content of individual cells, the y axis shows cell number. The first peaks, above channel 200 represent cells in G1, the second peak represents cells in G2 phase of the cell cycle. The purple line represents all cells analyzed, the green line represents those cells that are still viable and the pink line represents cells with an increased number of double strand breaks, which is indicative of apoptosis. Figure A: HL60 control, B: HL60 cells exposed to OD for 4 hours, C: HT29 control, and D: HT29 cells exposed to OD for 24 hours.

Figure 6.3.4 Cell cycle analysis of the HL60 cell line exposed to LD at 1:50 and 1:100 for 24, 48 and 72 hours

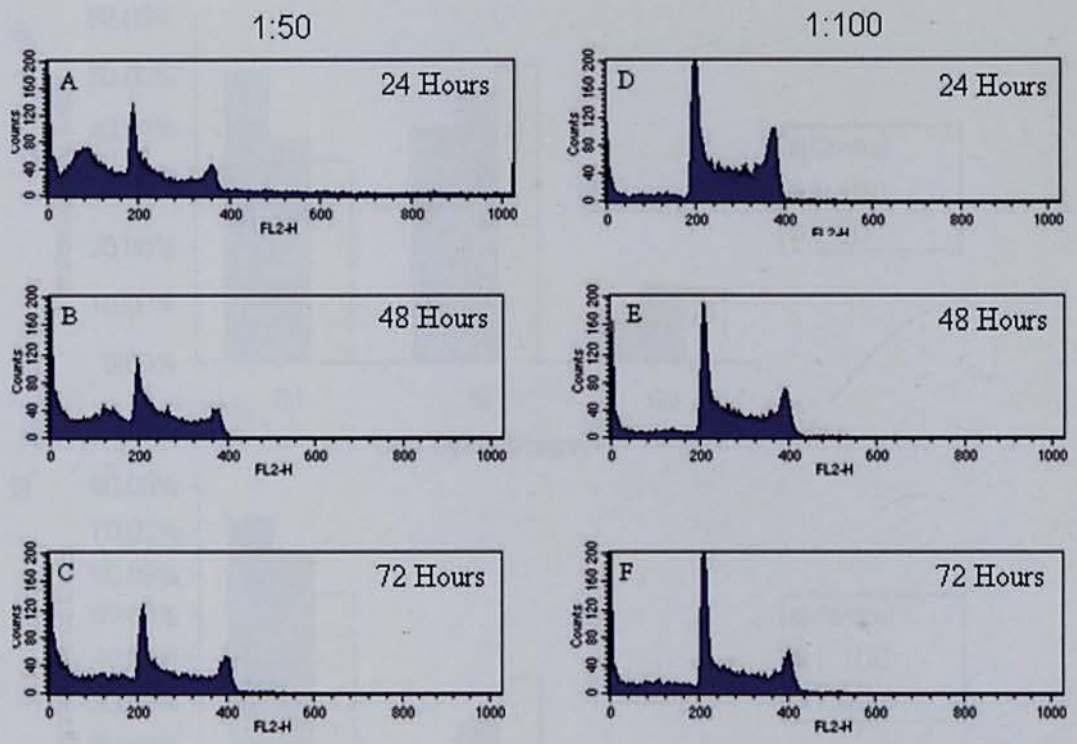


Figure 6.3.4 Cell cycle histograms of HL60 cells exposed to LD at 1:50 and 1:100. The peak in channel 200 shows cells in G1, and the peak in 400 cells in G2 of the cell cycle. The Sub-G1 region is apoptotic cells. Figures 5A-C show the effects of a LD at 1:50 on cell cycle characteristics after 24, 48 and 72 hours, respectively. Figures 5D-F show the effects of a 1:100 extract after 24, 48 and 72 hours, respectively.

Figure 6.3.5 Cell cycle analysis of HT29 cells exposed to 1:50 and 1:100 extracts of LD for 24, 48 and 72 hours compared to controls

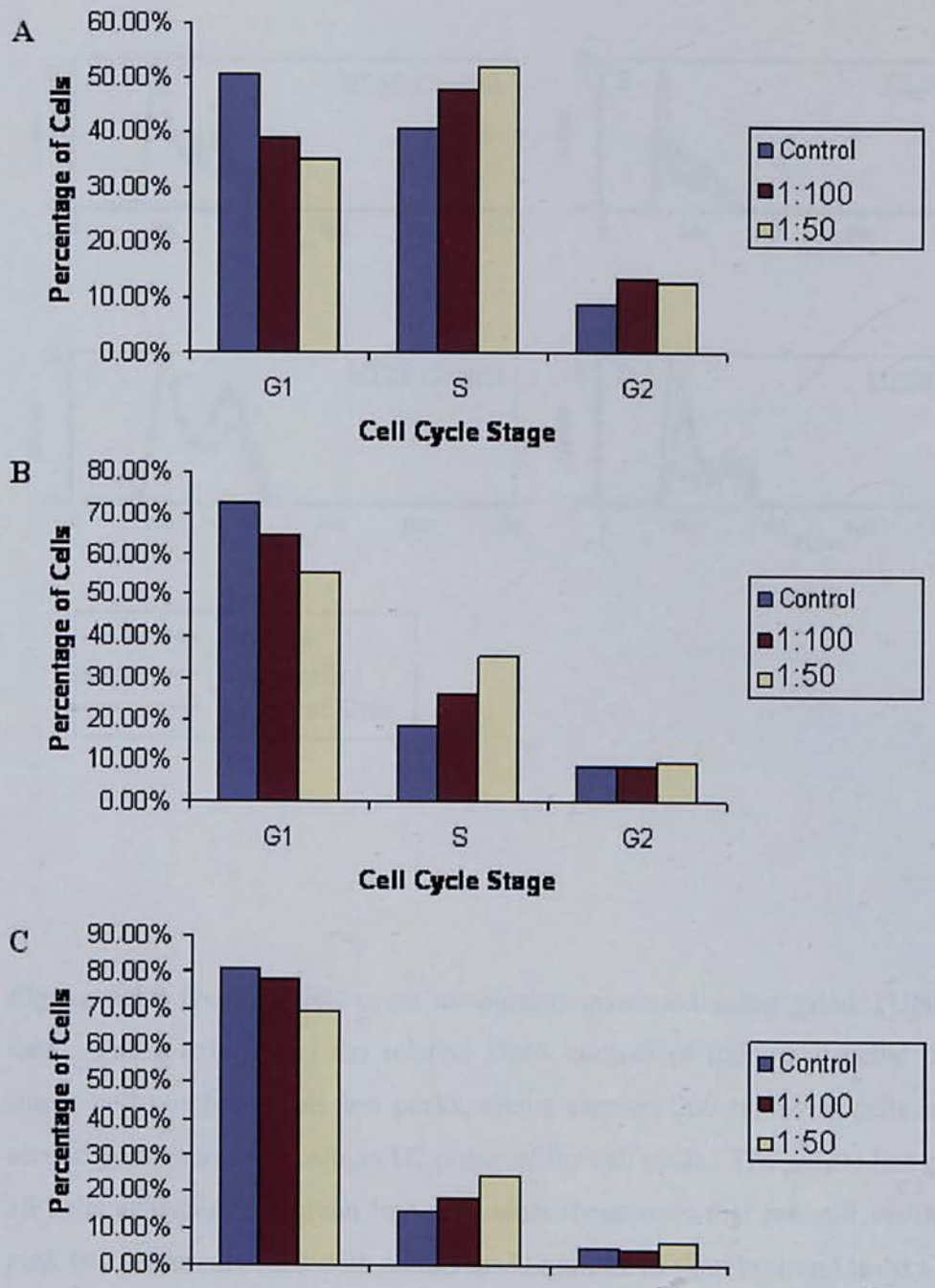


Figure 6.3.5 The effect of LD at 1:50 and 1:100 on the cell cycle characteristics of HT29 cells compared to controls. Figure 6A: the effect after 24 hours, 6B: the effect after 48 hours and 6C: the effect after 72 hours.

Figure 6.3.6 Overlaid histograms from TUNEL assay data showing where in the cell cycle HL60 and HT29 cells are apoptosing when exposed to LD at 1:10 for 4 hours and 24 hours, respectively

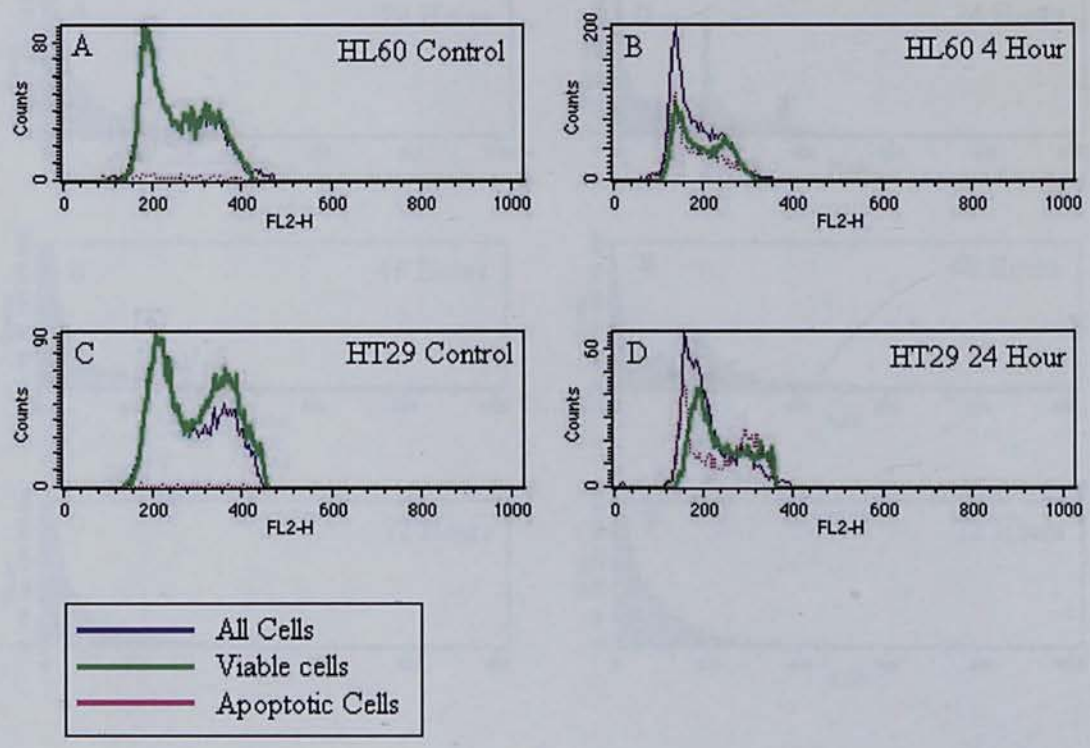


Figure 6.3.6 Overlaid cell cycle histograms generated using gated TUNEL assay data. The x axis shows the relative DNA content of individual cells, the y axis shows cell number. The first peaks, above channel 200 represent cells in G1, the second peak represents cells in G2 phase of the cell cycle. The purple line represents all cells analyzed, the green line represents those cells that are still viable and the pink line represents cells with an increased number of double strand breaks, which is indicative of apoptosis. Figure A: HL60 control, B: HL60 cells exposed to LD for 4 hours, C: HT29 control and D: HT29 cells exposed to LD for 24 hours.

Figure 6.3.7 Cell cycle analysis of the HL60 cell line exposed to PM at 1:50 and 1:100 for 24, 48 and 72 hours

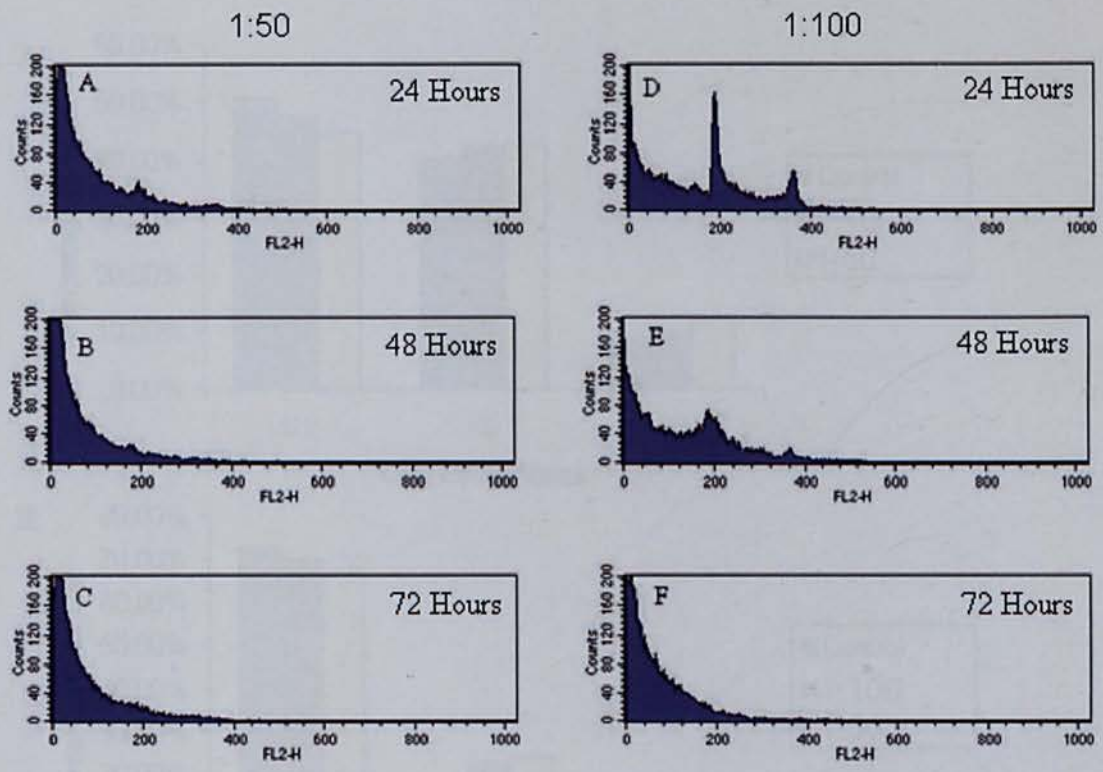


Figure 6.3.7 Cell cycle histograms of HL60 cells exposed to PM at 1:50 and 1:100. The peak in channel 200 shows cells in G1, and the peak in 400 cells in phase G2 of the cell cycle. The Sub-G1 region is apoptotic cells. Figures 5.A-C show the effects of a 1:50 extract on cell cycle characteristics after 24, 48 and 72 hours, respectively. Figures 5 D-F show the effects of a 1:100 extract after 24, 48 and 72 hours, respectively.

Figure 6.3.8 Cell cycle analysis of HT29 cells exposed to PM at 1:50 and 1:100 for 24, 48 and 72 hours compared to controls

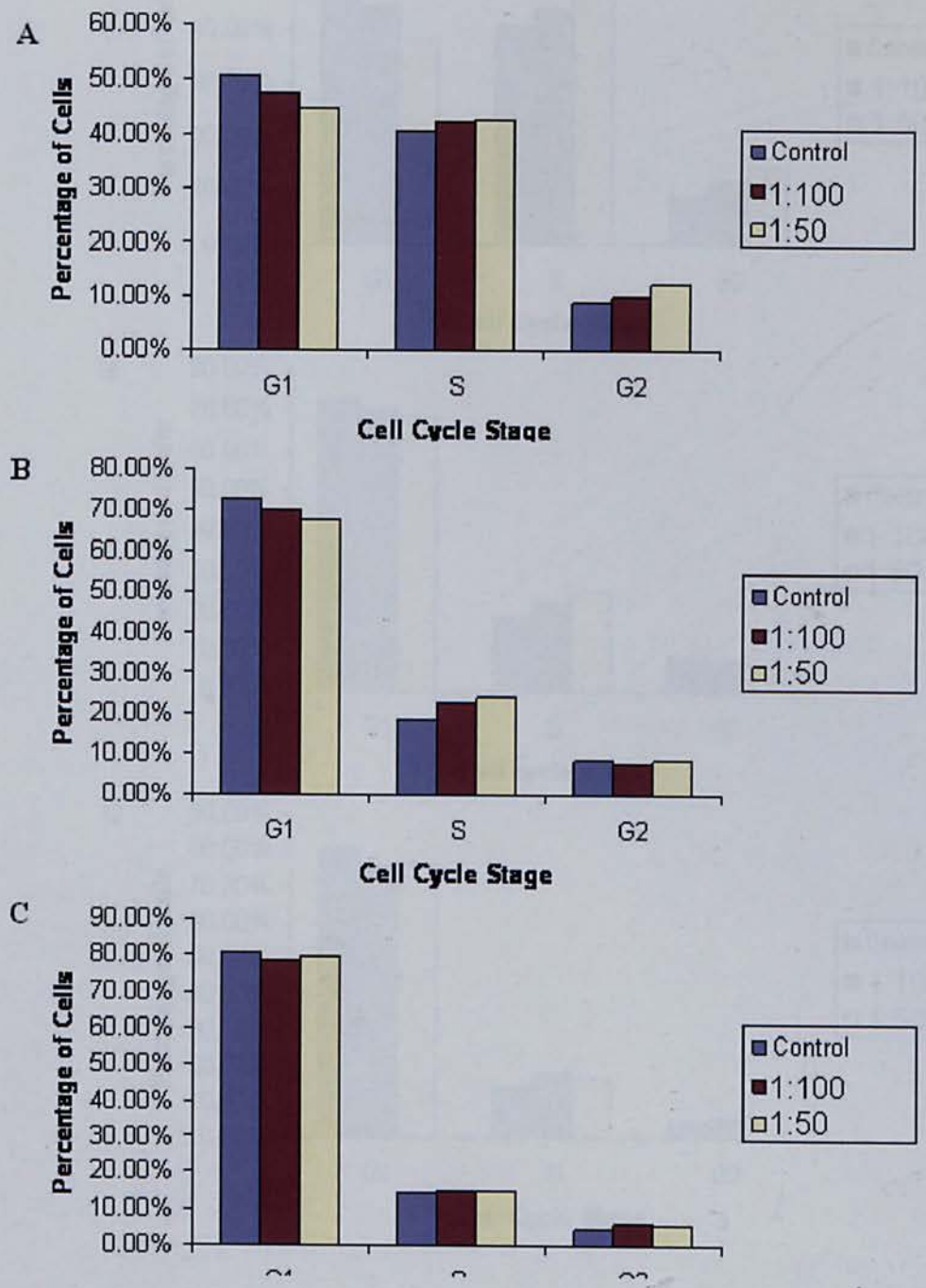


Figure 6.3.8 Shows the effect of PM on the cell cycle characteristics of HT29 cells compared to controls. Figure 6.A shows the effect after 24 hours, 6.B the effect after 48 hours and 6.C the effect after 72 hours.

Figure 6.3.2 Cell cycle analysis of HT29 cells exposed to OD at 1:50 and 1:100 for 24, 48 and 72 hours compared to controls

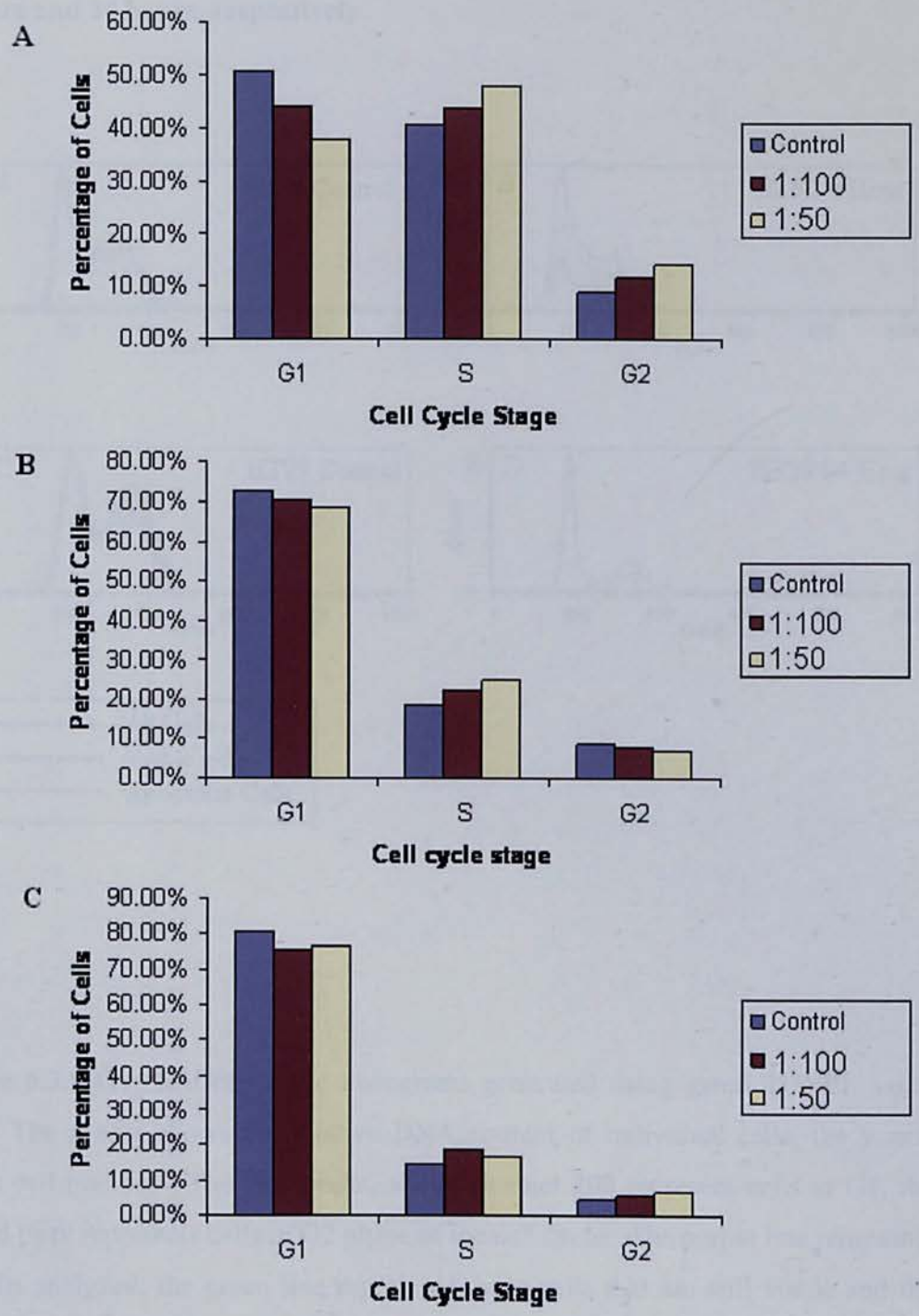


Figure 6.3.2 The effect of OD at 1:50 and 1:100 on the cell cycle characteristics of HT29 cells compared to controls. Figure 7A: the effect after 24 hours, 7B: the effect after 48 hours and 7C: the effect after 72 hours.

Figure 6.3.9 Overlaid histograms from TUNEL assay data showing where in the cell cycle HL60 and HT29 cells were apoptosing when exposed to PM at 1:10 for 4 hours and 24 hours, respectively

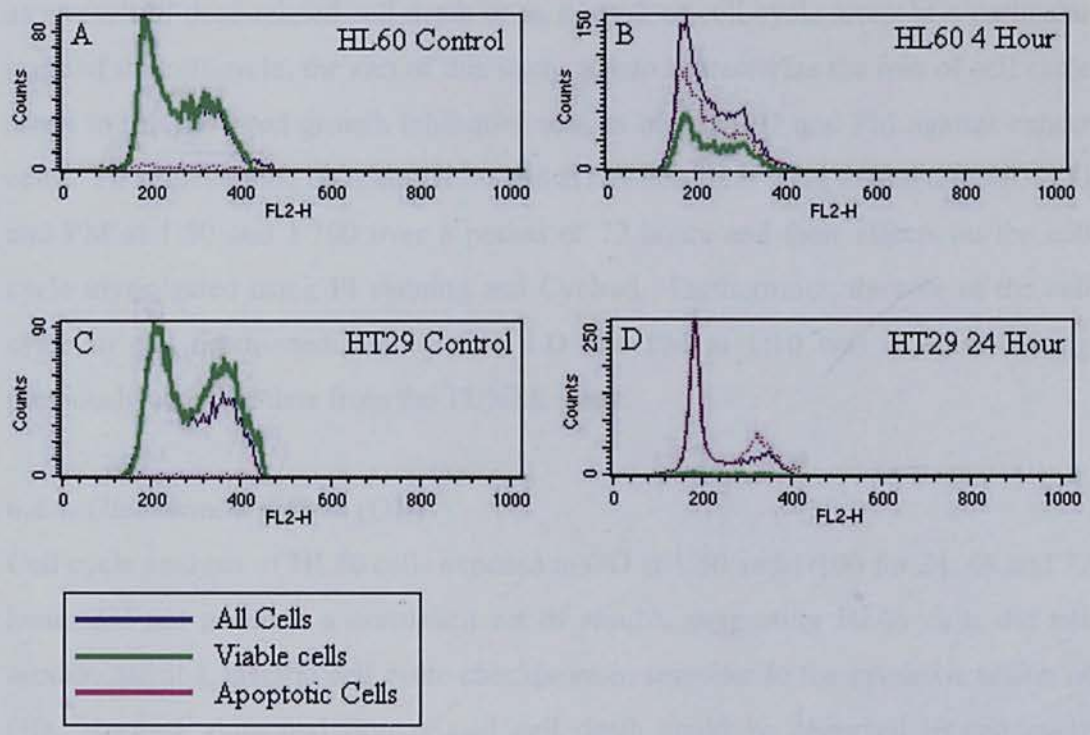


Figure 6.3.9 Overlaid cell cycle histograms generated using gated TUNEL assay data. The x axis shows the relative DNA content of individual cells, the y axis shows cell number. The first peaks, above channel 200 represent cells in G1, the second peak represents cells in G2 phase of the cell cycle. The purple line represents all cells analyzed, the green line represents those cells that are still viable and the pink line represents cells with an increased number of double strand breaks, which is indicative of apoptosis. Figure A: HL60 control, B: HL60 cells exposed to PM for 4 hours, C: HT29 control, and D: HT29 cells exposed to PM for 24 hours.

6.4. Discussion

The results of the growth inhibition study showed OD, LD and PM to be significantly toxic to a range of cancer cell lines at 1:10. Furthermore, OD, LD and PM were found to induce an apparent dose-related growth inhibitory action at lower concentrations. In order to determine whether this growth inhibitory action occurred as a result of dose-related cell death or as a result of cell cycle arrest at a particular stage of the cell cycle, the aim of this study was to characterize the role of cell cycle arrest in the observed growth inhibitory actions of OD, LD and PM against cancer cells. To achieve this, both the HL60 and HT29 cell lines were exposed to OD, LD and PM at 1:50 and 1:100 over a period of 72 hours and their effects on the cell cycle investigated using PI staining and Cyclred. Furthermore, the role of the cell cycle in cell death mediated by OD, LD and PM at 1:10 was examined using previously acquired data from the TUNEL assay.

6.4.1. *Oldenlandia diffusa* (OD)

Cell cycle analysis of HL60 cells exposed to OD at 1:50 and 1:100 for 24, 48 and 72 hours did not generate a consistent set of results, suggesting HL60 cells did not accumulate at a specific cell cycle checkpoint in response to the cytotoxic action of OD. Instead, dose and time-related cell death could be observed in cell cycle histograms of HL60 cells exposed OD at 1:50 and 1:100, characterized by distinct sub-G1 peaks. Thus, these results suggest that the dose-related growth inhibitory action of OD against HL60 cells which was observed in the growth inhibition study occurred as a result of dose-dependent apoptotic induction, and was not related to cell cycle arrest. In addition, overlaid cell cycle histograms of HL60 cells exposed to OD at 1:10 for 4 hours revealed apoptotic induction occurring in cells in every stage of the cell cycle, thus suggesting the cytotoxic action of OD at 1:10 in the HL60 cell line is independent of pro-apoptotic signalling from cell cycle checkpoints.

In contrast to the apparent cell cycle-independent action of OD on HL60 cells at 1:50 and 1:100, a consistent series of time and dose-related alterations in cycle characteristics were observed in HT29 cells exposed to OD at 1:50 and 1:100 (compared to control cell growth). This effect was most pronounced after 24 hours of exposure, at which time a dose-related decrease in cells in G1 could be observed,

with a concomitant increase in the percentage of cells in both S and G2 phases of the cell cycle. After 48 hours a dose-related increase in the number of cells in S-phase could still be observed, however at this time point there was a concomitant decrease in both the number of cells in G1 and G2 phases of the cell cycle. Finally, after 72 hours of exposure, cell cycle characteristics appeared to normalize. Thus, the results of this study suggest that OD inhibited the progression of HT29 cells through S phase of the cell cycle after 24 and 48 hours of exposure, and also induced some arrest at G2 after 24 hours of exposure, while having no effect on the G1 checkpoint, and that this activity may have contributed to the previously observed growth inhibitory action of OD in the HT29 cell line at 1:100 dilution.

The S phase checkpoint, which appeared to be triggered by OD in the HT29 cell line after 24 and 48 hours of exposure, is activated by genotoxic insults that occur during S phase, and causes transient, reversible delays in cell cycle progression, mainly by inhibition of new replicon initiation and thereby the slowing down of DNA replication (Lukas *et al*, 2004). S phase arrest can be triggered by compounds that directly damage DNA, compounds that inhibit DNA synthesis, and antimetabolites (compounds that resemble metabolites and interfere with physiological reactions involving it) (American Cancer Society, n.d.), therefore the results of this study suggest that water extracts of OD may posses one or more of these types of compounds, and these may be responsible for the ability of OD to transiently arrest S phase progression. However, to the authors knowledge compounds with these properties have not previously been identified in OD extracts.

The G2 checkpoint, which appeared to be triggered after 24 hours of exposure to OD in the HT29 cell line, prevents cells from initiating mitosis when they experience direct or indirect DNA damage during G2. Several classes of compounds cause G2 arrest, and include DNA damaging agents, topoisomerase inhibitors and inhibitors of proteins synthesis and degradation (Hung *et al*, 1996). G2 cell cycle arrest occurs as a result of a combination of mechanisms that operate via post-translational modifications of diverse effector proteins, and more delayed and sustained mechanisms that involve alterations of transcriptional mechanisms. A key downstream target of the G2 checkpoint is the major mitosis-promoting activity of the cyclin B/Cdk1 kinase. After DNA damage, activation of cyclin B/Cdk1 is

prevented through ATM/ATR and Chk1/(Chk2)- mediated subcellular sequestration and/or inhibition of the Cdc25C phosphatase that normally activates Cdk1 at the G2/M boundary (Lukas *et al*, 2004). As the results of this study suggest that OD may be inducing transient G2 arrest after 24 hours of exposure, it is possible that OD may posses compound that trigger G2 arrest in the HT29 cell line. It is also possible that OD may posses M-phase-specific arrest compounds that transiently interfere with cytoskeletal organization (Fischer *et al*, 2004), which would also result in the accumulation of cells in G2.

The results of this study also suggest that G1 arrest was not triggered by OD in the HT29 cell line at 1:50 or 1:100. The role of the G1 checkpoint is to prevent entry into S phase with damaged DNA, and this damage can be caused by compounds that directly damage DNA and compounds that interfere with DNA and protein synthesis and degradation (Hung *et al*, 1996). In response to the detection of DNA damage, cells passing through G1 activate the checkpoint transducing kinases ATM/ATR and Chk1/Chk2, which in turn target two effector proteins operating in distinct branches of the G1 checkpoint: Cdc25A phosphatase and the transcription factor p53. Phosphorylation of Cdc25A by Chk1 and Chk2 leads to enhanced ubiquitination and proteasome-mediated degradation of Cdc25A, thereby preventing Cdc25A mediated activatory dephosphorylation of Cdk2, the catalytic subunit of cyclin E/Cdk 2 and cyclin A/Cdk2 kinases. Inhibition of Cdk2 blocks loading onto chromatin of Cdc45, a protein required for recruitment of DNA polymerase α into assembled pre-replication complexes, thus preventing initiation of DNA synthesis. The checkpoint pathway that targets Cdc25A is implemented rapidly, operates independently of p53 status and is relatively transient, only delaying cell cycle for several hours.

In contrast, the prolonged maintenance of cell cycle arrest in response to DNA damage at the G1 checkpoint is dependent on p53. p53 is phosphorylated by Chk1/Chk2 and the upstream checkpoint kinases ATM/ATR. In addition, the ubiquitin ligase Mdm2 that normally binds p53 to ensure rapid turnover, is also targeted by ATM/ATR in response to DNA damage. Stabilization and accumulation of p53 protein results in its increased activity as a transcription factor. The key effector of p53-dependent transcription in relation to G1 arrest is the p21 (WAF1/Cip1) inhibitor of Cdk's. Increased levels of p21 after DNA damage blocks

G1/S. This takes several hours, and compliments the transient acute inhibition of Cdk2 (Lukas *et al*, 2004). The lack of G1-specific cell cycle arrest by OD in the HT29 cell line may be a result of OD not containing any compounds that induce G1 arrest. However, previous studies have revealed that the HT29 cell line is p53 mutant (with a point mutation (Arg-248→His)) (Rodrigues *et al*, 1990), and as a lack of functional p53 can prevent the prolonged arrest of cells with DNA damage at the G1 checkpoint, this could also account for the lack of G1 arrest observed in this study.

In contrast to the cell cycle-related action of OD on the HT29 cell line at 1:50 and 1:100, results from the TUNEL assay suggested that at 1:10 OD induced apoptosis in all cell cycle stages. This suggests that the cytotoxic action of OD at 1:10 is not related to pro-apoptotic signalling from cell cycle checkpoints at a particular cell cycle stage, as suggested above, and is instead triggered in all cell cycle stages through pathways not related to cell cycle checkpoints.

The ability of OD to augment cell cycle characteristics in cancer cell lines *in vitro* has not previously been examined. However, the observation that apoptosis is triggered in the HL60 cell line after exposure to OD at 1:50 and 1:100 while cell cycle arrest occurs in the HT29 cell line may be explained by previous studies that have shown HL60 cells to readily undergo apoptosis in response to a variety of stimuli, while HT29 cells are usually resistant to such pro-apoptotic stimuli (Shao *et al*, 1996).

6.4.2. Long Dan Xie Gan Wan (LD)

Cell cycle analysis of HL60 cells exposed to LD at 1:50 and 1:100 for 24, 48 and 72 hours using Cyclred did not generate consistent time or dose-dependent results. Instead, clear sub-G1 peaks could be observed in histograms of HL60 cells exposed to LD at 1:50 and 1:100. Thus, the results of this study suggest that the dose-related growth inhibitory action of LD at 1:100, as seen in the growth inhibition study, was related to apoptotic induction and not cell cycle arrest. Furthermore, overlaid histograms of TUNEL data showed HL60 cells were dying irrespective of their position in the cell cycle when exposed to LD at 1:10 for 4 hours, thus suggesting the cytotoxic action of LD at 1:10 is also cell cycle-independent.

In contrast to the lack of consistent cell cycle data obtained with the HL60 cell line, HT29 cells exposed to LD at 1:50 and 1:100 for 24, 48 and 72 hours exhibited a clear change in cell cycle characteristics, with a dose-related decrease in cells in G1 at all time points, an increase in cells in S phase at all time points, and a small change in the number of cells in G2 compared to the control. Thus, the results of this study suggest that the growth inhibitory action of LD at 1:50 and 1:100 in the HT29 cell line may be related to transient growth arrest during S-phase of the cell cycle. In contrast, overlaid histograms of TUNEL data revealed that at 1:10, LD induced apoptosis in a cell cycle-independent fashion. This may suggest that at high concentrations LD triggers apoptosis through the induction of large amounts of cellular damage, while at lower concentrations it induces less damage, and instead triggers S phase cell cycle arrest in the HT29 cell line.

As S phase arrest can be triggered by compounds that directly damage DNA, antimetabolites, and compounds that inhibit DNA synthesis (American Cancer Society, n.d.), the results of this study suggest that water extracts of LD may posses one or more of these types of compounds, and that these they may be responsible for the ability of LD to transiently arrest S phase progression in HT29 cells at 1:50 and 1:100.

The ability of LD in its entirety to augment the cell cycle characteristics of cancer cell lines *in vitro* has not previously been reported. However, other studies (published after the initial literature review, see Chapter 2) have examined the ability of some of its constituents to augment the cell cycle characteristics of cancer cell lines *in vitro*.

One of the major components found within *Fructvs gardeniae* (a constituent of LD) is the glycoside geniposide. As the isolation of glycosides is difficult due to their high polarity, the anticancer activity of the acetylated product of geniposide ($(Ac)_5GP$) has been examined (Peng *et al*, 2004). $(Ac)_5GP$ has been found cytotoxic to rat C6 glioma cells *in vitro*, inducing apoptosis and cell cycle arrest at G_0/G_1 by increasing levels of c-Myc, p53 and pro-apoptotic Bax while decreasing levels of anti-apoptotic Bcl-2 (Chang *et al*, 2002). In addition, a triterpene derivative,

saikosaponin D, isolated from *Radix bupleuri* (another constituent of LD), has been found to inhibit the growth of A549 lung cancer cells dose dependently, arresting cells in G1 phase of the cell cycle through activation of p53 and p21/WAF (Hsu *et al*, 2004). However, as the results of this study did not provide evidence of LD inducing cell cycle arrest at G1 in the HT29 cell line, it is unlikely that these compounds augmented cell cycle characteristics in this study. This may be due to the fact that these compounds were present in low levels, or absent, in this preparation of LD, or that they were present and did not affect cell cycle progression because the HT29 cell line is p53 negative (as discussed above). It is also possible that the cytotoxic actions of these compounds were counteracted by other compounds within this preparation of LD (Yuan & Lin, 2000).

In another study, acetone extracts of *Radix bupleuri* were found to induce cell cycle arrest at the G2/M checkpoint in A549 cells. A shift of soluble tubulin to a polymerized form suggested microtubules were stabilized by *Radix bupleuri*, which interfered with normal functioning of the mitotic spindle and contributed to the G2/M block (Cheng *et al*, 2005). However, only slight and transient G2/M arrest was seen in this study after 24 hours of exposure, which may suggest that those compounds in *Radix bupleuri* that induced G2/M arrest in the A549 cell line were absent in water extracts of LD, were present in levels too low to exert a significant growth inhibitory action, or were counteracted by other compounds.

A separate study showed that baicalein, isolated from *Radix scutellariae* (also an LD constituent) could inhibit the growth of human lung carcinoma CH27 cells through arrest in S phase of the cell cycle and induction of apoptosis (Lee *et al*, 2005). As S phase arrest was observed in this study, baicalein may have contributed to arrest observed in the HT29 cell line here. However, the levels of baicalein present in this CHR preparation are unknown, therefore the role of baicalein in the S phase inhibitory action of LD against the HT29 cell line remains to be elucidated.

The results of this study also showed LD was significantly toxic to the HL60 cell line at 1:50 and 1:100, and was less toxic to the HT29 cell line. Again, this may be explained by previous studies that have shown HL60 cells to readily apoptose in

response to a variety of stimuli, while HT29 cells are generally resistant to apoptosis (Shao *et al*, 1996).

6.4.3. *Polygonum multiflorum* (PM)

The results of this study suggest that PM does not induce cell cycle arrest in the HL60 cell line at 150 or 1:100. Furthermore, the results of this study suggest that the dose-related growth inhibitory action of PM against HL60 cells occurs as a result of dose related cell death: sub-G1 regions could be observed in cell cycle histograms of HL60 cells exposed to PM at 1:50 and 1:100 for 24, 48 and 72 hours. In addition, overlaid histograms of TUNEL data suggest that the cytotoxic action of PM against the HL60 cell line at 1:10 is also cell cycle independent. This trend could also be observed in HT29 cells exposed to PM at 1:10 for 24 hours.

In contrast, there was evidence of cell cycle arrest in HT29 cell populations exposed to PM at 1:50 and 1:100 after 24 and 48 hours of exposure (compared to control cell populations). After 24 hours of exposure to PM a dose-related decrease in the number of cells in G1 could be seen, along with a concomitant increase in the number of cells in both S and G2 phases of the cell cycle. After 48 hours a relative increase in the number of cells in S phase could still be observed, and after 72 hours cell cycle characteristics appeared to normalize. Thus, the results of this study suggest that PM may contain compounds that transiently inhibit the progression of cells through S phase, and may also trigger some cell cycle arrest at the G2 checkpoint within 24 hours of initial exposure. Thus, PM may elicit a growth inhibitory action in the HT29 cell line at 1:50 and 1:100 through the induction of transient cell cycle arrest, and not cell death, while 1:10 extracts of PM trigger cell death in a cell cycle-independent manner.

The ability of PM in its entirety to augment cell cycle characteristics has not previously been reported. However, a previous study has found that epicatechin gallate, a constituent of PM, could induce G1 arrest in the LoVo cell line (Xiaohua *et al*, 2000). However, as G1 arrest was not observed in this study, these results suggest epicatechin gallate did not exert any action in this study. This possible inaction of epicatechin gallate may be due to the fact that the HT29 cell line is p53 negative, and may therefore be resistant to agents that induce G1 arrest, or to the fact

that epicatechin gallate was not present in this preparation of PM, or was present at low levels, and did not exert an effect. It may also be possible that other compounds present within this preparation of PM may have counteracted the action of epicatechin gallate. That PM exerted a toxic action on the HL60 cell line and a moderate growth inhibitory action on the HT29 cell line may again be explained by the relative resistance of HT29 cell lines to apoptosis compared to HL60 cells (Shao *et al*, 1996).

6.4.4. Conclusion

The aim of this investigation was to characterize the role of cell cycle arrest in the previously observed growth inhibitory actions of OD, LD and PM against cancer cell lines *in vitro*. The results of this study showed that each CHR generally exerted a cytotoxic action against the HL60 cell line at 1:10, 1:50 and 1:100, but did not induce cell cycle arrest. In contrast, OD, LD and PM generally augmented the cell cycle characteristics of the HT29 cell line in a dose related fashion at 1:50 and 1:100, primarily through inhibition of S phase progression, thus suggesting that these CHRs may exert a growth inhibitory action in the HT29 cell line at lower concentrations through induction of cell cycle arrest. The ability of OD, LD and PM to augment the cell cycle characteristic of cancer cell lines *in vitro* have not previously been reported. However, the disparate results generated for the HL60 and HT29 cell lines may be explained through previous observations that the HT29 cell line is generally more resistant to apoptosis than the HL60 cell line (Shao *et al*, 1996). As such, these results suggest that the ability of OD, LD and PM to augment the cell cycle characteristics of other cancer cell types may vary depending on their relative resistance to pro-apoptotic stimuli. However, although these results suggest cell cycle arrest may have occurred in the HT29 cell line after exposure to these CHRs, they do not provide information regarding what compounds(s) were responsible for this activity, or which cell cycle arrest pathways were activated in response to the cytotoxic actions of those CHRs.

Chapter 7. The genotoxic potential of *Oldenlandia diffusa*, Long Dan Xie Gan Wan and *Polygonum multiflorum* against cancer cells

7.1. Introduction

Nuclear DNA carries a cell's genetic information and hereditary characteristics via its nucleotides and their sequence. Structural damage to the DNA molecule, which can occur spontaneously or as a result of interactions with its environment, can alter or eliminate a cell's ability to transcribe genes within affected areas, and can also lead to the induction of potentially harmful mutations in the cell's genome, which may lead to cancer. As such, the DNA of a cell is constantly monitored for sites of damage by a range of surveillance molecules. If damage is found in a cell as it progresses through the cell cycle arrest may occur to allow time for repair, or if that damage is too great apoptosis may be triggered through activation of the intrinsic signalling cascade (Turner *et al*, 2000).

Due to the strict surveillance mechanisms that ensure the integrity of the genome, DNA is a common target for chemotherapeutic agents. Furthermore, many naturally occurring cytotoxic compounds have been found to exert their biological action through the induction of DNA damage. Examples of compounds that damage DNA include alkylating agents, which add alkyl groups to nucleic acids, oxidising agents, which generate free oxygen radicals that can damage DNA, intercalators, which insert themselves between base-pairs and cause local unwinding of DNA, and topoisomerase inhibitors. The types of DNA damage induced by these agents include the generation of single strand breaks, double strand breaks, the addition of bulky adducts to nucleic acids, and DNA strand cross-linking (Drazin & Maltzman, 2003).

7.1.1. The genotoxic potential of OD, LD and PM

Previous results from this investigation suggested that water extracts of OD, LD and PM were significantly toxic to a range of cancer cell lines at a range of concentrations (Chapter 3). Furthermore, the results of this investigation suggested that OD and LD induced apoptosis in the HL60 and HT29 cell lines (Chapter 4), and that this occurred in the HL60 cell line as a result of activation of the intrinsic caspase cascade (which can be activated in response to the induction of DNA

damage) (Kroemer, 2002) (Chapter 5). In addition, the results of this investigation suggested that PM was inducing necrosis in the HL60 and HT29 cell lines at 1:10, and that the false-positive results of the TUNEL and PI staining assays may have occurred as a result of PM inducing large amounts of genomic fragmentation in those cell lines: these assays examine genomic fragmentation as a marker of apoptotic induction (Otsuki *et al*, 2003) (Chapter 4). Furthermore, analysis of HL60 and HT29 cell lines exposed to 1:50 and 1:100 concentrations of OD, LD and PM revealed that, while the apparent growth inhibitory action of OD, LD and PM occurred as a result of the induction of dose-related cell death in the HL60 cell line, HT29 cells exposed to lower concentrations of OD, LD and PM exhibited transient S phase (and to a lesser extent G2) cell cycle arrest, which can be triggered by the induction of DNA damage (Hung *et al*, 1996) (Chapter 6).

Thus, as all of the above characteristics can potentially occur as a result of the induction of DNA damage, the aim of this study was to determine whether water extracts of OD, LD and PM possessed the ability to induce DNA damage in cancer cell lines *in vitro*. The ability of OD, LD and PM in their entirety to induce DNA damage in cancer cells *in vitro* had not previously been reported, therefore this investigation had the potential to provide more information regarding the mode of cytotoxic action of these CHRs against cancer cell lines.

To examine the ability of OD, LD and PM to induce DNA damage in cancer cell lines *in vitro* the HL60 and HT29 cell lines were used. These cell lines were used because the cytotoxic actions of OD, LD and PM against these cell lines had been characterised in previous chapters.

To assess the genotoxic action of OD, LD and PM against the HL60 and HT29 cell lines, the pH>13 version of the single cell gel (comet) assay was used. This assay was selected as it is a commonly used, highly sensitive method of detecting low levels of various types of DNA damage in individual cells. For the comet assay, cells are exposed to cytotoxic test compounds then embedded in agarose, lysed, their DNA unwound under alkaline conditions and subject to electrophoresis. During electrophoresis negatively charged DNA will migrate to the cathode. Induction of double strand breaks (DSBs), single strand breaks (SSBs) and alkali labile sites

(ALS) results in an increased ability of DNA to migrate, while DNA-DNA and DNA-protein cross-linking reduces the ability of DNA to migrate (Tice *et al*, 2000). Thus, a change in tail length compared to unchallenged cell populations suggests the induction of some form of DNA damage by those test compounds.

In the case of this study, a change (increase) in the tail lengths of HL60 and HT29 cell lines exposed to OD, LD and PM will suggest that these CHRs induce DNA damage in these cell lines *in vitro*, and that this may account for their previously observed cytotoxic action.

7.2. Materials and Methods

7.2.1. Cell culture & CHR preparation

Cell culture and CHR preparation were performed as described in Chapter 3.

7.2.2. Identification of genotoxicity using the comet assay

The comet assay is a commonly used method of detecting low levels of DNA damage within individual cells. At the International Workshop on Genotoxicity Test Procedures (IWGTP) in 1999 an expert panel developed a series of guidelines for the comet assay. They concluded that, for optimum results, cells should be exposed to the test substance for 3-6 hours, and each experiment should include at least three analyzable concentrations, covering a range from maximal toxicity to little toxicity. Thus, for this experiment the effects of each CHR after 4 hours of exposure at 1:10, 1:50 and 1:100 were examined and compared to control cell populations (Tice *et al*, 2000).

Media (1.5ml) containing $1x10^5$ cells/ml was placed in 24 well multi-well plates. HL60 and HT29 cell lines were exposed to OD, LD and PM at 1:10, 1:50 and 1:100; a control was also set up. Cells were incubated at 37°C in an atmosphere containing 5% CO2 for 4 hours. To prepare slides for the comet assay 1% normal melting point (NMP) agarose (Promega, Southampton, UK) was pipetted on to glass slides and immediately covered with a cover slip. This was then left for 5 minutes at room temperature to allow the agarose to solidify. After 5 minutes the cover slip was removed and the slide placed in a fridge until samples were ready to be placed on the slides. After the 4-hour incubation, suspensions of the incubated cells were placed in a microcentrifuge tube and centrifuged at 800rpm for 5 minutes. The supernatant was then removed and cells re-suspended in PBS (50μl), yielding a solution containing 2000 cells/μl, 10μl of which was added to 75μl low melting point (LMP) agarose (Promega, Southampton, UK) (0.5%) heated to 37-42°C. The inoculated LMP agarose was then pipetted on to the NMP agarose covering the previously prepared slides. A cover slip was immediately added and the slides placed in the fridge for 5 minutes. The slides were then taken out of the fridge, the cover slips removed and the slides placed in lysis solution (2.5M NaCl, 100mM EDTA, 10mM

Tris and 1%v/v Triton® X-100 (added immediately before use)) for 1 hour at 4°C. The slides were then removed, washed in PBS and placed in electrophoresis solution (0.3M NaOH, 1mM EDTA, pH>13) for 30 minutes at 4°C. After this time cells were subject to electrophoresis for 30 minutes at 4°C at 25V/300ma. After electrophoresis, slides were washed in neutralisation buffer (0.4M Tris, pH 7.5 with 10M HCl) 3 times for 5 minutes each. Slides were then dried and EtBr (20μg/ml) (Sigma, Dorset, UK) was pipetted on to the gel, a cover slip was added and the slides incubated for at least 10 minutes before analysis by fluorescence microscopy using a Zeiss Axioskop microscope (Carl Zeiss LTD, Hertfordshire, UK) connected to a Nikon DN100 digital camera (Nikon, Kingston upon Thames, UK). Tail length was measured live at 1000x magnification using the Eclipse Net image analysis package (Nikon, Kingston upon Thames, UK) (a representative example is shown below, Figure 7.2.1).

Figure 7.2.1 Comet analysis of a control nucleus and the nucleus of a cell exposed to OD at 1:10 for 4 hours

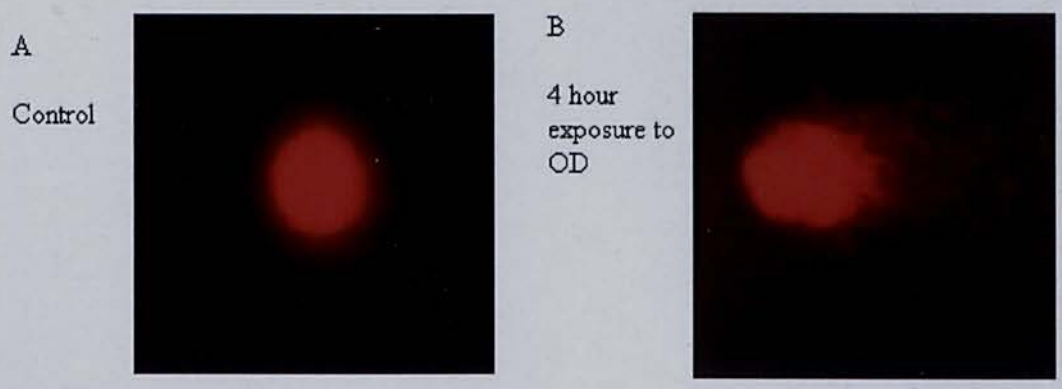


Figure 7.2.1 Typical results from the comet assay, showing a control nuclei and the nuclei of a cell exposed to OD at 1:10 for 4 hours.

In the comet assay, microscope images of necrotic and apoptotic cells are characterized as comets with small or non-existent heads and large diffuse tails, and are commonly referred to as hedgehogs, ghost cells or clouds (Hartmann *et al*,

2003). Due to these distinct morphological characteristics, apoptotic and necrotic cells were excluded from analysis.

The comet assay was carried out in duplicate and for each run 50 tails scored, yielding a total of 100 counts per experiment. For the effect of OD on HL60 cells 50 counts were made in one experiment and 20 in the other, yielding a total of 70 counts for that experiment. Average tail lengths were compared to the control using the paired sample T-test ($p \leq 0.05$ indicated a statistically significant difference) and data are represented as graphs showing average tail lengths.

7.3. Results

7.3.1. *Oldenlandia diffusa* (OD)

The comet assay showed a statistically significant (p=0.01) increase in genotoxic damage (compared to controls) in both HL60 and HT29 cell lines exposed to OD at 1:10 and 1:50, and at 1:100 in HT29 cells (Figure 7.3.1). For the HL60 cell line, the control tail length was 20.12μm, and increased to 33.27μm after exposure to OD at 1:10, 32.65μm after exposure to OD at 1:50, and 26.07μm after exposure to OD at 1:100. For the HT29 cell line, tail lengths increased from 25.61μm in the control to 56.04μm, 44.08μm and 29.98μm after exposure to OD at 1:10, 1:50 and 1:100, respectively.

7.3.2. Long Dan Xie Gan Wan (LD)

HL60 and HT29 cells exposed to LD for 4 hours at 1:10, 1:50 and 1:100 showed statistically significant increases in tail length when compared to controls (Figure 7.3.2). For the HL60 cell line, tail lengths increased from 18.97μm in the control to 46.29μm, 33.84μm and 32.23μm after exposure to LD for 4 hours at 1:10, 1:50 and 1:100, respectively, and for the HT29 cell line tail lengths increased from a control length of 13.06μm to 17.93μm, 20.58μm and 21.08μm at 1:10, 1:50 and 1:100, respectively. At all concentrations the observed genotoxic effect on the HL60 cell line was greater than that observed on the HT29 cell line.

7.3.3. *Polygonum multiflorum* (PM)

After 4 hours of exposure to a 1:10 extract of PM, tail lengths of HL60 cells were too long to measure, as they exceeded the visual field of the microscope. At 1:50 and 1:100, a statistically significant increase in tail lengths was observed, with tail lengths increasing from a control value of 10.44μm to 29.92μm at 1:50 and 13.2μm at 1:100 (Figure 7.3.3). For the HT29 cell line a statistically significant increase in tail length was observed at all concentrations, with the control at 19.8μm, and the tail lengths of cells exposed to PM at 1:10, 1:50 and 1:100 increasing to 46.39μm, 40.38μm and 35.25μm, respectively. At all concentrations the observed genotoxic effect of PM on the HL60 cell line was greater than that observed on the HT29 cell line.

Figure 7.3.1 Comet analysis of HL60 and HT29 cell lines exposed to OD at 1:10, 1:50 and 1:100 for 4 hours

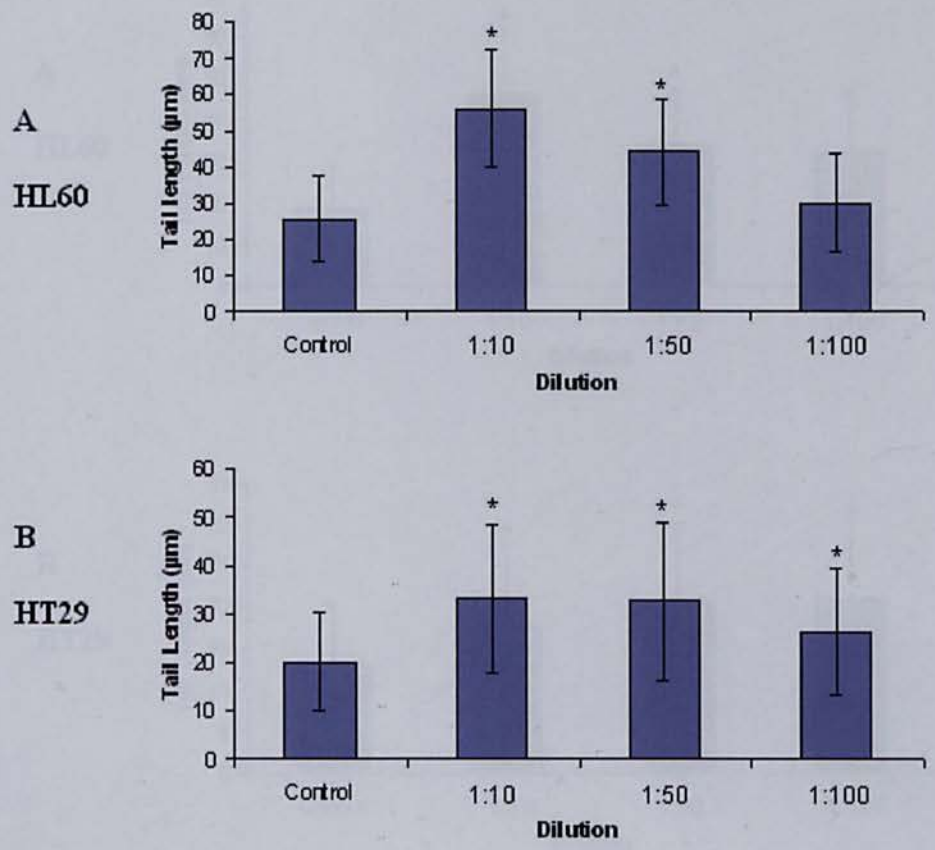


Figure 7.3.1 Average tail lengths (µm) of A: HL60 (n=70) and B: HT29 (n=100) cells exposed to aqueous extracts of OD for 4 hours at 1:10, 1:50 and 1:100 (compared to controls) ± standard deviation from the mean. * denotes statistical significance (p=0.01)

Figure 7.3.2 Comet analysis of HL60 and HT29 cell lines exposed to LD at 1:10, 1:50 and 1:100 for 4 hours

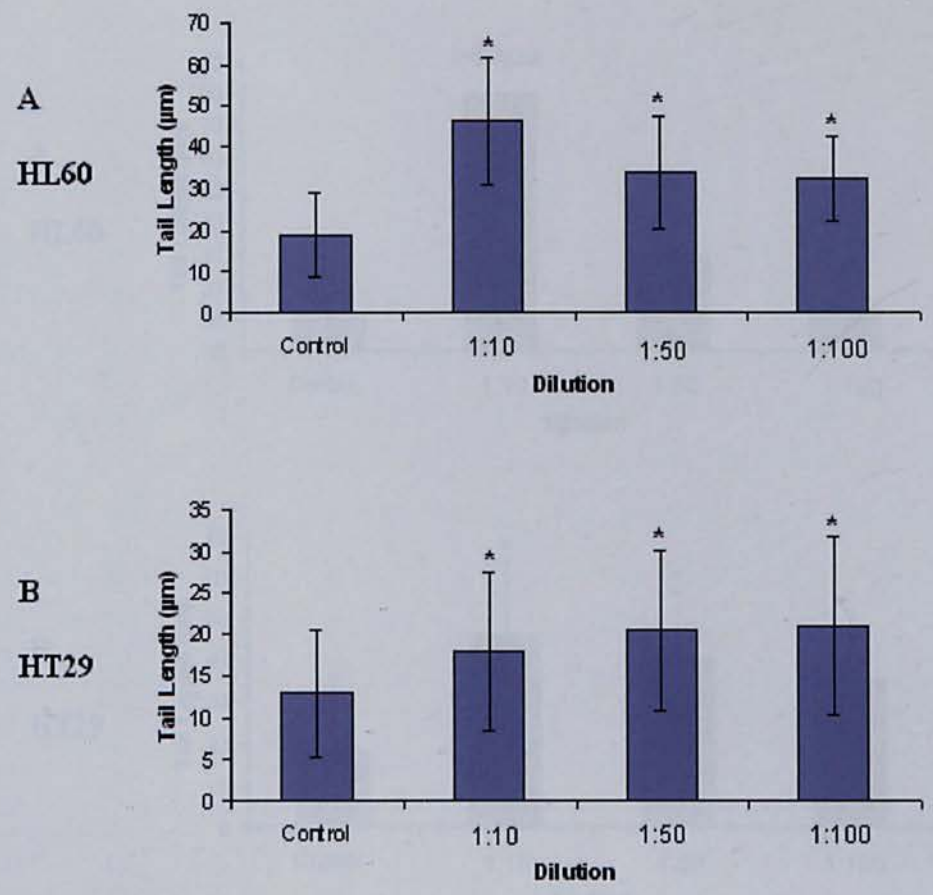


Figure 7.3.2 Average tail lengths (µm) of A: HL60 and B: HT29 cell lines exposed to aqueous extracts of LD for 4 hours at 1:10, 1:50 and 1:100 (compared to controls) ± standard deviation from the mean (n=100). * denotes statistical significance (p=0.01)

Figure 7.3.3 Comet analysis of HL60 and HT29 cell lines exposed to PM at 1:10, 1:50 and 1:100 for 4 hours

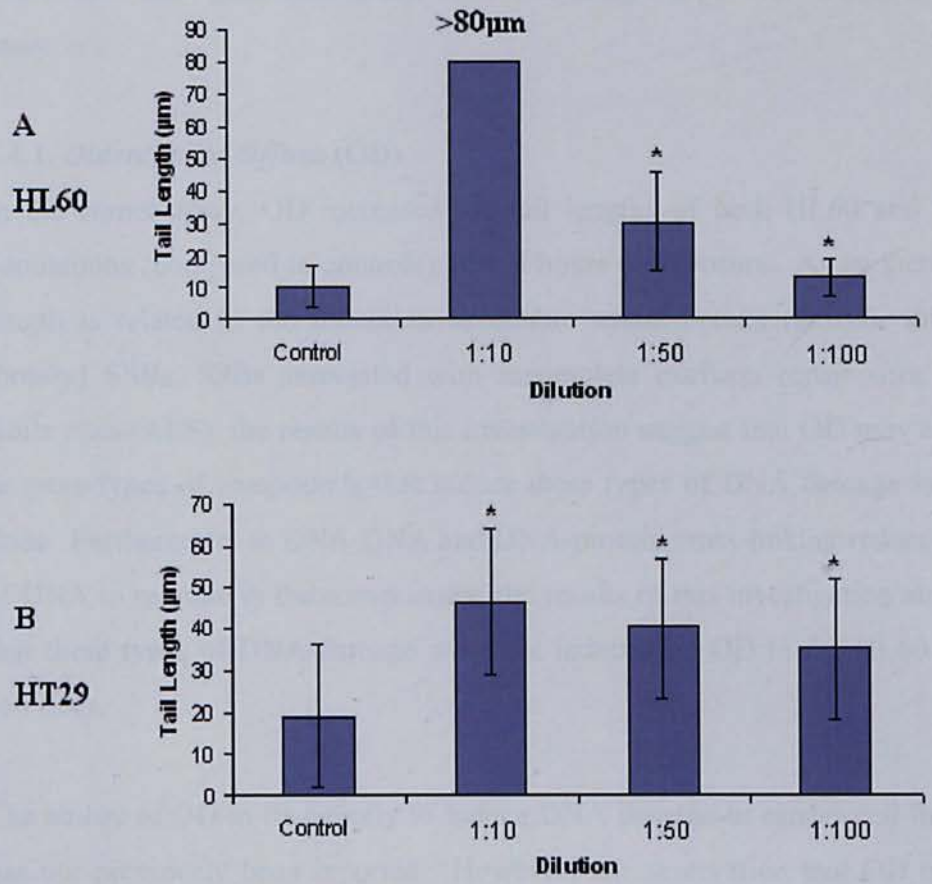


Figure 7.3.3 Average tail lengths (µm) of A: HL60 and B: HT29 cell lines exposed to aqueous extracts of PM at 1:10, 1:50 and 1:100 for 4 hours (compared to controls) ± standard deviation from the mean (n=100). * denotes statistical significance (p=0.01)

7.4. Discussion

The aim of this investigation was to determine whether the previously characterized cytotoxic actions of water extracts of OD, LD and PM against cancer cells occurred as a result of the induction of some form of DNA damage. To achieve this, HL60 and HT29 cells were exposed to OD, LD and PM at a range of concentrations for 4 hours, then their genotoxic action measured using the pH>13 version of the comet assay.

7.4.1. *Oldenlandia diffusa* (OD)

In the comet assay, OD increased the tail lengths of both HL60 and HT29 cell populations (compared to controls) after 4 hours of exposure. As an increase in tail length is related to the induction of double strand breaks (DSBs), single strand (breaks) SSBs, SSBs associated with incomplete excision repair sites and alkali labile sites (ALS), the results of this investigation suggest that OD may contain one or more types of compounds that induce these types of DNA damage in these cell lines. Furthermore, as DNA-DNA and DNA-protein cross-linking reduce the ability of DNA to migrate in the comet assay, the results of this investigation also suggests that these types of DNA damage were not induced by OD in the HL60 and HT29 cell lines.

The ability of OD in its entirety to induce DNA damage in cancer cell lines *in vitro* has not previously been reported. However, the observation that OD may induce DNA damage in the HL60 and HT29 cell lines may provide some insight into the cytotoxic actions of OD previously observed in this study: induction of DNA damage can result in cell death and growth inhibition (as seen against a range of cancer cell lines in Chapter 3), apoptotic induction (as seen in the HL60 and HT29 cell lines exposed to OD in Chapter 4), activation of the intrinsic caspase cascade (seen in HL60 cells exposed to OD in Chapter 5), and in cell cycle arrest (as seen in HT29 cells exposed to OD in Chapter 6).

The observation that OD exerted a greater genotoxic action against the HL60 cell line than the HT29 cell line is also consistent with the previous results obtained in this investigation, in that OD has generally been found to be more cytotoxic to the HL60 cell line than the HT29 cell line. That the HT29 cell line appears to be more

resistant to the genotoxic action of OD than the HL60 cell line may be explained by previous studies that have shown HT29s are generally more resistant to cytotoxic stimuli than the HL60 cell line (Shao *et al*, 1996). As OD was found to exert a genotoxic action against both the HL60 and HT29 cell lines, it is also possible that OD exerted a genotoxic action against the other cancer cells lines used in the initial screen (HCT-8, HeLa and CHO), and furthermore the results of this study could potentially account for the cytotoxic actions of OD observed against other cancer cell lines *in vitro* and *in vivo* reported in other studies (Sadava *et al*, 2000) (Gupta *et al*, 2004).

7.4.2. Long Dan Xie Gan Wan (LD)

The results of the comet assay showed LD induced a significant increase in the tail lengths of both HL60 and HT29 cell lines exposed to LD at 1:10, 1:50 and 1:100 after 4 hours (compared to the tail lengths of control cells). Thus, the results of this investigation suggest that LD induced DNA damage in those cell lines through the induction of DSBs, SSBs or ALS.

The ability of LD or its constituents to induce DNA damage in cancer cell lines *in vitro* has not previously been examined. However, the ability of LD to induce DNA damage is in-keeping with the previous results of this investigation, in that LD has been found to elicit a cytotoxic action against a range of cancer cell types (Chapter 3), to induce apoptosis in the HL60 and HT29 cell lines (Chapter 4), to activate the intrinsic caspase cascade in the HL60 cell line (Chapter 5) and to induce cell cycle arrest in the HT29 cell line (Chapter 6), actions which can all occur as a result of the induction of DNA damage. Furthermore, the results of this investigation suggest that LD exerts a greater genotoxic action on the HL60 cell line than the HT29 cell line, and this is also consistent with previous results obtained in this investigation, as LD has generally been found to exert a greater cytotoxic action against the HL60 cell line than the HT29 cell line. Again, this may be explained by previous studies that have shown HT29's to be more resilient to cytotoxic insult than HL60 cells (Shao *et al*, 1996). That LD induces genotoxic damage in both the HL60 and HT29 cell lines also suggests that LD may induce genotoxic damage in other cancer cell lines *in vitro*. Thus, the cytotoxic action of LD observed against the HCT-8, HeLa and CHO

cell lines in the initial growth inhibition study (Chapter 3) may also be related to LD exerting a genotoxic action.

7.4.3. *Polygonum multiflorum* (PM)

The results of this investigation showed that, after 4 hours of exposure, 1:10, 1:50 and 1:100 extracts of PM could significantly increase the tail lengths of HL60 and HT29 cell lines in the comet assay, thus suggesting PM induces DNA damage in those cell lines through the induction of DSBs, SSBs or ALS.

Strikingly, the levels of DNA damage induced in HL60 cells exposed to PM at 1:10 were so great that they exceeded the visual field of the microscope, and could therefore not be accurately measured. That such large amounts of DNA damage were induced may account for the previous false-positive results of the PI staining and TUNEL assays (Chapter 4): these assays look for evidence of genomic fragmentation as a marker of apoptotic induction, and while they gave positive results, the Annexin V assay (Chapter 4) did not, nor was there any evidence of caspase activation (Chapter 5), which is a key marker of apoptotic induction. Thus, the results of this study further suggest that PM does in fact induce necrosis in the HL60 cell line, and that this may be related to the induction of gross genomic fragmentation.

Significant DNA damage was also observed in HT29 cells exposed to PM at 1:10, 1:50 and 1:100, and this may again account for the cytotoxic action of PM observed in the growth inhibition study (Chapter 3) and in the Annexin V, PI staining and TUNEL assays (Chapter 4). Furthermore, this study suggested that PM induced more genotoxic damage in the HL60 cell line than the HT29 cell line, which is again consistent with the results of this investigation, in that PM was generally found to exert a greater cytotoxic action against this cell line than the HL60 cell line,. Furthermore, these results support the hypothesis that the HT29 cell line is generally more resistant to cytotoxic insult than the HL60 cell line (Shao *et al*, 1996). Finally, as PM induced genotoxic damage in both the HL60 and HT29 cell lines, it is also possible that PM exerted a genotoxic action against the other cell lines used in the growth inhibition study (HeLa, CHO and HCT-8), and that this activity may account for the observed cytotoxic action of PM against these cell lines.

7.4.4. Conclusion

The aim of this study was to determine whether OD, LD and PM induced DNA damage in the HL60 and HT29 cell lines. To achieve this, the comet assay was used. The results of this investigation suggested that OD, LD and PM did induce some form of genotoxic damage in both the HL60 and HT29 cell lines. Furthermore, greater genotoxic damage was observed in the HL60 cell line than the HT29 cell line, and as HT29 cells have consistently been more resistant to the cytotoxic actions of OD, LD and PM than the HL60 cell line in this study, these results appear to be consistent with those generated in previous chapters. As the ability of OD, LD and PM to induce DNA damage in cancer cell lines *in vitro* has not previously been reported, this study successfully provided more insight into the *in vitro* cytotoxic action of these CHRs against cancer cells. However, the comet assay cannot differentiate between the induction of SSBs, DSBs, ALS, or cytotoxic compounds that induce DNA damage directly or indirectly, thus further investigation would be required to determine the specific type of DNA damage induced by OD, LD and PM in cancer cell lines *in vitro*.

Chapter 8. The effect of *Oldenlandia diffusa*, Long Dan Xie Gan Wan and *Polygonum multiflorum* on non-stimulated and PHA-stimulated primary blood lymphocytes

8.1. Introduction

Effective cancer chemotherapeutic drugs exert a cytotoxic action against cancer cells while having little or no effect on normal cells within the body. However, as one of the underlying differences between cancer cells and the majority of normal cells is their rate of replication, many of the chemotherapeutic drugs in use today interfere with the processes of cell division, and as such also exhibit varying degrees of cytotoxicity to normal cells that rapidly divide (for example hair follicle cells and immune cells), thereby causing various side effects (Cancer Research UK, n.d.).

While the results of this investigation have suggested that OD, LD and PM are significantly toxic to a range of actively proliferating cancer cell lines *in vitro*, the cytotoxic action of these CHRs against normal proliferating and non-proliferating cell types have not been examined, and this information is essential in elucidating their chemotherapeutic potential, as effective anticancer agents are selectively toxic to cancer cells and not normal cells.

The initial literature review revealed that the cytotoxic potential of LD and PM against normal, non-cancerous cells had not previously been reported. However, a previous study, highlighted in the initial literature review, had shown that OD was significantly toxic to drug sensitive (H69) and multi-drug resistant small cell lung cancer cells (H69VP) while being significantly less toxic to a "normal" non-cancerous proliferating lung epithelial cell line (BEAS-2) at the same concentration (Sadava *et al*, 2002), thus suggesting that OD may exert a cancer cell-specific cytotoxic action. However, this study did not examine the cytotoxic potential of OD against other proliferating normal cell types, and did not examine the cytotoxic potential of OD against non-proliferating normal cell types *in vitro*.

Thus, due to the lack of information regarding the cytotoxic actions of OD, LD and PM against non-cancer cell types, the aim of this study was to devise and implement a strategy with which to examine the cytotoxic potential of OD, LD and PM against

non-cancer cells (using an *in vitro* test system) in an attempt to provide further information regarding their chemotherapeutic potential. To achieve this, human primary blood lymphocytes (PBLs) were isolated from whole blood, then cultured in the presence of OD, LD and PM and their toxicity determined. Furthermore, as the body is composed of both non-dividing and actively proliferating cell types, and as the cytotoxic action of many chemotherapeutic drugs are related to their ability to induce apoptosis in actively proliferating cells and not just cancer cells, the effects of OD, LD and PM on PBLS stimulated to proliferate (using the mitogen PHA) were also examined.

Propidium iodide (PI) staining and FACS analysis were used to examine the cytotoxic action of OD, LD and PM as they can be used to look for evidence of cell death (characterized by the induction of sub-G1 regions), and alterations in cell cycle characteristics, which is another marker of cytotoxicity.

8.2. Materials and Methods

8.2.1. CHR preparation

The CHRs OD, LD and PM were prepared as described in Chapter 3.

8.2.2. Isolation of PBLs

Primary human blood lymphocytes (PBLs) were isolated from 20ml heparinized venous whole blood using Ficoll-Paque Plus. Whole blood (15ml) was diluted 1:1 with PBS then layered on top of 20ml Ficoll in a 50ml falcon tube and centrifuged for 30 minutes at 800rpm. Lymphocytes were removed from the interface between the plasma and Ficoll layers.

8.2.3. Culture and stimulation of PBLs

Isolated PBLs were cultured in RPMI 1640 media containing 10% FBS supplemented with 100 U/ml penicillin and 100μg/ml streptomycin at 37°C in a 5% atmosphere. The effects of OD, LD and PM on non-proliferating and proliferating PBLs were examined. In order to stimulate isolated PBLs to proliferate *in vitro*, phytohaemagglutinin (PHA) was used. Phytohaemagglutinin (PHA) is a plant lectin isolated from the red kidney bean (*Phaseolus vulgaris*), and is a potent mitogen (Serke *et al*, 1987). To stimulate PBLs, 10mM PHA (Sigma, Dorset, UK) were added to appropriate flasks. Flasks were seeded at 10^5 cells/ml and incubated with OD, LD or PM at 1:10 for 24, 48 and 72 hours in the presence and absence of PHA. Controls for each time point were also made. Experiments were carried out in duplicate.

8.2.4. Analysis of cell death and alterations in cell cycle characteristics

Evidence of apoptotic induction and alterations in cell cycle characteristics were investigated using PI staining and FACS analysis (as previously described in Chapter 4). In order to analyse the cell cycle-related and apoptosis-inducing effects of each CHR on proliferating and non-proliferating PBLs using PI staining, singlets had to be gated and used for analysis (see appendix). Data are represented as histograms showing cell number (x-axis) versus relative PI fluorescence intensity (y-axis).

8.3. Results

8.3.1. Culture of PBLs

The control (Figure 8.3.1) showed non-stimulated and PHA stimulated primary blood lymphocytes successfully cultured *in vitro* for 24, 48 and 72 hours. For the non-stimulated control there were viable cells in G0 at all time points, and a small sub-G1 region. For PHA-stimulated PBLs there was no evidence of cell cycle progression after 24 hours of PHA exposure. However, after 48 hours a distinct cell cycle histogram was visible, which increased in size after 72 hours of exposure, thus suggesting PHA was stimulating the PBLs to proliferate.

8.3.2. *Oldenlandia diffusa* (OD)

Non-stimulated PBLs exposed to OD at 1:10 were still viable after 72 hours of exposure (Figure 8.3.2). There was no evidence of an increase in the sub-G1 region, however there was a decrease in the number of events relating to viable cells in G0 as time passed. In PHA-stimulated PBLs exposed to OD there was no evidence of cell cycle progression (as compared to the PHA-stimulated control). In addition, the relative number of cells in the G0/G1 peak was larger than in non-stimulated PBLs exposed to OD for the same period of time.

8.3.3. Long Dan Xie Gan Wan (LD)

For non-stimulated PBLs exposed to LD there was a decrease in the number of viable cells in G0 as time passed, however viable cells were still present after 72 hours of exposure (Figure 8.3.3). There was no evidence of cell cycle progression in PHA-stimulated PBLs exposed to LD, however viable cells were still present after 72 hours of exposure.

8.3.4. *Polygonum multiflorum* (PM)

For non-stimulated PBLs exposed to PM a sub-G1 shoulder appeared after 24 hours of exposure, and after 48 and 72 hours distinct sub-G1 peaks were observed (Figure 8.3.4). For PHA-stimulated PBLs exposed to PM sub-G1 peaks were also observed at all time points. Furthermore, cells were prevented from progressing into the cell cycle.

Figure 8.3.1 Control: *In vitro* incubation of non-stimulated and PHA-stimulated PBLS for 24, 48 and 72 hours

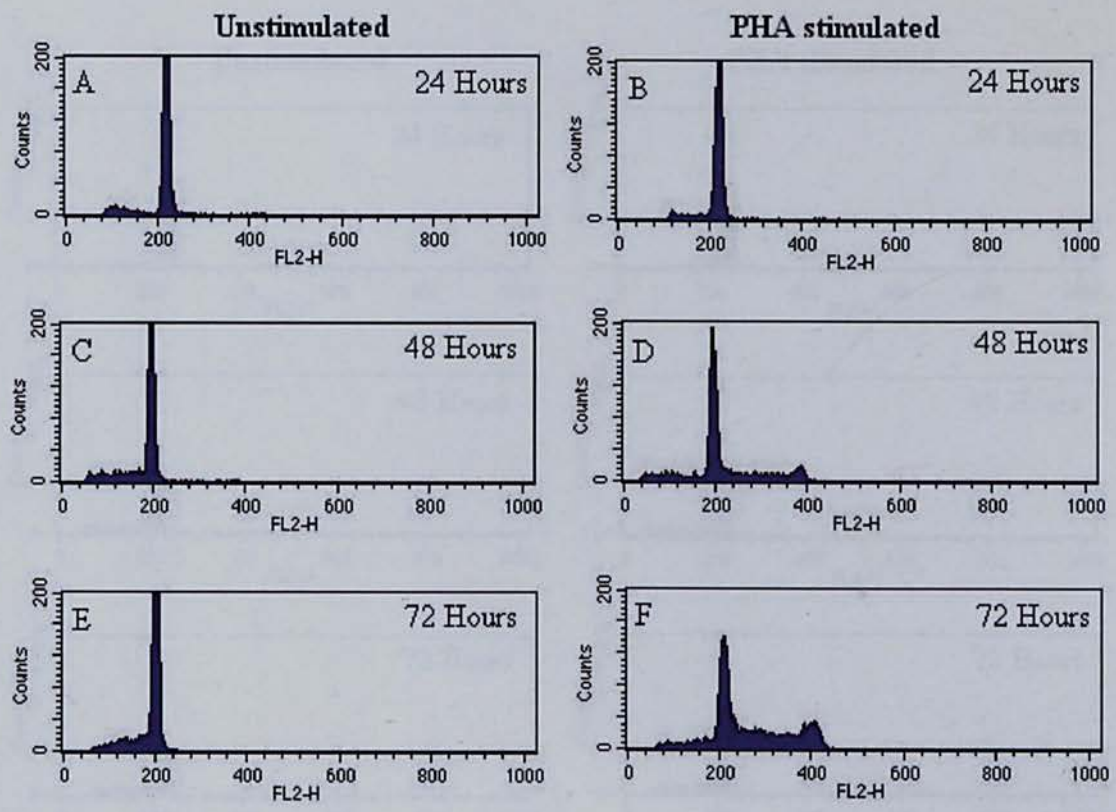


Figure 8.3.1 Control histograms showing PI stained non-stimulated and PHA-stimulated PBLs after 24, 48 and 72 hours of *in vitro* incubation. The x axis of each histogram shows relative DNA content, with cells in G0/G1 over channel 200 and cells in G2 over channel 400. The sub-G1 region represents apoptotic cells. The y axis represents cell number.

Figure 8.3.2 The effect of OD on non-stimulated and PHA stimulated primary blood lymphocytes after 24, 48 and 72 hours

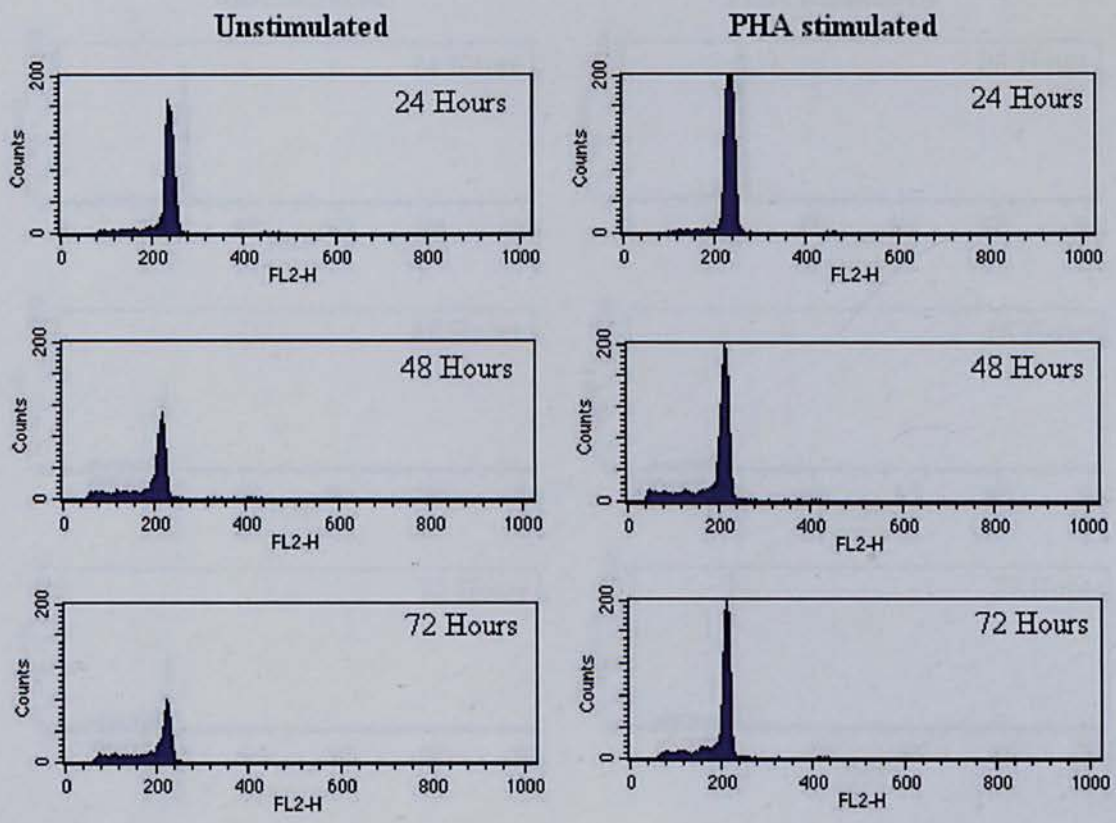


Figure 8.3.2 Histograms showing PI stained non-stimulated and PHA-stimulated PBLs exposed to OD at 1:10 for 24, 48 and 72 hours. The x axis of each histogram shows relative DNA content, with cells in G0/G1 over channel 200. The sub-G1 region represents apoptotic cells. The y axis represents cell number.

Figure 8.3.3 The effect of LD on non-stimulated and PHA stimulated primary blood lymphocytes after 24, 48 and 72 hours

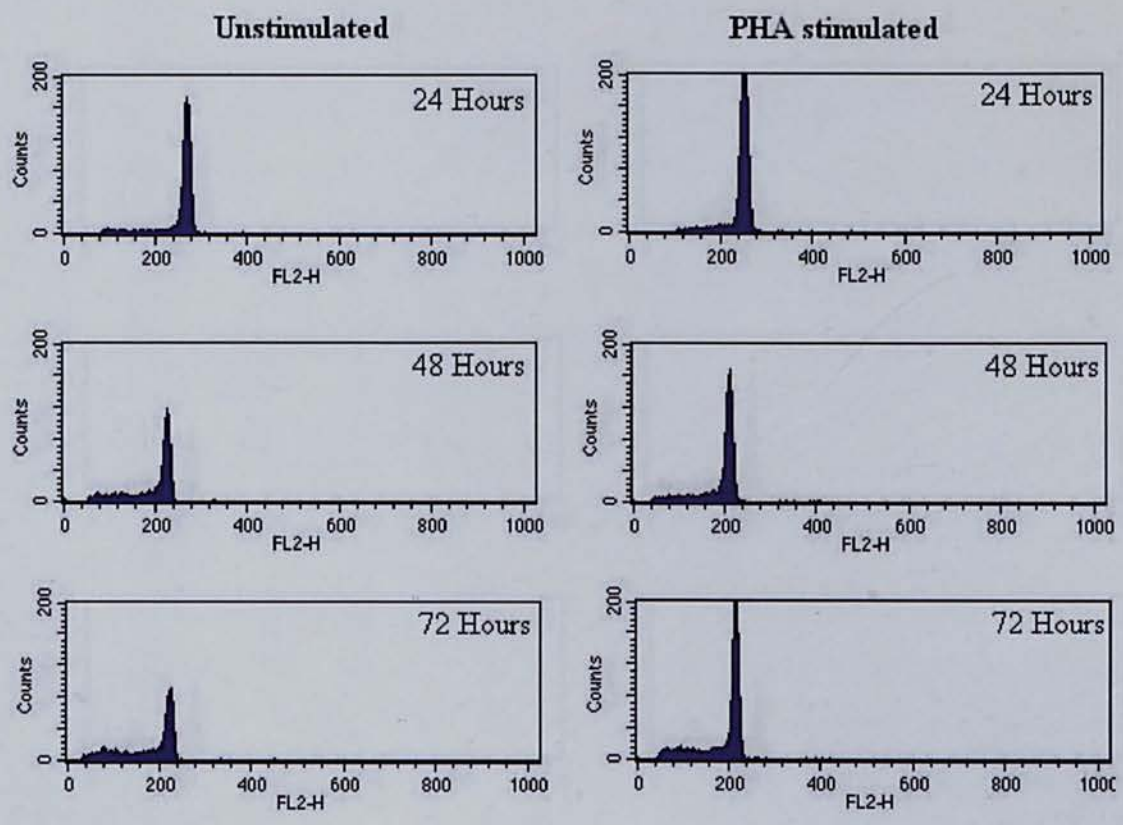


Figure 8.3.3 Histograms showing PI stained non-stimulated and PHA-stimulated PBLs exposed to LD at 1:10 for 24, 48 and 72 hours. The x axis of each histogram shows relative DNA content, with cells in G0/G1 over channel 200. The sub-G1 region represents apoptotic cells. The y axis represents cell number.

Figure 8.3.4 The effect of PM on non-stimulated and PHA stimulated primary blood lymphocytes after 24, 48 and 72 hours

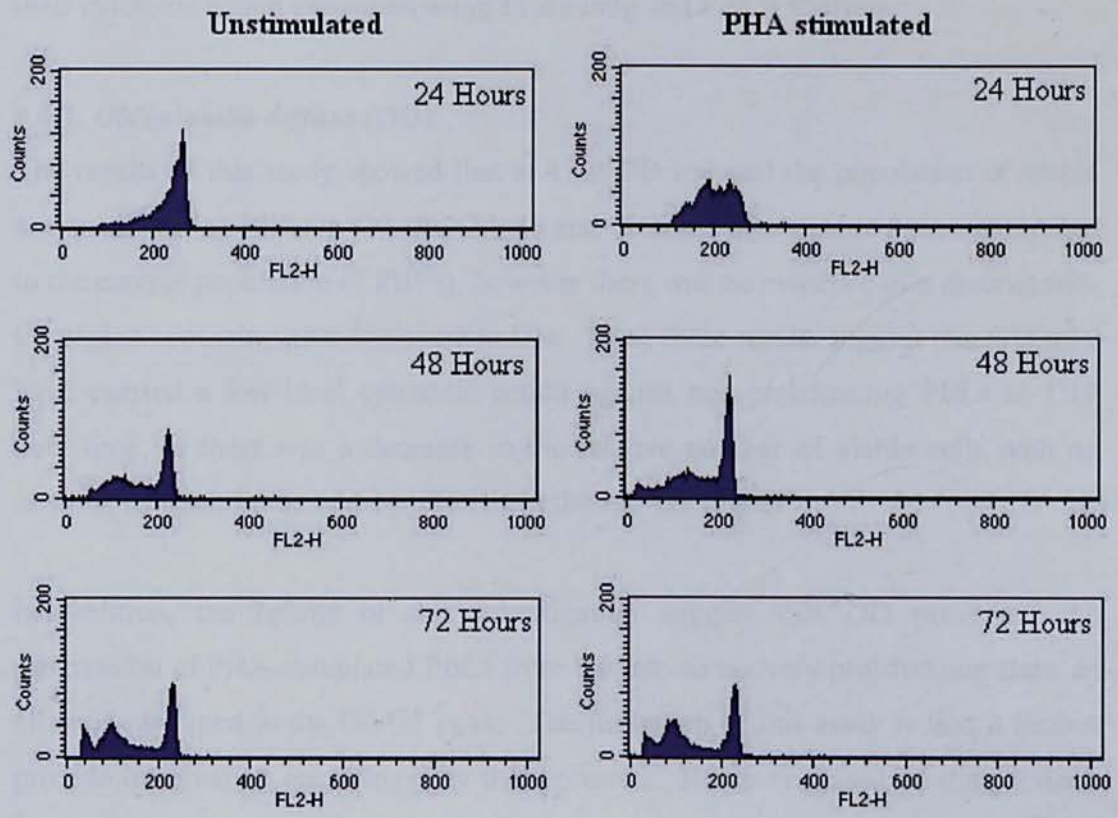


Figure 8.3.4 Histograms showing PI stained non-stimulated and PHA-stimulated PBLs exposed to PM at 1:10 for 24, 48 and 72 hours. The x axis of each histogram shows relative DNA content, with cells in G0/G1 over channel 200. The sub-G1 region represents apoptotic cells. The y axis represents cell number.

8.4. Discussion

The aim of this investigation was to examine the cytotoxic action of OD, LD and PM against non-cancerous cells in order to further elucidate their mechanism of action and to characterize their chemotherapeutic potential. To achieve this, both non-proliferating and proliferating PBLs were exposed to OD, LD and PM at 1:10 and their cytotoxic action examined using PI staining and FACS analysis.

8.4.1. *Oldenlandia diffusa* (OD)

The results of this study showed that at 1:10 OD reduced the population of viable non-proliferating PBLs in G0 after 24, 48 and 72 hours of exposure (when compared to the control population of PBLs), however there was no evidence of a distinct sub-G1 region appearing after exposure to OD. Thus, these results suggest that OD may have exerted a low-level cytotoxic action against non-proliferating PBLs at 1:10 over time, as there was a decrease in the relative number of viable cells with no obvious increase in the number of cells in the sub-G1 region.

In addition, the results of this investigation suggest that OD prevented the progression of PHA-stimulated PBLs from G0 into an actively proliferating state, as all cells remained in the G0/G1 peak. The limitation of this assay is that it cannot provide information regarding why this occurred. However, based on the previous results of this investigation (Chapter 7), it is possible that it occurred as a result of the induction of some form of genotoxic damage that may have prevented these cells from progressing through the G0/G1 checkpoint: in the comet assay OD was found to induce genotoxic damage in the HL60 and HT29 cell lines, and genotoxic damage can trigger arrest at the G1 checkpoint through activation of p53, thus preventing cell cycle progression (Lukas *et al*, 2004), and ultimately proliferation.

Thus, the results of this study suggest that at 1:10 OD may exert a cytotoxic action against both proliferating and non-proliferating PBLs. However, the observation that live PBLs were still present after 72 hours of exposure suggests that OD is more cytotoxic to cancer cells than normal cells, as in Chapter 3 at 1:10 OD killed a range of cancer cell lines within 24 hours of initial exposure, including the HL60, HeLa and CHO cell lines.

The suggestion that OD is more cytotoxic to cancer cells than normal cells appears to be consistent with the results of Sadava *et al* (2002), highlighted in the initial literature review, in which water extracts of OD were found to be significantly more toxic to lung cancer cell lines (H69 and H69VP) than the non-cancer BEAS-2 lung-epithelial cell line. This suggestion would also be consistent with a more recent *in vitro* study in which water extracts of OD were found to exhibit toxicity to a range of other cancer cell lines, including breast cancer cell lines (MCF-7 and MDA-MB-453), lung epithelial carcinoma (A5-49), human cervix carcinoma (C-33A), murine melanoma (B16-F10) and prostate cancer cell lines (Tsu-Pt1, Du-145 and Ln-Cap) while being significantly less toxic to normal pancreatic cell lines *in vitro* (Gupta *et al*, 2004). Furthermore, the observation that OD is more toxic to cancer cell lines *in vitro* than non-cancer cell lines is consistent with a study that has shown oral administration of OD can reduce B16-F10 cell growth in the lungs of C57/Bl/j mice while visual inspection shows OD exhibiting no toxicity to major organs (liver, heart stomach, kidney and intestines) (Gupta *et al*, 2004).

8.4.2. Long Dan Xie Gan Wan (LD)

The results of this study showed that LD decreased the number of viable non-proliferating PBLs in the G0 peak in a time-related fashion while not generating a significant sub-G1 region (when compared to the control), thus suggesting that at 1:10 LD may exert a moderate cytotoxic action against non-proliferating PBLs. Furthermore, the results of this study showed that LD inhibited the mitogenic activity of PHA, thus suggesting that LD may have induced some form of cytotoxic damage that prevented those cells from progressing through the G0/G1 checkpoint (as discussed above).

However, as LD was found to kill all HL60 leukaemic cells within 72 hours of exposure in the growth inhibition study (Chapter 3) at 1:10, the results of this study suggest that LD may exert a more significant cytotoxic action against this leukaemic cell line than normal PBLs, which would indicate that LD may exert a more significant cytotoxic action against cancer cells *in vivo* than normal cells.

The cytotoxic potential of LD against non-cancerous cells has not previously been reported. However, previous studies have shown that LD contains a number of

cytotoxic ingredients, which include *Radix bupleuri* (Cheng *et al*, 2003) (Hsu *et al*, 2004) (Cheng *et al*, 2005), *Radix scutellariae* (Ikemoto *et al*, 2000) (Sonoda *et al*, 2004), *Rhizoma alismatis* (Lee *et al*, 2001)) and *Fructvs gardeniae* (Peng *et al*, 2004) (for a full discussion see Chapter 3), therefore these may play a part in the cytotoxic action of LD against PBLs observed here.

8.4.3. *Polygonum multiflorum* (PM)

In this study, PM was found to be highly toxic to both non-proliferating and PHA-stimulated PBLs, characterized by the appearance of distinct sub-G1 peaks after 24, 48 and 72 hours of exposure. Interestingly, these sub-G1 peaks are a hallmark of apoptotic induction as they are distinct from the G0/G1 peak, while in previous studies PM was found to trigger necrosis in both the HL60 and HT29 cell lines (Chapter 4). Thus, the results of this study suggest that at 1:10 PM may trigger necrosis in cancer cell lines, while triggering apoptosis in normal PBLs cultured *in vitro*. In addition, PM was found to inhibit the mitogenic activity of PHA, thus suggesting that PM may be exerting some form of cytotoxic damage in PBLs that prevents the progression of cells through the G0/G1 checkpoint (Lukas *et al*, 2004). However, at 1:10 PM was found to kill a range of cancer cell lines, including the HL60, CHO and HCT-8 cell lines within 24 hours of exposure (Chapter 3), while in this study PBLs were still viable after 72 hours of exposure. Thus, the results of this study suggest that PM is more toxic to cancer cells than non-cancer cell lines *in vitro*.

The effect of PM on non-cancer cell lines has not previously reported. However, previous studies have found that a range of its constituents do exert pro-apoptotic activity: epigallocatechin gallate and epigallocatechin have been found to induce apoptosis in the human colon carcinoma LoVo cell line in a dose-dependent manner (Xiaohua *et al*, 2000), catechin has been shown to exhibit moderate cytotoxic activity to HSC-2 human carcinoma fibroblasts while being less toxic to normal HGF-2 fibroblasts (Babich *et al*, 2005), and emodin has been found to be cytotoxic to A549 cells *in vitro* (Su *et al*, 2005). Thus, it is possible that these compounds may have played a part in the pro-apoptotic action of PM against PBLs observed here.

8.4.4. Conclusions

The aim of this study was to determine whether OD, LD and PM exerted a cytotoxic action against non-cancerous cells. To achieve this, PBLs were exposed to OD, LD and PM at 1:10 and their cytotoxic potential examined using the PI staining assay to look for evidence of cell death and cell cycle arrest, which are both markers of cytotoxicity. The results of this study successfully showed that OD and LD exhibited a moderate cytotoxic action against PBLs, and that PM exhibited a significant cytotoxic action. Furthermore, this study showed that OD, LD and PM inhibited PHA-stimulated cell cycle progression, which is also a marker of toxicity.

Despite providing additional information concerning the cytotoxic action of these CHRs, there are limitations. This current study did not provide detailed information regarding why cells were prevented from entering the cell cycle after exposure to PHA, or which compounds were responsible for the observed actions of those CHRs. Furthermore, a more detailed study would be required to confirm observed pro-apoptotic action of PM against PBLs, and to confirm the modertate cytotoxic activity of OD and LD.

Chapter 9. Chemical analysis of *Oldenlandia diffusa*, Long Dan Xie Gan Wan and *Polygonum multiflorum* through activity-guided fractionation

9.1. Introduction

The efficacy of a CHR is traditionally attributed to the actions of various compounds within that remedy acting in combination in a synergistic or additive way against a single, or multiple biological targets (Yuan & Lin, 2000). Thus, in order to examine the efficacy of a CHR it is necessary to investigate the biological action of that remedy in its entirety (Rates, 2001). If a CHR is found to exert a biological action, investigations can then be conducted to determine if a single or multiple compounds are indeed responsible for their observed biological action(s) (Lee, 2000).

9.1.1. The cytotoxic constituents of OD, LD and PM

Previous results from this investigation suggested that crude water extracts of the CHRs OD, LD and PM were significantly toxic to range of cancer cell types *in vitro*. However, the results of these studies did not provide information regarding whether the cytotoxic activity of these CHRs was related to the action of a single type of cytotoxic compound, or to multiple cytotoxic compounds acting in a synergistic or combinatorial fashion against those cancer cell lines. Thus, the aim of this study was to determine whether a single or multiple compounds were responsible for the previously observed cytotoxic actions of water extracts of OD, LD and PM against cancer cells *in vitro*.

While previous studies have examined the cytotoxic action of crude extracts of OD against a range of cancer cell lines *in vitro* (Sadava *et al*, 2002) (Kim *et al*, 1998) (Gupta *et al*, 2004), the compound(s) that are responsible for the cytotoxic action of water extracts of OD are yet to be determined. A single study has found that ursolic acid (UA) is present in MeOH extracts of OD, and that this compound is cytotoxic against cancer cells in isolation (Kim *et al*, 1998). However, at the time of this investigation, no previous attempts had been made to determine whether a single or multiple compounds were responsible for the cytotoxic action of water extracts of OD against cancer cells.

The cytotoxic potential of water extracts of LD against cancer cells had not previously been examined at the time of this investigation. However, a range of studies have identified cytotoxic compounds within some of the herbs that are used to make LD (Table 9.1.1.1). Thus, it was possible that the cytotoxic action of LD previously observed in this study was related to the combined actions of these compounds, and not to a single cytotoxic constituent.

Table 9.1.1.1. Cytotoxic compounds within LD

Herbal Constituent	Compound	Reference
Radix bupleuri	Saikosaponin D Linoleic acid	Hsu *et al*, 2004
Radix scutellariae	Baicalin Baicalein Wogonin 2',3',5,7-tetrahydroxy flavone Aspigenin Viscidulin III Luteolin	Chan *et al*, 2000 Ikemoto *et al*, 2000 Sonada *et al*, 2004
Gardeniae Fructvs	Geniposide	Peng *et al*, 2004
Rhizoma alsimatis	Alisol B Alisol B acetate	Lee *et al*, 2001

The cytotoxic potential of crude water extracts of PM had not previously been examined against a range of cancer cell lines *in vitro* at the time of this investigation. However, previous studies had revealed that PM contains a range of compounds that elicit a range of cytotoxic activities against cancer cells when tested in isolation (Table 91.1.2). Thus, it was possible that the cytotoxic action of PM observed in this study may have been related to the combined actions of these compounds.

Table 9.1.1.2. Cytotoxic compounds within PM

Compound	Reference
Emodin Epicatechin Gallate Epigallocatechin Epigallocatechin Gallate	Koyama *et al*, 2003 Babich *et al*, 2005
Catechin Physcion	Lin *et al*, 2003

9.1.2. Determining the role of individual compounds in the cytotoxic action of crude water extracts of OD, LD and PM

To determine whether a single, or multiple compounds were responsible for the previously observed cytotoxic action of water extracts of OD, LD and PM against cancer cells *in vitro*, the compounds within each were split into fractions using High Performance Liquid Chromatography (HPLC), then the cytotoxic potential of each fraction tested against the HL60 cell line using the trypan blue exclusion assay.

In addition, those fractions that exerted a statistically significant growth inhibitory effect were analyzed by Dr Mirza at the Institute of Cancer Research (ICR, Surrey, UK) using Liquid Chromatography-Mass Spectrometry (LC-MS), thus providing the relative masses of the compounds within these active fractions. These masses were then compared to the known masses of cytotoxic compounds that had previously been reported in the respective CHRs in an attempt to determine whether the cytotoxic action of the CHRs was related to the actions of these previously reported compounds.

9.2. Materials and Methods

9.2.1. Cell culture and CHR preparation

Cell culture and CHR preparation were performed as described in Chapter 3.

9.2.2. Fractionation of CHRs by HPLC

The CHRs OD, LD and PM were split into fractions using a LichroCART® 250-10, LIChrospher® 100 RP-18e (10μm) HPLC column (Merck, Darmstadt, Germany). 500μl of each CHR was injected into the column and the flow rate set at 3ml/minute. Fractions (3ml) were collected at 1 minute intervals. Initially PBS was used as the mobile phase, later this was changed to water as PBS fractions could not be analyzed by LC-MS due to high levels of phosphate buffer present within the PBS. The HPLC prep column was attached to a Perkin-Elmer series 410 LC pump and a Perkin-Elmer LC-235 Diode array detector. Absorbance was measured at 253nm.

9.2.3. Examining the cytotoxic effects of CHR fractions

Individual fractions were sterile filtered using a 0.45μm sterile filter. HL60 cells at a starting concentration of 10^5/ml were exposed to CHR fractions for 48 hours then cell numbers compared to control cell growth using the trypan blue exclusion assay. Data are represented as graphs showing relative growth inhibition compared to control cell growth (± standard deviation).

9.2.4. Chemical analysis of biologically active fractions using LC-MS

Analysis of those fractions that exerted the strongest statistically significant (p=0.01) growth inhibitory effects was conducted by Dr Mirza at the Institute of Cancer Research (ICR, Surrey, UK) using Atmospheric Pressure Chemical Ionization (APCI) and sample detection using a Finnigan LCQ MS detector. Samples (1.5ml) were initially found to be too dilute, and along with a distilled water control were evaporated under vacuum at 55°C. These were then taken up in DMSO:Water 50:50 (200μl) and sonicated before analysis. Any remaining residue was taken up in MeOH:Chloroform 50:50 (200μl) and also sonicated before analysis.

9.2.5. Data Presentation and statistical analysis

Growth inhibition data are expressed as mean ± standard deviation from the mean. The mean growth inhibitory effect of each fraction was compared to control cell growth using ANOVA ($p=0.05$ and $p=0.01$).

9.3. Results

9.3.1 Initial analysis using PBS to fractionate the CHRs

Initially OD, LD and PM were split into fractions by HPLC using PBS as the running buffer. The growth inhibitory effect of each of these fractions was then tested on the HL60 cell line over a period of 48 hours using the trypan blue exclusion assay (Figure 9.3.1). Results showed OD contained two fractions that caused statistically significant growth inhibition; these were fractions 12 and 18. Two fractions of LD also exhibited a statistically significant toxic effect on the HL60 cell line; these were fractions 7 and 19. For PM a single fraction appeared to exert the same effect as PM in its entirety, this was fraction 5.

Further analysis of these fractions by LCMS was not possible due to the high levels of phosphate buffer in the PBS, therefore the experiment was repeated using water as a running buffer. As active PBS fractions of PM and LD were eluted early on, the first 10 water fractions of each CHR were subsequently tested (in duplicate), and are described below.

9.3.2. *Oldenlandia diffusa* (OD)

The absorbance (253nm) of OD as it passed through the HPLC column recorded a total of 21 distinct peaks, possibly relating to single compounds (Figure 9.3.2 A). The largest levels of absorbance were recorded between 2 and 8 minutes after injection of OD into the semi prep column, suggesting most of the constituents of OD were eluted in this time. The growth inhibitory effects of the first 10 fractions were examined using the HL60 cell line (Figure 9.3.2 B). Results showed fractions 1, 4 and 5 had a highly significant growth inhibitory effect ($p=0.01$), and fractions 3 and 7 had a reduced, yet still significant, growth inhibitory effect ($p=0.05$). Results from the LC-MS analysis showed a total of 2 compounds within fraction 4, and 5 compounds in fraction 5. The repeat experiment revealed 23 compounds in the HPLC trace (Figure 9.3.3 A), and fractions 1, 3, 4 ($p=0.01$) and 5 ($p=0.05$) were found to exert a significant growth inhibitory effect against the HL60 cell line. LC-MS analysis revealed a single compound in fraction 3, and 7 compounds in fraction 4.

9.3.3. Long Dan Xie Gan Wan (LD)

The HPLC trace for LD revealed a total of 24 peaks, each possibly relating to a single compound (Figure 9.3.4). Analysis of the cytotoxic effect of the first 10 fractions on the HL60 cell line after 48 hours of exposure revealed fractions 1 (p=0.01), 2, 4 and 7 (p=0.05) exerted a statistically significant toxic effect. A single compound was detected in fraction 2 by LC-MS, while 2 compounds were found in fraction 7. For the LD repeat a total of 25 peaks were recorded at 253nm (Figure 9.3.5). Fractions 2, 3, 4 and 7 exerted a toxic effect on the HL60 cell line after 48 hours (p=0.05). A single compound was detected by LC-MS in fraction 2, 2 compounds were found in fraction 4 and a single compound in fraction 7. None of the compounds detected by LCMS had the same molecular weights as previously reported compounds within the herbal constituents of LD (Table 9.4.2.1).

9.3.4. *Polygonum multiflorum* (PM)

For PM, a total of 17 peaks were recorded at 253nm (Figure 9.3.6). The growth inhibition study revealed fractions 1, 4, 5, 7 (p=0.01) and 3 (p=0.05) exerted a significant growth inhibitory effect. Analysis of fractions 4 and 7 by LC-MS revealed 1 compound in fraction 4, and 3 compounds in fraction 7. For the PM repeat, 13 peaks were recorded during HPLC separation (Figure 9.3.7). The cytotoxicity study of the first 10 fractions revealed fractions 5 (p=0.01), 3 and 6 (p=0.05) exerted a significant growth inhibitory effect. Analysis of fraction 5 by LC-MS revealed 4 compounds. The molecular weights of those compounds detected using LCMS did not correspond to the weights of cytotoxic compounds previously reported in PM (Table 9.4.3.1).

Figure 9.3.1 Growth inhibitory effects of PBS-eluted fractions of OD, LD and PM on the HL60 cell line after 48 hours of exposure.

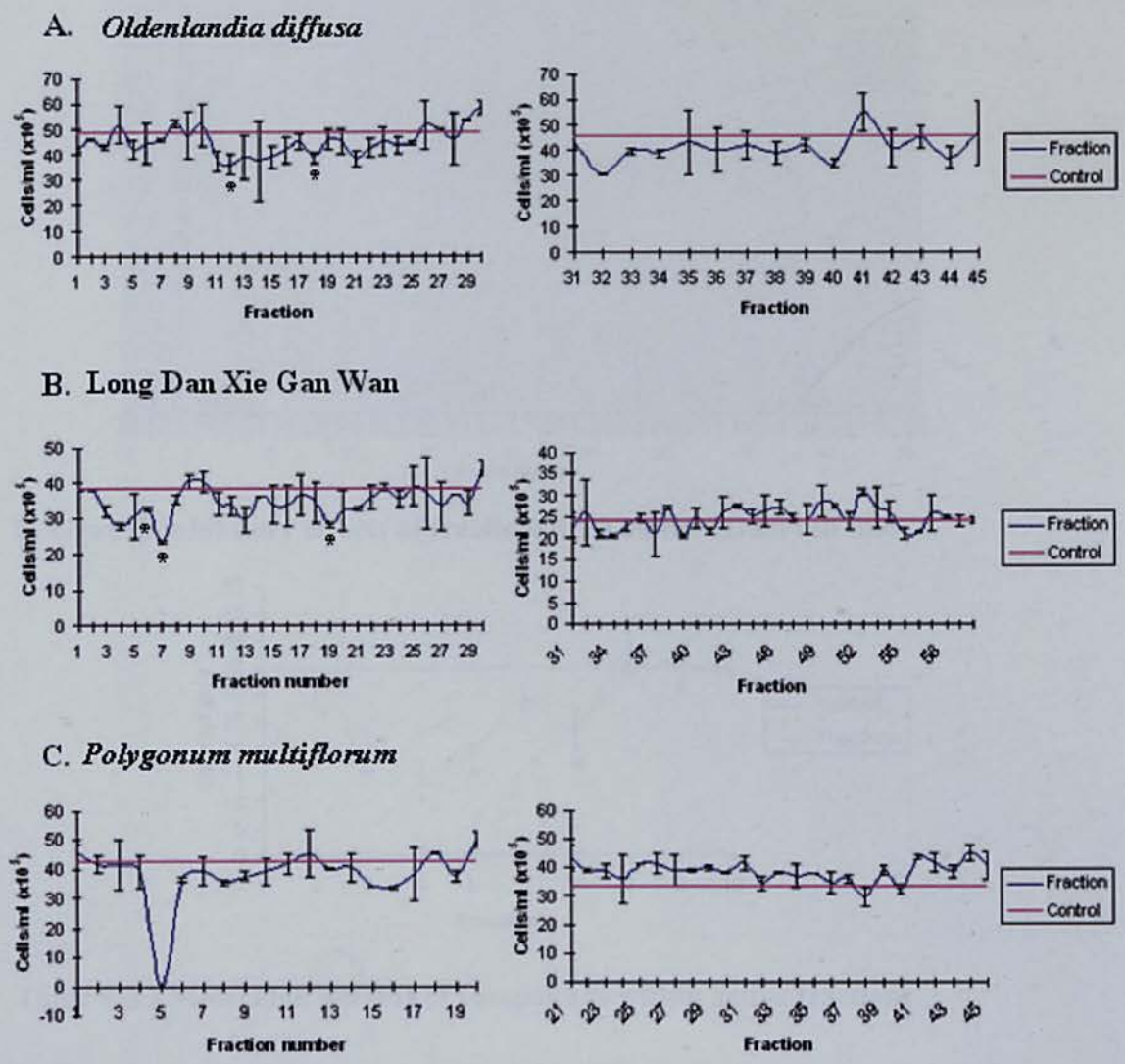
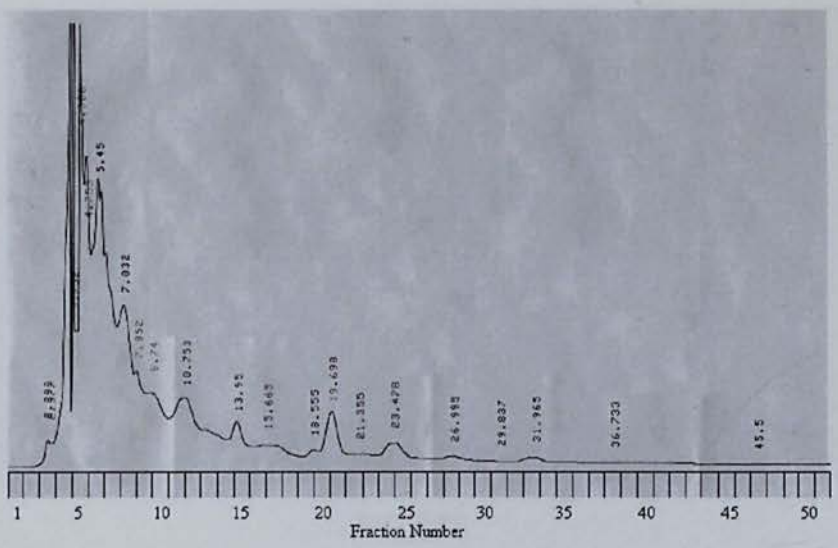


Figure 9.3.1 Shows the growth inhibitory effects of PBS eluted fractions of A: *Oldenlandia diffusa*, B: Long Dan Xie Gan Wan, and C: *Polygonum multiflorum* on the HL60 cell line after 48 hours of exposure. The pink line represents control cell growth, the blue line represents the number of cells/ml after 48 hours of exposure to CHR ± standard deviation form the mean, * denotes statistical significance p=0.05.

Figure 9.3.2 Activity-guided fractionation of *Oldenlandia diffusa* and chemical analysis of active fractions using LC-MS

A. HPLC readout



B. Growth inhibitory effects of fractions 1-10 on the HL60 cell line

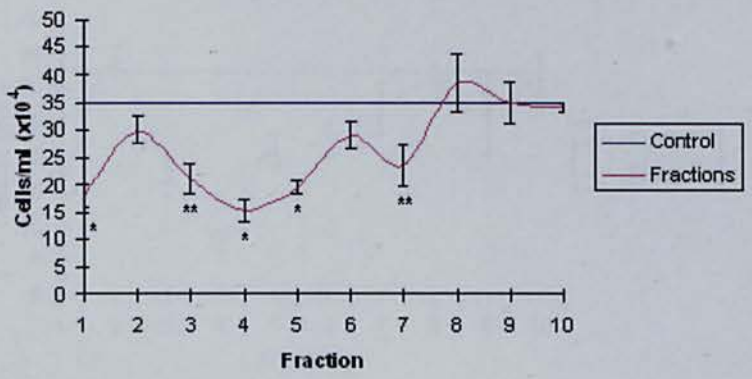


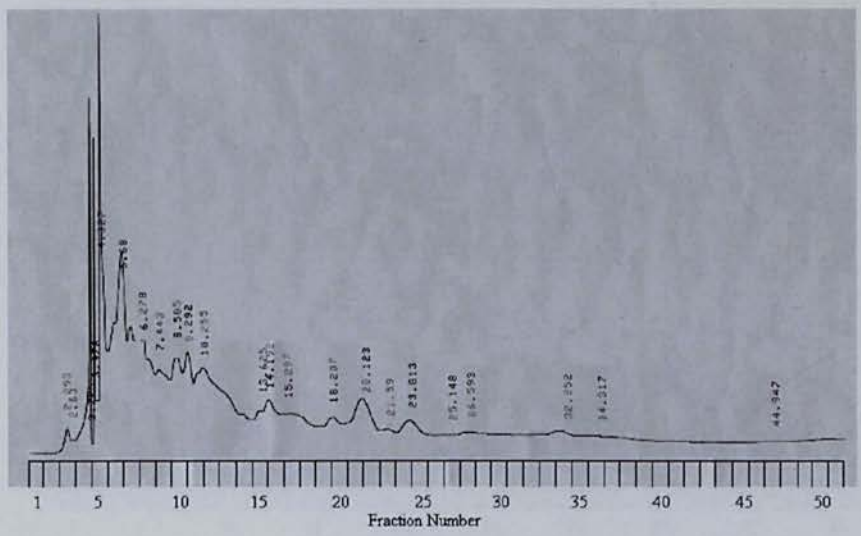
Table 9.3.1 Molecular weights of compounds within active fractions

Fraction 4 DMSO:Water (50:50)	213.8			
Fraction 4 MeOH:Chloroform (50:50)	222.9			
Fraction 5 DMSO:Water (50:50)	188	244.1	266.1	321.8
Fraction 5 MeOH:Chloroform (50:50)	188	266.1	362.1	

Figure 9.3.2 Figure A: HPLC readout (253nm) of OD eluted at 3ml/minute with the corresponding fraction number labelled on the y axis. Figure B: the growth inhibitory effect of each fraction on the HL60 cell line after 48 hours of exposure. **Table 9.3.1** The molecular weights of compounds isolated from those fractions by LC-MS. Each fraction was evaporated and taken up in DMSO:Water (50:50) and MeOH:Chloroform (50:50) for LC-MS analysis.

Figure 9.3.3 REPEAT: Activity-guided fractionation of *Oldenlandia diffusa* and chemical analysis of active fractions using LC-MS

A. HPLC readout



B. Growth inhibitory effects of fractions 1-10 on the HL60 cell line

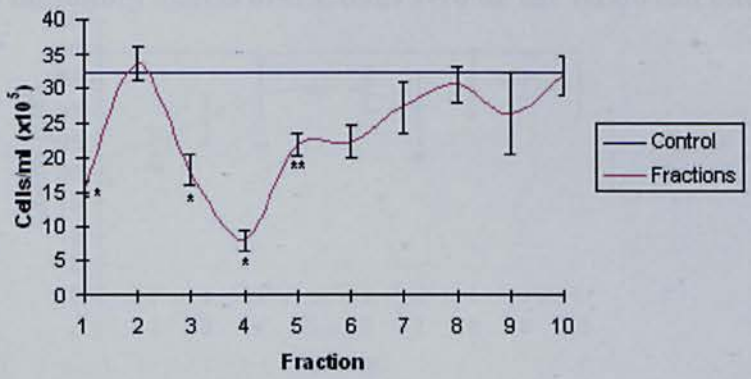


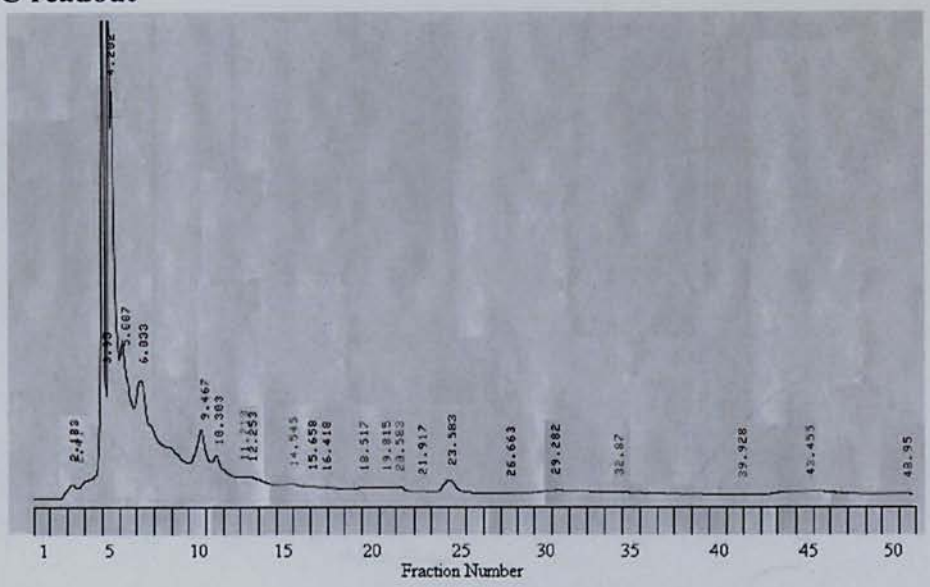
Table 9.3.2 Molecular weights of compounds within active fractions

Fraction 3 DMSO:Water (50:50)						
Fraction 3 MeOH:Chloroform (50:50)	362.1					
Fraction 4 DMSO:Water (50:50)	188	244.1	342.1			
Fraction 4 MeOH:Chloroform (50:50)	188	223	256.4	342.1	362	663.4

Figure 9.3.3 Figure A: HPLC readout (253nm) of OD eluted at 3ml/minute with the corresponding fraction number labelled on the y axis. Figure B: the growth inhibitory effect of each fraction on the HL60 cell line after 48 hours of exposure. **Table 9.3.2** The molecular weights of compounds isolated from those fractions by LC-MS. Each fraction was evaporated and taken up in DMSO:Water (50:50) and MeOH:Chloroform (50:50) for LC-MS analysis.

Figure 9.3.4 Activity-guided fractionation of Long Dan Xie Gan Wan and chemical analysis of active fractions using LC-MS

A. HPLC readout



B. Growth inhibitory effects of fractions 1-10 on the HL60 cell line

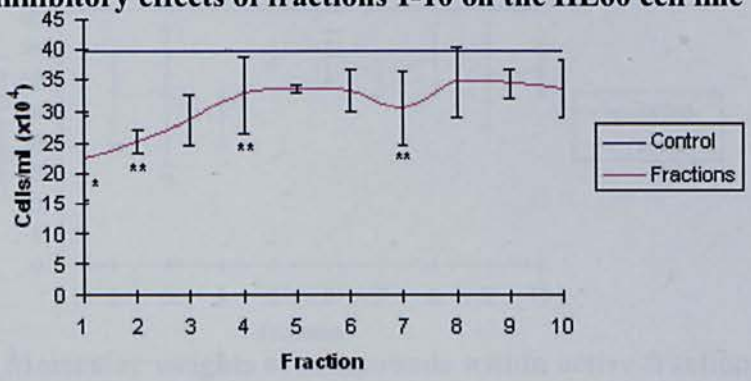


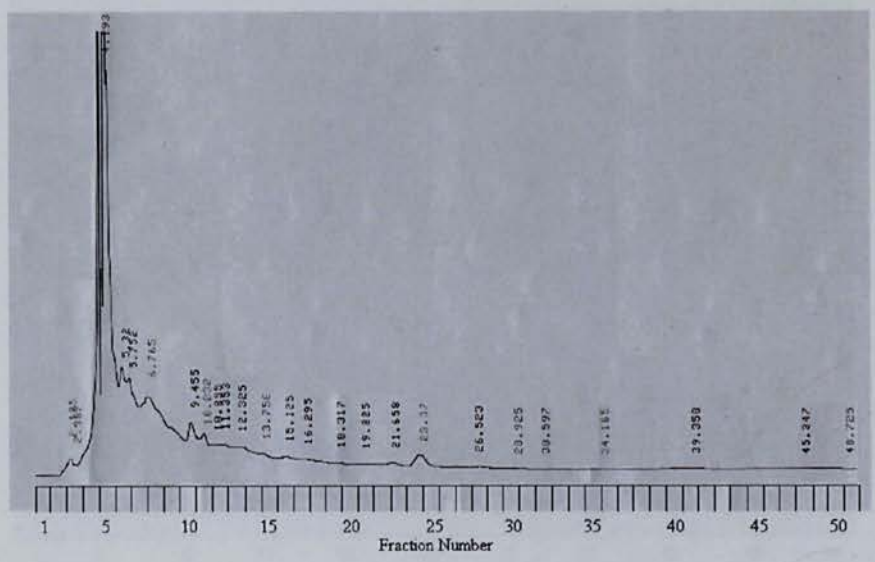
Table 9.3.3 Molecular weights of compounds within active fractions

Fraction 2 DMSO:Water (50:50)	
Fraction 2 MeOH:Chloroform (50:50)	362.1
Fraction 7 DMSO:Water (50:50)	278.1
Fraction 7 MeOH:Chloroform (50:50)	222.9

Figure 9.3.4 Figure A: HPLC readout (253nm) of OD eluted at 3ml/minute with the corresponding fraction number labelled on the y axis. Figure B: the growth inhibitory effect of each fraction on the HL60 cell line after 48 hours of exposure. **Table 9.3.3** The molecular weights of compounds isolated from those fractions by LC-MS. Each fraction was evaporated and taken up in DMSO:Water (50:50) and MeOH:Chloroform (50:50) for LC-MS analysis.

Figure 9.3.5 REPEAT: Activity-guided fractionation of Long Dan Xie Gan Wan and chemical analysis of active fractions using LC-MS

A. HPLC readout



B. Growth inhibitory effects of fractions 1-10 on the HL60 cell line

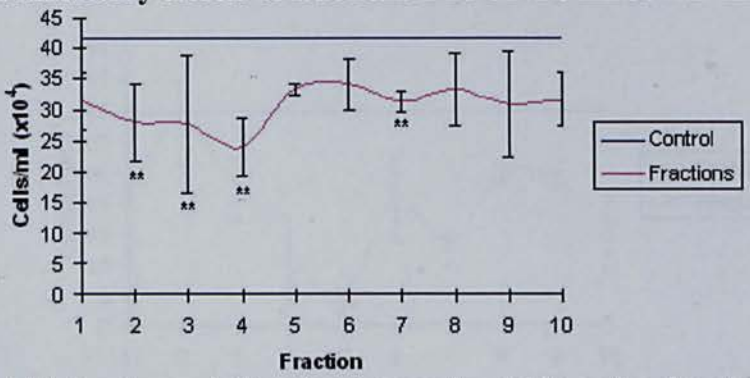


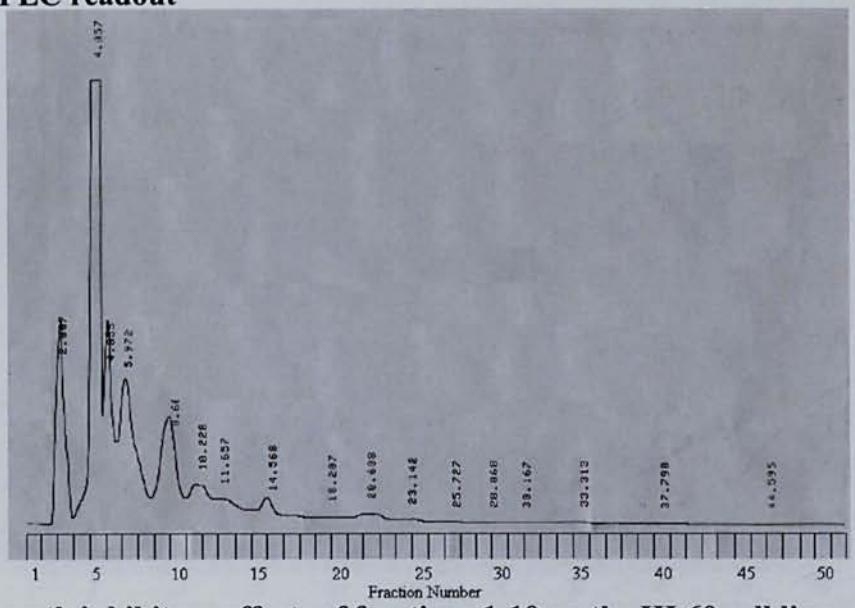
Table 9.3.4 Molecular weights of compounds within active fractions

Fraction 2 DMSO:Water (50:50)		
Fraction 2 MeOH:Chloroform (50:50)	418.3	
Fraction 4 DMSO:Water (50:50)		
Fraction 4 MeOH:Chloroform (50:50)	259.2	362.1
Fraction 7 DMSO:Water (50:50)	278.1	
Fraction 7 MeOH:Chloroform (50:50)		

Figure 9.3.5 Figure A: HPLC readout (253nm) of OD eluted at 3ml/minute with the corresponding fraction number labelled on the y axis. Figure B: the growth inhibitory effect of each fraction on the HL60 cell line after 48 hours of exposure. **Table 9.3.4** The molecular weights of compounds isolated from those fractions by LC-MS. Each fraction was evaporated and taken up in DMSO:Water (50:50) and MeOH:Chloroform (50:50) for LC-MS analysis.

Figure 9.3.6 Activity-guided fractionation of *Polygonum multiflorum* and chemical analysis of active fractions using LC-MS

A. HPLC readout



B. Growth inhibitory effects of fractions 1-10 on the HL60 cell line

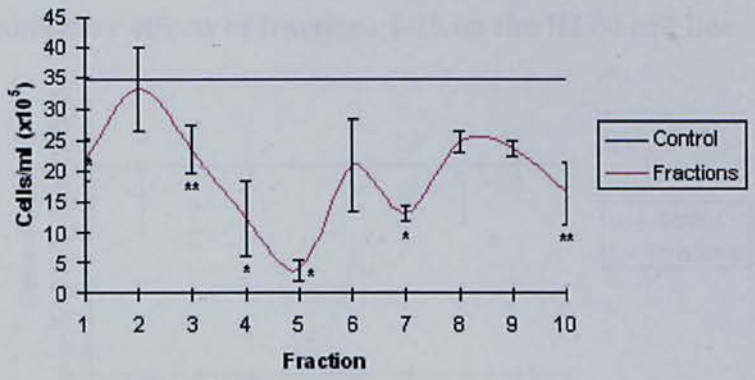


Table 9.3.5 Molecular weights of compounds within active fractions

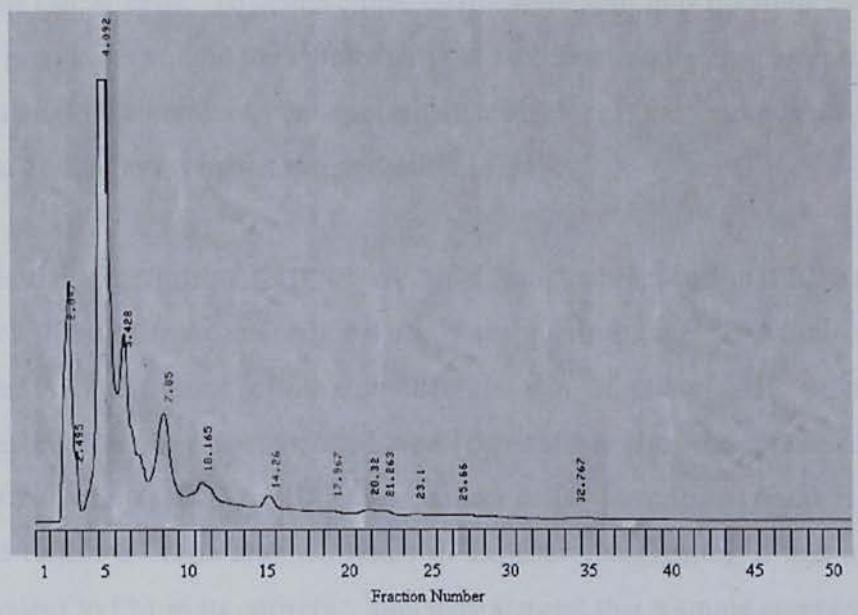
Fraction 4 DMSO:Water (50:50)				
Fraction 4 MeOH:Chloroform (50:50)	257.8			
Fraction 7 DMSO:Water (50:50)	278.1			
Fraction 7 MeOH:Chloroform (50:50)	160.4	207.8	278.1	362

Figure 9.3.6 Figure A shows an HPLC readout (253nm) of OD eluted at 3ml/minute with the corresponding fraction number labelled on the y axis. Figure B: the growth inhibitory effect of each fraction on the HL60 cell line after 48 hours of exposure.

Table 9.3.5 shows the molecular weights of compounds isolated from those fractions by LC-MS. Each fraction was evaporated and taken up in DMSO:Water (50:50) and MeOH:Chloroform (50:50) for LC-MS analysis.

Figure 9.3.7 REPEAT: Activity-guided fractionation of *Polygonum multiflorum* and chemical analysis of active fractions using LC-MS

A. HPLC readout



B. Growth inhibitory effects of fractions 1-10 on the HL60 cell line

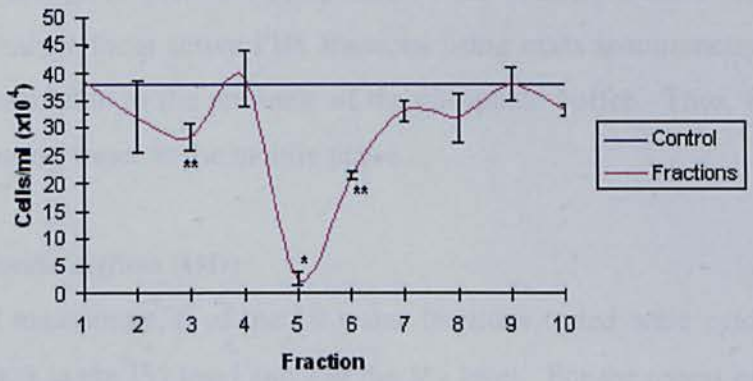


Table 9.3.5 Molecular weights of compounds within active fractions

Fraction 5 DMSO:Water (50:50)	223.8	259.8	
Fraction 5 MeOH:Chloroform (50:50)	223.8	193.8	362.1

Figure 9.3.6 Figure A shows an HPLC readout (253nm) of OD eluted at 3ml/minute with the corresponding fraction number labelled on the y axis. Figure B: the growth inhibitory effect of each fraction on the HL60 cell line after 48 hours of exposure. **Table 9.3.5** shows the molecular weights of compounds isolated from those fractions by LC-MS. Each fraction was evaporated and taken up in DMSO:Water (50:50) and MeOH:Chloroform (50:50) for LC-MS analysis.

9.4. Discussion

The aim of this study was to attempt to determine whether one or more compounds were responsible for the previously observed cytotoxic action of OD, LD and PM against cancer cells. To achieve this, CHRs were split into fractions using an HPLC semi prep column, and the cytotoxicity of each fraction determined using the HL60 cell line. Furthermore, the molecular weights of the compounds within each cytotoxic fraction were determined using LCMS.

For initial experiments CHRs were split into fractions using PBS as the mobile phase. Results from this original study revealed no single fraction of OD or LD exerted an anticancer action equivalent to that of either CHR in their entirety, suggesting no single compound was responsible for the previously observed cytotoxic action of OD or LD against cancer cells. In contrast, fraction 5 of PM was found to kill all cells after 48 hours of exposure, suggesting it had a toxic action equivalent to PM in its entirety. This may suggest that a single compound, eluted in fraction 5, was responsible for the cytotoxic action of PM. However, it is also possible that multiple cytotoxic compounds were eluted in this single fraction. Attempts to analyze these active PBS fractions using mass spectrometry techniques were unsuccessful due to the presence of the phosphate buffer. Thus, fractionation was repeated using water as the mobile phase.

9.4.1. *Oldenlandia diffusa* (OD)

For the initial experiment, 5 of the 10 water fractions tested were cytotoxic to the HL60 cell line, 3 at the 1% level and 2 at the 5% level. For the repeat experiment, 3 fractions exerted a significant growth inhibitory effect at the 1% level, and 1 fraction exerted a significant growth inhibitory effect at the 5% level. As multiple fractions exerted a cytotoxic action against the HL60 cell line, the results of this study suggest that the *in vitro* anticancer action of OD may be related to the combined actions of a number of cytotoxic compounds interacting in a synergistic or additive fashion.

The only directly cytotoxic compound that had previously been reported in OD at the time of the initial literature review was ursolic acid (UA, MW 456.7074) (Kim *et al*, 1997), however UA was not found in any of the active fractions in this study. This absence is likely to be due to UA being insoluble in water, therefore it was not

present in the aqueous extracts of OD used in this study. More recent investigations have found that the main components of OD are iridoid glucosides (Liang *et al*, 2006) (Kim *et al*, 2005). These types of compounds have been found to elicit a range of biological activities, including antitumour activity (Dinda *et al*, 2007). Thus, it is possible that the anticancer action of OD observed in this investigation may have been related to the actions of one of these types of compounds.

The results of this analysis of OD suggest that other compounds not previously described in OD may be responsible for its *in vitro* cytotoxic action against cancer cells.

9.4.2. Long Dan Xie Gan Wan (LD)

For LD a number of fractions were found to exert a significant growth inhibitory effect at the 5% level, however none of the fractions tested exerted a significant effect at the 1% level, which may suggest that LD contains a number of moderately cytotoxic compounds that interact to elicit a significant cytotoxic effect *in vitro*. LC-MS analysis of the cytotoxic fractions revealed that the molecular weights of the compounds within them did not correspond to the molecular weights of those cytotoxic compounds that have been previously isolated from some of the herbal constituents of LD (listed below).

Table 9.4.2.1. Previously reported anticancer compounds found within the herbal constituents of LD

Herbal Constituent	Compound	Molecular Weight	Reference
Radix bupleuri	Saikosaponin D Linoleic acid	781 280.4	Hsu *et al*, 2004
Radix scutellariae	Baicalin Baicalein Wogonin 2',3',5,7-tetrahydroxy flavone Aspigenin Viscidulin III Luteolin	270 270 284 286 270 346 286	Chan *et al*, 2000 Ikemoto *et al*, 2000 Sonada *et al*, 2004
Gardeniae Fructvs	Geniposide	388	Peng *et al*, 2004
Rhizoma alsimatis	Alisol B Alisol B acetate	472.71 514.75	Lee *et al*, 2001

Thus, the results of this study suggest that previously unreported compounds may be responsible for the *in vitro* cytotoxic action of LD on cancer cells previously observed in this investigation.

9.4.3. *Polygonum multiflorum* (PM)

Fraction 5 of PM appeared to exert a cytotoxic action similar to that of PM as a whole. This result may suggest that a single compound, eluted in fraction 5, is primarily responsible for the cytotoxic action of PM. However, other water fractions of PM also caused a growth inhibitory effect, most notably fractions 4 and 7 of the initial run, thus suggesting more than one compound within PM may exert a direct toxic action on the HL60 cell line. A number of compounds toxic to cancer cell lines have been previously reported in PM (below).

Table 9.4.3.1. Previously reported anticancer compounds isolated from PM

Compound	Molecular Weight	Reference
Emodin	270.2	Koyama *et al*, 2003
Epicatechin Gallate	442.4	Babich *et al*, 2005
Epigallocatechin	306.3	
Epigallocatechin Gallate	458.4	
Catechin	290.3	Lin *et al*, 2003
Physcione	284.3	

However, none of the molecular weights of compounds found in this study correspond to the molecular weights of those cytotoxic compounds previously described in PM. This may therefore suggest that those compounds exerting a cytotoxic action in this study have not previously reported.

9.4.4. Cytotoxic compounds within plants, fungi and medicinal herbs

The results of this study suggest that OD, LD and PM contain multiple compounds that exert a cytotoxic action against cancer cell lines *in vitro*, and the LCMS data discount the previously reported cytotoxic compounds within each CHR as being responsible for their cytotoxic activity. However, the LCMS data obtained for this study cannot be used to define what compounds were responsible for the cytotoxic actions of those fractions, and this information is essential in understanding how and why these CHRs elicit a cytotoxic action against cancer cells *in vitro*.

While this study has been unable to define what cytotoxic compounds were within the CHRs, those compounds, such as those listed in the table above, within plants that are typically found to be pharmacologically active are secondary plant metabolites (SM), and as such it is likely that compounds belonging to this generic family are responsible for the *in vitro* cytotoxic activity of OD, LD and PM against cancer cells.

Secondary plant metabolites are derived from primary metabolites (carbohydrates, lipids, nucleotides and peptides), and play a major role in the adaptation of plants to their environment. Their activities include antibiotic, antifungal and antiviral activities, and providing protection against insects and herbivores. Secondary metabolites (SM) are present in all higher plants, but as a general rule a single group of SM dominate within a given taxon. The distribution of SM within plants varies between tissues, organs and developmental stage. Secondary metabolites (SM) have been shown to exhibit a range of biological activities in humans, and as a result have been used for centuries in traditional medicine (Wink, 2003). Secondary metabolites (SM) are typically classified according to the biosynthetic pathway from which they are created. There are three main families of SM: nitrogen containing compounds, phenolics and terpenes (terpenoids) (Bourgaud *et al*, 2001).

The most important nitrogen containing SM are the alkaloids, one of the most diverse groups of SM found in plants. Many alkaloids are created to provide protection against herbivores, and are typically localized in areas where herbivorous attack would most affect plant fitness (e.g. growing tips and peripheral cell layers of stems and roots) (Lorence & Nessler, 2003). Many of the drugs used today are alkaloids from natural sources, and natural alkaloids have also been used as a basis to make more effective synthetic analogues. Alkaloids have been found to exhibit a range of biological activities, including antihypertensive effects (many indole alkaloids), antiarrhythmic effects (Quinidine, ajmaline, sparteine), antimalarial activity (quinine) and anticancer activity (dimeric indoles, vincristine, vinblastine) (Roberts & Wink, 1998).

Phenolic SM are characterized by the presence of a hydroxyl (-OH) group attached to a benzene ring or to other complex aromatic ring structures. Phenolic compounds range in size from phenol (MW 94, found in the oil of *Pinus sylvestris*), to polyphenols such as anthocyanin pigments (MW 2,000) and tannins (up to MW 20,000) (Forestry commission n.d.). The most widespread group of polyphenolic SM in the plant kingdom are the flavonoids. Flavonoids have a wide range of functions in plants, including pigmentation of flowers and fruit to attract insects and animals, protection against UV and defence against pathogenic organisms. Over 6000 flavonoids have been described. All share the same basic skeleton which includes the flavan nucleus, made of 2 aromatic rings with 6C atoms (ring A and B) interconnected by a heterocycle including 3C atoms (ring C). According to the modifications of the central C ring they can be divided into different structural classes: flavanones, isoflavanones, flavones, flavonols, flavanols and anthcyanins. In a single plant species, dozens of flavonoids may be present, many of which conjugated to sugar moieties. Flavonoids are responsible for much of the colour and flavour of fruits, vegetables and nuts, and form an integral part of the human diet. Flavanoids are suggested to protect against oxidative stress, coronary heart disease, cancers and other age-related disorders through their antioxidant free radical-scavenging activities (Schijlen *et al*, 2004).

Terpenes are small organic hydrocarbon molecules which can be cyclic or acyclic, saturated or unsaturated. Terpenes are made from 5 carbon isoprene units [$CH_2{=}C(CH_3)CH{=}CH_2$], and are subdivided into different classes according to the number of linked isoprene units they contain. C_5 terpenes (containing 1 isoprene unit) are hemiterpenes, C_{10} are monoterpenes, C_{15} are sesquiterpenes, C_{20} are diterpenes, C_{25} are sesterterpenes, C_{30} are triterpenes, C_{40} are tetraterpenes (also known as carotenoids) and C_{5n} are polyterpenes. Terpenoids are oxygenated derivatives of terpenes, and may contain hydroxyl or carbonyl groups (Smith *et al*, 2001). The terpenoid class is subdivided according to the number of carbon atoms in the same manner as terpenes. However, the skeleton of terpenoids may differ from strict additivity of isoprene units by the loss or shift of a fragment (Moss *et* al, 1994). Terpenes (terpenoids) are the largest group of natural products in plants, comprising essential oils, flavours, fragrances and lipid soluble plant pigments.

They are hydrophobic compounds, and are typically found in resin ducts, oil cells and glandular trichomes (Ormeno *et al*, 2007).

Thus, as the biological activities of CHRs are generally related to the actions of secondary plant metabolites (as described above), it is possible that the cytotoxic actions of OD, LD and PM observed in this study are related to the combined actions of these types of compounds.

9.4.5. Conclusions

The results of this study indicate that the cytotoxic actions of the CHRs previously highlighted in this investigation may be a result of multiple compounds interacting in a synergistic or additive fashion. Furthermore, the results of this investigation suggest that OD, LD and PM may contain cytotoxic compounds that have not previously been reported. However, the limitation of this study is that it does not characterize which compounds were present within these CHRs, and this information is essential in fully elucidating their cytotoxic potential against cancer cells.

Chapter 10. General Discussion

10.1. Examining the chemotherapeutic potential of Chinese herbal remedies (CHRs): summary of aims, objectives and findings

Chinese herbal remedies (CHRs) are commonly prescribed for the treatment of cancer, however their use is often based on the traditional belief systems of TCM, which include the theories of yin and yang and The Five Elements, and there is relatively little information regarding their efficacy or biological action (Ernst, 2002). Thus, the aim of this investigation was to select a range of CHRs with some suggestion of tumour modulatory activity and to devise and implement a strategy with which to investigate their direct toxicity to cancer cells in an attempt to elucidate their mode of action.

The first objective of this investigation was to select appropriate CHRs for analysis. The selection process involved a search of peer-reviewed literature (published between 1990-2003), investigation into the traditional uses of those remedies, and consultation with a TCM practitioner (as detailed in Chapter 1). The final criteria for the selection of CHRs for this investigation was primarily based on the preliminary findings of other research groups which suggested that certain CHRs (or their constituents) may elicit a direct action against cancer cells, but for which the cytotoxic potential of these CHRs in their entirety against a range of cancer cell lines had not yet been thoroughly examined (Sadava *et al*, 2002) (Kim *et al*, 1998) (Yoshida *et al*, 1997) (Wong *et al*, 1996) (Cheng *et al*, 2003) (Kok *et al*, 1995) (Ikemoto *et al*, 2000) (Chan *et al*, 2000) (Lee *et al*, 2001) (Horikawa *et al*, 1994) (Xiaohua *et al*, 2000) (Chiu *et al*, 2002) (Kuo *et al*, 1997) (Srinivas *et al*, 2003) (You *et al*, 1994) (Yang *et al*, 1994) (Yang *et al*, 1997). Based on these criteria, the CHRs *Oldenlandia diffusa* (OD), Long Dan Xie Gan Wan (LD), *Polygonum multiflorum* (PM) and *Polyporus umbellatus* (PU) were selected for this investigation.

The second objective of this investigation was to assess the direct cytotoxic potential of the selected CHRs against cancer cells. To achieve this, an *in vitro* cell based system was used. In order to examine the spectrum and specificity of activity of these CHRs a range of cancer cell lines were used, and included a leukamic cell line (HL60), gut associated cancer cells (HT29 and HCT-8), cervical cancer cells (HeLa)

and a non-human cancer cell line (CHO). The cytotoxic actions of water extracts of each CHR were examined to determine whether traditional preparations of these remedies exhibited a direct cytotoxic potential against cancer cells, and the effect of ethanol extracts were investigated to determine whether smaller, volatile compounds within the CHRs possessed anticancer activity.

The results of this study suggested that water extracts of OD, LD and PM were significantly toxic to a range of cancer cell types *in vitro*, generally killing cancer cells at high concentrations (1:10) and exerting a growth inhibitory action at lower concentrations (1:100 and 1:1000), thus suggesting that the preparations of OD, LD and PM used in this study contained water soluble compounds that exerted a dose related cytotoxic action against a range of cancer cell types. In contrast, ethanol extracts of these CHRs generally exerted a less significant cytotoxic action, thus suggesting these preparations did not contain smaller, polar compounds that exerted an *in vitro* anticancer action.

In contrast to the significant cytotoxic action of water extracts of OD, LD and PM against cancer cells observed in this study, water extracts of PU were found to exert very little cytotoxic effect on any cell line at any concentration. Ethanol extracts also exhibited very little effect at any concentration. Thus, these results suggested that PU did not contain water soluble or polar compounds that were directly toxic to the cancer cell lines used in this investigation.

The third objective of this study was to then characterize the chemotherapeutic potential of those CHR extracts found to exert a direct cytotoxic action against cancer cells. This was initially explored by determining the mode of cell death (apoptosis or necrosis) triggered in cancer cells exposed to those CHRs at 1:10. The mode of cell death induced by water extracts of OD, LD and PM in cancer cells *in vitro* was examined using the Annexin V, PI staining and TUNEL assays. Two cell lines (HL60 and HT29) were used to examine the chemotherapeutic potential of OD, LD and PM and to look for evidence of cancer cell line-specific effects.

The results of the Annexin V, PI staining and TUNEL assays consistently suggested that water extracts of OD and LD induced apoptosis in both the HL60

and HT29 cell lines. However, these assays provided a series of inconsistent data for the mode of cell death induced by PM, with the TUNEL assay suggesting PM was inducing apoptosis, and the Annexin V and PI staining assays suggesting it was inducing necrosis. Thus, further investigation was required to confirm the mode of cell death induced in cancer cell lines by PM.

Having characterized the mode of cell death triggered in cancer cells by water extracts of OD and LD, and with data suggesting that water extracts of PM may be inducing necrosis, the fourth objective of this investigation was to further characterize the cytotoxic action of water extracts of OD, LD and PM against cancer cells. This involved determining the role of the intrinsic and extrinsic pro-apoptotic signalling pathways in cell death mediated by OD, LD and PM, examining the role of cell cycle arrest in the growth inhibitory actions of the CHRs at lower concentrations, and examining their genotoxic action against cancer cells.

The role of the intrinsic and extrinsic pro-apoptotic pathways in cell death mediated by water extracts of OD, LD and PM were examined because these are distinct pro-apoptotic signalling cascades that are activated in response to distinct pro-apoptotic stimuli, with the extrinsic cascade activated by signalling through death receptors, and the intrinsic cascade activated by the detection of intracellular damage (Schimmer *et al*, 2001). Thus, determining which pro-apoptotic signalling cascade was activated by the CHR extracts would provide information regarding their mechanism of biological action on the cancer cell lines *in vitro*.

The extrinsic apoptotic pathway is characterized by the activation of caspase 8, and the intrinsic apoptotic pathway characterized by activation of caspase 9. Thus, activation of caspase 8 and 9 were examined in HL60 cells exposed to water extracts of OD and LD. In addition, caspase 3 activation was investigated because caspase 3 is a key executioner caspase that is activated by both pathways, and its activation is a hallmark response to apoptotic induction (Schimmer *et al*, 2001).

The results of this study revealed that water extracts of OD and LD activated caspases 9 and 3 but not 8 in the HL60 cell line, thus suggesting that water extracts of OD and LD induced apoptosis through activation of the intrinsic caspase cascade.

Furthermore, as the intrinsic caspase cascade is activated in response to the detection of intracellular damage, the results of this investigation suggested that OD and LD may have induced some form of intracellular damage that led to apoptotic induction.

This study also revealed that PM did not activate any of the caspases, thus suggesting that PM induced necrosis in the HL60 cell line. This finding was consistent with the results of the Annexin V and PI staining assays, and provided further evidence that PM was inducing necrosis and not apoptosis in the HL60 and HT29 cell lines.

While the above studies helped characterize the cytotoxic action of water extracts of OD, LD and PM at 1:10, as was observed in the growth inhibition study, they did not address the issue of whether the apparent growth inhibitory actions of these CHRS at lower concentrations (1:100 and 1:1000, as observed in the growth inhibition study) was occurring as a result of dose-related cell death or the induction of cell cycle arrest, and this information was essential in characterizing their cytotoxic action against cancer cells at a range of concentrations. Thus, their ability to augment cell cycle characteristics and trigger cell death at 1:50 and 1:100 were examined.

The results of this study revealed that, for the HL60 cell line, the apparent growth inhibitory actions of OD, LD and PM were related to dose-related cell death and not cell cycle arrest. In contrast, dose-related augmentation of cell cycle characteristics appeared to occur in HT29 cells exposed to OD, LD and PM, primarily during S phase of the cell cycle and to a lesser extent G2.

The role of the cell cycle in death mediated by OD, LD and PM at 1:10 in both the HL60 and HT29 cell lines was also examined. Using previously acquired data from the TUNEL assay, cell cycle histograms of viable and dead cells were overlaid and revealed that both HL60 and HT29 cells were dying irrespective of their position in the cell cycle. Thus, these data indicated that the cytotoxic actions of these CHRs at 1:10 were triggering cell death in a manner that was not related to signalling from cell cycle checkpoints.

Having examined the role of the intrinsic pro-apoptotic cascade and cell cycle arrest in the cytotoxic actions of OD, LD and PM against cancer cells, their genotoxic potential was characterized. The genotoxic potential of OD and LD against cancer cells was examined because the induction of DNA damage can result in cell death, apoptotic induction, activation of the intrinsic caspase cascade and cell cycle arrest, all of which had been observed during this investigation. For PM, genotoxic potential was examined because it had been found to kill a range of cancer cell lines in the growth inhibition study, to trigger cell cycle arrest in the HT29 cell line, and to generate false-positive results in the TUNEL and PI staining assays (for which genomic fragmentation is measured as a marker of apoptotic induction), and while PM was not found to induce apoptosis, genomic fragmentation was observed.

The results of this study revealed that OD and LD exerted a dose-related genotoxic action against the HL60 and HT29 cell lines, thus suggesting that the cytotoxic action of these CHRs may have been related to the induction of some form of genotoxic damage that triggered the intrinsic caspase cascade, resulting in apoptotic induction. Furthermore, the induction of DNA damage by these CHRs may have resulted in the cell cycle arrest that was observed in the HT29 cell line.

The results of this study also revealed that PM exerted a significant genotoxic action against both the HL60 and HT29 cell lines. This effect was most pronounced against the HL60 cell line, and at 1:10 the amount of DNA damage generated was so great it could not be accurately measured. The observation that PM did induce DNA damage within these cell lines was consistent with those results obtained earlier in this investigation, and supported the suggestion that those results generated for the TUNEL and PI staining assays were related to the induction of DNA damage through the direct action of PM against DNA, and not an indirect consequence of genomic fragmentation occurring as part of the apoptotic cascade.

The fifth objective of this investigation was to determine whether the cytotoxic action of the selected CHRs was cancer cell specific: effective chemotherapeutic agents are selectively toxic to cancer cells and not normal cells. Thus, the cytotoxic potential of OD, LD and PM was investigated against primary blood lymphocytes

(PBLS), both proliferating and non-proliferating, as the body is comprised of both non-dividing and actively proliferating cell types.

The results of this study suggested that water extracts of OD, LD and PM were more toxic to cancer cell lines *in vitro* than non-proliferating PBLs. The results of this study also showed that OD, LD and PM prevented the progression of phytohemagglutinin (PHA) stimulated PBLs from G0 in to G1/S of the cell cycle, thus suggesting that these CHRs may have been inducing some form of damage that was preventing those cells from progressing through the G0/G1 checkpoint.

For OD and LD there was only a small increase in the number of PBLS actively apoptosing at any time when compared to the control population of PBLs, suggesting that little apoptosis was occurring in both un-stimulated and PHA-stimulated PBL populations. However, for PBLs exposed to PM there was a large sub-G1 region characteristic of apoptotic induction, thus suggesting that PM triggered apoptosis in both non-proliferating and PHA-stimulated PBLs (in contrast to its inducing necrosis in cancer cell lines).

The final objective of this investigation was to assess the role of individual compounds in the observed actions of the selected CHRs. This was primarily investigated because the efficacy of any CHR could be attributed to the combined actions of all the compounds within that remedy and the manner in which it is prepared (Lee, 2000).

To examine the role of individual compounds in the observed actions of OD, LD and PM, an activity-guided fractionation study was performed, in which CHR extracts were split into fractions and the cytotoxic potential of each examined. Those extracts found to exert a significant toxic action were then subject to chemical analysis in order to determine whether a single, or multiple compounds were found to be responsible for the observed actions of those CHR extracts.

The results of this study revealed that multiple fractions of each CHR exerted a cytotoxic action against the HL60 cell line, thus suggesting that each of the CHRs contained more than one type of cytotoxic compound. This suggestion was further

supported by chemical analysis of those fractions, which showed that they contained compounds of differing molecular weights. Furthermore, the molecular weights of those compounds previously identified as cytotoxic in OD, LD and PM in previous studies did not correspond to the molecular weights of those compounds found in the cytotoxic fractions of this study, thus suggesting that the preparations of OD, LD and PM used in this investigation contained cytotoxic compounds that have not previously been reported in these CHRs (Kim *et al*, 1997) (Hsu *et al*, 2004) (Chan *et al*, 2000) (Ikemoto *et al*, 2000) (Sonada *et al*, 2004) (Peng *et al*, 2004) (Lee *et al*, 2001) (Koyama *et al*, 2003) (Babich *et al*, 2005) (Lin *et al*, 2003).

This chemical analysis study did not reveal the identities of those compounds found within the cytotoxic fractions of OD, LD and PM. However, those compounds within plants that are typically found to be pharmacologically active are secondary plant metabolites (SM). There are three main families of SM: nitrogen containing compounds, phenolics and terpenes (terpenoids) (Bourgaud *et al*, 2001). Thus, it is likely that the cytotoxic constituents of OD, LD and PM identified in this study are members of one of these families.

Overall, the results of this investigation suggested that water extracts of OD, LD and PM were significantly toxic to a range of cancer cell lines *in vitro* while being less toxic to non-cancer cells, and that the cytotoxic actions of water extracts of these CHRs were related to the combined actions of multiple compounds and not to a single cytotoxic constituent. For OD and LD, their cytotoxic action appeared to be related to activation of the intrinsic pro-apoptotic signalling cascade, possibly through induction of genomic damage, leading to cell death via apoptosis. For PM, cytotoxicity to cancer cells appeared to be related to the induction of necrosis. Furthermore, the results of this study suggested that PU did not elicit a direct cytotoxic action against cancer cells *in vitro*.

10.2 Addressing the limitations of the strategy used

The aim of this investigation was to select a range of CHRs with some suggestion of tumour modulatory activity and to investigate their direct toxicity to cancer cells in an attempt to elucidate their mode of action.

While the strategy devised for this investigation did provide information regarding the direct cytotoxic action of OD, LD, PM and PU against cancer cells, there are limitations in the approach used, and a number of these are directly related to common issues regarding CHR research. Amongst these common issues are a lack of standardization of CHR preparations, variability in the actions of herbs depending on where they were grown and their time of harvest (Yuan & Lin 2000), accidental contamination and adulteration, and the incorrect identification of herbs (Ernst 2002) affecting results. In order to address some of these issues within this study, CHRs were all purchased from a single reputable source in a single lot and used throughout the investigation. However, these factors must, whenever possible, be taken into consideration when carrying out research in this area, and must also be taken into consideration when comparing results from different studies.

Furthermore, when considering the results of this investigation, it is important to note that a traditionally Western, or mechanistic, approach has been used to examine the anticancer potential of remedies that were devised using traditional Eastern philosophies. Thus, while this mechanistic approach has provided information regarding the cytotoxic potential of the selected CHRs against cancer cells in a Western scientific context, it must be noted that the theoretical foundations of TCM are based on the belief that the efficacy of a CHR is related to it exerting multiple biological activities that interact synergistically in the treatment of disease, and therefore mechanistic studies (such as this one) do not help explain or predict the *in vivo* actions of CHRs (Jin-Ling Tang, 2006).

For this reason, some TCM practitioners argue that efficacy-based approaches, in which the activities of CHRs are directly tested on patients, are the only way of determining the potential effectiveness of a CHR. Furthermore, they would also argue that if efficacy-based studies show that a CHR is effective, there is no need to conduct mechanistic studies to characterize their mode of biological activity, as these would not alter their effectiveness, they would simply provide another theory with which to explain their mode of action.

Thus, proponents of efficacy-based research may argue that any mechanistic studies are fundamentally flawed, as they ignore the fundamental ethos of TCM and therefore

do not contribute to the understanding of the efficacy of those remedies, while proponents of Western methodologies may argue that mechanistic studies are central to our understanding of the biological actions of such medicines. However, there is no right answer to this contentious issue, and it is up to an individual to formulate their own opinion regarding the relative merits of mechanistic and efficacy-based research.

Despite its limitations, this mechanistic investigation has provided some insight into the direct cytotoxic action of OD, LD, PM and PU against cancer cells *in vitro*, and furthermore the results of this investigation may provide some insight into their *in vivo* anticancer potential.

10.3. Elucidating the *in vitro* and *in vivo* anticancer potential of *Oldenlandia diffusa*

Oldenlandia diffusa (OD) is a popular CHR that is traditionally prescribed as a tea for the treatment of a variety of diseases, including lung liver and rectal cancers (Chu, 2003). The results of this *in vitro* investigation suggested that water extracts of OD were selectively toxic to a range of cancer cell types, triggering apoptosis through activation of the intrinsic apoptotic pathway, possibly as a result of inducing some form of genotoxic damage. The suggestion that OD may elicit a cytotoxic action against a range of cancer cell lines *in vitro* is consistent with previous and subsequent studies that have shown extracts of OD elicit a direct cytotoxic action against other cancer cell lines *in vitro* (Sadava *et al*, 2002) (Kim *et al*, 1998) (Gupta *et al*, 2004) (Yadav & Lee, 2006). The suggestion that OD may elicit an anticancer action through induction of the intrinsic pro-apoptotic pathway is consistent with studies that have shown OD can activate the caspase cascade in other cell lines (Sadava *et al*, 2004) (Yadav & Lee, 2006). Furthermore, the findings of this investigation that OD is more toxic to cancer cells than non-cancer cells *in vitro* are consistent with a study that has shown OD to be significantly more toxic to lung cancer cells than normal lung epithelial cells (Sadava *et al*, 2002). Thus, this present investigation provides support to the theory that OD may elicit a cancer cell-specific directly cytotoxic action against a range of cancer cell types *in vitro*, and not just against those cancer cell types for which it is prescribed.

In addition, this present investigation has suggested for the first time, as far as the author is aware, that the cytotoxic action of OD may be related to its ability to induce DNA damage in cancer cell lines, and that this activity can potentially lead to the induction of cell cycle arrest (as seen in the HT29 cell line). The results of this present investigation also suggest that the cytotoxic action of OD against cancer cells *in vitro* may be related to a number of cytotoxic compounds that have not previously been reported (as far as the author is aware), as the chemical analysis study revealed that the cytotoxic fractions of OD contained compounds of previously un-reported molecular weights.

Furthermore, as the results of this and other *in vitro* investigations suggest that OD is significantly toxic to a range of cancer cell lines while being less toxic to non-cancer cells, it is possible that OD may exert an anticancer action *in vivo* through direct toxicity to cancer cells, and that this may be related to the selective activation of the intrinsic caspase cascade in cancer cells (and not normal cells) and the induction of apoptosis.

The suggestion that OD may elicit an anticancer action *in vivo* is supported by studies in mice that have shown water extracts of OD can inhibit the growth of Renca (Wong *et al* 1996) and B16-F10 tumours in mice, while having no toxic effect on the major organs (liver, heart, stomach, kidney and intestines) (Gupta *et al*, 2004). However, studies have shown that, in addition to exerting a direct cytotoxic action against cancer cells, CHRs can also exert an indirect anticancer action through immune-enhancing activity: CHRs have been shown to affect acquired immunity and (more commonly) innate immunity. Changes observed in acquired immunity include a mitogenic effect on B lymphocytes and increased antibody production. Stimulatory effects on innate immunity include an increase in Natural Killer (NK) and Lymphocyte Activated Killer (LAK) cell number and activity, macrophage activation, an increase in phagocytic activity, and proliferation of specific T cell subsets (Block & Mead, 2003).

A number of studies have examined the immune-enhancing potential of OD against tumours in mice and human immune cells using both *in vitro* and *in vivo* test systems: in one study, OD was found to promote specific lethal activity of human

and mice NK cells against tumour cells, increase B cell antibody production, increase the production of cytokines by monocytes and promote phagocytosis against tumour cells. In this study, a 90 kD glycoprotein was believed to be responsible for the action of OD (Shan *et al*, 2001).

In another study, water extracts of OD were shown to act as an accelerator of murine peritoneal macrophage activation by rIFN-γ *in vitro*, increasing production of nitric oxide (NO) and TNF-α via NF-κB activation. It is believed this may (in part) explain the effects of OD on tumours *in vivo*, as NO is a macrophage derived effector molecule against tumours (Chung *et al*, 2002). In addition, OD has been found to restore suppressed immune function in tumour-bearing mice (Yoshida *et al*, 1997).

An investigation in to the effect of OD on human lymphocytes *in vitro* found OD weakly stimulated PBL's to proliferate. Cytotoxic T lymphocyte activity induced by incubating PBLs with allogenic tumour cells (MT-2) was enhanced by the presence of OD. *Oldenlandia diffusa* (OD) was also found to significantly enhance IgG production, although it did not elicit a proliferative response. In addition, OD was found to stimulate IL-1 production by monocytes. Furthermore, OD treated monocytes had tumour cell growth inhibitory activity in *in vitro* culture (Shan *et al*, 1999).

Overall, this and other studies provide evidence that OD may possess anticancer activity when used in its traditional manner, and that this activity may be related to the combined actions of directly toxic and immune-enhancing compounds. Furthermore, this current investigation suggests that OD may be a source of novel chemotherapeutic compounds that could potentially be used in Western medicine. However, as the results of this investigation suggest that the efficacy of OD is related to the combined actions of a number of compounds, it may not be possible to isolate any individual compounds that exert a significant anticancer action while exerting little toxicity to other cell types.

10.4. Elucidating the *in vitro* and *in vivo* anticancer potential of Long Dan Xie Gan Wan (LD)

Long Dan Xie Gan Wan (LD) is a CHR that is made of 10 separate herbs, including *Radix Scutellariae* (Huang Qin), *Fructvs Gardeniae* (Zhi zi), *Radix glycyrrhizae* (Gan cao), *Medulla tetrapanacis* (Tong cao), *Radix rehmanniae* (Di huang), *Radix Gentianae* (Long dan), *Radix angelicae sinensis* (Dang gui), *Semen Plantaginis* (Che qian zi), *Radix Bupleuri* (Cai hu) and *Rhizoma alismatis* (Ze xie) (RxList, 2004). Long Dan Xie Gan Wan (LD) was primarily selected for this investigation because a TCM practitioner recommended it for study, and because previous studies had found that its constituents possess anticancer activity in isolation (Cheng *et al*, 2003) (Kok *et al*, 1995) (Hsu *et al*, 2004) (Cheng *et al*, 2005) (Lee *et al*, 2005) (Ikemoto *et al*, 2000) (Sonoda *et al*, 2004) (Lee *et al*, 2001) (Peng *et al*, 2004).

To the authors knowledge, this is the first investigation to suggest that LD may elicit a cytotoxic action against a range of cancer cell lines *in vitro*. The results of this *in vitro* investigation suggested that water extracts of LD were selectively toxic to a range of cancer cell types, triggering apoptosis through activation of the intrinsic apoptotic pathway, possibly as a result of inducing some form of genotoxic damage, and that this activity was related to the actions of a number of compounds whose molecular weights did not correspond to those previously reported within its herbal constituents (Hsu *et al*, 2004) (Chan *et al*, 2000) (Ikemoto *et al*, 2000) (Sonada *et al*, 2004) (Peng*et al*, 2004) (Lee *et al*, 2001).

Based on the findings of this investigation, it is possible that LD may exert an anticancer action *in vivo* through a directly cytotoxic action against cancer cells. However, the *in vivo* anticancer action of LD has not previously been examined, and as such there is no direct evidence to support this theory.

Yet while the *in vivo* anticancer action of LD in its entirety has not been examined, studies have focused on the *in vivo* anticancer potential of its constituents. For example, daily administration of *Radix bupleuri* has been shown to inhibit the growth of A549 cells in mice while having no effect on mouse body weight (Cheng *et al*, 2005), thus suggesting that LD may contain compounds that possess an *in vivo* anticancer action. In a separate study, BALB/c mice implanted with WEHI-164

tumour cells were given i.p. injections of selected fractions of *Radix bupleuri*, and tumour cell growth was found to be significantly inhibited. Furthermore, i.p. injection of *Radix bupleuri* significantly increased the number of tumour infiltrating lymphocytes within implanted tumours and elevated NK toxicity in splenocytes. *In vitro*, water extracts of *Radix bupleuri* stimulated the cytolysis of tumour cells co-cultured with macrophages, induced LAK cytotoxicity to tumour cells and increased IFN-γ cytokine release by T and NK cells (which prime macrophages for non-specific tumouricidal activity). However, little direct toxic activity on cell lines was observed (Kok *et al*, 1995). Thus, the results of this investigation suggest that this constituent of LD may elicit an anticancer action *in vivo* through its immune-enhancing potential. Another constituent of LD, *Radix scutellariae*, has also been shown to elicit an *in vivo* anticancer action, inhibiting the growth of MBT-2 tumour cells implanted in C3/HeN mice (Ikemoto *et al*, 2000).

Overall, the results of this and other investigations suggest that LD may contain a number of compounds that interact to elicit an anticancer activity, potentially through a combination of direct toxicity to cancer cells and through indirect immune-enhancing activity. Thus, there may be some scientific basis for the use of LD in the treatment of cancer. Furthermore, there may be compounds within LD that may be of chemotherapeutic value. However, the efficacy of any CHR is traditionally attributed to the combined actions of its constituents, and this study suggests that LD may possess a number of compounds that act in combination in a synergistic or additive way.

10.5. Elucidating the *in vitro* and *in vivo* anticancer potential of *Polygonum multiflorum* (PM)

Polygonum multiflorum (PM) is a medicinal vine plant that is commonly prescribed to the elderly by TCM practitioners to restore vitality. The cytotoxic potential of PM against cancer cell lines *in vitro* has not previously been reported. However, PM has been found to contain compounds that elicit a pro-apoptotic action against cancer cell lines in isolation *in vitro*, and include emodin (Koyama *et al*, 2003), epicatechin gallate (Babich *et al*, 2005), epigallocatechin gallate, catechin and physcion (Lin *et al*, 2003). As PM contained these pro-apoptotic compounds, it was hypothesized that in its entirety it can elicit a pro-apoptotic action against cancer cells.

However, the results of this investigation showed that, instead of PM triggering apoptosis in cancer cell lines *in vitro* through the combined actions of these compounds, water extracts of PM triggered necrosis in cancer cells, possibly through the induction of DNA damage, and this activity was related to the combined actions of compounds whose molecular weights did not correspond to those of the pro-apoptotic compounds previously described in PM. Furthermore, PM was found to induce apoptosis in cultured PBLs instead of necrosis, thus suggesting that the mode of cell death triggered by PM varied between cancerous and non-cancerous cell types. Although the reason for this is unknown, it may be related to the functional status of p53 in the PBLs, and their ability to evade necrotic cell death (Koyama *et al*, 2003) (Babich *et al*, 2005) (Lin *et al*, 2003).

Thus, while other studies have shown that PM contains compounds that induce apoptosis in cancer cell lines in isolation (Xiaohua *et al*, 2000) (Chiu *et al*, 2002) (Kuo *et al*, 1997) (Srinivas *et al*, 2003), the results of this study suggest that, in its entirety, water extracts of PM trigger necrosis in cancer cell lines *in vitro*, and apoptosis in PBLs. Furthermore, as effective cancer chemotherapeutic agents selectively induce apoptosis in cancer cells and not normal cells, thereby facilitating their removal from the body without eliciting an immune response, the results of this investigation suggest that PM is not an effective anticancer remedy. However, it is possible that the cytotoxic constituents of PM could potentially be effective chemotherapeutic agents when used in isolation.

10.6. Elucidating the *in vitro* and *in vivo* anticancer potential of *Polyporus umbellatus* (PU)

Polyporus umbellatus (PU) is a medicinal mushroom that is traditionally prescribed for the treatment of difficult urination, bladder cancer and diarrhoea (Dharmananda, 2002). The direct cytotoxic actions of PU against cancer cell lines *in vitro* had not been reported at the time of this study. However, previous studies had suggested that PU could elicit an anticancer action in tumour bearing mice (You *et al*, 1994), and prevent the post-operative recurrence of bladder cancer in humans (Yang *et al*, 1997) (Yang *et al*, 1999), thus suggesting that PU could potentially possess a genuine anticancer action.

The results of this present investigation suggest that PU does not possess a directly cytotoxic action against cancer cell lines *in vitro*. Thus, this investigation suggests that the previously reported *in vivo* anticancer action of PU may be related to the actions of compounds that can enhance immune function against tumours and not compounds that are directly cytotoxic to cancer cells. This theory is supported by histopathological studies performed by You *et al* (1994) which showed that oral administration of PU stimulated infiltration of transplanted tumours in mice with lymphocytes, and with a more recent investigation that showed PU extracts could reverse the immunosuppressive properties of S180 tumour cells against mouse splenocytes (Yang *et al*, 2004).

10.7. Conclusion

This investigation has provided an insight into the *in vitro* directly cytotoxic potential of the CHRs OD, LD, PM and PU against cancer cells, and also provided information regarding their *in vivo* chemotherapeutic potential. However, due to common variation in the efficacy of individual herbs depending on where they are grown, their time of harvest, their purity, the manner in which they are prepared and variation in the constituents of common CHRs (Yuan & Lin, 2000), there is a long way to go before the modes of action that explain their purported effectiveness are fully characterized.

However, the results of this investigation do provide information regarding the anticancer actions of the CHRs OD, LD, PM and PU and thus do go some way to facilitate elucidating their mode of action. Furthermore, as the biological actions of these CHRs appear to be related to the combined actions of a range of compounds within them, this investigation has highlighted the relevance of examining the efficacy of CHRs in their entirety, and of examining the actions of CHRs in a variety of biological settings (as these CHRs have been shown to exhibit a range of cytotoxic and immune-enhancing activities). In addition, the results of this investigation suggest that CHRs may be a source of novel chemotherapeutic agents, and that there may be some scientific basis for their traditional use in the treatment of cancer. It is clear, however, that further research is needed to determine the potential of these CHRs as novel chemotherapeutic agents.

Future work

A range of strategies need to be employed to further elucidate that anticancer potential of the CHRs OD, LD, PM and PU. These strategies may include further investigation into their *in vitro* cytotoxic action, investigation into their immune enhancing potential, *in vivo* studies examining their chemotherapeutic potential, and chemical analysis studies to determine the compounds responsible for their biological action(s).

1. Future work examining the direct cytotoxic action(s) of water extracts of the CHRs against cancer cells *in vitro*

The results of this current investigation suggested that the CHRs OD and LD exerted a significant cytotoxic action against cancer cell lines *in vitro*, possibly through the induction of genotoxic damage and activation of the intrinsic pro-apoptotic signalling cascade. Thus, further investigation may be warranted to further characterize the genotoxic and pro-apoptotic activity of OD and LD against cancer cells *in vitro*.

This further investigation may include defining the specific type of DNA damage induced by OD and LD. It may also include characterization of the role of Bcl 2 family members in the pro-apoptotic activity of the CHRs, as the intrinsic pro-apoptotic cascade is regulated through interactions of Bcl2 family members. In addition, the ability of OD and LD to trigger cell cycle arrest in the HT29 cell line, as observed in this current investigation, may have also been related to their genotoxic action. Thus, further investigation into the ability of these CHRs to augment cell cycle characteristics within this cell line would provide more information regarding their cytotoxic activity against cancer cells over a range of concentrations. Furthermore, examining the cytotoxic action of OD and LD against a range of other cancer cell lines would provide further information regarding whether or not their actions are cancer cell type-specific. Finally, further characterization of the growth inhibitory actions of OD and LD against PBLs and other non-cancerous cell types cultured *in vitro* may provide further information regarding their cancer cell-specific cytotoxic potential and a greater knowledge and understanding of their effect on non-cancer cell lines.

2. Future work to investigate the immune-enhancing potential of the CHRs against cancer cells

While this investigation focused on the direct cytotoxic action of the CHRs OD, LD, PM and PU against cancer cells, some of these CHRs may, or have been shown to elicit anticancer activity through their immune-enhancing activity. Thus, an investigation into their ability to enhance immune function against cancer cells may provide further information regarding their *in vivo* anticancer potential.

3. Future work to elucidate the *in vivo* anticancer potential of the CHRs OD, LD and PU

The efficacy of any CHR is traditionally related to the belief that it exerts multiple biological activities. Thus, *in vivo* animal studies and human trials may provide information regarding their true anticancer potential.

4. Future work regarding chemical analysis of those CHRs

While the above studies would further characterize the efficacy of the CHRs in their entirety, chemical analysis studies would provide information that may contribute to identifying compounds that are key in the cytotoxic effects observed in this investigation. Then, having isolated and characterized the nature of these compounds, it may be possible to determine which combinations of ingredients were responsible for the actions of those CHRs in their entirety. In addition, chemical analysis may lead to the discovery of novel compounds which could potentially be useful for the treatment of cancer when used in isolation.

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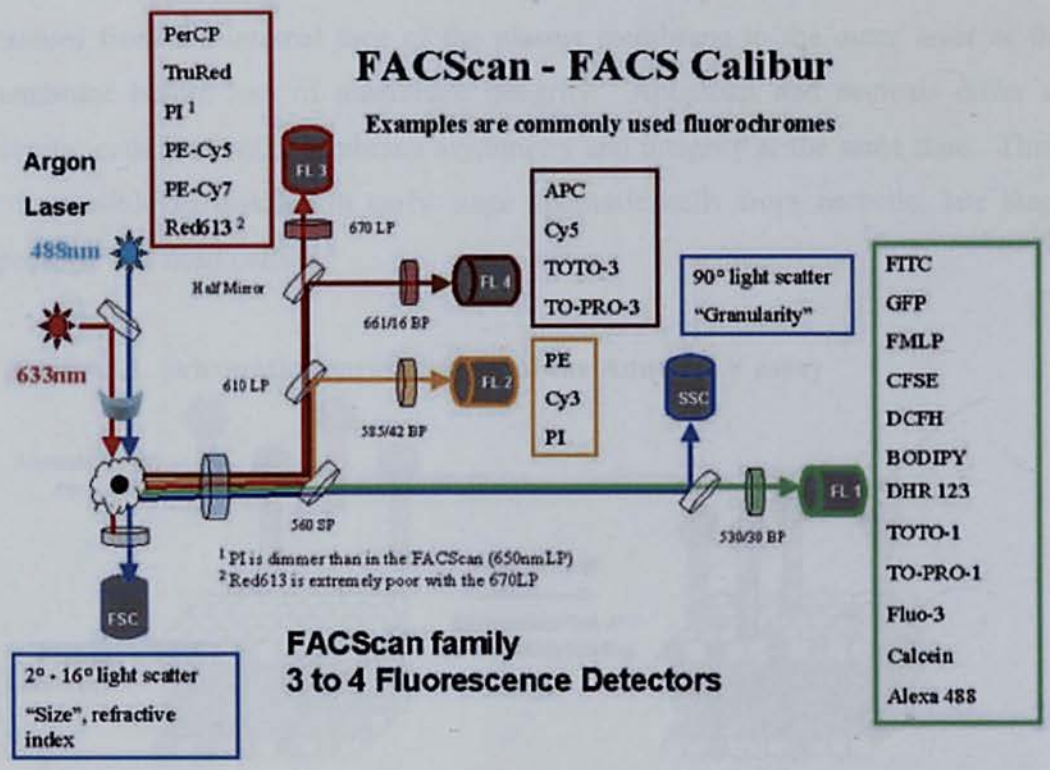
Appendix

A1. Flow cytometry

A flow cytometer measures the characteristics of individual cells in suspension as they are actively passed through a sensing point (the flow cell). Flow cytometers consist of a sample injection port, a light source, collection optics and a computer that translates signals to data. When a cell suspension is inserted into a flow cytometer, cells are drawn through the flow cell in single file and pass through the light source (in a FACSCalibur this light source consists of a 488nm blue laser and a 635nm red laser). The collection optics within a flow cytometer measure the physical characteristics of individual cells and light emitted from fluorochromes attached to individual cells as they are excited by the light source (summarized in Figure A1).

The physical characteristics of cells measured by a flow cytometer are their size and granularity. The size of a cell is characterized by the amount of laser light that passes through that cell; this is known as Forward Scatter (FSC). The granularity of a cell is characterized by the amount of light that is diffracted as it passes through a cell, and is known as Side Scatter (SSC). The FACSCalibur also contains four fluorescence detectors (FL1, 2, 3 and 4) that detect specific ranges of light (the wavelengths of light detected by each are determined by the filters placed in front of those detectors). The FL detectors are used to detect light emitted from fluorochromes bound to specific targets within cells as they pass through the laser shining through the flow cell. These flurochromes can be bound to specific targets within cells as antibody conjugates or through the fluorochrome itself binding to cellular components (for example propidium iodide (PI) binds to double stranded DNA and RNA). A flow cytometer can measure up to 1000 cells per second, and 10,000-15,000 events are typically scored.

Figure A1. Detector arrangement of the FACSCalibur flow cytometer and commonly used fluorochromes



Taken from facs.scripps.edu/ facslab.html,

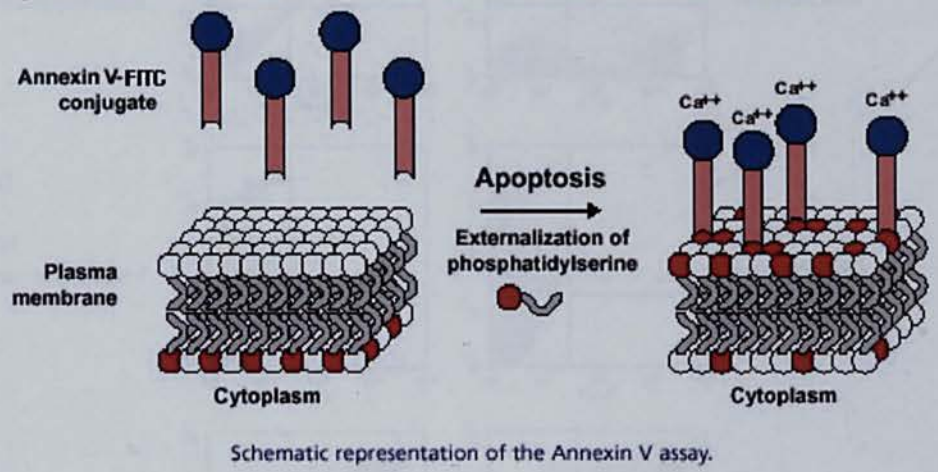
retrieved 21 November 2005

Figure A1 Diagram showing the detector arrangement of the FACSCalibur flow cytometer. When individual cells pass through the flow cell they pass through an argon laser. The Forward Scatter (FSC) measures the amount of light that has passed through that cell, and is proportional to its size, while the Side Scatter (SSC) detector measures the light scatter generated by that cell, which is proportional to its granularity. In addition, any fluorochromes bound to cells will be excited and emit light as they pass through the laser. This light will be detected by one of the four fluorochrome detectors (FL1, 2, 3 or 4), depending on the wavelength of light that fluorochrome emits. Commonly used fluorohromes are listed next to the relevant FL detector.

A2. Annexin V assay compensation

The Annexin V assay is used to detect one of the early morphological characteristics unique to apoptotic cell death, which is translocation of phosphatidylserine (PS) residues from the internal face of the plasma membrane to the outer layer of the membrane before loss of membrane integrity. Apoptosis and necrosis differ as necrotic cells lose both membrane asymmetry and integrity at the same time. Thus, it is possible to distinguish early stage apoptotic cells from necrotic, late stage apoptotic and dead cells.

Figure A2.1. Schematic representation of the Annexin V assay



Taken from BDbiosciences.com

Figure A2.1 Shows translocation of PS residues from the inner plasma membrane to the outer membrane during apoptosis. Externalized PS residues are bound by FITC conjugated Annexin V.

Annexin V itself is a calcium-dependent phospholipid-binding protein with a high affinity for PS residues. For the Annexin V assay, annexin V protein is conjugated with the fluorochrome FITC. When incubated with a cell population, annexin V-FITC will bind to early stage apoptotic, late stage apoptotic, necrotic and dead cells. To differentiate between these cell populations, the vital dye propidium iodide (PI) is also added, which stains late stage apoptotic, necrotic and dead cells while not staining early stage apoptotic cells (Vermes *et al*, 1995).

The fluorochrome FITC, whilst primarily detected by FL1, is also detected by the FL2 detector (due to the wide spectra emitted by the FITC fluorochrome), which in this experiment was used to measure the PI signal. Therefore the FITC signal was subtracted from FL2 using compensation before acquisition of data, and the PI signal subtracted from FL1, as summarized below.

Figure A2.2. Calibration of the flow cytometer for the Annexin V assay

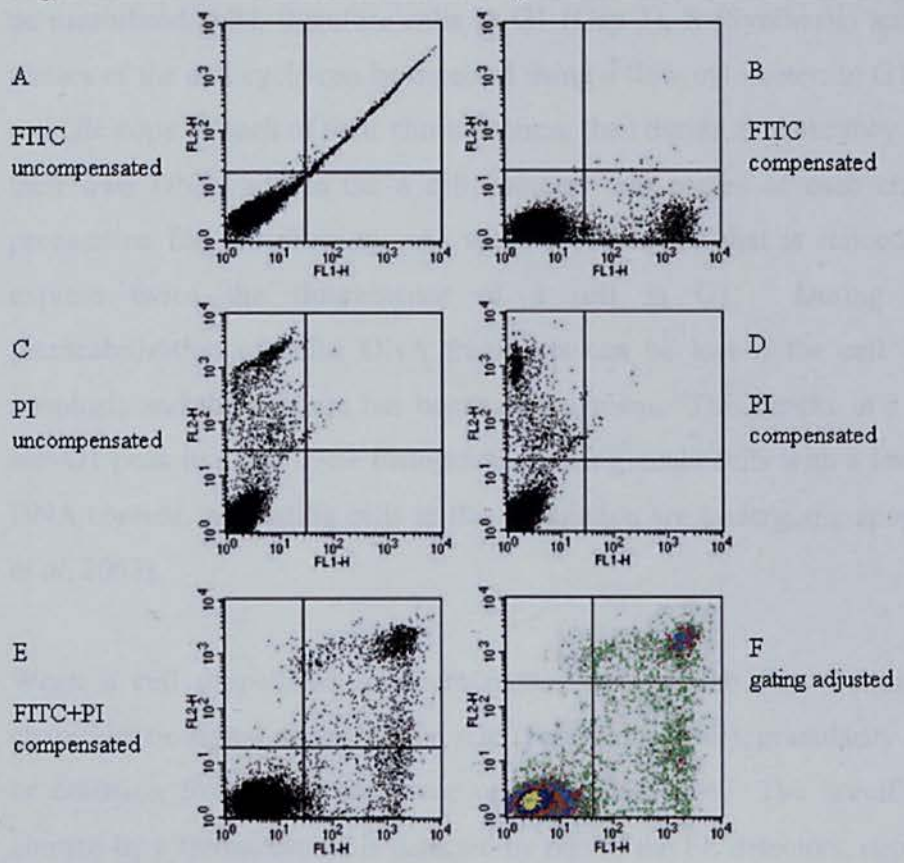


Figure A2.2. Compensation of FL1 and FL2 detectors and regions gated before data acquisition of the Annexin V assay. The x axis represents Annexin V-FITC detected by the FL1 detector. The y axis represents PI staining of cells detected by FL2.

To calibrate the flow cytometer before acquisition of data a cell population was stained with FITC only (Figure A2.A) and analyzed. The diagonal line represents FITC positive cells whose signal is being detected by both FL1 and FL2 detectors. After compensation (Figure B) the signal is detected by FL1 only, and not FL2. Another population of cells was stained with PI only (Figure C) and the compensation adjusted so PI was not detected by FL1 (Figure D). As shown, the spectral overlap is much less for PI in FL1 than FITC in FL2. After calibration,

control flasks containing both fluorochromes were analyzed (Figure E) and quadrants readjusted for analysis of results (Figure F).

A3. PI staining compensation

This technique specifically provides evidence of one of the later characteristics of apoptosis, which is fragmentation of the genome. Propidium iodide (PI) is a fluorescent dye that binds stochiometrically to double stranded DNA within permeabilised cells, therefore cells in G1 (Gap 1), S (Synthesis) and G2 (Gap 2) phases of the cell cycle can be detected using a flow cytometer: in G1 cells contain a single copy of each of their chromosomes, then during S phase they begin to copy their own DNA, and in G2 a cell contains two copies of each chromosome in preparation for cell division. As such, a cell in G2 that is stained with PI will express twice the fluorescence of a cell in G1. During fixation and permeabilization of cells, DNA fragments can be lost if the cell is undergoing apoptosis and the genome has begun to fragment. This results in a characteristic sub-G1 peak in a cell cycle histogram, showing intact cells with a less than normal DNA content, suggesting cells in this population are undergoing apoptosis (Otsuki *et al*, 2003).

When a cell or cellular fragment passes through the flow cell it generates a characteristic signal depending on size (Forward Scatter), granularity (Side-Scatter) or emission from a fluorochrome or fluorescent dye. The specific wavelength emitted by a fluorochrome is detected by one of the FL detectors, depending on the wavelength of emitted light (either FL1, 2, 3 or 4). Propidium iodide is detected by the FL2 detector. When an event is registered it generates a signal which is recorded as a peak with a characteristic height (FL-Height), width (FL-Width) and area (FL-Area). FL-Height is typically used to plot data. Using PI staining, the intensity of an FL2-Height signal from a cell in G2 is approximately double that of a cell in G1. However, when two cells in G1 pass through the flow cell at the same time (known as a doublet) this is recorded as a single event, and gives a signal similar to that of a single cell in G2. Cells in G2 and doublets can be separated by creating a dot plot showing FL2-Area versus FL2-Width. Although both types of event give the same FL2-Height and FL2-Area signals, the width of the signal is greater. Therefore it is possible to gate single events and exclude doublets (Figure

A3.A), then use this gate to show only single events in cell cycle histograms. Figure B shows an un-gated histogram, with a doublet peak shown between channels 320-360. Figure C shows a gated histogram, containing only single cells that have passed through the flow cell. Gating is necessary to compare the percentage of viable cells to the apoptotic population, it is also necessary when analysing the percentage of cell in each stage of the cell cycle. Gating singlets was also used for the TUNEL assay (see below).

Figure A3. Gating of single cells for analysis of cell populations using PI staining

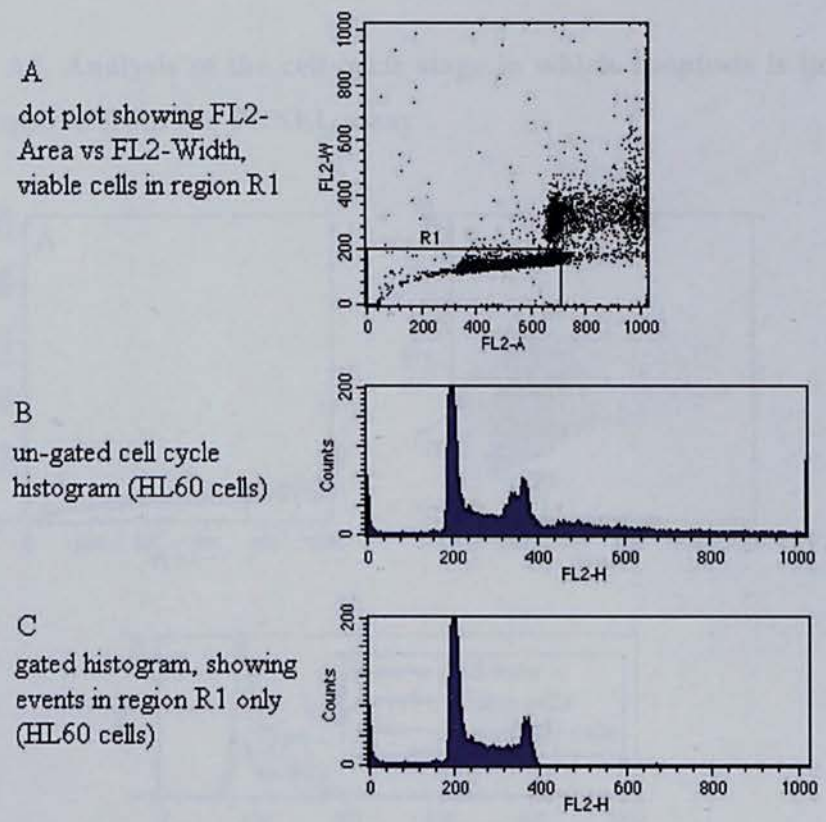


Figure A3. Gating of cell populations for analysis using FL2 area versus FL2 width to discriminate between single cells that have passed through the flow cytometer and doublets.

A4. TUNEL assay compensation

The TUNEL (TdT-mediated dUTP Nick-End Labelling) assay is used to detect 3′ hydroxyl-termini DNA strand breaks within individual cells: these breaks are characteristic of DNA cleaved by endonucleases during apoptosis. In the TUNEL

assay, these DNA strand breaks are labelled through the catalytic incorporation of fluorescin-12 dUTP at the 3'-hydroxyl ends of fragmented DNA using the enzyme Terminal Deoxynucleotidyl Transferase (TdT). Thus, cells undergoing apoptosis become fluorescently labelled, while non-apoptotic cells remain unlabelled (Otsuki *et al*, 2003).

Analysis of the cell cycle stage in which apoptosis was being induced are expressed as overlaid histogram plots showing all cells, viable cells and apoptotic cells, with data gated from TUNEL density plots and FL2-Area versus FL2-Width plots, as shown below in Figure A4.

Figure A4. Analysis of the cell cycle stage in which apoptosis is induced using data acquired from the TUNEL assay

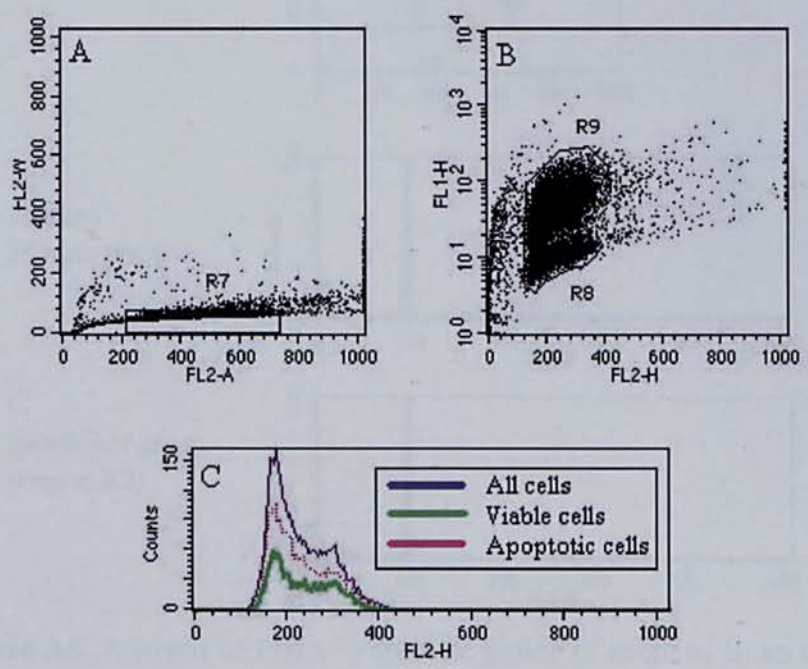


Figure A4. Figure A: FL2-A versus FL2-W. Region R7 of this plot gates single events that have passed through the flow cell. This region is shown as the purple histogram line, representing all cells. Figure B: dot plot showing DNA content on the x axis and the relative number of strand breaks on the y axis. Region R8 contains viable cells, represented as the green line in the histogram, region R9 gates apoptotic cells, represented as the pink line in the histogram. This example is HL60 cells exposed to PM for 4 hours.

A5. Positive control compensation

For non-proliferating PBLs cells are only found resting in G0, and not S or G2 phases of the cell cycle. For un-gated cell populations a peak appears over the G2 region of the cell cycle histogram, however this region represents doublets. These can be removed by gating for single events only using FL2-Area versus FL2-Width dot plots (described in Figure A5). In addition to doublets, triplets can also be observed between channels 600 and 800.

Figure A5. Gating of PBLs for analysis using PI staining and flow cytometry

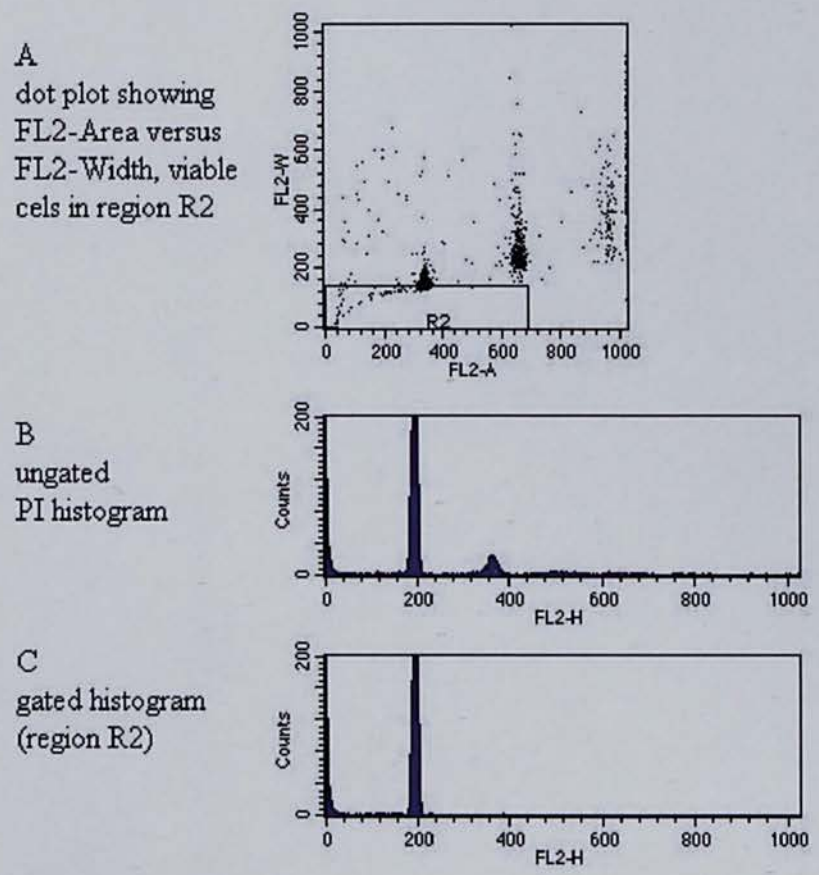


Figure A5. Analysis of PBLs. Figure A: gating of singlets, B: an ungated cell cycle histogram, showing doublets in channel 400, where G2 cells would be found in an actively proliferating cell population, C: a gated histogram showing only cells in G0.