THE EFFECTS OF NOVEL APOPTOTIC AGENTS EXTRACTED FROM *COMMIPHORA* SPECIES ON CANCER CELL LINES

by

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Dedication

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Abstract

The apoptotic effects of various concentrations of ethanol-, hexane-, and ethyl acetate-extracted molmol (from *Commiphora molmol*) and haddi (from *Commiphora gudiotti*) on two cancer cell lines, S180 and HT1080, and on a normal chick fibroblasts cell line, were studied. In addition, the effects of two commercially obtained molmol ethanol (Flavex) and haddi essential oil extracts (Aldrich) were also studied, and results compared and contrasted with results obtained using freshly extracted molmol and haddi preparations. Furthermore, the effects of two of the chemical constituents of haddi, *Trans-Beta-o*cimene and *Gamma*-Bisabolene (obtained commercially) were also studied, using the above-mentioned cell lines.

Various techniques were employed to study these effects, including growth assays, MTT viability assay, histological and morphological techniques for the detection of apoptosis (haematoxylin-eosin staining and scanning and transmission electron microscopy), TUNEL technique which is the detection of DNA fragmentation, and Annexin V assay to detect apoptotic plasma membrane change, using fluorescence microscopy.

The results showed that ethanol-extracted molmol and hexane-extracted haddi are apoptosis inducers in the murine S180 sarcoma cells and HT1080 human fibrosarcoma cells. Morphological analysis showed that both S180 cells and HT1080 cells exposed to these extracts exhibited characteristics signs of apoptosis, including the formation of pyknotic nuclei, apoptotic cells, apoptotic bodies, blebbing of outer membrane, vacuolation, breaking-up of outer membrane, and loss of microvilli and filopodial attachments, as well as signs of chromatin margination and chromatin condensation alongside the nuclear membrane. Some secondary necrosis was also seen when cells were treated with extracts and tested with Annexin-V, and showed positive signs for changes in membrane phospatidylserine asymmetry. Annexin V assay also demonstrated that apoptosis and death of S180 cells was faster than in HT1080 cells.

The results showed that both molmol and haddi extracts performed better at higher concentrations than at lower concentrations, and that apoptotic changes in S180 cells and HT1080 cells were found to be time-dependent.

The study also demonstrated that commercial preparations of molmol (Flavex) and haddi essential oil (Aldrich) were effective, but less so than the natural extracts.
Molmol extracts at particular concentrations targeted cancer cells, in contrast to haddi extracts, which targeted both cancer and normal cell lines.

The results also demonstrated that *Trans-β*-ocimene was more effective than γ-bisabolene in inducing apoptosis in S180 cells than in HT1080 cells. However, SEM results showed that these two chemicals acted on cancer cells in a similar way to that of haddi extracts. γ-bisabolene was more effective at LC_{50} concentration (5 mM) in bringing about apoptosis in S180 cells and HT1080 cells than *Trans-β*-ocimene, at LC_{50} concentration (100 mM).

Both chemicals were very potent inducers of apoptosis.

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Chapter One

General Introduction

1.1 Cancer: An Overview

Kirsch & Kustan (1998) described cancer, in terms of neoplasia, as a disorder that allows unchecked proliferation of individual mutant cells to form tumour(s). However, despite the fact that the development of cancer is normally associated with uncontrolled proliferation, it should also be perceived as abnormal differentiation (Sporn & Roberts, 1984). The tumour cell survives by invading the surrounding tissue, which finally ends up in the destruction of the whole organism.

Tumours are divided into two types: benign and malignant. The former consist of a slow growing mass of cells that does not generally spread into the surrounding tissues. Conversely, malignant tumours can give rise to secondary tumours at different sites (meta-astasis), consequently making the cancer difficult to eliminate. Nonetheless, cancers are generally divided into three types, according to the tissues they arise from: (i) **carcinomas**, the most common form of human cancer, representing around 90 per cent of all cancers which arise from the epithelial cells; (ii) **sarcomas**, representing 5 per cent of all cancers arising from connective tissues or muscle cells; and (iii) **leukaemias**, or **lymphomas**, also representing 5 per cent of all cancers, which arise from haemopoietic and lymphatic cells (Alberts *et al.*, 1994; Kleinsmith & Kish, 1995).

Alberts *et al.* (1994) and Raven & Johnson (1999) have shown that somatic mutation in an individual cell is mainly responsible for the initiation of the cancer. However, the progeny of the mutated cells may undergo further spontaneous induced mutation

prior to becoming cancerous. Genetic change in cells has been reported to be responsible for cancer formation, and the cells in a particular cancer can often share common abnormalities in their DNA (Ames *et al.*, 1973). The majority of such changes are acquired, for instance, through viral infections, toxic agents (carcinogens) or ionising radiation. According to Möller (1995), potentially oncogenic viruses, genotoxic agents and high energy radiation are integral parts of the environment, yet cancers caused by acquired genomic alterations tend to appear late in life. Hence, compensatory mechanisms must exist, for example the immune system that plays an important role in the elimination of cancerous cells in an organism. Furthermore, Möller (1995) indicated that the affected cells may attempt to repair their own genomic damage, and if repair fails, the cell may commit suicide (apoptosis) for the welfare of the organism. Accordingly, a cancerous growth is often seen as the abnormal activation of the mitotic programme and/or the inactivation of programmes for growth-inhibition and cell death.

Liu *et al.* (1998) reported that head and neck squamous cell carcinoma (HNSCC) is one type of cancer that represents a major health problem worldwide, being the fourth most frequent tumour (Parkins *et al.*, 1993). HNSCCs are principally defined as being those cancers in the oral cavity, pharynx and larynx. According to Vokes *et al.* (1993), the majority of head and neck squamous cell carcinoma occur in the oral cavity: tongue, floor of the mouth, hard palate, buccal mucosa, and lip. The largest percentage of head and neck cancers are those squamous cell carcinoma of the tongue (Mineta *et al.*, 2000).

Farber (1984) proposed some two decades ago that head and neck carcinogenesis is a multi-step process that is driven by the accumulation of genetic alterations. Due to

the fact that cancerous or pre-cancerous cells acquire proliferation-promoting alterations in the structure or expression of cellular oncogens, they also tend to lose gene functions which serve to limit cellular proliferation.

Bishop (1991) indicated the associated gain of growth-promoting proto-oncogene function and the loss of negative regulations of cell cycle as a result of inactivation of tumour: suppressor genes activate the cell towards increasingly aberrant cell-cycle control at the molecular level and histopathogenic changes at an anatomic level.

Oral cancer and HNSCC appear as a consequence of multiple molecular genetic events in many chromosomes and genes. The consequence of such chromosomal (that is, genetic) damage is cell dysregulation, with disruption in all signalling, the cell growth cycle, and/or mechanism of repair cell damage or eliminate dysfunctional cells. Impaired or enhanced function of genes or their products is involved in this carcinogenesis; cell regulatory processes are affected through transcribed proteins. Carcinogenesis is a multistep process associated with the expression of defects in the integrated control of cellular differentiation and proliferation (Weinstein, 1987; Sparks *et al.*, 1988). The accumulation of these genetic changes, often over a period of time, seems to lead, in some instances, to dysregulation, to the extent that growth becomes autonomous and invasive mechanisms develop, leading to carcinoma (Lyons & Clark, 1997; Scully *et al.*, 2000).

Studies relating to the cell kinetics of human tumours have shown that the mean cycle time of cells within these tumours is typically between two to four days, which is a great deal shorter than the volume-doubling of the tumour, which is typically two to three months for common solid tumours. Two factors may help to explain this discrepancy: (i) a high proportion of non-proliferating cells; and (ii) a high rate of

selective cell death, which may be apoptotic or non-apoptotic. Steel (1977) and Tannok (1989) indicated that the presence of necrosis or pyknotic cells is evidence for cell death in tumours, and the rate of cell death or loss from human tumour can be greater than cell production.

1.2 Cell Death in Tumours

Studies relating to cell death showed that controlled/programmed cell death is crucial for the normal development of multicellular organisms, as well as for maintenance of tissue and organ size (Bowen & Lockshin, 1981; Wyllie, 1987; Walker *et al.*, 1988).

Cell death as a whole can be grouped into two types: (i) pathological and accidental cell death, or **necrosis**; and (ii) death by design; that is, **programmed cell death (PCD)** (Bowen, 1993; Kiechle & Zhang, 2002a). PCD usually forms part of a normal physiological programme. The term 'programmed cell death' was originally coined by Lockshin (1969) to describe a genetically controlled process in the context of insect development. PCD is a significant developmental and homeostatic process in multicellular organisms, which Bowen (1998) described as an 'altruistic suicide' that shapes and structures tissues and organs, and has a key role in normal embryological development, morphogenesis and metamorphosis. The term 'apoptosis', that is, 'a falling away', was coined by Kerr *et al.* (1972) to indicate this programmed and controlled cell death, and to define the morphologic features, including cytoplasmic blebbing, chromatin condensation, cell shrinkage, nuclear fragmentation, cell rounding, i.e., loss of adhesion, and cells shrinkage, and finally, the fragmenting of the cell into membrane-bound apoptotic bodies which are ingested by neighbouring phagocytic cells. Such morphologic changes can be observed in fixed and stained tissues or in cells grown in culture (Kiechle & Zhang, 2002a). This process of cell

death, according to Geske & Gerschenson (2001) and Kiechle & Zhang (1998, 2002a), requires time to take place after the initial insult, in contrast to necrosis, which has none of the cellular morphologic changes reported above for apoptosis, and occurs rapidly after the initial insult Bowen (1998) also indicates that apoptosis is an equal and opposite force to mitosis with its own distinctive morphology that occurs during early development and continuously through metazoan life.

Apoptosis and necrosis are two extremes of one continuous spectrum of death (Wyllie *et al.*, 1984).

However, some authors argue that PCD is not an appropriate synonym for apoptosis (Steel, 2001; Sloviter, 2002). For example, Sloviter (2002) indicates that PCD refers to well-controlled intracellular biochemical pathways that ultimately lead to the morphologic features defined by the term apoptosis, and recommends that necrosis be replaced with the term 'passive cell death', and apoptosis with the term 'active cell death', and active cell death be divided into two subgroups, immediate or delayed active cell death, depending on the time of onset following the initial triggering event. Nonetheless, Snider *et al.* (1999) argue that in some pathological conditions, the two forms of cell death may co-exist in the same tissues, and elements of both processes may occur in the same cells. For instance, it has been thought that necrosis is the mode of myocardial cell death following ischaemia for many years, and since 1994, apoptotic morphologic features have been described to be associated with myocardial ischaemia and infarction (Ohno *et al.*, 1998; Yaoita *et al.*, 2000; Knaapen *et al*, 2001). In addition, both apoptosis and necrosis may share common mediators. Bonfoco *et al.* (1995) reported that low levels of glutamate receptor over-activation can trigger both neuronal apoptosis and necrosis. A high concentration of intracellular ATP favours

apoptosis, whereas low concentration of ATP shifts cell death toward necrosis (Los *et al.*, 2002).

1.2.1 Necrosis

Necrosis is caused by physical, chemical, toxic or anoxic injury, often to the cell membrane. The injury brings about an alteration of the cell environment and a functional deficit. Necrotic cells are known to swell rather than shrink, due to the fact that during the early stages of necrosis the cell membrane pumps fail, leading to an influx of water, sodium and calcium. This is followed by acidosis and osmotic shock. Increased acidosis gives rise to pyknotic nuclei as the chromatin precipitates, and nuclei ultimately experience karyoplysis. Bowen *et al.* (1998) indicated that there is no early chromatin margination or activation of endonuclease activity as observed in apoptosis. Bowen (1998) reported that calcium influx during necrosis triggers phospholipase A, which affects the internal mitochondria membranes and this, in turn, causes a terminal drop in ATP production leading to an autolytic deterioration in the cells. Finally, the endoplasmic reticulum and lysosomes swell and burst, the latter releasing hydrolytic enzymes, which cause further autolytic destruction of the cells, which ultimately break up and produce an inflammatory response.

1.2.2 Apoptosis

Reed (2000) and Sjöström & Bergh (2001) described programmed cell death (PCD), that is, apoptosis, as an evolutionary conserved pathway needed for embryonic development and tissues homoeostasis. Meier *et al.* (2000) also maintain that apoptosis plays an important role in the regulation of cell numbers during development and tissue homoeostasis. Sjöström & Bergh (2001) refer to apoptosis as the normal physiological response to many stimuli, including irreparable DNA

damage. Thompson (1995), on the other hand, reports that various diseases evolve as a consequence of hyperactivation or suppression of programmed death, the former including neurodegenerative diseases, immunodeficiency and ischaemia-reperfusion injury, and the latter including cancer and autoimmune disorders. Many other authors also indicated that that the dysregulation of apoptosis that results in increased activity is associated with a variety of clinical disorders, such as cancer, autoimmunity, neurodegenerative diseases, haematopoietic disorders and infertility (see Kiechle & Zhang, 1998, 2002b; Yuan & Yanker, 2000; Hanlou *et al.*, 2001; Mirkes, 2001; Müllauer *et al.*, 2001; Ameisen, 2002; Markstrom *et al.*, 2002; Ramos *et al.*, 2002; Rathmel & Thompson, 2002).

According to Sjöström & Bergh (2001), in cancer, the balance between proliferation and PCD is disturbed, and defects in apoptotic pathways allow cells with genetic abnormalities to survive. They add that the majority of cytotoxic and hormonal treatments, and radiation, eventually kill cancer cells by triggering irreparable cellular damage which triggers apoptosis. Accordingly, the effectiveness of cancer treatment depends not only on the cellular damage they cause, but also on the cell's ability to respond to damage and by prompting the apoptotic machinery. Researchers view apoptosis as a powerful kinetic concept, being an equal and opposite force to mitosis (e.g., see Kerr *et al.*, 1972), and as such it plays a vital role in the maintenance of renewable tissues (Bowen, 1993).

Sarraf & Bowen (1986, 1988) have reported that apoptosis occurs in tumours, and is probably a consequence of residual, intrinsic auto-regulatory mechanisms which operate during the early stages of neoplastic transformation that finally fail to control malignant changes.

Apoptosis is essential for the development and the maintenance of the population balance of cells within an organism. Apoptosis leads to cell condensation and shrinkage. Cells tend to lose water early on in apoptosis, leading to an increase in density. Bowen *et al.* (1998) indicated that cell membrane pumps continue functioning during apoptosis, and the apoptotic cells can exclude vital dyes. Apoptosis involves ATP production, and is usually accompanied by new mRNA and *de novo* protein synthesis. The nuclear envelope dilates during apoptosis, thus forming blebs that contain marginating chromatin. An important feature of apoptosis is that of the fragmentation of the chromatin by non-lysosomal nuclear endonuclease, which cuts the double-stranded DNA into oligonucleotide fragments of 180 to 200 bp which appear as 'ladders' when run on gel electrophoresis (Bowen, 1993; Bowen *et al.*, 1998). However, not all apoptotic cells show DNA laddering. For example, it has been reported that nuclear changes during apoptosis can occur without endonuclease activation of oligonucleotide production (Oberhammer *et al.*, 1993). Bowen (1998) showed that at later stages of apoptosis, the plasma membrane becomes active and convoluted to form blebs, followed by the fragmentation of the cell into several membrane-bound spheres or apoptotic bodies of different sizes that are rapidly phagocytosed by neighbouring cells of phagocytes, without inducing an inflammatory response.

Tumour suppressor gene *p53*, the anti-apoptotic gene *Bcl-2*, and the pro-apoptotic gene *bax* are the most studied genes that are related to apoptosis (Sjöström and Bergh, 2002). Wallace-Brodeur & Lowe (1999) reported that normal wild type *p53* can limit cell proliferation after DNA damage through two mechanisms: arresting the cell cycle or activating apoptosis. *P53* plays a dual and complex role in chemosensitivity, in that it can either increase apoptosis or arrest growth and in that way increase drug

resistance (Sjöström and Bergh, 2002), and this may explain why promising pre-clinical data which indicate the presence of wild type *p53* would predict chemosensitivity have translated into more conflicting clinical data (Schmitt & Lowe, 1999; Hamilton & Piccart, 2000). Furthermore, drugs which do not cause DNA damage, such as taxanes and vinca alkaloids, may induce apoptosis through pathways independent of *p53* (Sjöström and Bergh, 2002).

1.3 Morphological Techniques for Detection of Apoptosis

Apoptotic cells can be easily identified using routine histological staining methods, such as the haematoxylin and eosin (H&E) dyes. Histological assessments of cells and tissues are in the main based on rather delicate membrane and pH changes that result in changes in membrane permeability, and in various staining patterns (Bowen, 1981).

One of the main characteristics of classical apoptosis is the presence of darkly stained hyperchromatic nuclei, known as pyknotic nuclei. Sandritter & Riede (1975) provided a detailed discussion of nuclear pyknosis in level cells. During the early stages of apoptosis, the nuclear chromatin generally clumps and aggregates at the nuclear membrane. This is followed by dilation of the nuclear membrane and chromatin condensation typical of pyknosis. In late apoptotic cells, the pyknotic chromatin becomes progressively less visible and is dispersed or lost. Bowen *et al.* (1991) reported nuclear pyknosis in cells undergoing necrosis, although the necrotic cells do not show characteristic chromatin margination.

Sandritter & Riede (1975) stated that the first changes leading to necrosis include degenerative swelling, caused by failure of the sodium pump, and ATP activity, which in turn causes inflow of charged ions such as sodium, calcium and water, and outflow

of potassium ions into the extracellular space. This leads to changes in the cell membrane permeability, which at the light microscopy level could be reflected in the cell's reaction to dyes. In contrast, plasma membrane changes during apoptosis are related to membrane bending and induction of blebbing and fragmentation. Furthermore, the apoptotic cells and fragments tend to lose rather than gain water that might lead to changes in the pH and surface permeability of the cells. Such changes are reflected through the reactions to histological stains (Bowen, 1981). Ballard has illustrated that apoptotic cell nuclei are intensively chromophilic, and could be easily identified in iron/haematoxylin stained sections. This hyperchromasia of pyknotic nuclei is caused by chromatin condensation and also by exposure to negative charge phosphate groups. Sandritter & Riede (1975), for instance, found that pyknotic nuclei bind more eosin, due to the increase in basic histone groups.

1.3.1 Haematoxylin-Eosin Staining

Haematoxylin and eosin dyes were used in the present study, since they are among the most widely employed routine histological stains. Haematoxylin-eosin staining is a relatively simple technique used to demonstrate clearly different tissue structures from different body areas which are prepared in various ways. These two stains provide an excellent contrast between the nuclear material and the cytoplasm. While haematoxylin stains cell nuclei blue-black with good intra-nuclear detail, the eosin stains cell cytoplasm in various shades of pink and red (Stevens, 1986). A number of researchers have adapted this technique for morphological analysis in different substrates, such as paraffin-embedded materials, haema-embedded materials, LR White resin and cell smears (Moffit, 1994; Al-Hazzaa & Bowen, 1998; Bowen, 1998; Szegedi *et al.*, 1998).

Haematoxylin-eosin staining is also routinely used in clinical and cancer research for the recognition and semi-quantitative assessment of apoptotic bodies. It helps detect low frequency apoptosis in cell cultures, which is not possible using flow cytometry (Fujita *et al.*, 1997).

Haematoxylin is adequate as a nuclear stain only in the presence of a mordant, the most effective of which include salts of aluminium, iron, tungsten, and occasionally lead (Stevens, 1986). In the present study, Mayer's haematoxylin was used, which belongs to the group of alum haematoxylins. The mordant used was aluminium potassium sulphate (potash alum). Haematoxylin stains nuclei red, which is then changed into a blue-black colour when washed in tap water, which is slightly alkaline. This method is referred to as 'blueing'.

Eosin is widely used in conjunction with alum haematoxylin to demonstrate the general histology structure of cells or tissues. The eosins are xanthene dyes of which eosin Y is the most widely used. As a cytoplasmic stain, eosin is used as 0.5-1.0% solution in distilled water with a crystal of thymol added to prevent the growth of fungi (Stevens, 1986). Tap water wash differentiates the eosin stain in the cytoplasm and the subsequent intensity of both the haematoxylin and eosin stains.

1.3.2 Scanning Electron Microscopy

The morphological changes that occur during programmed cell death can be analysed during scanning electron microscopy (SEM). SEM allows for the collection of secondary electrons in the scanning mode and produces topographical images. Bowen (1981) indicated that fine structural changes in cells observed at electron microscopy level can reflect underlying biochemical changes, such as changes of the plasma membrane and cytomembranes which can provide reliable and early signs as

to the probability of cell death. SEM is also employed for the cytochemical localisation of enzymatic activity by means of backscattered electron imaging (BEI), which provides atomic number contrast, and can be used to distinguish between the two spatially separated products of differing atomic numbers. BEI is also employed for the study of sub-cellular localisation, to some extent based on the backscattered electrons that emerge from under the surface of the specimen, that can be collected and analysed (Bowen *et al.*, 1988).

SEM is regularly employed in the laboratory to study the morphological characteristics of cells both in solid tumours and *in vitro.* It is widely employed to study and time events in apoptotic cell cultures. In general, cells experiencing apoptosis primarily round off and lose contact with the neighbouring cells as they condense. Then, the cytoplasm becomes convoluted and blebs outwards, eventually giving rise to apoptotic bodies. Bowen *et al.* (1993) reported a substantial degree of membrane bending taking place during apoptotic body formation, accompanied by localised changes in the calcium concentration.

1.4 TUNEL Technique for Detection of DNA Fragmentation

Detection of *in situ* DNA fragmentation is at present one of the most frequently used techniques with which to highlight apoptotic cells in tissue. DNA fragmentation during apoptosis is induced by caspase-mediated cleavage of ICAD (inhibitor of caspase-activated DNAase) and ensuing activation of CAD (caspase-activated DNAase) which subsequently leads to the characteristic internucleosomal DNA double strands breaks (Enari *et al.*, 1998). DNA fragmentation into multiples of 180-200 base pairs (BP) is regarded as a feature of programmed cell death (Kerr *et al.*, 1972; Wyllie *et al.*, 1980; Arends *et al.*, 1990). Nonetheless, according to Bortner *et*

al. (1995) large fragments in the range of 50-150 kbp are also generated. Oberhammer *et al.* (1993) and Zakeri *et al.* (1993) also reported forms of programmed cell death that lack the prototypical internucleosomal pattern of DNA fragmentation. In contrast, necrotic cell death is accompanied by late random DNA fragmentation by release of lysosomal DNAase, though this concept has been challenged. Techniques detecting fragmentation are therefore not specific for apoptosis, but also detect DNA damage in a variety of cell injury and cell death paradigms. In addition to the characteristic, orderly oligonucleosomal fragmentation, it is mainly the enormous amount of DNA strand breaks that is the basis for the relative specificity of *in situ* DNA fragmentation techniques for apoptotic cell death (Ansari *et al.*, 1993).

There are a number of kits available (that can be used with flow cytometric or histochemical context) to assay DNA fragmentation. For example, Boehringer Mannheim and Oncor (Apoptosis kit), based on the TUNEL reaction, which may use fluoresein, alkaline phosphatase or peroxidase for immunohistological reporting.

1.5 Cancer Treatment

There are four main modes of treatment of cancer: surgery, radiotherapy, chemotherapy, and experimental gene therapy. These can be used singly or in combination (Alison & Sarraf, 1997). Natural plant or animal products have also been used in anticancer therapy (da Rocha *et al.*, 2001).

Surgery involves the removal of the primary tumour alone or combined with the removal of its draining lymphatics and lymph nodes, either selectively or in the presence of nodal metastasis (Schantz *et al.*, 1993). Radiotherapy is often used in combination with surgery or as an alternative to it (Peters, 1998). Radiotherapy is administered in two ways; by an external beam, using either (i) photons or X-rays

produced by a linear accelerator, or as gamma rays from a cobalt source, or (ii) implant of radioactive source (Brach therapy). Surgery and radiotherapy often eliminate primary or localised disease but may eventually fail if cancer has metastasised to other areas of the body. Under such circumstances, chemotherapy, if used properly, has the potential to control or eliminate metastatic disease and reduce mortality. In some clinical trials, **adjuvant therapy**, that is, combining chemotherapy with surgery or radiotherapy or both, has increased survival rates for a number of solid tumours that have formerly been treated by only one therapeutic modality.

In several local multimodality treatment protocols, treatment often starts as local control, either by surgery or irradiation, followed by chemotherapy to treat metastatic tumour foci. However, an approach that utilises chemotherapy as the first-line treatment has been recently employed, which is followed by surgery or radiation therapy. The rationale for this so-called neoadjuvant chemotherapy, a term coined by Frei (1988), is that the primary tumour can be shrunk before local eradication is attempted, and micrometastatic foci can be attacked initially, without waiting until local treatment is completed.

1.6 Natural Products in Cancer Therapy

Farnsworth *et al.* (1985) and Cragg *et al.* (1997) reported that the role of natural products as a source for remedies has been recognised since ancient times. A survey of plants used against cancer carried out over three decades has listed a large number of such plants (Hartwell, 1968). Bindseil *et al.* (2001) reported that natural products are the most constantly successful source of drug lead, and that 39% of the 520 new drugs approved between 1983 and 1994 were either natural products or derived from natural products (Cragg *et al.*, 1997).

A number of plant-derived compounds are at present successfully employed in cancer treatment (da Rocha *et al.*, 2001), as shown in Table 1.1

The vinca alkaloid family isolated from the periwinkle *Catharanthus roseus*, found in the rain forest of Madagascar, is one of the most significant examples (Noble, 1990). DeVita *et al.* (1970) reported that the introduction of the vinca alkaloid vincristine was responsible for an increase in the cure rates for Hodgkin's disease and some forms of leukaemia. This alkaloid is reported to inhibit microtubule assembly, inducing tubulin self-association into coiled spiral aggregates (Noble, 1990; Wood *et al.*, 2001).

Table 1.1 Plant-derived anticancer agents

Compound	Cancer Use	Status
Vincristine	Leukaemia, lymphoma, breast, lung, paediatric solid cancers and others	Phase III/IV
Vinblastine	Breast, lymphoma, germ-cell and renal cancer	Phase III/IV
Paclitaxel	Ovary, breast, lung, bladder, and head and neck cancer	Phase III/IV
Docetaxel	Breast and lung cancer	Phase III
Topotecan	Ovarian, lung and paediatric cancer	Phase II/III
Irinotecan	Colorectal and lung cancer	Phase II/III
Flavopiridol	Experimental	Phase I/II
Acronyciline	Experimental	Phase II/III
Bruceantin	Experimental	Preclinical/phase I
Thalicarpin	Experimental	Preclinical/phase I

Source: da Rocha *et al.* (2001, p. 366).

Etoposide, an epipodophyllotoxin, is another example of a highly active agent, derived from the mandrake plant *Podophyllum peltatum* and the wild chervil *P. emodi* (Stähelin, 1973), which has produced high cure rates in testicular cancer when used in

combination with bleomycin, which is also derived from a natural product, and cisplatin (Williams *et al.*, 1987). Etoposide is also reported to show significant activity against small-cell lung carcinoma (Chabner, 1991; Perry, 1992; Harvey, 1999). Liu (1989) reported that it is a topoisomerase II inhibitor stabilising enzyme-DNA cleavable complexes leading to DNA breaks.

1.6.1 Flavones and Isoflavones

Flavones and isoflavones have been reported to play a prominent role in cancer prevention due to their existence in numerous plants associated with reduced cancer rates (Birt *et al.*, 2001). Epidemiological research has always shown an inverse relationship between consumption of vegetables and fruits and the risk of human cancers at many sites (Block *et al.*, 1992; Messina *et al.*, 1998; Steinmetz & Potter, 1991a). There are a number of probable mechanisms by which intake of vegetables and fruit may prevent carcinogenesis (Birt *et al.*, 2001). Research showed that plant foods contain a wide variety of anticancer phytochemicals with many prospective biological activities that may reduce cancer susceptibility (Adlercreutz, 1990; Steinmetz & Potter, 1991b; Wattenberg, 1992a, b; Waladkhani & Clemens, 1998). Adlercreutz (1995), Knight & Eden (1996), Potter & Steinmetz (1996), Hollman *et al.* (1997), Knekt *et al.* (1997), Kou (1997) and Bravo (1998) showed that flavonoids and isoflavonoids are notably promising candidates for cancer prevention.

Flavonoids are plant secondary metabolites found in all terrestrial vascular plants, and defined chemically as substances composed of a common phenylchromanone structure (C6-C3-C6), with one or more hydroxyl substituents, including derivatives (Birt *et al.*, 2001). Isoflavonoids, in sharp contrast to flavonoids, possess a 3-phenylchroman skeleton which is biologically derived from the 2-phenylchroman

skeleton of the flavonoids. Isoflavonoids are found in plants of the subfamily Papilionoideae of the Leguminosae, including soyabeans (Harborne, 1989; Birt *et al.*, 2001). Harborne (1989) indicated that both groups occur commonly as esters, ether, or glycoside derivatives, or mixtures thereof, embracing over 4,000 compounds. In mammals, both occur only through dietary intake (Birt *et al.*, 2001).

There are several mechanisms for flavonoid and isoflavonoid inhibition of cancer, including oestrogenic and anti-oestrogenic activity, antiproliferation, cell cycle arrest and apoptosis, antioxidation, induction of detoxification enzymes, regulation of host immune function, and other mechanisms (Birt *et al.*, 2001). These other mechanisms include the association of changes in protein phosphorylation of cancer cell lines with growth inhibition by flavonoids and isoflavonoids (see Kuo *et al.*, 1994; Kuo & Yang, 1995; Gamet-Payrastre *et al.*, 1999), and topoisomerase antagonism by flavonoids and isoflavonoids (see Constantinou *et al.*, 1994).

Dietary flavonoids and isoflavonoids are usually non-toxic. However, Czeczot *et al.* (1990) and Friedman & Smith (1984) reported some flavonoids (e.g., quercetin) to be toxic, and Miksicek (1993, 1995) identified some flavonoids and isoflavonoids, e.g., kampherol and genistein, as oestrogenic agonists at 1 μM.

In a recent review of cancer preventive effects of flavonoids, Le Merchand (2002) concluded that data accumulated, particularly in recent years, have demonstrated a wide variety of biological actions for flavonoids that may be beneficial against cancer. Nonetheless, he added that it is not clear whether these effects would also be present at physiological concentrations, and for the metabolites that are likely to be most relevant to humans.

The epidemiological evidence that would support a cancer protective effect is still limited and has so far been rather inconsistent. Nonetheless, Le Marchand (2002) maintains that a few relationships have emerged, for example possible inverse association between soya intake (possibly early in life) and premenopausal breast cancer, green tea consumption and stomach cancer, and onion and apple intakes and lung cancer. More data are therefore required before any of such associations can be used to support specific health recommendations.

1.6.2 Propolis

Recently, propolis, a resinous hive product collected by honeybees from various plant sources, has been reported to have a broad spectrum of biological activities, namely anticancer, antioxidant, antiflammatory, antibiotic, antifungal activities, cytotoxic, hepatoprotective and free radical scavenging effects, or an antiproliferative activity (Burdock, 1998; Marcucci, 1995; Banskota *et al.*, 2000a,b, 2001a,b, 2002). The composition of propolis depends on the time, vegetation and the area of collection, thus quality evaluation is important before its use in food and drinks (Banskota *et al.*, 2000a). Marcucci (1995) found that propolis from temperate zones contains mainly phenolic compounds, including flavonoids and cinnamic acid derivatives. Bankova *et al.* (2000), on the other hand, found that diterpenes and prenylated compounds are present in tropical propolis of the South-American continent, which are virtually absent in temperate propolis, together with lignans, flavonoids, and other classes of compounds. This difference in composition between the two zones is mainly due to their different vegetations, but even with this difference, propolis from both regions possessed similar biological properties (Burdock, 1998; Banskota *et al.*, 2001a). Basnet *et al.* (1996) and Banskota *et al.* (2000b, 2001b) isolated 31 different

constituents from Brazilian propolis, three of which were new, and 15 others isolated for the first time from propolis, together with either antiproliferative activity or hepatoprotective activity.

1.6.3 Marine Organisms

Marine organisms have also been used as a source of new anticancer agents (Schwartsmann *et al.*, 2001). C-nucleosides isolated from the Caribbean sponge, *Cryptotheca crypta*, about forty years ago, provided the basis for the synthesis of cytarabine, the first marine-derived anticancer agent to be developed for clinical trials, which is used at present in the routine treatment of patients with leukaemia and lymphoma (Schwartsmann *et al.*, 2001). Didemnin B, apilidine and ET-743 are derived from tunicates, which have entered phase I and II trials as antitumour agents (Rinehart, 2000). Didemnin B, a cyclic depsipeptide isolated from *Tridemnum solidum*, a tunicate, has shown impressive antitumour activity in human tumour models *in vitro* and in tumours growing in athymic mice (Geldof *et al.*, 1999). Nonetheless, despite the observed antitumour effects, clinical trials of didemnin B were discontinued due to severe neuromuscular and cardiotoxic effects (Shin *et al.*, 1991, Weiss *et al.*, 1994). Dehudrodidemnin B was then isolated from the Mediterranean tunicate, *Aplidium albicans*, which was later called aplidine. Antitumour activity was observed in patients with advanced solid tumours (renal-cell carcinoma, malignant melanoma, tumours of neuroendocrine origin, and medullary carcinoma of the thyroid (Izquierdo *et al.*, 2000; Maroun *et al.*, 2000; Raymond *et al.*, 2000). The main toxic effects include nausea and vomiting, myalgia, transient disturbance of liver function, and local irritation at the injection site. However, muscular toxicity was circumvented by the concomitant administration of L-carnitine

(Schwartsmann *et al.*, 2001). It will start phase II trials in various solid tumours in the near future. Other marine-derived anticancer agents include ecteinascidins, derived from the Caribbean tunicate *Ecteinascidia turbinate,* dolastatins, cytotoxic cyclic and linear peptides derived from the sea hare, *Dolabella auricularia* (a mollusc found in the Indian Ocean), bryostatin, a macrocyclic natural lactone from the marine bryzoan, *Bugula neritina*, and many others. Schwartsmann *et al.* (2001) review provides further information about these antitumour agents.

1.7 Plants and Biochemicals in Cancer Therapy

A number of plants, and plant extracts, have been screened for their anticarcinogenic properties, one of which is the *Salvia* plant species.

1.7.1 *Salvia* spp.

Several studies have reported the *Salvia miltiorrhiza*, a herbal plant widely used in traditional Chinese medicine for the treatment of chronic hepatitis and liver fibrosis (Liu *et al.*, 2000). Substances isolated from this plant have been documented to exhibit antioxidant capabilities. Recent studies also indicated that this plant has anti-tumour potential. Extract of *S. miltiorrhiza* noticeably prolonged the survival period of Ehrlich ascites carcinoma-bearing mice, and tanshione II-A sodium sulphonate, a compound isolated from this plant, could potentiate the cytotoxic action of hydroxycamptothecine against Ehrlich ascites carcinoma (see Chang & But, 1986).

Yang *et al.* (1981) found that pruzmaquinone A and B, two components extracted from *S. miltrorrhiza*, have anti-tumour activity in mice, while Wu *et al.* (1991) and Ryu *et al.* (1997) reported on the action of extracts of this plant on transformed cells *in vitro*. Ryu *et al.* (1997) found that methanolic extract of *S. miltiorrhiza* showed a

significant anti-proliferation effect against cultured human cancer cell lines. Wu *et al.* (1991) tested fifteen tanshinone analogues isolated from chloroform extract for their cytotoxic activities against four types of cell lines derived from human carcinoma. More recently, Liu *et al.* (1999) suggested that *S. miltiorrhiza* is a potent anti-oxidant in protecting primary hepatocytes from oxidative damage induced by aflatoxin B_1, a well recognised hepatocarcinogen.

Liu *et al.* (2000) evaluated the molecular mechanism of *S. miltiorrhiza* (earlier studies had not investigated mechanism of action) in human hepatoma line, $HepG_2$, and showed that this plant exerted clear cytotoxic effects, strongly inhibiting the proliferation of $HepG_2$ cells. They also observed that treatment with this plant caused apoptotic cell death as evaluated by (i) morphological changes by using acridine orange/ethidium bromide staining; (ii) DNA fragmentation by TdT-mediated dUTP nick end labelling (TUNEL); and (iii) sub-G_1 cell analysis. Liu *et al.* (2000) also found depletion of intracellular gluthione (GSH) and eduction of mitochondrial membrane potential to be involved in the initiation of apoptosis.

Frusciante *et al.* (2000) reported that several epidemiological studies have illustrated the importance of fruit and vegetables in the human diet so as to prevent the onset of cardiovascular disease and several forms of cancer. They reviewed two of the most widely grown and genetically well-known species in the world, the tomato and the potato.

Tomatoes are important sources of vitamin C, potassium, folic acid and carotenoids such as lycopene and β-carotene. Frusciante *et al.* (2000) indicated that it has been demonstrated that lycopene has anti-oxidant properties and interferes with the growth of cancerous cells.

Quasney *et al.* (2001) demonstrated that human gastric cancer cells can be killed by micromolar concentrations of the plant sulpolipid sulfoquinovosyldiacylglycerol (SQDG), which is present in most photosynthetic organisms.

1.7.2 *Allium* spp.

The species *Allium sativum*, commonly known as garlic, has been widely studied for its anticancer properties. Kwon *et al.* (2002) reported that diallyl disulphide (DADS), a component of garlic (*Allium sativum*), has been known to exert effective chemopreventive activity against colon, lung, and skin cancer. They demonstrated that DADS induces apoptosis of human leukaemia HL-60 cells in a concentration- and time-dependent manner with an IC_{50} for cell viability of less than 25 μM. They found that DADS activated caspase-3 as evidenced by both the proteolytic cleavage of the proenzyme and increased protease activity, and that activation of caspase-3 was maximal at 3 hr and led to the cleavage of 116 kDa poly (ADP-ribose) polymerase (PARP), which resulted in the accumulation of an 85 kda cleavage product. They found that their results indicated that DADS-induced apoptosis is triggered by the generation of hydrogen peroxide, activation of caspase-3, degradation of PARP, and fragmentation of DNA. They concluded that the induction of apoptosis by DADS may be the key mechanism by which its chemopreventive action against cancer is based.

1.7.3 Aspirin and Salicylic Acid

Aspirin has been studied in terms of its anticarcinogenic effect, mostly in the large bowel. Baron (1995) indicated that most of the studies published have reported an inverse association, though a few indicated otherwise. Piqué *et al.* (2000) found that aspirin and other non-steroidal anti-inflammatory drugs induce apoptosis in many cell types. They concluded that their results demonstrated that aspirin-induced apoptosis

involves release of cytochrome *c* from mitichondria preceding caspase activation and loss of $\Delta\Psi_m$. They recommended undertaking further investigation.

Stark *et al.* (2001) found that aspirin induces activation of NF-κB, which is required for its anti-tumour activity and may contribute to the protective effects of aspirin observed in clinical trials. They added that the NF-κB and death response to aspirin appear relatively cell-specific since they were evident in numerous colon cancer cell lines, but not in cell lines of non-colonic origin.

1.7.4 Triterpenes

Recently, Ukiya *et al.* (2002) evaluated fifteen pentacyclic triterpene diols and triols, including six taraxanstanes (faradiol, heliantriol B_0, heliantriol C, 22α-methoxyfaradiol, arnibiol, and faradiol α-epoxide), five oleananes (manildiol, erythrodiol, longispisnogenin, coflodiol, and heliantriol A_1), two ursanes (brein and uvaol), and two lupines (cakenduladiol and heliantriol B_2), isolated from the non-saponifiable lipid fraction of the edible flower extract of *Chrysanthemum morifolium*, for their inhibitory effects on Epstein-Barr virus early antigen (EBV-EA) activation induced by the tumour promoter, 12-*O*-teradecanoylphorbol-13-acetate, in Raji cells, as a primary screening test for antitumour promoters. They found that all of the compounds tested exhibited inhibitory effects against EBV-EA activation, with potencies either comparable with or stronger than those of glycerrhetic acid, a known natural anti-tumour-promoter. They also reported that the evaluation of the cytotoxic activity of six compounds, faradiol, helantriol B_0, heliantriol C, arnidiol, faradiol-α-epoxide, and maniladiol, against human cancer cell lines revealed that faradiol-α-epoxide possesses a wide range of cytotoxicity, with GI_{50} (concentration that yields 50% growth) values of mostly less than 6μM.

Lopéz *et al.* (2002) reported that a crude ethanolic extract of *Cupressus lusitanica* Mill. leaves exhibited cytotoxicity in a panel of cancer cell lines. They also reported that cell death was due to apoptosis, assessed by morphologic features and specific DNA fragmentation detected *in situ* end-labelling of DNA breaks (TUNEL). They also showed that apoptotic cells death was induced timely in a dose-dependent manner, and despite the absence of changes in the expression levels of antiapoptotic protein Bcl-2, proapoptotic Bax protein variants ω and δ were increased. They recommended that their results warrant further research of possible antitumour compounds in this plant.

Saleem *et al.* (2002) studied the inhibition of cancer growth by crude extract and the phenolics of *Terminalia chebula* retz. Fruit. This plant, known as black myroblans, is indigenous in Pakistan and India, and has been studied for its homoeostatic, antitussive, laxative, diuretic and cardiotonic activities. Saleem *et al.* (2002) studied the effects of a 70% methanol extract of this fruit for its effect on growth in several malignant cells lines, including human (MCF-7) and mouse (S115) breast cancer cell line, a human osteosarcoma cell line (HOS-1), a human prostate cancer line (PC-3), and a non-tumorigenic, immortalised human prostate cell line (PNT1A). They found that the extract decreased cell viability, inhibited cell proliferation, and induced cell death in a dose-dependent manner in all cell lines. They also found that flow cytometry and other analyses indicated that some apoptosis was induced by the extract at lower concentrations, though at higher concentrations necrosis was the major mechanism of cell death. Three components, chebulinic acid, tannic acid and allagic acid were found to be the most growth inhibitory phenolics of *T. chebula* fruit.

1.7.5 Isoprenoids

Tatman & Mo (2002) found that volatile isoprenoid constituents of fruits, vegetables and herbs cumulatively suppress the proliferation of cancer cells lines (murine B16 melanoma and human HL-60 leukaemia cells).

Melnykovych *et al.* (1992) and Haug *et al.* (1994) reported that isoprenoid farnesol triggers growth inhibition and apoptotic death of human leukaemia CEM-C1 cells. Yazlovitskaya & Melnykovych (1995) studied the selective farnesol toxicity amd translocation of protein kinase C in neoplastic HeLa-S3K and non-neoplastic CF-3 cells. They pointed to the possible involvement of protein kinase C (PKC) in the toxic effect of farnesol, which occurs with some degree of selectivity depending on cell origin.

Miquel *et al.* (1996) tested the effects of several isoprenoids on A549 human lung adenocarcinoma cells, but only farnesol and geranylgeraiol induced actin cytoskeleton disorganisation, growth inhibition, and apoptosis. They showed that all isoprenoids tested are potent inhibitors of HMG CoA reductase activity, the sterols being the most powerful, while they induce neither F-actin disorganisation nor apoptosis. They concluded that the molecular mechanisms induced by farnesol and geranylgeraniol appear independent of reductase regulation.

Flach *et al.* (2000) found apomine (SR-45023A), a mevalonate/isoprenoid pathway inhibitor, to be an antiproliferative, and that it induces apoptosis similar to farnesol, and reported that apomine triggered apoptosis in HL50 cells in less than two hours. They concluded that the overall similar profile on mevalonate synthesis inhibition, cell growth inhibition, and apoptosis suggests that apomine acts as a synthetic mimetic of farnesol.

Hamada *et al.* (2002) found that 1-farnesylpyridinium (FPy), an analogue of farsenol, initially initiated morphological changes similar to those of typical apoptosis in human leukaemia HL-60 cells. However, FPy-treated cells were characterised by the complete absence of final apoptotic events, for example fragmentation into apoptotic bodies. These authors regarded FPy-induced cell death to be apoptotic on the basis of the induction of DNA fragmentation and the protection against these events by the co-addition of a pan-caspase inhibitor. Hamada *et al.* (2002) also reported that the increase in the cytoplasmic cytochrome *c* level substantiated the possibility that PFy-treated cells should have the ability to complete the whole apoptotic process, ending in cell fragmentation and apoptotic body formation. They also found that PFy, applied at too low concentrations to induce apoptosis, could suppress the induction of apoptotic body formation in HL-60 cells by typical inducers of apoptosis, for example actinomycin D or ansiomycin. It was also found that PFy showed a cytochalasin-like effect on spatial arrangement of actin filament independent of its apoptosis-inducing activity.

1.7.6 *Burseraceae*

The *Burseraceae* are a family of trees or shrubs which are generally spiny and often release aromatic resins when wounded. There are some 17 genera and 560 species widespread in the tropic regions of both hemispheres.

In Africa, the chief economic products from this family are the gum-resins, of the two genera *Commiphora* and *Boswellia.*

1.7.6.1 *Boswellia* spp.

The gum resin of *Boswellia serrata* is another plant product used against human leukaemia. The alcoholic extracts of the resin (boswellic acids) are reported to have an inhibitory activity against human leukaemia HL-60 cells in culture (Shao *et al.*, 1998). Shao *et al.* (1998) isolated four major triterpene acids, including β-boswellic acid, 3-*O*-acetyl-β-boswellic acid, 11-keto-β-boswellic acid, and 3-*O*-acetyl-11-keto-β-boswellic acid, from the gum of *B. serrata*, and examined them for their *in vitro* antitumour activity. They found that these acids inhibited the synthesis of DNA, RNA and protein in human leukaemia HL-60 cells in a dose-dependent manner, with IC_{50} values ranging between 0.6 and 7.1 μM. They also found that 3-*O*-acetyl-11-keto-β-boswellic acid induced the most pronounced inhibitory effects of DNA, RNA and protein synthesis, with IC_{50} values of 0.6, 0.5, and 4.1 μM, respectively. The effect of this acid on DNA was found to be irreversible, and that it significantly inhibited the cellular growth of HL-60 cells, but did not affect cell viability.

Huang *et al.* (2000) also studied the antitumour and anticarcinogenic activities of β-boswellic acid, extracted from *B. serrata*, and reported that their results showed that β-boswellic acid and its derivatives have anticarcinogenic, anti-tumour, and anti-hyperlipidemic activities.

Jing *et al.* (1999) reported that boswellic acid acetate (BC-4), a compound isolated from the herb *Boswellia carterii* Birds., can induce differentiation and apoptosis of leukaemia cells. They found that BC-4 induced monocytic differentiation of myeloid leukaemia HL-60, U937 and ML-1 cells at a dose less than 12.5 μg/ml (24.2 μM). They also reported that BC-4 was a potent inducer, with 90% of the cells showing morphologic changes, and 80% to 90% of cells showing NBT reduction. BC-4 also

led to an increase in specific and non-specific esterase, but based on benzidine staining assay, it failed to induce erythroid leukaemia DS-19 and K562 cell differentiation. Nonetheless, in contrast to its selective differentiation effects, BC-4 strongly inhibited growth of all cell lines tested. Growth inhibition effect was dose- and time-dependent. Jing *et al.* (1999) also maintained that in HL-60 cells, 20 μg/ml (38.8 μM) of BC-4 decreased viable cell numbers by 60% at 24 hours, whereas at three days there were almost no viable cells. Morphologic and DNA fragmentation analysis proved that BC-4 induced apoptosis, and concluded that the dual apoptotic and differentiation effects of BC-4 suggest that it may be a powerful agent in treating leukaemia.

1.7.6.2 *Commiphora* spp.

The most famous of these plants is known as the myrrh tree, and belongs to the species known as *Commiphora molmol. C. molmol* has been reported to have an anti-inflammatory activity. Tariq *et al.* (1985) reported that administering petroleum ether extract of oleo-gum resin of *C. molmol,* at a dose of 500 mg/kg body weight, produced significant inhibition of carrageenan-induced inflammation and cotton pellet granuloma. They also reported its significant antipyretic activity in mice.

Qureshi *et al.* (1993) studied the genotoxic, cytotoxic and antitumour properties of olego gum resin of *Commiphora* spp. in normal and Ehrlich ascites carcinoma cell-bearing mice. They evaluated the genotoxic and cytotoxic activity in normal mice on the bases of the frequency of micronuclei and the ratio of polychromatic to normochromatic cells in bone marrow, substantiated by the biochemical changes in hepatic cells. These authors also evaluated the antitumour activity of *Commiphora* from the total count, and the viability of Ehrlich ascites carcinoma cells and their

nucleic acid, protein, malondialdehyde and elemental concentrations, in addition to observations on survival and the trend of changes in body weight. Qureshi *et al.* (1993) also reported that results obtained in the Ehrlich ascites carcinoma cell-bearing mice revealed the cytotoxic and antitumour activity of Myrrh, which, as they claimed, was equivalent to that of the standard cytotoxic drug cyclophosphamide, and that on the basis of the nonmutagenic, antioxidative, and cytotoxic potential of *Commiphora*, its use in cancer therapy seems to be appropriate, and suggested further investigations.

The same research group (Al-Harbi *et al.*, 1994) carried out further research on the anticarcinogenic effect of *Commiphora molmol* on solid tumours induced by Ehrlich carcinoma cells in mice. The antitumour activity was evaluated at the end of 25 and 50 days of treatment, and it was found that treatment of *C. molmol* (250 and 500 mg/kg/day) was cytotoxic in Ehrlich solid tumour. They also reported that the antitumour potential of *C. molmol* was comparable to cyclophosphamide. Nonetheless, they found that this effect was less evident after 50 days of treatment. They also indicated that their results confirmed the cytotoxicity and anticarcinogenic potential of *C. molmol*, and recommended undertaking further studies to investigate its mode and safety for medicinal use in cancer therapy.

More recently, Al-Harbi *et al.* (1997) studied the gastric antiulcer and cytoprotective effect of *C. molmol* in rats. They found that treatment of rats with 1 ml of 80% ethanol caused depletion of stomach wall mucus, and reduction in the concentration of protein, nucleic acids and non-protein (NP)-SH groups in the stomach wall. Ethanol treatment also caused histopathological lesions such as necrosis, erosion, congestion and haemorrhage of the stomach wall. They reported that pre-treatment with *C. molmol* offered a dose-dependent protection against all these effects. Likewise, it

affected the malondialdehyde concentration altered by ethanol treatment, and offered protection against mucosal damage caused by indomethacin and its combination with ethanol. Al-Harbi *et al.* (1997) attributed the protective effect of *C. molmol* to its effect on mucus production, increase in nucleic acid and NP-SH concentration, which seemed to be mediated through its free radical-scavenging, thyroid-stimulating and prostaglandin-inducing properties.

Rao *et al.* (2001) carried out toxicity studies of *C. molmol* oleo-gum-resin in mice. They found that there was no difference in mortality in acute or chronic treatment of mice as compared to controls. They found that at the end of the treatment, weight gain in the treated and control groups was significant. They also found a significant increase in weight of testes, caudae epididymides, and seminal vesicles in the molmol-treated group. Haematological studies revealed a significant increase in red blood cells and haemoglobin levels compared to the control group. They found that molmol failed to show any spermatotoxic effect.

Tipton *et al.* (2003) investigated the *in vitro* cytotoxic and anti-inflammatory effects of myrrh oil on human gingival fibroblasts and epithelial cells. They reported that fibroblasts resident in the gingival tissue can participate in local inflammation through the production of cytokines, for example IL-6 and IL-8. Their results suggest that myrrh oil may reduce fibroblast production of such proinflammatory cytokines, and consequently reduce the participation of these cells in gingival inflammation associated with gingivitis and periodontitis. They recommended that more *in vitro* and clinical studies are required regarding herbal remedies traditionally used to treat periodontal diseases.

1.8 Aims and Objectives

The main aims of this project are to investigate the anticarcinogenic potential of two species of *Commiphora*, *C. molmol* (molmol) and *C. guidotti* (haddi), and some of the constituent biochemicals of these two plants, against three cells lines, one normal cell line, chick normal fibroblast cell line, and two cancer cell lines, S180 murine sarcoma cell line, and HT1080 human fibrosarcoma cell line, and to find out whether these plants contain apoptotic agents, and whether they induce apoptosis or not.

The objectives specified are:

1. To investigate appropriate extracts of the two plants, using ethanol, hexane and ethyl acetate as the extractants.

2. To investigate the effects of ethanolic extracted molmol and hexane extracted haddi on the growth and proliferation of the three cell lines mentioned above.

3. To test the cytotoxicity of the two extracts on the three cell lines, with special reference to apoptosis.

4. To investigate the effects of two commercially available extracts of the two plants, Flavex molmol and haddi essential oil (Aldrich), on tumour cell growth and apoptosis.

5. To investigate the effects of two commercially available biochemical components of both plants, *trans*-β-ocimene and γ-bisabolene on tumour cell growth and apoptosis.

In order to achieve these objectives, several methods and techniques were used, as explained in the following chapter.

Chapter Two

Materials and Methods

2.1 Introduction

Many researchers have tested a large number of plants and their extracts for their anti-carcinogenic properties (Tariq *et al.*, 1985; Qureshi *et al.*, 1993; Al-Harbi *et al.*, 1994). Of these plants are species belonging to the Genus *Commiphora* (Myrrh); for example *C. molmol* has been reported to have an anti-inflammatory activity (Tariq *et al.*, 1985).

In this study, two species of molmol, *C. molmol* (molmol) and *C. guidotti* (haddi) were used for the treatment of three cell lines to investigate their anti-carcinogenic effects. Extracts of each plant were prepared using four solvents. Methods of preparation are explained later in this chapter.

2.2 Cell Lines

Three types of cell lines were used to test the cytotoxic effects of the extracts obtained from the two experimental plants: S180 murine sarcoma cell line, HT1080 human fibrosarcoma cell line, and chick normal fibroblasts (NF) cell line. These three cell lines proved to be excellent models for the investigation of cell death-inducing effects of the molmol and haddi plant extracts.

2.2.1 S180 Murine Sarcoma Cell Line

The S180 tumour, also referred to as Crocker's sarcoma, arose spontaneously in the axial region of a mouse, and was discovered in 1914. Although this is commonly treated as an epithelioma, there is much uncertainty with respect to the original

phenotypic nature of this tumour (Zuckerberg, 1973). The tumour is entirely undifferentiated and bears no characteristics that allow it to be classified categorically (Dunham, 1952). S180 is highly malignant, slightly immunogenic, and is used extensively as an experimental tumour model in chemotherapy screening studies.

This cell line was obtained from the European Collection of Cell Culture (EEACC). Results obtained using extracts from two African plants (*C. molmol* and *C. guidotti*) were compared with results obtained using HT1080 fibrosarcoma and chick normal fibroblast (NF) cell lines.

S180 sarcoma cells were maintained in Minimum Essential Medium with Earle's salts, with and without L-glutamine (GibcoBRL, UK), supplemented with 10% v/v foetal calf-serum (FCS, GibcoBRL, UK), and 1% v/v penicillin/streptomycin solution, containing 100 IU/ml penicillin and 100 μg/mg streptomycin, and 1% non-essential amino acids, in a humidified atmosphere of 95% trypsin and 5% CO_2. Cells were split every 5 to 7 days after trypsinisation with trypsin-EDTA (1X) (Gibco, UK), and were seeded at a concentration of 5 x 10^4 per 25 cm^2 Costar flask.

All materials and biochemicals used were pre-warmed in a water bath maintained at 37°C prior to their use.

Cells were grown and maintained using Costar flasks, with a surface area of either 25 cm^2 or 75 cm^2.

2.2.2 HT1080 Human Fibrosarcoma Cell Line

HT1080 cell line was developed by Rasheed *et al.* in 1974. They represent a pseudodiploid line with the model number of 46 chromosomes. HT1080 cell lines are epithelial-like adherent cells developed from a fibrosarcoma which arose adjacent to the acetabulum of a 35 year-old white male. This kind of tumour can spread rapidly

to the bone through the blood stream or by direct invasion. The most commonly affected areas include the lower jaw, nasal sinuses, vertebrae, spine and radius. Nonetheless, femur and tibia are also frequently affected. Fibrosarcomas can later on spread or metastasise to other soft tissues such as kidney and lymph nodes.

The cells can give rise to tumours in immunosuppressed NIH Swiss mice susceptible to infection by RNA tumour viruses, and which are particularly well suited for the production of Moloney murine leukaemia virus, and are therefore used as helper cells, viral vector producer cells (VPCs), or retroviral packaging cells. This packaging cell line offers the advantage of producing high titres of retroviral vectors' resistance to inactivation by human serum, enabling their use in human therapy. Reducing the viral sequences has also reduced the risk of producing replication component retroviruses. Consequently, this cell line offers great potential for the development of human gene therapy.

HT1080 cells do not grow well in semi-solid medium, but require Dulbecco's modified Eagle medium with a high glucose and L-glutamine supplement with 10% FCS. The cells are highly tumorigenic in nude mice due to their possession of a mutant hyperactive N-*ras* allele which initiates a linear signal transduction cascade culminating in the transcriptional activation of genes involved in mitogenesis, and gives rise to continuous mitogenesis (Reuter *et al.*, 1995). The hyperactive N-*ras* extracellular signal-regulated kinase is the first in a constitutively expressed pathway of Raf, MEK, and MPAK (also known as ERK), which activate ELK-1 in the nucleus, leading to gene expression and the consequential neoplastic activity and phenotype (disorganised actin filament) of HT1080 cells.

This cell line was kindly provided by Dr. Alvin Kwan (School of Biosciences, Cardiff University). They were also grown and maintained in Dulbecco's Modified Eagle

Medium (DMEM) without sodium pyruvate, but with L-glucose (GibcoBRL, UK). 0.5 ml gentamycin, 10% FCS, antibiotics, L-glutamine, 1% sodium pyruvate (GIBCO, UK), 1% HEPES buffer (GIBCO, UK), and vitamin C also added.

All materials and biochemicals used were pre-warmed in a water bath maintained at 37°C prior to their use.

Cells were also grown and maintained using Costar flasks, as explained above.

2.2.3 Normal Chick Fibroblast (NF) Cell Line

The chick fibroblasts were kindly provided by Dr. Jim Ralphs (School of Biosciences, University of Cardiff). The preparation of chick fibroblasts involved the dissection of flexor tendons from the feet of 6-day-old chickens and internal fibroblasts extracted by a modification of the methods of Banes *et al.* (1988). Tendons were treated successively with 0.5% bacterial collagenase I for 3 minutes and 0.25% trypsin for 15 minutes at 37°C, and then scraped with a rubber policeman to remove surface cells. The remaining tendons were chopped and digested successively with 1% protease for 1 hour and 0.5% collagenase I for 2 hours. Cells were then centrifuged at 1000 rpm for 5 minutes and were resuspended and plated in high glucose DMEM containing 5% foetal calf serum (FCS) and antibiotic, grown to confluence and passaged.

2.3 Thawing of Frozen Cell Stocks

Cell stocks obtained for the three cell lines were kept in foetal calf serum (FCS) in Epindorff tubes and stored in a freezer at –80°C. The tubes containing the cells were thawed rapidly, when required, in a 37°C water bath for 2-3 minutes. The tube contents were then shaken thoroughly and added to 25 cm^2 flasks containing 5-7 ml of the growth medium. The flasks were then left for about three days, after which period the medium was changed in order to remove dead cells. Flasks were then sub-

cultured normally when cell density in the flasks had increased after about five days. The cells were then cultured, as described in the following section.

2.4 Maintenance of Adherent Cell Cultures

Cell stocks of all three cell lines were maintained by continuous sub-cultures from 25 cm^2 and 75 cm^2 flasks when about 80% confluency was observed. The following procedure was employed for sub-culturing. The media were removed from the flask, using a large volume pipette, and the adherent cell monolayer was washed twice with 1 to 2 ml phosphate buffered saline (PBS). (PBS: 1.5 mM KH_2PO_4, 6.5 mM Na_sHPO_s, 137 mM NaH_2PO_4, 137 mM NaCl, 2.7 mM KCl, and pH adjusted to pH 7.5). The prepared buffer was filtered through a 0.22 μm porosity membrane filter (Millipore, UK) to sterilise and remove any insoluble residue. Phosphate buffered saline was prepared by dissolving one PBS tablet (GIBCO, UK) in 500 ml of distilled water, and the solution was then filtered through a 500 ml Millipore Stericup vacuum-driven disposable filtration system. The phosphate buffer was removed, and 2 ml of pre-warmed (37°C) Trypsin-EDTA solution (1X) (GibcoBRL) were added to the cell monolayer in order to rinse the flasks and to remove any residual growth medium, and the majority of this volume was removed. 2 to 5 ml of Trypsin-EDTA solution were then added to the adherent cells, depending on cell number and flask size (25 cm^2 or 75 cm^2), and the flasks were incubated at 37°C for 3 to 5 minutes to allow cells to detach. The cultures were examined using an inverted microscope (Zeiss ID03, Germany) to make sure that all cells had detached, and the cell suspension was dispersed by repetitive pipetting in order to obtain a single cell suspension, free of clumps, and the cell number was estimated using a haematocytometer (Sigma Chemical Co, USA). An appropriate volume was then removed such that the seeding density of the new culture would be $5x10^4$ cells (S180, HT1080 and NF) per 25 cm^2

flask in 5 to 7 ml fresh growth medium, and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air (Stericult incubator). The lids of the culture flasks were loosened to allow gas exchange. S180 cultures were refed after 4 days, and HT1080 cultures for 5 to 8 days, and were left in the incubator until reaching 80% confluency, when subculture was repeated as described above. Morphological changes and counting of the cells were performed as described above.

2.4.1 Determination of Cell Numbers

The improved Neubauer haematocytometer is a microscopic counting chamber with defined areas and a known depth. The cell number within this area can therefore be used to calculate cell concentration, using the following formula:

$$c = n/v,$$

where c is the cell concentration (cells/ml), n is the number of cells counted, and v is the chamber volume counted (ml). For the improved Neubauer slide, the depth of the chamber is 0.1 mm, and the central area used is 1 mm^2. Accordingly, v equals 0.1 mm^3, or $1x10^{-4}$ ml. The formula then becomes:

$$c = n/10^{-4} = n \times 10^4 \text{ (cells/ml)}.$$

2.5 Preparation of Molmol (*Commiphora molmol*) Extracts

Molmol was extracted using ethanol, hexane, ethyl acetate and Flavex extract. Flavex, a commercially available aqueous alcoholic extract of *C. molmol* (20%w/v in 96% ethanol) was obtained from Flavex International Limited (UK). It is referred to in this thesis as Flavex molmol.

2.5.1 Ethanol Extracts

Ethanolic extracts were prepared by adding 20 g of molmol to 100 ml of absolute ethanol. The mixture was stirred for a few minutes and left for seven days at room temperature in a dark cupboard. After that period, the mixture was stirred for a few minutes to ensure complete suspension of the extract in the extracting solution, and the solution was filtered using a 150 ml Millipore stericups filter. To aid filtration, extracts were filtered under suction using a 250 ml Buchner flask. The filtrate was then evaporated under vacuum using a rotary evaporator (BÜCHI, Germany) at a constant temperature of 70°C.

2.5.2 Hexane Extracts

Molmol was also extracted with hexane, by adding 20 g of molmol to 100 ml of hexane. The mixture was then treated as described for ethanolic extraction. However, because the Millipore filter becomes damaged when organic solvents such as hexane and ethyl acetate, the extractants, are involved, the mixture was not filtered through a Millipore filter, and instead filtration was carried out under vacuum using a 250 ml Buchner flask.

2.5.3 Ethyl Acetate Extracts

Molmol was also extracted with ethyl acetate in a similar way as described for hexane extraction, with 100 ml of ethyl acetate replacing hexane as the extractant, and vacuum was used to aid filtration.

2.5.4 Flavex Extract

Flavex is a commercially available aqueous ethanolic extract of molmol obtained from Flavex International Limited.

All extracts were prepared at 20%w/v. All chemicals used for extraction were obtained from Fisher Chemicals, UK.

2.6 Preparation of Haddi (*Commiphora guidotti*) Extracts

Haddi is the local name in Somalia for *Commiphora guidotti*. Haddi was extracted using ethanol, hexane and ethyl acetate solvents, and prepared as described for *C. molmol*. An essential oil extract (Opoponax oil) was obtained commercially from Aldrich, USA.

2.7 Preparation of Stock Extracts

For each extract, 60 mg of the plant extract was added to, and dissolved in, 120 µl of ethanol to give a total volume of 180 µl. From this total volume, 144 µl was added to 36 ml of the growth medium and vortex-mixed using a Whirlmixer (Fisons). This was then centrifuged at 20,000 rpm for five minutes, and filtered using a 50 ml Millipore Stericup vacuum-driven disposable filtration system. This procedure resulted in a stock solution of 1333 µg/ml.

It has been noticed that when the oil was dissolved in ethanol and then added to the medium, a cloudy emulsion was formed.

2.8 S180 and HT1080 Cell Growth Assays

Three replicates of growth curves experiments, each containing three batches, were run simultaneously, as follows: control, treatment with an ethanolic molmol extract, and treatment with hexane haddi extract for S180 cell line and HT1080 cell line.

The growth curves help to visualise any effects that the treatments mentioned above may have on the S180 and the HT1080 cell lines, by investigating the differences between the control and treatment curves. For each run, 24 flasks, with 25 cm^2

surface area each, were prepared with a seeding density of 4 x 10^4 cells per flask. Cell numbers in each flask were then counted twice per day, every day, using a haematocytometer, and an average was then calculated. This counting procedure was carried out for all experiments, control and treatments (nine trials for each treatment). For the S180 cell line growth experiment, ethanolic molmol and hexane haddi extracts were added to the culture on Day 4 for a concentration of 366.63 µg/ml for ethanolic molmol, and 366.63 µg/ml for hexane haddi. The control medium was only replaced with fresh medium. Results were taken every 24 hours for 8 days for the S180 cell line and 14 days for the HT1080 cell line. HT1080 medium was changed and treated at Day 7, instead of at Day 4 as for the S180 treatments.

2.9 S180, HT1080 and Normal Fibroblast MTT Assays

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay procedure adopted for the S180, HT1080 and normal cell lines was as follows:

Cells were seeded at seeding density of $2x10^5$ cells/ml.

100 µl of cells at $2x10^5$ cells/ml were added to a 96-well microtiter plate (avoiding the outer edge of the plate as cells would dry out, and PBS was added to outer wells to prevent evaporation of the media in the test wells).

The plate was then left for 4 hours in an incubator in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

A 100 µl of medium with cells to the 1st row of the plate as it is the control (untreated cells).

A 100 µl of plant extract at five different concentrations. The plate was kept in an incubator for 24, 48 and 72 hours. 20µl of MTT were added to each well. The plate was wrapped in foil, as MTT is sensitive to light. Then the plate was placed into the

incubator for 4 hours at 37°. The media were removed. 200µl of DMSO were added to each well. The plate was read on ELISA at 575 nm.

Calculate percentage toxicity and percentage viability using the following formulae:

$$\% \text{ Cytotoxicity} = \frac{a - b}{a} \times 100$$

where: a = mean absorbance of nine wells with culture medium only.
b = mean absorbance of nine wells with plant extract dilution.
% Viability was measured using the following formula:

$$\% \text{ Viability} = 100 - \% \text{ Cytotoxicity}$$

LC_{50} concentrations for 666.6 µg/ml extract were calculated using the following formula:

$$LC_{50} = (666.60 * 50)/\text{percentage toxicity}$$

The same steps were repeated, after 48 hours and 72 hours, to find out the percentage of toxicity and percentage of viability after 48 and 72 hours of incubation.

For each treatment, three wells were used per replicate, and a total of 3 triplicate runs were attempted, giving a total of 9 wells per experiment. In each treatment, a control and five test concentrations were set up.

MTT assays were carried out and repeated three times for five different concentrations of the following extracts:

Molmol: Ethanolic, hexane, ethyl acetate, and Flavex extracts.

Haddi: Ethanolic, hexane, ethyl acetate, and essential oil (Aldrich) extracts.

Results of the MTT viability test showed that molmol-ethanolic and haddi-hexane extracts had the optimum effect on the cell lines used. Accordingly, the remaining

experiments were undertaken using molmol-ethanolic and haddi-hexane extracts.

2.9.1 Preparation of Concentrations Used in MTT Assays

Different concentrations of molmol and haddi extracts were prepared for various purposes of the study. The 666.60 µg/ml to 266.45 µg/ml concentrations of extracts were prepared as illustrated in Table 2.1.

Table 2.1 266.45-666.60 µg/ml treatment concentrations of ethanolic-, hexane-, ethyl acetate-, flavex-, and essential oil Aldrich-extracts.

Medium Volume (µl)	Final Volume (µl)	Oil Concentration (%v/v)	mg/ml	µg/ml	Final Concentration (µg/ml)
12,000	12,000	0.133	1.333	1333.2	666.6
600	4,000	0.113	1.133	1133.2	566.6
620	3,500	0.093	0.932	932.4	466.2
645	3,000	0.073	0.731	732.	366.0
680	2,500	0.053	0.533	532.8	266.4

In the event of greater total volume of treatment being required, multiples of this table were used

The 666.60 µg/ml to 0.65 µg/ml concentrations of extracts were prepared as illustrated in Table 2.2.

A further dilution series was prepared, covering the range of 366.60 and 466.6 µg/ml of the extract (Table 2.3).

Table 2.2 0.65-666.60 µg/ml treatment concentrations of ethanolic-, hexane-extracted molmol and haddi.

Final Volume (µl)	Oil Concentration (%v/v)	mg/ml	µg/ml	Final Concentration (µg/ml)
12,000	0.133	1.333	1333.2	666.6
2,000	0.067	0.667	666.6	333.3
2,000	0.017	0.167	166.6	83.3
2,000	0.002	0.021	20.8	10.4
2,000	0.000	0.001	1.3	0.065

Table 2.3 366-466 µg/ml treatment concentrations of ethanolic molmol

Final Volume (µl)	Oil Concentration (%v/v)	mg/ml	µg/ml	Final Concentration (µg/ml)
12,000	0.093324	0.933	933.2	466.6
2,000	0.088268	0.883	882.6	441.3
2,000	0.083344	0.833	833.4	416.7
2,000	0.078224	0.782	782.2	391.1
2,000	0.073336	0.733	733.3	366.6

A higher concentration series of extracts were prepared for the treatment with haddi essential oil (Aldrich) (Table 2.4)

Table 2.4 Higher treatment concentrations for haddi essential oil (Aldrich).

Final Volume (µl)	Oil Concentration (%v/v)	mg/ml	µg/ml	Final Concentration (µg/ml)
36,000	0.133	1.333200	1333.200	1333.2
18,000	0.113	1.133220	1133.220	1133.2
12,150	0.093	0.932691	932.691	932.9
8,260	0.073	0.733958	733.958	733.9
8,250	0.053	0.533788	533.788	533.7

2.9.2 Preparation of MTT

The concentration used in the present study was 5 mg/ml, and this is the established optimal concentration of MTT (Cedilo-Rivera *et al.*, 1992; Hegyesi & Csaba, 1997; Al-Hazzaa, 2002). MTT powder was dissolved and the stock MTT was prepared at a concentration of 5 mg/ml in phosphate buffered saline, then the solution was filtered using a 0.22 μm membrane filter (Millipore, UK) to remove any insoluble residue. The stock solution was then stored in bottles wrapped with foil because MTT is photosensitive.

Viable cell growth was determined by the MTT dye reduction microassay (Boehrenger Mannheim, UK). MTT [3-(4,5-dimethylthaizol-2-yl),-2,5-diphenyltetrazolium bromide] is a yellow, water-soluble tertrazolium dye which is reduced by live cells to a purple formazan product, insoluble in aqueous solutions. Tetrazolium reactions have a wide application in the indirect demonstration of dehydrogenase activity. The reaction is used as the end point in a rapid drug-screening assay (Plumb *et al.*, 1989). The MTT method has been adapted for use with microtitre plates as a cellular viability assay (Bowen, 1998).

2.10 Haematoxylin-Eosin Staining

A. S180 Cell Culture Preparation

S180 cells seeded at cell density of $2x10^5$ cells/ml were exponentially grown on 13 mm glass coverslips in 12-well Corning plates, incubated in Mini Essential Culture Medium containing 10% FCS at 37°C and 5% CO_2. The cells were then treated with two different concentrations, 666 μg/ml and LC50 concentrations of ethanolic molmol or hexane haddi, and a control (untreated), for the following time points:

Ethanolic molmol, 666.6 μg/ml: 6, 12, 24 hours.

Ethanolic molmol, 234 μg/ml: 6, 12, 24 hours.

Hexane haddi, 666.6 μg/ml: 6, 12, 24 hours.

Hexane haddi, 201 μg/ml: 6, 12, 24 hours.

Control – untreated: 6, 12, 24 hours.

After each treatment, which was carried out in duplicates, the cells on the cover slips were fixed with 10% neutral buffer formalin for ten to twenty minutes. The fixed cells were then stained in Mayer's haematoxylin for two to three minutes, followed by three rinsings in tap water, and once in distilled water. The cells were then counterstained with eosin for 45 to 60 seconds, followed by another three rinsings in tap water, and once in distilled water. Finally, the cells were air dried and mounted in Xam. The morphology of the cells was studied using a Leitz DMRB light microscope at X40 and X100 magnifications.

B. HT1080 Cell Culture Preparation

$2x10^5$ HT1080 cells were exponentially grown on 13 mm glass coverslips in 12-well Corning plates, incubated in DMEM medium containing 10% FCS at 37°C and 5% CO_2. The cells were then treated with 666 μg/ml and LC_{50} concentrations of ethanolic molmol or hexane haddi, and a control (untreated), for the following time points:

Ethanolic molmol, 666.6 μg/ml: 6, 12, 24 hours.

Ethanolic molmol, 234 μg/ml: 6, 12, 24 hours.

Hexane haddi, 666.6 μg/ml: 6, 12, 24 hours.

Hexane haddi, 201 μg/ml: 6, 12, 24 hours.

Control – untreated: 6, 12, 24 hours.

The cells were then fixed in neutral buffer formalin, stained with haematoxylin, counterstained with eosin, and their morphology examined, following the procedure described for S180 cells.

C. Normal Fibroblast Cell Culture Preparation

Normal fibroblast, seeded at $2x10^5$ cells/ml, were also exponentially grown on 13 mm glass coverslips in 12-well Corning plates, incubated in DMEM containing 5% FCS at 37°C and 5% CO_2. The cells were then treated with 666 μg/ml and LC_{50} concentrations of ethanolic molmol and hexane haddi, and a control (untreated), for the following time points:

Ethanolic molmol, 666.6 μg/ml: 6, 12, 24 hours.

Ethanolic molmol, 234 μg/ml: 6, 12, 24 hours.

Hexane haddi, 666.6 μg/ml: 6, 12, 24 hours.

Hexane haddi, 201 μg/ml: 6, 12, 24 hours.

Control – untreated: 6, 12, 24 hours.

The cells were then fixed, stained, and examined as described above.

2.11 Detection of DNA Fragmentation (TUNEL)

2.11.1 Procedure

A. HT1080 Cell Culture Preparation

$2x10^5$ HT1080 cells were exponentially grown on 13 mm glass coverslips in 12-well Corning plates, incubated in DMEM containing 10% FCS at 37°C and 5% CO_2. The cells were then treated with 666.6 μg/ml and LC_{50} concentrations of ethanolic molmol or hexane haddi, and a control (untreated), for the following time points:

Ethanolic molmol, 666.6 μg/ml: 3, 6, 12, 24, 48 hours.

Ethanolic molmol, 234 μg/ml: 3, 6, 12, 24, 48 hours.

Hexane haddi, 666.6 μg/ml: 3, 6, 12, 24, 48 hours.

Hexane haddi, 201 μg/ml: 3, 6, 12, 24, 48 hours.

Control – untreated: 3, 6, 12, 24, 48 hours.

The cells were washed with PBS, and were then fixed with 4 % formaldehyde for 10 minutes at room temperature.

2.11.2 Klenow Fragmentation Procedure

The following procedure was underaken:

1. Rehydration

The coverslips were immersed in 1X TBS for 15 minutes at room temperature, and the glass slides around the specimens, were carefully dried.

2. Permeabilisation of specimens

1μL of 2mg/ml proteinase K (1:100 in 10mM Tris PH 8) was mixed with 99μl 10mM Tris per specimen). The entire specimen was covered with 50-100μl of 20μg/ml proteinase K and incubated at room temperature for 5 minutes. The slide was dipped 2-3 times into a beaker of 1X TBS. The excess liquid was gently tapped off and the glass slides around the specimen carefully dried.

3. Inactivation of endogenous peroxidases

10μl of 30% H_2O_2 was mixed with 90μl methanol per specimen. The entire specimen was covered with 100μl of 30 %H_2O_2 and incubated at room temperature for 5 minutes. The slides were rinsed with 1X TBS, the excess liquid gently tapped off, and the glass slides around the specimens were carefully dried.

4. Equilibration and Labelling Reaction

10X Klenow equilibration buffer was diluted 1 in 10 with distilled water by mixing 10µl of 10X buffer with 90µl of distilled water per specimen.The entire specimen was covered with 100µl of 1X Klenow equilibration buffer and incubated at room temperature for 10 to 30 minutes, while preparing the labelling reaction mixture. The working Klenow Labelling Reaction mixture was prepared as follows:

The contents of the Klenow labelling reaction mix tube was lightly vortexed. The Klenow enzyme vial was pulse–spun in a microcentrifuge. The sample was transferred to a clean microfuge tube, placed on ice, and 58.4µl Klenow labelling reaction and 1.6µl Klenow enzyme were mixed gently. The 1X Klenow equilibration buffer from the specimens was carefully blotted, taking care not to touch the specimens. The specimens were covered with a piece of parafilm cut slightly larger than the specimen, the slides placed in a humidified chamber, and incubated at 37°C for 1.5 hours.

5. Termination of Labelling Reaction

The stop buffer was prewarmed to 37°C for 5 minutes. The slides were rinsed with 1X TBS and the entire specimen covered with 100µl of stop solution. These were incubated at room temperature for 5 minutes. The slides were then rinsed with 1X TBS. The excess liquid was gently tapped off, and the glass slides around the specimens carefully dried.

6. Detection

The entire specimen was covered with 100µl of blocking buffer and incubated at room temperature for 10 minutes. The 5X conjugate was diluted 1 in 50 in blocking buffer by mixing 2µl of 50X conjugate with 98µl blocking buffer per specimen. The

blocking buffer was carefully blotted from the specimens,taking care not to touch them, and 100µ of diluted 1X conjugate was immediately applied. The slides were placed in a humidified chamber and incubated at room temperature for 30 minutes. Five minutes before concluding incubation, the DAB solution was prepared by dissolving one tablet of DAB and one tablet of H_2O_2/ urea in 1 ml of tap water. The slides were rinsed with 1X TBS.

The excess liquid was gently tapped off and the glass slides around the specimens carefully dried. The entire specimen was covered with 100µl of DAB solution, incubated at room temperature for 10-15 minutes, and the slides were rinsed with distilled water.

7. Counterstain

The entire specimen was covered with 100µl of Methyl Green counterstains solution, immediately covered, and incubated at room temperature for 3 minutes. The edge of the slides was pressed against an absorbent towel to draw off the most of the counter stain.

B. Normal Fibroblast Cell Culture Preparation

Normal fibroblasts, seeded at $2x10^5$ cells/ml, were also exponentially grown on 13 mm glass coverslips in 12-well Corning plates, incubated in DMEM containing 5% FCS at 37°C and 5% CO_2. The cells were then treated with LC_{50} concentration of ethanolic molmol and hexane haddi, and a control (untreated), for the following time points:

Ethanolic molmol, 234 µg/ml: 3, 6, 12, 24, 48 hours.

Hexane haddi, 201 µg/ml: 3, 6, 12, 24, 48 hours.

Control – untreated: 3, 6, 12, 24, 48 hours.

The cells were then fixed, stained, and examined as described above.

2.12 Scanning Electron Microscopy

In the present study, changes in the cell surface morphology of treated cells were analysed and compared with the cell surface morphology of untreated cells (controls).

2.12.1 Preparation of Fixatives

Sodium cacodylate buffer. A 0.1M solution of sodium cacodylate (supplied by Agar Scientific Ltd., UK) buffer was prepared by adjusting 50 ml of 0.2M buffer to pH 7.3-7.4 with 1M HCl, and then the volume was made up to 100 ml with distilled water.

Gluteraldehyde fixative. A solution of 1.25% gluteraldehyde (supplied by Agar Scientific Ltd., UK) containing 0.1M buffer (pH 7.4) was prepared by adding 10 ml of 0.25% v/v gluteraldehyde solution to 40 ml sodium cacodylate buffer solution (0.2M). 6 ml of this stock were then further diluted with 6 ml of the DMEM growth medium.

2.12.2 Preparation of Cell Culture

S180 murine sarcoma cells, HT1080 fibrosarcoma cells and chick normal fibroblasts were exponentially grown on 13-mm thermanox plastic coverslips in 12-well Corning plates incubated in growth medium containing 10% FCS (5% FCS) at 37°C and 5% carbon dioxide. This procedure was carried out for cells treated with 366 μg/ml ethanolic-molmol extract (two coverslips) or for cells treated with 733 μg/ml hexane-haddi extract (two coverslips). As hexane haddi was not available, haddi essential oil (Aldrich) was used at this concentration. The remaining coverslip was left untreated as a control. The cover slips were then placed in 12-well Corning plates in 1 ml of growth medium. Next, the plates were incubated for 4 hours at 37°C to allow cells to adhere to the cover slips. The plates were then left for 12 hours before fixation.

2.12.3 Fixation

All types of cells used in the present study were fixed in 1.25% gluteraldehyde in cacodylate buffer for one hour at room temperature, and then washed twice with 0.1M cacodylate buffer for 10 minutes each to remove excess fixative. The cells were then further fixed in 1% osmium tetroxide (Agar Scientific, UK) in 0.1M cacodylate buffer for one hour, in order to improve conductivity in the electron beam within the SEM, and also to protect the cells from possible beam damage during microscopy.

2.12.4 Dehydration

The cover slips containing fixed cells were gradually dehydrated with various concentrations of ethanol, starting at 50% concentration, and ending with four successive rinsings with absolute ethanol, using the following regime:

a) 50% ethanol for 5 minutes at room temperature.

b) 70% ethanol for 5 minutes at room temperature.

c) 90% ethanol for 5 minutes at room temperature.

d) 100% ethanol for 10 minutes at room temperature.

e) 100% ethanol for 10 minutes at room temperature.

f) 100% ethanol for 10 minutes at room temperature.

g) 100% ethanol for 10 minutes at room temperature.

2.12.5 Microscopy

The cover slips were then transferred to a Critical Point Dryer (CPD) (Balzers CPD 30). The drying process was carried out under high pressure (1300 psi) using CO_2, and allowing the temperature to rise to 45°C (critical point drying temperature). Under these conditions, the liquid CO_2 was completely evaporated within 2 hours,

leaving dried crystalline cells. The coverslips were then mounted on aluminium stubs, using double-sided carbon tape, and were placed in an Alcatel Gold Sputterer Coater (EITI Scope, England) and coated with a 100 nm layer of gold. The samples were then examined under a Philips XL20 Scanning Electron Microscope at a 20kV accelerating voltage and a range of magnifications.

2.13 Transmission Electron Microscopy

HT1080 cells treated as for transmission electron microscopy preparation method were fixed with 1% gluteraldehyde for 24 hours at 4°C, and then washed in fresh cacodylate buffer overnight. The cells were removed and gradually dehydrated by washing in alcohol dehydrating series, as explained in Section 2.11.4. The cells were then removed from the thermanox using a plastic scraper, transferred into microcentrifuge tubes containing Araldite preparation (300 g/ml), and were spun down in an MSE centrifuge at 1000 rpm for five minutes to obtain a firm pellet. The resulting pellet was embedded in capsules with freshly prepared resin and polymerised in an oven at 60°C for 24 hours. Polymerised resin blocks were cut into 90 nm thick sections using a diamond knife and a Reichert Ultracut OMU4 microtome (Reichert-Jung, Austria). The ultrathin sections were collected on pioloform-coated copper grids (10 squares), counterstained in 2% uranyl acetate for one hour, and then in lead citrate for 20 minutes. Finally, the grids were washed in three changes of 0.02M NaOH solution, followed by three rinses in double-distilled water. The grids were examined using a JEOL 1200EXelectron microspcope operated at 80 kV accelerating voltage, using magnifications ranging from x2000 to x10,000. Transmission electron micrographs were recorded on Kodak 4489 EM film plates (3.5x4 in) and developed in Kodak ID 19 solution.

2.14 Fluorescence Microscopy

Immunohistochemical confirmation was carried out using the Apoptosis Detection Kit (Oncogene Research Products U.K.). This assay has been found to detect the apoptotic cells significantly earlier that DNA-based assay (Amin *et al.*, 2000). In this work, S180 cells, HT1080, and chick normal fibroblast cells were washed by PBS after staining, in order to avoid any subsesequent background fluorescence, and that resulted in loss of the dead cells that had been detached from the monolayer. Fixation with paraformaldehyde is not recommended, as the fixing would result in cell death, and all the cells will appear stained only with propidium iodide. In this assay, a fluorescein isothiocyanate (FITC) conjugate of Annexin V is used, which allows for the detection of apoptosis. Necrotic cells also bind Annexin V-FITC due to membrane permeabilisation. Propidium iodide (PI) is employed to differentiate between viable, early apoptotic, and late apoptotic or necrotic cells. While necrotic cells will bind Annexin V-FITC and stain with propidium iodide, propidium iodide will be excluded from viable (FITC negative) and early apoptotic (FITC positive) cells. Late apoptotic cells will acquire both labels due to the necrotic-like disintegration of the cell and the absence of phagocytosis *in vitro* (Oncogen Research Products, 1998).

A RAPID protocol has been developed by Annexin V-FITC binding *directly* in tissue culture media. This prevents the need for tedious centrifugation and wash steps that increase the occurrence of mechanical membrane disruption. Furthermore, since apoptosis is a dynamic process that is ongoing once cells are removed from culture conditions and continues throughout experimental processing, the RAPID protocol is recommended for the detection of cells in early apoptosis (Oncogyne Research Products, 1998).

A. S180 Cell Culture Preparation

S180 cells seeded at cell density of $2x10^5$ cells/ml were exponentially grown on 13 mm glass coverslips in 12-well Corning plates, incubated in Mini Essential Culture Medium containing 10% FCS at 37°C and 5% CO_2. The cells were then treated with two different concentrations, 666 μg/ml and LC50 concentrations of ethanolic molmol or hexane haddi, and a control (untreated), for the following time points:

Ethanolic molmol, 666.6 μg/ml: 12, 24 hours.

Ethanolic molmol, 234 μg/ml: 12, 24 hours.

Hexane haddi, 666.6 μg/ml: 12, 24 hours.

Hexane haddi, 201 μg/ml: 12, 24 hours.

Control – untreated: 12, 24 hours.

B. HT1080 Cell Culture Preparation

$2x10^5$ HT1080 cells were exponentially grown on 13 mm glass coverslips in 12-well Corning plates, incubated in DMEM containing 10% FCS at 37°C and 5% CO_2. The cells were then treated with 666.6 μg/ml and LC_{50} concentrations of ethanolic molmol or hexane haddi, and a control (untreated), for the following time points:

Ethanolic molmol, 666.6 μg/ml: 12, 24 hours.

Ethanolic molmol, 234 μg/ml: 12, 24 hours.

Hexane haddi, 666.6 μg/ml: 12, 24 hours.

Hexane haddi, 201 μg/ml: 12, 24 hours.

Control – untreated: 12, 24 hours.

C. Normal Fibroblast Cell Culture Preparation

Normal fibroblast cells, seeded at $2x10^5$ cells/ml, were also exponentially grown on 13 mm glass coverslips in 12-well Corning plates, incubated in DMEM containing

5% FCS at 37°C and 5% CO_2. The cells were then treated with 666.6 μg/ml and LC_{50} concentrations of ethanolic molmol and hexane haddi, and a control (untreated), for the following time points:

Ethanolic molmol, 666.6 μg/ml: 12, 24 hours.

Ethanolic molmol, 234 μg/ml: 12, 24 hours.

Hexane haddi, 666.6 μg/ml: 12, 24 hours.

Hexane haddi, 201 μg/ml: 12, 24 hours.

Control – untreated: 12, 24 hours.

Following the incubation after the treatment time, the medium in each well was pipetted off, and each well was washed twice with 1 ml of PBS. Each coverslip was covered with 50μl of 1X binding buffer. 10 μl of Media Binding Reagents were added, followed by 3 μl of Annexin V-FITC. The cells were incubated for 15 minutes in the dark at room temperature. 10 μl of propidium iodide were added. The coverslips were inverted on glass slides contained mounting medium (vectra shelid) . The slides were examined directly by fluorescent microscopy (Leitz, Loborolux 12). Images of random fields of cells were captured using a digital camera (Roper Scientific CoolSnap, digital camera) connected to the fluorescence microscope.

2.15 *Trans-β*-Ocimene and γ-Bisabolene

Trans-β-ocimene and γ-bisabolene are two of the biochemical components of haddi plant. These two components were identified by Mr. Ahmed Ali, School of Biosciences, Cardiff University, using gas chromatography-mass spectroscopy technique.

Once these two biochemicals were identified, they were ordered from R.C Treatt, Suffolk. Both were then confirmed to be those identified by Mr. Ahmed Ali by analysis of their retention times and mass spectra.

These biochemicals were selected since they make up a reasonable proportion of the overall extract. Accordingly, they may be the active components of the haddi extracts.

2.15.1 Preparation

The molecular weights of *trans-β*-ocimene and *γ*-bisabolene were first determined. For each biochemical, the respective weight (in mg, corresponding to the molecular weight) was added to 900 µl of growth medium and 100 µ of DMSO, to give an overall stock concentration of 1M, which was further diluted to the concentrations required, for the various assays. When a larger amount of stock was required, multiples of each weight were used.

2.15.2 MTT Assay

Five concentrations of each of ocimene and bisabolene were used. The dilutions of the overall stock solution used to obtain the five final concentrations used in the assays: 50, 100, 150, 200 and 250 µM of both biochemicals, were estimated as shown in Tables 2.5 and 2.6.

Table 2.5 Concentrations of *trans-β*-ocimene used in MTT assays

	Volume of Stock (µL)	Volume of Media (µL)	Total Vol. (µL)	Conc. (mM)	Final Conc. (mM)
Conc. 1	100	900	1000	100	50
Conc. 2	200	800	1000	200	100
Conc. 3	300	700	1000	300	150
Conc. 4	400	600	1000	400	200
Conc. 5	500	500	1000	500	250

Table 2.6 Concentrations of *γ-b*isaolene used in MTT assays

	Volume of Stock (µL)	Volume of Media (µL)	Total Vol. (µL)	Conc. (mM)	Final Conc. (mM)
Conc. 1	25	475	500	100	50
Conc. 2	50	450	500	200	100
Conc. 3	75	425	500	300	150
Conc. 4	100	400	500	400	200
Conc. 5	125	375	500	500	250

The MTT procedure described in Section 2.9 was used, replacing the plant extracts with either ocimene or bisabolene at the concentration referred to in Tables 2.5 and 2.6. Only S180 and HT1080 cells were used, and not the normal fibroblast cell line.

2.15.3 Time Course Assay

For the time course assay, ocimene was added to S180 cells and HT1080 cells at two concentrations of 65 and 95 Mm. Bisabolene was added at two concentrations of 10 and 20 Mm. Calculation of these concentrations is presented in Tables 2.7 and 2.8.

Table 2.7 Concentrations of *trans-β*-ocimene used in time course assays

	Volume of Stock (µL)	Volume of Media (µL)	Total Vol. (µL)	Conc. (mM)	Final Conc. (mM)
Conc. 1	130	870	1000	130	65
Conc. 2	190	810	1000	190	95

Table 2.8 Concentrations of *γ*-bisabolene used in time course assays

	Volume of Stock (µL)	Volume of Media (µL)	Total Vol. (µL)	Conc. (mM)	Final Conc. (mM)
Conc. 1	20	980	1000	20	10
Conc. 2	40	960	1000	40	20

LC_{50} values for both ocimene and bisabolene were obtained from the results of the time-course. LC_{50} concentrations were then used in Annexin-V assay and SEM.

2.15.4 Annexin-V Assay and Scanning Electron Microscopy

Procedures for Annexin-V and SEM used earlier (Sections 2.12 and 2.14, respectively) were also followed with either ocimene or bisabolene instead of the plant extracts.

2.16 Statistical Analysis

Data were tested for normality of distribution using the Anderson-Darling test. In all cases, it was found that data were not normally distributed. Therefore, the data were tested by the non-parametric equivalent of ANOVA, the Kruskal-Wallis tests, followed by pairwise comparison by Mann-Whitney, using Minitab Version 13.1. The statistical findings are tabulated in Appendices I to V. Excel 2002 was used to present raw data, calculate mean readings, standard deviation and standard error, and to present these data graphically.

Chapter Three

Results

3.1 Introduction

Growth curves of S180 sarcoma cells grown in DMEM growth medium on their own, and after the addition of ethanolic molmol and hexane haddi extracts, are analysed in this chapter. The growth curves of HT1080 grown alone, and after the addition of ethanolic molmol and hexane haddi extracts, are also analysed. The results for the MTT viability tests for the above are also illustrated, and the results of further parallel experiments, for example morphological techniques, electron microscopy and flow cytometry, are also presented here.

The S180 sarcoma and HT1080 fibrosarcoma cell lines were chosen as they are representative of cancerous cells that can adhere to the walls of the containers they are kept in (see Chapter Two), and can be easily recovered and counted. Therefore, they were suitable for the purposes of the present study.

A normal chick fibroblast cell line was selected as representative of normal, non-cancerous cells that have the capability to divide. The effects of the extracts of the two experimental plants (*Commiphora molmol* and *C. guidotti*) were studied on both cancerous and non-cancerous cells in order to compare these effects on both cell types.

An increase in the number of cells in a proliferating cell line can be used as an index of normal behaviour, and growth curves can be determined.

3.2 S180 Growth Assay

The S180 cells were found to grow exponentially, reaching their maximum cell number (2,289,583±51,054) on Day 6 (see Figure 3.1). The growth then started declining exponentially, reaching an average total of 1,573,333 on Day 8. The initial seeding density of S180 cells at the start of the experiment (Day 0) was 4×10^4.

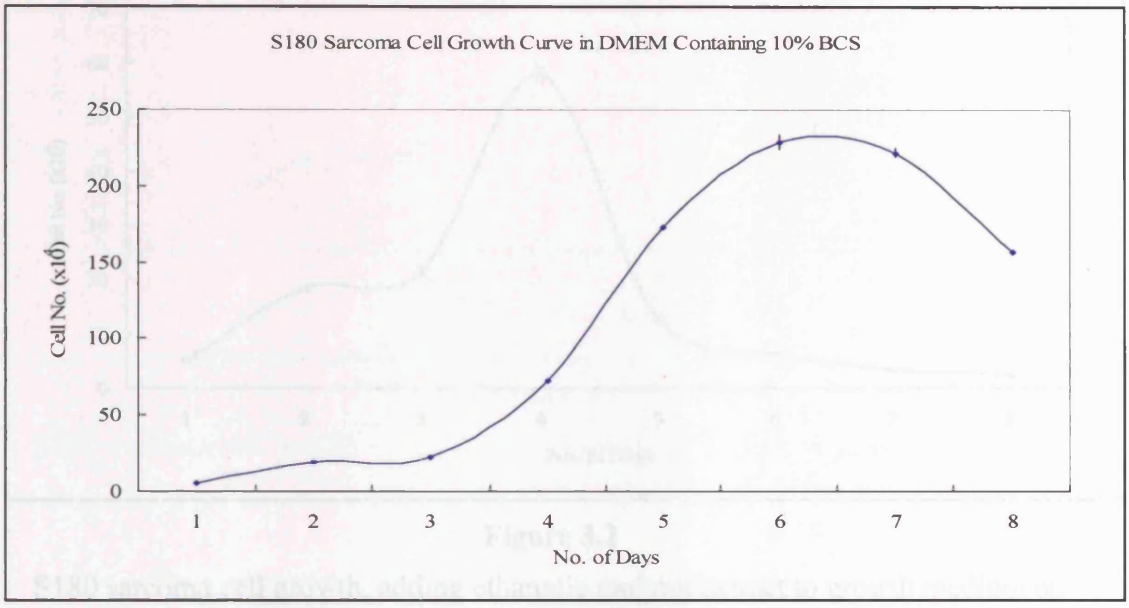


Figure 3.1

S180 sarcoma cell growth in control medium (n=9, bars=standard error) (K-W test, $p<0.001$).

S180 cells were left to grow in the medium for four days, upon which (Day 4) ethanolic molmol extract and hexane haddi extract were added to the growing cells. The growth in both extracts declined sharply ($p<0.001$) (see Figures 3.2, 3.3 and 3.4).

The experiment using S180 cells and ethanolic molmol extract lasted for eight days. Three experiments were carried out, each with three replicates, hence a total of nine trials were attempted, and the average cell number represents the average of nine trials. A similar experimental set-up was run, using hexane haddi extract instead of ethanolic molmol extract. In the case of adding ethanolic molmol extract on Day 4

(Figure 3.2), the cell number dropped from an average of 579,900±12,584 cells on Day 4 to 129,400±4,760 cells on Day 5, declining to 25,300±1,229 cells on Day 8 ($p<0.001$). This illustrated the cytotoxic action of molmol extract on S180 sarcoma cells.

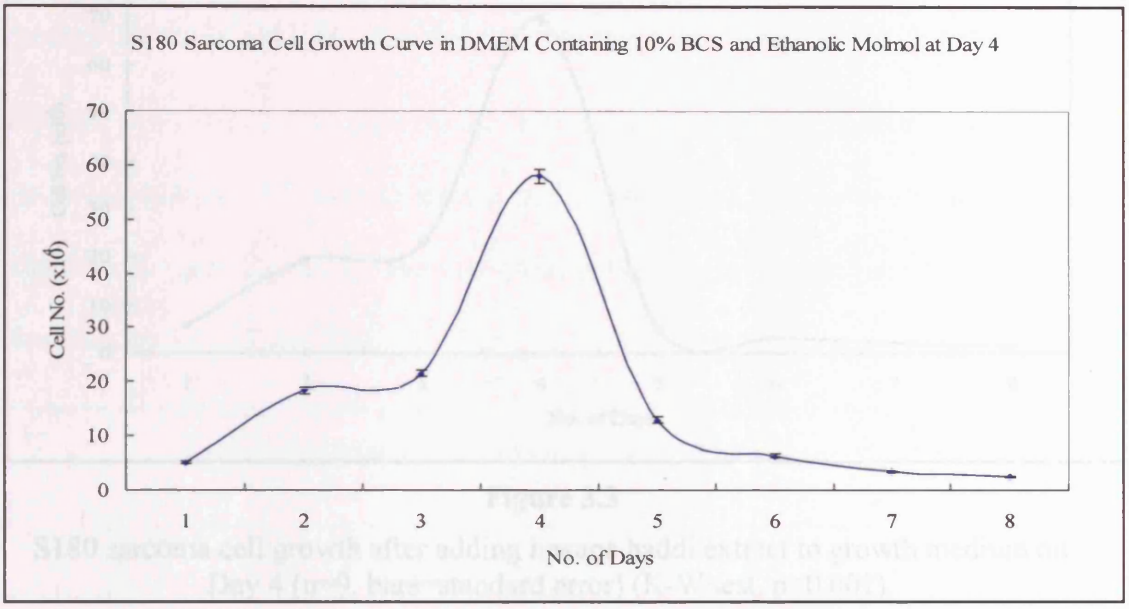


Figure 3.2

S180 sarcoma cell growth, adding ethanolic molmol extract to growth medium on Day 4 (n=9, bars=standard error) (K-W test, $p<0.001$).

In the case of adding hexane haddi extract on Day 4 (Figure 3.3), the cell number dropped from an average of 695,000±11,659 cells on Day 4 to 49,444±3,651 cells on Day 5, declining to 15,833±1,021 cells on Day 8. This pattern of rapid decline in cell number also illustrates the rapid action of haddi extract in killing S180 sarcoma cells.

Figure 3.4 shows the effect of adding ethanolic molmol and hexane haddi extracts on the growth of S180 sarcoma cells, compared with their growth without adding them. Figure 3.4 also shows that the growth of S180 sarcoma cells during the first four days of the experiment, that is, before the addition of the plant extracts, was very similar in all experiments.

Kruskal-Wallis test shows that there are significant differences between the results of the control and those of adding experimental plant extracts ($p<0.001$).

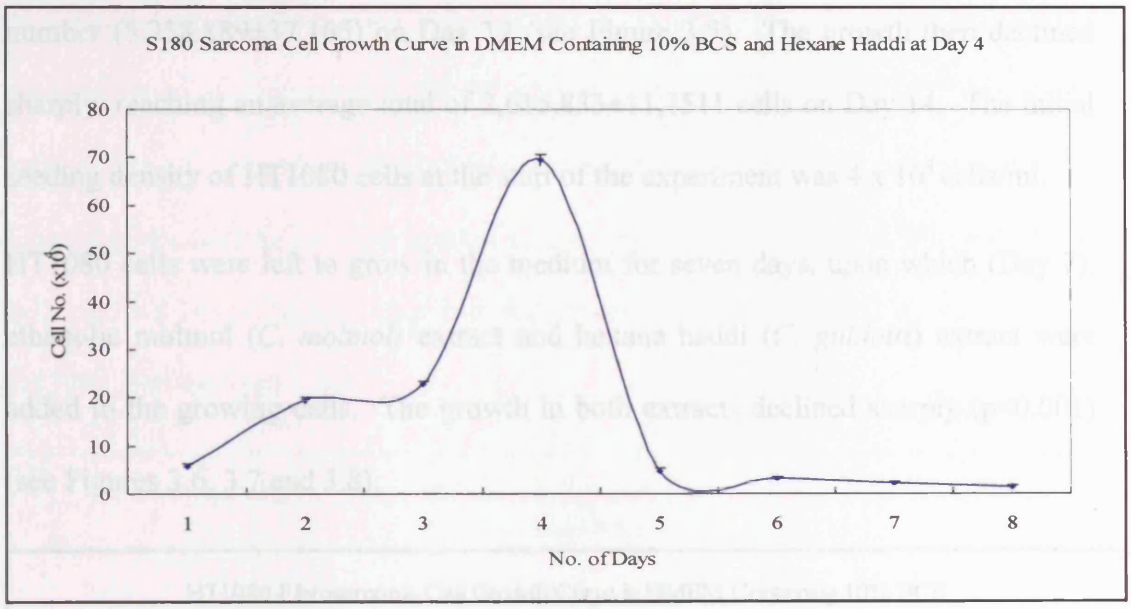


Figure 3.3

S180 sarcoma cell growth after adding hexane haddi extract to growth medium on Day 4 (n=9, bars=standard error) (K-W test, $p<0.001$).

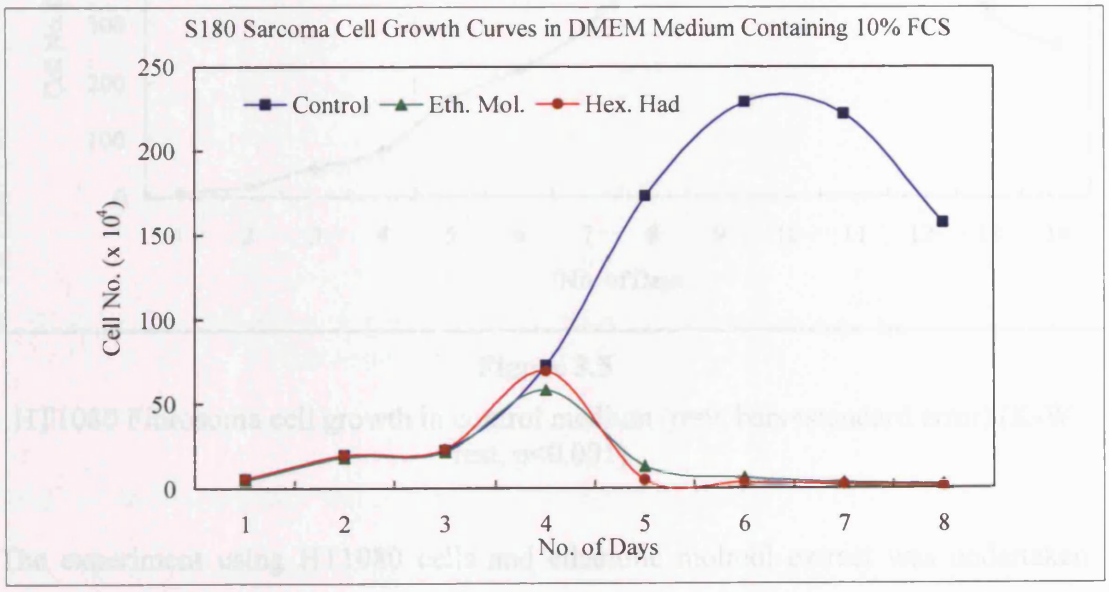


Figure 3.4

Comparison of growth curves of S180 cells with and without addition of molmol and haddi extracts to growth medium.

3.3 HT1080 Growth Assay

The HT1080 cells were found to grow exponentially, reaching their maximum cell number (5,258,889±37,165) on Day 12 (see Figure 3.5). The growth then declined sharply, reaching an average total of 2,615,833±11,1511 cells on Day 14. The initial seeding density of HT1080 cells at the start of the experiment was 4 x 10^4 cells/ml.

HT1080 cells were left to grow in the medium for seven days, upon which (Day 7), ethanolic molmol (*C. molmol)* extract and hexane haddi (*C. guidotti*) extract were added to the growing cells. The growth in both extracts declined sharply ($p<0.001$) (see Figures 3.6, 3.7 and 3.8).

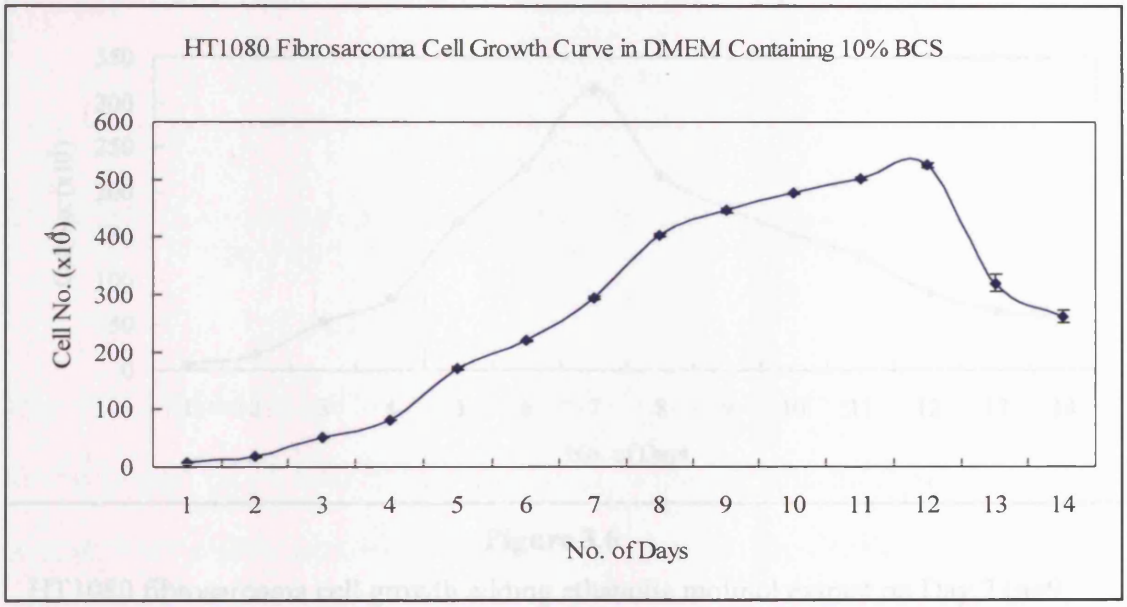


Figure 3.5

HT1080 Fibrosoma cell growth in control medium (n=9, bars=standard error) (K-W test, $p<0.001$).

The experiment using HT1080 cells and ethanolic molmol extract was undertaken over a period of 14 days. Three experiments were carried out, each with three replicates, that is, a total of nine trials were attempted, and the average cell number

represents the average of nine trials. A similar experimental set-up was also undertaken using hexane haddi extract instead of ethanolic molmol extract.

In the case of adding ethanolic haddi extract on Day 7 (Figure 3.6), the cell number dropped from an average of 3,158,333±22,197 cells on Day 7 to 2,187,778±12,849 cells on Day 8. The sharp decline in the number of HT1080 cells continued for the remaining six days of the experimental period, reaching a low count of 596,667±7.784 cells on Day 14 ($p<0.001$). This is an indication that molmol extract is cytototoxic to HT1080 fibrosarcoma cells.

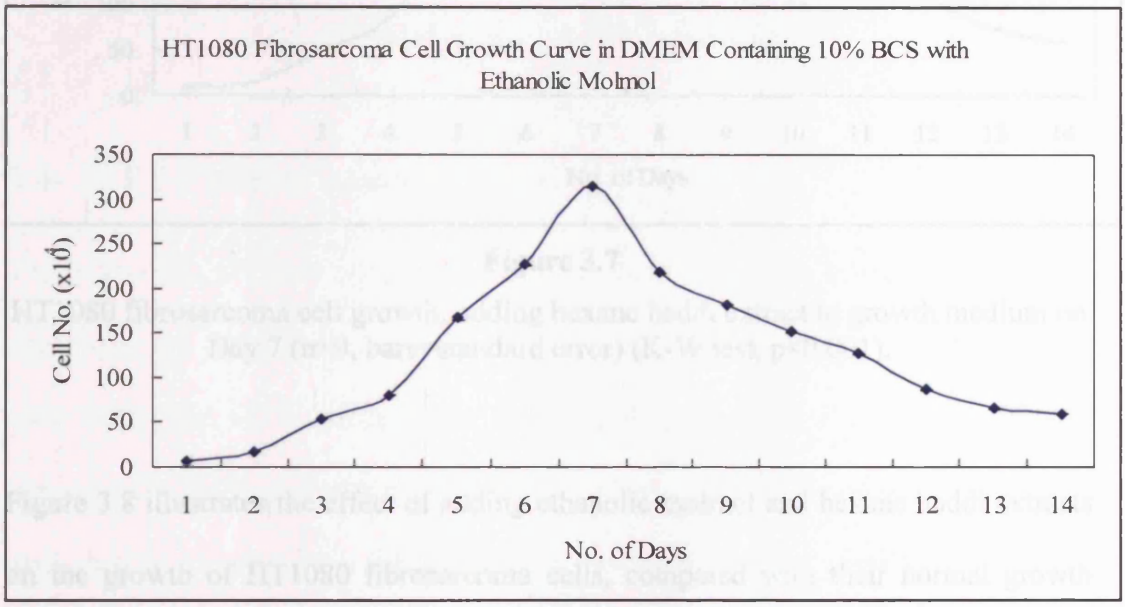


Figure 3.6

HT1080 fibrosarcoma cell growth adding ethanolic molmol extract on Day 7 (n=9, bars=standard error) (K-W test, $p<0.001$).

Prior to adding hexane haddi extract to medium containing HT1080 fibrosarcoma cells, the cells were left to grow exponentially for seven days, on which day the hexane extract of haddi was added (Figure 3.7). The cell number then declined on Day 8 to 2,688,444±22,079 cells from 2,970,833±10,467 cells Day 7. The drop in the number of HT1080 cells continued to the end of the experiment, when a total number

of 508,556±6,572 cells were counted. This pattern of rapid decline in cell number also illustrates the rapid action of haddi extract in killing the HT1080 fibrosarcoma cells.

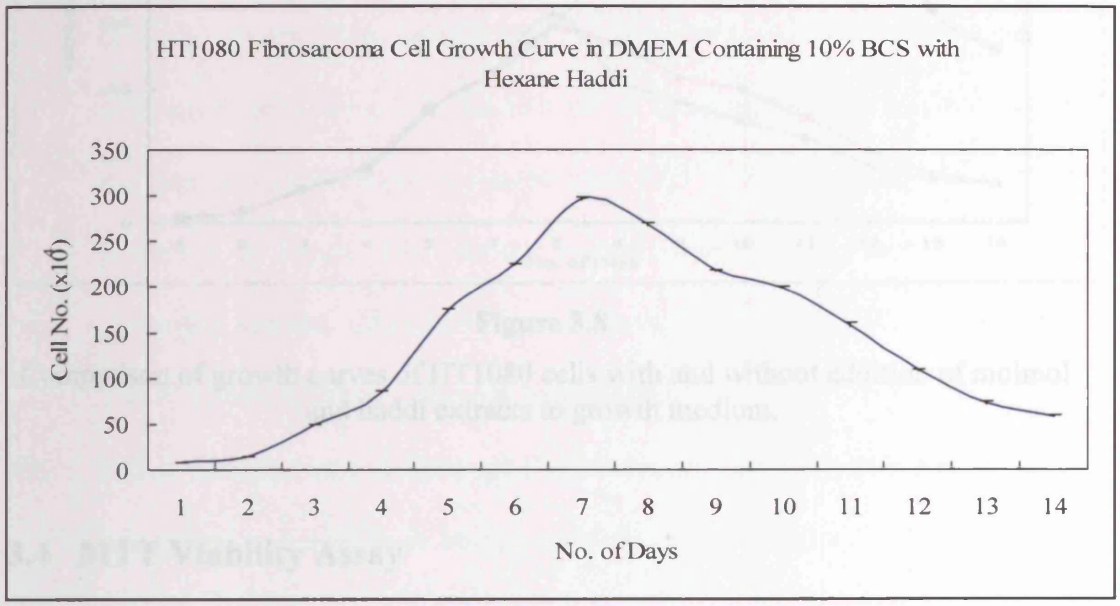


Figure 3.7

HT1080 fibrosarcoma cell growth, adding hexane haddi extract to growth medium on Day 7 (n=9, bars=standard error) (K-W test, $p<0.001$).

Figure 3.8 illustrates the effect of adding ethanolic molmol and hexane haddi extracts on the growth of HT1080 fibrosarcoma cells, compared with their normal growth without adding these extracts (control). Figure 3.8 also shows that the growth of HT1080 cells during the first seven days of the experiment, that is, before the addition of either ethanolic molmol or hexane haddi extracts, was very similar in all experiments.

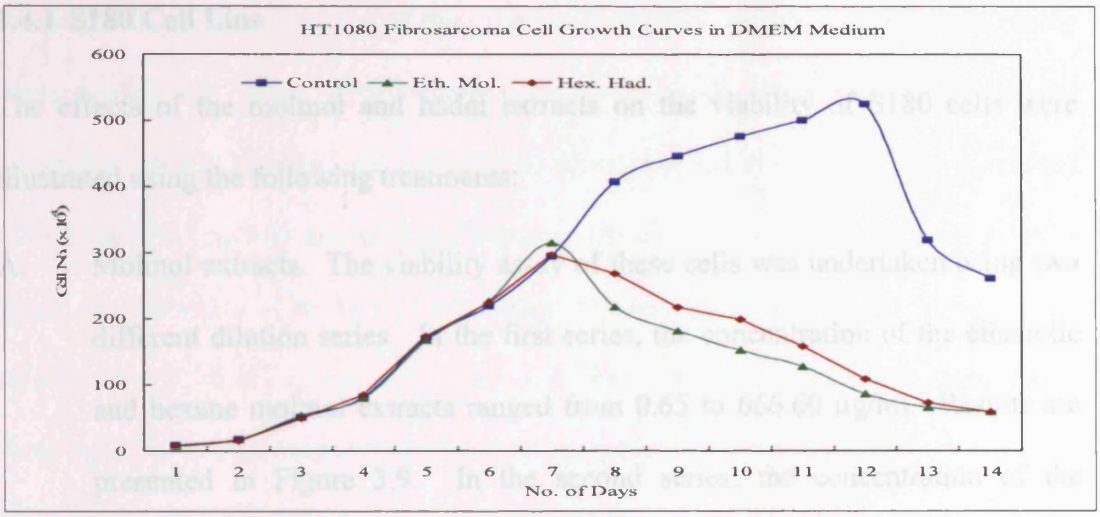


Figure 3.8

Comparison of growth curves of HT1080 cells with and without addition of molmol and haddi extracts to growth medium.

3.4 MTT Viability Assay

The S180 cells, HT1080 cells and normal fibroblast cells were treated with different concentrations of molmol and haddi extracts over a period of 24, 48 and 72 hours, after which the MTT solution was added to the growth medium to measure the percentage viability to establish viability. Calculation of percentage viability is explained in Chapter Two (Section 2.9.3). Mean percentage viabilities have been plotted against control and concentrations used 24, 48 and 72 hours after treatment, to illustrate whether or not the addition of the various extracts at various concentrations may have any effect on the growth of the S180, HT1080 and normal fibroblast cells. All experiments were conducted against a control consisting of the cells and the growth medium.

3.4.1 S180 Cell Line

The effects of the molmol and haddi extracts on the viability of S180 cells were illustrated using the following treatments:

A. Molmol extracts. The viability assay of these cells was undertaken using two different dilution series. In the first series, the concentration of the ethanolic and hexane molmol extracts ranged from 0.65 to 666.60 μg/ml. Results are presented in Figure 3.9. In the second series, the concentration of the ethanolic, hexane, ethyl acetate and Flavex molmol extracts ranged from 266.45 to 666.60 μg/ml. Results are presented in Figures 3.10 to 3.14.

B. Haddi extracts. The viability of S180 cells was tested in ethanolic, hexane, ethyl acetate and Aldrich hexane extracts, also in two series as for molmol extracts. Results are illustrated in Figures 3.15 to 3.20.

A. Molmol Extracts

A.1 Ethanolic Molmol Extract (0.65-666.60 μg/ml)

Figure 3.9 clearly shows that the lowest viability percentage was obtained using ethanolic molmol extract at 666.60 μg/ml concentration, thus indicating low survival rates, or high toxicity rates of S180 cells at this concentration. In contrast, higher mean viability than the control was obtained using the extract at 10.42 and 0.65 μg/ml concentrations ($P<0.001$), indicating that there were more viable cells in these two treatments than in the control. This suggests that these two concentrations are low enough not to have any impact on the cells, rather, growth or mitogenecity seemed to be enhanced at these two low levels of molmol. The percentage viability results (Figure 3.9) substantiate this trend, showing higher percentage viability figures than the control, that is, more cell survival at these two concentrations of molmol than in

the control. Figure 3.9 also shows that cell viability decreased with time, being highest after 72 hours of treatment, particularly at the highest concentration of ethanolic molmol extract (666.60 µg/ml). However, the two lowest concentrations (10.42 and 0.65 µg/ml) did not have any toxic effects on the S180 cells, even after 24 hours of treatment. The 83.33 µg/ml concentration level had little or insignificant effect of the S180 cells, especially after 24 hours of treatment. The data show that at these two low extract concentrations a mitogenic effect can be obtained.

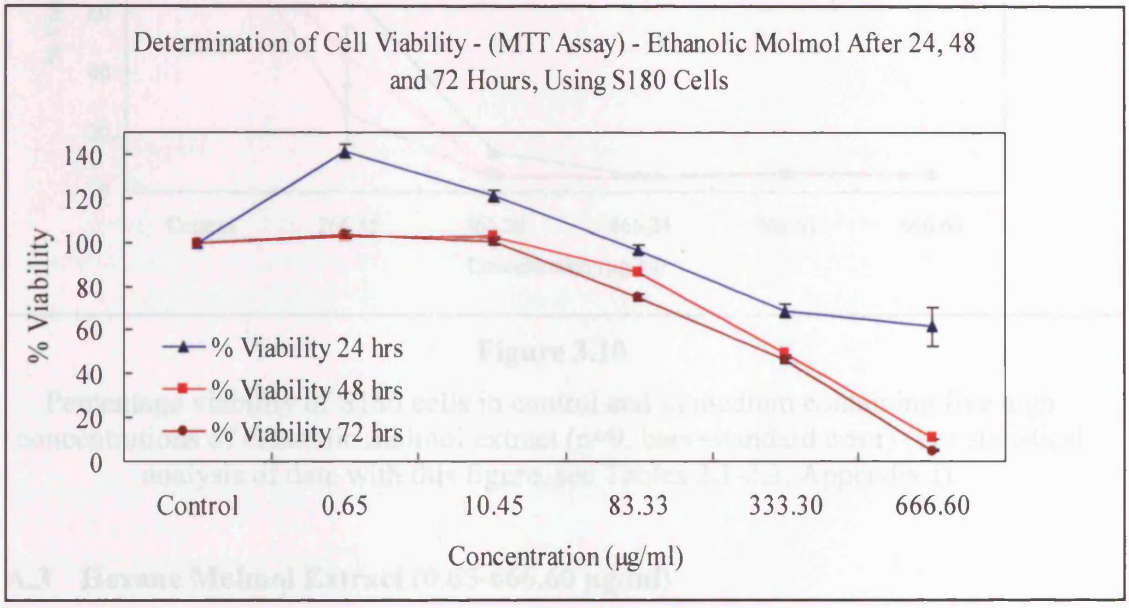


Figure 3.9

Percentage viability of S180 cells in control and in medium containing five different concentrations of ethanolic molmol extract (n=9, bars=standard error) (For statistical analysis of data with this figure, see Tables 1.1-1.3, Appendix I).

A2 Ethanolic Molmol Extract (266.45-666.65 µg/ml)

In these treatments, adding high concentrations of ethanolic molmol shows that this range of concentrations was effective in killing S180 cells, particularly the three highest concentrations of 666.60, 566.61 and 466.24 µg/ml, which resulted in almost 5% viability within 24 hours of treatment (see Figure 3.10) ($P<0.001$). Lowest viability of S180 cells treated with 366.00 µg/ml ethanolic molmol was attained after

72 hours of treatment. However, treatment of S180 cells with 266.45 µg/ml was less effective than the other higher concentrations, resulting in about 74%, 52% and 30% cell viability after 24, 48 and 72 hours, respectively.

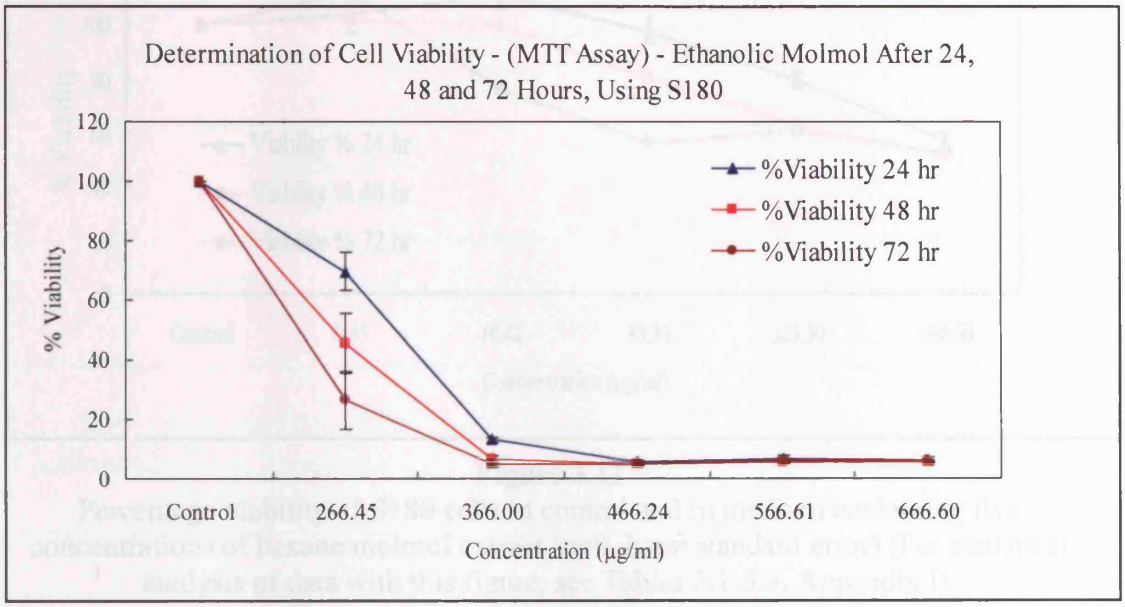


Figure 3.10

Percentage viability of S180 cells in control and in medium containing five high concentrations of ethanolic molmol extract (n=9, bars=standard error) (For statistical analysis of data with this figure, see Tables 2.1-2.3, Appendix I).

A.3 Hexane Molmol Extract (0.65-666.60 µg/ml)

Hexane molmol extract using this range of concentrations was not effective in its toxicity, compared to ethanolic molmol extract of similar concentrations (Figure 3.11). In fact, at 10.42 µg/ml concentration of hexane molmol, growth of S180 cells was enhanced (mitogenetic effect) after 24 and 48 hours, as illustrated by higher percentage viability than the control, but showed some effect after 72 hours, when about 77% viability was calculated. At 0.65 µg/ml concentration, growth was enhanced throughout the experimental period, and no toxicity was detected in the S180 cell.

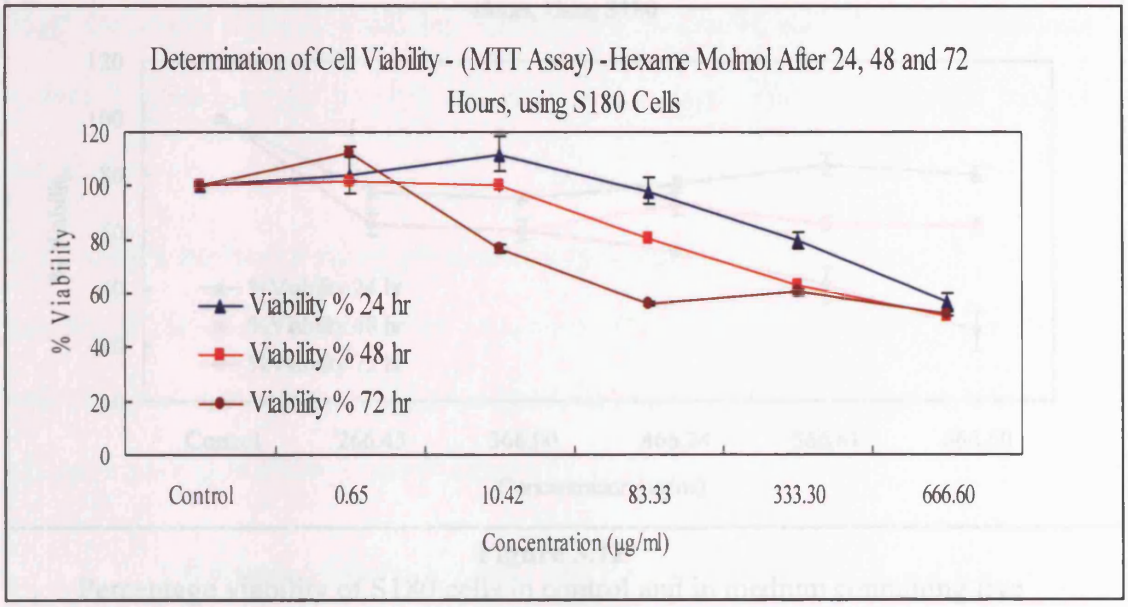


Figure 3.11

Percentage viability of S180 cells in control and in medium containing five concentrations of hexane molmol extract (n=9, bars=standard error) (For statistical analysis of data with this figure, see Tables 3.1-3.3, Appendix I).

A.4 Hexane Molmol Extract (266.45-666.60 µg/ml)

Hexane molmol extract was less effective in reducing the viability of S180 cells than ethanolic molmol extract using a similar concentration range. Addition of 666.60 µg/ml hexane molmol extract produced the lowest percentage viability (about 25%) after 72 hours (Figure 3.12).

A.5 Ethyl Acetate Molmol Extract (266.45-666.60 µg/ml)

Treatment of S180 cells with ethyl acetate molmol extract (with the same concentration ranges as those of ethanolic and hexane extract) (Figure 3.13) shows that the lowest concentration (266.45 µg/ml) was also not as effective as the other concentrations, only resulting in about 69%, 46% and 12% cell viability after 24, 48 and 72 hours, respectively.

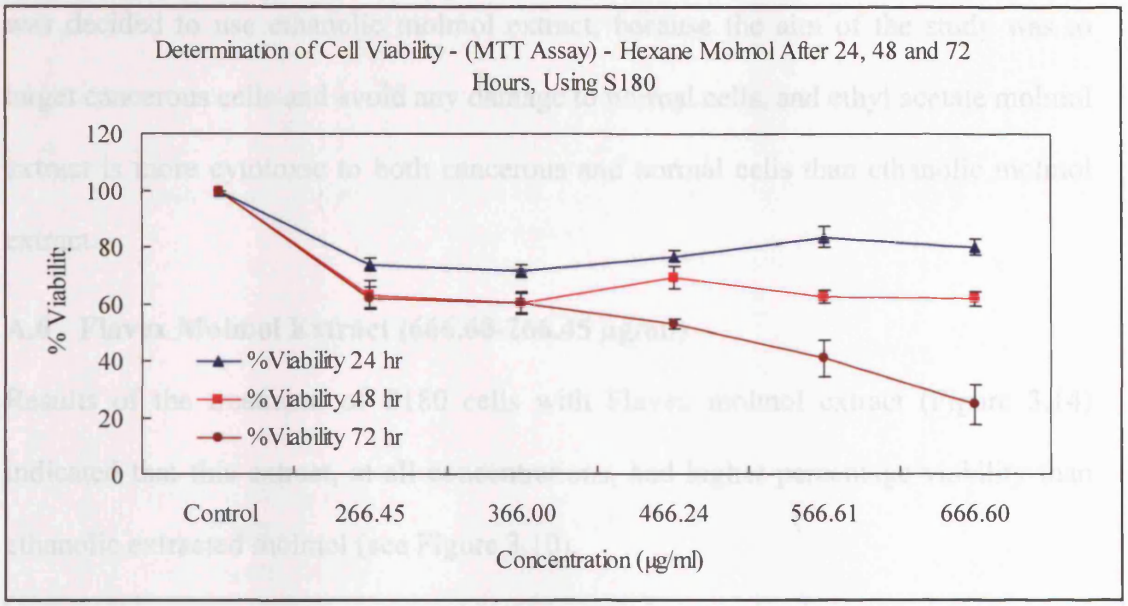


Figure 3.12

Percentage viability of S180 cells in control and in medium containing five concentrations of hexane molmol extract (n=9, bars=standard error) (For statistical analysis of data with this figure, see Tables 4.1-4.3, Appendix I).

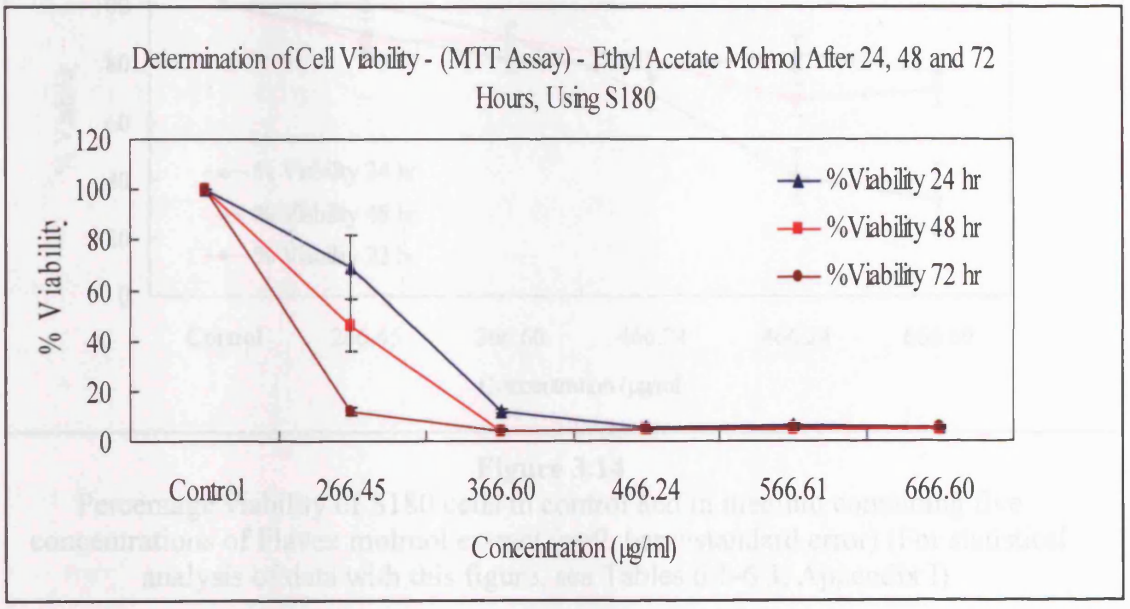


Figure 3.13

Percentage viability of S180 cells in control and in medium containing five concentrations of ethyl acetate molmol extract (n=9, bars=standard error) (For statistical analysis of data with this figure, see Tables 5.1-5.3, Appendix I)

It is noted that the ethyl acetate molmol extract resulted in lower percentage viability of the S180 cells than did ethanolic molmol extract, using similar concentrations ($P<0.001$). Similar observations were made regarding normal fibroblast cells treated with ethyl acetate and ethanolic molmol acetate (see Figure 3.40). Accordingly, it

was decided to use ethanolic molmol extract, because the aim of the study was to target cancerous cells and avoid any damage to normal cells, and ethyl acetate molmol extract is more cytotoxic to both cancerous and normal cells than ethanolic molmol extract.

A.6 Flavex Molmol Extract (666.60-266.45 µg/ml)

Results of the treatment of S180 cells with Flavex molmol extract (Figure 3.14) indicated that this extract, at all concentrations, had higher percentage viability than ethanolic extracted molmol (see Figure 3.10).

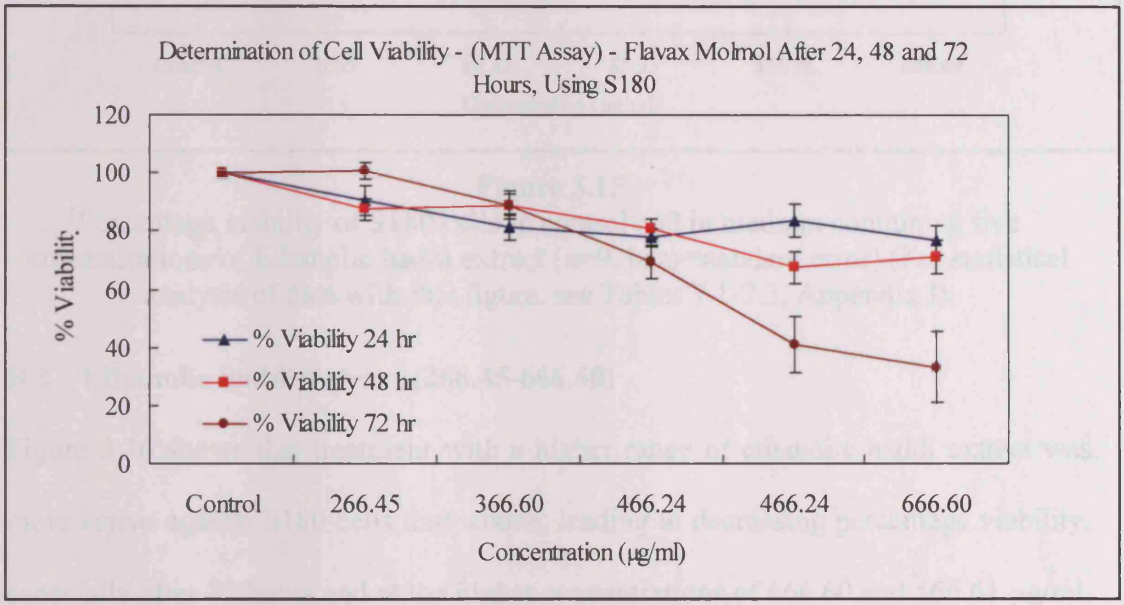


Figure 3.14
Percentage viability of S180 cells in control and in medium containing five concentrations of Flavex molmol extract (n=9, bars=standard error) (For statistical analysis of data with this figure, see Tables 6.1-6.3, Appendix I).

B. Haddi Extracts

B.1 Ethanolic Haddi Extract (0.65-666.6 µg/ml)

Figure 3.15 clearly demonstrates that lower concentrations of ethanolic haddi extract were not very active against S180 cells. These higher means than the control indicate growth promotion, as evidenced by higher percentage viability than in the control, that is, an increased mitogenesis has occurred at these low concentrations, resulting in

more viable cells under these treatments than in the control. The lowest percentage viability was about 68% after 72 hours, and at the highest concentration.

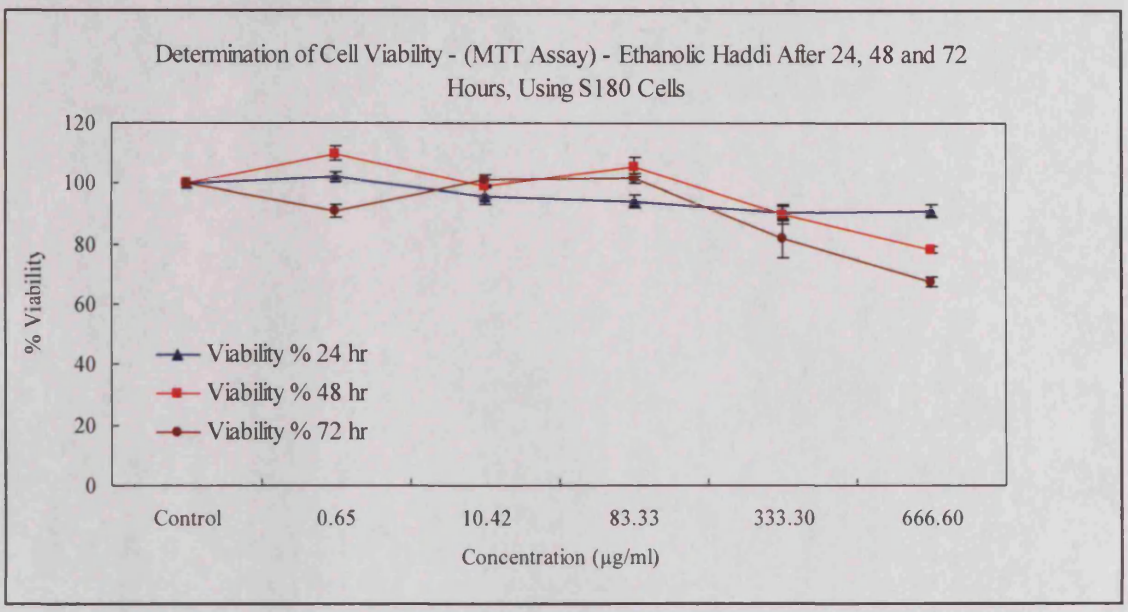


Figure 3.15
Percentage viability of S180 cells in control and in medium containing five concentrations of Ethanolic haddi extract (n=9, bars=standard error) (For statistical analysis of data with this figure, see Tables 7.1-7.3, Appendix I).

B.2 Ethanolic haddi Extract (266.45-666.60)

Figure 3.16 shows that treatment with a higher range of ethanolic haddi extract was more active against S180 cells than above, leading to decreasing percentage viability, especially after 72 hours and at the higher concentrations of 666.60 and 566.61 µg/ml. It is also noted that the results of the highest concentration did not match that used in this experiment, possibly due to different experimental batches of the plant extracts.

B.3 Hexane Haddi Extract (0.65-666.6 µg/ml)

Hexane haddi extract (Figure 3.17) performed better than ethanolic haddi extract, even at the lower end of the concentration spectrum (Figure 3.15) ($P<0.001$), except after 72 hours of treatment with 0.65 µg/ml concentration treatment, where some S180 cell growth led to increased percentage viability than in the control, that is, increased mitogenesis after 72 hours of exposure to the extract (Figure 3.17).

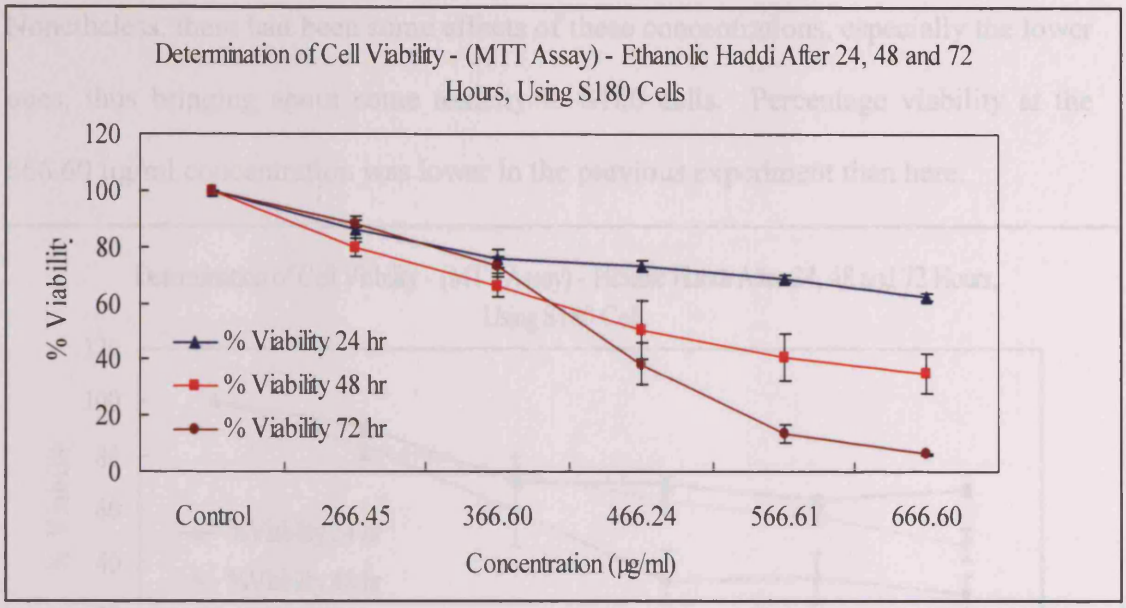


Figure 3.16

Percentage viability of S180 cells in control and in medium containing five concentrations of ethanolic haddi extract (n=9, bars=standard error) (For statistical analysis of data with this figure, see Tables 8.1-8.3, Appendix I).

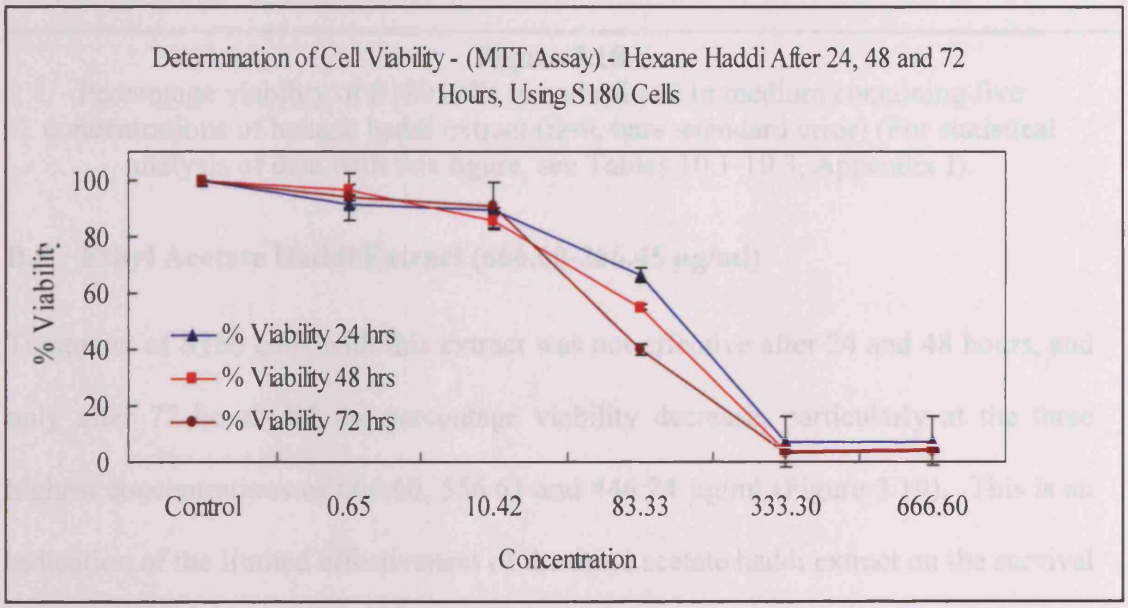


Figure 3.17

Percentage viability of S180 cells in control and in medium containing five concentrations of hexane haddi extract (n=9, bars=standard error) (For statistical analysis of data with this figure, see Tables 9.1-9.3, Appendix I).

B.4 Hexane Haddi Extract (266.45-666.60 µg/ml)

Results obtained (Figure 3.18) clearly show that increasing the concentration of the hexane haddi extracts seemed not effective in decreasing percentage viability.

Nonetheless, there had been some effects of these concentrations, especially the lower ones, thus bringing about some toxicity to S180 cells. Percentage viability at the 666.60 µg/ml concentration was lower in the previous experiment than here.

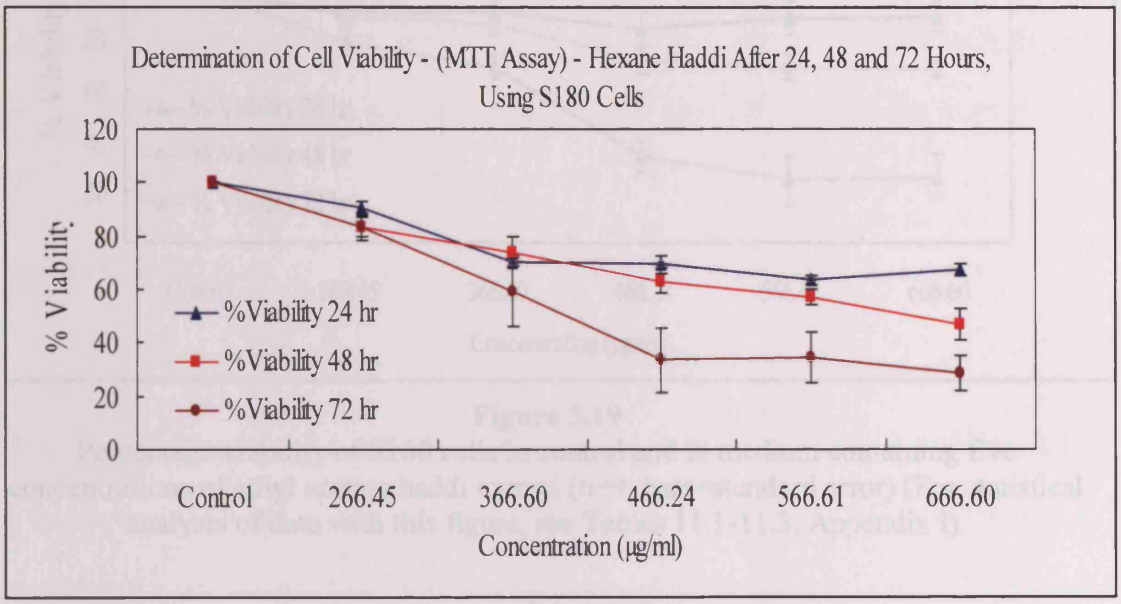


Figure 3.18
Percentage viability of S180 cells in control and in medium containing five concentrations of hexane haddi extract (n=9, bars=standard error) (For statistical analysis of data with this figure, see Tables 10.1-10.3, Appendix I).

B.5 Ethyl Acetate Haddi Extract (666.60-266.45 µg/ml)

Treatment of S180 cells with this extract was not effective after 24 and 48 hours, and only after 72 hours did the percentage viability decrease, particularly at the three highest concentrations of 666.60, 556.61 and 446.24 µg/ml (Figure 3.19). This is an indication of the limited effectiveness of the ethyl acetate haddi extract on the survival of the S180 cells, compared to ethyl acetate molmol (see Figure 3.13).

B.6 Haddi Essential Oil (Aldrich) Extract (266.45-666.60 µg/ml)

Haddi essential oil (Aldrich) extract was also not very effective in bringing about low percentage viability (Figure 3.20). Percentage viability values were around 94% at the lower concentration. Nonetheless, some toxicity was noted in all concentrations used.

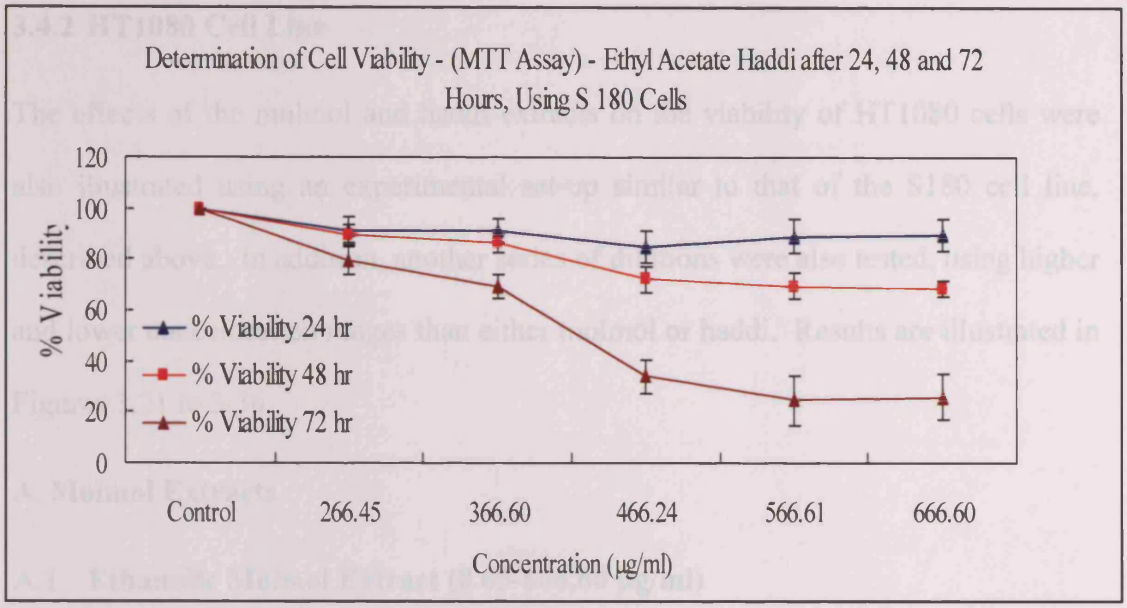


Figure 3.19

Percentage viability of S180 cells in control and in medium containing five concentrations of ethyl acetate haddi extract (n=9, bars=standard error) (For statistical analysis of data with this figure, see Tables 11.1-11.3, Appendix I).

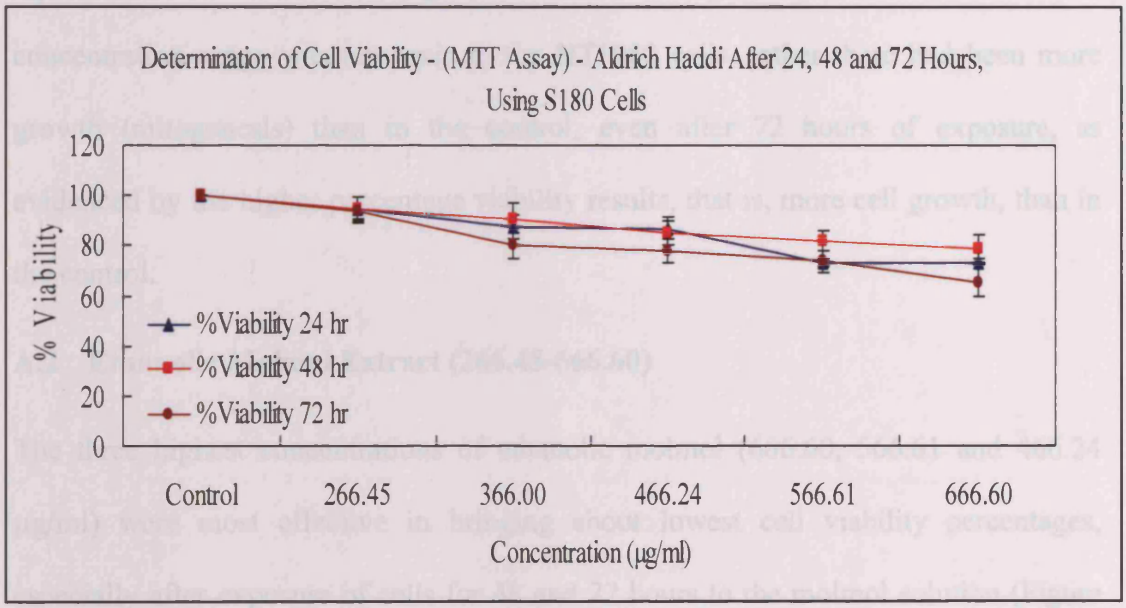


Figure 3.20

Percentage viability of S180 cells in control and in medium containing five concentrations of Aldrich hexane haddi extract (n=9, bars=standard error) (For statistical analysis of data with this figure, see Tables 12.1-12.3, Appendix I).

3.4.2 HT1080 Cell Line

The effects of the molmol and haddi extracts on the viability of HT1080 cells were also illustrated using an experimental set-up similar to that of the S180 cell line, described above. In addition, another series of dilutions were also tested, using higher and lower concentration ranges than either molmol or haddi. Results are illustrated in Figures 3.21 to 3.36.

A. Molmol Extracts

A.1 Ethanolic Molmol Extract (0.65-666.60 μg/ml)

Using this concentration range resulted in the lowest percentage viability after exposure to the highest concentration (666.60 μg/ml) (Figure 3.21), similar to that reported for the 666.60-266.6 μg/ml concentration range (see A2 below). The lower concentration range was not toxic to the HT1080 cells, rather there had been more growth (mitogenesis) than in the control, even after 72 hours of exposure, as evidenced by the higher percentage viability results, that is, more cell growth, than in the control.

A.2 Ethanolic Molmol Extract (266.45-666.60)

The three highest concentrations of ethanolic molmol (666.60, 566.61 and 466.24 μg/ml) were most effective in bringing about lowest cell viability percentages, especially after exposure of cells for 48 and 72 hours to the molmol solution (Figure 3.22) ($P<0.001$). The two lower concentrations were less toxic to the HT1080 cells, especially after a short period of exposure (24 hours).

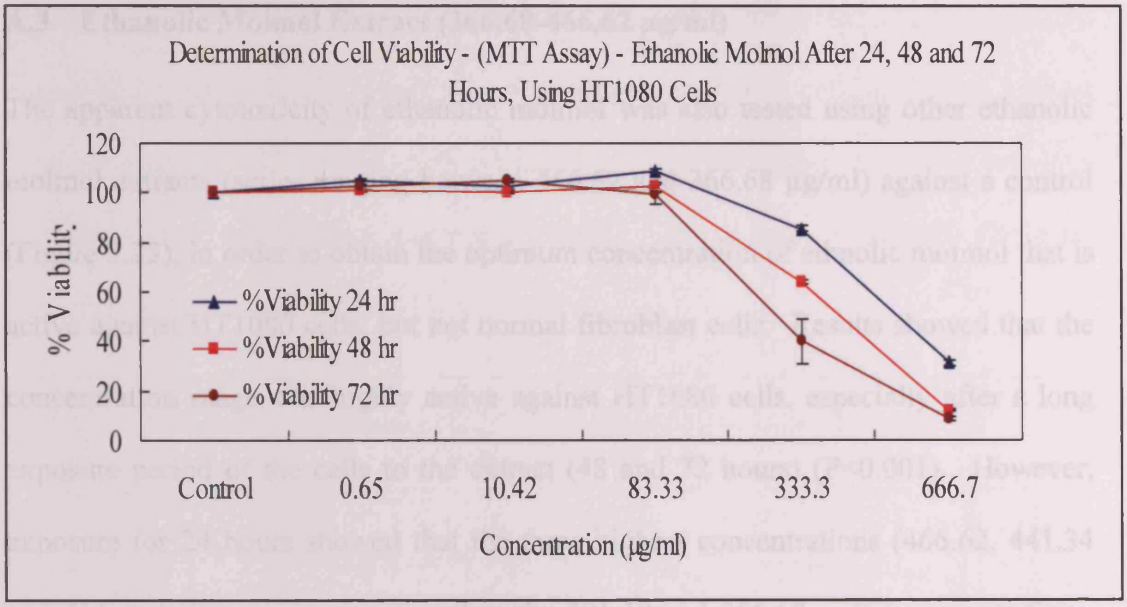


Figure 3.21

Percentage viability of HT1080 cells in control and in medium containing five concentrations of ethanolic molmol extract (n=9, bars=standard error) (For statistical analysis of data with this figure, see Tables 1.1-1.3, Appendix II).

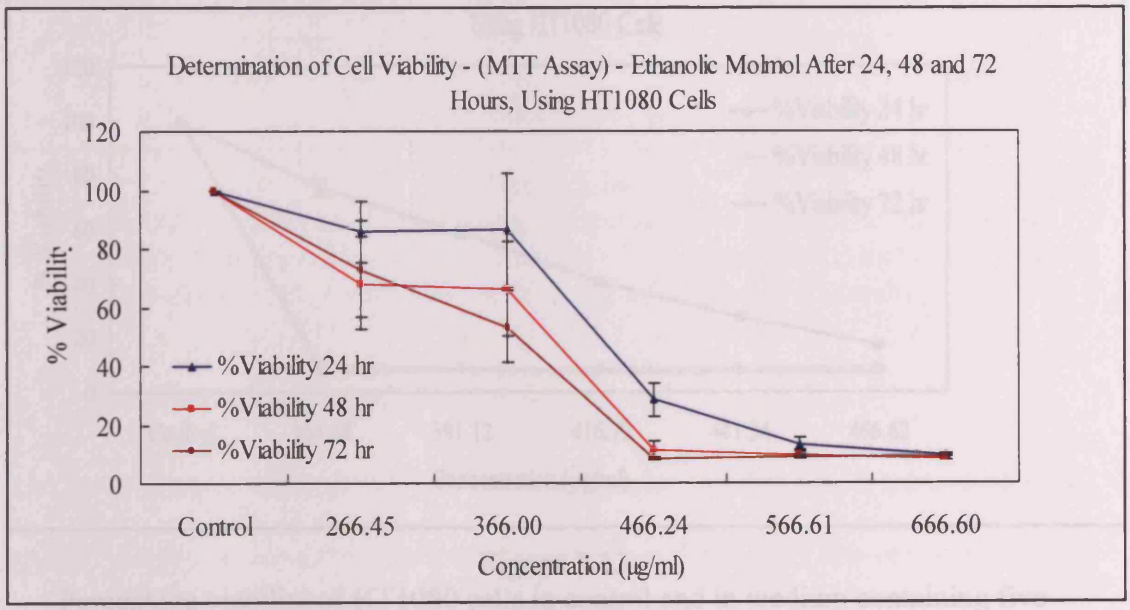


Figure 3.22

Percentage viability of HT1080 cells in control and in medium containing five concentrations of ethanolic molmol extract (n=9, bars=standard error) (For statistical analysis of data with this figure, see Tables 2.1-2.3, Appendix II).

A.3 Ethanolic Molmol Extract (366.68-466.62 µg/ml)

The apparent cytotoxicity of ethanolic molmol was also tested using other ethanolic molmol extracts (series ranging between 466.62 and 366.68 µg/ml) against a control (Figure 3.23), in order to obtain the optimum concentration of ethnolic molmol that is active against HT1080 cells, but not normal fibroblast cells. Results showed that the concentration range was highly active against HT1080 cells, especially after a long exposure period of the cells to the extract (48 and 72 hours) ($P<0.001$). However, exposure for 24 hours showed that the three highest concentrations (466.62, 441.34 and 416 µg/ml) were more active than the 391.12 and 366.68 µg/ml concentrations, resulting in about 17% to 40% cell viability.

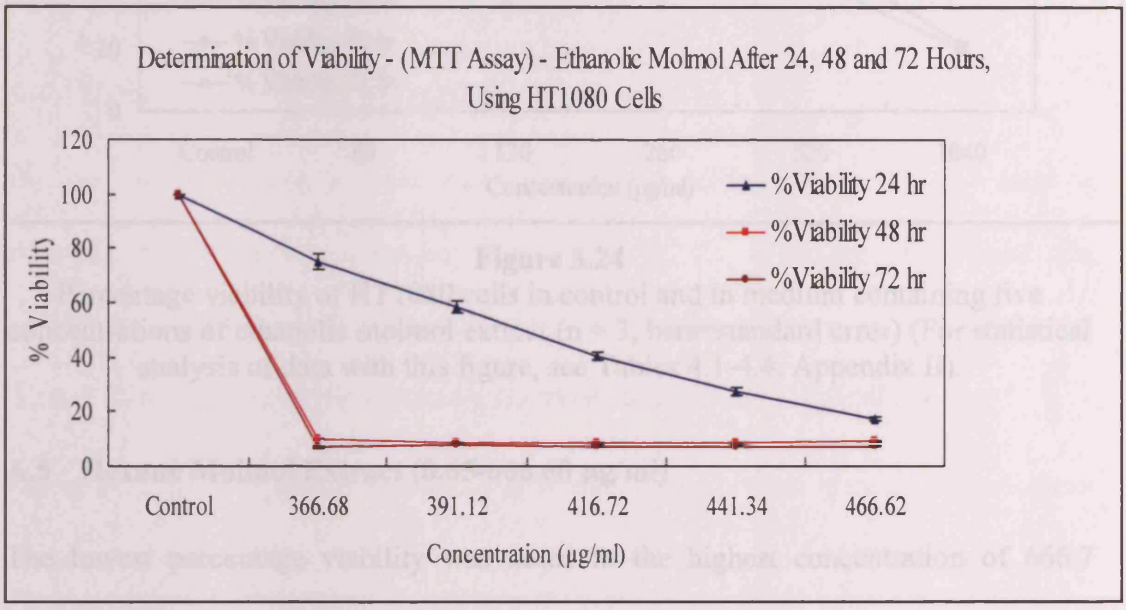


Figure 3.23
Percentage viability of HT1080 cells in control and in medium containing five concentrations of ethanolic molmol extract (n=9, bars=standard error) (For statistical analysis of data with this figure, see Tables 3.1-3.3, Appendix II).

A.4 Ethanolic Molmol Extract (65-1040 µg/ml)

Here, a wider range of concentrations was used as a preliminary exercise. Results for this ethanolic molmol concentration range were recorded after subjecting HT1080

cells to 12, 24, 48 and 72 hours of exposure. Figure 3.24 shows that ethanolic molmol extract at this concentration range was active against HT1080 cells at the two highest concentrations of 1040 and 520 µg/ml, which were higher than the previous concentrations. Nonetheless, percentage viability at the lower end of the spectrum was very high, around 95% to 97% viability.

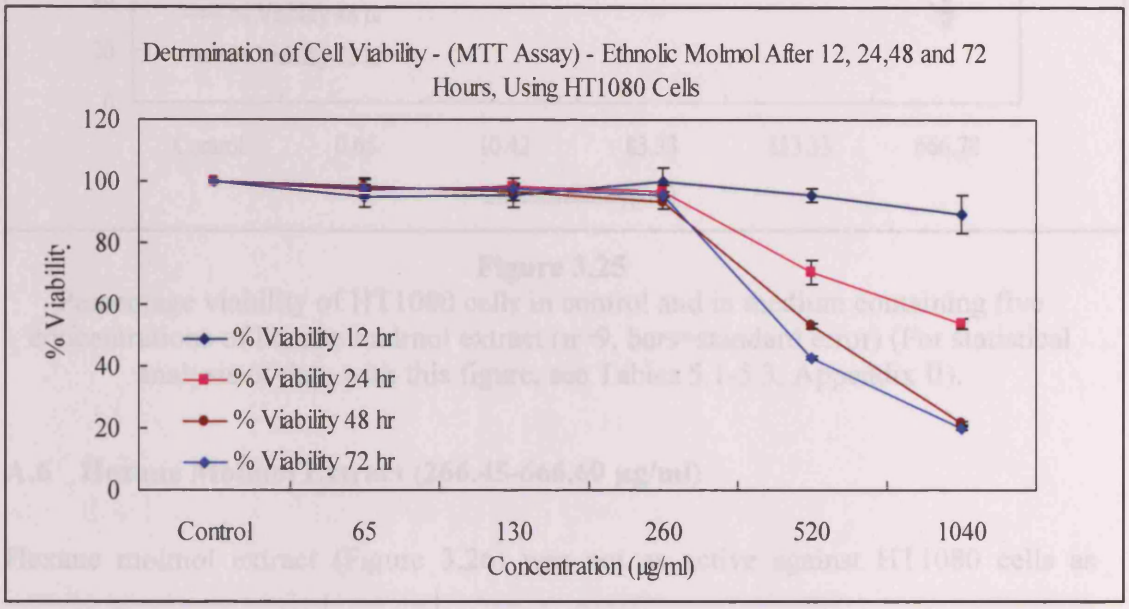


Figure 3.24
Percentage viability of HT1080 cells in control and in medium containing five concentrations of ethanolic molmol extract (n = 3, bars=standard error) (For statistical analysis of data with this figure, see Tables 4.1-4.4, Appendix II).

A.5 Hexane Molmol Extract (0.65-666.60 µg/ml)

The lowest percentage viability was noted in the highest concentration of 666.7 µg/ml. In the lower concentration, it seems that the extract has enhanced growth (mitogenesis), particularly at the end of the experiment, when higher percentage viability values, that is, growth and survival of cells, than in the control were noted (Figure 3.25).

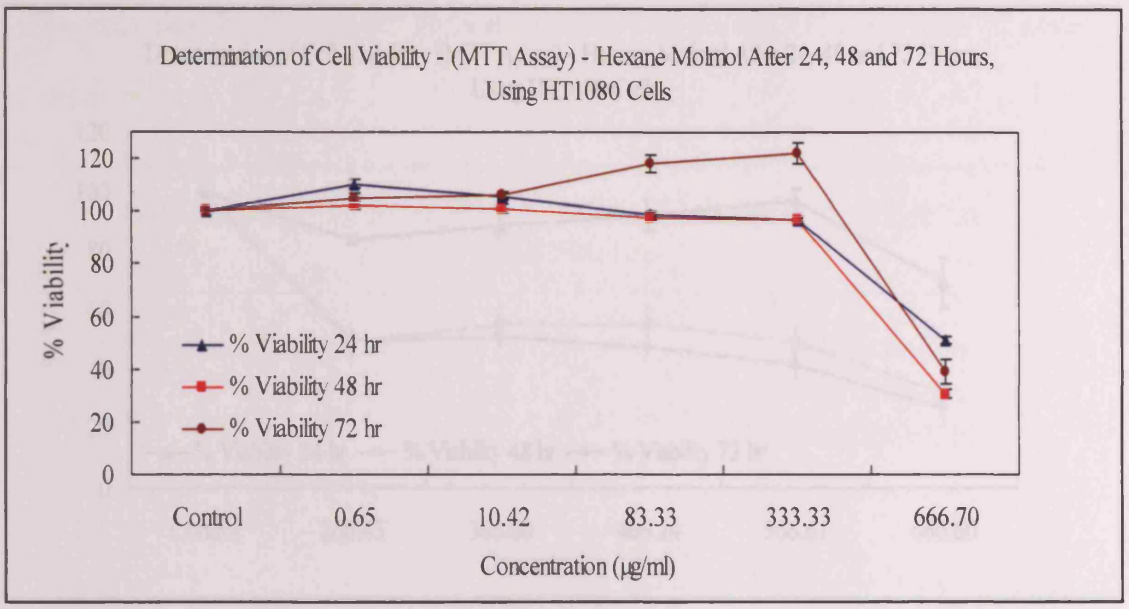


Figure 3.25
Percentage viability of HT1080 cells in control and in medium containing five concentrations of hexane molmol extract (n=9, bars=standard error) (For statistical analysis of data with this figure, see Tables 5.1-5.3, Appendix II).

A.6 Hexane Molmol Extract (266.45-666.60 µg/ml)

Hexane molmol extract (Figure 3.26) was not as active against HT1080 cells as ethanolic molmol extract (compare with Figure 3.22). A similar observation was noted with regard to using S180 cells. This is further evidence of its activity against normal fibroblast, using the same concentration range as for S180 cells.

Figure 3.26 clearly shows that the percentage viability was lowest at the highest concentration (666.60 µg/ml), but only after longer exposure of HT1080 cells to the extract. However, after 24 hours of exposure, viability was very high, ranging between around 69% and 97%. Even at longer exposure to the extract, that is, 72 hours, viability ranged between 26% and 51%, in contrast to using ethanolic molmol extract, when viability ranged between 9% and 73% after 72 hours of exposure, and between 9% and 86% after 24 hours. Results of using these ethanolic extract concentrations also support the fact that ethanolic molmol extracts out-performed hexane molmol extracts.

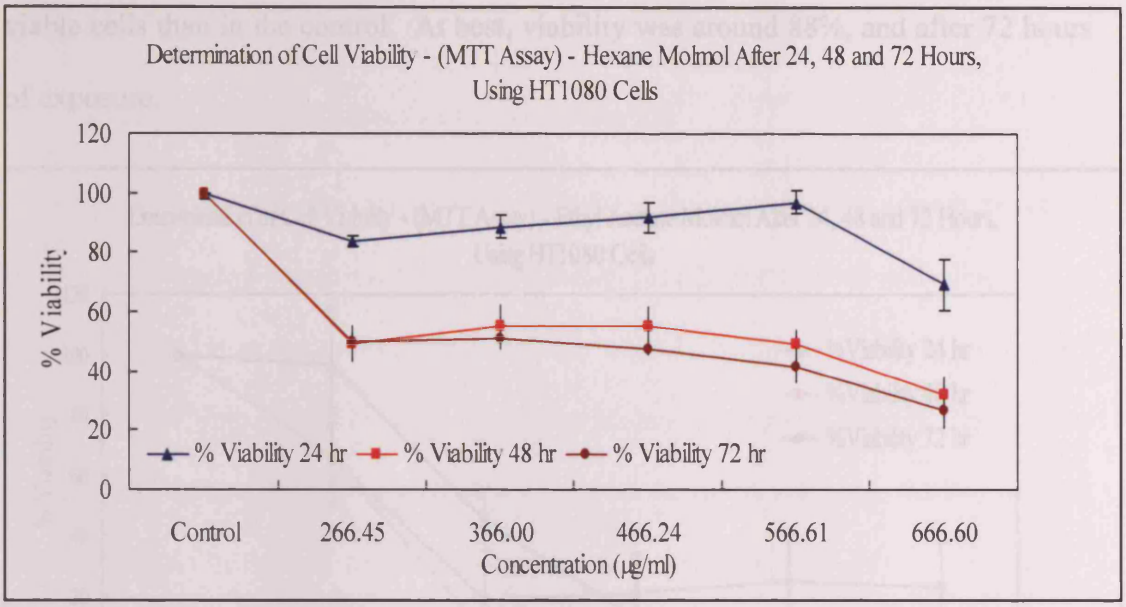


Figure 3.26
Percentage viability of HT1080 cells in control and in medium containing five concentrations of hexane molmol extract (n=9, bars=standard error) (For statistical analysis of data with this figure, see Tables 6.1-6.3, Appendix II).

A.7 Ethyl Acetate Molmol Extract (266.45-666.60 µg/ml)

Results using ethyl acetate molmol extract (Figure 3.27) produced lower percentage viability than using hexane molmol extract (Figure 3.26), and matched that using ethanolic molmol extract (Figure 3.22).

Figure 3.27 clearly shows that percentage viability values were far lower than in the control, even after 24 hours of exposure, except for the lowest concentration (266.45 µg/ml), when it seems that there was high percentage viability (96%).

A.8 Flavex Molmol Extract (266.45-666.60 µg/ml)

Flavex molmol extract was less effective in its activity against H1080 (Figure 3.28). Flavex molmol extract also had the least effect on S180 cells. On a few occasions, this extract had no apparent effect; instead, it seemed to enhance growth, that is, mitogenesis, leading to higher percentage viability than in the control, that is, more

viable cells than in the control. At best, viability was around 88%, and after 72 hours of exposure.

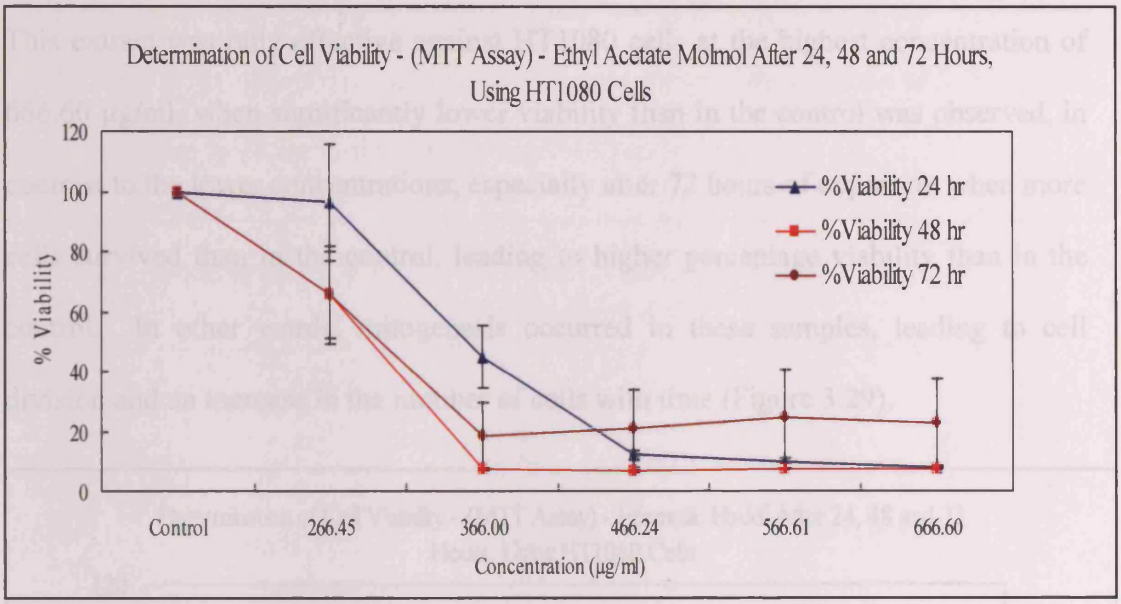


Figure 3.27
Percentage viability of HT1080 cells in control and in medium containing five concentrations of ethyl acetate molmol extract (n=9, bars=standard error) (For statistical analysis of data with this figure, see Tables 7.1-7.3, Appendix II).

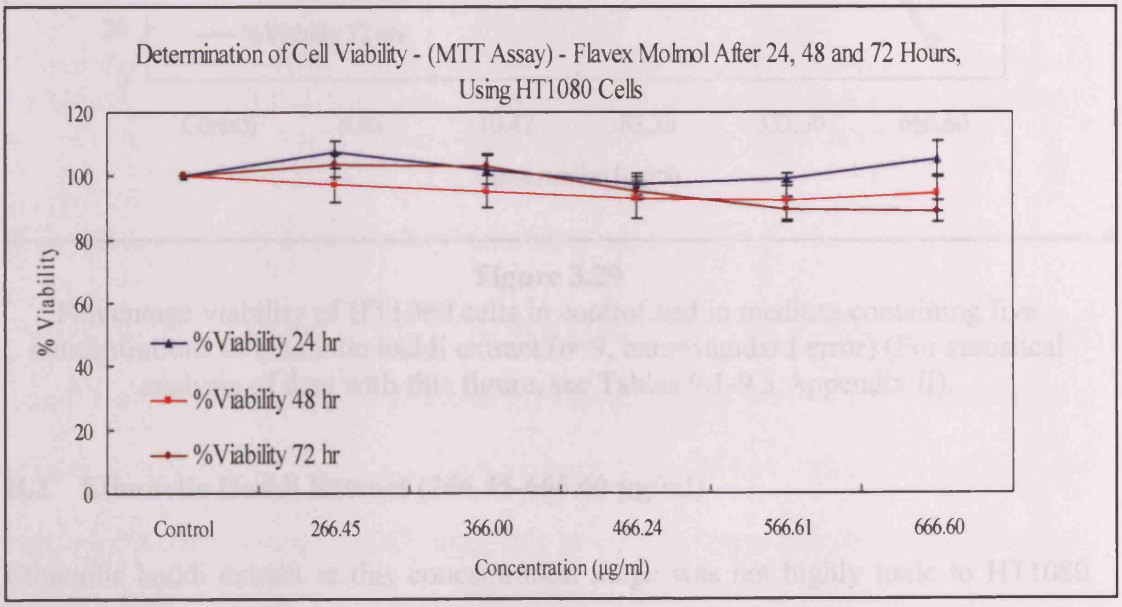


Figure 3.28
Percentage viability of HT1080 cells in control and in medium containing five concentrations of Flavex molmol extract (n=9, bars=standard error) (For statistical analysis of data with this figure, see Tables 8.1-8.3, Appendix II).

B. Haddi Extracts

B.1 Ethanolic Haddi Extract (0.65-666.60 µg/ml)

This extract was only effective against HT1080 cells at the highest concentration of 666.60 µg/ml, when significantly lower viability than in the control was observed, in contrast to the lower concentrations, especially after 72 hours of exposure, when more cells survived than in the control, leading to higher percentage viability than in the control. In other words, mitogenesis occurred in these samples, leading to cell division and an increase in the number of cells with time (Figure 3.29).

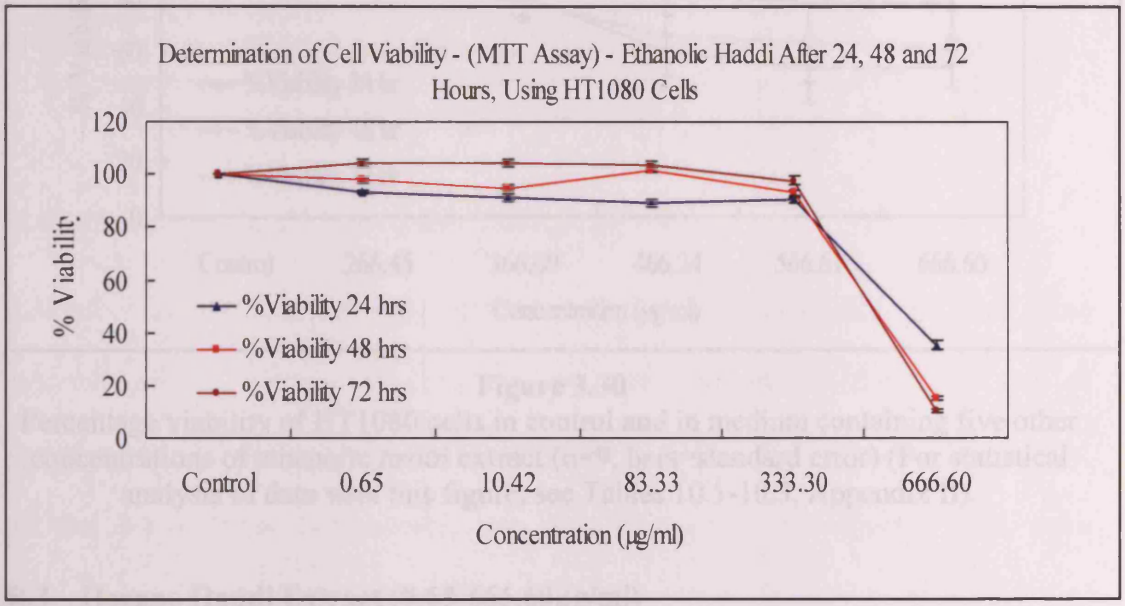


Figure 3.29
Percentage viability of HT1080 cells in control and in medium containing five concentrations of ethanolic haddi extract (n=9, bars=standard error) (For statistical analysis of data with this figure, see Tables 9.1-9.3 Appendix II).

B.2 Ethanolic Haddi Extract (266.45-666.60 µg/ml)

Ethanolic haddi extract at this concentration range was not highly toxic to HT1080 cells, even after 72 hours of exposure. The percentage viability ranged between 109% at 266.45 µg/ml extract after 24 hours, and 53.0% at 566.61 µg/ml after 72 hours of exposure. Ethanolic haddi extracts of the same concentrations were more effective in

bringing about lower viability percentages when used against S180 cells than they were against HT1080 cells (see Figure 3.16 for comparison). This indicates that ethanolic haddi extract at these concentrations has resulted in mitogenesis in the case of HT1080 cells, and proves that S180 cells are more sensitive than HT1080 cells, which are more resistant to the extract than S180 cells.

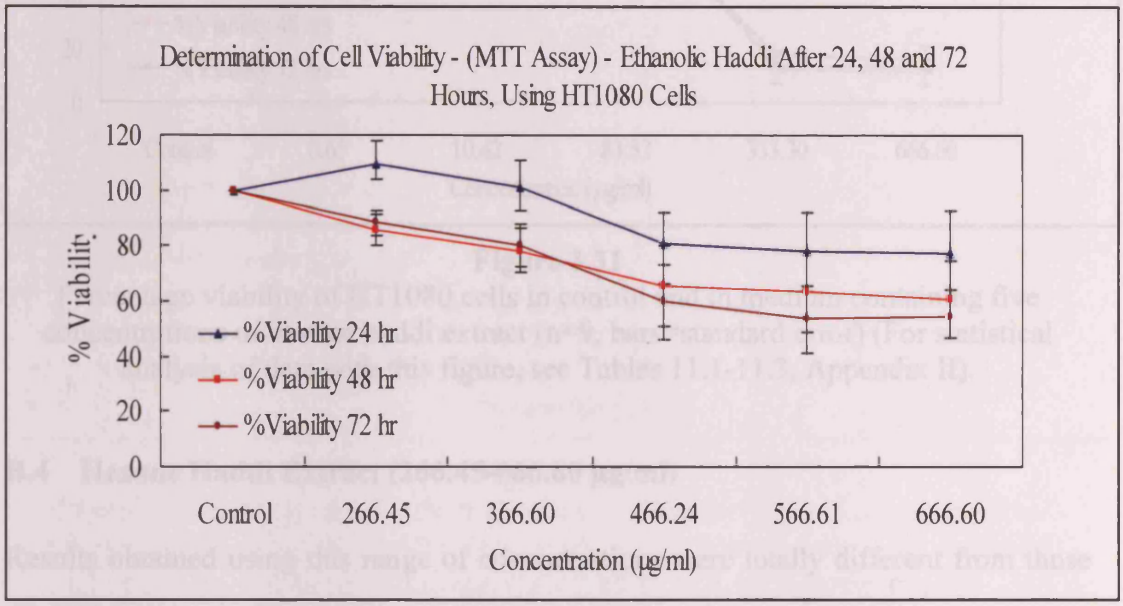


Figure 3.30
Percentage viability of HT1080 cells in control and in medium containing five other concentrations of ethanolic haddi extract (n=9, bars=standard error) (For statistical analysis of data with this figure, see Tables 10.1-10.3, Appendix II).

B.3 Hexane Haddi Extract (0.65-666.60µg/ml)

Hexane haddi extract was more toxic to HT1080 cells than ethanolic haddi extract, using a similar range of concentrations, though at the two lowest concentrations results did not differ. The two highest concentrations (666.90 and 333.30 µg/ml) were highly effective against HT1080 cells, and resulted in lower percentage viability than in the control (Figure 3.31) ($P<0.001$).

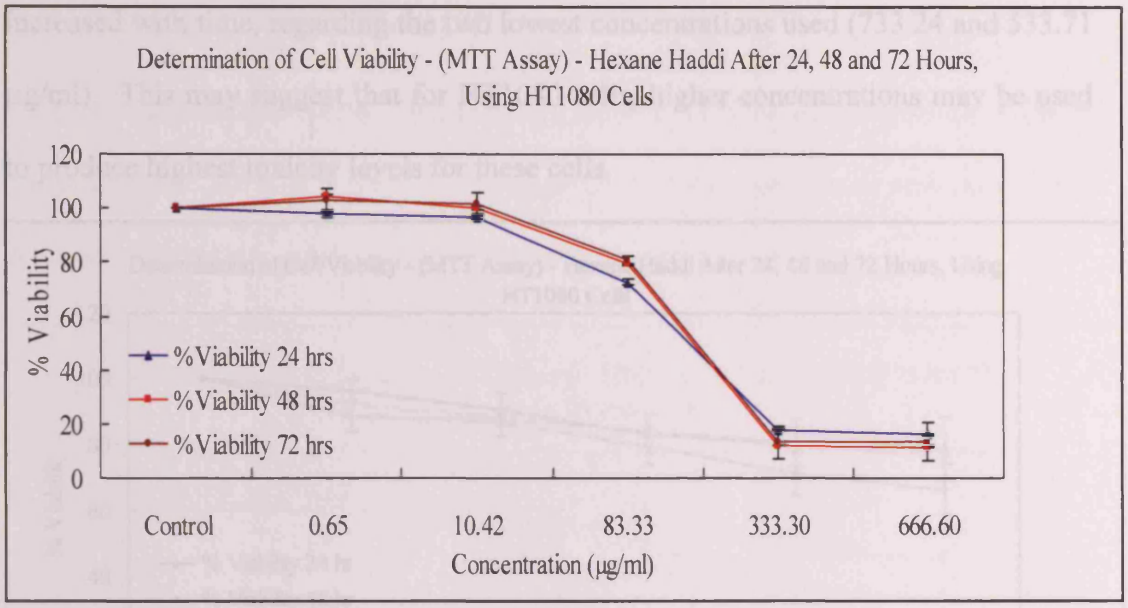


Figure 3.31
Percentage viability of HT1080 cells in control and in medium containing five concentrations of hexane haddi extract (n=9, bars=standard error) (For statistical analysis of data with this figure, see Tables 11.1-11.3, Appendix II).

B.4 Hexane Haddi Extract (266.45-666.60 µg/ml)

Results obtained using this range of concentrations were totally different from those reported above. This difference was persistent throughout the three sets of experiments. Percentage viability was high (Figure 3.32). To investigate further, another set of three experiments, also consisting of three replicates each, were undertaken, using a higher range of hexane haddi extract, in order to make sure that these differences were also persistent in the new set of experiments.

B.5 Hexane Haddi Extract (533.71-1333.20 µg/ml)

This high range of concentration was also used in order to obtain the optimum concentration of hexane haddi extract that is effective against HT1080 cells. Using higher concentrations of hexane haddi extract resulted in low percentage viability throughout the range used (Figure 3.33) ($P<0.001$), thus indicating high mortality, compared with the other concentrations used above (Figure 3.32). Apparent toxicity

increased with time, regarding the two lowest concentrations used (733.24 and 533.71 µg/ml). This may suggest that for HT1080 cells, higher concentrations may be used to produce highest toxicity levels for these cells.

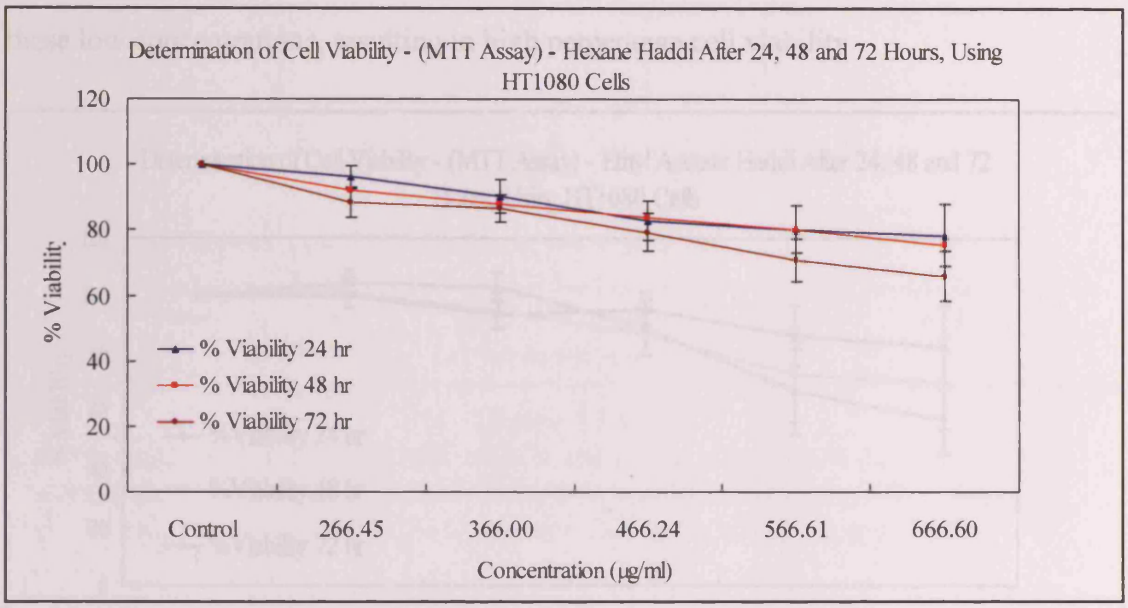


Figure 3.32
Percentage viability of HT1080 cells in control and in medium containing five concentrations of hexane haddi extract (n=9, bars=standard error) (For statistical analysis of data with this figure, see Tables 12.1-12.3, Appendix II).

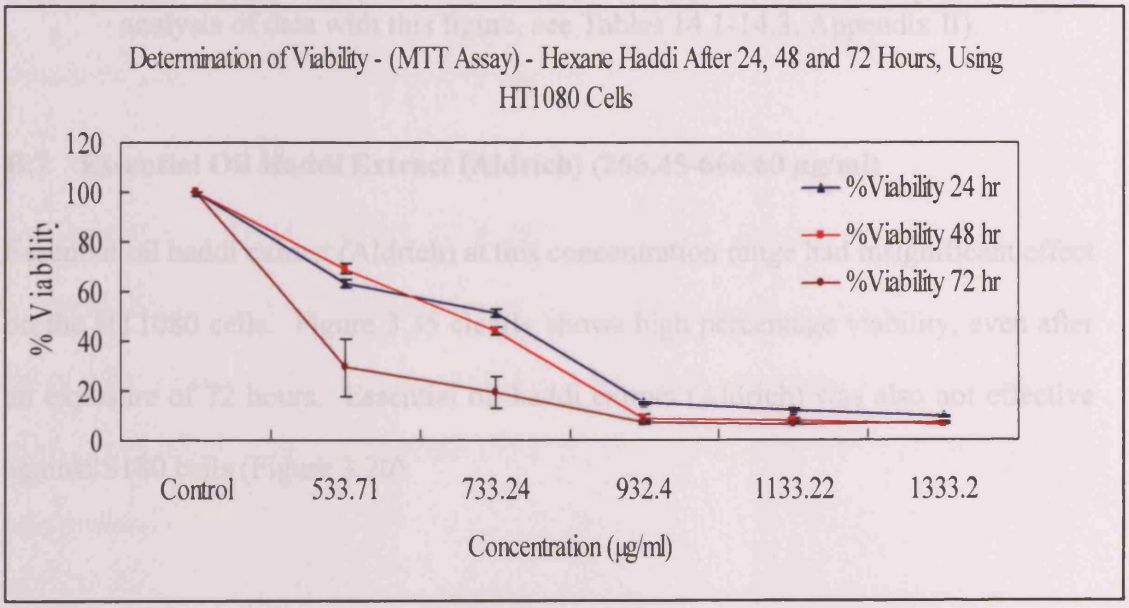


Figure 3.33
Percentage viability of HT1080 cells in control and in medium containing five concentrations of hexane haddi extract (n=9, bars=standard error) (For statistical analysis of data with this figure, see Tables 13.1-13.3, Appendix II).

B.6 Ethyl Acetate Haddi Extract (266.45-666.60 µg/ml)

Ethyl acetate haddi extract was not effective against HT1080 cells, especially at lower concentrations. Results (Figure 3.34) suggest a high survival rate of cells exposed to these low concentrations, resulting in high percentage cell viability.

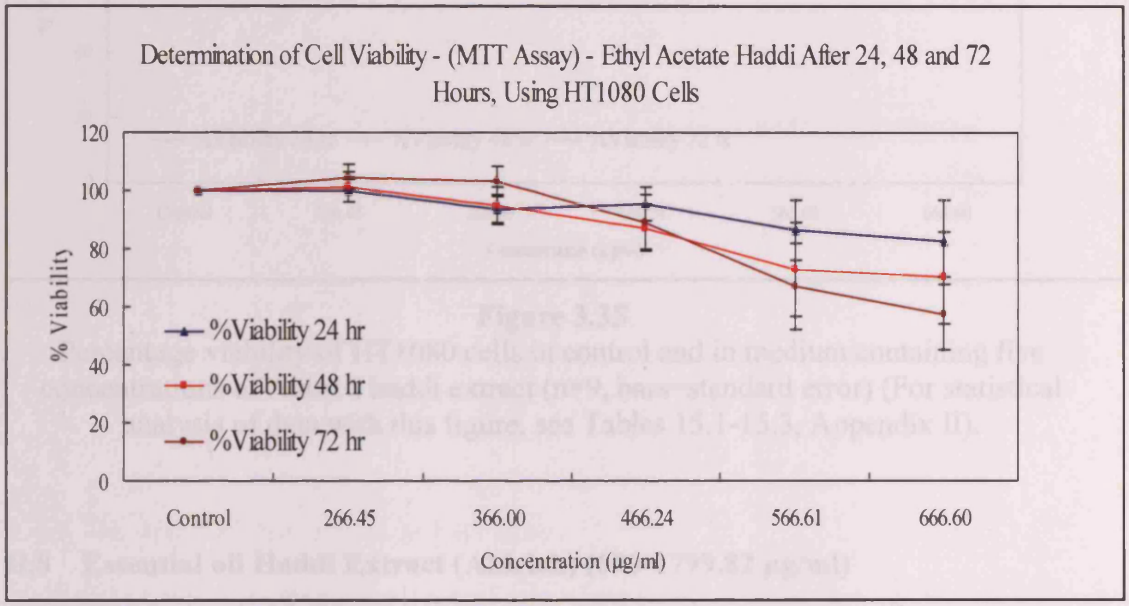


Figure 3.34
Percentage viability of HT1080 cells in control and in medium containing five concentrations of ethyl acetate haddi extract (n=9, bars=standard error) (For statistical analysis of data with this figure, see Tables 14.1-14.3, Appendix II).

B.7 Essential Oil Haddi Extract (Aldrich) (266.45-666.60 µg/ml)

Essential oil haddi extract (Aldrich) at this concentration range had insignificant effect on the HT1080 cells. Figure 3.35 clearly shows high percentage viability, even after an exposure of 72 hours. Essential oil haddi extract (Aldrich) was also not effective against S180 cells (Figure 3.20).

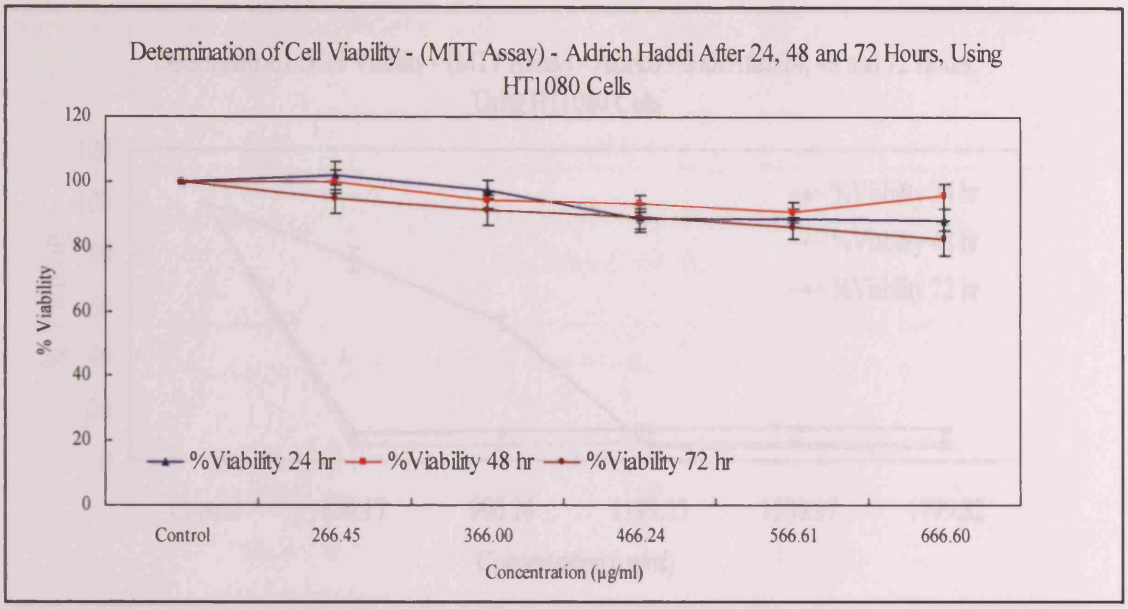


Figure 3.35
Percentage viability of HT1080 cells in control and in medium containing five concentrations of Aldrich haddi extract (n=9, bars=standard error) (For statistical analysis of data with this figure, see Tables 15.1-15.3, Appendix II).

B.8 Essential oil Haddi Extract (Aldrich) (600-1799.82 µg/ml)

The HT1080 cells were also exposed to a stronger essential oil haddi extract (Aldrich) concentration range (Figure 3.36). Results indicate that percentage viability decreased substantially, with time of exposure ($P<0.001$).

Nonetheless, it seems that HT1080 cells, initially affected by the two lowest concentrations (600.17 and 900.26 µg/ml) resulting in about 6% viability after an exposure for 24 and 48 hours, reversed this reaction when signs of recovery were noted after 72 hours of exposure, as evidenced by high percentage viability compared to the previous two days of exposure, that is, survival of cells and their proliferation was evident.

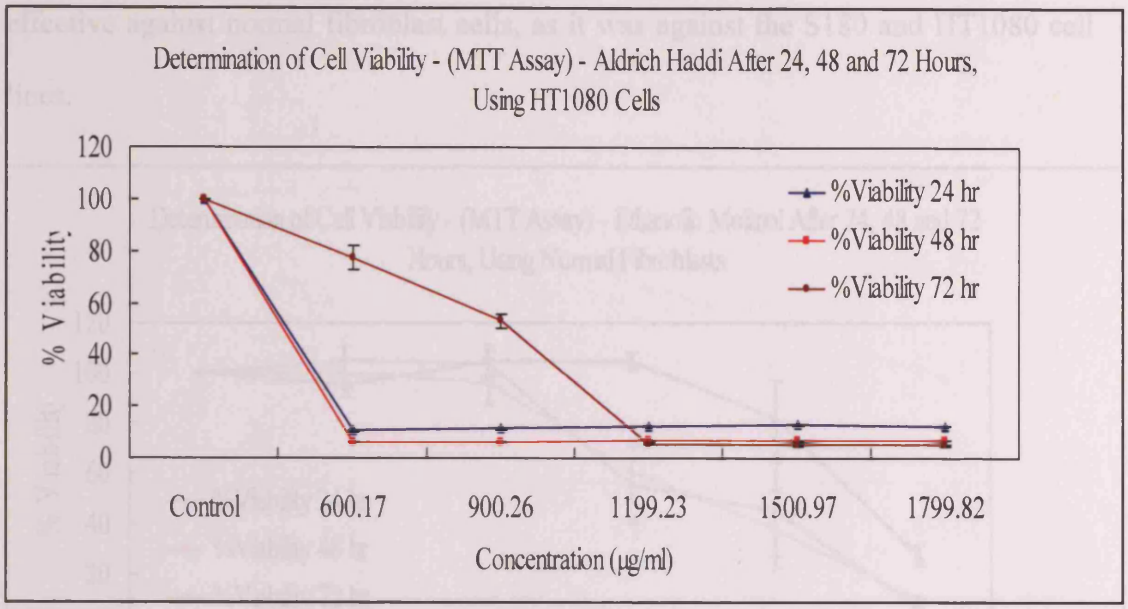


Figure 3.36
Percentage viability of HT1080 cells in control and in medium containing five higher concentrations of Aldrich haddi extract (n=9, bars=standard error) (For statistical analysis of data with this figure, see Tables 16.1-16.3, Appendix II).

3.4.3 Normal Chick Fibroblast Cell Line

The effect of the molmol and haddi extracts on the viability of normal chick fibroblast cells were also investigated using similar treatment protocols to those used before for S180 and HT1080 cell lines. Results are illustrated in Figures 3.37 to 3.46.

A. Molmol Extracts

A.1 Ethanolic Molmol Extract (266.45-666.60 µg/ml)

The three highest concentrations were most effective against normal fibroblast cells (Figure 3.37) ($P<0.001$). Nonetheless, for the 466.24 µg/ml concentration of ethanolic molmol, mitogenesis occurred after 24 hours of exposure, followed by a substantial cell death after 48 and, especially, after 72 hours of exposure. The other lower concentrations were ineffective, resulting in enhanced growth, that is, mitogenesis, and higher percentage viability than in the control, especially after 72 hours of exposure. Results show that the 366.00 µg/ml extract concentration was not

effective against normal fibroblast cells, as it was against the S180 and HT1080 cell lines.

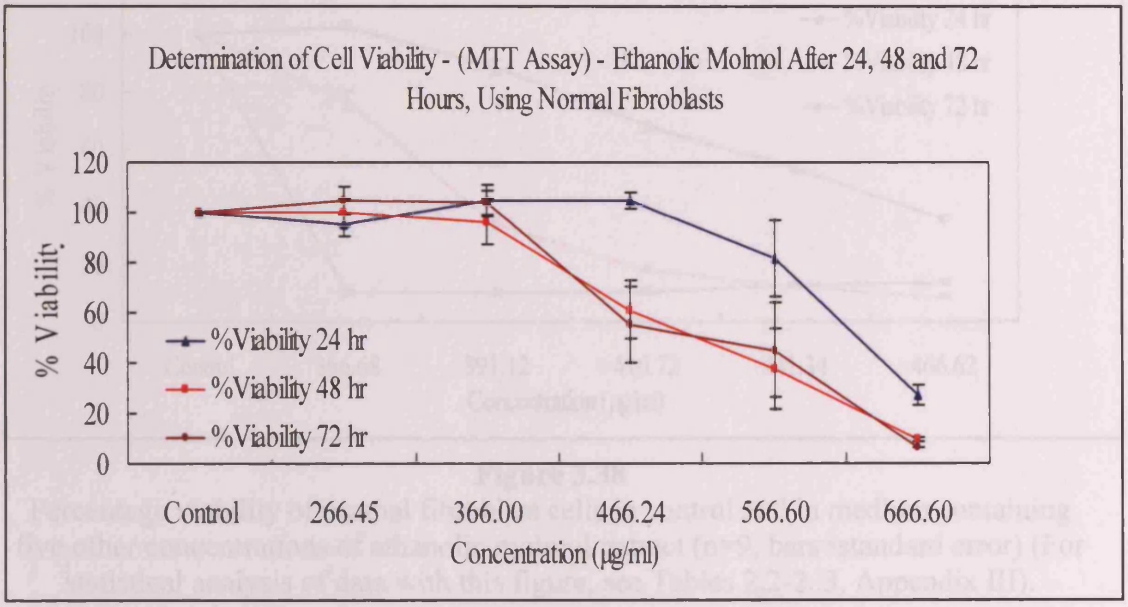


Figure 3.37
Percentage viability of normal fibroblast cells in control and in medium containing five concentrations of ethanolic molmol extract (n=9, bars=standard error) (For statistical analysis of data with this figure, see Tables 1.1-1.3, Appendix III).

A.2 Ethanolic Molmol Extract (366.68-466.62 µg/ml)

Because 466.24 µg/ml extract was not highly effective against normal fibroblast cells, illustrated above, it was decided to run a test using a concentration range between 366.68 and 466.62 µg/ml, in order to decide the actual concentration that is least effective against the normal fibroblast cells. The lowest concentration (366.68 µg/ml) lagged behind the others after 24 hours of exposure, when mitogenesis was noted (higher percentage viability than in the control) (Figure 3.38). Also, a high percentage cell viability (77%) was noted after 48 hours of exposure to 366.68 µg/ml.

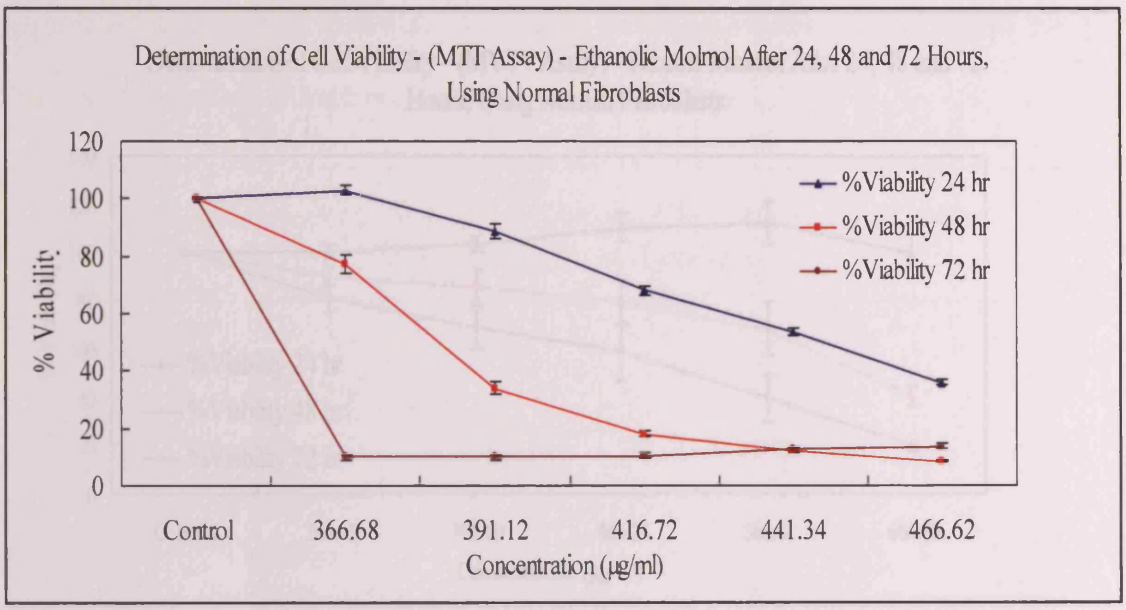


Figure 3.38
Percentage viability of normal fibroblast cells in control and in medium containing five other concentrations of ethanolic molmol extract (n=9, bars=standard error) (For statistical analysis of data with this figure, see Tables 2.2-2..3, Appendix III).

A.3 Hexane Molmol Extract (266.45-666.60 µg/ml)

This range of concentrations was only highly toxic to normal fibroblast cells towards the end of the experiment. After 24 hours of exposure, growth was enhanced in cultures exposed to all concentrations, indicating mitogenesis, but a definite activity was shown after 72 hours of exposure, especially at the two highest concentrations (Figures 3.39).

A.4 Ethyl Acetate Molmol Extract (666.60-266.50 µg/ml)

All concentrations used, except the lowest (266.50 µg/ml), were effective against normal fibroblast cells, particularly after 48 and 72 hours of exposure ($P<0.001$). In the lowest concentration, growth enhancement (mitogenesis) was noted, as shown by the higher percentage viability than in the control (Figures 3.40).

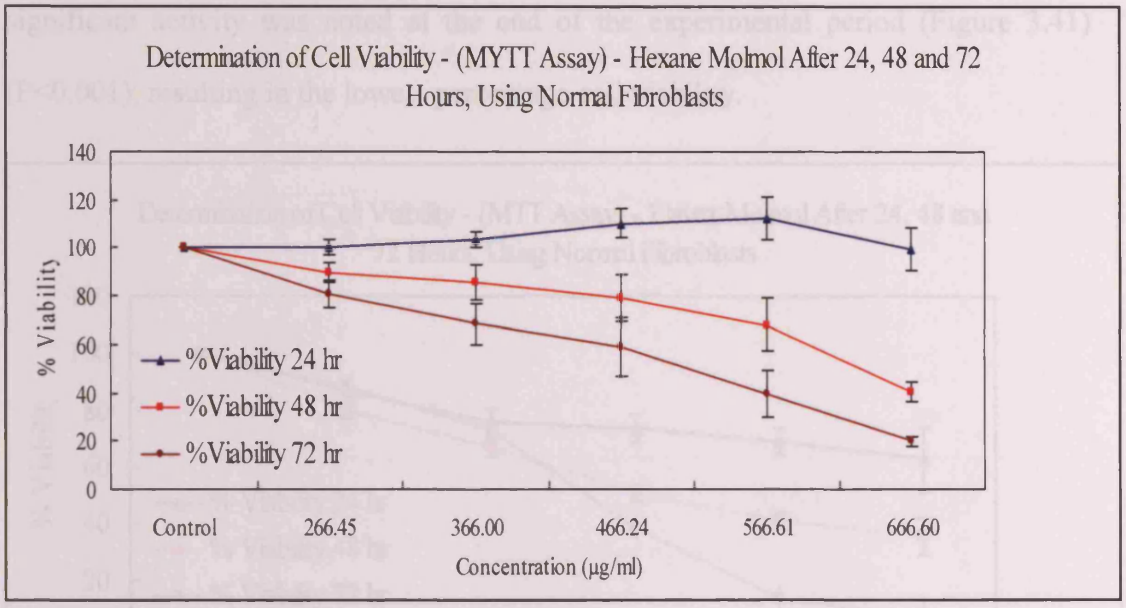


Figure 3.39

Percentage viability of normal fibroblast cells in control and in medium containing five concentrations of hexane molmol extract (n=9, bars=standard error) (For statistical analysis of data with this figure, see Tables 3.1-3.3, Appendix III).

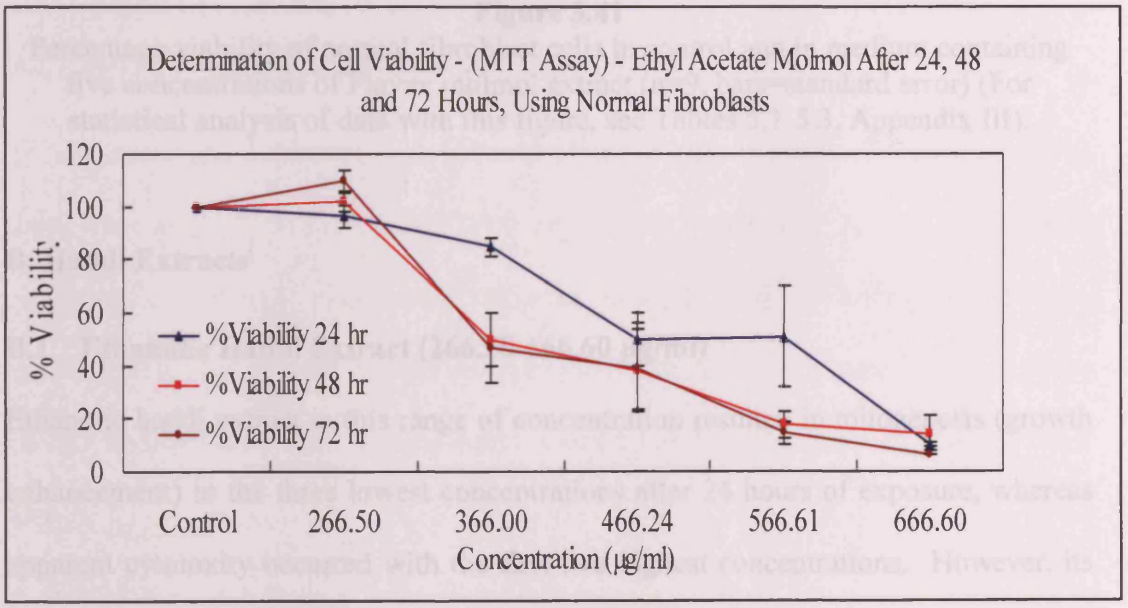


Figure 3.40

Percentage viability of normal fibroblast cells in control and in medium containing five concentrations of ethyl acetate molmol extract (n=9, bars=standard error) (For statistical analysis of data with this figure, see Tables 4.1-4.3, Appendix III).

A.5 Flavex Molmol Extract (266.45-666.60 µg/ml)

Flavex molmol extract employed at this concentration range was also effective, especially at the two highest concentrations (566.61 and 666.6 µg/ml), when

significant activity was noted at the end of the experimental period (Figure 3.41) ($P<0.001$), resulting in the lowest percentage cell viability.

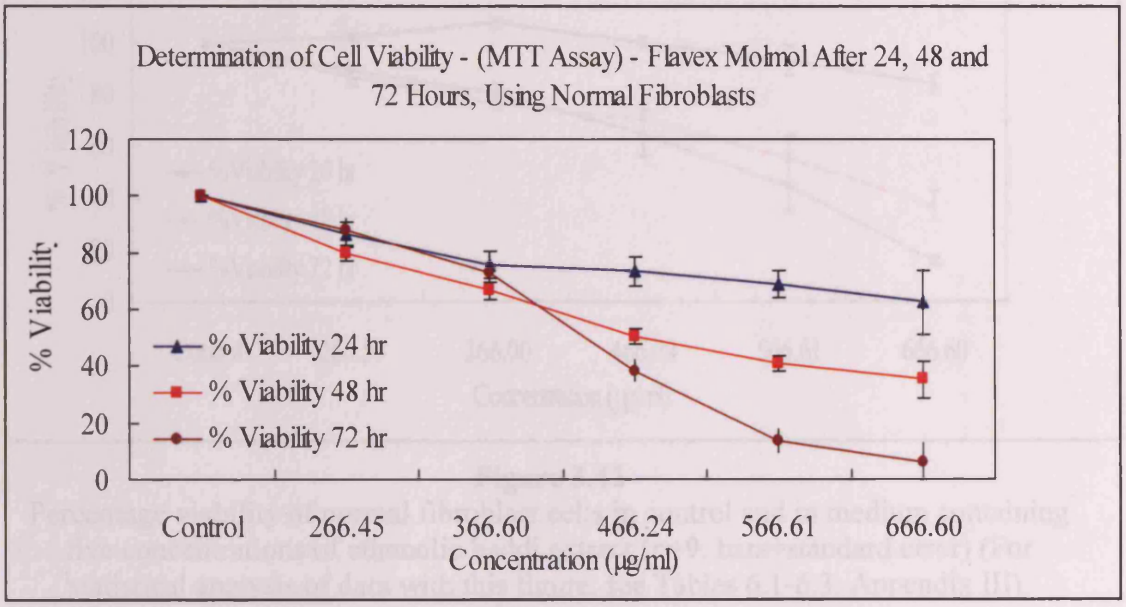


Figure 3.41

Percentage viability of normal fibroblast cells in control and in medium containing five concentrations of Flavex molmol extract (n=9, bars=standard error) (For statistical analysis of data with this figure, see Tables 5.1-5.3, Appendix III).

B. Haddi Extracts

B.1 Ethanolic Haddi Extract (266.50-666.60 µg/ml)

Ethanolic haddi extract in this range of concentration resulted in mitogenesis (growth enhancement) in the three lowest concentrations after 24 hours of exposure, whereas apparent cytotoxity occurred with the first two highest concentrations. However, its activity was more evident later in the experiment, though the only significant lowest percentage viability was also illustrated by the two highest concentrations (666.60 and 566.61 µg/ml), particularly with the former, after 48 and 72 hours of exposure (Figure 3.42).

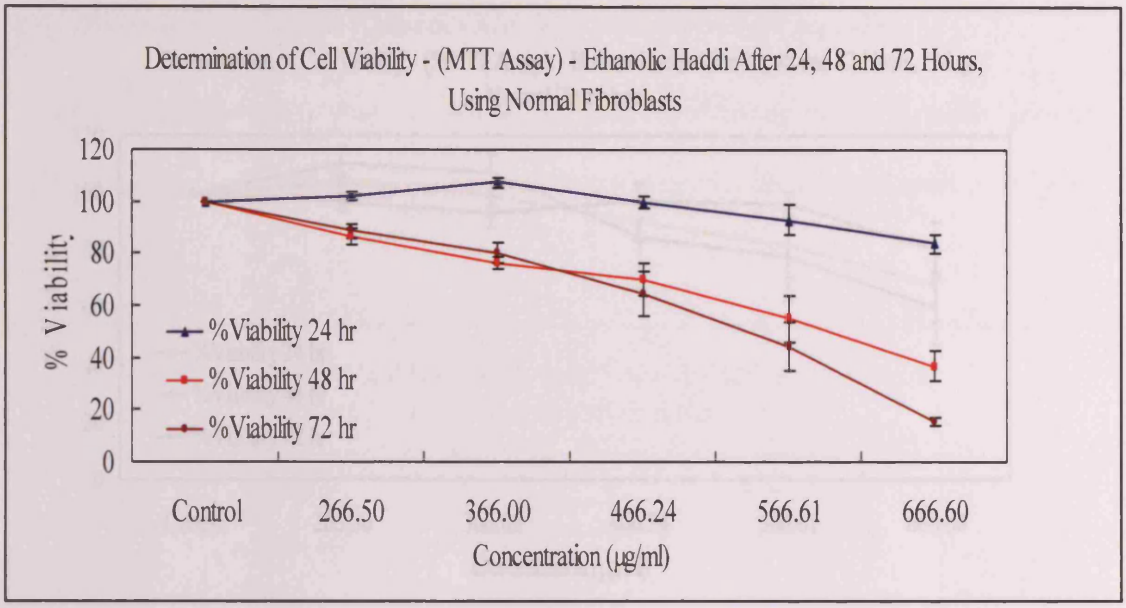


Figure 3.42
Percentage viability of normal fibroblast cells in control and in medium containing five concentrations of ethanolic haddi extract (n=9, bars=standard error) (For statistical analysis of data with this figure, see Tables 6.1-6.3, Appendix III).

B.2 Hexane Haddi Extract (266.50-666.60 µg/ml)

These hexane haddi extracts were not very effective against normal fibroblast cells, as they were against S180 and HT1080 cell lines, particularly in the two lowest concentrations where mitogenesis occurred after 48 and 72 hours of exposure, resulting in higher percentage cell viability than in the control (Figure 3.43). Because of this differential effect, hexane haddi extract has been selected to run the other assays.

B.3 Ethyl Acetate Haddi Extract (266.50-666.60µg/ml)

Ethyl acetate haddi extract at this concentration range was also highly ineffective in terms of cytotoxicity throughout the experimental period, resulting in most cases in higher percentage viability than in the control, that is, mitogenesis (growth enhancement), though there had been insignificant toxicity effect at the two highest concentrations at the end of the experiment (Figure 3.44).

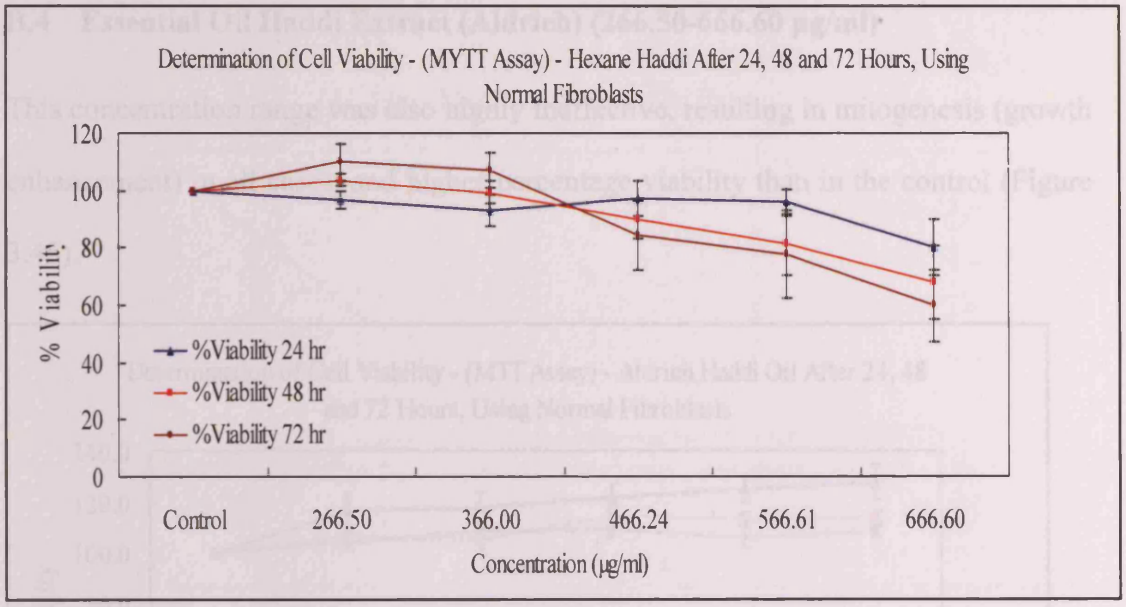


Figure 3.43

Percentage viability of normal fibroblast cells in control and in medium containing five concentrations of Hexane haddi extract (n=9, bars=standard error) (For statistical analysis of data with this figure, see Tables 7.1-7.3, Appendix I).

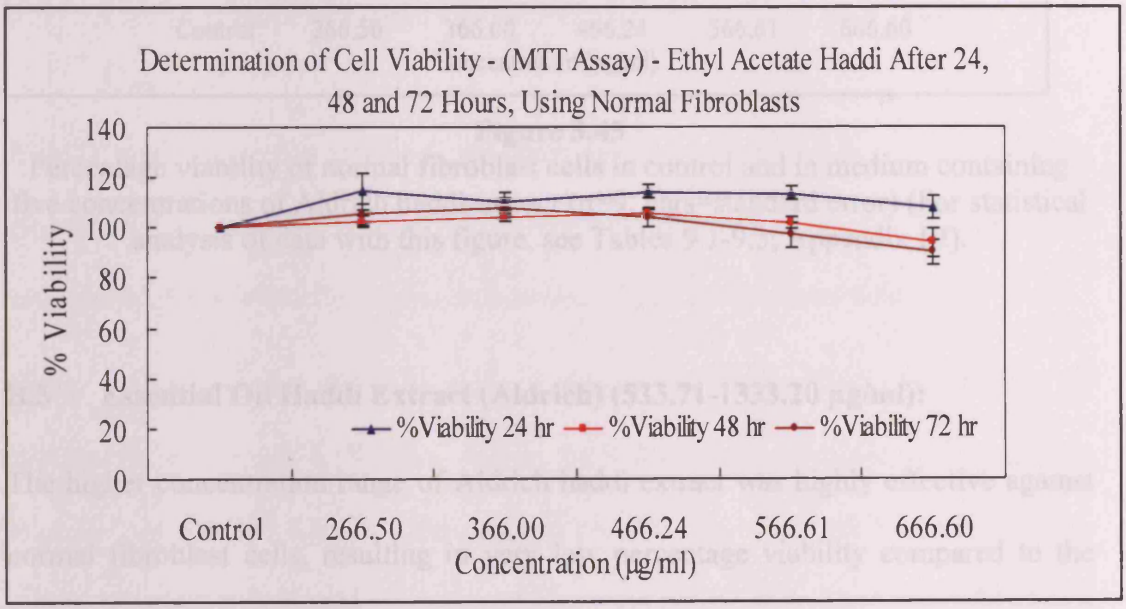


Figure 3.44

Percentage viability of normal fibroblast cells in control and in medium containing five concentrations of ethyl acetate haddi extract (n=9, bars=standard error) (For statistical analysis of data with this figure, see Tables 8.1-8.3, Appendix III).

B.4 Essential Oil Haddi Extract (Aldrich) (266.50-666.60 µg/ml)

This concentration range was also highly ineffective, resulting in mitogenesis (growth enhancement) in all cases, and higher percentage viability than in the control (Figure 3.45).

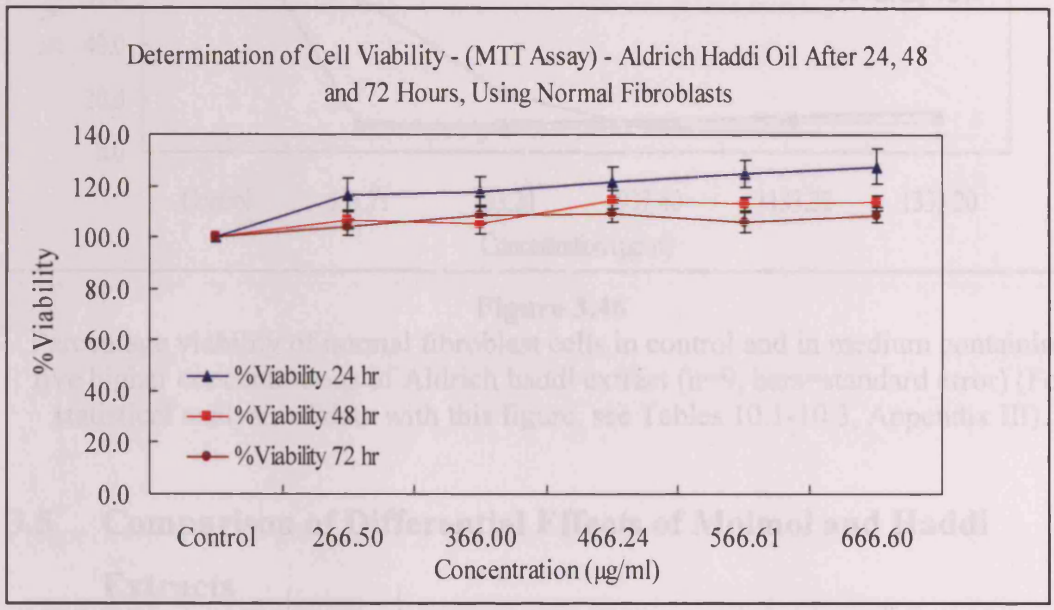


Figure 3.45
Percentage viability of normal fibroblast cells in control and in medium containing five concentrations of Aldrich haddi extract (n=9, bars=standard error) (For statistical analysis of data with this figure, see Tables 9.1-9.3, Appendix III).

B.5 Essential Oil Haddi Extract (Aldrich) (533.71-1333.20 µg/ml):

The higher concentration range of Aldrich haddi extract was highly effective against normal fibroblast cells, resulting in very low percentage viability compared to the control (Figure 3.46) ($P<0.001$).

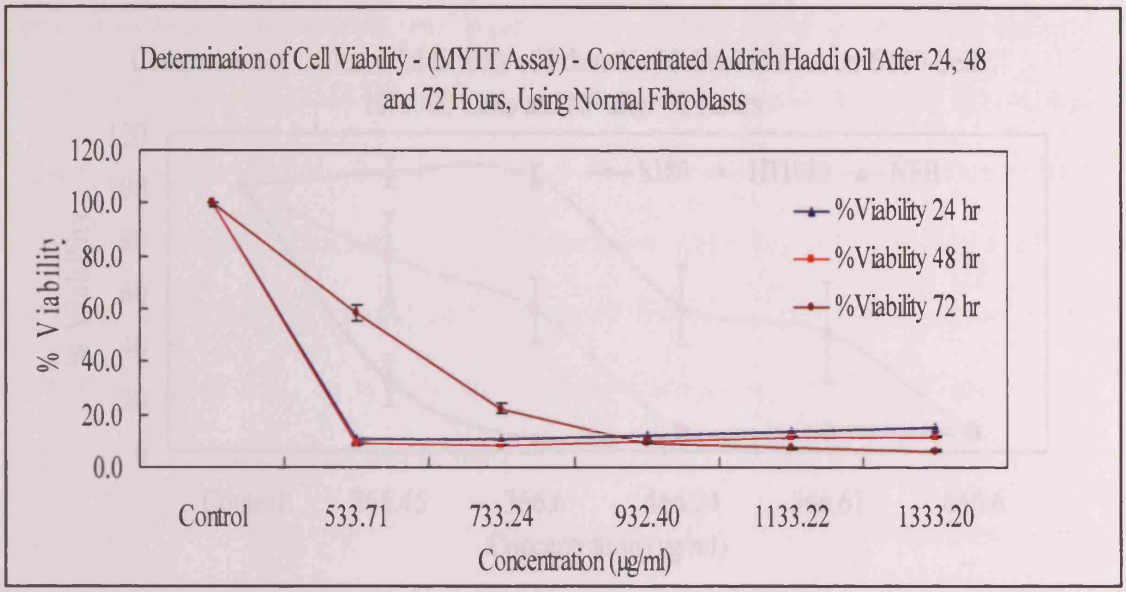


Figure 3.46
Percentage viability of normal fibroblast cells in control and in medium containing five higher concentrations of Aldrich haddi extract (n=9, bars=standard error) (For statistical analysis of data with this figure, see Tables 10.1-10.3, Appendix III).

3.5 Comparison of Differential Effects of Molmol and Haddi Extracts

Results analysed earlier showed that extracts of molmol and haddi plants, using ethanol, hexane and ethyl acetate, had differential effects on S180 cells, HT1080 cells, and normal fibroblast cells. These differential effects are analysed below.

Figure 3.47 shows that ethanolic molmol extract was most effective in terms of its cytotoxic effect against S180 cells. While the S180 viability declines to around 5.0% after 72 hours at 366.6 µg/ml, compared to around 50% of the HT1080 cells, where this concentration had a mitogenetic effect on the normal fibroblast cells ($P<0.001$). Higher concentrations of ethanolic molmol extracts were active against all cell lines used, especially the S180 and HT1080. It can possibly be said that using this concentration to treat cancerous cells is appropriate, as it appears to target only cancerous cells rather than normal cells.

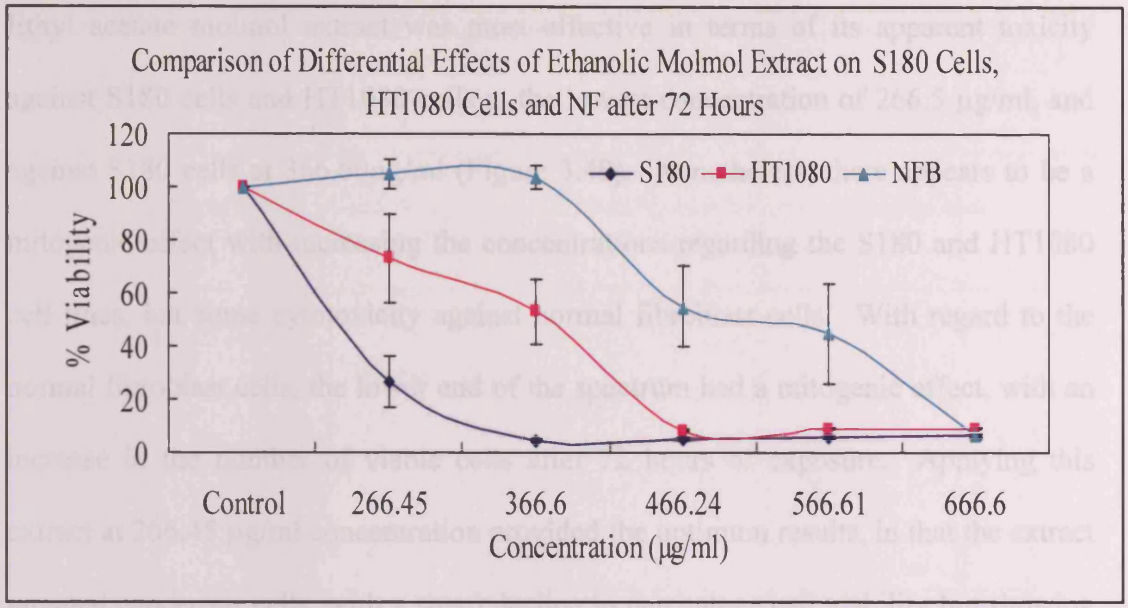


Figure 3.47

Differential effects of ethanolic molmol extracts on S180, HT1080 and normal fibroblast cell line after 72 hours of exposure (n=9, bars=standard error) (For statistical analysis of data with this figure, see Table 1.1, Appendix IV).

Figure 3.48 shows that hexane molmol extract is effective, in terms of its apparent cytotoxicity, against all cell lines used. It is therefore not suitable to treat cancerous cells because it targets normal cells and cancerous cells alike.

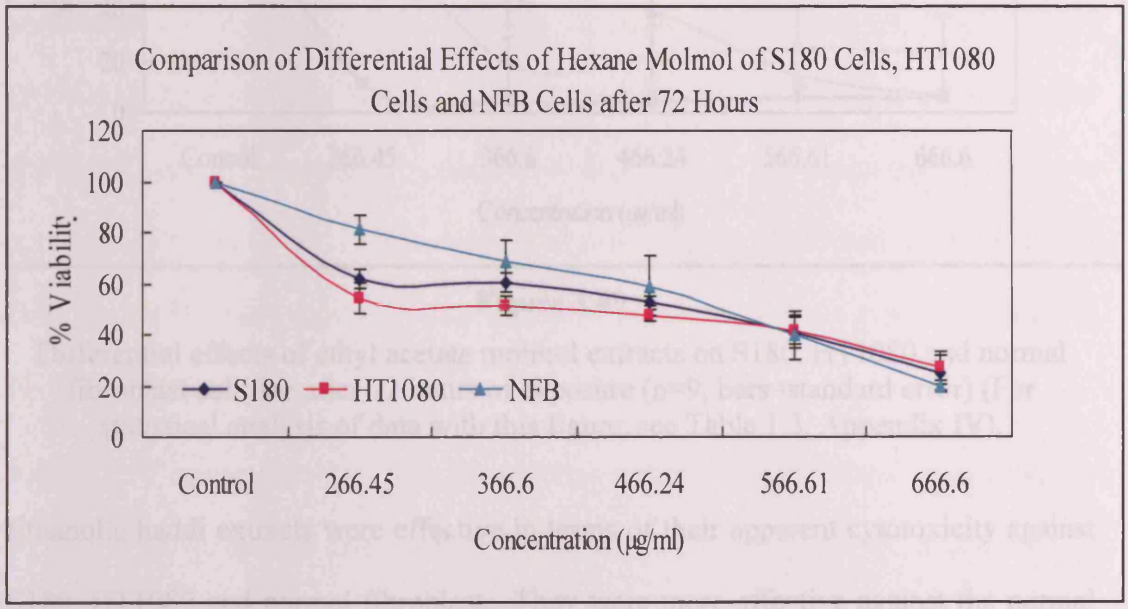


Figure 3.48

Differential effects of hexane molmol extracts on S180, HT1080 and normal fibroblast cell line after 72 hours of exposure (n=9, bars=standard error) (For statistical analysis of data with this figure, see Table 2.1, Appendix IV).

Ethyl acetate molmol extract was most effective in terms of its apparent toxicity against S180 cells and HT1080 cells at the lowest concentration of 266.5 µg/ml, and against S180 cells at 366.60µg/ml (Figure 3.49). Nonetheless, there appears to be a mitogenic effect with increasing the concentrations regarding the S180 and HT1080 cell lines, but some cytotoxicity against normal fibroblast cells. With regard to the normal fibroblast cells, the lower end of the spectrum had a mitogenic effect, with an increase in the number of viable cells after 72 hours of exposure. Applying this extract at 266.45 µg/ml concentration provided the optimum results, in that the extract targeted cancerous cells, with a sharp decline in percentage cell viability but showing a mitogenic effect on the normal fibroblast cells.

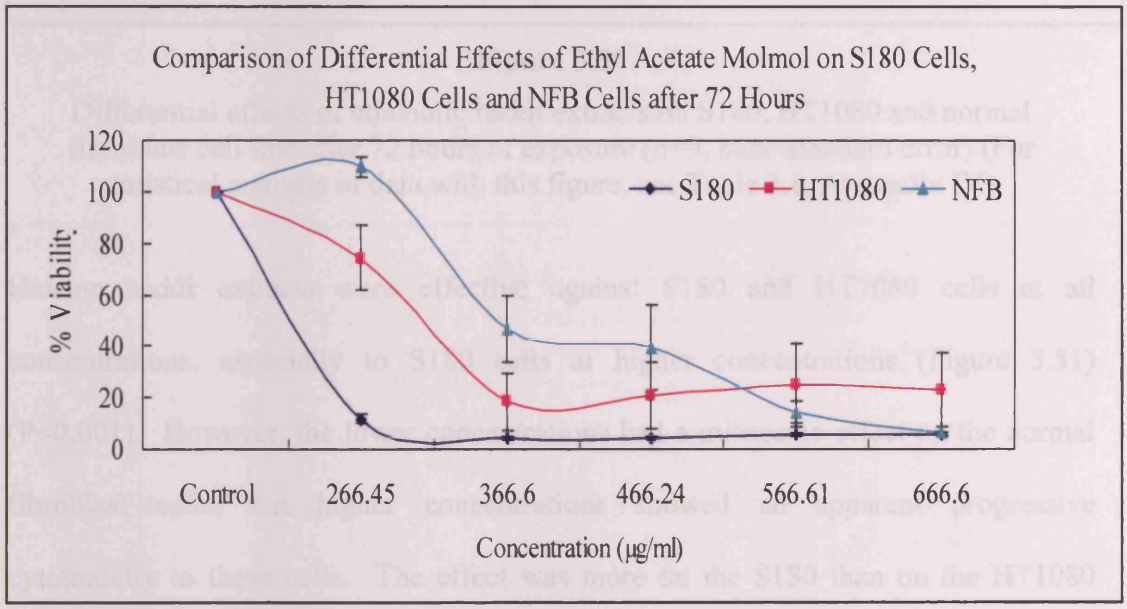


Figure 3.49

Differential effects of ethyl acetate molmol extracts on S180, HT1080 and normal fibroblast cell line after 72 hours of exposure (n=9, bars=standard error) (For statistical analysis of data with this figure, see Table 1.3, Appendix IV).

Ethanolic haddi extracts were effective in terms of their apparent cytotoxicity against S180, HT1080 and normal fibroblast. They were more effective against the normal fibroblast cells at the higher concentration ranges than against HT1080 cells (Figure 3.50). However, they were more effective preparations against S180 cells than

against HT1080 and fibroblast cells, resulting in considerably lower percentage cell viability in S180 than in the control.

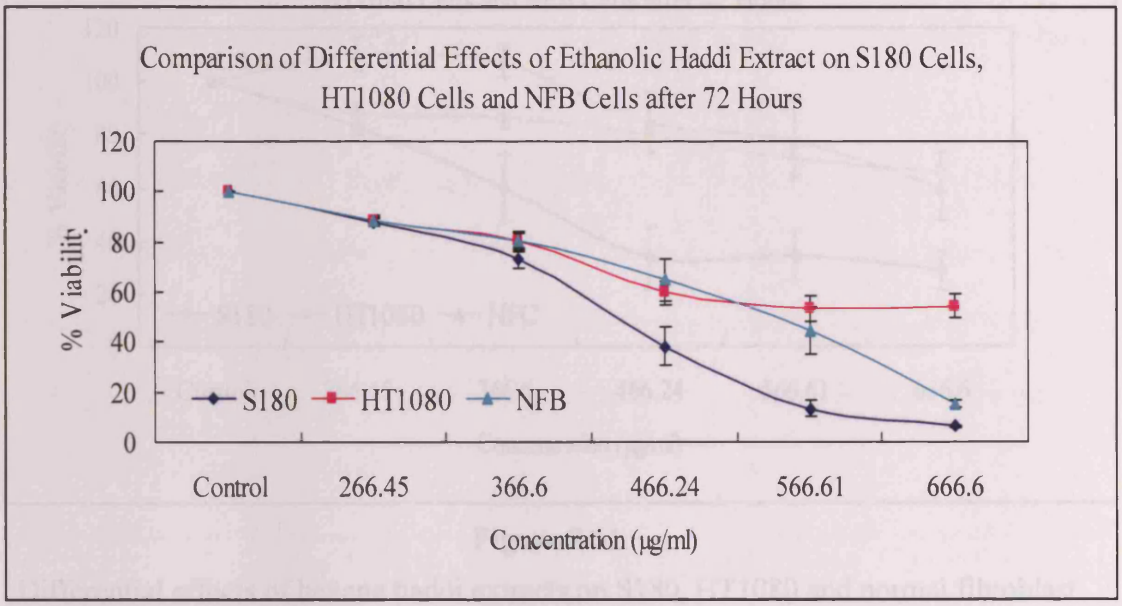


Figure 3.50

Differential effects of ethanolic haddi extracts on S180, HT1080 and normal fibroblast cell line after 72 hours of exposure (n=9, bars=standard error) (For statistical analysis of data with this figure, see Table 2.1, Appendix IV).

Hexane haddi extracts were effective against S180 and HT1080 cells at all concentrations, especially to S180 cells at higher concentrations (Figure 3.51) ($P<0.001$). However, the lower concentrations had a mitogenic effect on the normal fibroblast cells, but higher concentrations showed an apparent progressive cytotoxicity to these cells. The effect was more on the S180 than on the HT1080 cells, which indicates that S180 cells are more sensitive to these treatments than the HT1080 cells.

Figure 3.52 shows that the two lower ethyl acetate concentrations were effective against S180 cells, but had a mitogenic effect on the HT1080 cells and normal fibroblast cells. These extracts can be used at the lower concentrations to target only S180 cells, but not HT1080 cells.

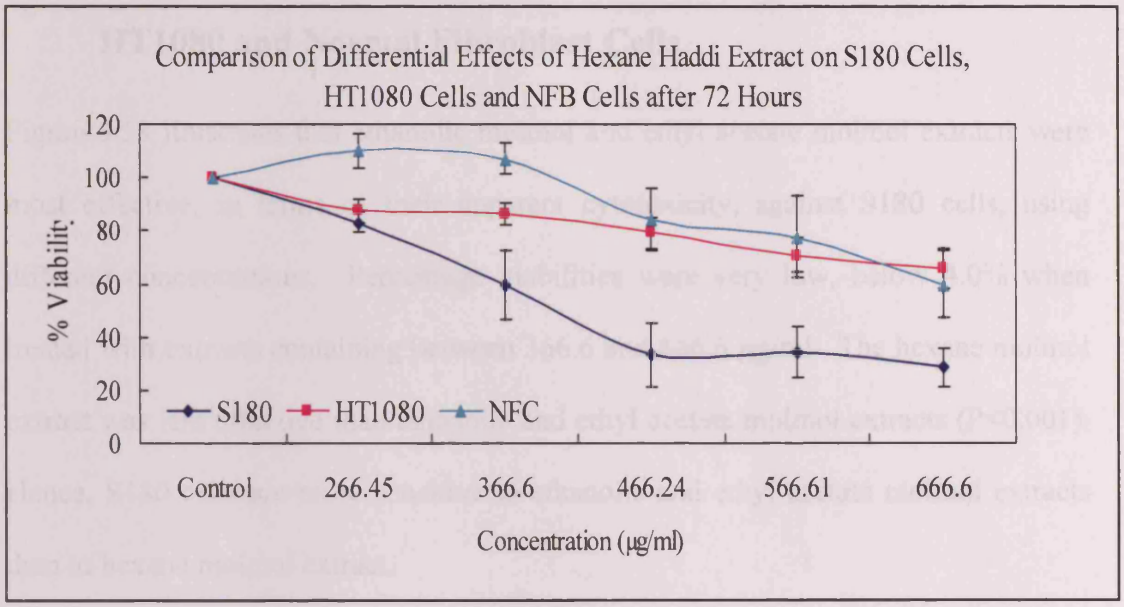


Figure 3.51

Differential effects of hexane haddi extracts on S180, HT1080 and normal fibroblast cell line after 72 hours of exposure (n=9, bars=standard error) (For statistical analysis of data with this figure, see Table 2.2, Appendix IV).

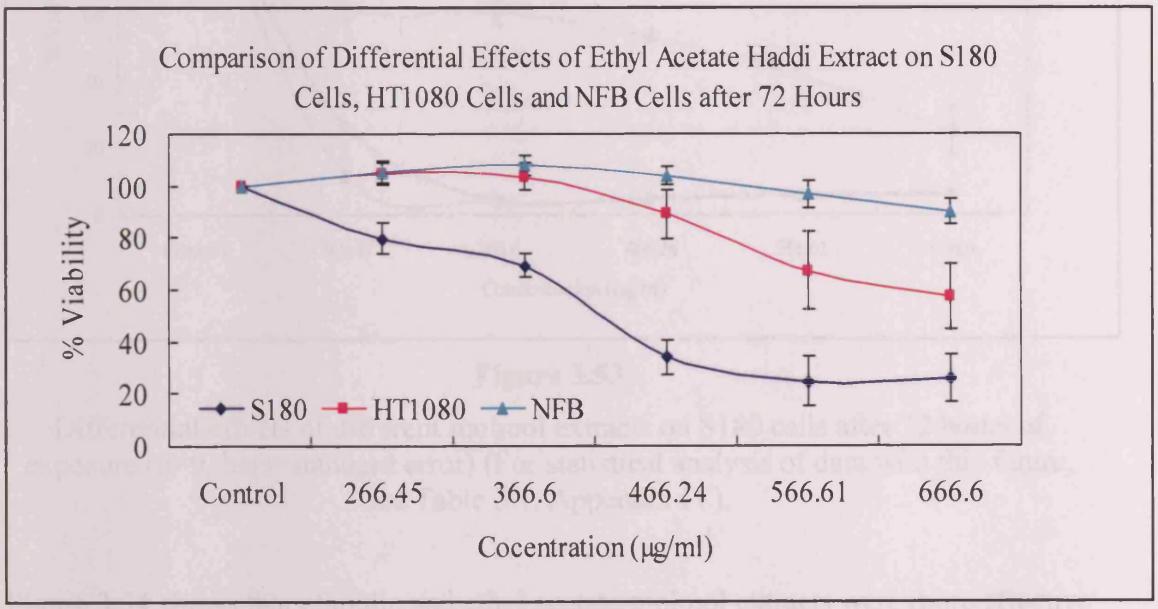


Figure 3.52

Differential effects of ethyl acetate haddi extracts on S180, HT1080 and normal fibroblast cell line after 72 hours of exposure (n=9, bars=standard error) (For statistical analysis of data with this figure, see Table 2.3, Appendix IV).

3.6 Comparison of Differential Effects of Molmol Extract on S180, HT1080 and Normal Fibroblast Cells

Figure 3.53 illustrates that ethanolic molmol and ethyl acetate molmol extracts were most effective, in terms of their apparent cytotoxicity, against S180 cells, using different concentrations. Percentage viabilities were very low, below 4.0% when treated with extracts containing between 366.6 and 666.6 μg/ml. The hexane molmol extract was less effective than ethanolic and ethyl acetate molmol extracts ($P<0.001$). Hence, S180 cells are more sensitive to ethanolic and ethyl acetate molmol extracts than to hexane molmol extract.

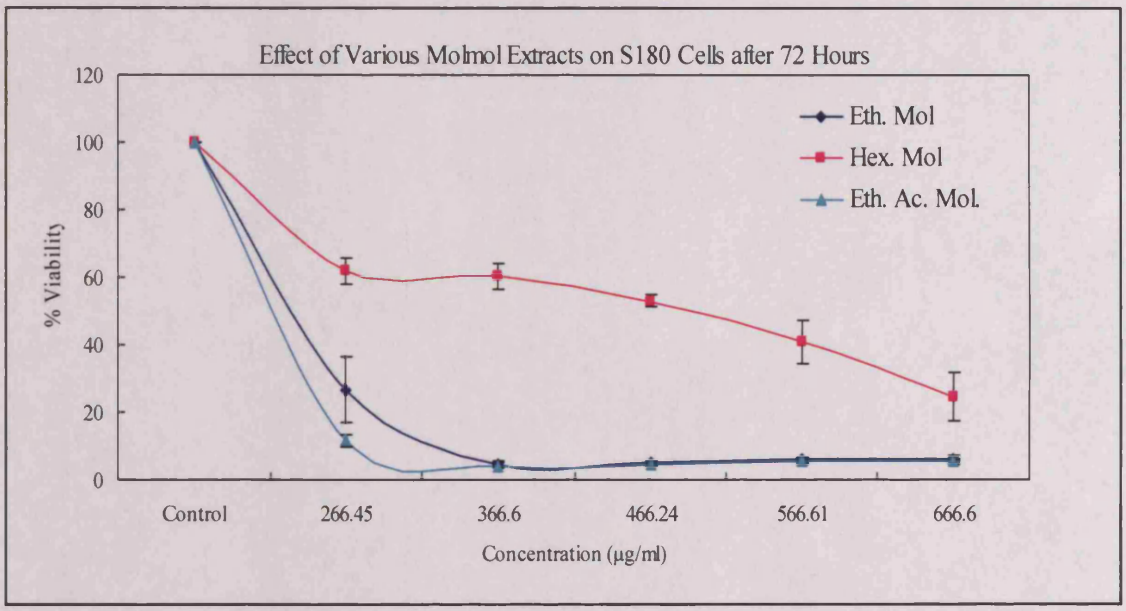


Figure 3.53

Differential effects of different molmol extracts on S180 cells after 72 hours of exposure (n=9, bars=standard error) (For statistical analysis of data with this figure, see Table 3.1, Appendix IV).

Figure 3.54 shows that ethnolic and ethyl acetate molmol extracts were more effective against HT1080 cells than hexane molmol extract ($P<0.001$). However, HT1080 cells were sensitive to ethanolic molmol extract, though less sensitive than S180 cells to this extract.

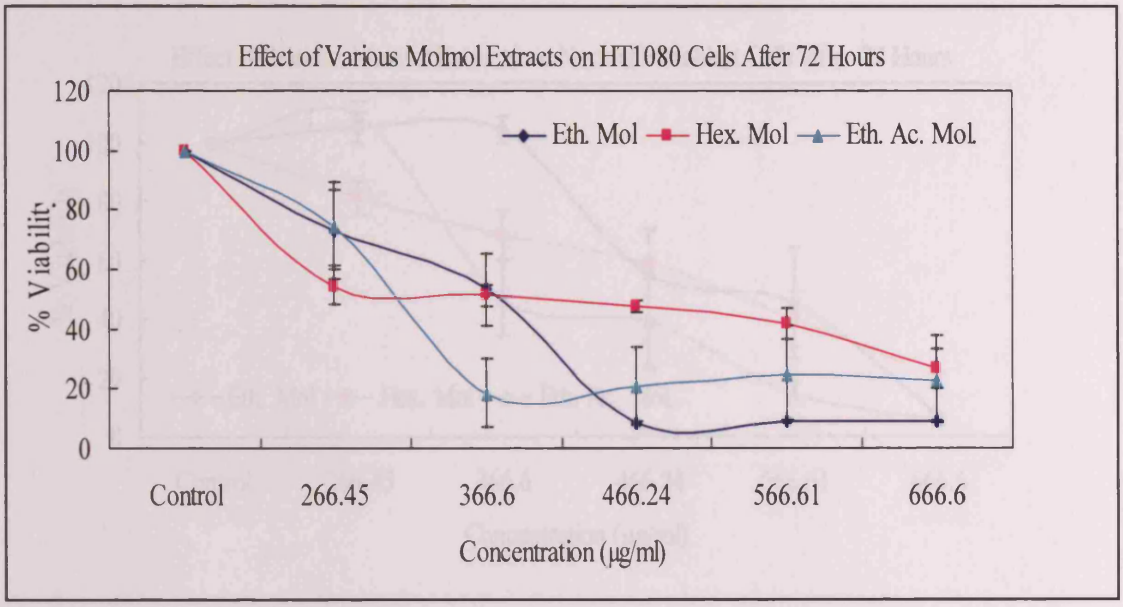


Figure 3.54

Differential effects of different molmol extracts on HT1080 cells after 72 hours of exposure (n=9, bars=standard error) (For statistical analysis of data with this figure, see Table 3.2, Appendix IV).

Normal fibroblast cells showed increased viability when treated with ethanolic and ethyl acetate molmol extracts at lower concentrations, even indicating mitogenetic effects at such low concentrations. However, they were very sensitive to all concentrations of hexane molmol extracts, and higher concentrations of ethnolic molmol than ethyl acetate molmol extract (Figure 3.55).

Normal fibroblast cells showed increased viability, when treated with ethanolic and ethyl acetate molmol extracts at lower concentrations, even indicating mitogenetic effects at such low concentrations. However, they were very sensitive to all concentrations of hexane molmol extracts, and higher concentrations of ethyl acetate molmol extract than ethanolic molmol extract. (Figure 3.55).

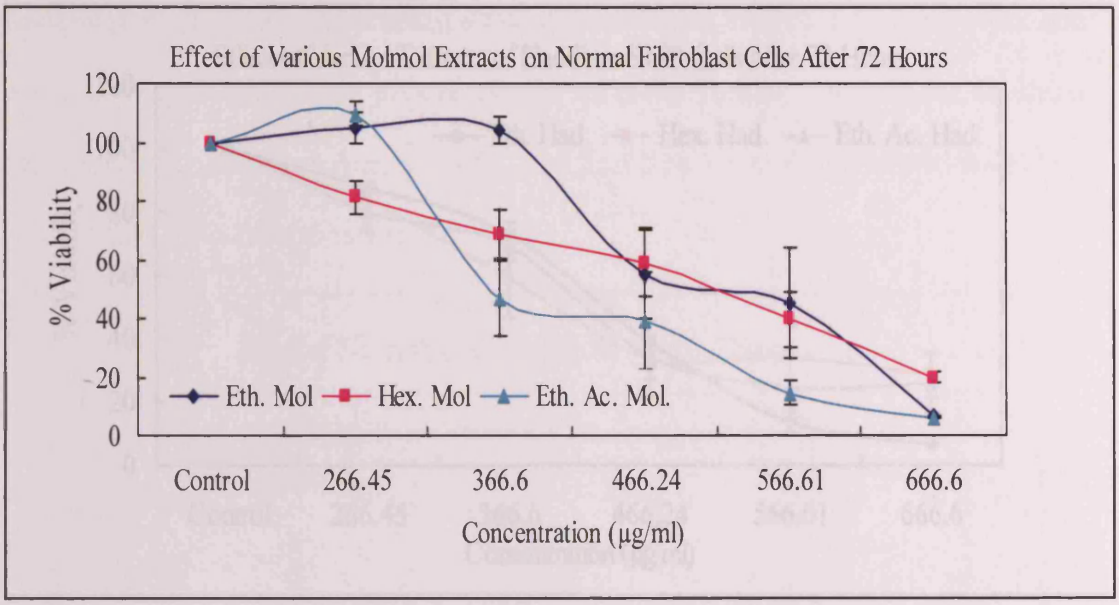


Figure 3.55

Differential effects of different molmol extracts on normal fibroblast cells after 72 hours of exposure (n=9, bars=standard error) (For statistical analysis of data with this figure, see Table 3.3, Appendix IV).

3.7 Comparison of Differential Effects of Haddi Extracts on S180, HT1080 and Normal Fibroblast Cells

S180 cells were sensitive to various extracts of haddi (Figure 3.56), where cell viability declined at all concentrations, especially ethanolic haddi extract at the highest concentration. However, viability was almost similar in the case of the three extracts at the lowest concentration, and which was much higher than at the other higher concentrations.

Figure 3.57 shows that HT1080 cells were less sensitive to ethanolic haddi extract than other haddi extracts, in contrast to its apparent cytotoxic effect on S180 cells (Figure 3.56). Ethyl acetate haddi extract had a mitogenic effect at the two lower concentrations, but was more effective than hexane haddi extracts at the two highest concentrations.

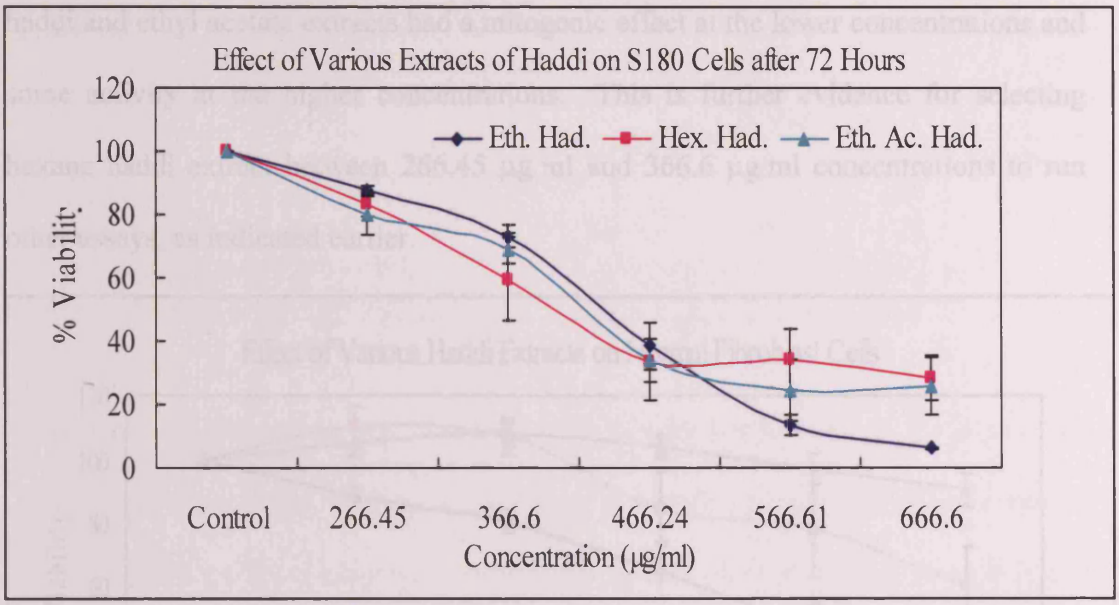


Figure 3.56

Differential effects of different haddi extracts on S180 cells after 72 hours of exposure (n=9, bars=standard error) (For statistical analysis of data with this figure, see Table 4.1, Appendix IV).

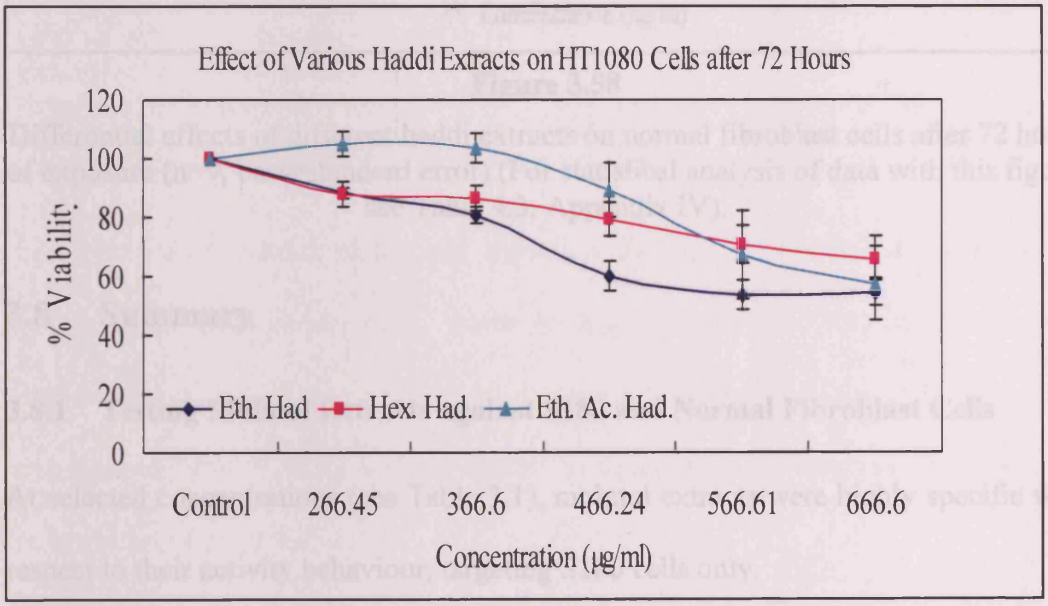


Figure 3.57

Differential effects of different haddi extracts on HT1080 cells after 72 hours of exposure (n=9, bars=standard error) (For statistical analysis of data with this figure, see Table 4.2, Appendix IV).

Normal fibroblast cells (Figure 3.58) were more sensitive to ethanolic haddi extract than to the other two extracts, especially at higher concentrations ($P<0.001$). Hexane

haddi and ethyl acetate extracts had a mitogenic effect at the lower concentrations and some activity at the higher concentrations. This is further evidence for selecting hexane haddi extract between 266.45 µg/ml and 366.6 µg/ml concentrations to run other assays, as indicated earlier.

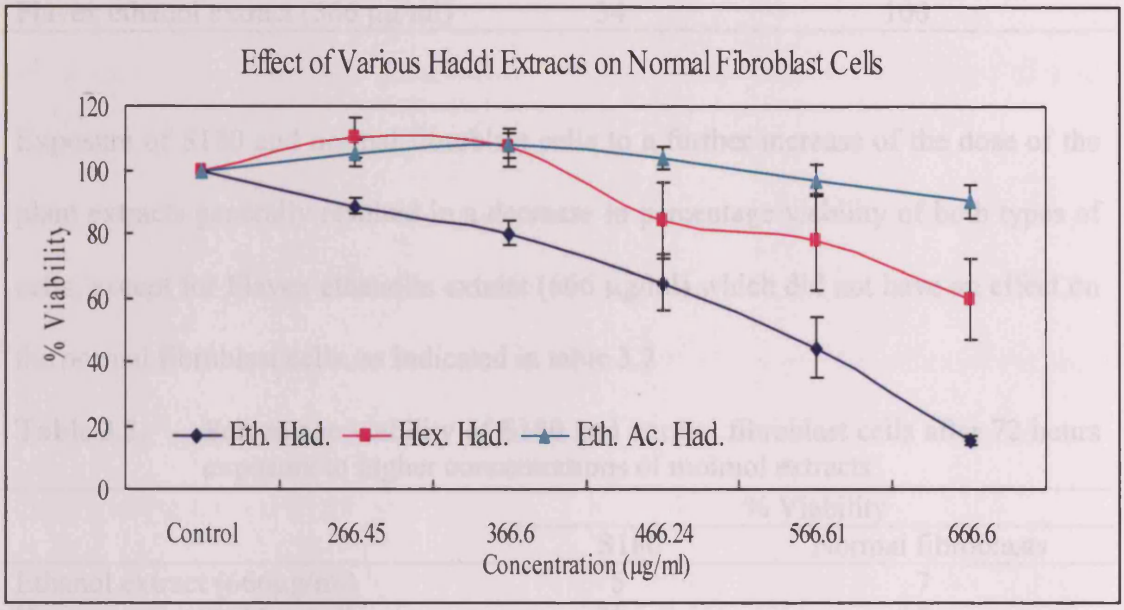


Figure 3.58

Differential effects of different haddi extracts on normal fibroblast cells after 72 hours of exposure (n=9, bars=standard error) (For statistical analysis of data with this figure, see Table 4.3, Appendix IV).

3.8 Summary

3.8.1 Testing Molmol Extracts against S180 and Normal Fibroblast Cells

At selected concentrations (see Table 3.1), molmol extracts were highly specific with respect to their activity behaviour, targeting S180 cells only.

At these concentrations, all the molmol extracts, except hexane extracted molmol, showed potent cytotoxic properties against the S180 murine carcinoma cells, but were not detrimental to the normal fibroblast cells.

Table 3.1. Percentage viability of S180 and normal fibroblast cells after 72 hours exposure to molmol extracts

	% Viability	
	S180	Normal fibroblasts
Ethanol extract (366 μg/ml)	4	100
Hexane extract (366 μg/ml)	63	78
Ethyl acetate extract (266μg/ml)	12	100
Flavex ethanol extract (566 μg/ml)	34	100

Exposure of S180 and normal fibroblast cells to a further increase of the dose of the plant extracts generally resulted in a decrease in percentage viability of both types of cells, except for Flavex ethanolic extract (666 μg/ml) which did not have an effect on the normal fibroblast cells, as indicated in table 3.2

Table 3.2. Percentage viability of S180 and normal fibroblast cells after 72 hours exposure to higher concentrations of molmol extracts

	% Viability	
	S180	Normal fibroblasts
Ethanol extract (666μg/ml)	5	7
Hexane extract (666μg/ml)	20	27
Ethyl acetate extract (366 μg/ml)	4	48
Flavex ethanol extract (666 μg/ml)	24	100

Only the Flavex extract of molmol showed a differential concentration (666μg/ml), which killed S180 cancer cells, whilst having no apparent cytotoxic effect on the normal fibroblasts. At the higher concentrations, both the ethanolic and ethyl acetate extracts exhibited a strong activity against S180 and normal fibroblast cells, resulting in lower percentage viability.

An increase in the dose application of molmol ethyl acetate extract from 266 to 366 μg/ml resulted in a sharp increase in cell death in both cell types.

The normal fibroblast cells did not tolerate larger doses of molmol ethanolic extracts (666μg/ml) compared to the nil effect of the 366 μg/ml concentration.

On the basis of these experiments, the ethanol extract of molmol was chosen for further biological testing, using selected bioassays for evaluating programmed cell death (see further results in this chapter relating to these bioassays).

3.8.2 Testing Haddi Extracts against S180 and Normal Fibroblast Cells

At selected concentrations (see Table 3.3), the haddi plant extracts were clearly specific with respect to their activity, targeting S180 cells only.

At these concentrations, only the hexane and ethyl acetate extracts of haddi showed potent cytotoxic properties, specifically targeting the S180 murine carcinoma cells, resulting in comparatively reduced percentage viability. Normal fibroblasts were only affected by ethanolic haddi extract, resulting in 82% viability, whereas other extracts did not target normal fibroblasts

Table 3.3 Percentage viability of S180 and normal fibroblast cells after 72 hours exposure to haddi extracts

	% Viability	
	S180	Normal fibroblasts
Ethanol extract (366µg/ml)	75	82
Hexane extract (366µg/ml)	4	100
Ethyl acetate extract (466µg/ml)	33	100
Essential oil extract (666 µg/ml)	62	100

Increasing the dose of haddi extracts had an apparent cytotoxic effect on the normal fibroblasts, resulting in a low percentage viability. The decrease in cell viability is shown in Table 3.4.

Table 3.4 Percentage viability of S180 and normal fibroblast cells after 72 hours exposure to higher concentrations of haddi extracts

	% Viability	
	S180	Normal fibroblasts
Ethanol extract (666µg/ml)	6	21
Hexane extract (666µg/ml)	4	67
Ethyl acetate extract (666µg/ml)	25	97
Essential oil extract	77	100

Increasing the dose of the plant extracts applied to the S180 cells resulted in a decrease in percentage viability of both the S180 and the normal fibroblast cells, as shown in Table 3.2. At these higher doses, only the ethyl acetate extracts of molmol (666 μg/ml) showed differential concentrations which specifically killed S180 cancer cells whilst having negligible cytotoxic effects on the normal fibroblast cells.

3.8.3 Testing Molmol Extracts against HT1080 and Normal Fibroblast Cells

At selected concentrations, only the ethanolic and ethyl acetate extracts of molmol, showed both potent and specific cytotoxic properties against the HT1080 human fibrosarcoma cells, whereas they did not have detrimental effects on the normal fibroblast cells (see Table 3.5).

Table 3.5 Percentage viability of HT1080 and normal fibroblast cells after 72 hours exposure to molmol extracts

	% Viability	
	HT1080	Normal fibroblasts
Ethanol extract (366μg/ml)	7	100
Hexane extract (366μg/ml)	51	83
Ethyl acetate extract (366μg/ml)	7	100
Flavex ethanol extract (666 μg/ml)	88	100

Increasing the dose of molmol extracts increased the apparent cytotoxic behaviour against HT1080 cells, but decreased the viability of the normal fibroblast cells, as shown in Table 3.6. Although low percentage viability was observed for the ethanolic and ethyl acetate plant extracts, tested at the higher concentrations, their apparent cytotoxic activities were not specific to HT1080 cells, as they also affected normal fibroblast cells.

Table 3.6 Percentage viability of HT1080 and normal fibroblast cells after 72 hours exposure to higher concentrations of molmol extracts

	% Viability	
	HT1080	Normal fibroblasts
Ethanol extract (666µg/ml)	9	7
Hexane extract (366µg/ml)	51	61
Ethyl acetate extract (366µg/ml)	7	6
Flavex ethanol extract (666 µg/ml)	88	100

3.8.4 - Testing Haddi Extracts against HT1080 and Normal Fibroblast Cells

At selected concentrations (see Table 3.7), only the hexane plant extract of haddi showed potent properties against the HT1080 human fibrosarcoma cells. None of the concentrations tested had significant cytotoxic properties against the normal fibroblast cells.

Table 3.7 Percentage viability of HT1080 cells and normal fibroblast cells after 72 hours exposure to haddi extracts

	% Viability	
	HT1080	Normal fibroblasts
Ethanol extract (366µg/ml)	79	87
*Hexane extract (333 and 366µg/ml)	7	100
Ethyl acetate extract (666µg/ml)	54	93
Essential oil extract (666 µg/ml)	80	100

Increasing the dose of haddi extracts increased the cytotoxic potential against HT1080 cells, as well as increasing the incidence of cell death in normal fibroblast cells, as shown in Table 3.8. Again, the hexane extract of the haddi showed both potent and somewhat specific cytotoxic targeting of the HT1080 cells.

Table 3.8 Percentage viability of HT1080 cells and normal fibroblast cells after 72 hours exposure to higher concentrations of haddi extracts

	% Viability	
	HT1080	Normal Fibroblasts
Ethanol extract (666µg/ml)	9	12
Hexane extract (666µg/ml)	7	54
Ethyl acetate extract (666µg/ml)	54	93
Essential oil extract (900µg/ml)	52	9

3.9 Haematoxylin-Eosin Staining.

S180, HT1080 and normal fibroblast cell lines were grown exponentially, and treated with either ethanolic molmol or hexane haddi extracts for 6, 12 and 24 hours, as described in Chapter Two, Section 2.10.2

3.9.1 S180 Cells

A. Control Treatment

The control S180 cells were epithelial-like, exhibiting a rounded or polygonal morphology (Figure 3.59A). The cells also appeared to be a confluent monolayer. The cells remained attached to the substratum, using their pseudopodial attachments (Figure 3.59B). The cells were uniformly stained, and nuclei appeared healthy.

B. Ethanolic Molmol Extract

The earliest changes in S180 cells during apoptosis were visible within 6 hours of treatment with ethanolic molmol. In cells at this stage of treatment, the cytoplasm became condensed, and eventually broke up into several membrane-bound apoptotic bodies, with smooth surfaces (Figure 3.60). Some dying cells showed pyknotic nuclei. Some of the apoptotic bodies consisted mainly of nuclear fragments (deeply stained), while others consisted of plasma membrane surrounding cytoplasm (lightly stained).

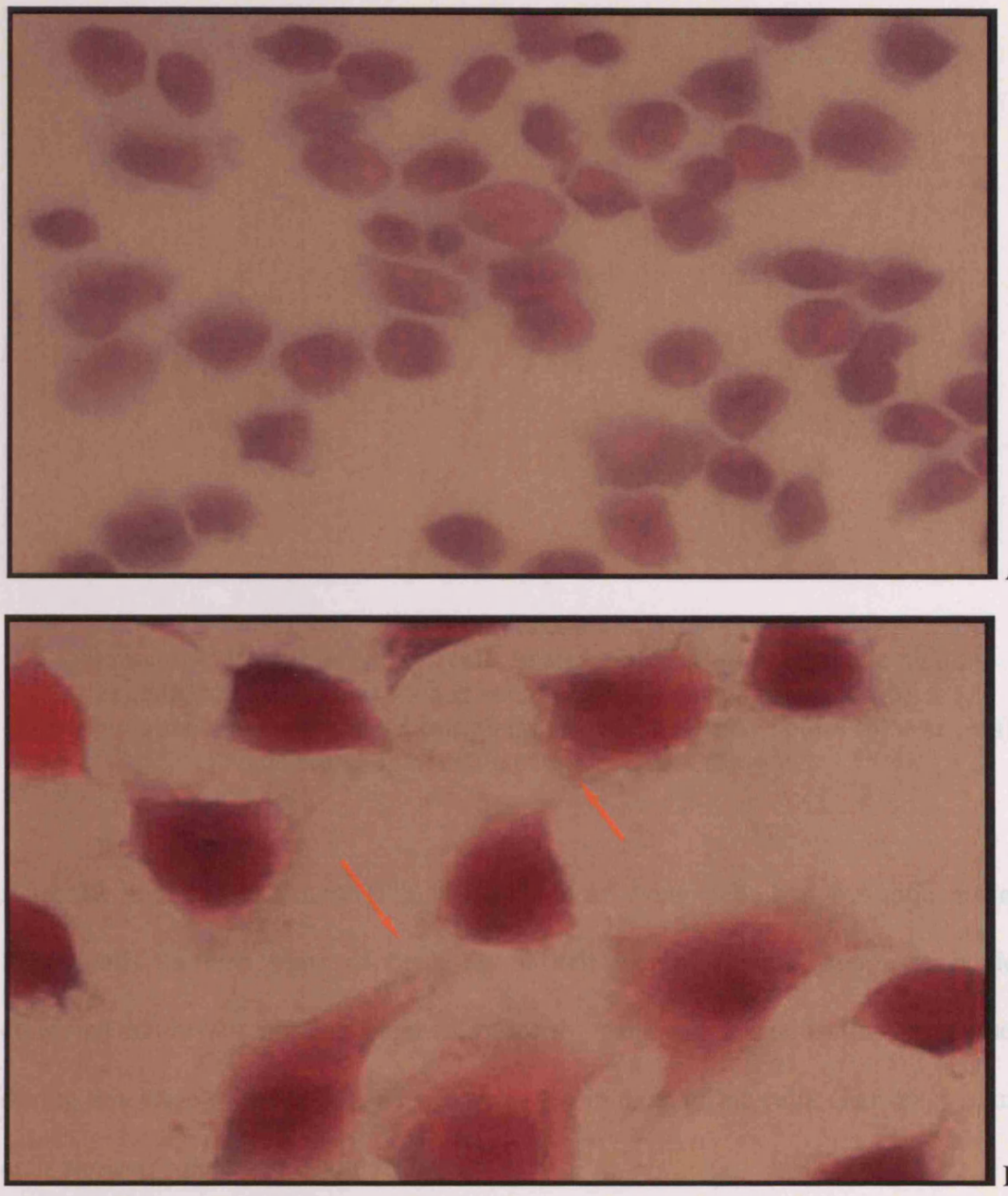


Figure 3.59
Light microscopy images of S180 cells stained with haematoxylin and eosin.
(A: X300, B: X750). (Orange arrows: Pseudopodia).

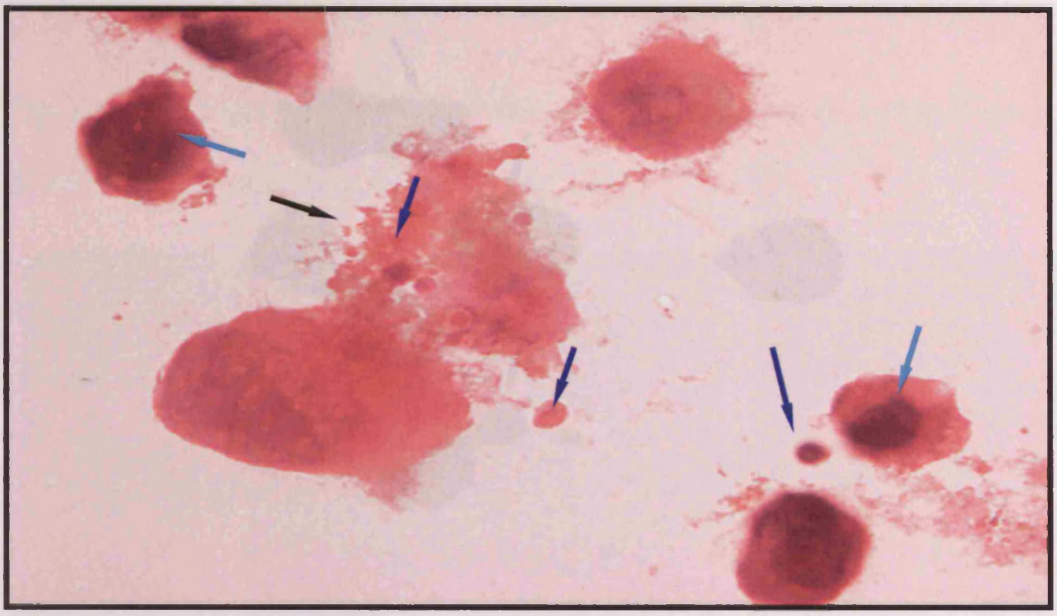


Figure 3.60
Light microscopy images of S180 cells treated with 234μg/ml ethanolic molmol extract for 6 hours and stained with haematoxylin and eosin (X750).
Dying cells show pyknotic nuclei (turquoise arrows), apoptotic bodies (blue arrows) and break-up of cell membrane (black arrow)

After 12 hours of treatment (Figure 3.61), most dying cells had pyknotic nuclei. Some cells showed signs of break-up of cell membrane, and apoptotic bodies consisted mainly of nuclear fragments (deeply stained). Vacuolisation progressed during this stage of treatment and was observed in most of the cells. However, some cells showed condensed cytoplasm.

After 24 hours of treatment (Figure 3.62), most of the dying cells had pyknotic nuclei, and were highly vacuolated. Apoptotic cells broke up, forming membrane-bound apoptotic bodies, consisting of either nuclear fragments (deeply stained) or only plasma membrane surrounding cytoplasm.

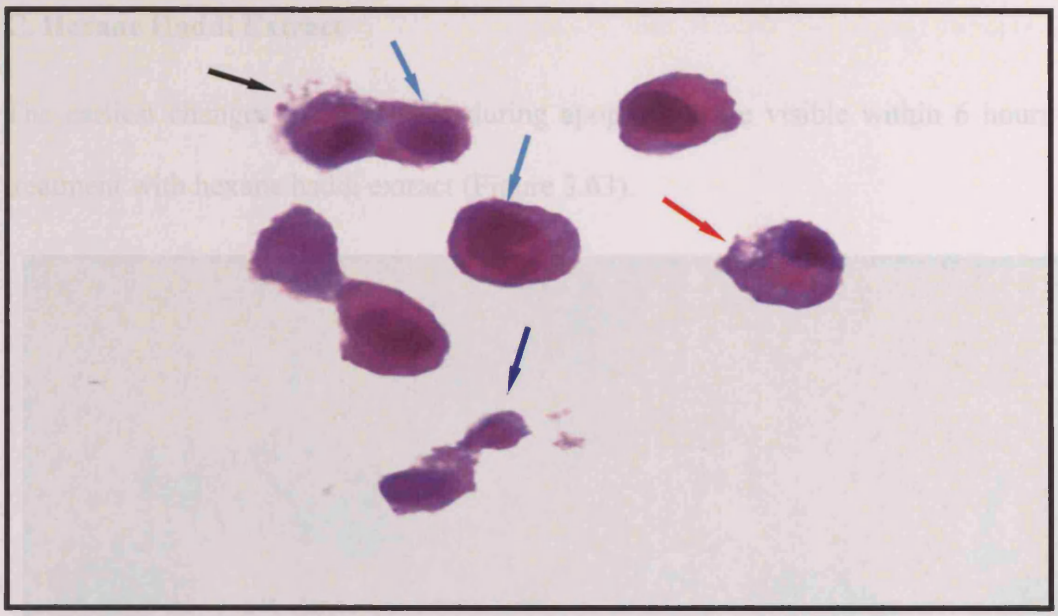


Figure 3.61
Light microscopy images of S180 cells treated with 234µg/ml ethanolic molmol extract for 12 hours and stained with haematoxylin and eosin (X750).
Dying cells show pyknotic nuclei (turquoise arrows), apoptotic bodies (blue arrows), break-up of cell membrane (black arrow) and vacuolisation (red arrow)

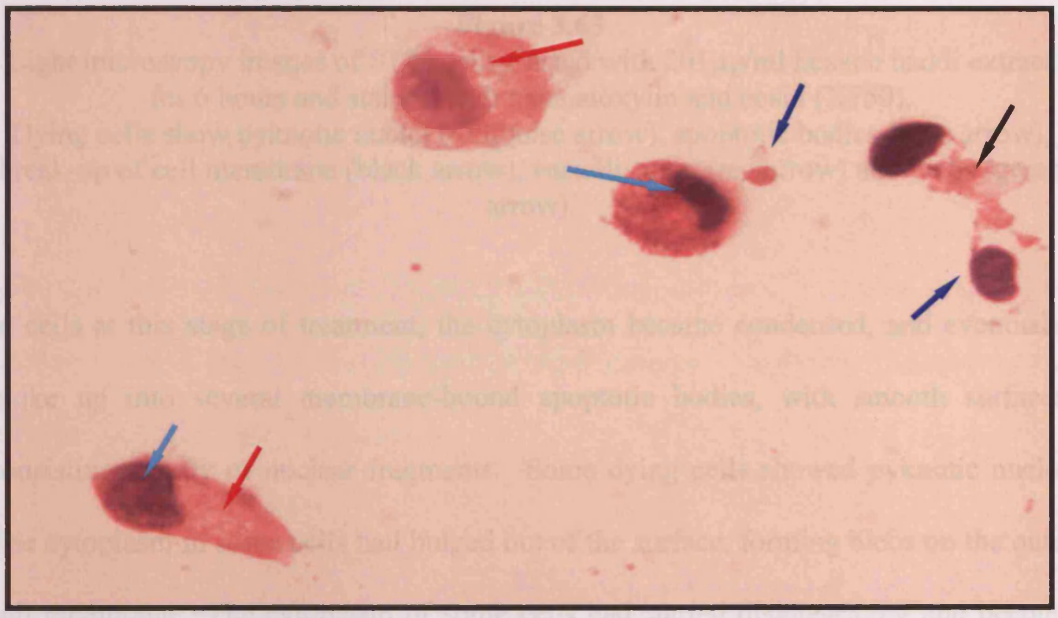


Figure 3.62
Light microscopy images of S180 cells treated with 666.6µg/ml ethanolic molmol extract for 24 hours and stained with haematoxylin and eosin (X750).
Dying cells show pyknotic nuclei (turquoise arrows), apoptotic bodies (blue arrows), break-up of cell membrane (black arrow) and vacuolisation (red arrow)

C. Hexane Haddi Extract

The earliest changes in S180 cells during apoptosis were visible within 6 hours of treatment with hexane haddi extract (Figure 3.63).

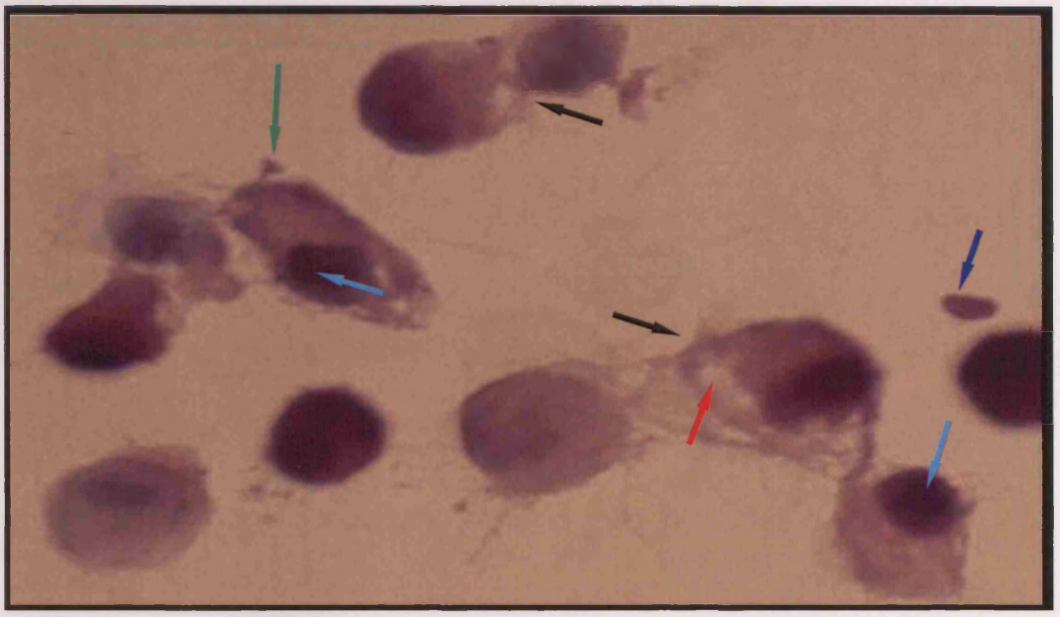


Figure 3.63
Light microscopy images of S180 cells treated with 201µg/ml hexane haddi extract for 6 hours and stained with haematoxylin and eosin (X750).
Dying cells show pyknotic nuclei (turquoise arrow), apoptotic bodies (blue arrow), break-up of cell membrane (black arrow), vacuolisation (red arrow) and blebs (green arrow).

In cells at this stage of treatment, the cytoplasm became condensed, and eventually broke up into several membrane-bound apoptotic bodies, with smooth surfaces, consisting mainly of nuclear fragments. Some dying cells showed pyknotic nuclei. The cytoplasm in some cells had bulged out of the surface, forming blebs on the outer cell membrane. The cytoplasm of some cells had started disintegrating and become vacuolated.

After 12 hours of treatment (Figure 3.64), dying cells had pyknotic nuclei, some showing signs of break-up of cell membrane, and apoptotic bodies consisting mainly

of nuclear fragments (deeply stained) were formed. Vacuolisation was observed in some cells, whereas others had condensed cytoplasm.

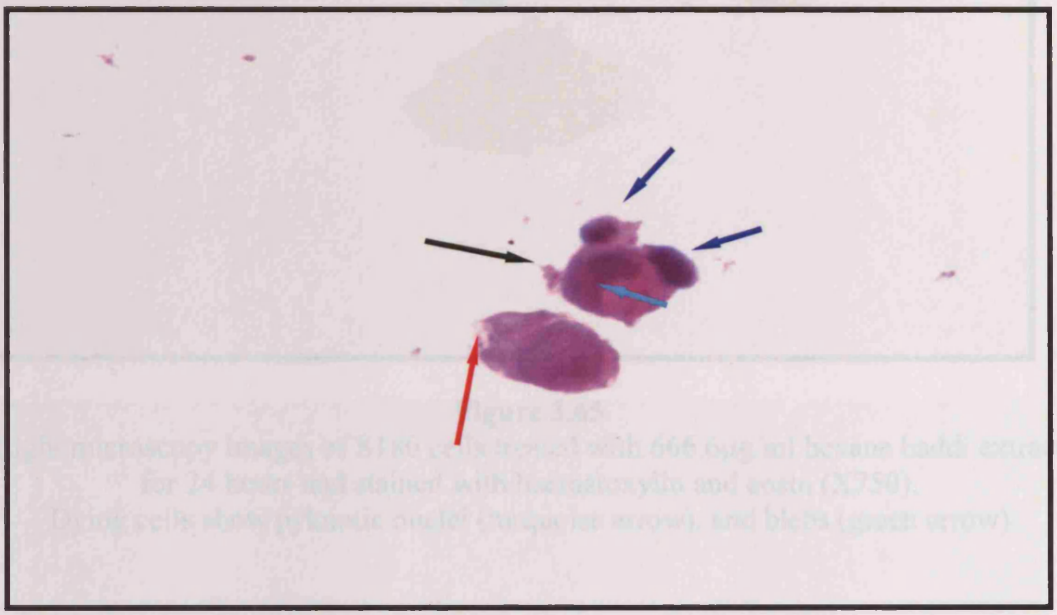


Figure 3.64
Light microscopy images of S180 cells treated with 666.6µg/ml hexane haddi extract for 12 hours and stained with haematoxylin and eosin (X750).
Dying cells show pyknotic nuclei (turquoise arrow), apoptotic bodies (blue arrow), break-up of cell membrane (black arrow) and vacuolisation (red arrow)

After 24 hours of treatment (Figure 3.65), dying cells had pyknotic nuclei, and showed signs of cytoplasm disintegration (light staining with eosin). Apoptotic cells had blebs on their outer membrane.

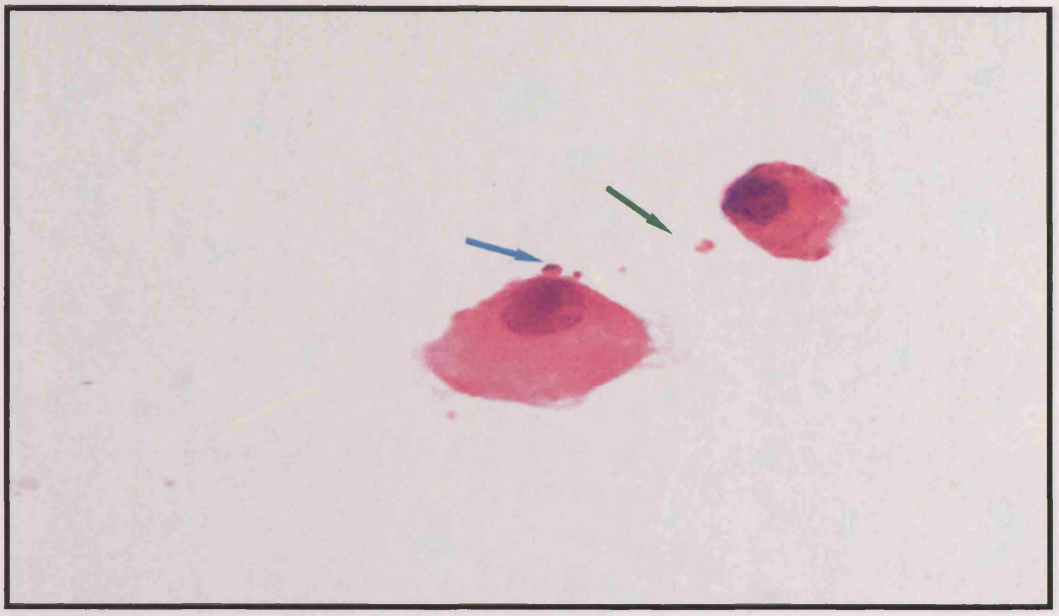


Figure 3.65
Light microscopy images of S180 cells treated with 666.6µg/ml hexane haddi extract for 24 hours and stained with haematoxylin and eosin (X750).
Dying cells show pyknotic nuclei (turquoise arrow), and blebs (green arrow)

3.9.2 HT1080 Cells

A. Control Treatment

The control HT1080 cells examined were epithelial-like, exhibiting polygonal morphology. The cells appeared as a confluent monolayer, remaining attached to the substratum by their pseudopodial attachments (Figure 3.66A). The nuclei appeared rounded or ellipsoidal. Cells undergoing mitosis could also be observed in the culture (Figure 3.66B)

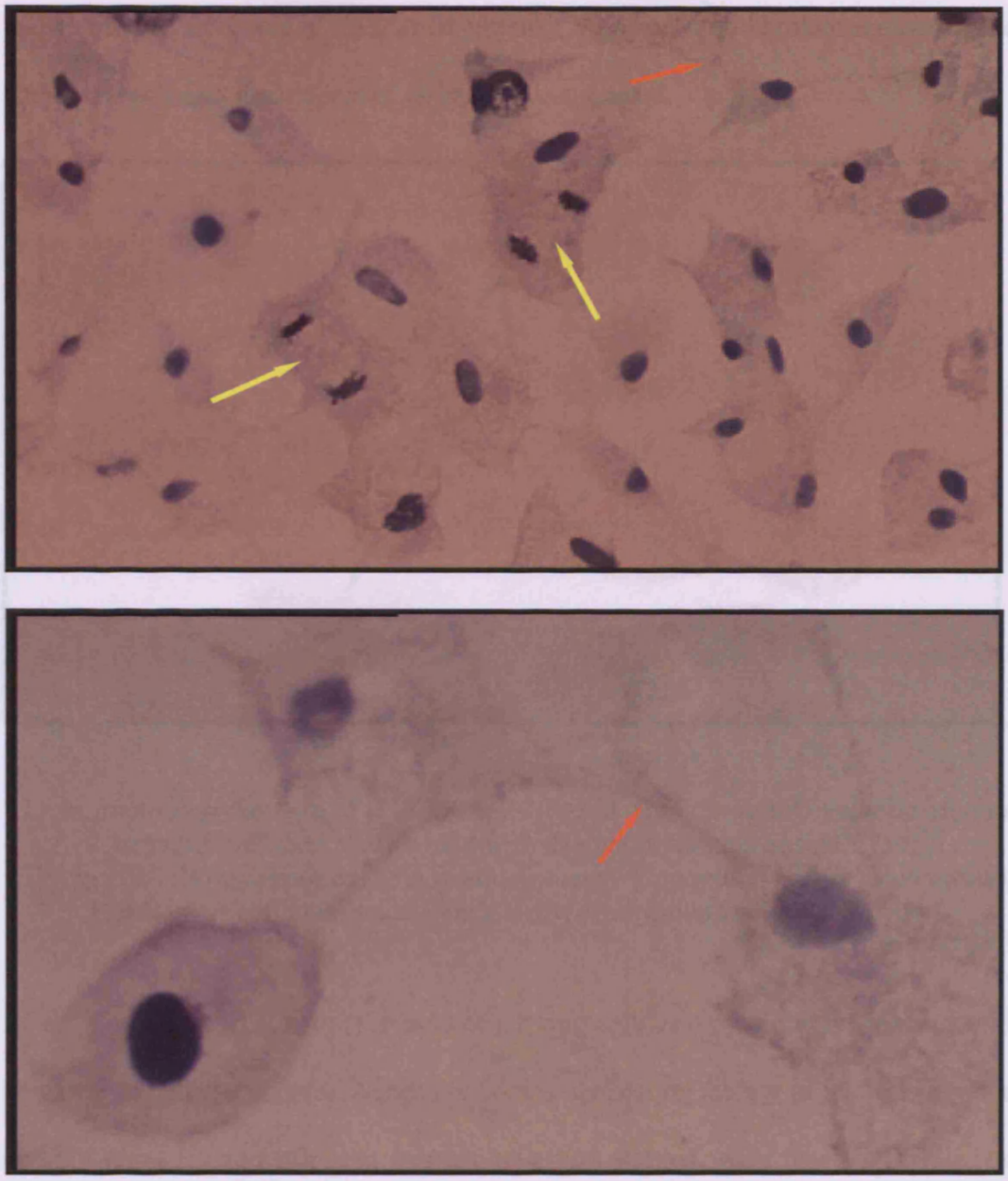


Figure 3.66
Light microscopy images of HT1080 cells stained with haematoxylin and eosin. (A: X300, B: X750). (Yellow arrows: mitosis; Orange arrows: pseudopodia).

B. Ethanolic Molmol Extract

The earliest changes in HT1080 cells during apoptosis were visible within 6 hours of treatment with ethanolic molmol (Figure 3.67). Some cells at this stage of treatment had pyknotic nuclei, whereas other nuclear material had disintegrated, and apoptotic

bodies mainly consisted of nuclear fragments. Some cells had broken-up membranes, and cytoplasm had disintegrated, or become vacuolated.

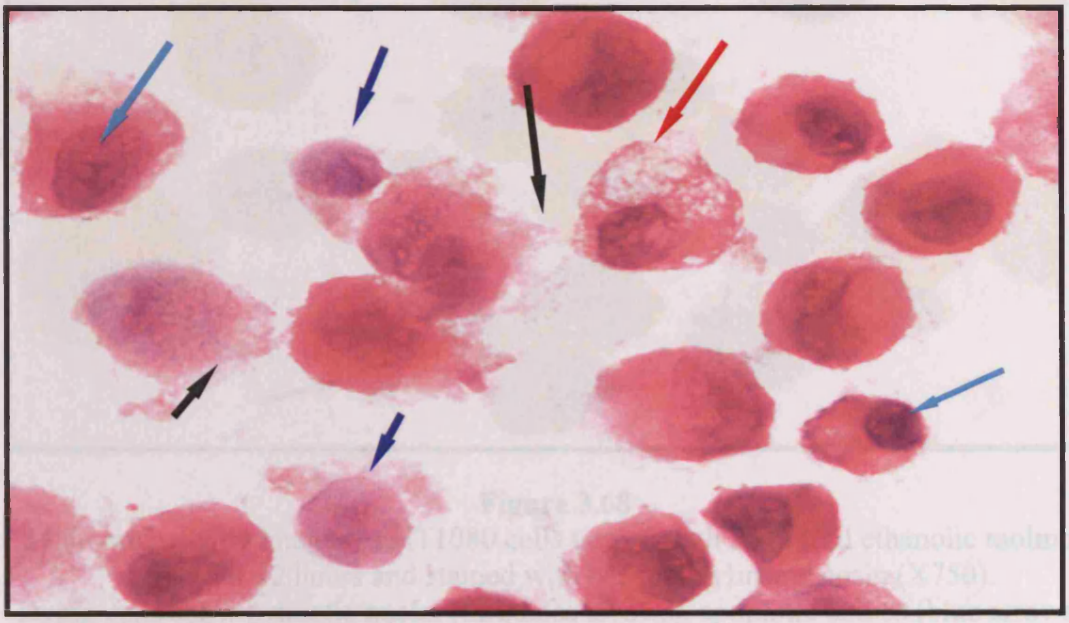


Figure 3.67
Light microscopy images of HT1080 cells treated with 234µg/ml ethanolic molmol extract for 6 hours and stained with haematoxylin and eosin (X750).
Dying cells show pyknotic nuclei (turquoise arrow), apoptotic bodies (blue arrow), break-up of cell membrane (black arrow) and vacuolisation (red arrow).

After 12 hours of treatment (Figure 3.68), dying cells had pyknotic nuclei, some with break-up of cell membrane. Apoptotic bodies, consisting mainly of nuclear fragments (deeply stained) and cytoplasm fragments (lightly stained) were formed. While some cells showed condensed cytoplasm, some of which did not have pyknotic nuclei, others showed signs of cytoplasm disintegration (lightly stained with eosin).

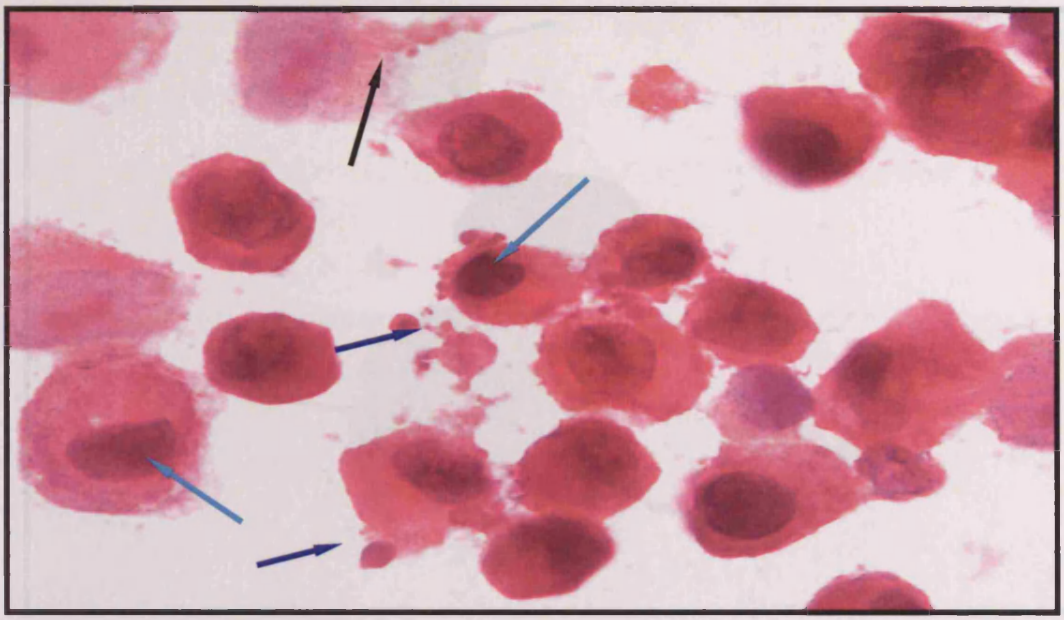


Figure 3.68
Light microscopy images of HT1080 cells treated with 234µg/ml ethanolic molmol extract for 12 hours and stained with haematoxylin and eosin (X750).
Dying cells show pyknotic nuclei (turquoise arrows), apoptotic bodies (blue arrows), and break-up of cell membrane (black arrow)

After 24 hours of treatment with ethanolic molmol (Figure 3.69), the dying cells showed cytoplasmic disintegration with the formation of vacuoles, some with blebs on the outer cytoplasmic membrane. Apoptotic cells with pyknotic nuclei, but no apoptotic bodies, were found.

C. Hexane Haddi Extract

The earliest changes in HT1080 cells during apoptosis were visible within 6 hours of treatment with hexane haddi (Figure 3.70). Dying cells showed pyknotic nuclei. Apoptotic bodies consisted mainly of nuclear fragments (deeply stained). Dying cells also showed cytoplasmic disintegration with the formation of vacuoles, some with blebs on the outer cytoplasmic membrane.

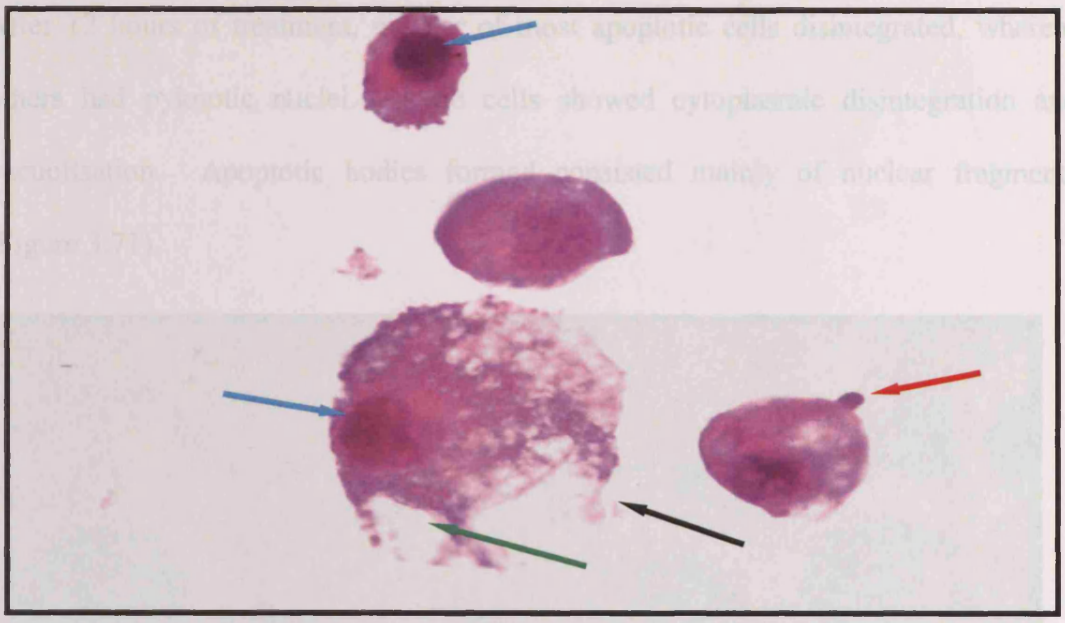


Figure 3.69

Light microscopy images of HT1080 cells treated with 666.6µg/ml ethanolic molmol extract for 24 hours and stained with haematoxylin and eosin (X750).
Dying cells show pyknotic nuclei (turquoise arrows), break-up of cell membrane (black arrow) with high vacuolisation and blebs (green arrow)

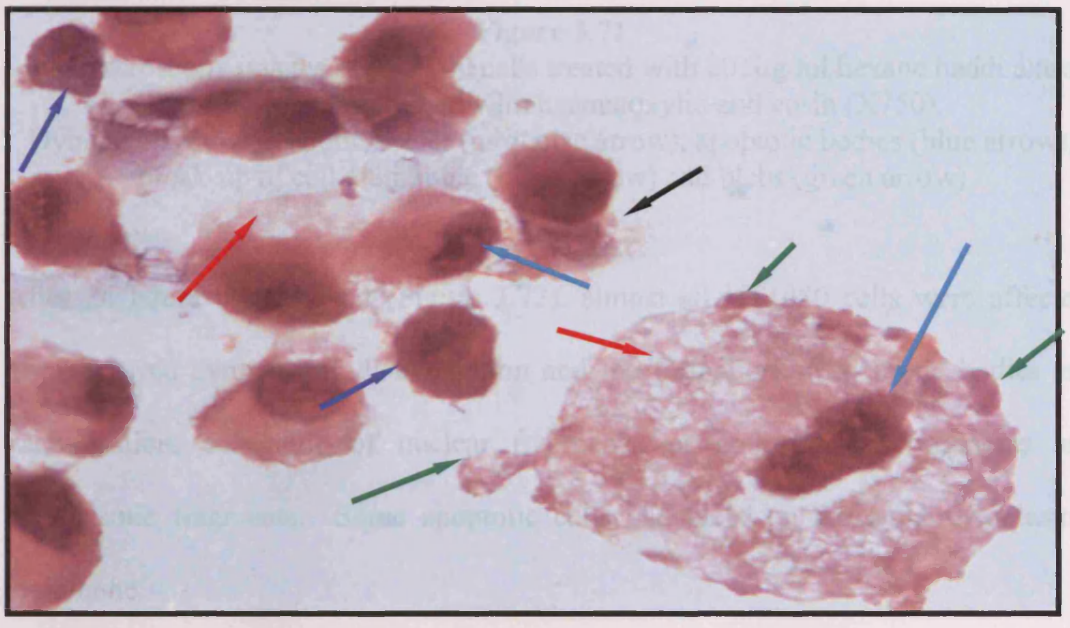


Figure 3.70

Light microscopy images of HT1080 cells treated with 201µg/ml hexane haddi extract for 6 hours and stained with haematoxylin and eosin (X750).
Dying cells show pyknotic nuclei (turquoise arrows), apoptotic bodies (blue arrows), break-up of cell membrane (black arrow) and blebs (green arrow).

After 12 hours of treatment, nuclear of most apoptotic cells disintegrated, whereas others had pyknotic nuclei. Some cells showed cytoplasmic disintegration and vacuolisation. Apoptotic bodies formed consisted mainly of nuclear fragments (Figure 3.71).

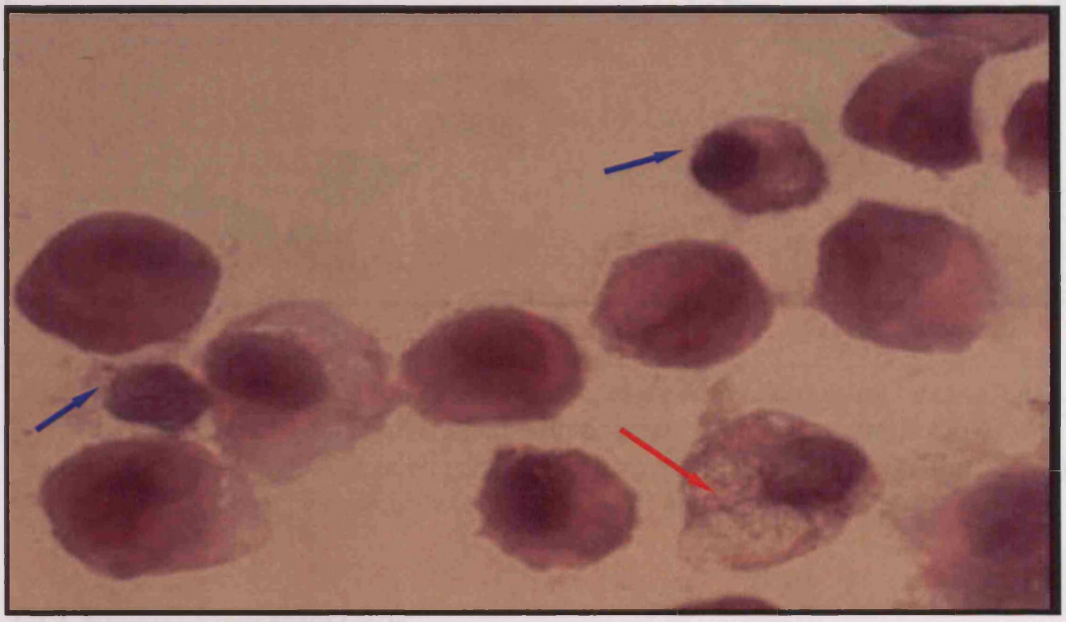


Figure 3.71
Light microscopy images of HT1080 cells treated with 201µg/ml hexane haddi extract for 12 hours and stained with haematoxylin and eosin (X750).
Dying cells show pyknotic nuclei (turquoise arrow), apoptotic bodies (blue arrow), break-up of cell membrane (black arrow) and blebs (green arrow)

After 24 hours of treatment (Figure 3.72), almost all HT1080 cells were affected. They showed cytoplasmic disintegration and the formation of apoptotic bodies and vacuolisation, consisting of nuclear fragments, or both nuclear fragments and cytoplasmic fragments. Some apoptotic cells had blebs on the outer cytoplasmic membrane.

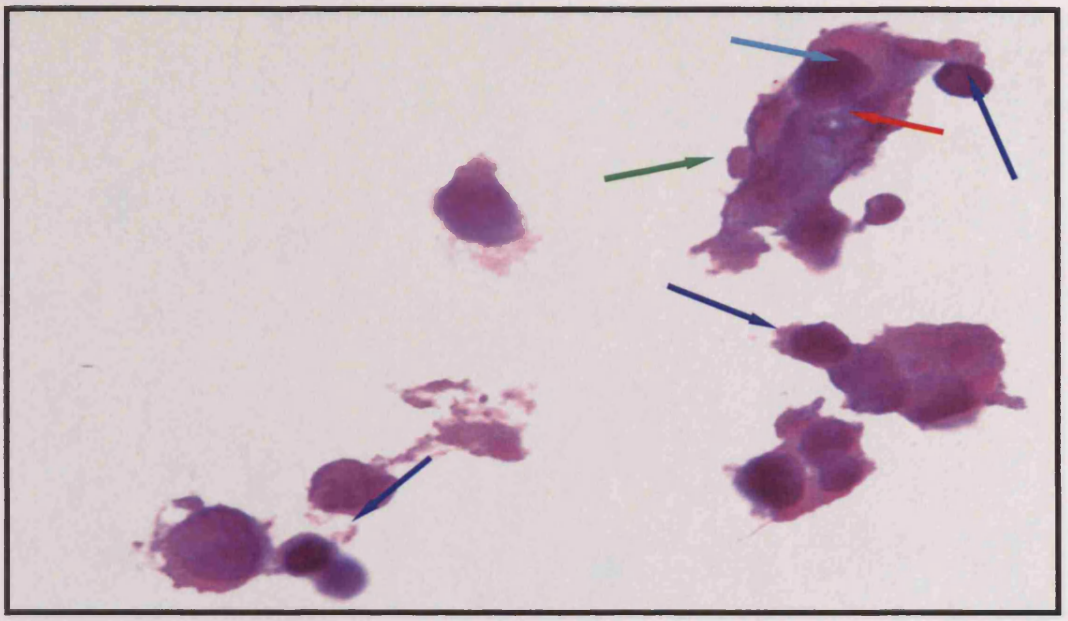


Figure 3.72
Light microscopy images of HT1080 cells treated with 666.6µg/ml hexane haddi extractfor 24 hours and stained with haematoxylin and eosin (X750).
Dying cells show pyknotic nuclei (turquoise arrow), apoptotic bodies (blue arrow), break-up of cell membrane (black arrow) and blebs (green arrow).

3.9.3 Normal Fibroblast Cells

A. Control Treatment

The control normal fibroblast cells examined were epithelial-like, exhibiting polygonal morphology. The cells appeared as a confluent monolayer, remaining attached to the substratum by their pseudopodial attachments (Figure 3.73). The nuclei appeared rounded or ellipsoidal.

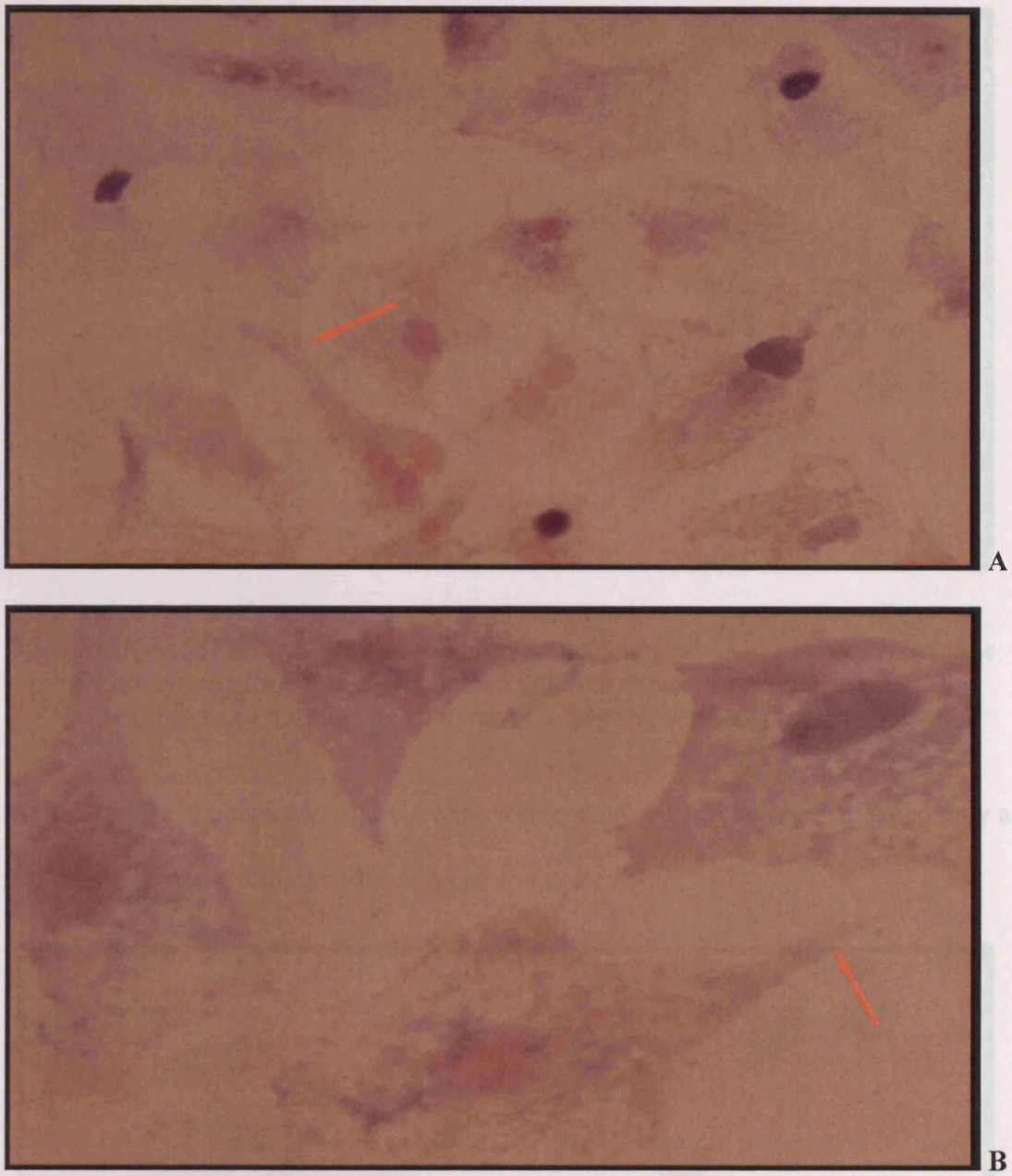


Figure 3.73
Light microscopy images of normal fibroblast cells stained with haematoxylin and eosin.
(A: X300, B: X750). (Orange arrows: pseudopodia).

B. Ethanolic Molmol Extract

After 6 hours of treatment (Figure 3.74), all normal fibroblast cells remained intact and unchanged, all with normal nuclei, some possibly showing mitosis.

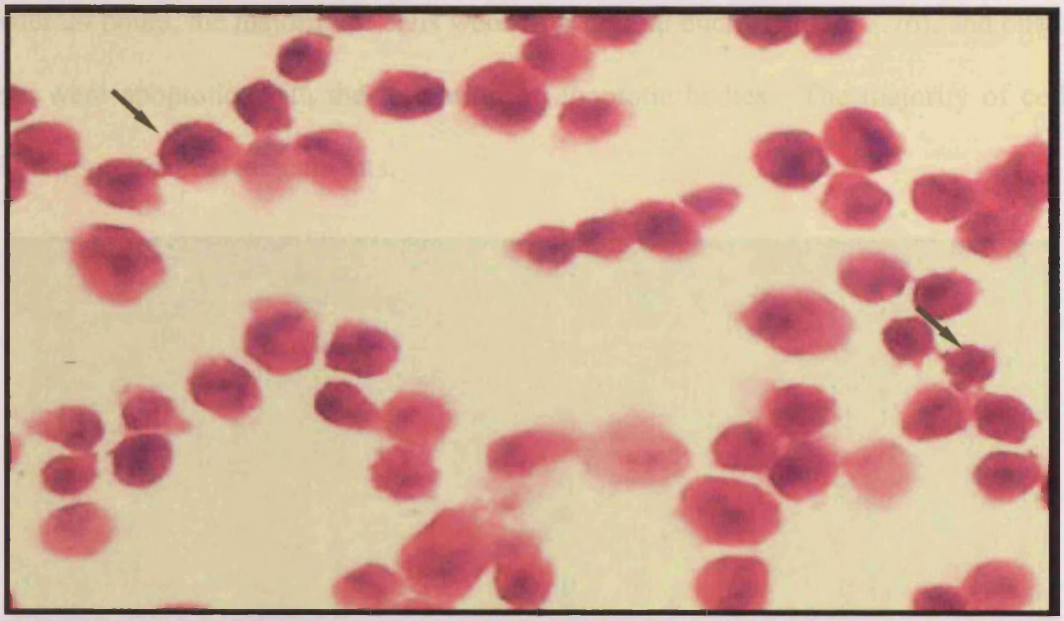


Figure 3.74
Light microscopy images of normal fibroblast cells treated 234µg/ml with ethanolic molmol extract for 6 hours and stained with haematoxylin and eosin (X300). Cells show normal nuclei, some possibly show mitosis (arrows).

After 12 hours, the majority of cells were with normal nuclei (Figure 3.75), and only a few were apoptotic, with the formation of apoptotic bodies.

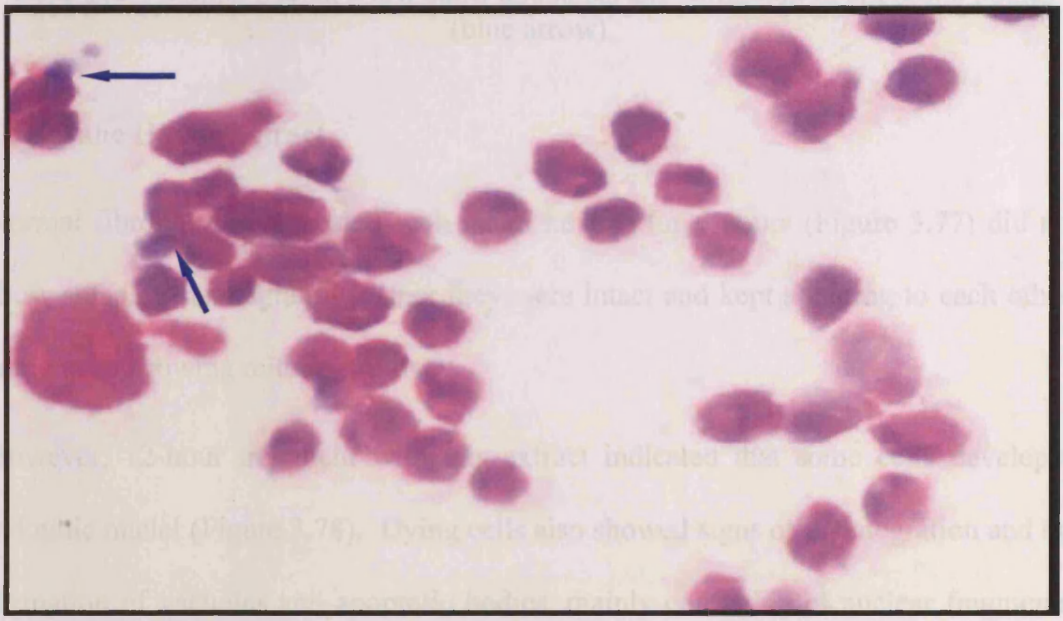


Figure 3.75
Light microscopy images of normal fibroblast cells treated with 234µg/ml ethanolic molmol extract for 12 hours and stained with haematoxylin and eosin (X300). Cells show normal nuclei, a few show apoptosis and formation of apoptotic bodies (blue arrow).

After 24 hours, the majority of cells were with normal nuclei (Figure 3.76), and only a few were apoptotic, with the formation of apoptotic bodies. The majority of cells show pseudopodial attachments.

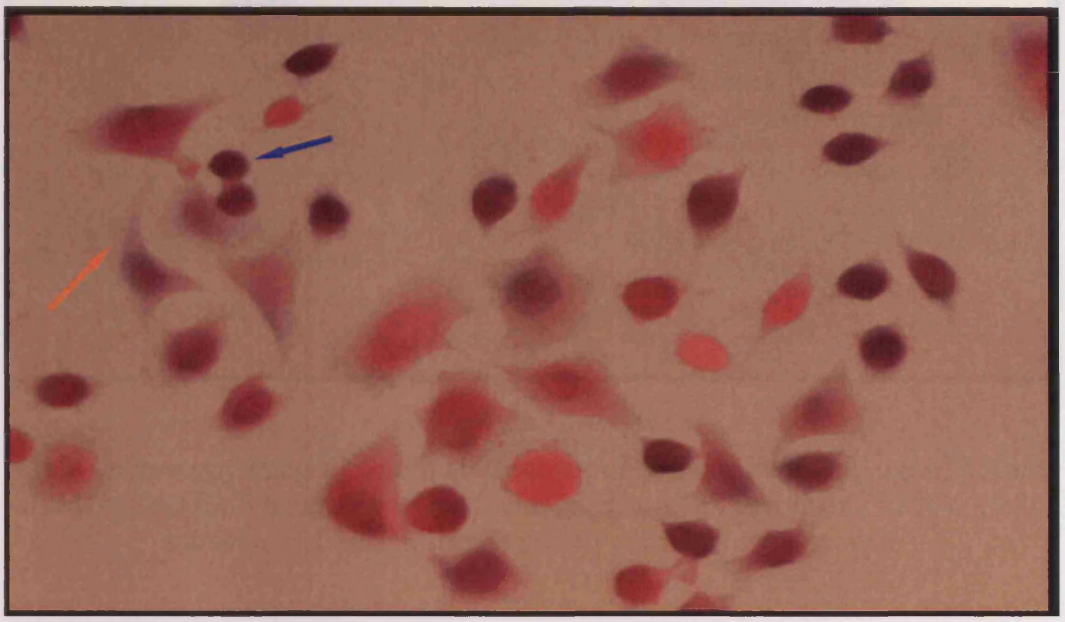


Figure 3.76
Light microscopy images of normal fibroblast cells treated with 234μg/ml ethanolic molmol extract for 24 hours and stained with haematoxylin and eosin (X300). Cells show normal nuclei, a few show apoptosis and formation of apoptotic bodies (blue arrow).

C. Hexane Haddi Extract

Normal fibroblast cells treated with haddi hexane for 6 hours (Figure 3.77) did not show signs of disintegration, rather they were intact and kept adhering to each other, some even showing mitotic activity.

However, 12-hour treatment with this extract indicated that some cells developed pyknotic nuclei (Figure 3.78). Dying cells also showed signs of disintegration and the formation of vacuoles and apoptotic bodies, mainly consisting of nuclear fragments. Some dying cells had blebs on the outer cell membrane.

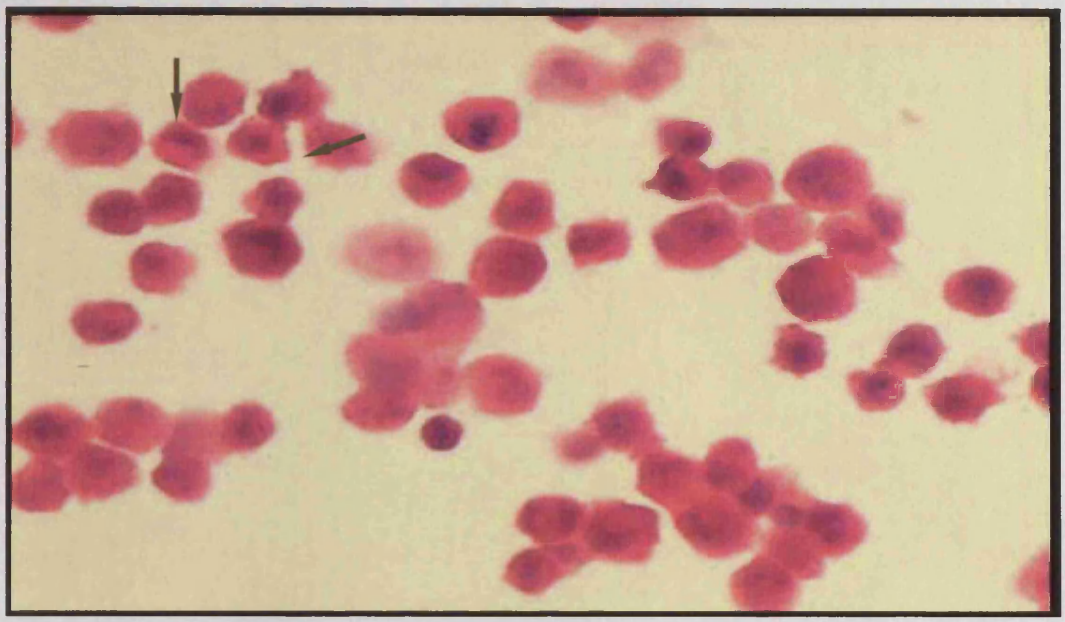


Figure 3.77
Light microscopy images of normal fibroblast cells treated with 201µg/ml hexane haddi extract for 6 hours and stained with haematoxylin and eosin (X300). Cells show normal nuclei, some possibly show mitosis (arrows).

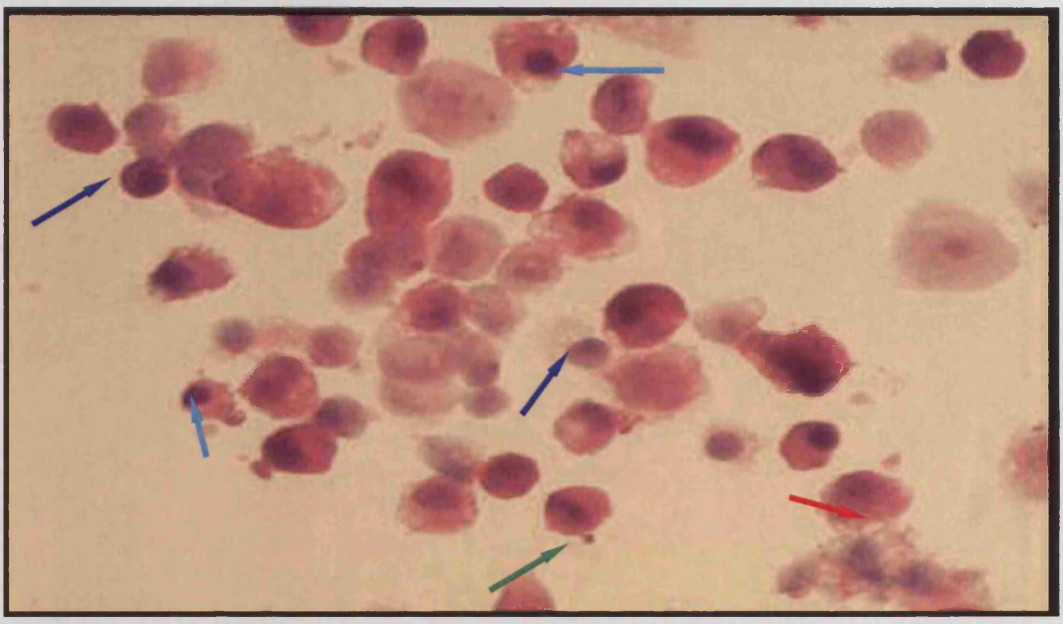


Figure 3.78
Light microscopy images of normal fibroblast cells treated with 201µg/ml hexane haddi extract for 12 hours and stained with haematoxylin and eosin (X300). Cells show normal nuclei, a few show apoptosis with pyknotic nuclei (turquoise arrow), vacuolisation (red arrow), formation of apoptotic bodies (blue arrow) and blebs on the outer cell membrane (green arrow).

24-hours treatment indicated that some cells developed pyknotic nuclei (Figure 3.79). Dying cells also showed signs of disintegration and the formation of vacuoles and apoptotic bodies, mainly consisting of nuclear fragments. Some dying cells had blebs on the outer cell membrane.

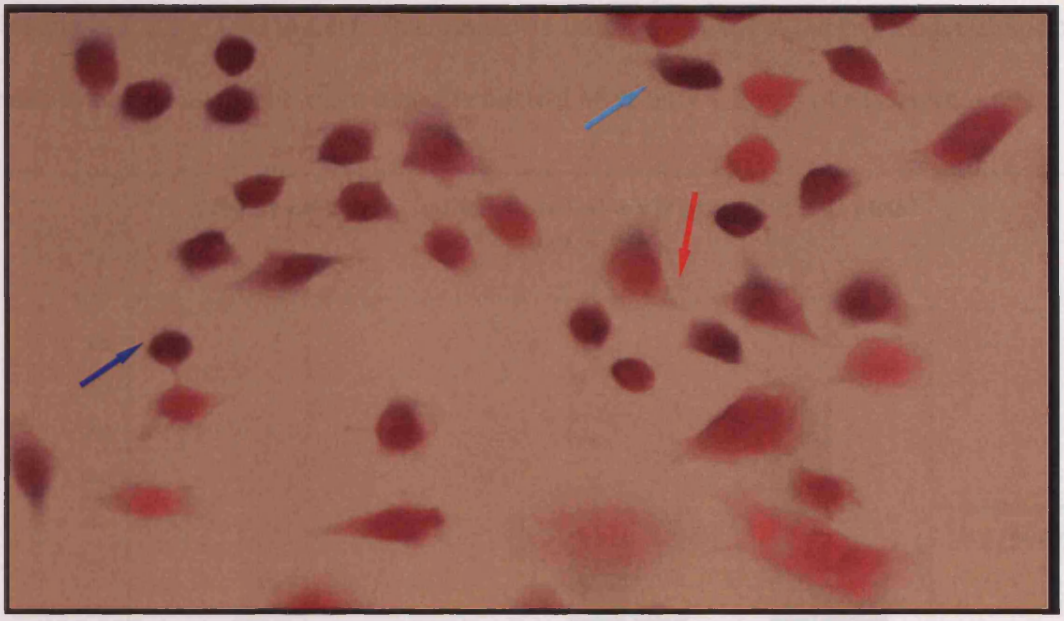


Figure 3.79
Light microscopy images of normal fibroblast cells treated with 201µg/ml hexane haddi extract for 24 hours and stained with haematoxylin and eosin (X300). Cells show normal nuclei, a few show apoptosis with pyknotic nuclei (turquoise arrow), vacuolisation (red arrow), and formation of apoptotic bodies (blue arrow).

3.10 Apoptotic Cells Counting in Culture with Haematoxylin and Eosin Technique

3.10.1 S180 Cells

S180 cells grown in a control culture showed slight occurrence of apoptotic cells, a mean of 2.25% of a total 400 cells counted, in contrast to 97.75% normal cells . However, when these cells were grown in a medium containing 666 µg/ml ethanolic molmol extract, there was a tendency to develop more apoptotic cells with time, increasing from 20.75% cells after 6 hours of incubation to 76.25% after 24 hours.

This tendency was also observed when cells were grown in medium containing 234 µg/ml ethanolic molmol extract, though lower percentages of apoptotic cells were observed, increasing from 12.25% after 6 hours of incubation to 41.75% after 24 hours (Figure 3.80).

Results in Figure 3.80 indicate that ethanolic molmol extract at the two concentrations leads to higher apoptotic rates than the control after only 6 hours of exposure.

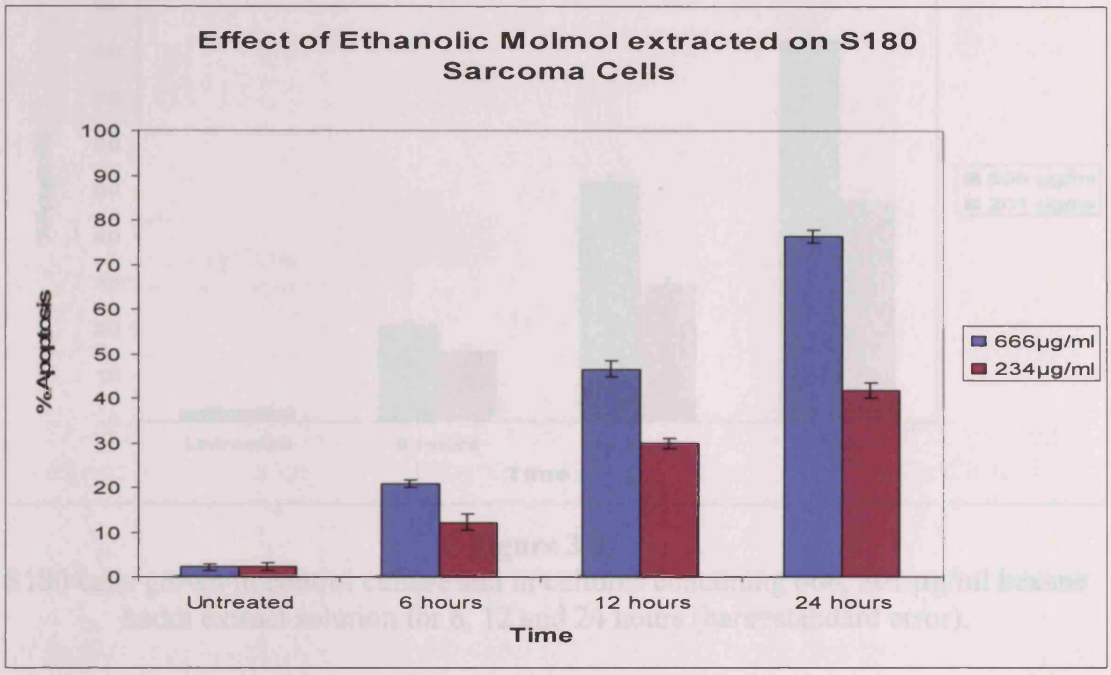


Figure 3.80
S180 cells grown in control culture and in cultures containing 666, 234 µg/ml ethanolic molmol extract solutions for 6, 12 and 24 hours (bars=standard error).

S180 cells grown in a control culture showed a low occurrence of apoptotic cells, a mean of 2.25% of a total 400 cells counted. The remaining 97.75% of cells were non-apoptotic (normal) cells. When S180 cells were grown in a medium containing 666 µg/ml hexane haddi, the percentage of apoptotic cells after 6 hours of exposure (20.75%) was higher than in the control . However, after longer periods of exposure, the percentages of apoptotic cells increased to 51.75% and 82.5% after 12 and 24-hour exposure, respectively (Figure 3.81). When these results are compared with

those of S180 cells grown in medium containing 666 µg/ml ethanolic molmol extract, the percentages of apoptotic cells exposed to hexane haddi extract were higher than those exposed to ethanolic molmol extract after 12 and 24 hours, but equal in both treatments after 6 hours.

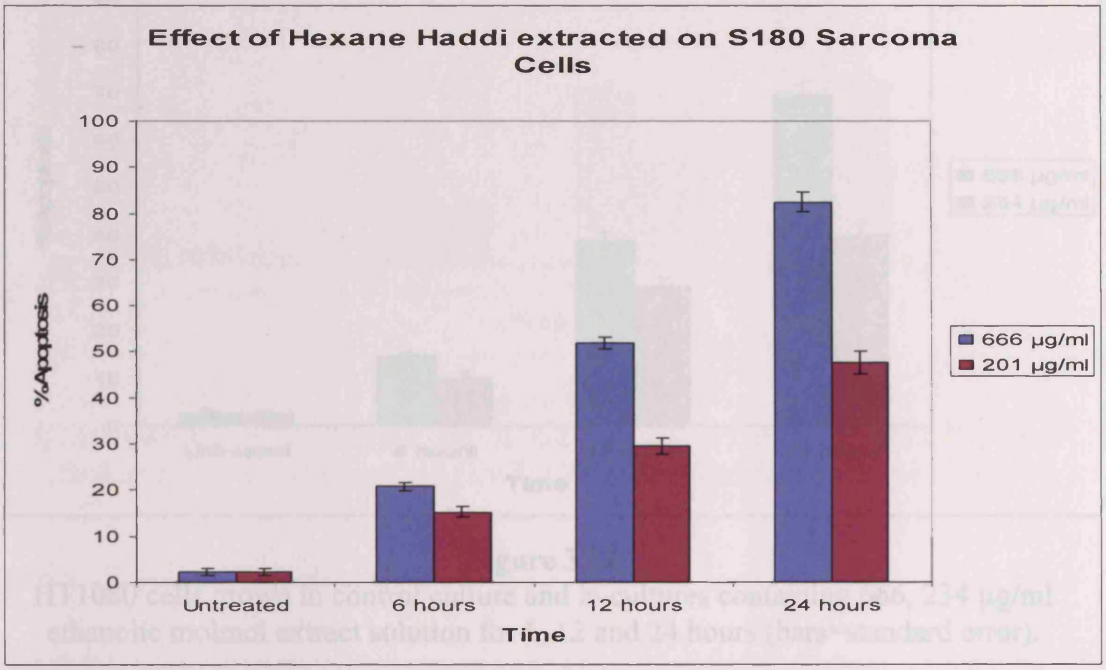


Figure 3.81
S180 cells grown in control culture and in cultures containing 666, 201 µg/ml hexane haddi extract solution for 6, 12 and 24 hours (bars=standard error).

3.10.2 HT1080 Cells

HT1080 cells grown in a control culture showed a slight occurrence of apoptotic cells, a mean of 2.5% of a total 400 cells counted, in contrast to 97.5% normal cells. However, when these cells were grown in a medium containing 666 µg/ml ethanolic molmol extract, there was a tendency to develop more apoptotic cells with time, increasing from 14.5% apoptotic cells after 6 hours of incubation to 69.5% after 24 hours. This tendency was also observed when cells were grown in medium containing 234 µg/ml ethanolic molmol extract, though lower percentages of

apoptotic cells were observed, increasing from 12.25% after 6 hours of incubation to 41.75% after 24 hours (Figure 3.82).

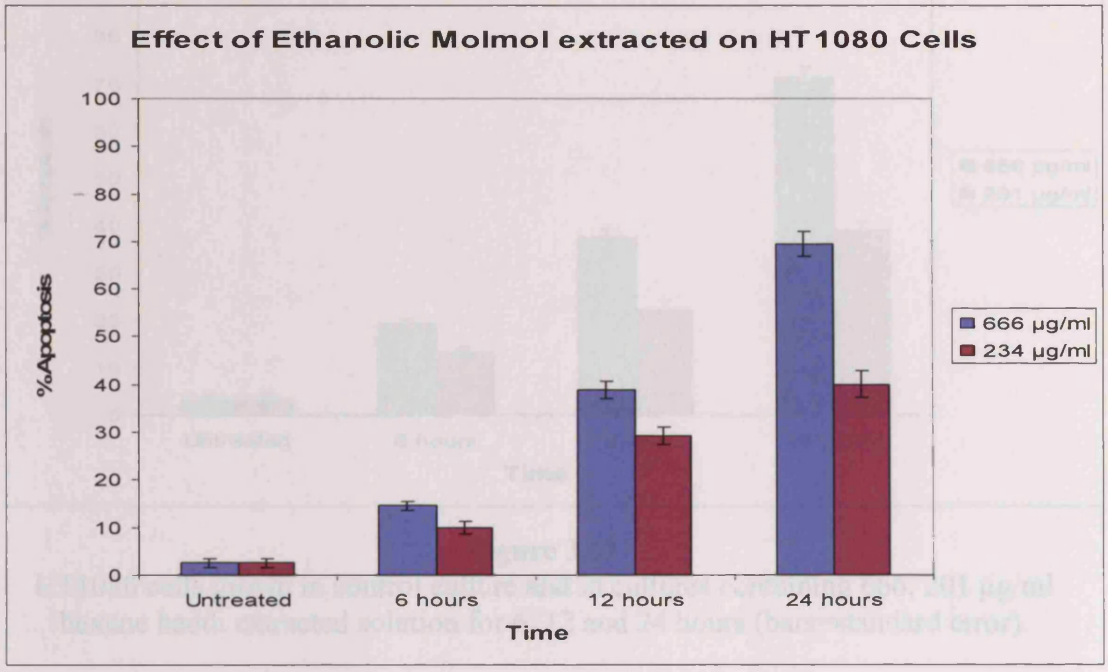


Figure 3.82
HT1080 cells grown in control culture and in cultures containing 666, 234 µg/ml ethanolic molmol extract solution for 6, 12 and 24 hours (bars=standard error).

Results in Figure 3.82 indicate that ethanolic molmol extract at the two concentrations led to higher apoptotic rates than the control after only 6 hours of exposure.

HT1080 cell grown in a control culture showed slight occurrence of apoptotic cells, a mean of 3% of a total 400 cells counted, in contrast to 97% non-apoptotic cells. However, when HT1080 cells were grown in a medium containing 666 µg/ml hexane haddi, there was a tendency to develop more apoptotic cells with time, increasing from 19% apoptotic cells after 6 hours of incubation to 71.25% after 24 hours. A similar observation was noted when cells were grown in media containing 201 µg/ml hexane haddi, though lower percentages of apoptotic cells were observed, increasing from 13.25% after 6 hours of incubation to 39% after 24 hours (Figure 3.83).

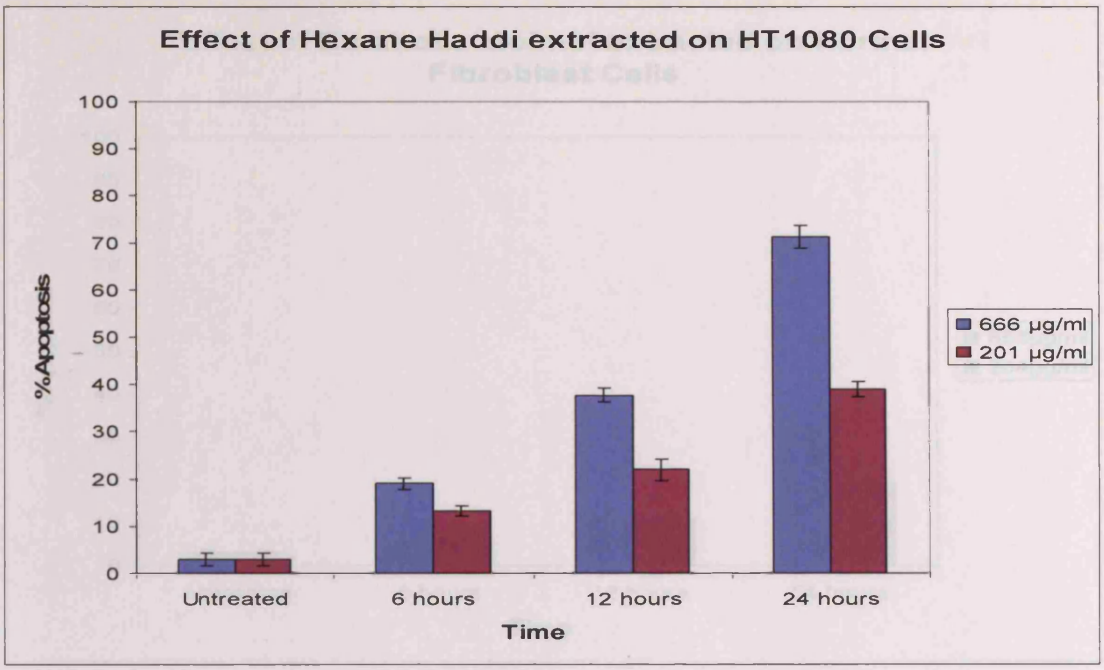


Figure 3.83
HT1080 cells grown in control culture and in cultures containing 666, 201 µg/ml hexane haddi extracted solution for 6, 12 and 24 hours (bars=standard error).

3.10.3 Normal Fibroblast Cells

Normal fibroblast cells grown in a control culture showed slight occurrence of apoptotic cells, a mean of 3% of a total 400 cells counted, in contrast to 97% non-apoptotic cells. When normal fibroblast cells were grown in a medium containing 666 µg/ml ethanolic molmol, the production of apoptotic cells was much less than in S180 and HT1080 cell lines, increasing from 4.5% apoptotic cells after 6 hours of incubation to 32% after 24 hours. A similar observation was noted when cells were grown in media containing 234 µg/ml ethanolic molmol, though lower percentages of apoptotic cells were observed, increasing from 6.25% after 6 hours of incubation to 19.25% after 24 hours (Figure 3.84).

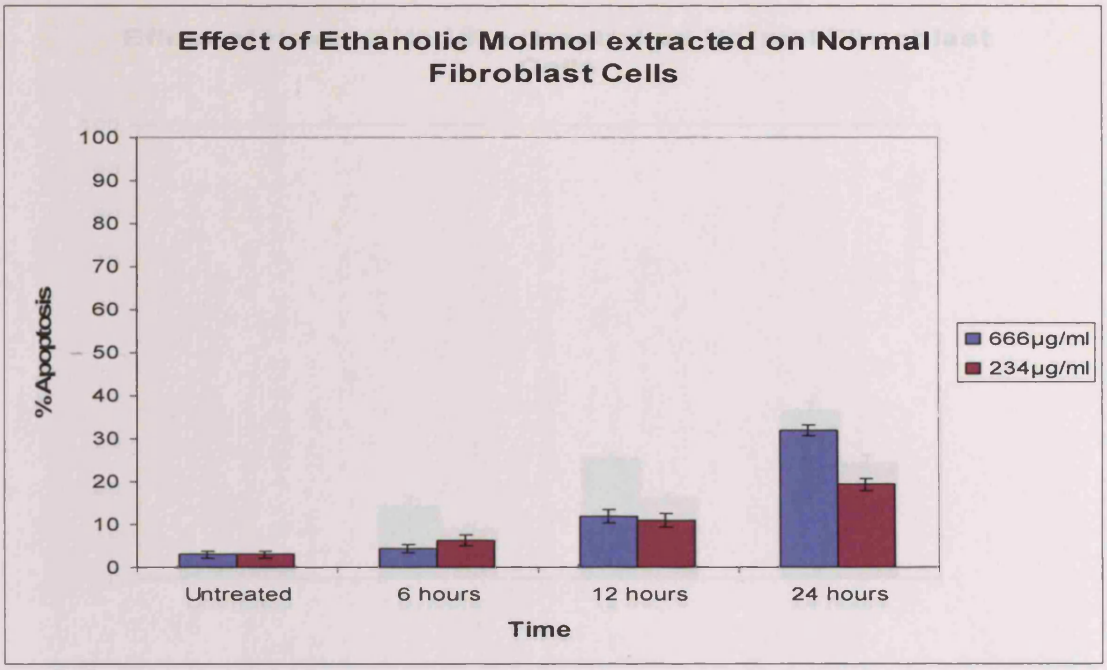


Figure 3.84

Normal fibroblast cells grown in control culture and in cultures containing 666, 234 µg/ml ethanolic molmol extract solution for 6, 12 and 24 hours (bars=standard error).

Normal fibroblast cells grown in a control culture also showed slight occurrence of apoptotic cells, a mean of 2.25% of a total 400 cells counted, in contrast to 97.75% non-apoptotic cells. When normal fibroblast cells were grown in a medium containing 666 µg/ml hexane haddi extract, the production of apoptotic cells was also much less than in S180 and HT1080 cell lines, increasing from 16% apoptotic cells after 6 hours of incubation to 37% after 24 hours. A similar observation was noted when cells were grown in medium containing 201 µg/ml hexane haddi extract, though lower percentages of apoptotic cells were observed, increasing from 11% after 6 hours of incubation to 25.5% after 24 hours (Figure 3.85).

It is noted that hexane haddi extract performed slightly better in producing apoptosis in S180 cells than ethanolic molmol, which showed that hexane haddi extract is more potent than ethanolic molmol extract.

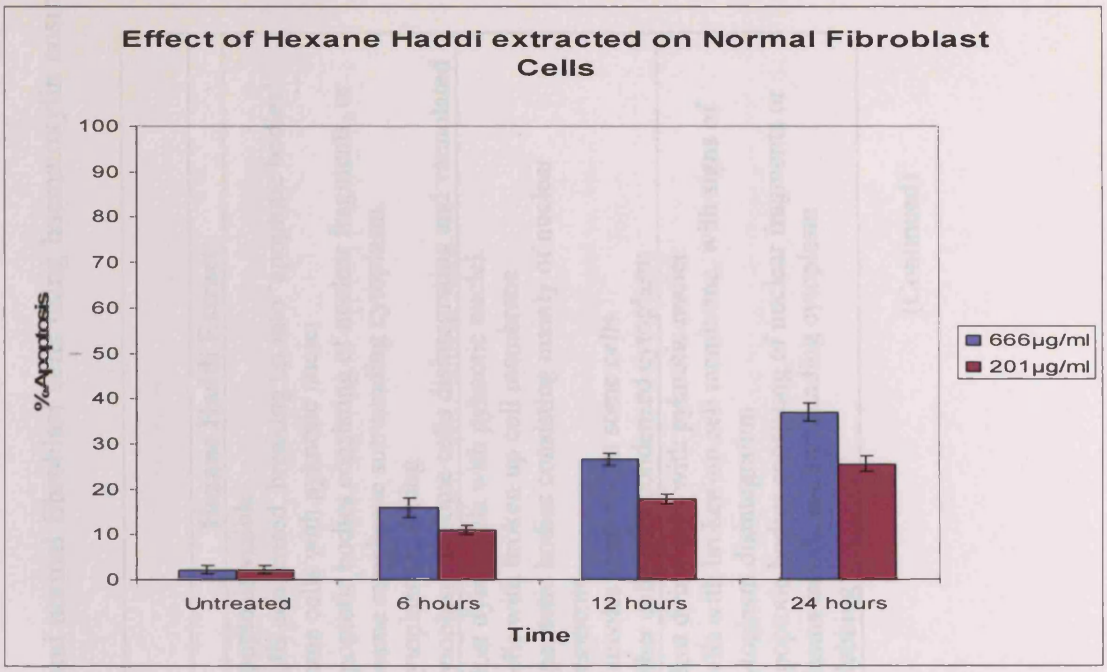


Figure 3.85
Normal fibroblast cells grown in control culture and in cultures containing 666, 234 µg/ml hexane haddi extract solution for 6, 12 and 24 hours (bars=standard error).

Ethanolic molmol and hexane haddi extract treatments produced very similar results in terms of the percentages of apoptotic cells in HT1080. The difference was not significant.

Both solutions were not as effective against normal fibroblast cells as they were against S180 cells and HT1080 cells. Hence, it can be said that molmol and haddi extracts targeted cancerous cells more than normal cells.

3.11 Summary

The effects of molmol and haddi extracts on the S180, HT1080 and normal fibroblast cell lines used in this study can be illustrated as follows (Table 3.9).

Table 3. 9 Summary of effects of molmol and haddi extracts on S180, HT1080 and normal fibroblast cells using haematoxylin eosin technique

S180 Cells			
Untreated	**Exposure**	**Ethanolic Molmol Extract**	**Hexane Haddi Extract**
Cells appeared normal	6 Hours	▪ Apoptosis visible. ▪ Cells condensed, breaking up into 'apoptotic bodies'. ▪ Some cells with pyknotic nuclei ▪ Apoptotic bodies consisting of nuclear fragments, or plasma membrane surrounding cytoplasm.	▪ Apoptosis visible. ▪ Cells condensed, breaking up into 'apoptotic bodies'. ▪ Some cells with pyknotic nuclei ▪ Apoptotic bodies consisting of nuclear fragments, or plasma membrane surrounding cytoplasm. ▪ Cytoplasm blebbing ▪ Cytoplasm in some cells disintegrating and vacuolated
	12 Hours	▪ Most dying cells with pyknotic nuclei. ▪ Cells with broken-up cell membrane ▪ Apoptotic bodies consisting mainly of nuclear fragments. ▪ Vacuoles observed in most cells ▪ Some cells with condensed cytoplasm	▪ Most dying cells with pyknotic nuclei. ▪ Cells with broken-up cell membrane ▪ Apoptotic bodies consisting mainly of nuclear fragments. ▪ Vacuoles observed in some cells ▪ Other cells with condensed cytoplasm
	24 Hours	▪ Most dying cells with pyknotic nuclei. ▪ Cells with broken-up cell membrane ▪ Apoptotic bodies consisting of nuclear fragments or plasma membrane surrounding cytoplasm ▪ Cells with highly vacuolated cytoplasm	▪ Most dying cells with pyknotic nuclei. ▪ Cells with broken-up cell membrane, with signs of cytoplasm disintegration ▪ Apoptotic bodies consisting of nuclear fragments or plasma membrane surrounding cytoplasm ▪ Blebbing

(Continued)

Table 3.9 (Continued)

HT1080 Cells			
Untreated	**Exposure**	**Ethanolic Molmol Extract**	**Hexane Haddi Extract**
Cells appeared normal, some dividing	6 Hours	▪ Apoptosis visible. ▪ Cells condensed, breaking up into 'apoptotic bodies'. ▪ Some cells with pyknotic nuclei, some cells with nuclear material disintegrating ▪ Apoptotic bodies consisting mainly of nuclear fragments ▪ Some cells with broken-up cell membrane, cytoplasm disintegrating, or vacuolated	▪ Apoptosis visible. ▪ Dying cells with pyknotic nuclei ▪ Apoptotic bodies mainly consisting of nuclear fragments ▪ Cytoplasm blebbing ▪ Cytoplasm in some cells disintegrating and vacuolated
	12 Hours	▪ Most dying cells with pyknotic nuclei. ▪ Cells with broken-up cell membrane ▪ Apoptotic bodies consisting mainly of nuclear fragments, and cytoplasm fragments. ▪ Some cells with condensed cytoplasm, some with no pyknotic cells, and others with disintegrated cytoplasm	▪ Most apoptotic cells with disintegrated cytoplasm, others with pyknotic nuclei. ▪ Apoptotic bodies consisting mainly of nuclear fragments, and cytoplasm fragments. ▪ Some cells with disintegrated cells and vacuolated
	24 Hours	▪ Apoptotic cells with pyknotic nuclei ▪ Apoptotic bodies not formed. ▪ Dying cells with disintegrated cytoplasm ▪ Cells vacuolated ▪ Blebbing	▪ Almost all cells affected ▪ Apoptotic bodies formed ▪ Cytoplasm disintegration ▪ Apoptotic bodies consisting of nuclear fragments or plasma membrane surrounding cytoplasm ▪ Blebbing

(Continued)

Table 3.9 (Continued)

Normal Fibroblast Cells			
Untreated	**Exposure**	**Ethanolic Molmol Extract**	**Hexane Haddi Extract**
Cells appeared normal	6 Hours	▪ All cells intact and unchanged ▪ Some cells dividing	▪ As for ethanolic molmol.
	12 Hours	▪ Majority of cells normal. ▪ A few apoptotic cells. ▪ Some cells forming apoptotic bodies	▪ Some cells developing pyknotic nuclei ▪ Dying cells disintegrating ▪ Vacuolation. ▪ Formation of apoptotic bodies. ▪ Some cells blebbing
	24 Hours	▪ Majority of cells normal. ▪ A few apoptotic cells. ▪ Some cells forming apoptotic bodies ▪ Majority of cells with pseudopodial attachments	▪ Some cells developing pyknotic nuclei ▪ Dying cells disintegrating ▪ Vacuolation ▪ Formation of apoptotic bodies, consisting of nuclear fragments ▪ Some cells blebbing

Results illustrated in Table 3.9 clearly show that both plant extracts were effective against S180 and HT1080 cell lines, even after a short time of exposure (6 hours). Nonetheless, hexane extracted haddi was more effective on normal fibroblast than ethanolic extracted molmol, an indication that the latter mainly targets cancer cells, whereas the former seemed to have targeted all three cell lines.

3.12 DNA Fragmentation Assay (Klenow DNA Fragmentation Detection TUNEL)

Only HT1080 and normal fibroblast cell lines were used in this experiment to show the effect of ethanolic molmol and hexane haddi extracts on the survival of these cells.

The cells were treated with these extracts for the following periods: 3, 6, 12, 24 and 48 hours, against a control containing no plant extract.

3.12.1 HT1080 Cell Line

Cells grown in control medium, that is, untreated with molmol or haddi extracts, showed normal cells, with intact nuclear material, as shown in Figure 3.86

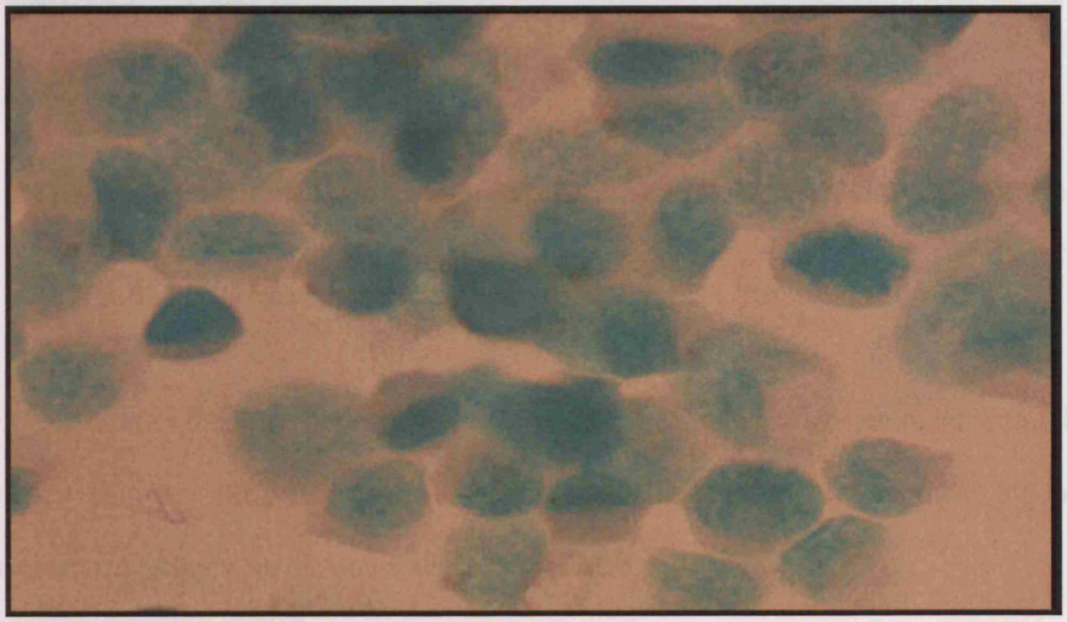


Figure 3.86
Untreated HT1080 cells (Mag. X750).

A. Ethanolic Molmol Extract (234 µg/ml)

HT1080 cells treated with ethanolic molmol for 3 hours did not show any sign of DNA break strands, which is the positive reaction indicating this break-up (Figure 3.87).

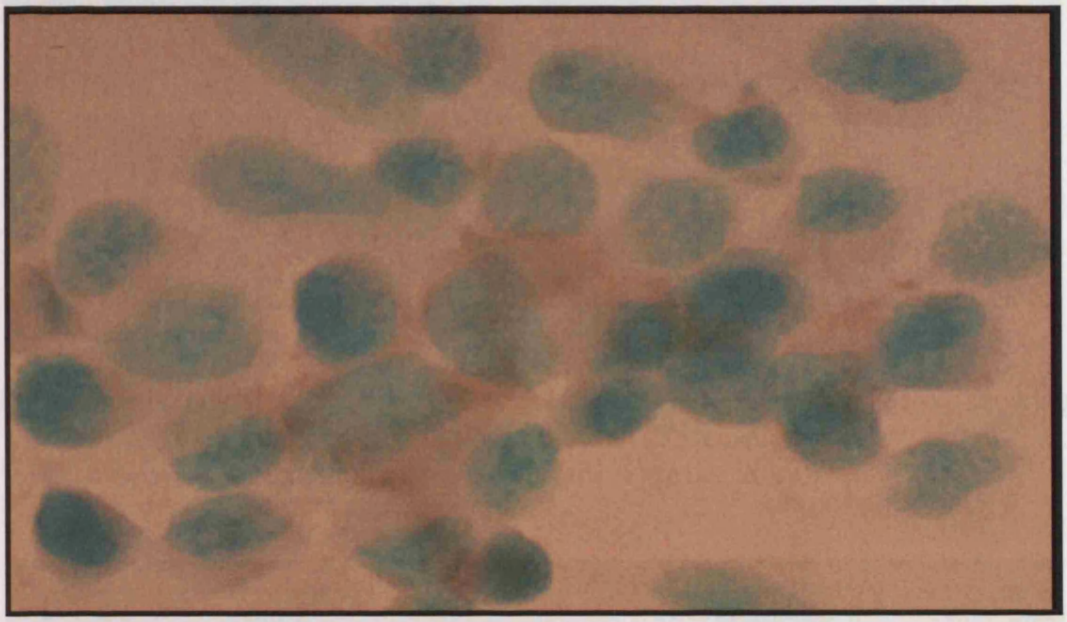


Figure 3.87
HT1080 cells treated with ethanolic molmol extract (234 µg/ml) for 3 hrs (Mag.X750).

However, after 6 and 12 hours, a few cells can be seen, while after treatment with this extract (Figure 3.88), cells showed signs of disintegration, as illustrated by the appearance of blebs on the outer wall of the treated cells.

Treatment with ethanolic molmol for 24 hours resulted in further cell disintegration and fragmentation of DNA material, as illustrated by the appearance of more blebs on the outer wall of the treated cells (Figure 3.89).

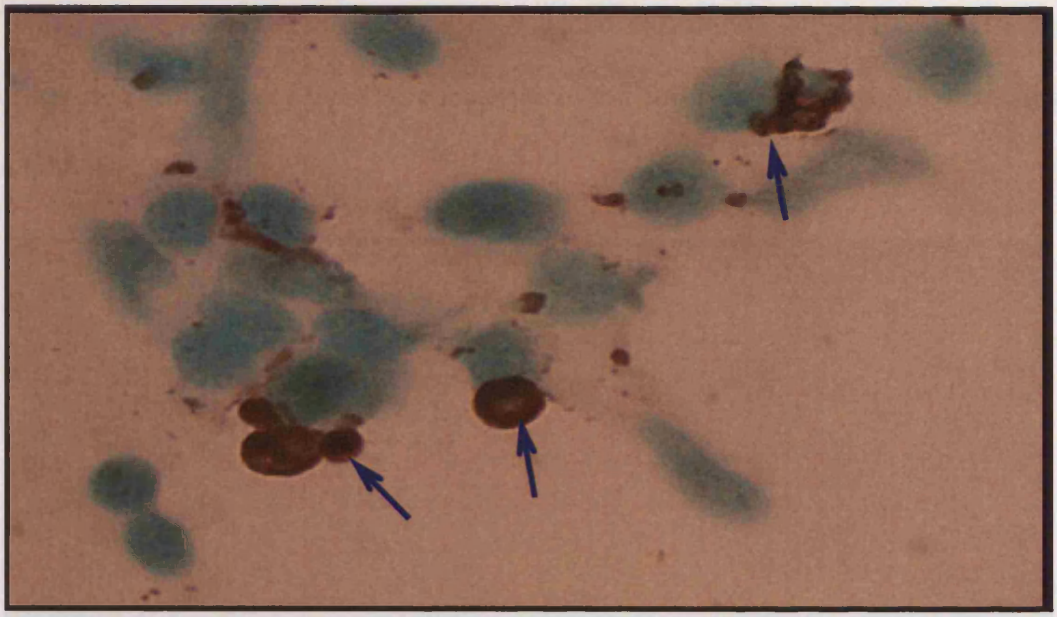


Figure 3.88
HT1080 cells treated with ethanolic molmol extract (234 µg/ml) for 12 hrs , showing signs of DNA fragmentation in the form of DAB stained peroxidase activity (formation of blebs, blue arrow) (Mag. X750).

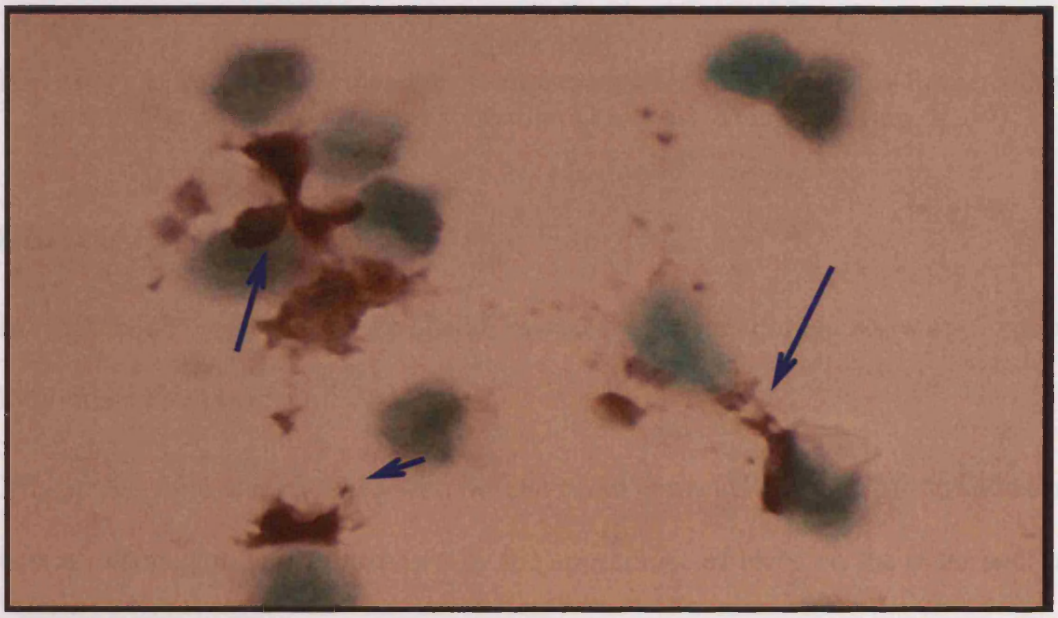


Figure 3.89
HT1080 cells treated with ethanolic molmol extract (234 µg/ml) for 24 hrs, showing further signs of DNA fragmentation (blue arrows) (Mag. X750).

Treatment for 48 hours resulted in complete disintegration of HT1080 cells, and further disintegration of the DNA material in the form of DAB stained peroxidase activity (Figure 3.90).

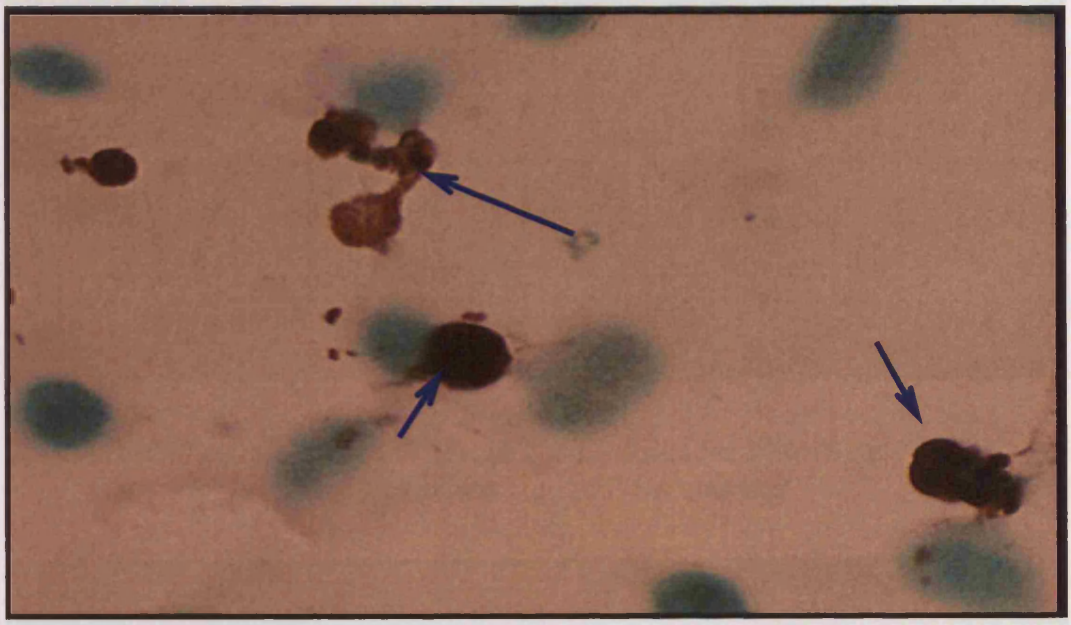


Figure 3.90
HT1080 cells treated with ethanolic molmol extract for 48 hrs, showing further signs of DNA fragmentation (further formation of blebs, blue arrow) (Mag. X750).

B. Hexane Haddi Extract

HT1080 cells treated with hexane haddi extract for 3 hours did not show any sign of DNA strand breaks (Figure 3.91).

Cells treated for 6 and 12 hours with hexane haddi extract (Figure 3.92) showed clear signs of disintegration, as illustrated by the appearance of blebs on the outer wall of the treated cells.

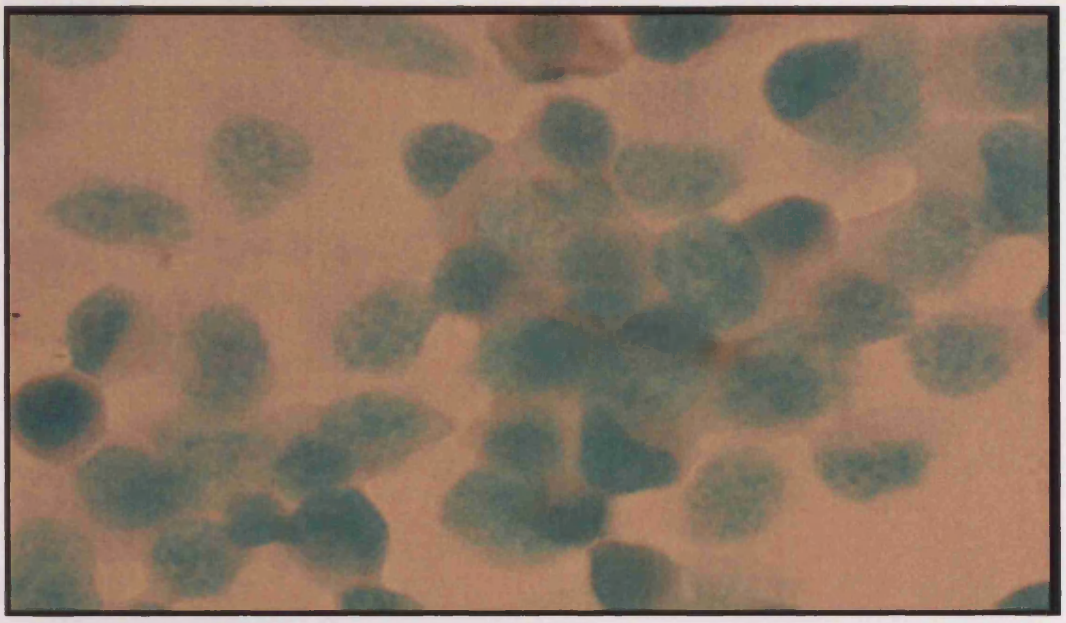


Figure 3.91
HT1080 cells treated with hexane haddi extract for 3 hrs (Mag. X750).
Note lack of any sign of DNA staining

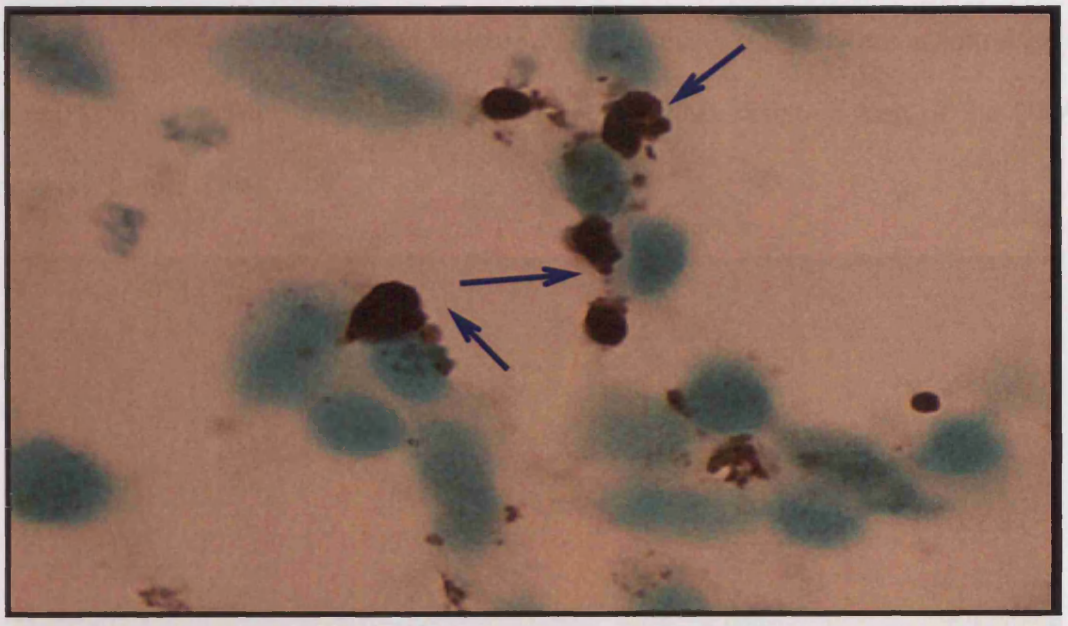


Figure 3.92
HT1080 cells treated with hexane haddi extract for 12 hrs, showing signs of DNA fragmentation (formation of blebs, blue arrow) (Mag. X750), in the form of DAB positive staining.

Treatment with hexane haddi extract for 24 hours resulted in further cell disintegration and fragmentation of DNA material, as illustrated by the appearance of more blebs on the outer wall of the treated cells (Figure 3.93).

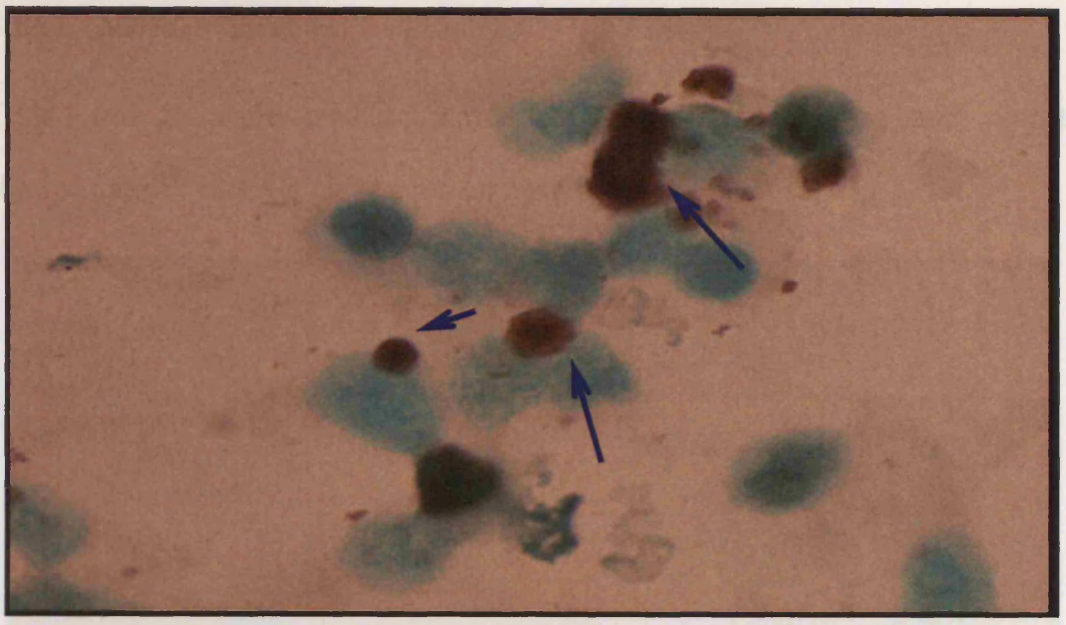


Figure 3.93
HT1080 cells treated with hexane haddi extract for 24 hrs, showing further signs of DNA fragmentation (further formation of blebs, blue arrow) (Mag. X750), and DAB staining.

Treatment of HT1080 cells with hexane haddi extract for 48 hours resulted in a complete disintegration of HT1080 cells and the further disintegration of the DNA material (Figure 3.94).

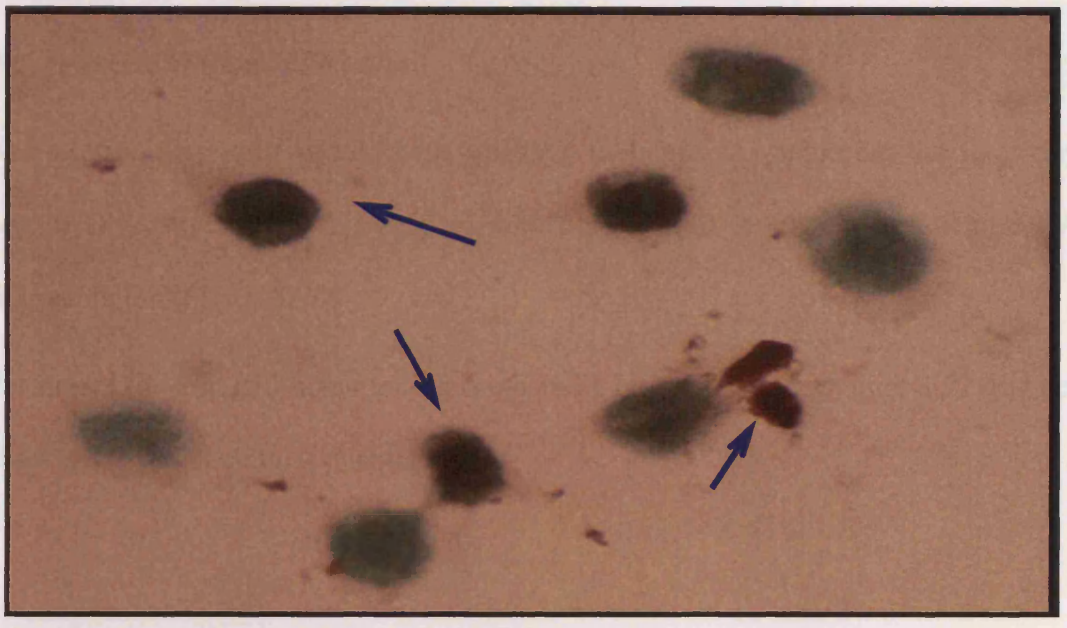


Figure 3.94
HT1080 cells treated with hexane haddi extract for 48 hrs, showing further signs of DNA fragmentation (further formation of blebs, blue arrow) (Mag. X750) in terms of DAB staining.

3.12.2 Normal Fibroblast Cell Line

Cells grown in control medium, that is, untreated with molmol or haddi extracts showed normal cells, with intact nuclear material, as in Figure 3.95.

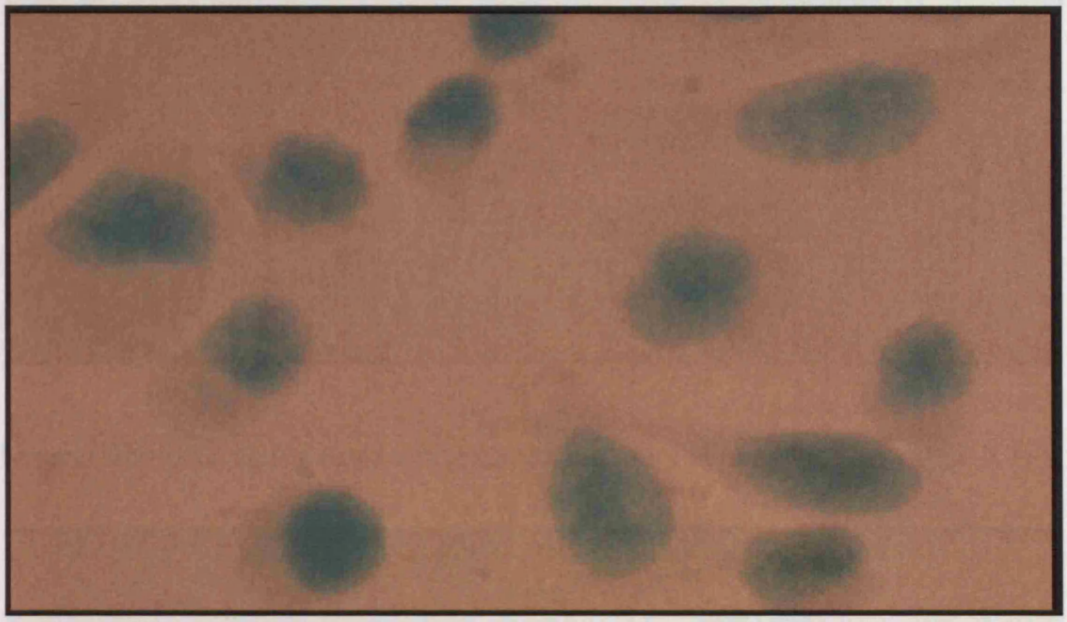


Figure 3.95
Untreated normal fibroblast cells (Mag. X750), showing no signs of DNA fragmentation and staining.

A. Ethanolic Molmol (234 µg/ml)

Normal fibroblast cells treated with ethanolic molmol for 3 hours did not show any sign of DNA break and DAB staining, which is the positive reaction indicating DNA fragmentation (Figure 3.96).

Treatment for 6 and 12 hours with this ethanolic molmol extract (Figure 3.97) did not show any signs of cellular disintegration.

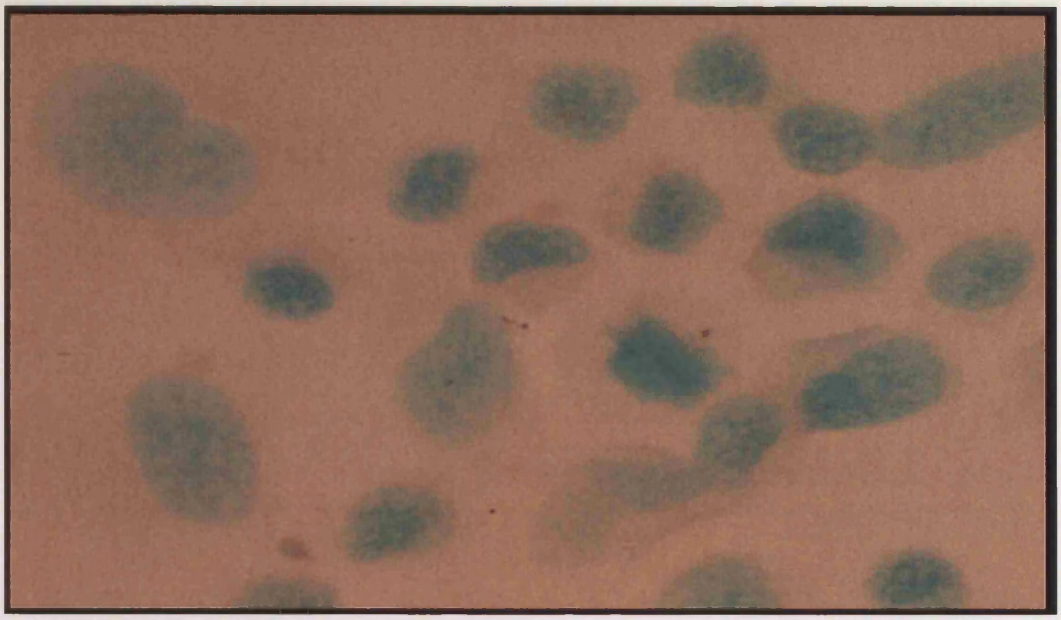


Figure 3.96
Normal fibroblast cells treated with ethanolic molmol extract for 3 hrs (Mag. X750).

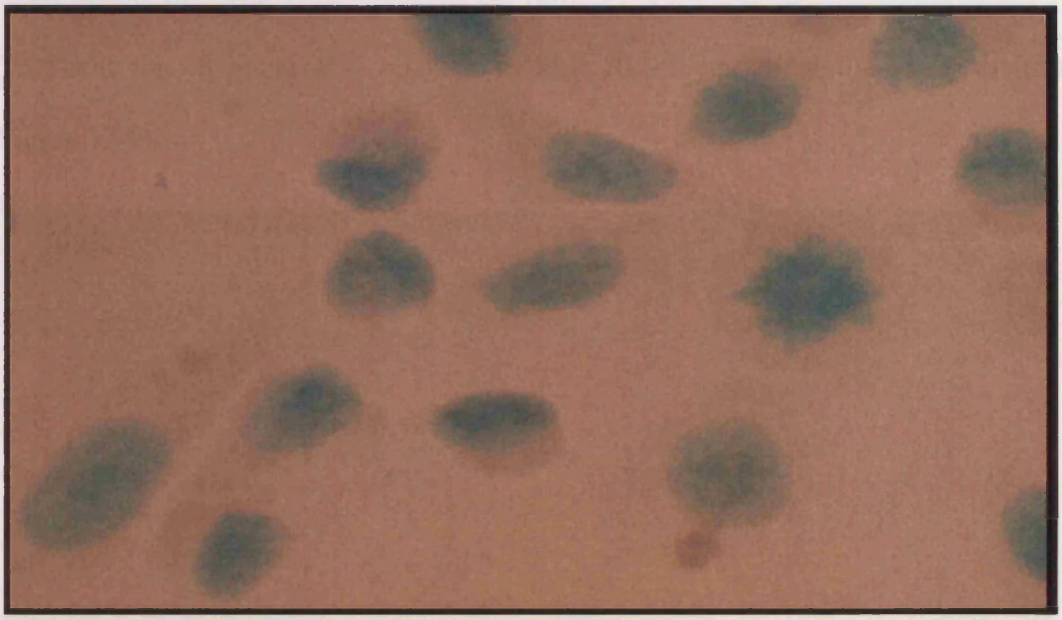


Figure 3.97
Normal fibroblast cells treated with ethanolic molmol extract for 12 hrs. No signs of DNA fragmentation and no lack of DAB staining (Mag. X750).

Treatment with ethanolic molmol for 24 hours only resulted in minor signs of fragmentation of DNA material, as illustrated by the appearance of few blebs on the outer wall of the treated cells (Figure 3.98).

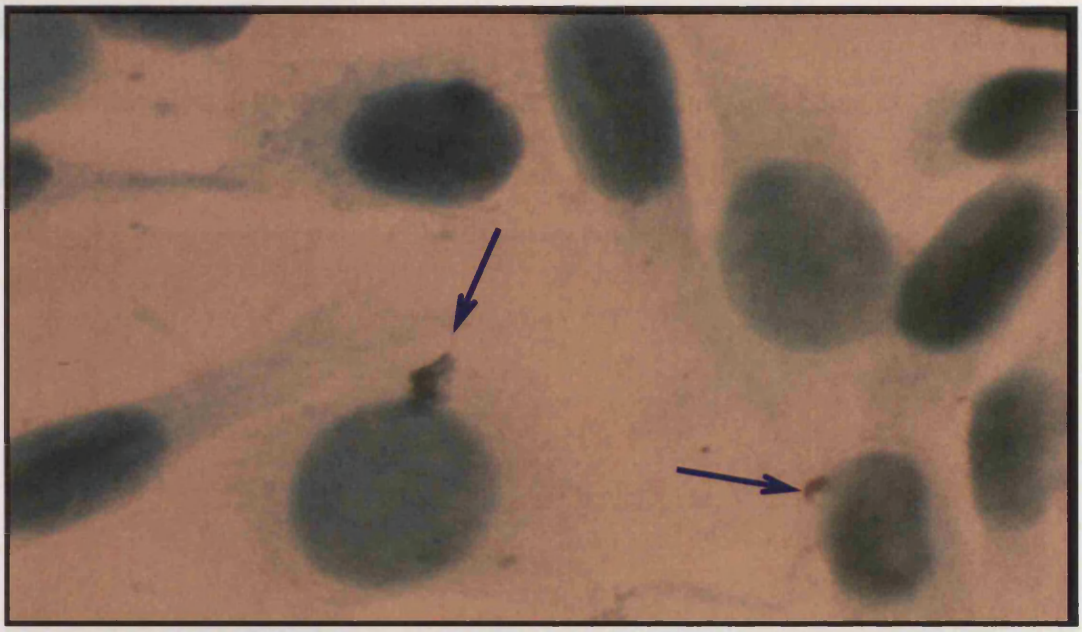


Figure 3.98
Normal fibroblast cells treated with ethanolic molmol extract for 24 hrs, showing few signs of DNA fragmentation (formation of blebs, blue arrow). (Mag. X750).

Treatment for 48 hours also resulted in little disintegration of the DNA material (Figure 3.99).

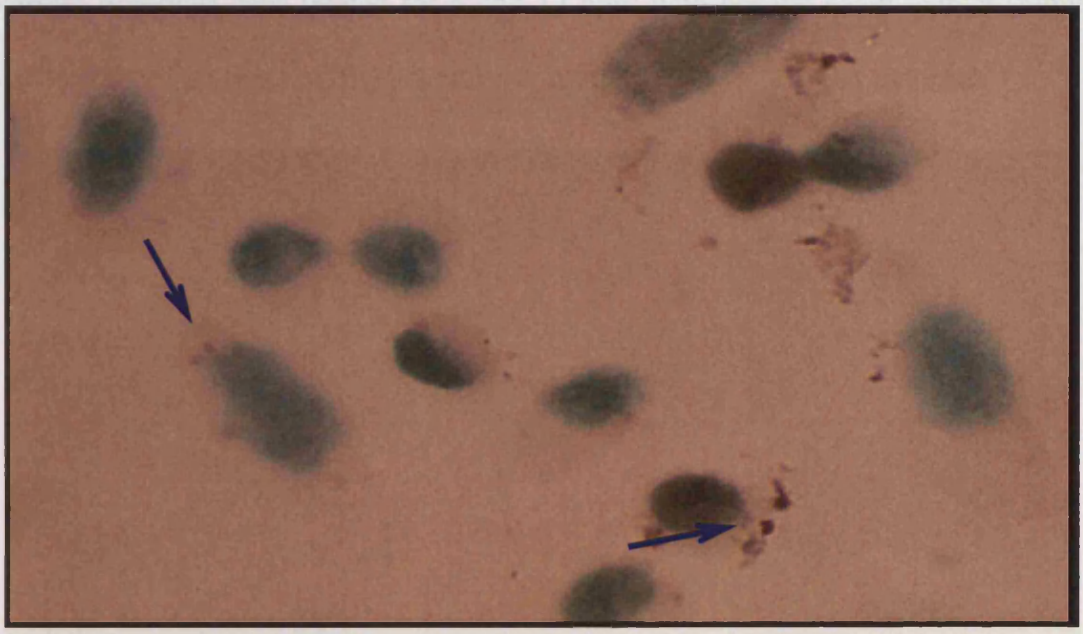


Figure 3.99
Normal fibroblast cells treated with ethanolic molmol extract for 48 hrs, showing few signs of DNA fragmentation in terms of brown colour (formation of blebs, blue arrow). (Mag. X750)

B. Hexane Haddi Extract

Normal fibroblast cells treated with hexane haddi extract for 3 hours did not show any sign of DNA fragmentation and DAB staining (Figure 3.100).

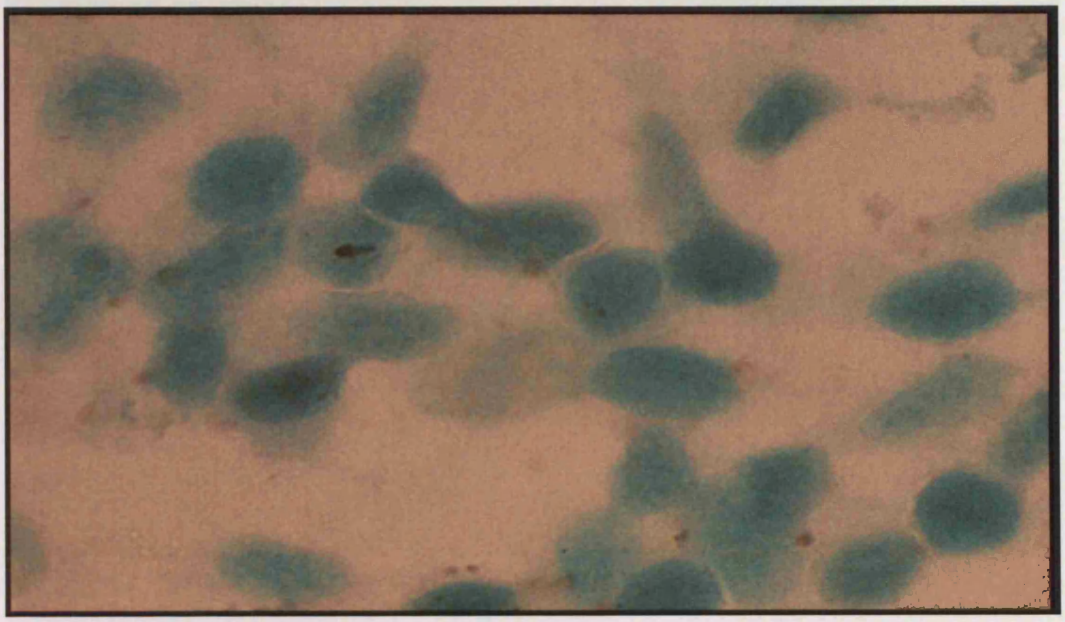


Figure 3.100
Normal fibroblast cells treated with hexane extracted haddi for 3hrs. (Mag. X750).

Normal fibroblast cells treated for 6 and 12 hour with hexane haddi extract (Figure 3.101) showed no signs of disintegration and fragmentation of DNA material.

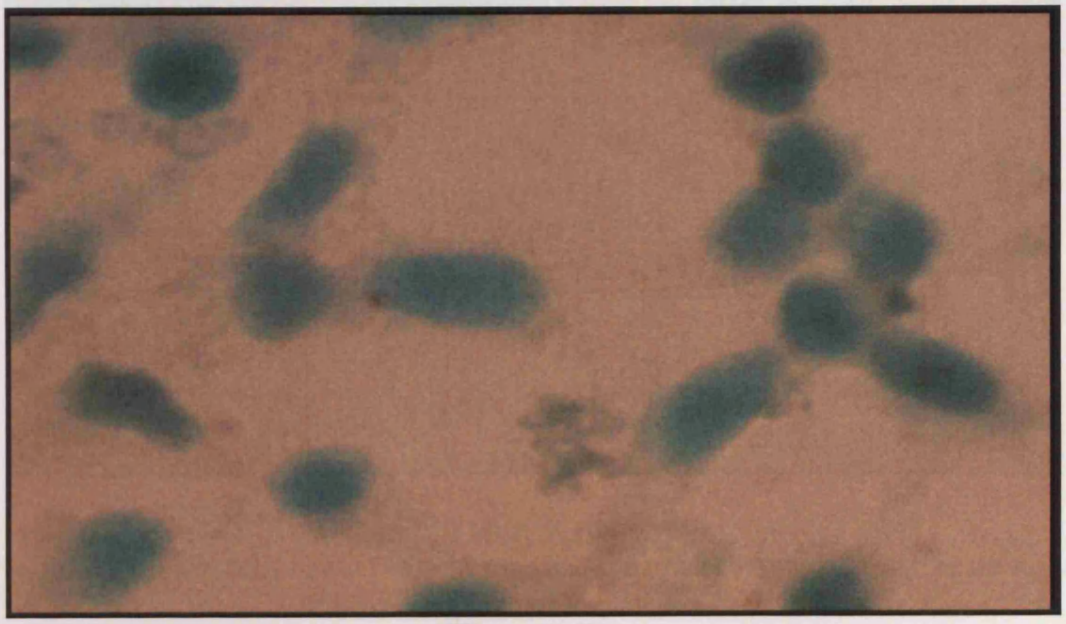


Figure 3.101
Normal fibroblast cells treated with hexane haddi extract for 12hrs, showing no signs of DNA fragmentation. (Mag. X750).

Treatment with hexane haddi extract for 24 hours did not result in any cell disintegration and fragmentation of DNA material (Figure 3.102).

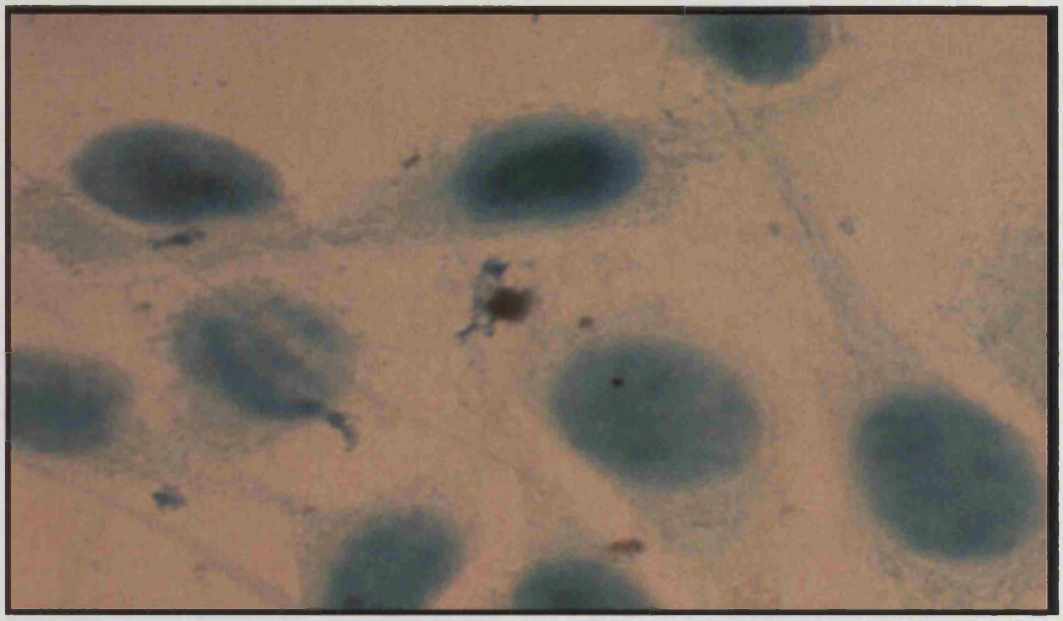


Figure 3.102
Normal fibroblast cells treated with hexane haddi extract for 24 hrs, showing no signs of DNA fragmentation (Mag. 750X).

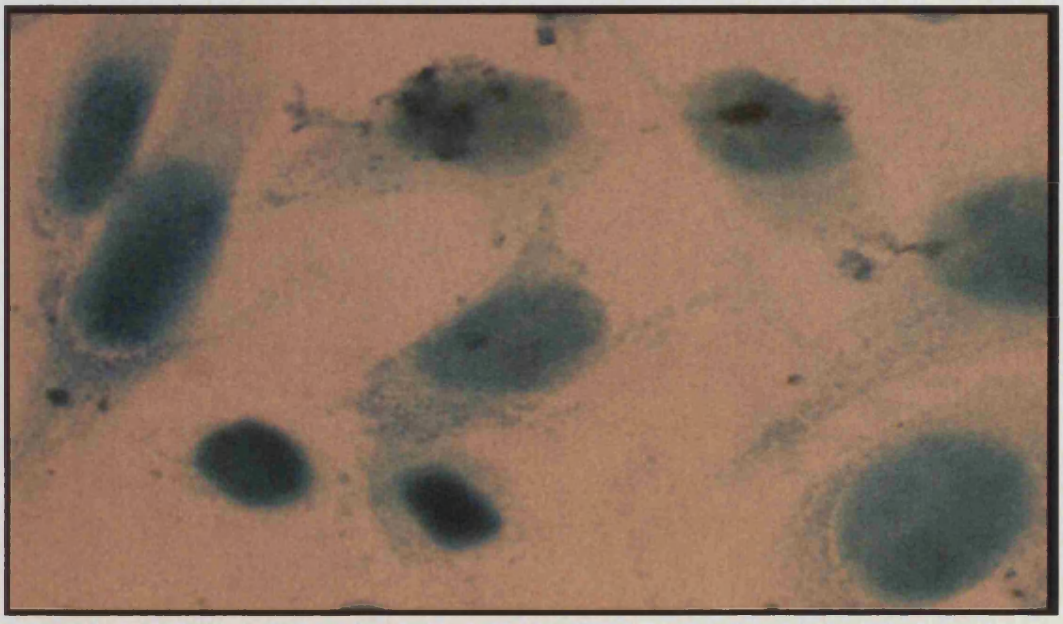


Figure 3.103
Normal fibroblast cells treated with hexane haddi extract for 48 hrs, showing no signs of DNA fragmentation (Mag. X750).

Treatment of normal fibroblast cells with hexane haddi extract for 48 hours also did not result in any disintegration of normal fibroblast cells and disintegration of the DNA material (Figure 3.103).

To sum up, the TUNEL assay showed signs of DNA fragmentation after 12 hours of treatment of HT1080 cells with ethanolic molmol and hexane haddi extracts, which increased further with time. Nonetheless, these extracts did not induce any DNA fragmentation in the normal fibroblast cells.

3.13 Summary

The effects of molmol and haddi extracts on HT1080 and normal fibroblast cells can be illustrated as follows (Table 3.10).

Table 3.10 Summary of effects of molmol and haddi extracts

	Untreated	**Ethanolic Molmol**	**Hexane Haddi**
HT1080	Normal	▪ No DNA break strands after 3 hours ▪ Cells disintegrated after 6 and 12 hours (blebbing). ▪ Further disintegration after 24 hours (more blebbing) ▪ Complete disintegration after 48 hours	▪ No DNA break strands after 3 hours ▪ Cells disintegrated after 6 and 12 hours (blebbing). ▪ Further disintegration after 24 hours (more blebbing) ▪ Complete disintegration after 48 hours
Normal Fibroblast	Normal	▪ No DNA break strands after 3, 6 and 12 hours ▪ No fragmentation of DNA after 24 hours ▪ No disintegration after 48 hours	▪ No DNA break strands after 3, 6 and 12 hours ▪ Minor fragmentation of DNA after 24 hours (little blebbing) ▪ Little disintegration after 48 hours

However, when these cells were grown in a medium containing 666 µg/ml ethano molmol extract, there was a tendency to develop more apoptotic cells with tir increasing from 10 % apoptotic cells after 6 hours of incubation to 79.75% after hours. This trend was also observed when cells were grown in medium containi 234 µg/ml ethanolic molmol extract, though lower percentages of apoptotic cells w observed, increasing from 9 % after 6 hours of incubation to 65 % after 48 hou (Figure 3.104).

Results in Figure 3.104 indicate that ethanolic molmol extract at the t concentrations led to higher apoptotic rates than the control after only 6 hours exposure. However, there was no effect after 3 hours at both concentrations, whi gave 6% and 4.75%, respectively.

HT1080 cell grown in a control culture showed slight occurrence of apoptotic cells mean of 3% of a total 400 cells counted, in contrast to 97% non-apoptotic ce However, when HT1080 cells were grown in a medium containing 666 µg/ml hexa haddi extract, there was a tendency to develop more apoptotic cells with tir increasing from 13.5% apoptotic cells after 6 hours of incubation to 88.25% after hours. A similar observation was noted when cells were grown in a mediu containing 201 µg/ml hexane haddi, though lower percentages of apoptotic cells w observed, increasing from 11.25% after 6 hours of incubation to 67% after 48 hours

(Figure 3.105). The results showed that there was no increased response at 3 hours for both 666µg/ml and 201µg/ml concentrations, which gave 4.75% and 6.5%, respectively.

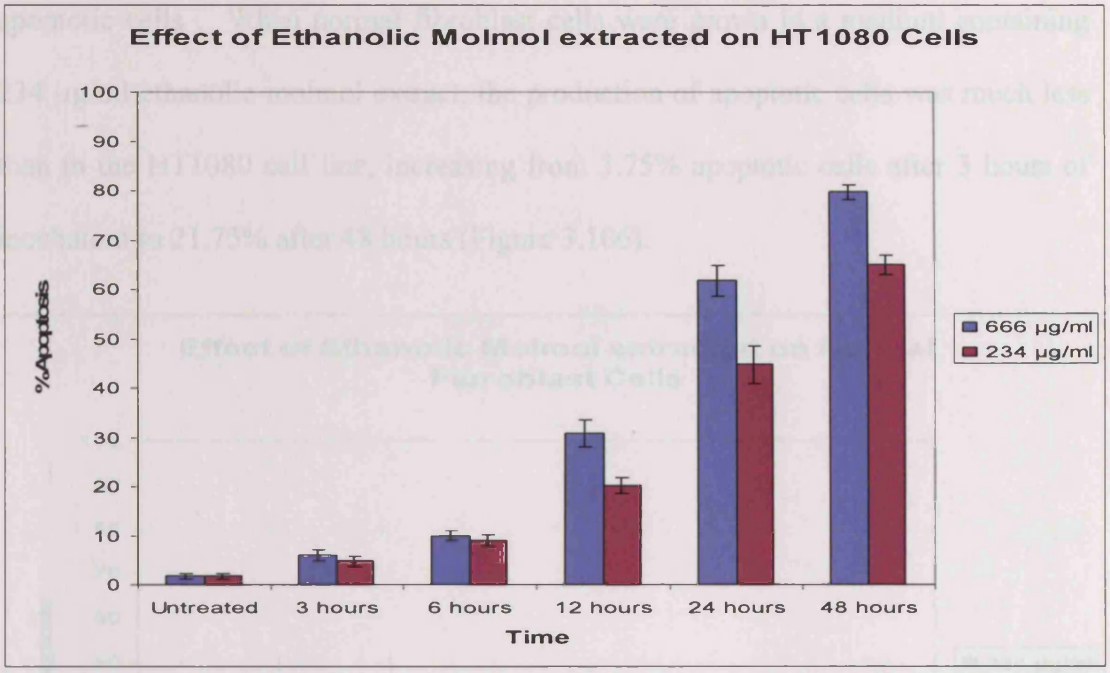


Figure 3.104

HT1080 cells grown in control culture and in cultures containing 666, 234 µg/ml ethanolic molmol extract solution for 3, 6, 12, 24 and 48 hours (bars=standard error).

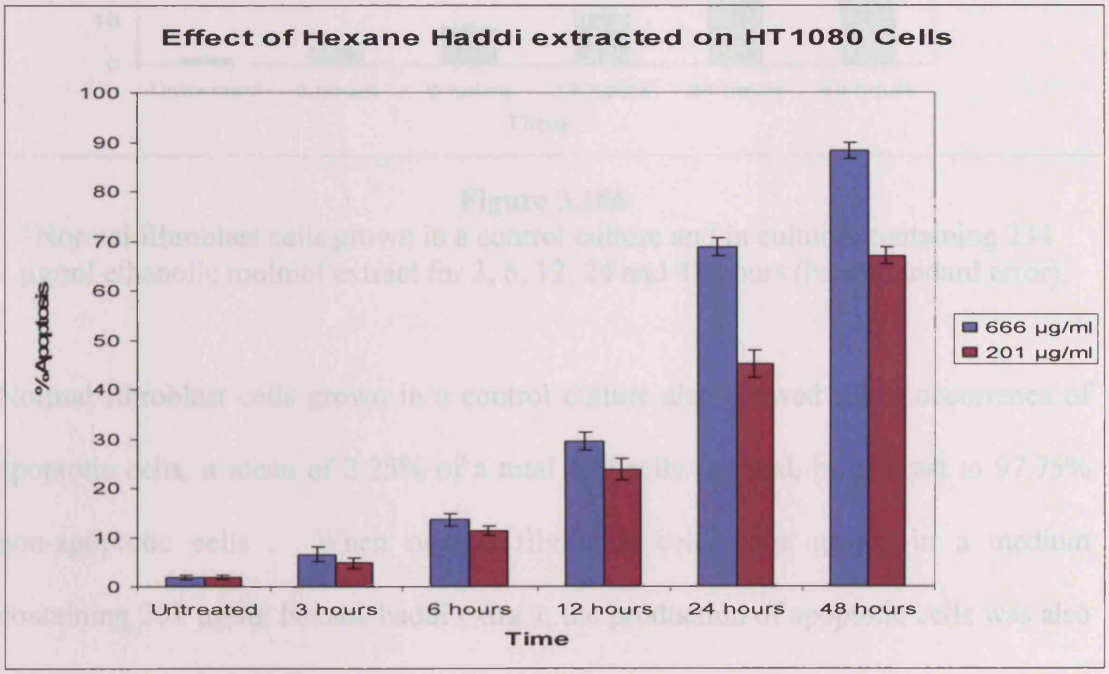


Figure 3.105

HT1080 cells grown in control culture and in cultures containing 666, 201 µg/ml hexane haddi extract solution for 3, 6, 12, 24 and 48 hours (bars=standard error).

3.14.2 Normal Fibroblast Cells

Normal fibroblast cells grown in a control culture showed slight occurrence of apoptotic cells, a mean of 1.5% of a total 400 cells counted, in contrast to 98.5% non-apoptotic cells . When normal fibroblast cells were grown in a medium containing 234 µg/ml ethanolic molmol extract, the production of apoptotic cells was much less than in the HT1080 cell line, increasing from 3.75% apoptotic cells after 3 hours of incubation to 21.75% after 48 hours (Figure 3.106).

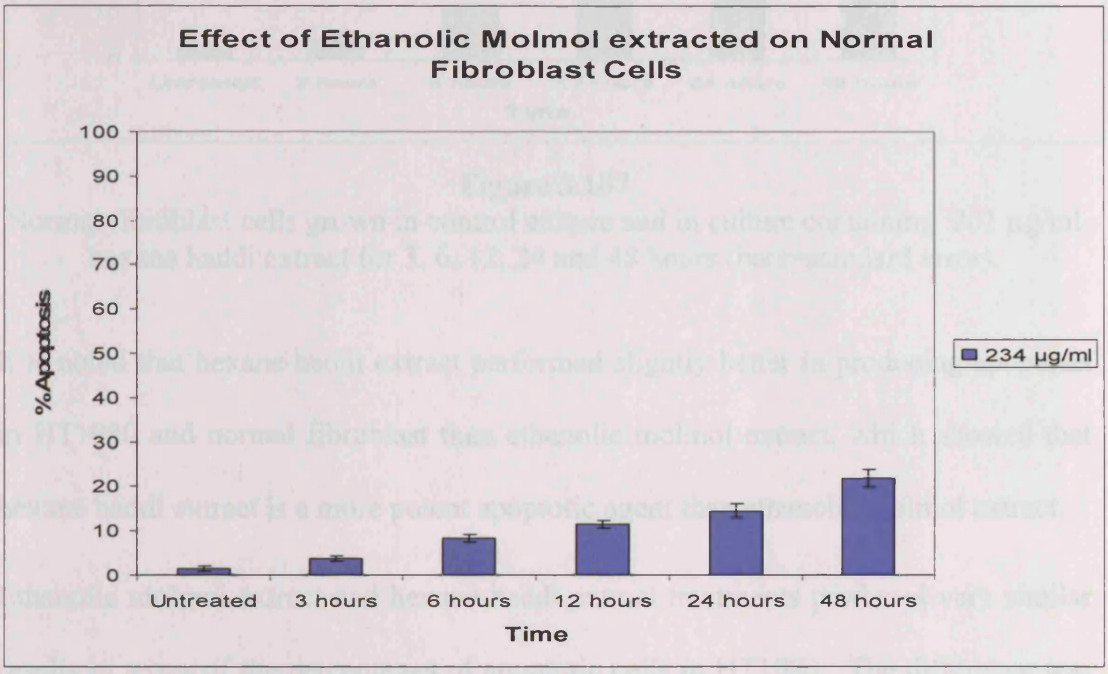


Figure 3.106
Normal fibroblast cells grown in a control culture and in cultures containing 234 µg/ml ethanolic molmol extract for 3, 6, 12, 24 and 48 hours (bars=standard error).

Normal fibroblast cells grown in a control culture also showed slight occurrence of apoptotic cells, a mean of 2.25% of a total 400 cells counted, in contrast to 97.75% non-apoptotic cells . When normal fibroblast cells were grown in a medium containing 201 µg/ml hexane haddi extract, the production of apoptotic cells was also much less than in the HT1080 cell line, increasing from 4% apoptotic cells after 3 hours of incubation to 22.5% after 48 hours (Figure 3.107).

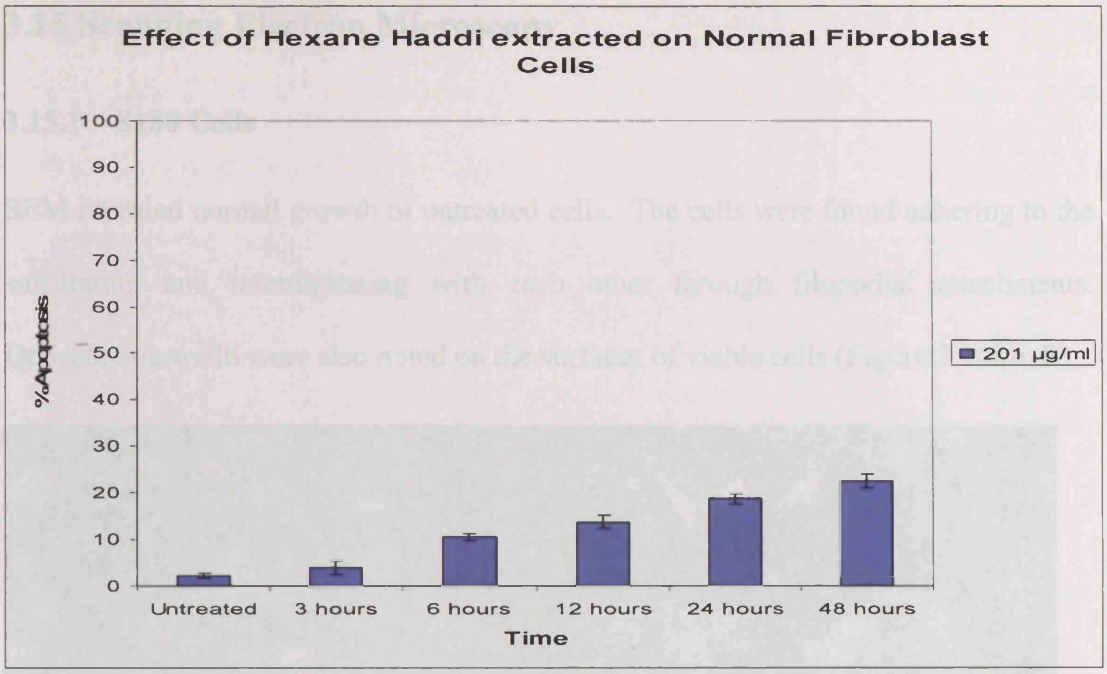


Figure 3.107
Normal fibroblast cells grown in control culture and in culture containing 201 µg/ml hexane haddi extract for 3, 6, 12, 24 and 48 hours (bars=standard error).

It is noted that hexane haddi extract performed slightly better in producing apoptosis in HT1080 and normal fibroblast than ethanolic molmol extract, which showed that hexane haddi extract is a more potent apoptotic agent than ethanolic molmol extract.

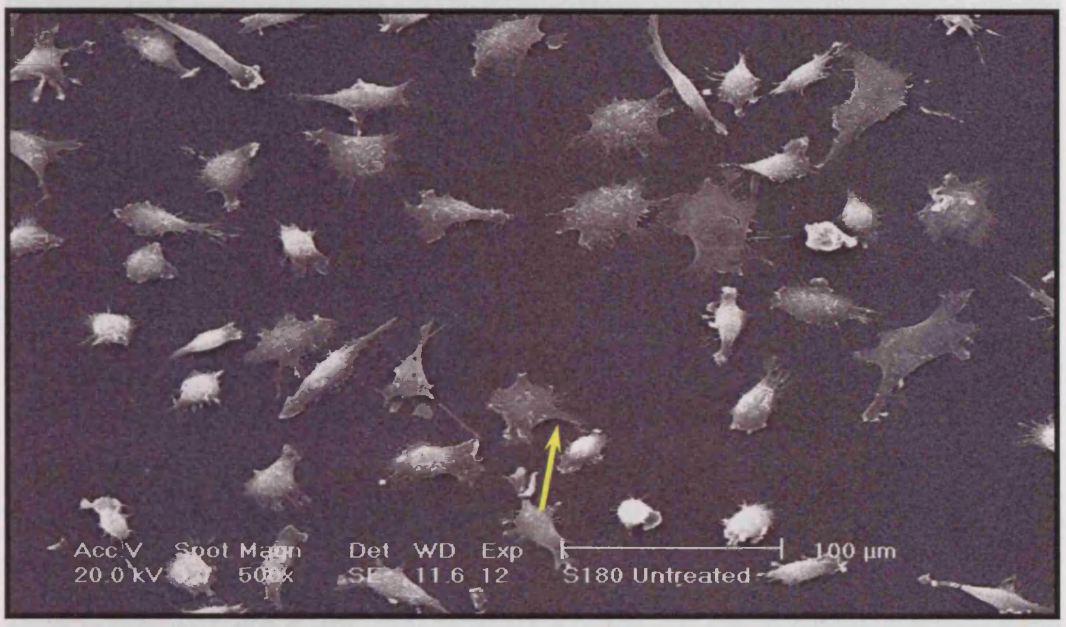
Ethanolic molmol extract and hexane haddi extract treatments produced very similar results in terms of the percentages of apoptotic cells in HT1080. The difference was not significant.

Both solutions were not as effective against nornal fibroblast cells as they were against HT1080 cells. Hence, it can be said that in terms of apoptosis, molmol and haddi extracts targeted cancerous cells more than normal cells.

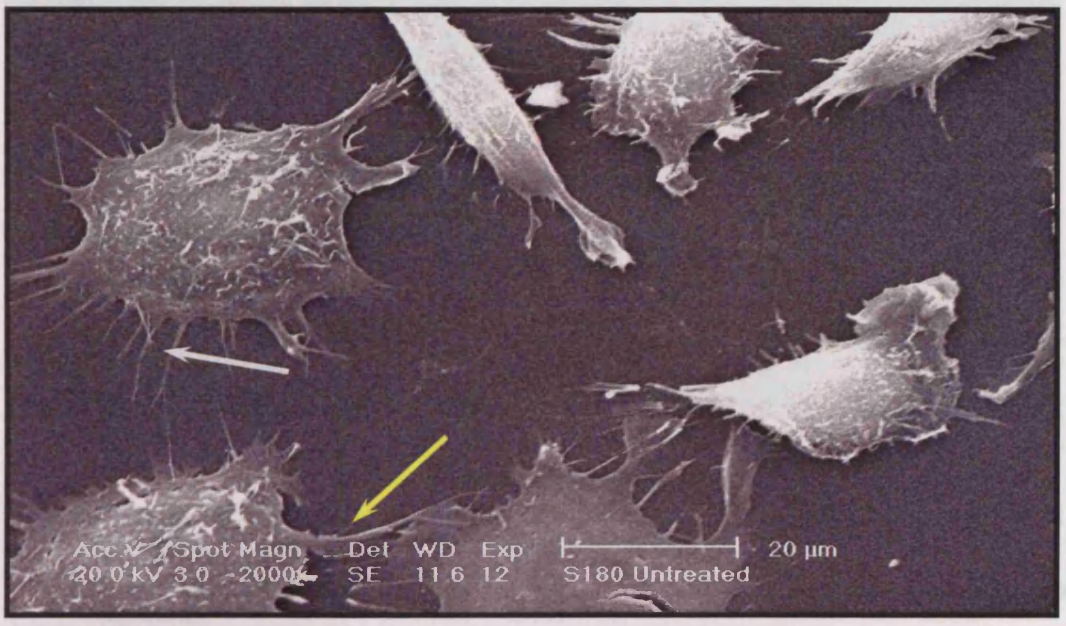
3.15 Scanning Electron Microscopy

3.15.1 S180 Cells

SEM revealed normal growth of untreated cells. The cells were found adhering to the substratum and interdigitating with each other through filopodial attachments. Delicate microvilli were also noted on the surfaces of viable cells (Figure 3.108A-B).



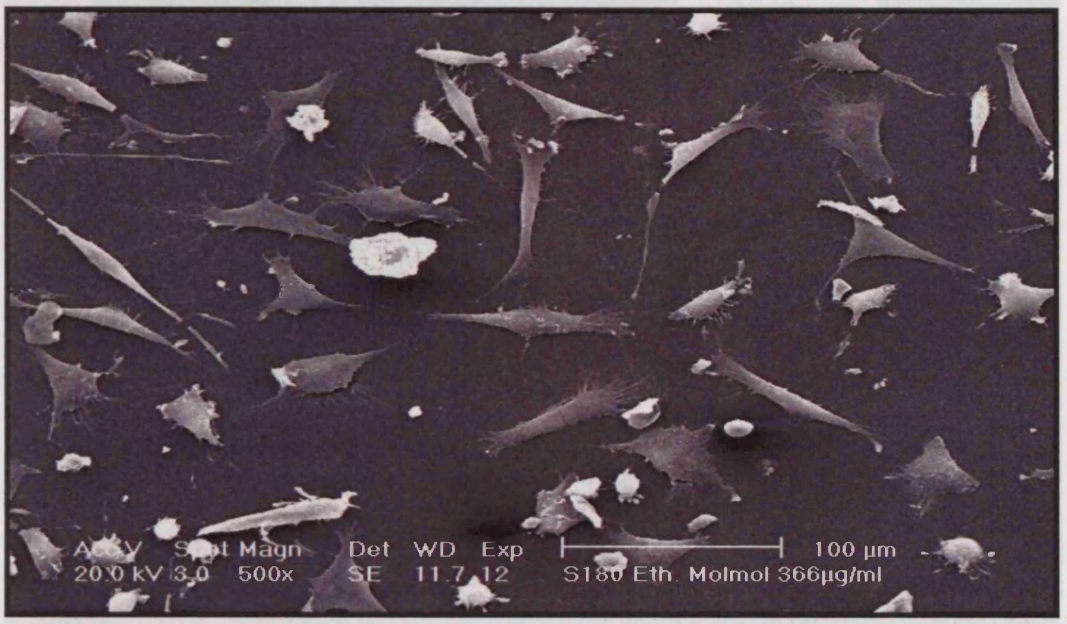
A



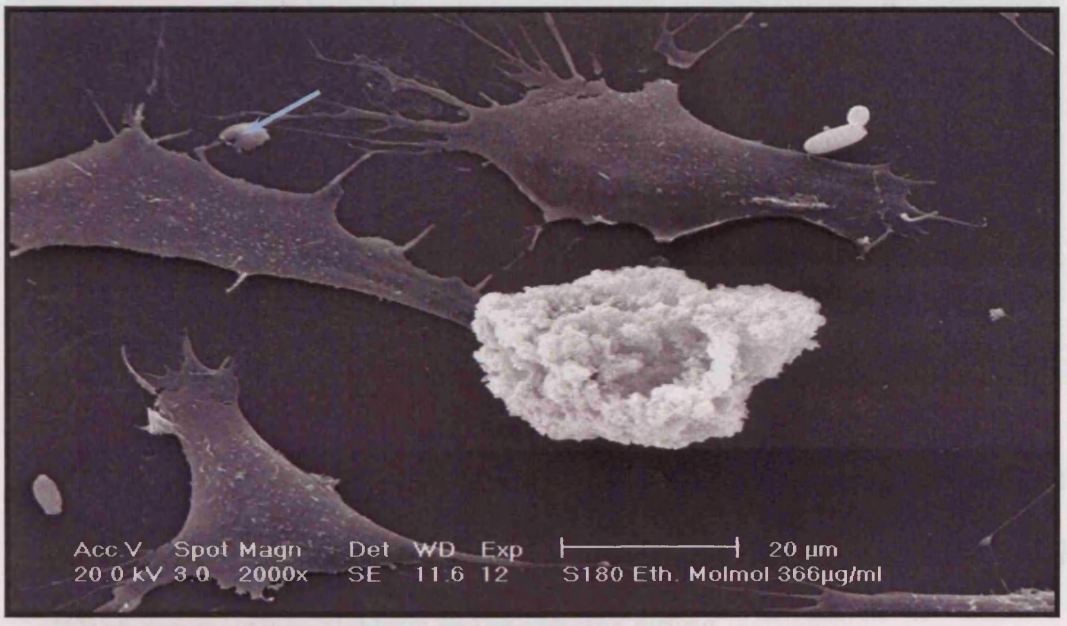
B

Figure 3.108
SEM images of untreated S180 cells. Cells show surface microvilli (white arrow) and filopodial attachments (yellow arrow).

Exposure of S180 cells for 12 hours to an ethanolic molmol extract at a concentration of 366 µg/ml resulted in symptoms of apoptosis, including signs of fragmentation (blebbing), and apoptotic bodies (Figure 109A,B,C,D).



A



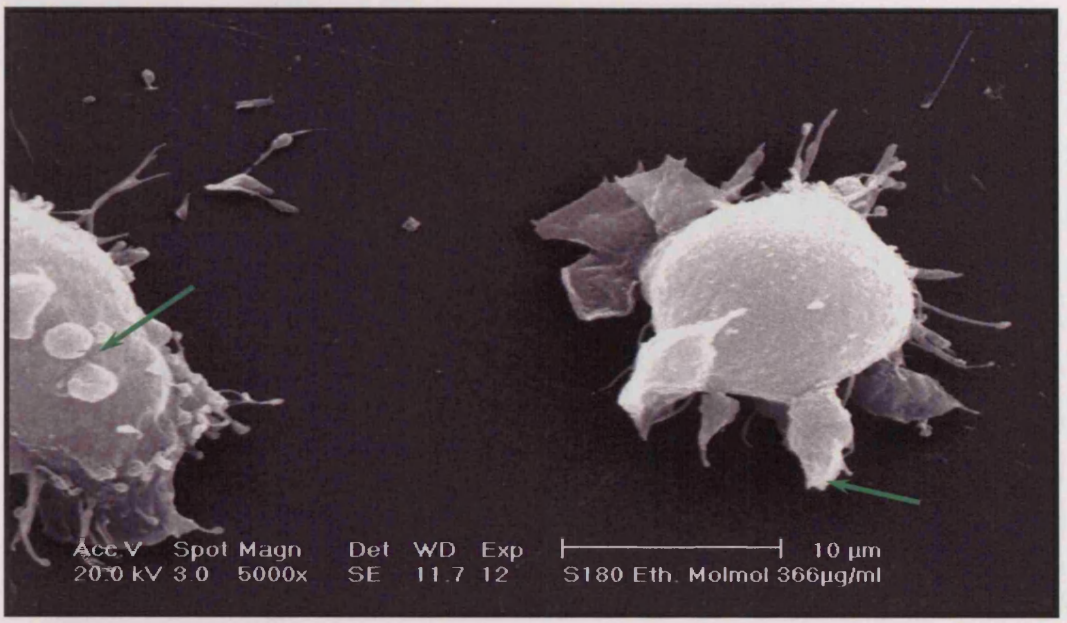
B

Figure 3.109 (A-B)

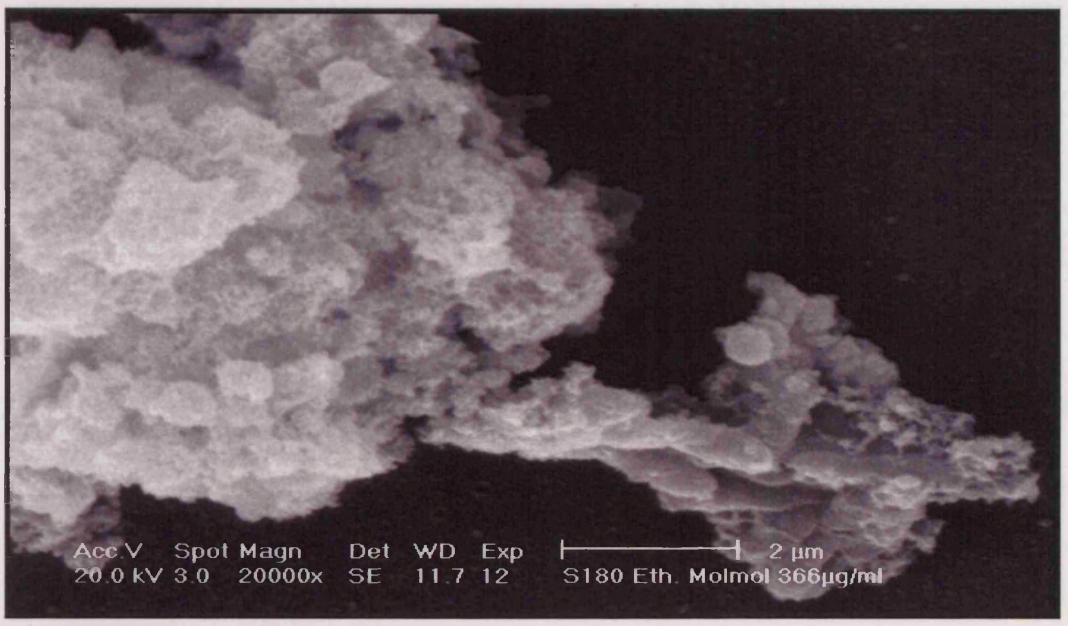
SEM images of S180 cells treated for 12 hours with 366 µg/ml ethanolic molmol extract.

(A) Showing loss of most microvilli, loss of filopodial attachments and membrane blebbing.

(B) Showing loss of most microvilli, loss of filopodial attachments, and formation of apoptotic bodies (turquoise arrow).



C



D

Figure 3.109 (C-D)

SEM images of S180 cells treated for 12 hours with 366 µg/ml ethanolic molmol extract.

(C) Showing loss of microvilli, loss of filopodial attachments, membrane blebbing (green arrow).

(D) Showing surface blebbing and membrane pitting.

Exposure of S180 cells for 12 hours to a hexane haddi extract at a concentration of 733 µg/ml also resulted in symptoms of apoptosis, including signs of fragmentation (blebbing), and apoptotic bodies (Figure 3.110A-D).

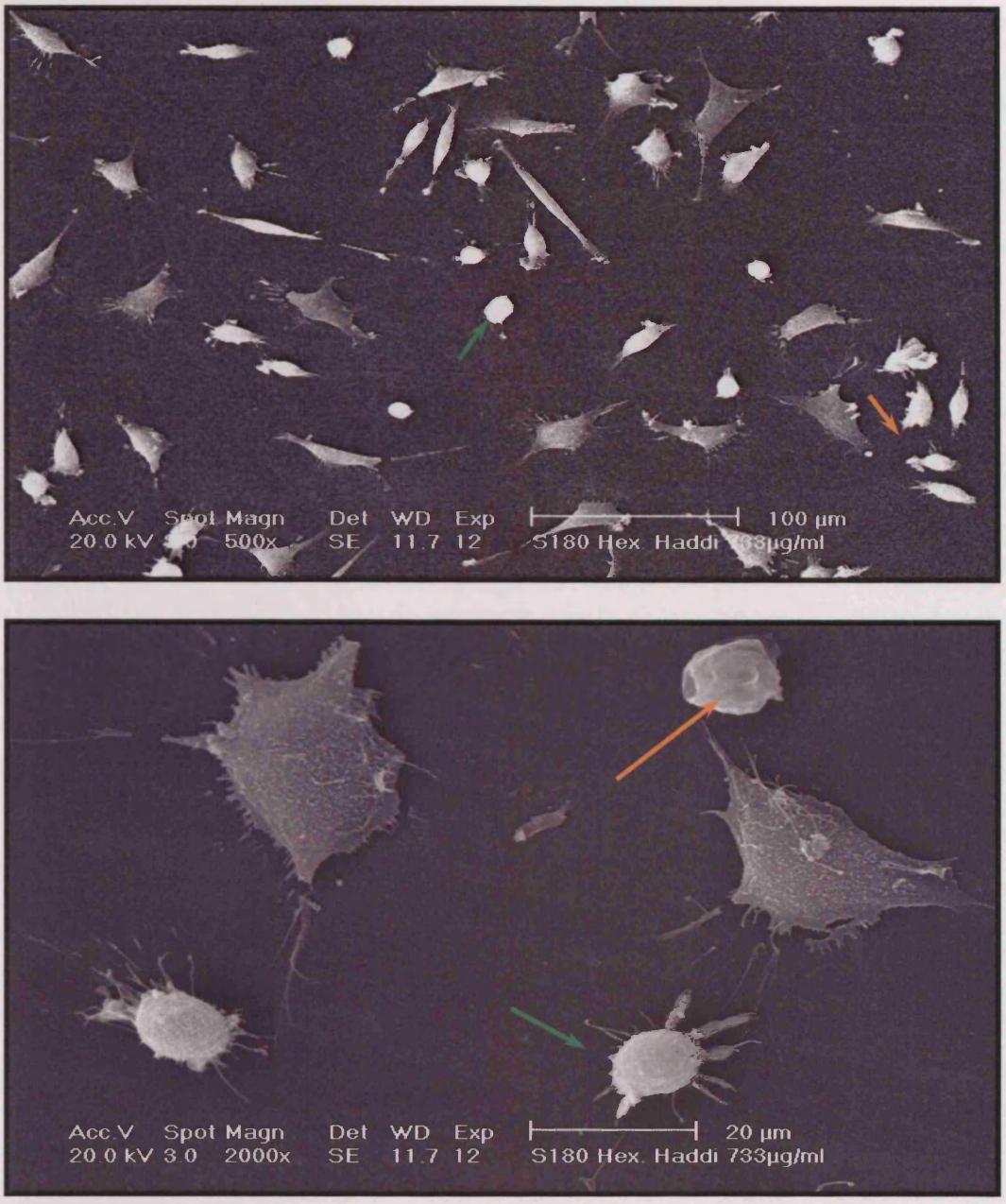
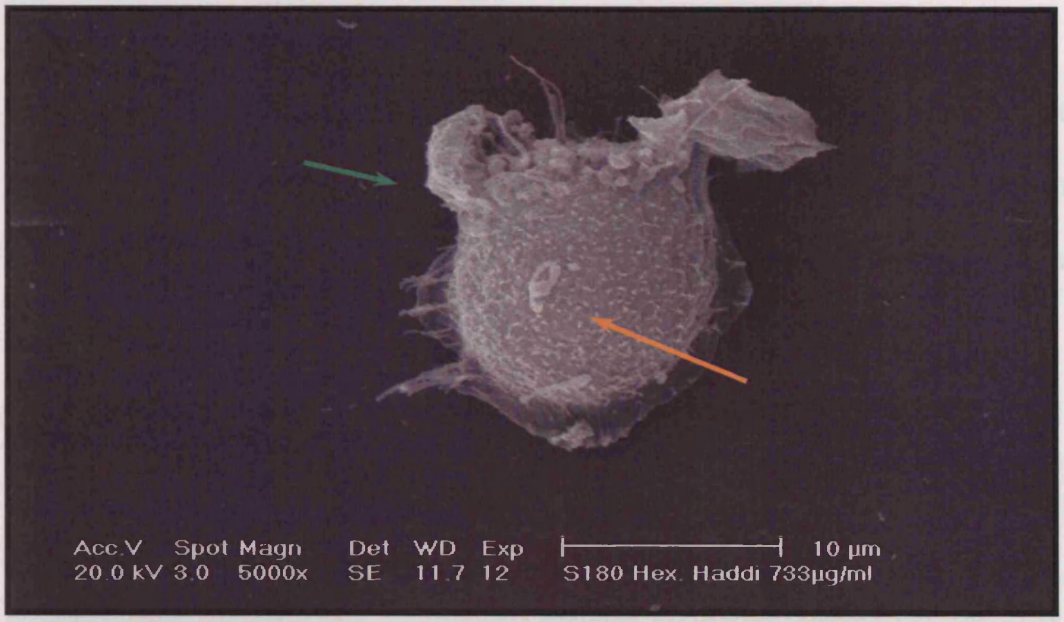


Figure 3.110 (A-B)

SEM images of S180 cells treated for 12 hours with 733 µg/ml hexane haddi extract.

(A) Showing loss of most microvilli, loss of filopodial attachments, formation of apoptotic cells (orange arrow) and apoptotic bodies (green arrow).

(B) Showing loss of most microvilli, loss of filopodial attachments, and formation of apoptotic cells (orange arrow).



C

D

Figure 3.110 (C-D)

SEM images of S180 cells treated for 12 hours with 733 µg/ml hexane haddi extract.

(C) Showing loss of microvilli, loss of filopodial attachments, membrane blebbing (green arrow) and apoptotic cells (orange arrow).

(D) Showing surface blebbing and membrane pitting.

3.15.2 HT1080 Cells

SEM revealed normal growth of untreated cells. The cells were found adhering to the substratum and interdigitating with each other through filopodial attachments. However, delicate microvilli were not noted on the surfaces of viable cells (Figure 3.111A,B,C).

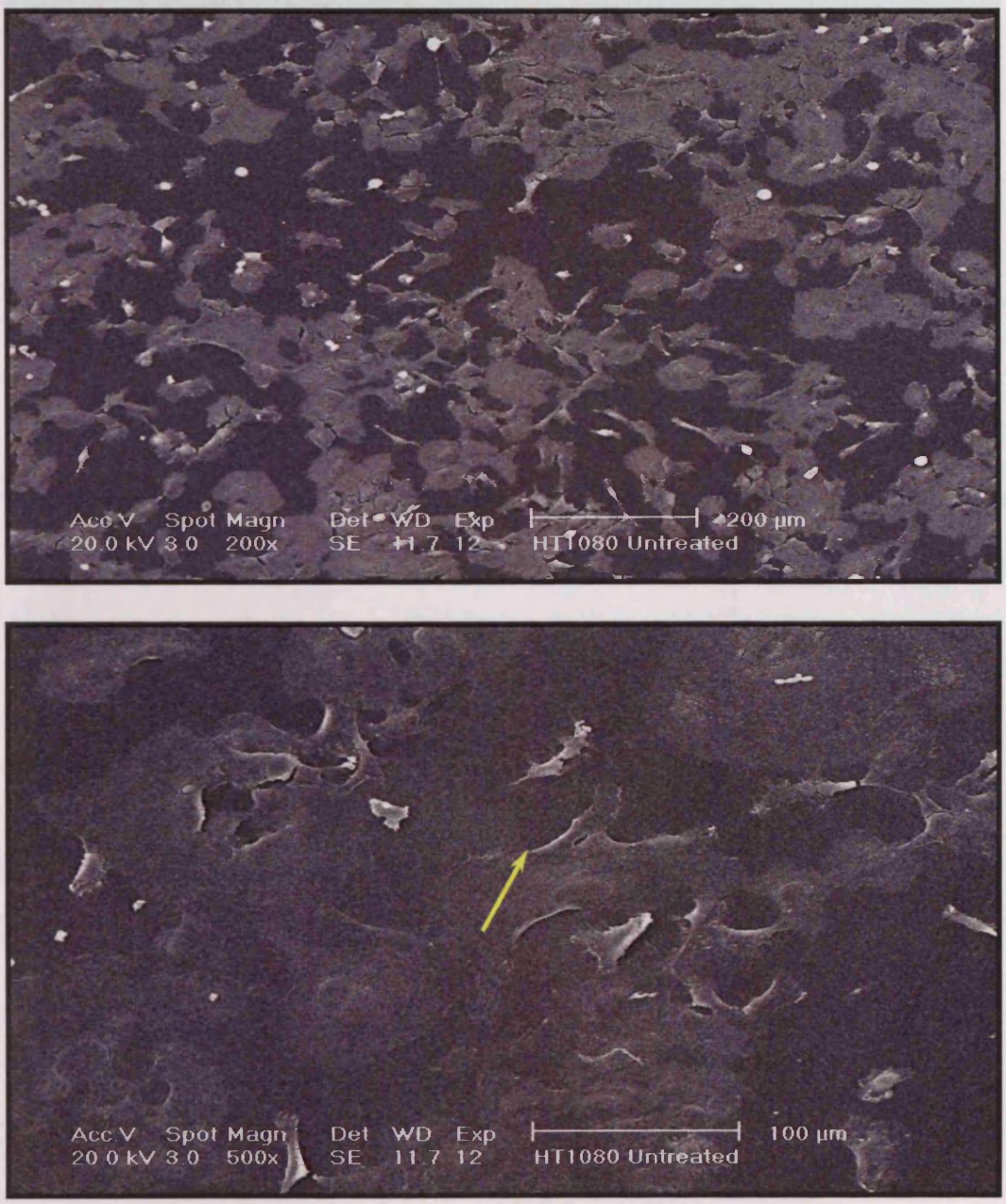
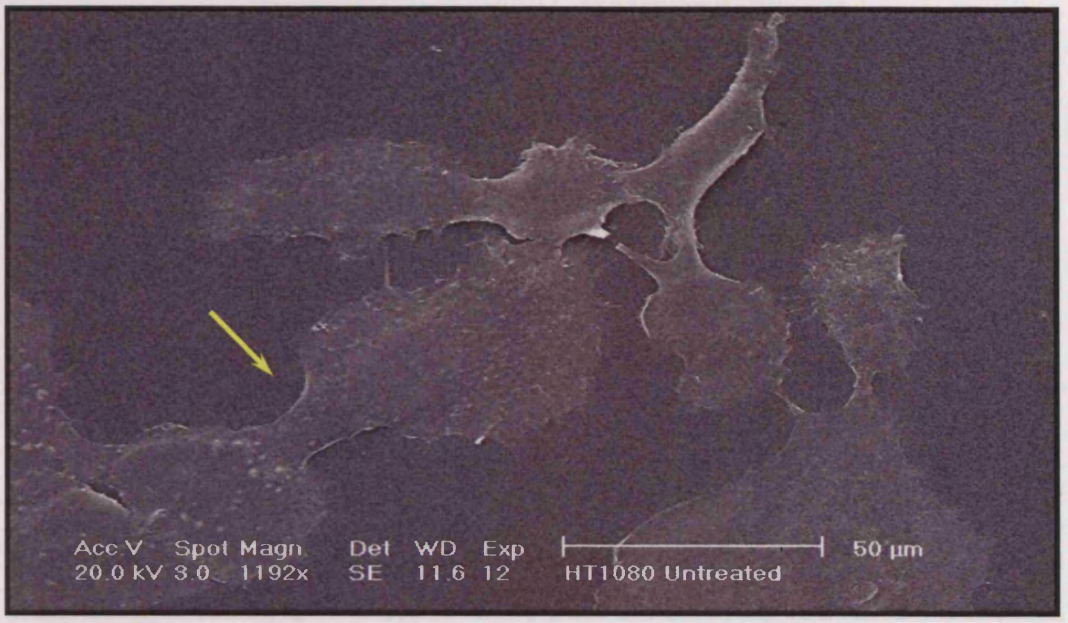


Figure 3.111 (A-B)
SEM images of untreated HT1080 cells. Cells are attached to the surface and show filopodial attachments (yellow arrow).



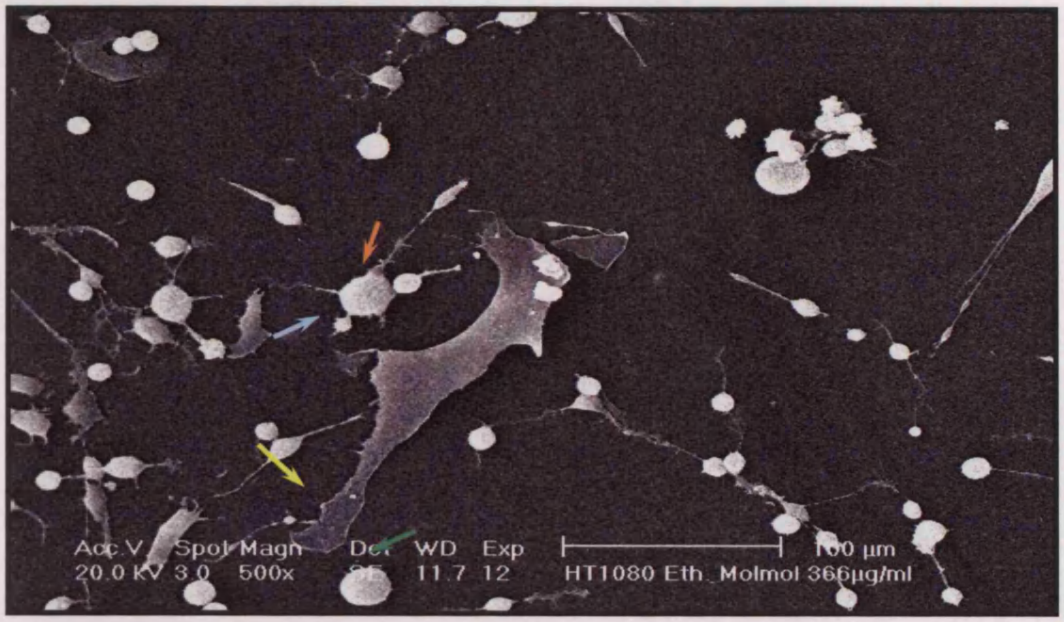
C

Figure 3.111 (C)
SEM images of untreated HT1080 cells. Cells are attached to the surface and show filopodial atachments (yellow arrow).

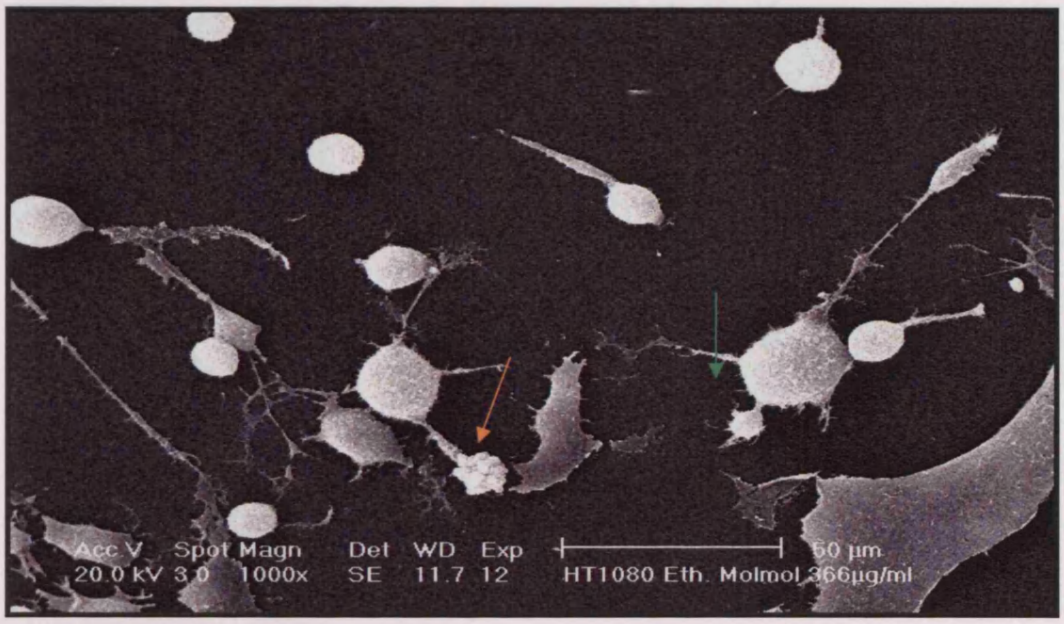
HT1080 cells exposed to ethanolic molmol extract at a concentration of 336 µg/ml for 12 hours showed symptoms of apoptosis, including the formation of apoptotic cells and apoptotic bodies, and fragmentation (blebbing) (Figure 3.112A-E).

A

Figure 3.112 (A)
SEM images of HT1080 cells treated for 12 hours with 366 µg/ml ethanolic molmol extract.



B

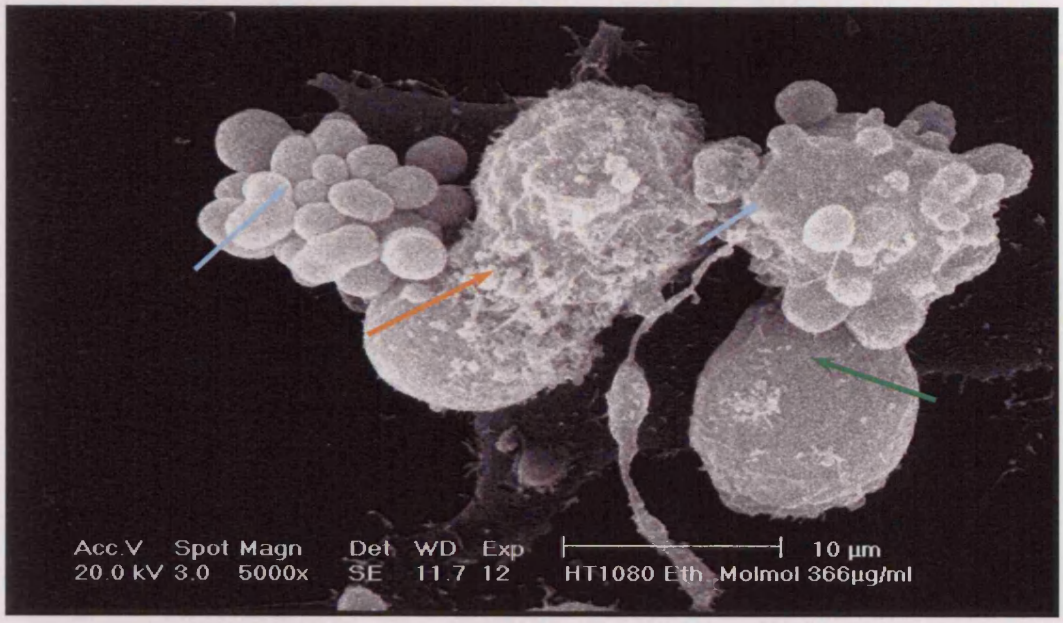


C

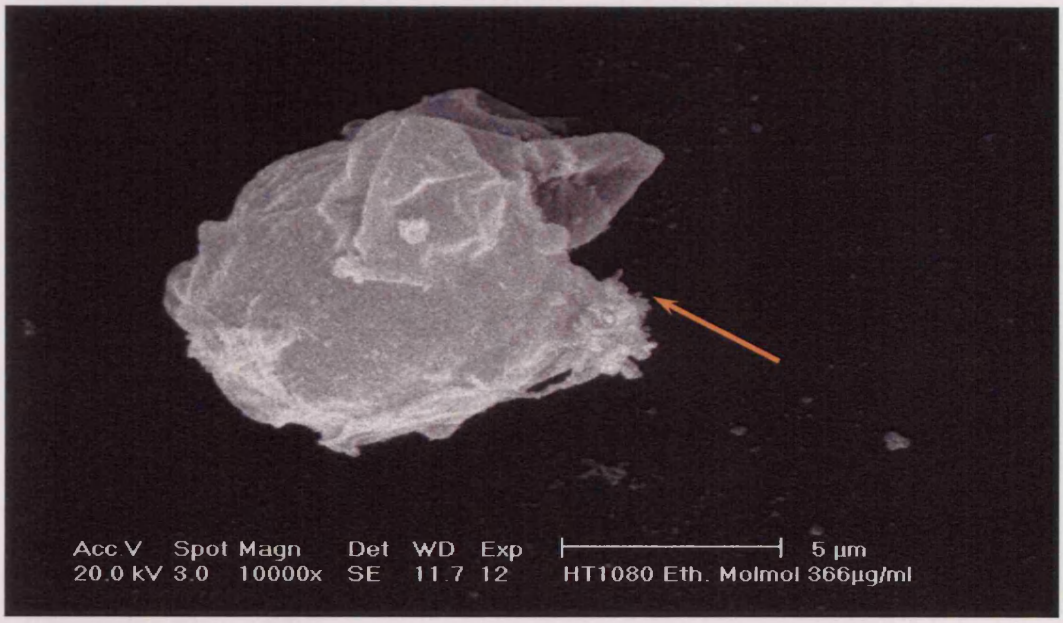
Figure 3.112 (B-C)

SEM images of HT1080 cells treated for 12 hours with 366 µg/ml ethanolic molmol extract.

(A&B) Showing formation of apoptotic cells (orange arrow) and apoptotic bodies (green arrow), and membrane blebbing (turquoise arrow). One cell shows a filopodial attachment (yellow arrow).



D



E

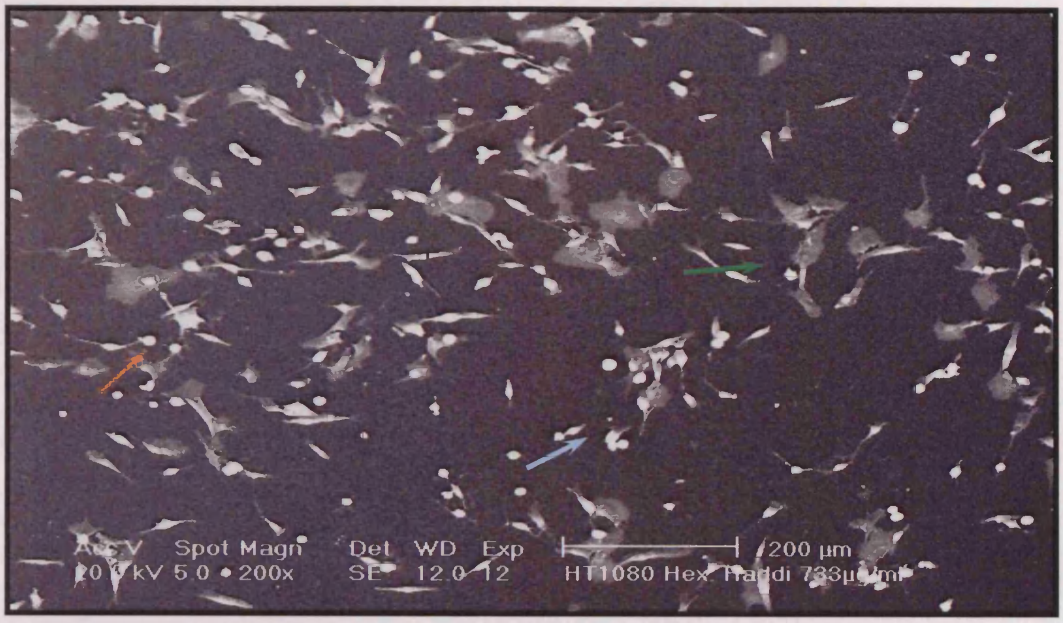
Figure 3.112 (D-E)

SEM images of HT1080 cells treated for 12 hours with 366 µg/ml ethanolic molmol extract.

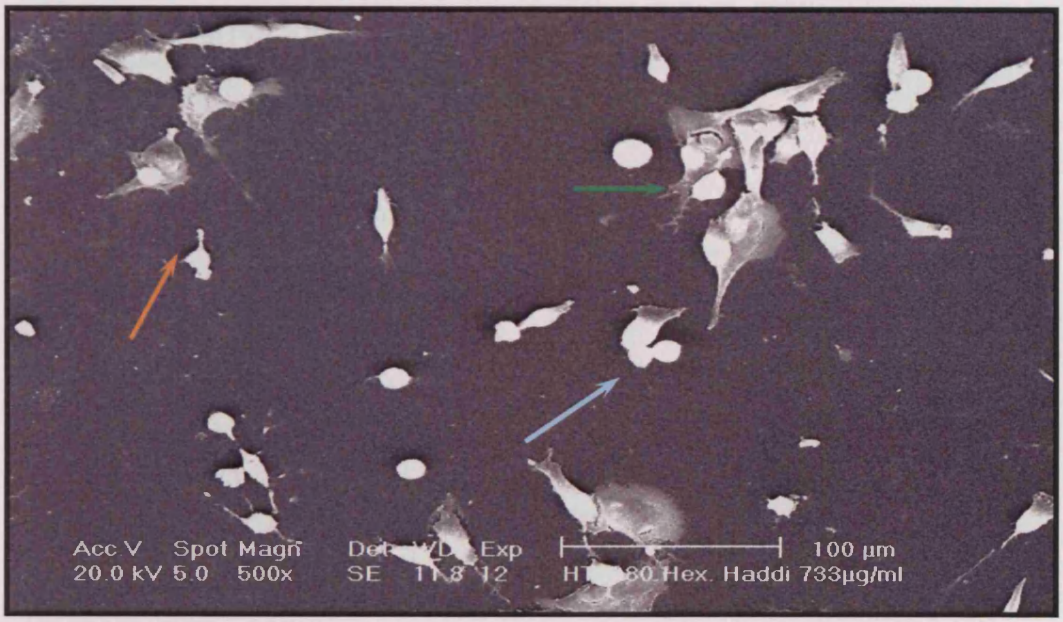
(C&D) Showing apoptotic cells (orange arrow) and apoptotic bodies (green arrow), and membrane blebbing (turquoise arrow).

(E) Showing apoptotic cell (orange arrow).

Exposure of HT1080 cells to hexane haddi extract (733 µg/ml) also resulted in symptoms of apoptosis, including the formation of apoptotic cells and apoptotic bodies, loss of filopodial attachments, and surface blebbing (Figure 3.113A-E).

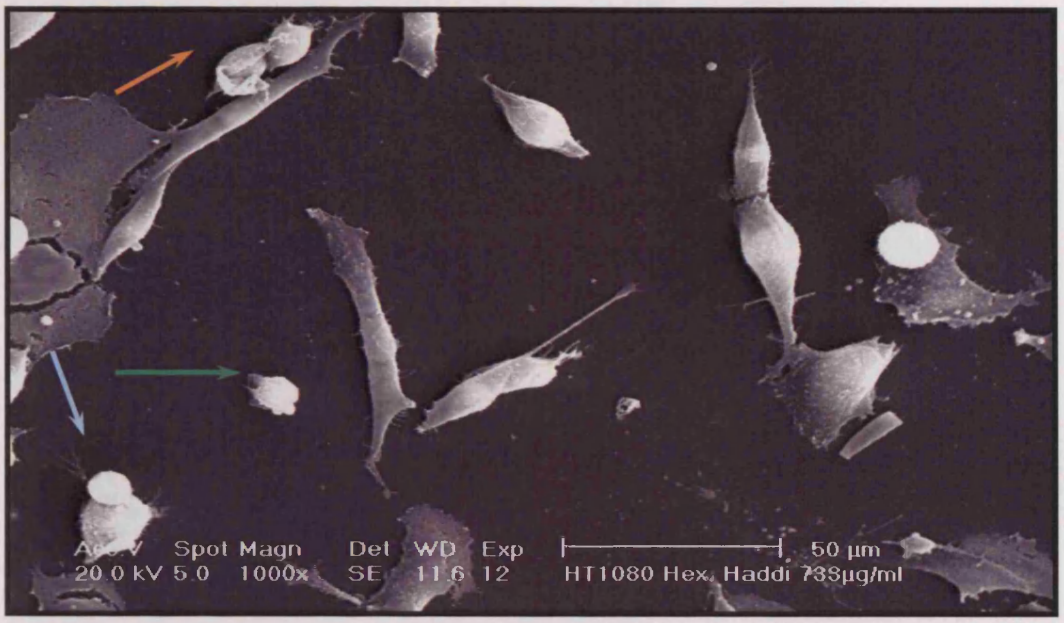


A

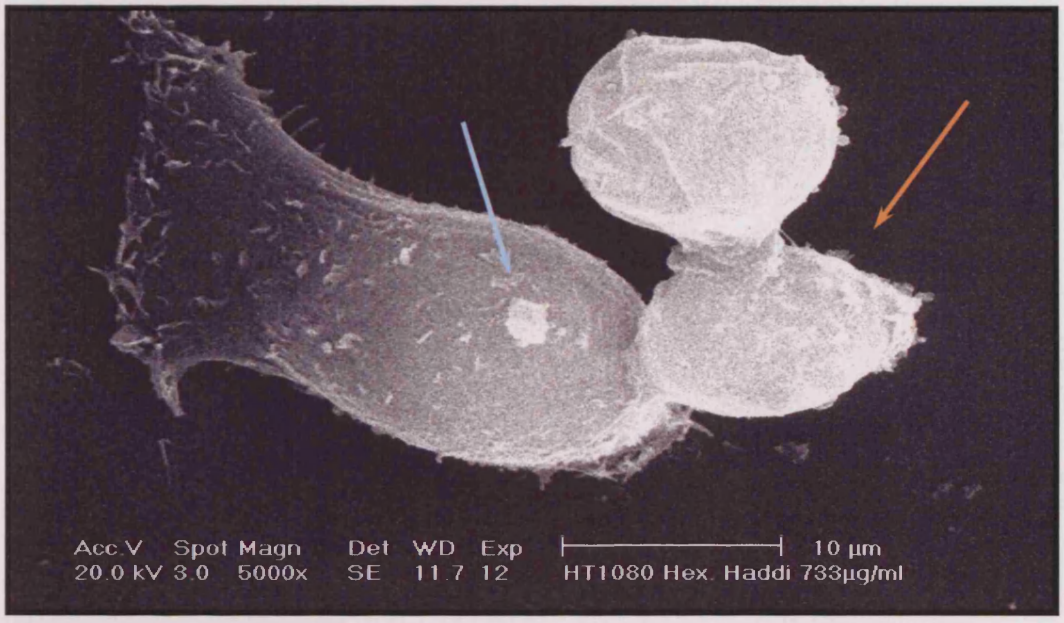


B

Figure 3.113 (A-B).
SEM images of HT1080 cells treated for 12 hours with 733 µg/ml hexane haddi extract.
(A&B) Showing formation of apoptotic cells (orange arrow) and apoptotic bodies (green arrow), and membrane blebbing (turquoise arrow).



C



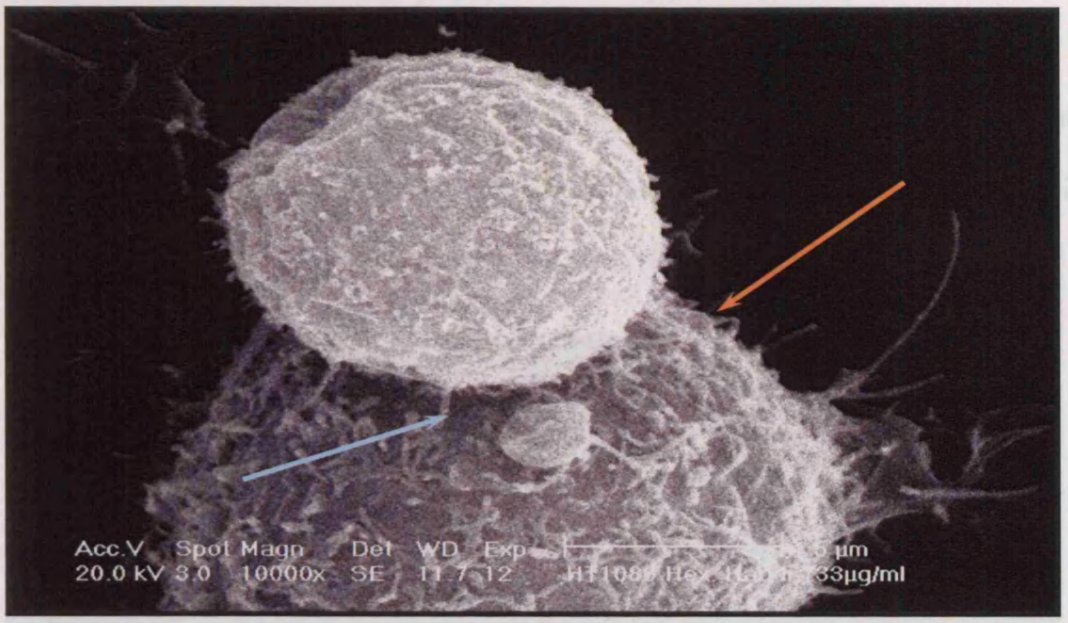
D

Figure 3.113 (C-D)

SEM images of HT1080 cells treated for 12 hours with 733 µg/ml hexane haddi extract.

(C) Showing apoptotic cells (orange arrow) and apoptotic bodies (green arrow), and membrane blebbing (turquoise arrow).

(D) Showing apoptotic cell (orange arrow) and surface blebbing (turquoise arrow).



E

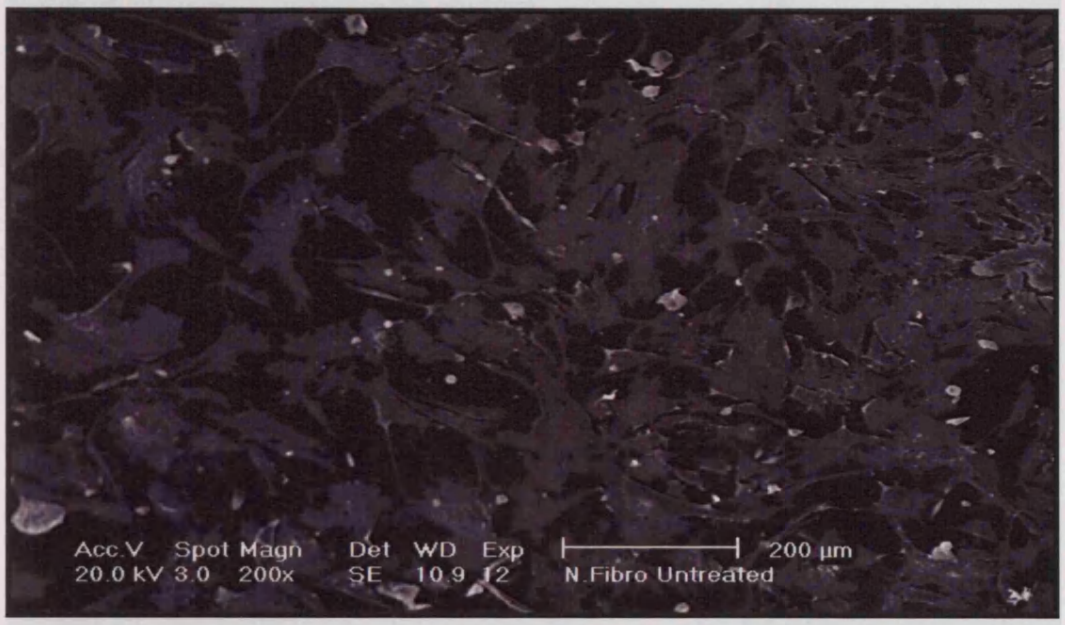
Figure 3.113 (E)

SEM images of HT1080 cells treated for 12 hours with 733 µg/ml hexane haddi extract.

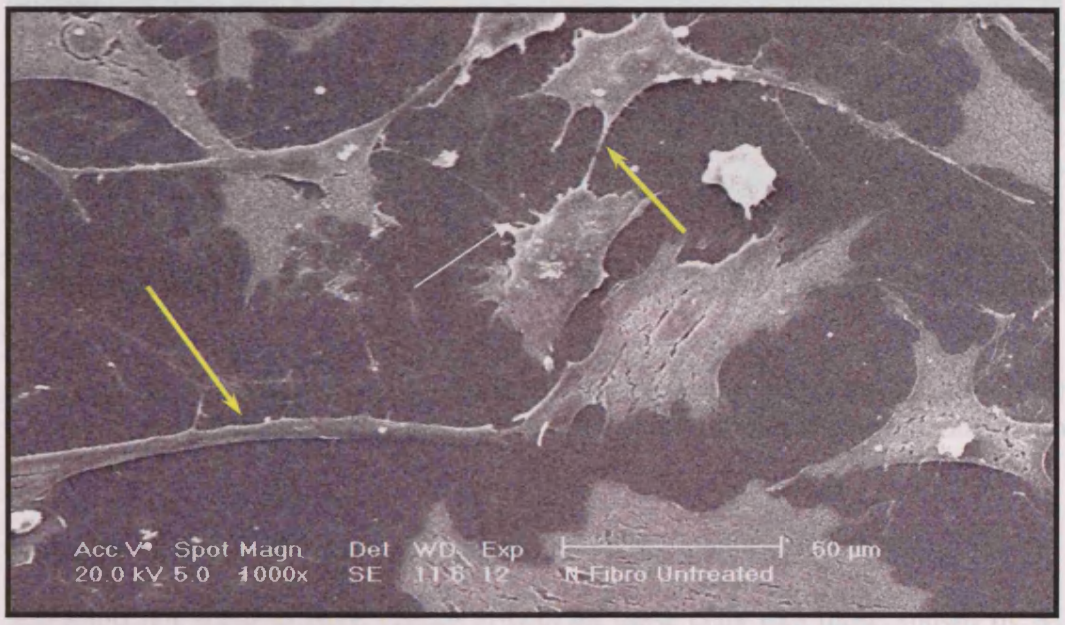
(E) Showing apoptotic cell (orange arrow) and surface blebbing (turquoise arrow).

3.15.3 Normal Fibroblast Cells

Untreated normal fibroblast cells were found adhering to the substratum and interdigitating with each other through filopodial attachments. Delicate microvilli were also observed on the surfaces of viable cells (Figure 114A-D).

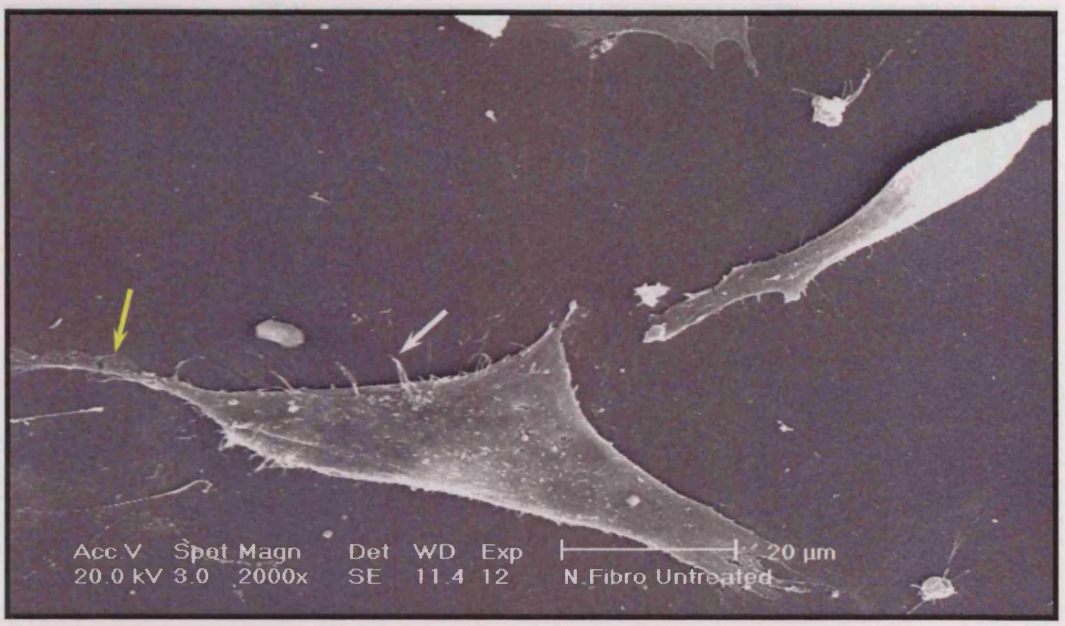


A

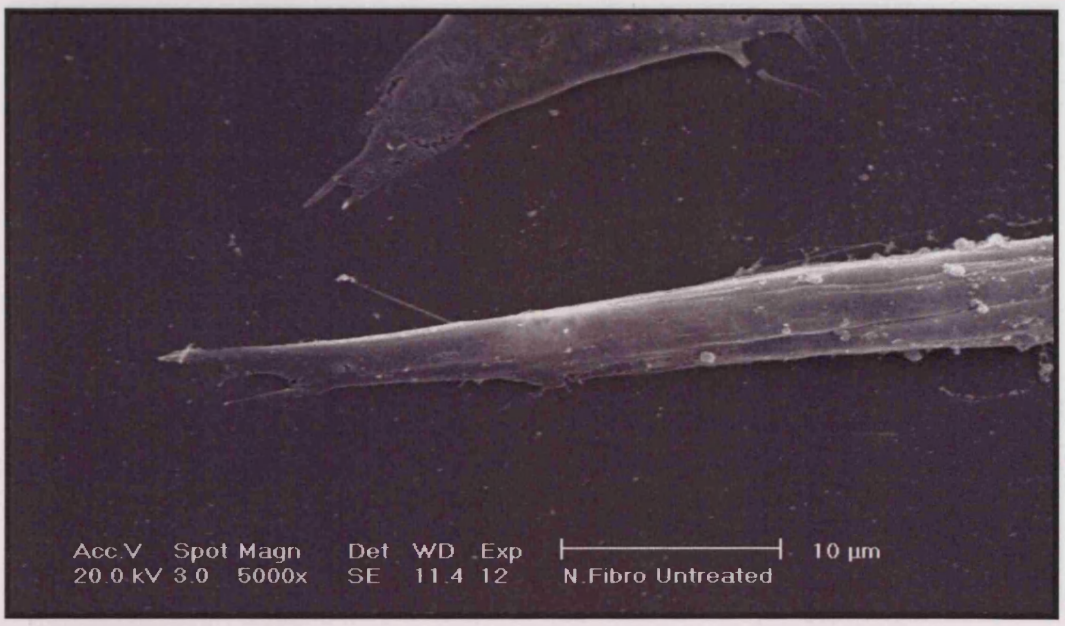


B

Figure 3.114 (A-B)
SEM images of untreated normal fibroblast cells, showing cell attachment to the substratum (A), surface microvilli (white arrow) and filopodial attachments (yellow arrow).



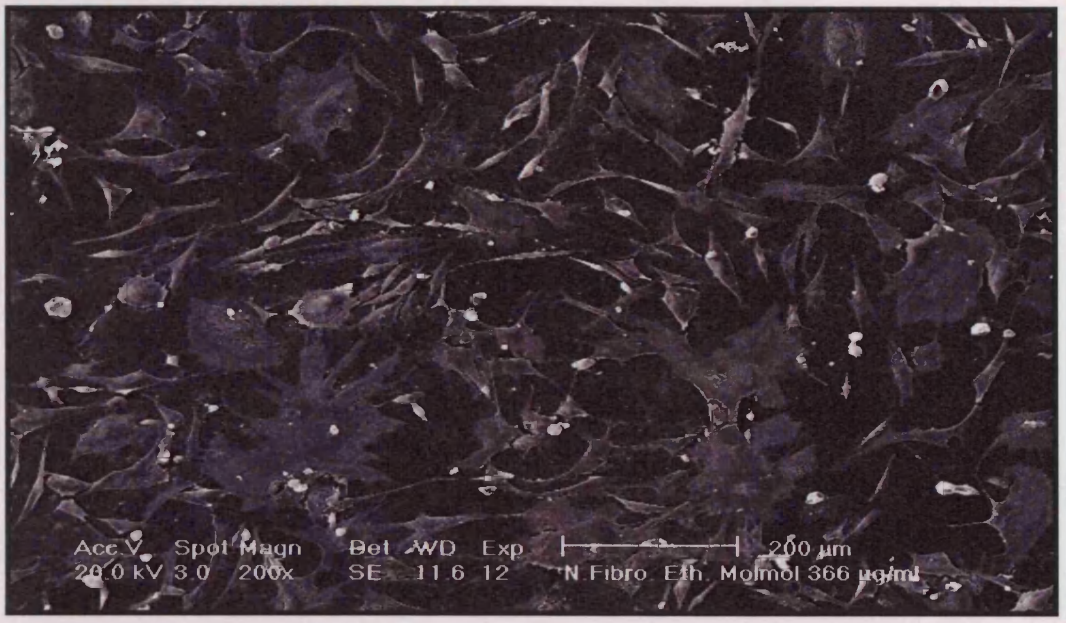
C



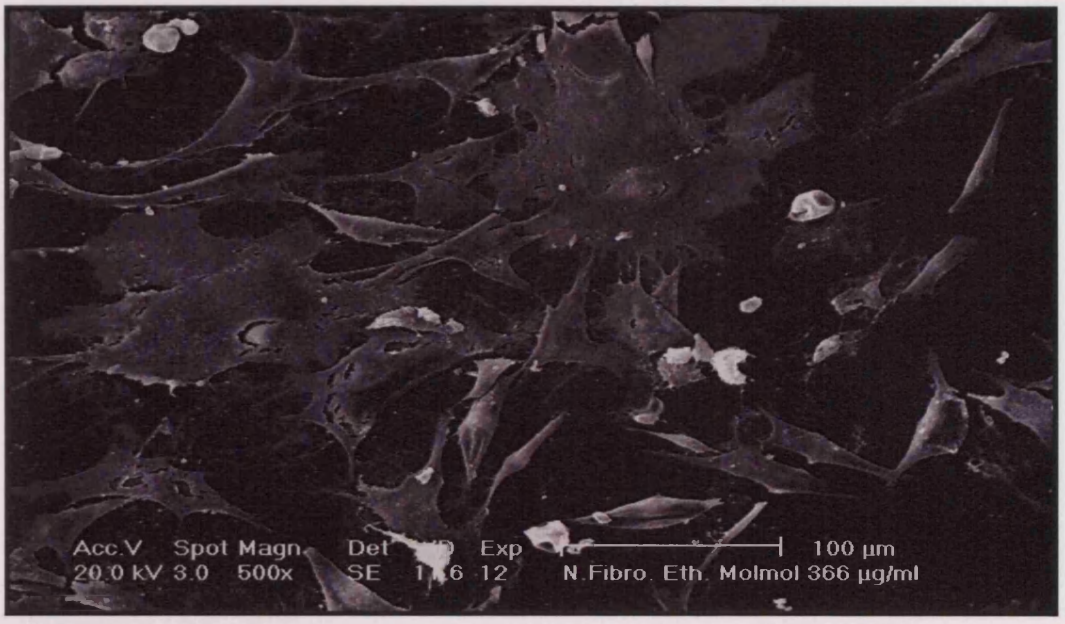
D

Figure 3.114 (C-D)
SEM images of untreated normal fibroblast cells, showing cell attachment to the substratum (A), surface microvilli (white arrow) and filopodial attachments (yellow arrow).

Exposure of normal fibroblast cells to an ethanolic molmol extract (366 µg/ml) for 12 hours did not seem to have visible effects on these cells like those shown by S180 cells and HT1080 cells treated with this extract for a similar period of time (Figure 3.115A-D).

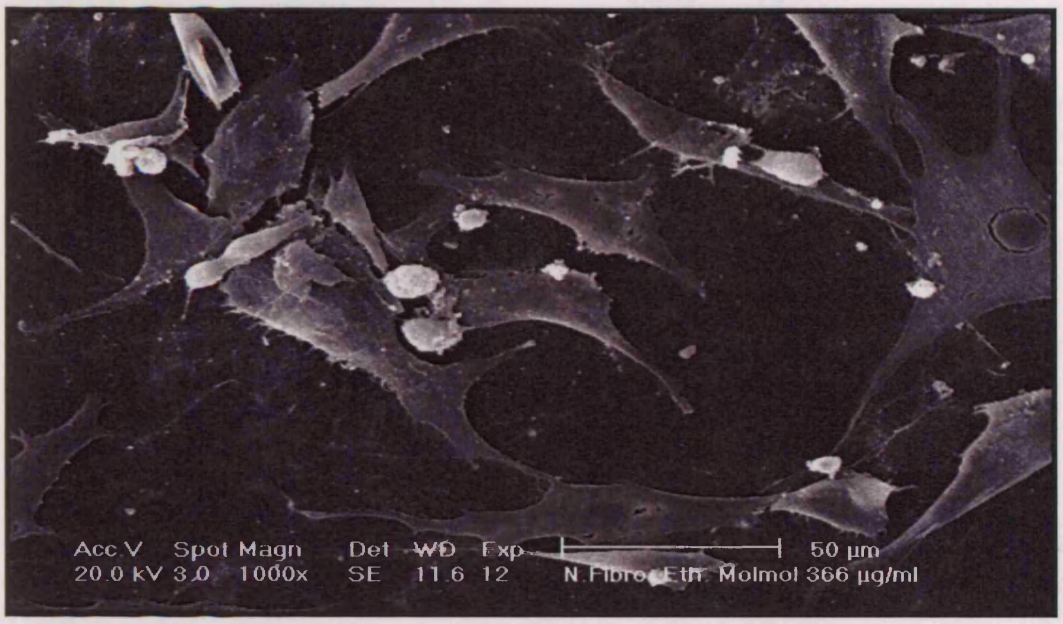


A

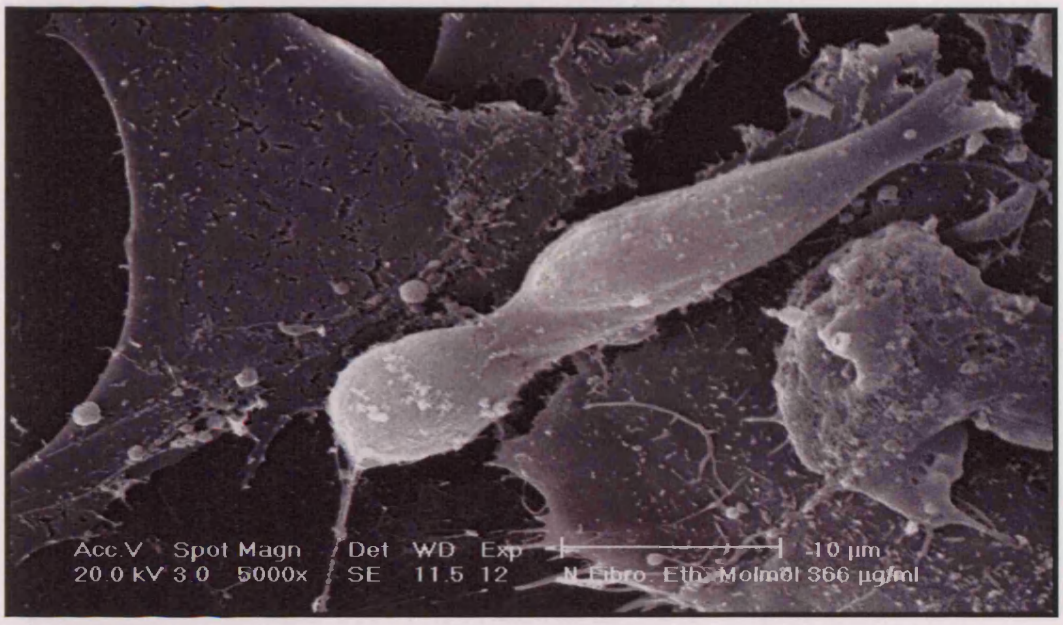


B

Figure 3.115 (A-B)
SEM images of normal fibroblast cells treated with ethanolic molmol extract (366 µg/ml), showing cell attachment to the substratum, and filopodial attachments.



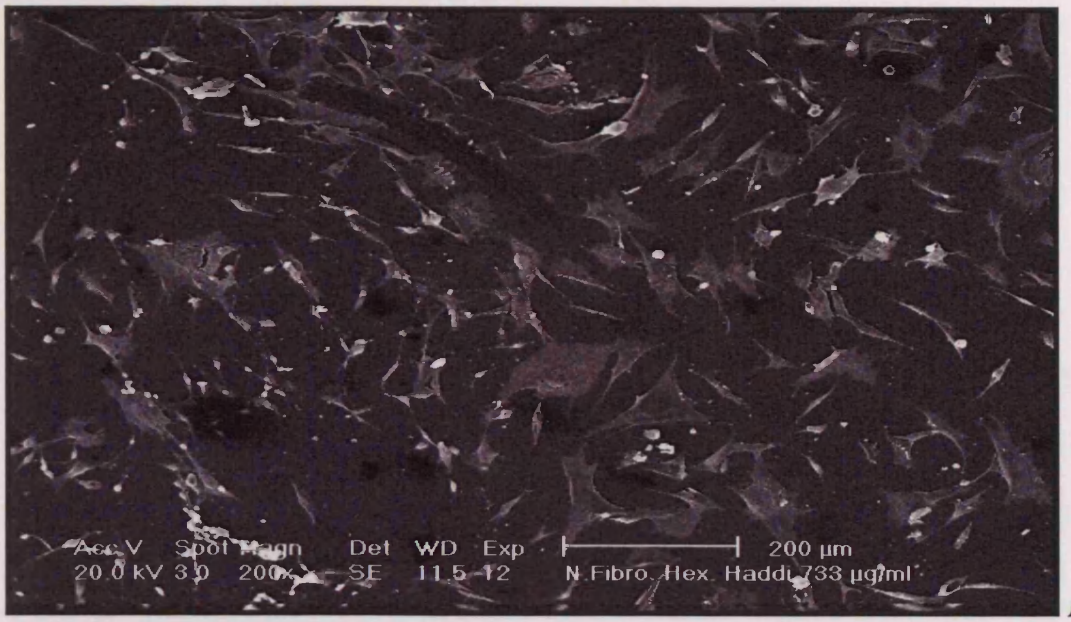
C



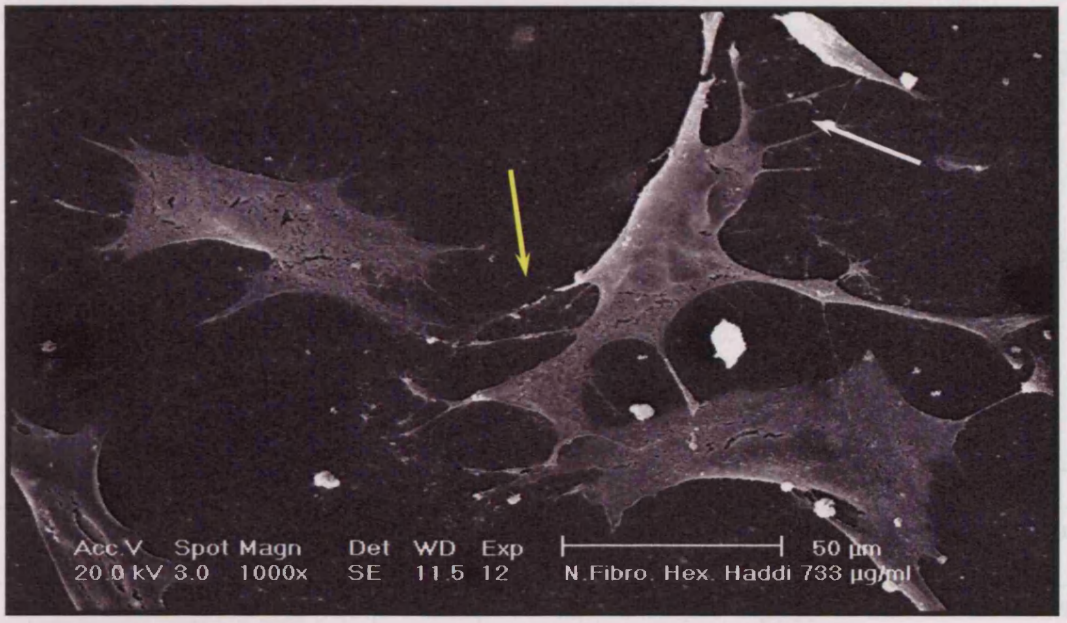
D

Figure 3.115 (C-D)
SEM images of normal fibroblast cells treated with ethanolic molmol extract (366 µg/ml), also showing cell attachment to the substratum, and filopodial attachments.

Exposure of normal fibroblast cells to hexane haddi extract (733 µg/ml) for 12 hours also did not seem to have visible effects on these cells like those shown by S180 cells and HT1080 cells treated with this extract for a similar period of time (Figure 3.116A-C).

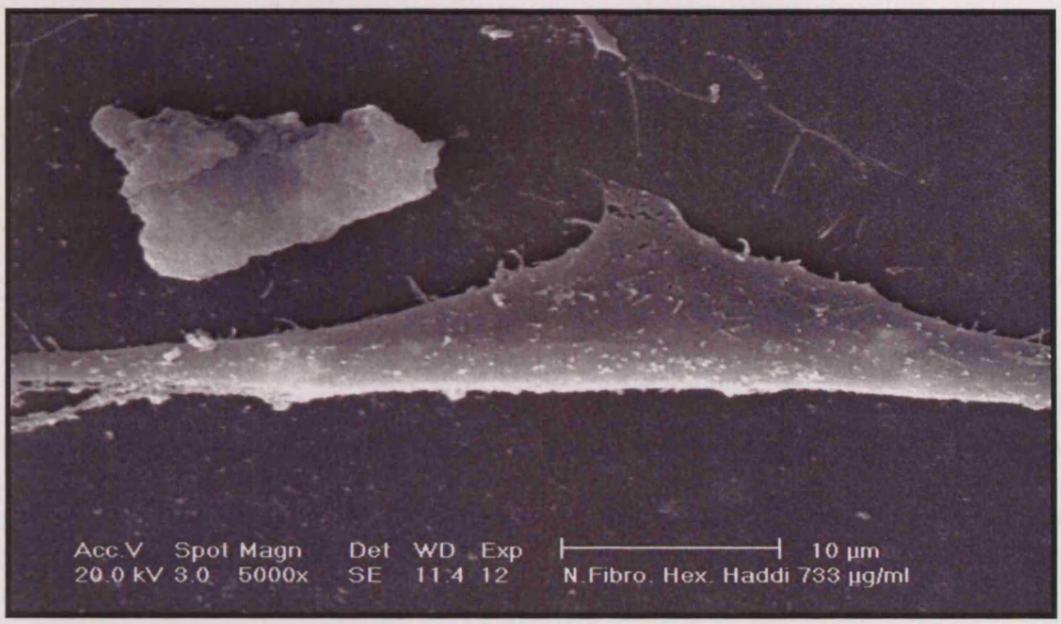


A



B

Figure 3.116 (A-B)
SEM images of normal fibroblast cells treated with hexane haddi extract (733 µg/ml), showing cell attachment to the substratum, and filopodial attachments.



C

Figure 3.116 (C)
SEM images of normal fibroblast cells treated with hexane haddi extract (733 µg/ml), showing cell attachment to the substratum, and filopodial attachments.

3.16 Summary

The effects of molmol and haddi extracts on the three cell lines used in this study can be illustrated as follows (Table 3.11).

Table 3.11. Summary of effects of molmol and haddi extracts

	Treatments in Respect of Apoptosis Formation		
Cell Line	**Untreated**	**Ethanolic Molmol**	**Hexane Haddi**
Normal Fibroblasts	None	• Some low level of apoptosis	• Low level of apoptosis
S180	None	• Loss of most microvilli • Loss of filopodial attachements • Blebbing and membrane pitting • Hence, medium apoptosis	• Loss of most microvilli • Loss of filopodial attachements • Blebbing and membrane pitting • Formation of apoptotic cells and apoptotic bodies • Hence, high apoptosis
HT1080	None	• Formation of apoptotic bodies • Blebbing • Loss of most filopodial attachments • Hence, high apoptosis	• Formation of apoptotic bodies • Blebbing • Loss of most filopodial attachments • Hence, high apoptosis

3.17 Transmission Electron Microscopy (TEM): HT1080 Cells

3.17.1 Effects of Ethanolic Molmol

TEM revealed normal growth of untreated cells. The cells were found adhering to the substratum, and looked healthy, with clearly defined internal organelles. The cells interdigitated with each other through filopodial attachments, but delicate microvilli were not noted on the surfaces of viable cells. At higher magnification nucleus, nucleolus, mitochondria and lipids, as well as nuclear membrane, are clearly identified.

However, HT1080 cells exposed to ethanolic molmol extract at a concentration of 234 μg/ml for 12 hours showed symptoms of apoptosis, including the formation of apoptotic bodies, and fragmentation (blebbing) (Figure 3.17). Cells also showed chromatin margination and loss of filopodial attachments.

Higher magnification of HT1080 cells treated with ethanolic molmol at 234 μg/ml (Figure 3.18) clearly illustrates the formation of apoptotic cells, blebs on these cells, and the formation of apoptotic bodies. It also shows the condensation of chromatin bodies alongside the nuclear membrane.

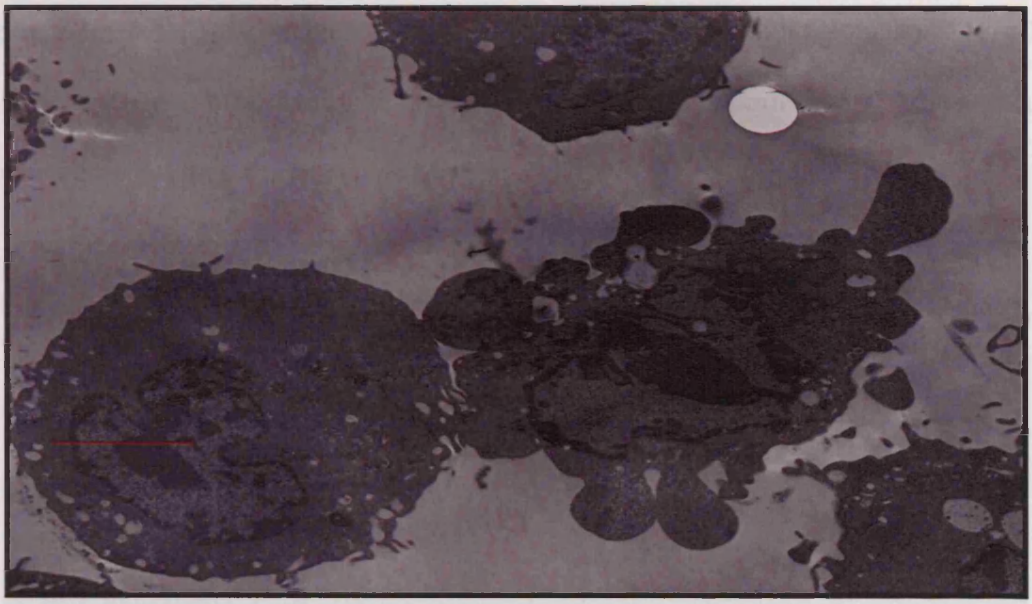


Figure 3.117

Low magnification TEM showing treated HT1080 cells and an apoptotic cell with blebs and nucleus exhibiting highly condensed chromatin alongside the nuclear membrane.

Magnification : 2500X **Treatment : Ethanolic Molmol**

Scale bar : 3μm **Time :12hrs Concentration 234 μg/ml**

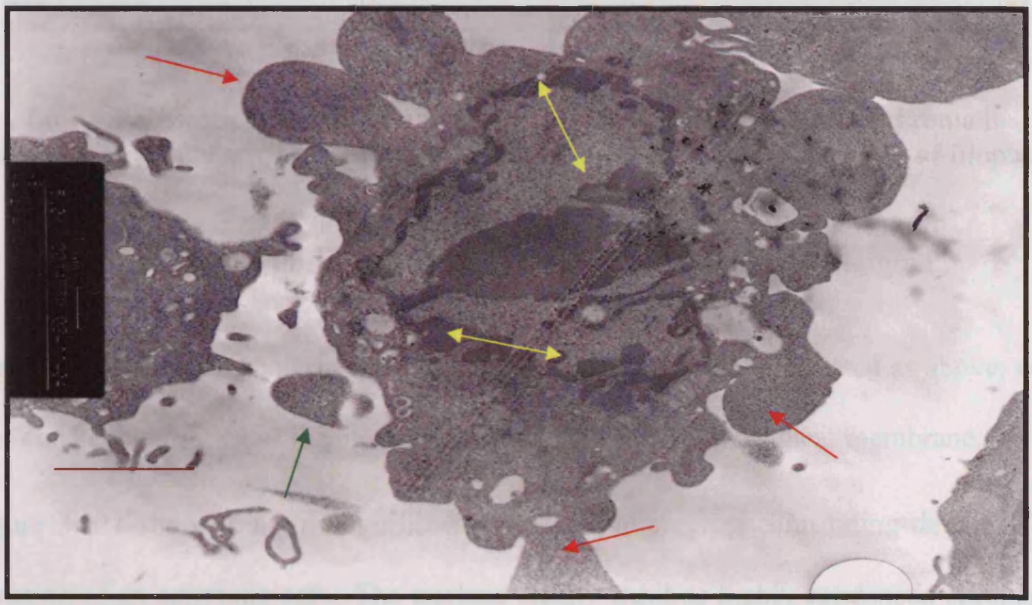


Figure 3.118

High magnification TEM of Figure 3.117 showing an apoptotic cell with blebs (red arrows) and apoptotic body (green arrow). Nucleus exhibits highly condensed chromatin alongside the nuclear membrane (yellow arrows).

Magnification : 5000X **Treatment : Ethanolic Molmol**

Scale bar : 1μm **Time :12hrs Concentration 234 μg/ml**

Figure 3.119 clearly shows the margination of the chromatin within the nuclei of HT1080 cells treated with ethanolic molmol at a concentration of 234 µg/ml for 12 hours. It also shows the vacuolation of the cytoplasm, and the loss of filaments.

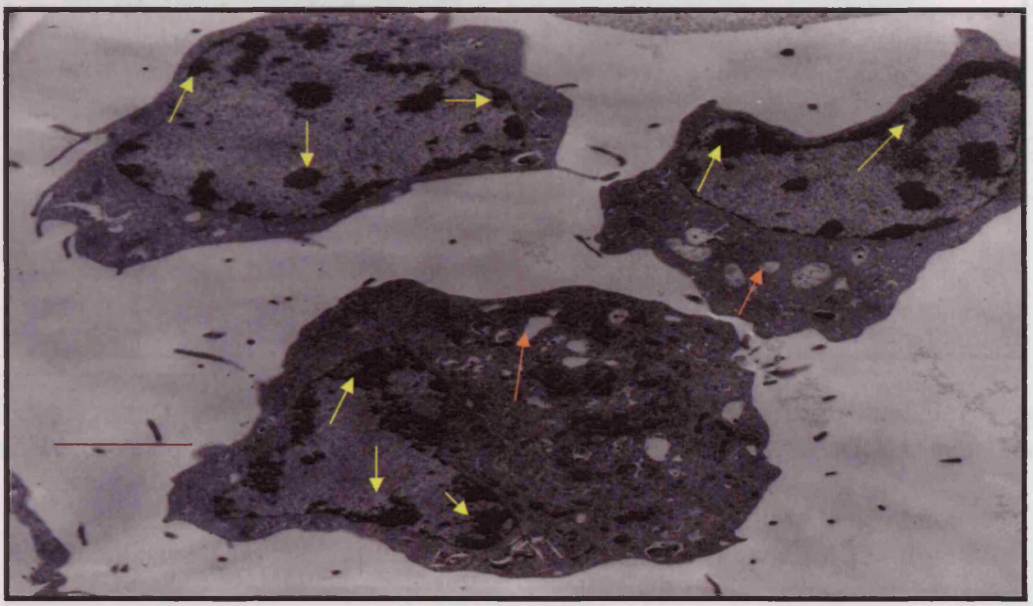


Figure 3.119
Low magnification TEM showing treated HT1080 cells with signs of chromatin margination (yellow arrows), cytoplasm vacuolation (orange arrow) and loss of filopodial filaments.
Magnification : 2000X **Treatment : Ethanolic Molmol**
Scale bar : 2µm **Time :12hrs Concentration 234 µg/ml**

Figure 3.120 shows an enlarged view of an apoptotic HT1080 cell treated as above, with blebs and condensation of the chromatin material alongside the nuclear membrane.

Figure 3.121 shows a high magnification electron micrograph illustrating detailed fine structure of an apoptotic cell. The nucleus clearly exhibits highly condensed chromatin margination alongside the nuclear membrane, swollen mitochondria, and some debris. The latter might be a sign of necrosis.

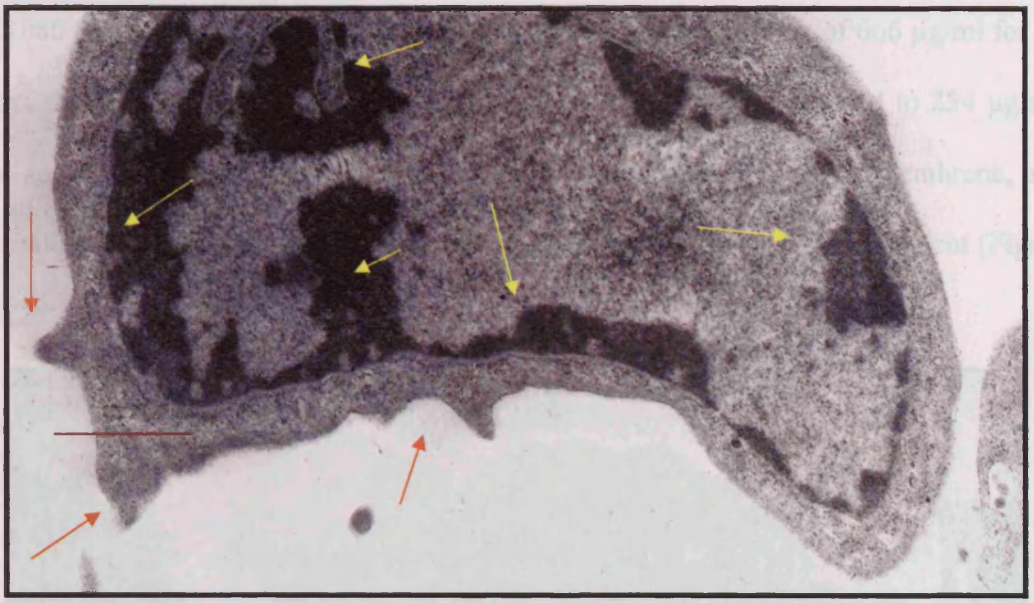


Figure 3.120

High magnification TEM of Figure 3.119 showing an apoptotic cell with blebs (orange arrows). Nucleus exhibits highly condensed chromatin alongside the nuclear membrane (yellow arrows).

Magnification : 5000X **Treatment : Ethanolic Molmol**
Scale bar : 1μm **Time: 12hrs Concentration 234 μg/ml**

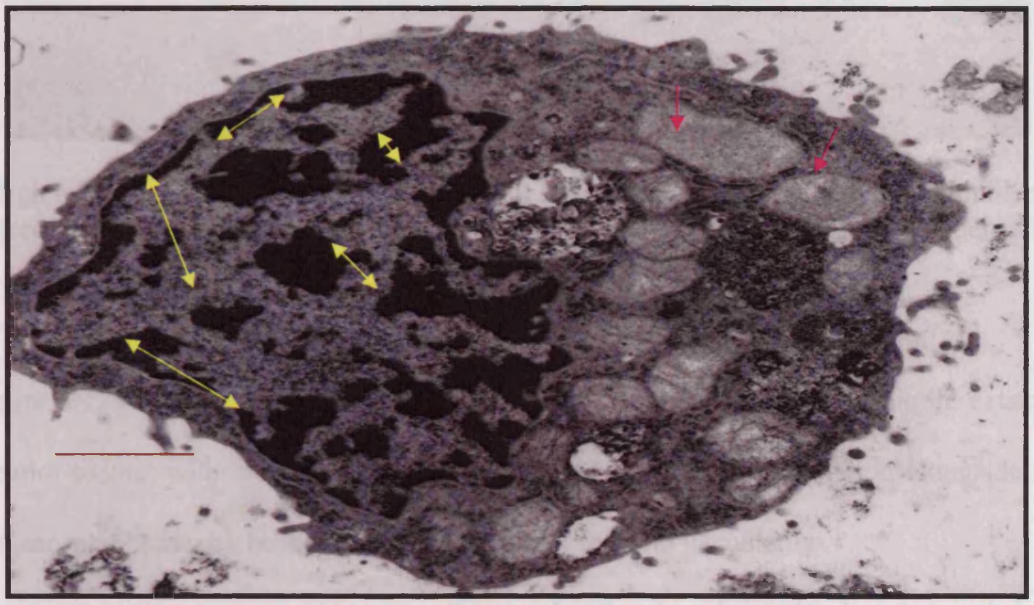


Figure3.121

High magnification TEM showing detailed fine structure of an apoptotic cell. Nucleus exhibits highly condensed chromatin margination alongside the nuclear membrane (yellow arrows). Mitochondria appear swollen (pink arrows). Some debris can also be noticed.

Magnification : 8000X **Treatment : Ethanolic Molmol**
Scale bar : 1μm **Time : 12hrs Concentration 234 μg/ml**

HT1080 cells exposed to ethanolic molmol extract at a concentration of 666 µg/ml for 12 hours showed more advanced symptoms of apoptosis than those exposed to 234 µg/ml. The apoptotic cells were covered with blebbings all over the outer cell membrane, and several apoptotic bodies can be observed. Cytoplasmic vacuoles are also evident (Figure 3.122).

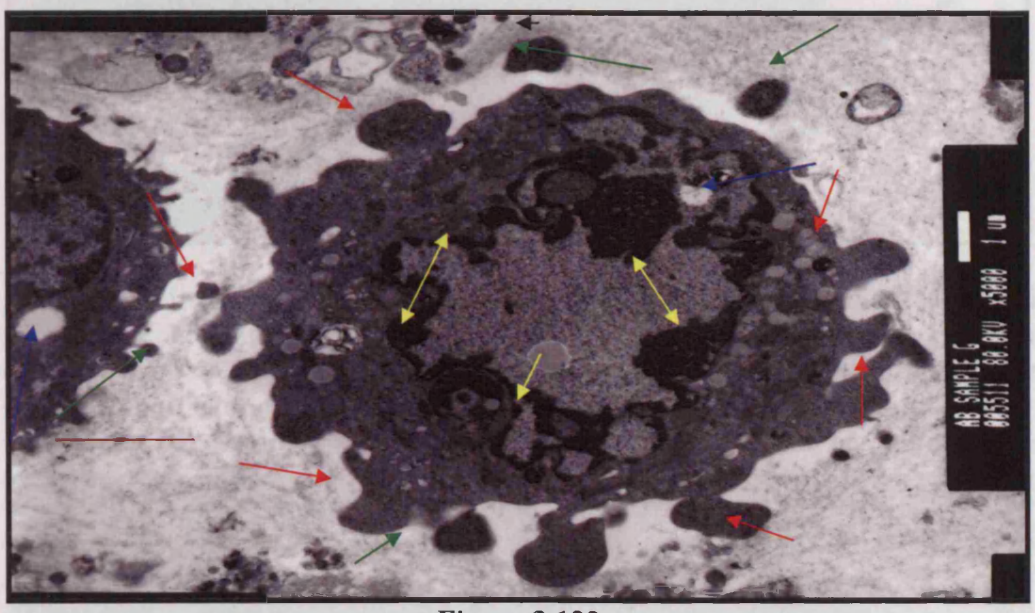


Figure 3.122
High magnification TEM of treated HT1080 cells showing an apoptotic cell with blebs (red arrows), apoptotic bodies (green arrows), vacuoles (blue arrows) and condensed chromatin margination (yellow arrows).
Magnification : 5000X **Treatment : Ethanolic Molmol**
Scale bar : 1µm **Time :12hrs Concentration 666 µg/ml**

Figure 3.123 shows a damaged HT1080 cell treated with 666 µg/ml ethanolic extacted molmol extract with clear indication of condensed chromatin margination alongside the nuclear membrane, blebbings of the outer membrane, and vacuolation.

Figure 3.124 illustrates an HT1080 cell treated with ethanolic molmol at 666 µg/ml for 12 hours showing indications of damage to the cell membrane and evident vacuolation of the cytoplasm. The nucleus shows some condensed chromatin. All these indications suggest apoptosis.

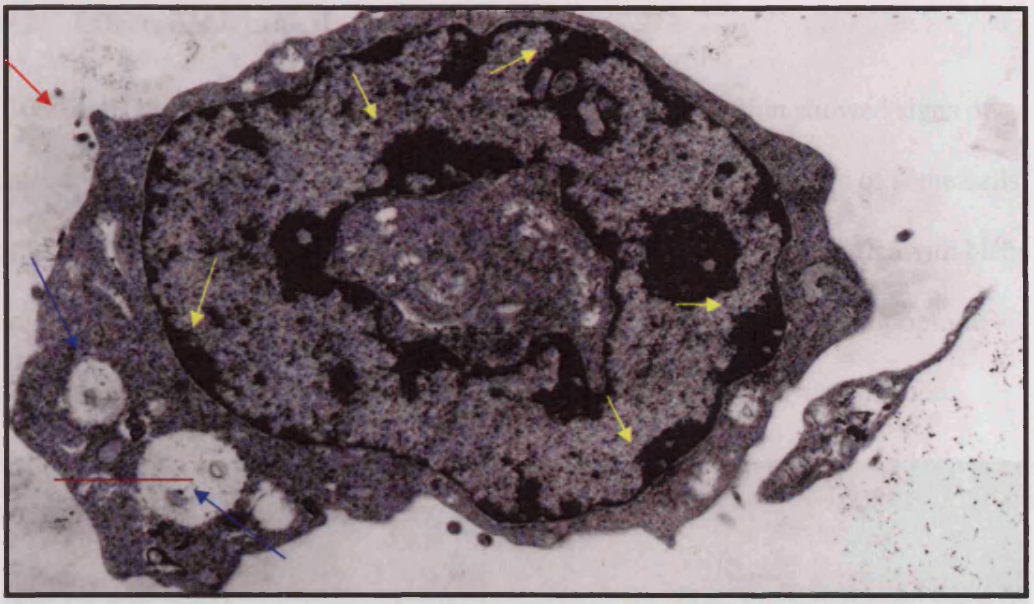


Figure 3.123

High magnification TEM of HT1080 cells showing signs of damage with clear indication of condensed chromatin margination alongside the nuclear membrane (yellow arrows), and blebbings of the outer membrane (red arrows). Vacuolation is evident (blue arrows).

Magnification : 6300X **Treatment : Ethanolic Molmol**
Scale bar : 1μm **Time : 12hrs Concentration 666 μg/ml**

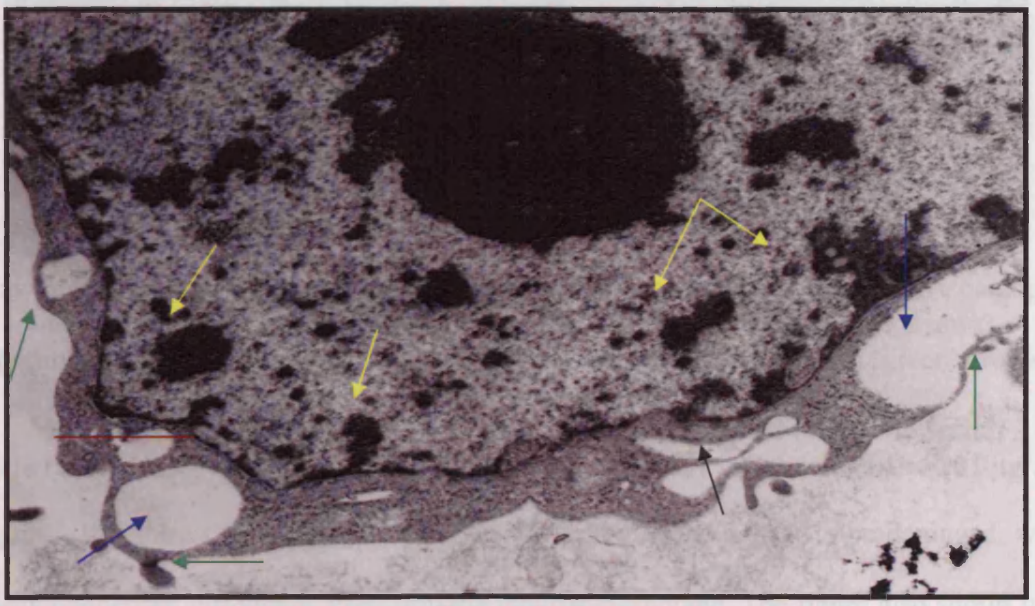


Figure 3.124

High magnification TEM showing a treated cell with some indications of damage to the cell membrane (light green arrows) and vacuolation (blue arrows) of the rough endoplasmic reticulum (black arrow). Nucleus shows some condensed chromatin (yellow arrows) that may suggest that the cell is apoptotic.

Magnification : 6300X **Treatment : Ethanolic Molmol**
Scale bar : 1μm **Time : 12hrs Concentration 666 μg/ml**

3.17.2 Effects of Hexane Haddi

HT1080 cells treated with 201 µg/ml hexane haddi extract solution showed signs of extensive damage to the cells (Figure 3.125). The outer cell membranes of some cells show signs of disintegration, suggesting necrosis. Other cells are apoptotic, with blebs on the outer cell membrane. This treatment also resulted in the formation of apoptotic bodies. Condensed chromatin alongside the nuclear membrane can also be seen.

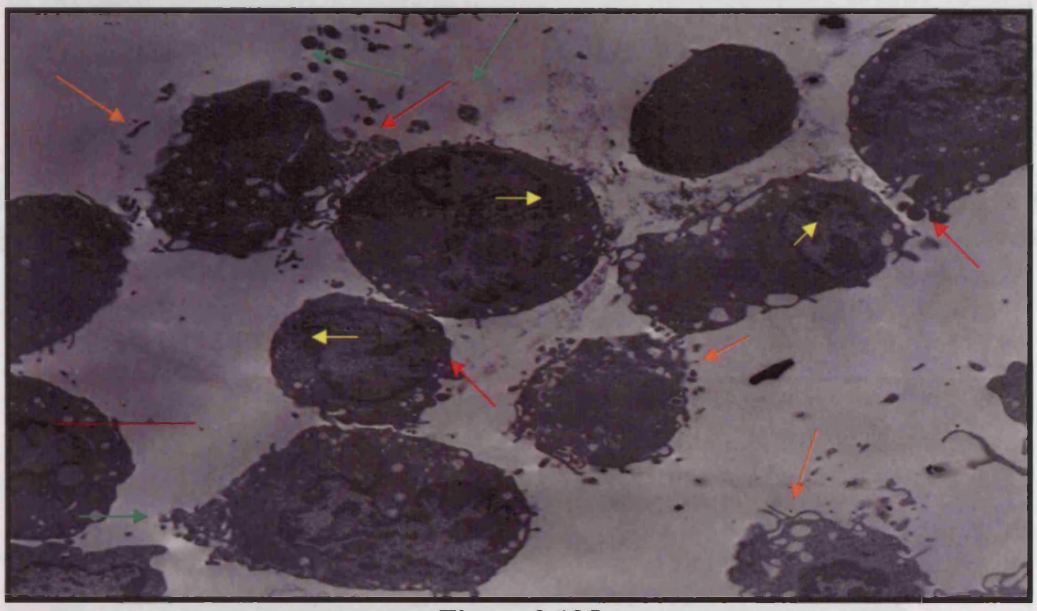


Figure 3.125
Low magnification TEM showing treated HT1080 cells with signs of extensive damage to cells. Some are necrotic with disintegrated cell membranes (orange arrows). Apoptotic cells, with blebs (red arrows) and apoptotic bodies are formed (green arrows). Condensed chromatin (yellow arrows) alongside the nucleus membrane.
Magnification : 2000X **Treatment : Hexane Haddi**
Scale bar : 3µm **Time : 12hrs Concentration 201 µg/ml**

Figure 3.126 shows two apoptotic cells, in which the nuclei appear to be damaged, with highly condensed chromatin alongside the nuclear membrane. The figure also shows loss of filaments and the formation of vacuoles.

Figure 3.127 shows a damaged cell, with highly condensed chromatin alongside the nuclear membrane. The mitochondria seem to have swollen.

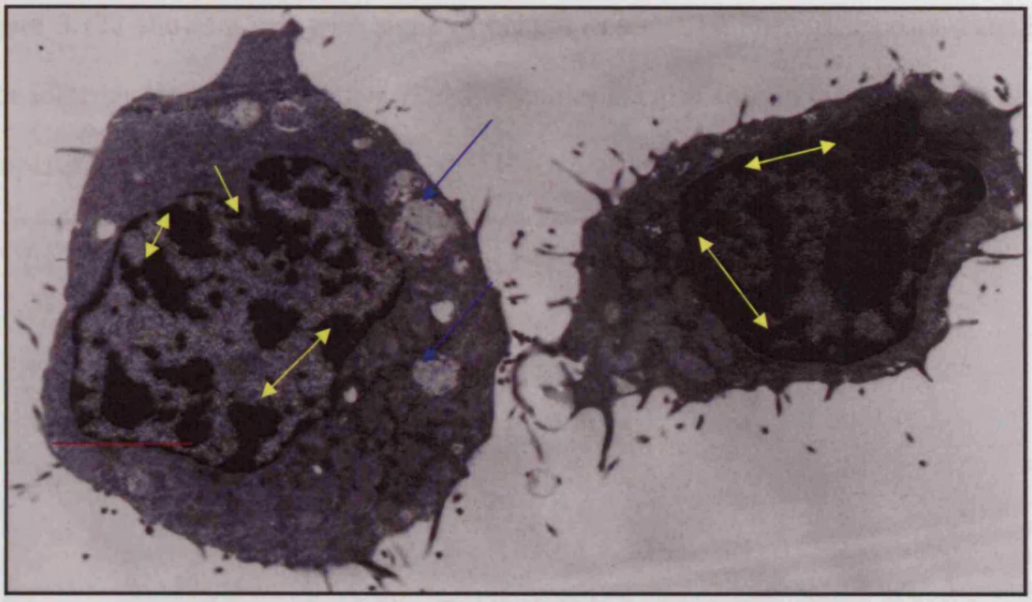


Figure 3.126

High magnification TEM showing apoptotic cells. The nucleus appears damaged, with highly condensed chromatin alongside the nuclear membrane (yellow arrows). There are signs of loss of filopodia and the formation of vacuoles (blue arrows)

Magnification : 5000X **Treatment : Hexane.Haddi**

Scale bar : 1μm **Time :12hrs Concentration 201 μg/ml**

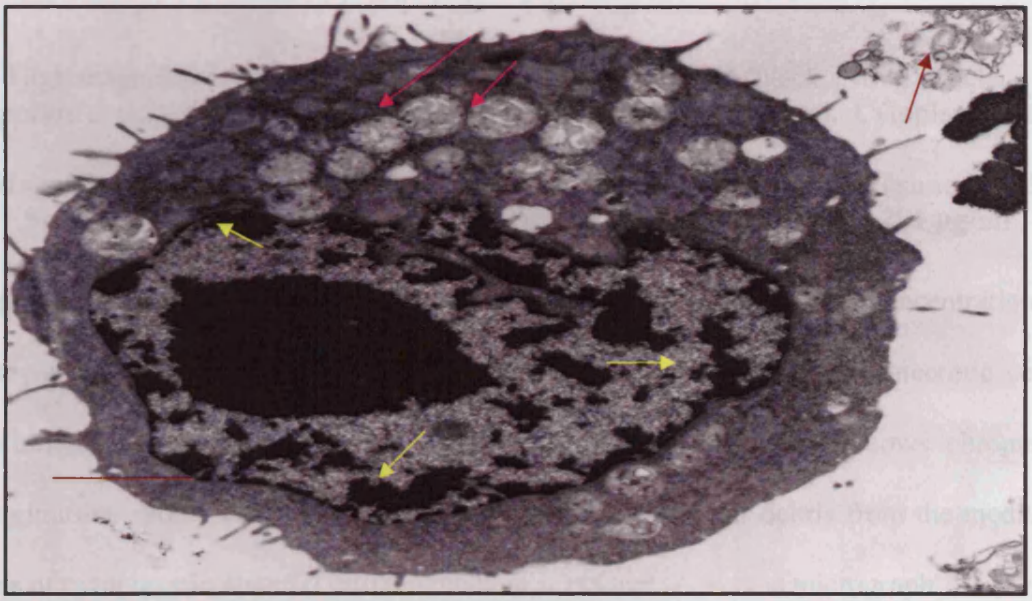


Figure 3.127

High magnification TEM showing a damaged cell. The nucleus appears damaged with highly condensed chromatin alongside the membrane (yellow arrows). The mitochondria are swollen (pink arrows). Note adjacent cell debris (dark red arrow)

Magnification : 5000X **Treatment : Hexane Haddi**

Scale bar : 1μm **Time : 12hrs Concentration 201 μg/ml**

Figure 3.128 shows a cell with signs of phagocytosis. The nucleus appears damaged, with some chromatin margination. Small vacuoles are also seen in the outer layer of the cytoplasm.

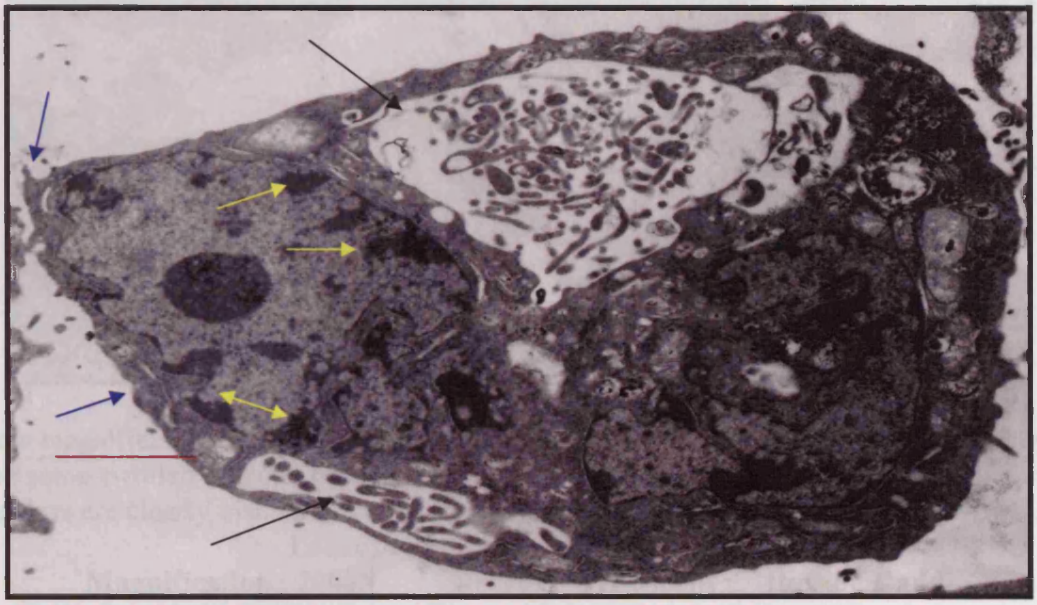


Figure 3.128
High magnification TEM showing a cell with phagocytosis (black arrow). Nucleus appears damaged, with some chromatin margination (yellow arrows). Cytoplasm with small vacuoles

Magnification : 4000X **Treatment : Hexane Haddi**
Scale bar : 1µm **Time :12hrs Concentration 201 µg/ml**

Figure 3.129 shows HT1080 cells treated with hexane haddi extract at a concentration of 666 µg/ml. Cells show signs of extensive damage, some of which are necrotic cells. Vacuolation and some debris are clearly visible, and the nucleus shows chromatin margination. Some cells appear to have phagocytosed cellular debris from the medium. Loss of cytoplasmic material into the medium is evident across the micrograph

Figure 3.130 shows an enlarged damaged HT1080 cell treated with hexane haddi extract at 666 µg/ml. The cytoplasm is highly vacuolated, and the nucleus shows chromatin margination.

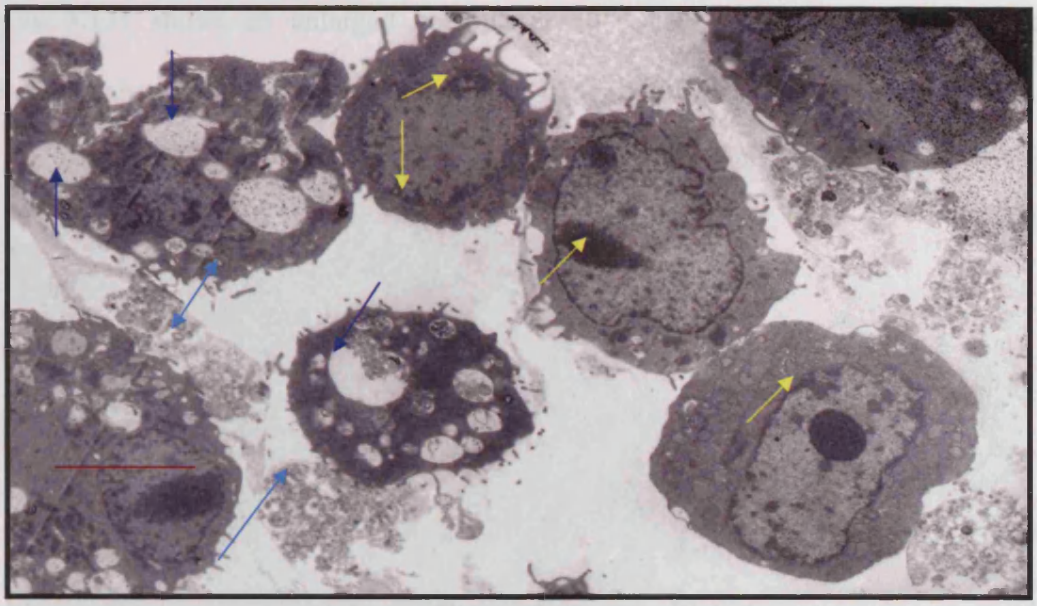


Figure 3.129

Low magnification TEM showing treated HT1080 cells with signs of extensive damage, and some swollen necrotic cells (light blue arrows). Vacuolation (blue arrows) and some debris are clearly visible. Some chromatin margination can be seen (yellow arrows). Loss of cytoplasmic material evident.

Magnification : 2000X **Treatment : Hexane Haddi**
Scale bar : 2µm **Time : 12hrs Concentration 666 µg/ml**

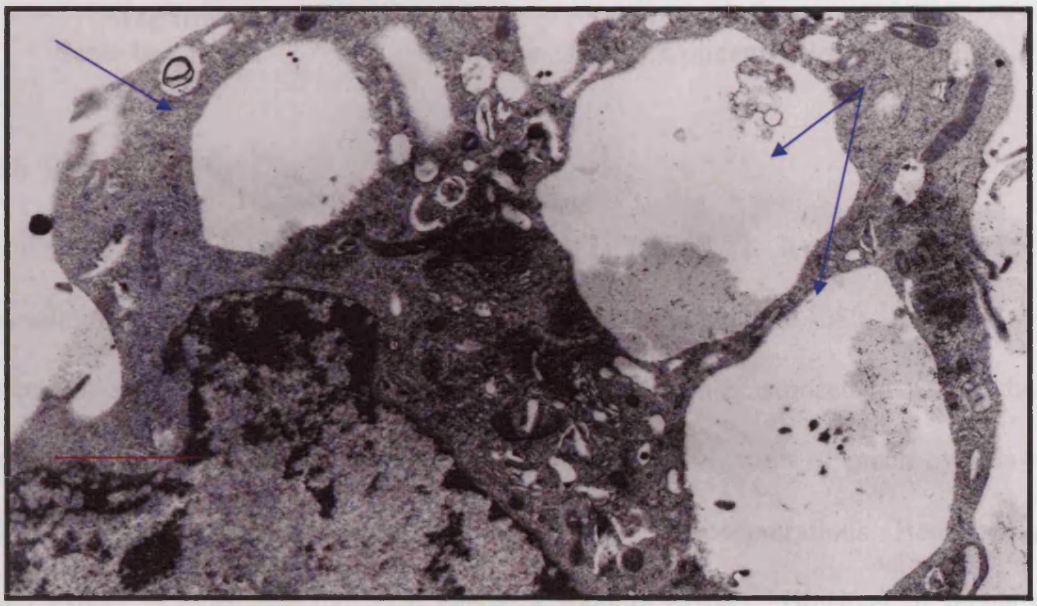


Figure 3.130

High magnification TEM showing a damaged cell with large clear vaculations (blue arrows).

Magnification : 4000X **Treatment : Hexane Haddi**
Scale bar : 1µm **Time :12hrs Concentration 666 µg/ml**

Figure 3.131 shows an enlarged necrotic HT1080 cell with some debris. The cell membrane also shows signs of disintegration. Cytoplasmic debris is also evident.

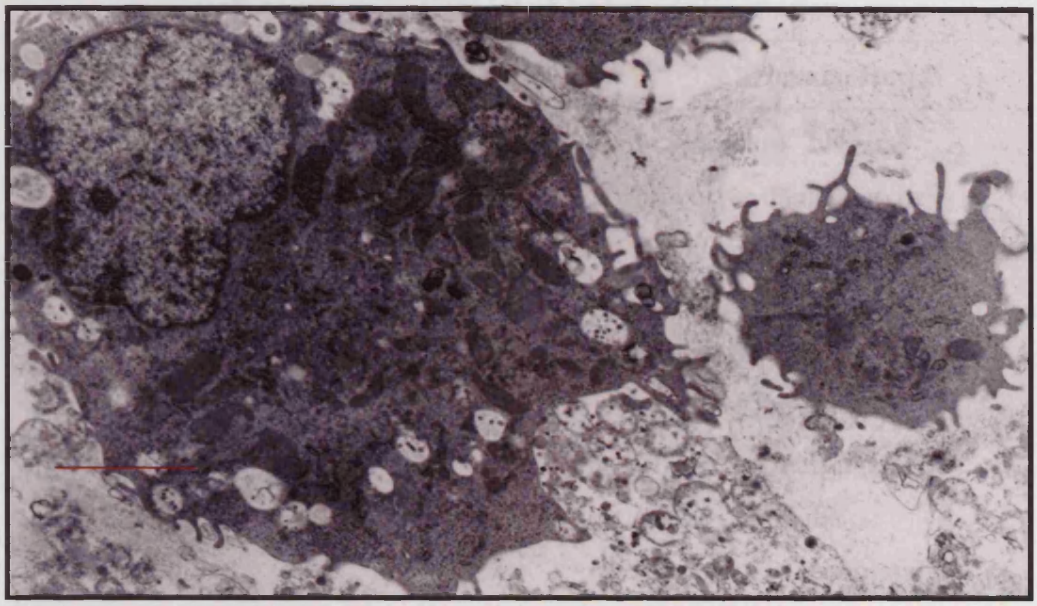


Figure 3.131
High magnification TEM of Figure 3.130 showing an enlarged necrotic cell with some debris. The cell membrane is disintegrated. Cytoplasmic debris is clearly seen
Magnification : 5000X **Treatment : Hexane Haddi**
Scale bar : 1µm **Time :12hrs Concentration 666 µg/ml**

3.18 Summary

Results of the TEM investigation are summarised in Table 3.12. It can be concluded that ethanolic molmol extract resulted in damaging treated HT1080 cells at both concentrations used. However, damage to these cells when exposed to hexane haddi extract seemed to have been more extensive, with the formation of much cytoplasmic debris and disintegration of cells membranes also, at both concentrations. Hence, hexane haddi extract is more effective than ethanolic molmol extract. However, it seems that even the lower haddi extract concentration is very potent, leading to a potentially necrotic impact on the cells.

Table 3.12. Summary of effects of ethanolic molmol and hexane haddi at two concentrations on HT1080 cells

Treatments in Respect of Apoptosis Formation		
Untreated	**Ethanolic Molmol**	**Hexane Haddi**
None	**Concentration 234 µg/ml** • Formation of apoptotic cells • Blebbing • Condensation of chromatin alongside nuclear membrane (chromatin margination) • Formation of apoptotic bodies • Cytoplasmic vacuolation • Loss of filopodia • Swollen mitochondria • Cytoplasmic debris	**Concentration 201g/ml** • Extensive damage to cells • Disintegration of cell membranes • Formation of apoptotic cells • Blebbing • Formation of apoptotic bodies • Chromatin margination • Loss of filopodia • Cytoplasmic vacuolation • Swollen mitochondria • Formation of cell debris • Phagocytosis • Probably secondary necrosis
	Concentration 666 µg/ml • Formation of apoptotic cells • Blebbing • Formation of apoptotic bodies • Cytoplasmic vacuolation • Chromatin margination. • Damaged cell membranes	**Concentration 666 µg/ml** • Extensive damage to cells • Cytoplasmic vacuolation • Formation of cell debris • Chromatin margination • Disintegration of cell membranes • Necrosis

3.19 Annexin V Assay to Detect Apoptotic Plasma Membrane Changes

3.19.1 S180 Cells

Control live untreated S180 cells did not show changes in the membrane phosphatidylserine asymmetry. Accordingly, they were not labelled with Annexin V-FITC (Figure 3.132A-B). Only a few cells showed positive Annexin V-FITC reaction, appearing bright apple green on the cell membrane surface. This is a very low percentage of apoptosis shown to occur in cells grown in control cultures. Control cells did not show signs of necrosis, as none appeared red with propidium iodide.

S180 cells grown in culture containing 666 μg/ml ethanolic molmol extract for 12 and 24 hours also showed signs of induced apoptosis (Figure 3.133A-B), and showed positive results for changes in membrane phosphatidylserine asymmetry. The majority of these cells appeared green with Annexin V-FITC, showing positive labelling on the cell surface and possibly cytoplasm and nuclear envelope. Some cells appeared red with PI, possibly indicating some secondary necrosis after the death of apoptotic cells and changes in permeability of the plasma membrane, which allowed the penetration of PI into the cells, staining them red.

S180 cells grown in cultures containing 234 μg/ml (LC_{50}) ethanolic molmol extract for 12 and 24 hours showed signs of apoptosis (Figure 3.134A-B) and also showed positive results for changes in membrane phosphatidylserine asymmetry. Most of the cells exposed to Annexin V-FITC showed positive labelling on the cell surface. Some apoptotic cells also showed Annexin V distribution within the cytoplasm. Only a few cells appeared red, possibly indicating some secondary necrosis, as explained above.

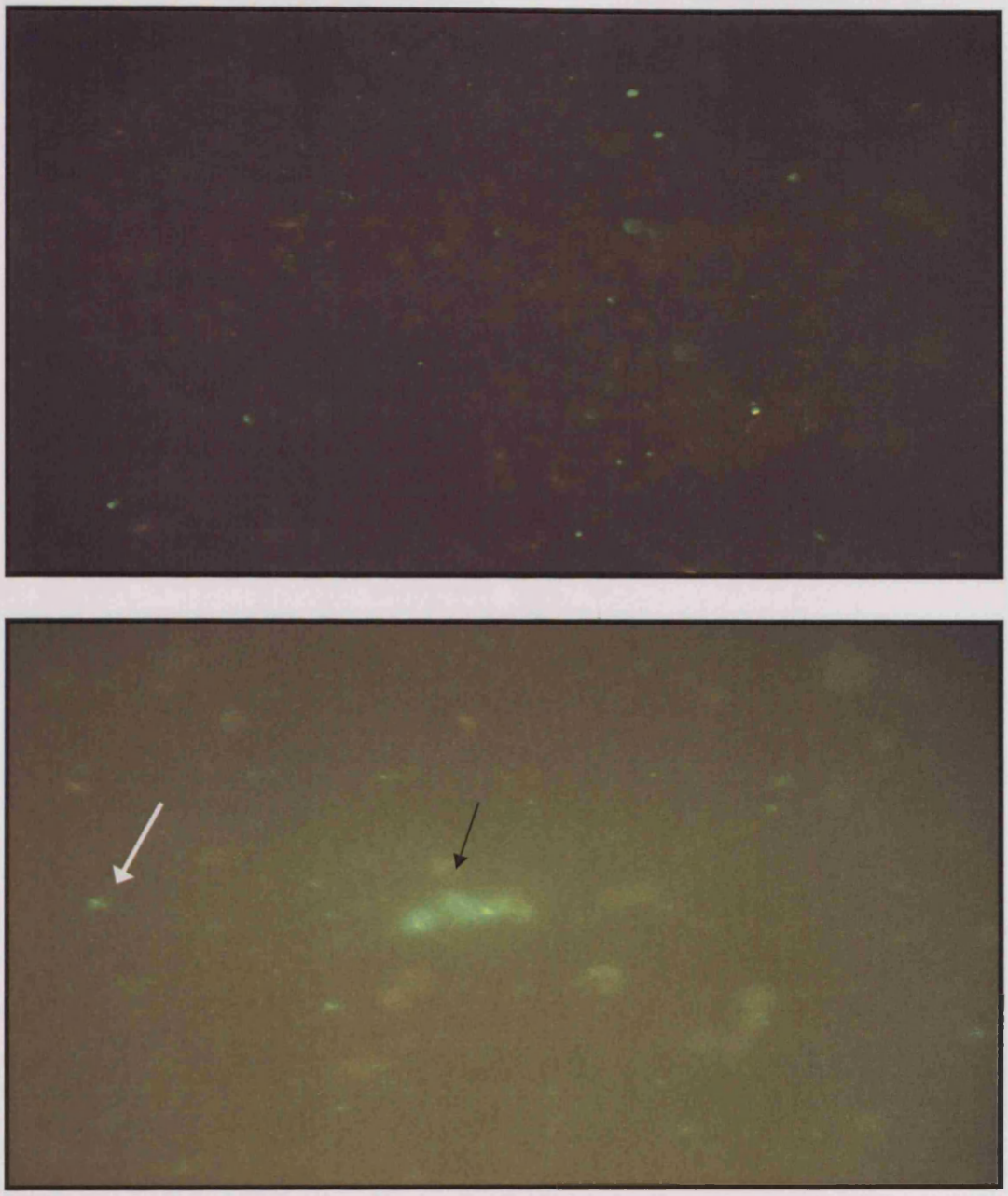
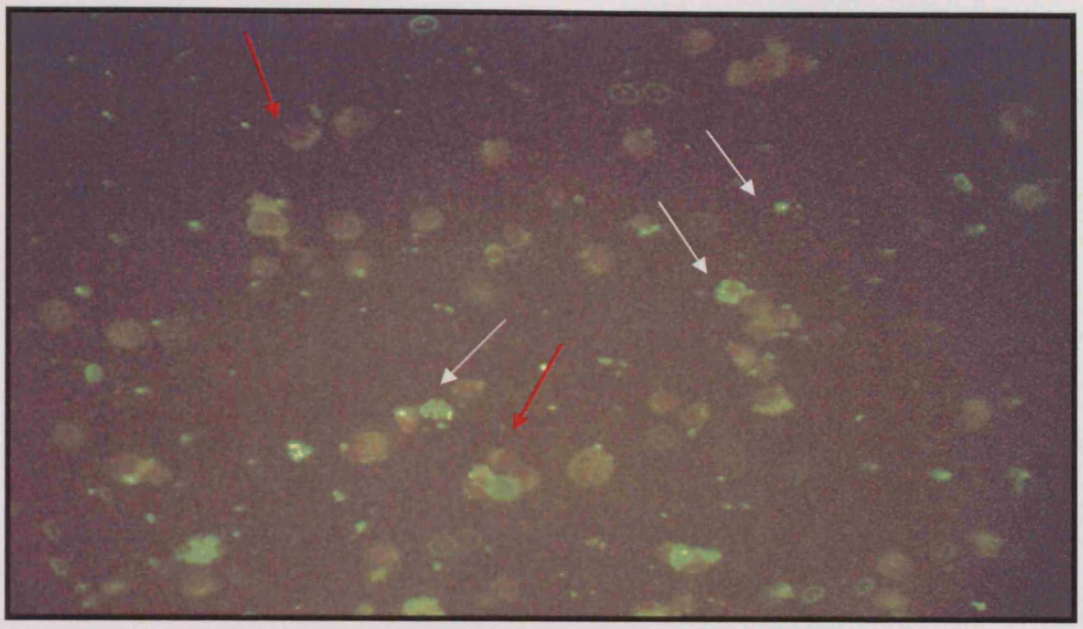
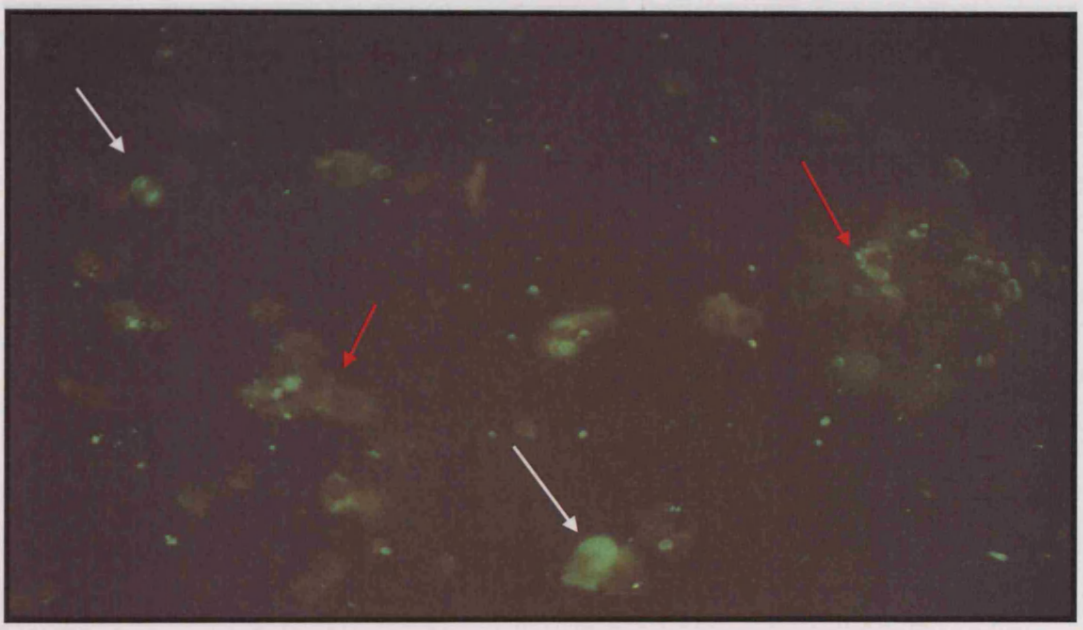


Figure 3.132
Fluorescence microscope images of untreated (control) S180 cells after treatment with fluorescein isothyiocyanate (Mag.X75) and propidium iodide (PI). A few late apoptotic cells can be seen labelled with FITC and appearing bright apple green on the cell membrane surface (white arrow). Artefact (black arrow)



A



B

Figure 3.133

Fluorescence microscope images of S180 cells treated with 666 µg/ml ethanolic molmol extract for 12 hours **(A)** and 24 hours **(B)** after treatment with fluorescein isothyiocyanate and propidium iodide (IP) (Mag. X120).

(A) 12 hour treatment: Apoptotic cells (white arrows) showing even Annexin V distribution on the cell membrane. A few cells show signs of secondary necrosis (red stain with PI) (red arrows).

(B) 24 hour treatment: Apoptotic cells (white arrow), but some showing signs of secondary necrosis (red arrows).

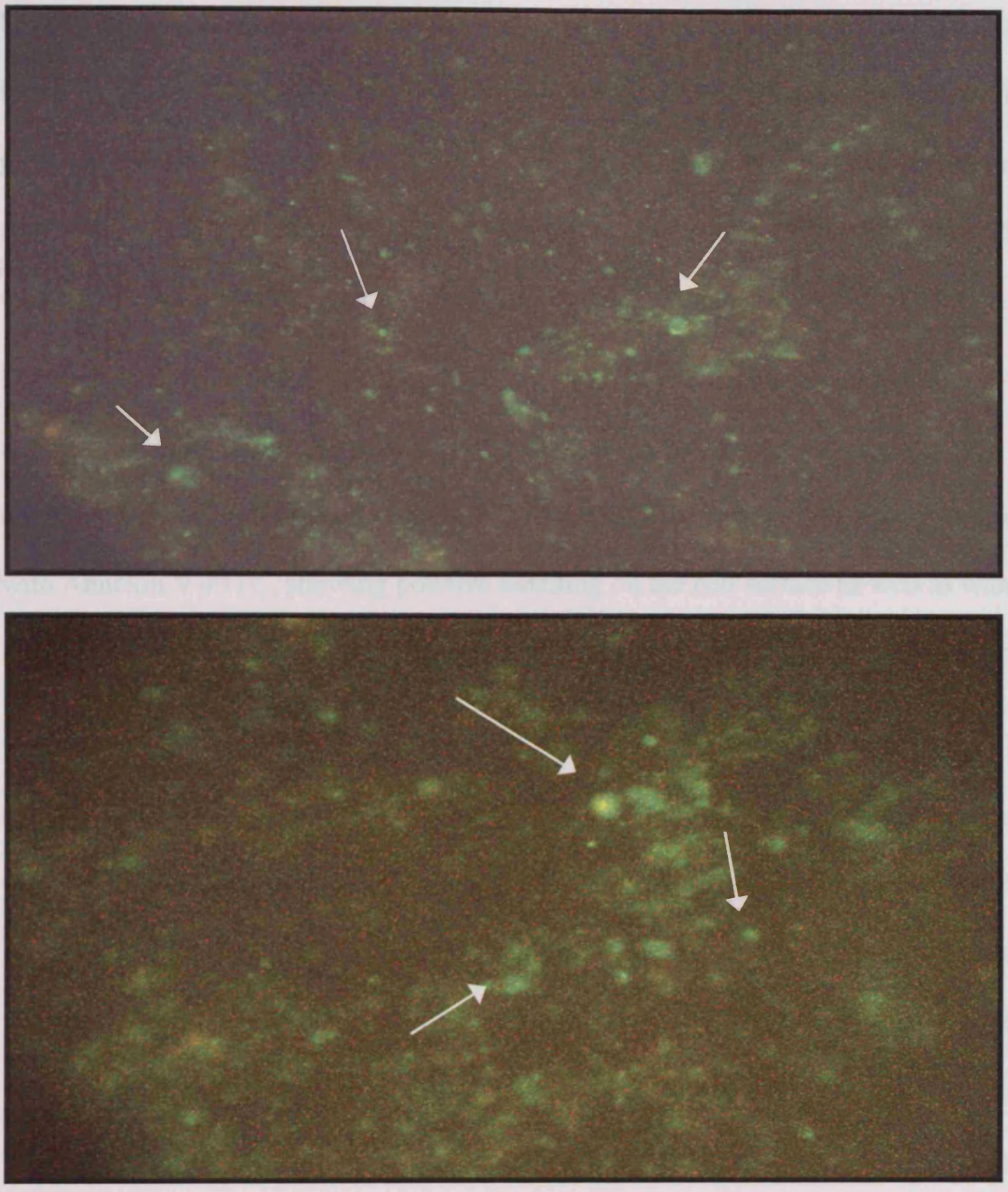


Figure 3.134

Fluorescence microscope images of S180 cells treated with 234 μg/ml ethanolic molmol extract for 12 hours (A) and 24 Hours (B) after treatment with fluorescein isothyiocyanate (FITC) and propidium iodide (PI). (Mag.X75)

(A) 12 hour treatment. Apoptotic cells (white arrows) showing concentration of Annexin V on the cell membrane. One cell shows signs of secondary necrosis (labelled red with PI) (red arrows).

(B) 24 hour treatment. Apoptotic cells (white arrows) showing concentration of Annexin V on the cell membrane and apparent distribution within the cytoplasm in some cells. Only a few cells show signs of secondary necrosis (red arrows) and 'rafting'

S180 cells grown in culture containing 666 μg/ml hexane haddi extract for 12 and 24 hours (Figures 3.135A-B) showed positive results for changes in membrane phosphatidylserine asymmetry. The majority of these cells appeared green with Annexin V-FITC, showing positive labelling on the cell surface and within the cytoplasm. Some cells stained red with PI, possibly indicating secondary necrosis.

S180 cells grown in culture containing 201 μg/ml (LC50) hexane haddi extract for 12 and 24 hours (Figures 3.136A-B) also showed positive results for changes in membrane phosphatidylserine asymmetry. The majority of these cells appeared green with Annexin V-FITC, showing positive labelling on the cell surface as well as within the cytoplasm. Some cells stained red with PI, possibly indicating secondary necrosis.

3.19.2 HT1080 Cells

Control live untreated HT1080 cells did not show changes in the membrane phosphatidylserine asymmetry. Hence, they were not labelled with Annexin V-FITC (Figures 3.137A-B). Only a few cells showed positive Annexin V-FITC labelling, appearing bright apple green on cell membrane surface. This reflects a very low percentage of natural apoptosis, which has also been shown to occur in these cells grown in control cultures. No control cells showed signs of necrosis, since none was stained red with propidium iodide.

HT1080 cells grown in culture containing 666 μg/ml ethanolic molmol extract for 12 and 24 hours (Figure 3.138A-B), hence undergoing induced apoptosis, showed positive results for changes in membrane phosphatidylserine asymmetry. Cells appeared yellow green with Annexin V-FITC, showing positive labelling on the cell surface and possibly within the cytoplasm. None was stained red, indicating negative test for PI.

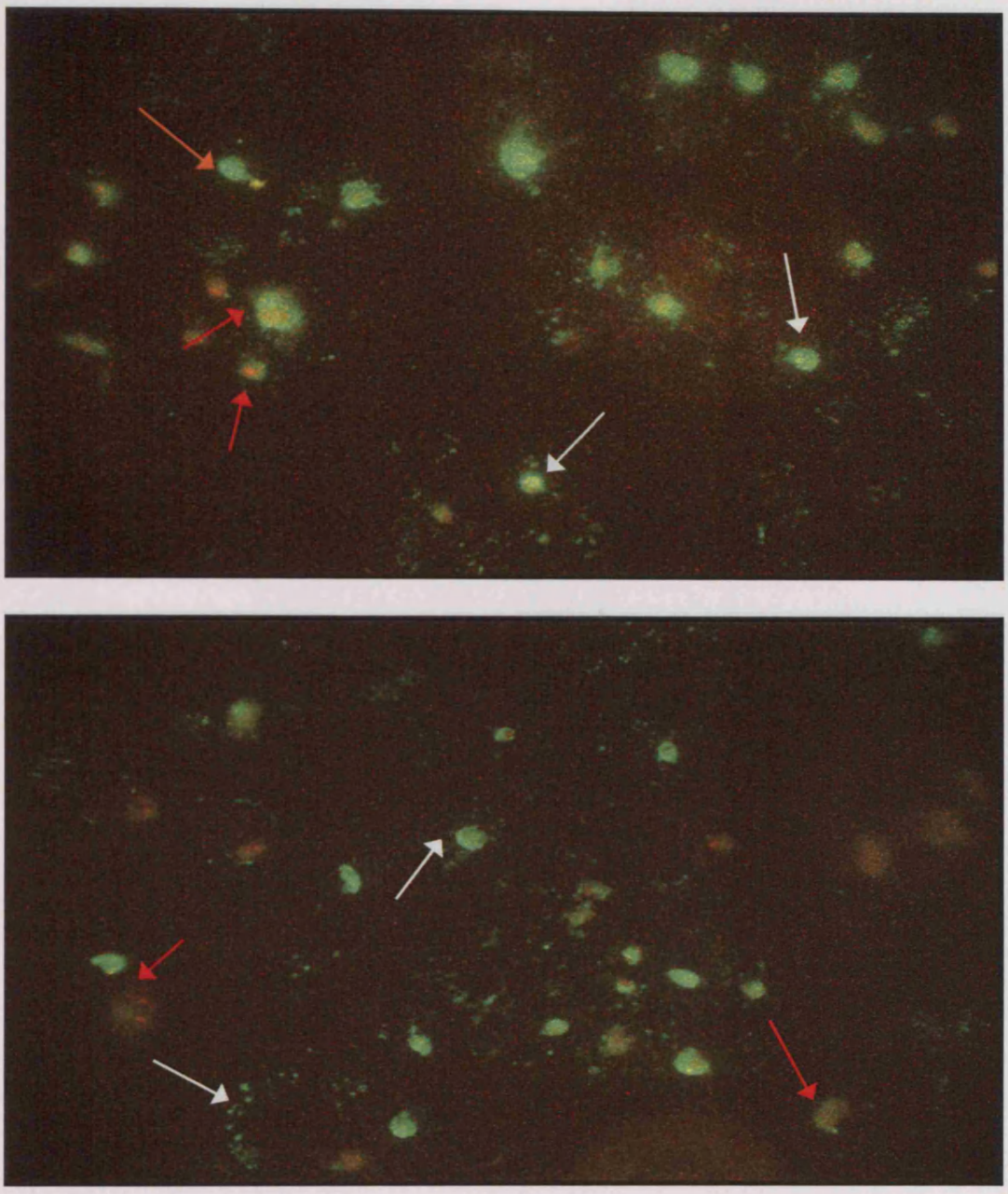
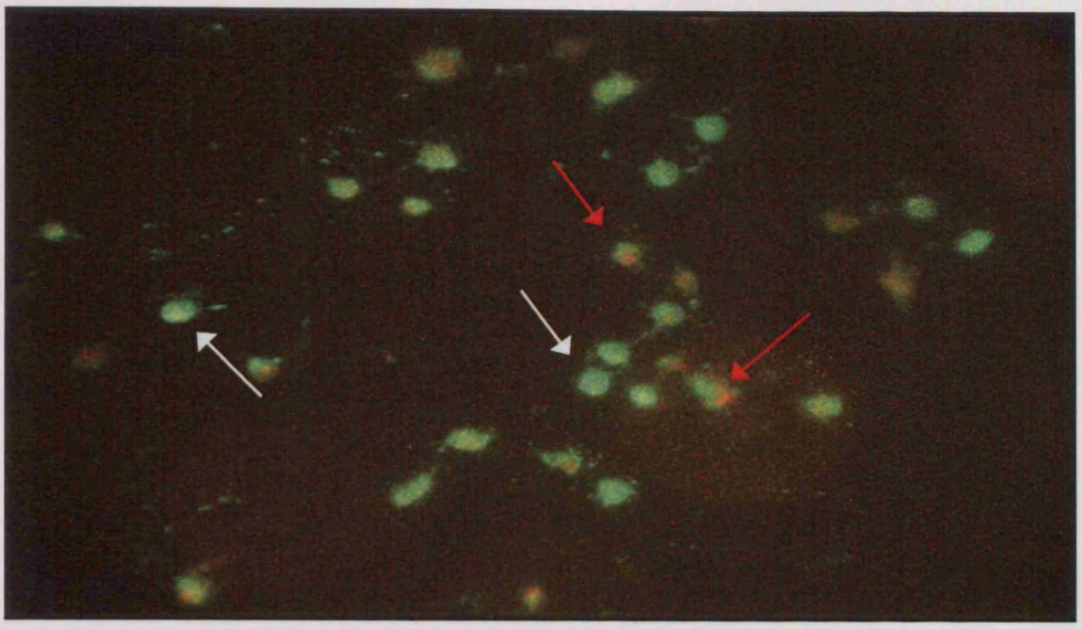


Figure 3.135

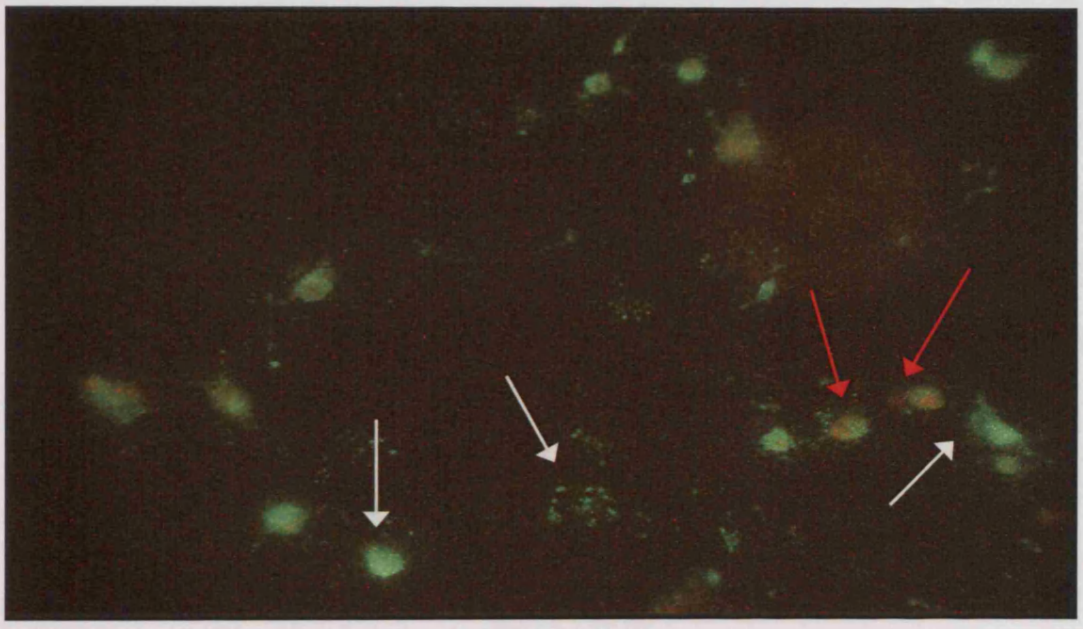
Fluorescence microscope images of DNA in S180 cells treated with 666 µg/ml hexane haddi extract for 12 hours **(A)** and 24 hours **(B)** after treatment with fluorescein isothyiocyanate and propidium iodide (PI). Mag.X120

(A) 12 hour treatment: Apoptotic cells (white arrows) showing concentration of Annexin V on the cell membrane. A few cells show signs of secondary necrosis (red labelling with PI) (red arrows).

(B) 24 hour treatment: Apoptotic cells (white arrows), a few showing signs of secondary necrosis (red arrows).



A



B

Figure 3.136

Fluorescence microscope images of S180 cells treated with 201 µg/ml hexane haddi extract for 12 hours **(A)** and 24 hours **(B)** after treatment with fluorescein isothyiocyanate (FITC) and propidium iodide (PI). (Mag.X120)

(A) 12 hour treatment: Apoptotic cells (white arrows) showing concentration of Annexin V on the cell membrane. A few cells show signs of secondary necrosis (red labelling with PI) (red arrows).

(B) 24 hour treatment: Apoptotic cells (white arrows), a few showing signs of secondary necrosis (red arrows).

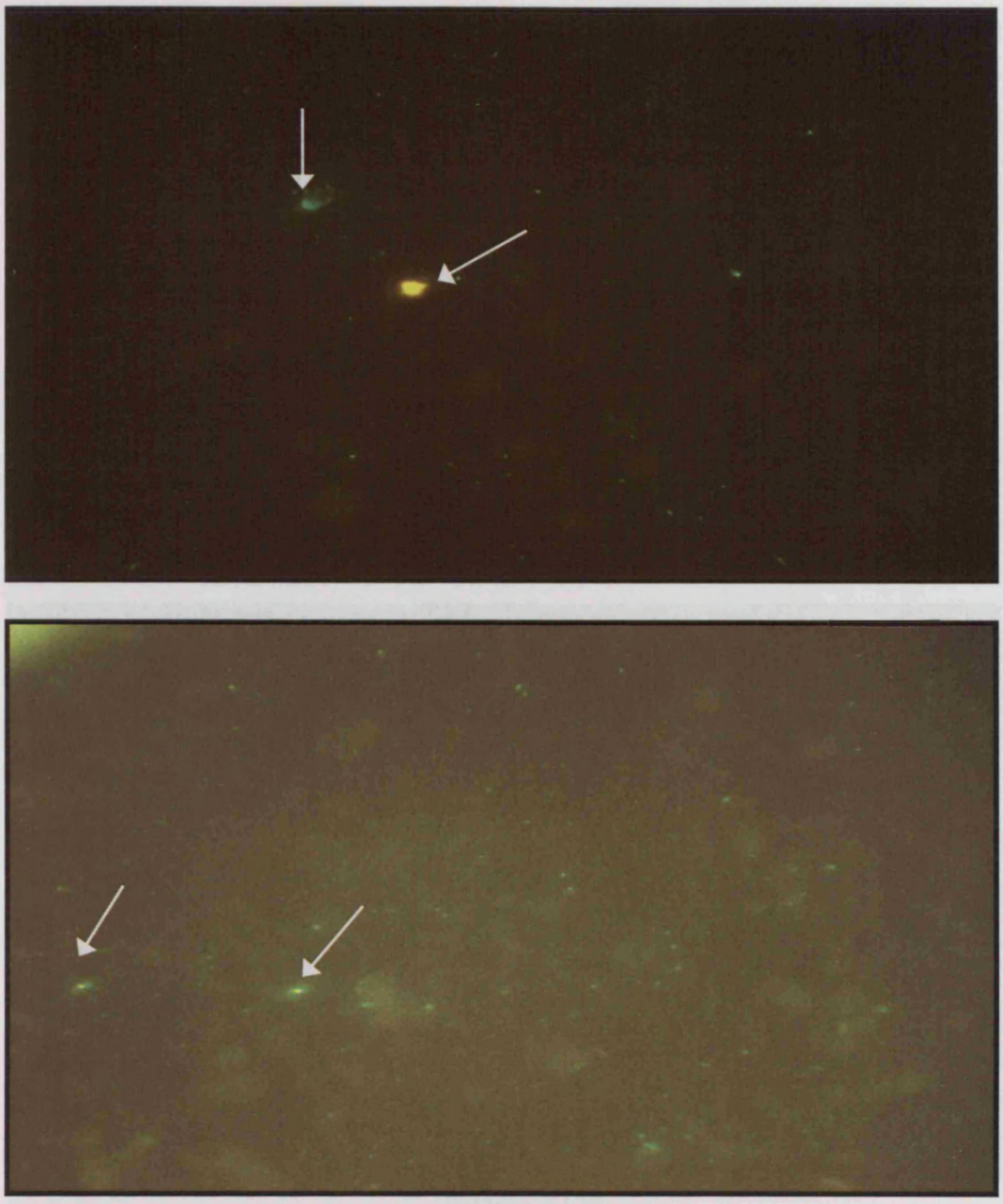
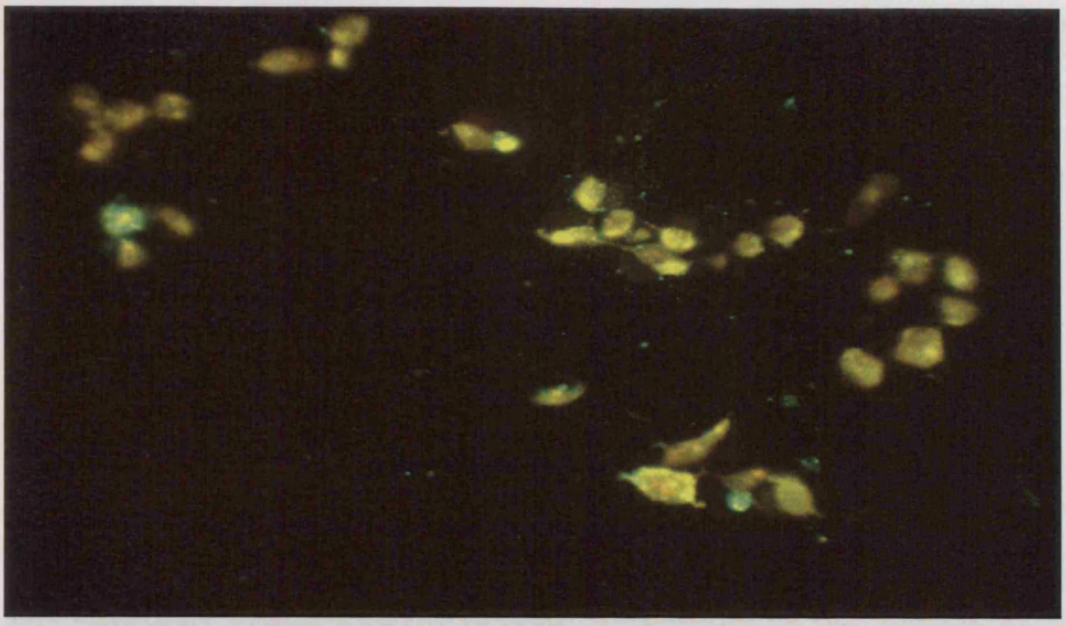
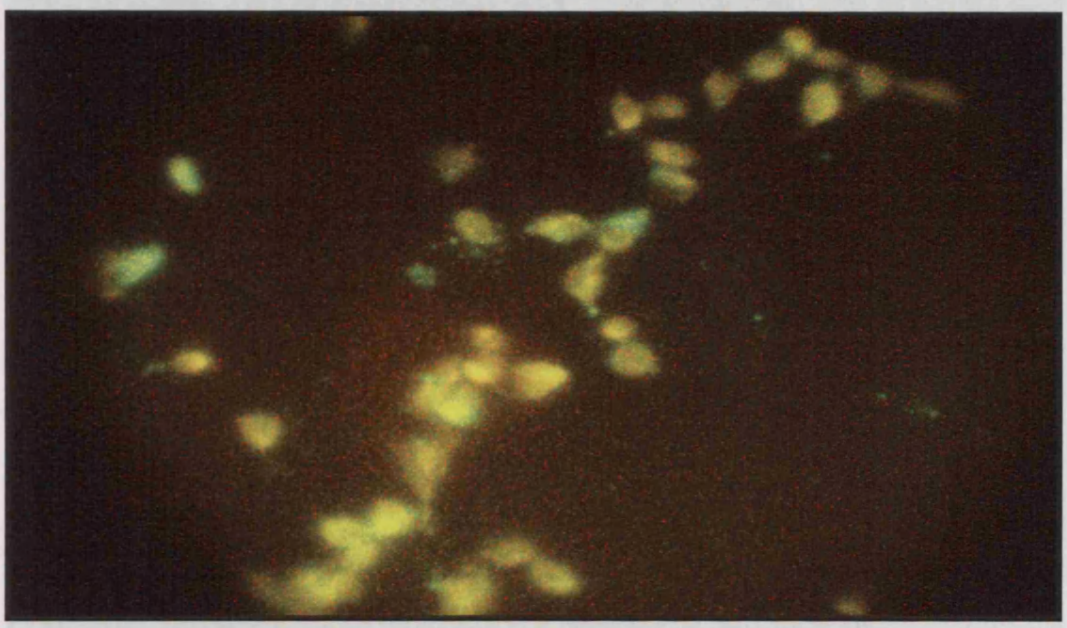


Figure 3.137
Fluorescence microscope images of untreated (control) HT1080 cells after treatment with fluorescein isothyiocyanate (FITC) and propidium iodide. (Mag.X75).
A few apoptotic cells can be seen labelled with FITC, appearing bright apple green on cell membrane surface (white arrow).



A



B

Figure 3.138

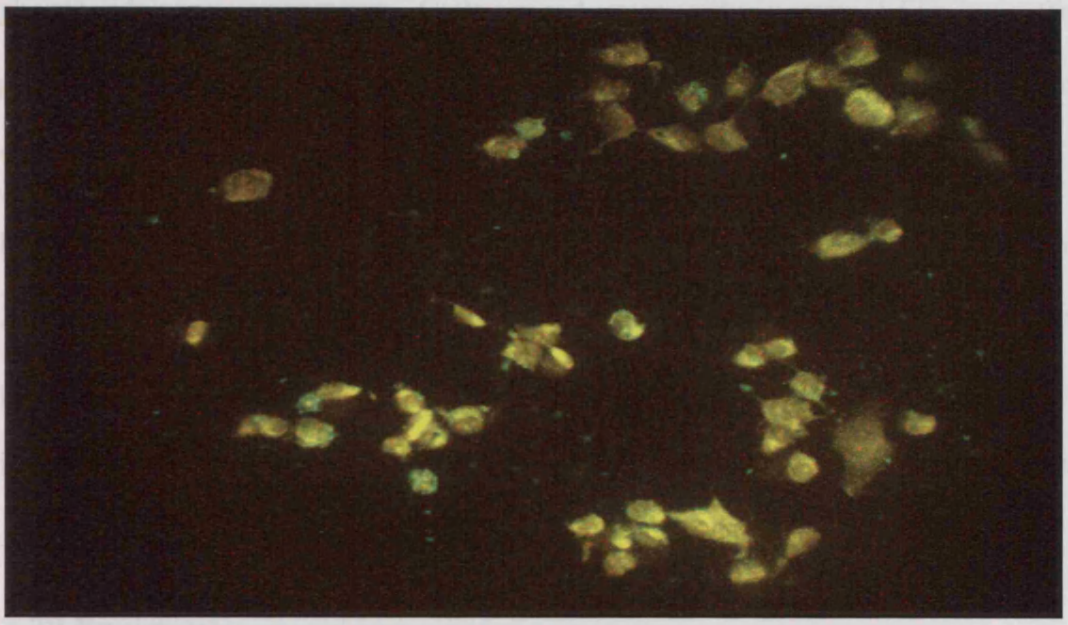
Fluorescent microscope images of HT1080 cells treated with 666 μg/ml ethanolic molmol extract for 12 hours **(A)** and 24 hours **(B)** after treatment with fluorescein isothyiocyanate (FITC) and propidium iodide (PI). (Mag.X120)

(A) 12 hour treatment: Apoptotic cells show Annexin V distribution on the cell membrane, but mainly apparent distribution within the cytoplasms.

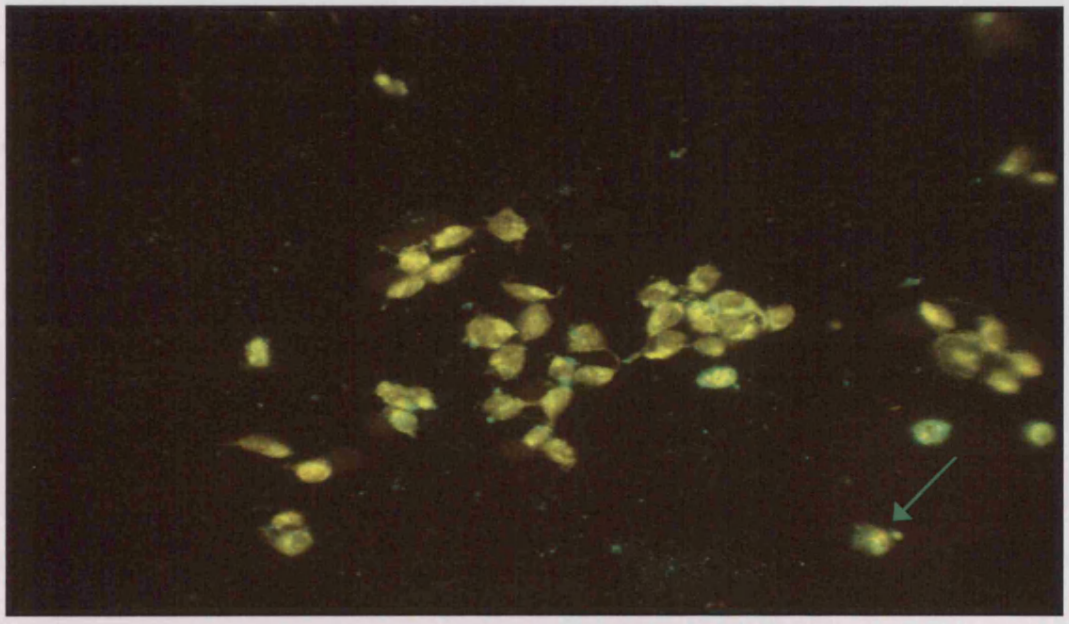
(B) 24 hour treatment: As in **A.**

HT1080 cells grown in culture containing 234 μg/ml ethanolic molmol extract for 12 and 24 hours (Figures 3.139) also showed positive results for changes in membrane phosphatidylserine asymmetry. Cells stained yellow green with Annexin V-FITC showing positive labelling on the cell surface, and the majority showed even

AnnexinV distribution within the cytoplasm. None showed positive test for PI, that is, necrosis was not noted. Some cells showed blebs on the outer membrane.



A



B

Figure 3.139

Fluorescence microscope images of HT1080 cells treated with 234 μg/ml ethanolic molmol extract for 12 hours **(A)** and 24 hours **(B)** after treatment with fluorescein isothyiocyanate (FITC) and propidium iodide (PI). (Mag.X120).

(A) 12 hour treatment: Apoptotic cells showing Annexin V distribution on the cell membrane, but mainly apparent distribution within the cytoplasm.

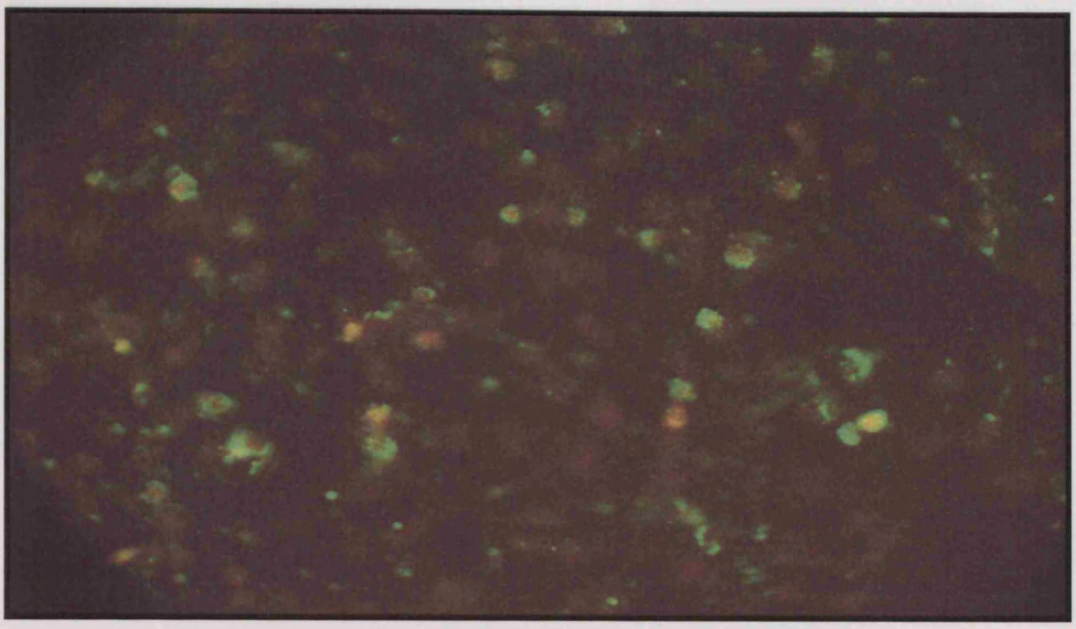
(B) 24 hour treatment: As in **A.** Some cells show blebs on the outer membrane (green arrow).

HT1080 cells grown in culture containing 666 μg/ml hexane haddi for 12 and 24 hours (Figure 3.140A-B) also showed positive results for changes in membrane phosphatidylserine asymmetry. Cells stained green with Annexin V FITC, showing positive labelling on the cell surface. None showed positive for PI, that is, necrosis was not noted.

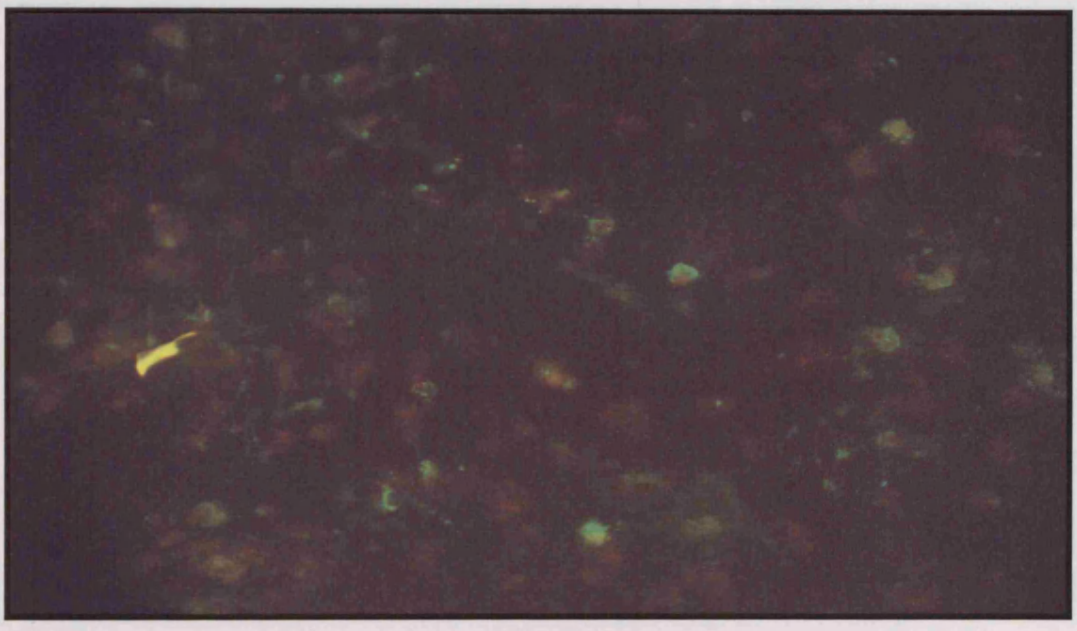
HT1080 cells grown in culture containing 201 μg/ml hexane haddi extract for 12 and 24 hours (Figures 3.141A-B) also showed positive results for changes in membrane phosphatidylserine asymmetry. Cells stained green with Annexin V FITC showing positive labelling on the cell surface. A number of cells (around 2-3% of the total) showed positive test for PI, suggesting secondary necrosis.

3.20 Summary

The effects of molmol and haddi extracts on the S180 and HT1080 cell lines used in this study are presented in Table 3.13. Results clearly show that the extracts of both plants, even at the LC_{50} concentrations, were very effective in initiating apoptosis in S180 and TH1080 cell lines.



A



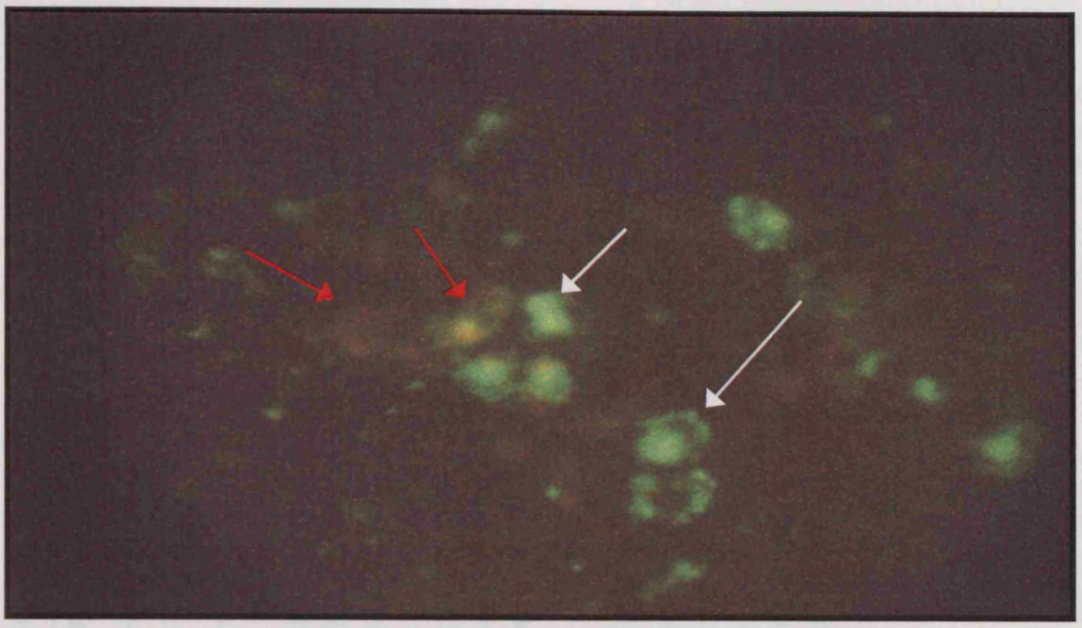
B

Figure 3.140

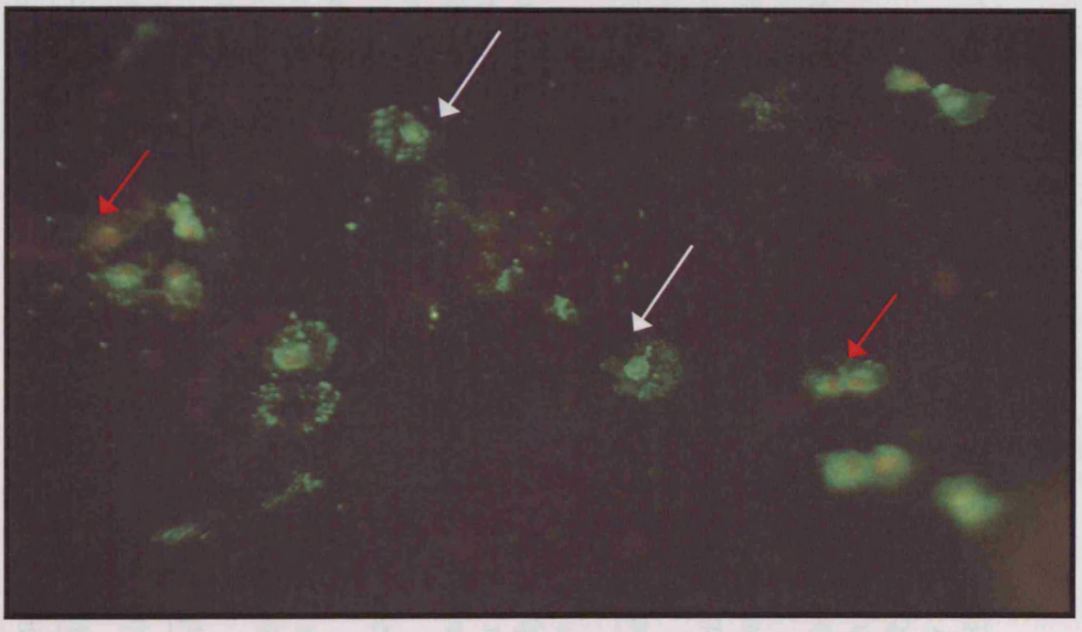
Fluorescence microscope images of HT1080 cells treated with 666 µg/ml hexane haddi extract for 12 hours **(A)** and 24 hours **(B)** after treatment with fluorescein isothyiocyanate (FITC) and propidium iodide (PI). (Mag.75X)

(A) 12 hour treatment: Apoptotic cells showing Annexin V distribution on cell membrane

(B) 24 hour treatment: Apoptotic cells mainly showing apparent distribution of Annexin V within cytoplasm.



A



B

Figure 3.141

Fluorescence microscope images of HT1080 cells treated with 201 µg/ml hexane haddi extract for 12 hours **(A)** and 24 hours **(B)** after treatment with fluorescein isothyiocyanate (FITC) and propidium iodide (PI). (Mag.120X)

(A) 12 hour treatment: Apoptotic cells showing Annexin V distribution on cell membrane (white arrows). Some cells show positive PI test (red arrows).

(B) 24 hour treatment: As in **A**.

Table 3.13 Summary of effects of molmol and haddi extracts on S180 and HT1080 cells using an Annexin V assay.

	Untreated	**Ethanolic Molmol 666 µg/ml**	**Ethanolic Molmol 234 µg/ml (LC_{50})**	**Hexane Haddi 666 µg/ml**	**Hexane Haddi 201 µg/ml (LC_{50})**
S180	▪ No changes	▪ Signs of induced apoptosis. ▪ Positive labelling on cell surface (green yellow fluorescence). ▪ Secondary necrosis/changes. ▪ Changes in phsophatidylserine asymmetry.	▪ Signs of induced apoptosis. ▪ Positive labelling on cell surface (green yellow fluorescence). ▪ Some secondary necrosis. ▪ Changes in phsophatidylserine asymmetry.	▪ Signs of induced apoptosis. ▪ Positive labelling on cell surface (green yellow fluorescence). ▪ Some secondary necrosis. ▪ Changes in phosphatidylserine asymmetry.	▪ Signs of induced apoptosis. ▪ Positive labelling on cell surface (green yellow fluorescence). ▪ Some secondary necrosis. ▪ Changes in phosphatidylserine asymmetry. ▪ Positive test for PI (red fluorescence).
HT1080	▪ No changes	▪ Signs of induced apoptosis. ▪ Some positive labelling on cell surface. ▪ No necrosis. ▪ Changes in phsophatidylserine asymmetry. ▪ Negative test for PI	▪ Positive labelling on cell surface. ▪ No necrosis ▪ Changes in phsopha-tidylserine asymmetry. ▪ Negative test for PI	▪ Positive labelling on cell surface. ▪ No necrosis ▪ Changes in phsopha-tidylserine asymmetry. ▪ Negative test for PI	▪ Positive labelling on cell surface. ▪ Secondary necrosis ▪ Changes in phsophatidylserine asymmetry. ▪ Positive test for PI. (red fluorescence).

3.21 MTT Viability Assay Using *Trans-β*-Ocimene and γ-Bisabolene

The S180 cells and HT1080 cells were treated with different concentrations of *trans-β*-ocimene and γ-bisabolene, both components of haddi (*C. guidotti)* extracts, over periods of 24, 48 and 72 hours. After this time period, the MTT solution was added to the growth media to measure the mean optical density, and also to measure the percentage viability. The mean optical density was used to calculate the percentage toxicity, as explained in Chapter Two (Section 2.9.3), then the percentage viability was estimated by subtracting percentage toxicity from 100 (see Section 2.9.3). Therefore, mean percentage viability has been plotted against control and concentrations used 24, 48 and 72 hours after treatment, in order to illustrate whether or not the addition of the various concentration of *trans-β*-ocimene and γ-bisabolene at various concentrations will have any effect on the growth of the S180 and HT1080 cell lines. All experiments were conducted against a control consisting of the cells and the growth medium.

All experiments were run three times in triplicates, i.e., nine readings were taken.

A preliminary study of the chemistry of the haddi (*C. guidotti*) extracts was performed by Mr Ahmed Ali using gc-ms (Personal communication in the School of Biosciences, 2003). Identification of individual peaks was made by comparing their mass spectra to those stored in the two libraries (NIST and WILEY). Gc-ms analysis revealed a difference in the chemistry of the hexane and ethanol extracts of haddi. He found the major components in the hexane extract to be *trans-β*-ocimene, α-santalene, *trans-α*-bergamotene and α-bisabolene. The ethanol extract of haddi revealed α-santalene, *trans-α*-bergamotene and α-bisabolene to be the major compounds. The difference in the two

total ion current (TIC) chromatographs is the omission of the *trans*-β-ocimene peak in the ethanol extract, as shown in Figure 3.142.

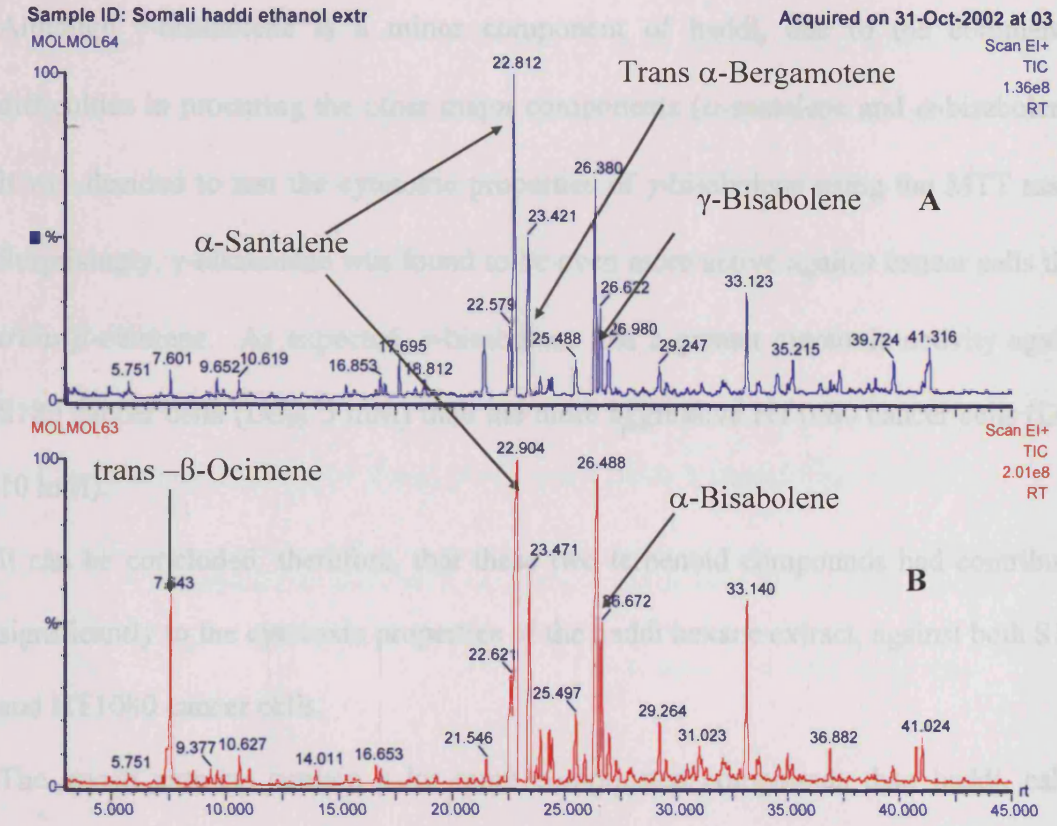


Figure 3.142
Total Ion Current Chromatograms of (A) Haddi Ethanol Extract, and (B) Haddi Hexane Extract
(Reproduced by kind permission of Mr Ahmed Ali, 2003).

By comparing the two TIC chromatograms and using a process of elimination, it could be deduced that the presence of *trans-β*-ocimene may play a significant role in the higher cytotoxic activity of the hexane extract of haddi compared to the ethanol extract. This was found to be the case for both the S180 and the HT1080 cancer cell cultures (see Tables 3.14 to 3.17).

Using the MTT assay enabled us to confirm the cytotoxic properties of *trans-β*-ocimene against both the S180 murine sarcoma and the human fibrosarcoma cancer cells.

As expected, *trans-β*-ocimene was found to be more active against the less aggressive S180 cancer cells, with a LC_{50} of 65mM, compared to 95mM for the HT1080 cancer cells.

Although γ-bisabolene is a minor component of haddi, due to the commercial difficulties in procuring the other major components (*α*-santalene and *α*-bisabolene), it was decided to test the cytotoxic properties of *γ*-bisabolene using the MTT assay. Surprisingly, γ-bisabolene was found to be even more active against cancer cells than *trans-β*-ocimene. As expected, *γ*-bisabolene had a greater cytotoxic activity against S180 cancer cells (LC_{50} 5 mM) than the more aggressive HT1080 cancer cells (LC_{50} 10 mM).

It can be concluded, therefore, that these two terpenoid compounds had contributed significantly to the cytotoxic properties of the haddi hexane extract, against both S180 and HT1080 cancer cells.

The myrrh extracts contain a lot more complicated compounds than haddi, called furano-sesquiterpenes (Mr Ahmed Ali, Personal communication, 2003). Again, these are not commercially available, and hence were unavailable for testing.

3.21.1 S180 Cell Line

The effect of *trans-β*-ocimene and *γ*-bisabolene on the viability of S180 cells were illustrated using the following treatments:

A. *Trans -β*-ocimene. The viability assay of these cells was undertaken using five different dilutions: 50, 100, 150, 200 and 250 mM. Results are presented in Figure 3.143.

B. *γ*-bisabolene. The viability of S180 cells was also tested in five concentrations, as indicated above. Results are presented in Figure 3.144.

A. *Trans-β*-Ocimene

Figure 3.143 clearly illustrates that the apparent cytotoxicity of *trans-β*-ocimene to S180 cells increased with increasing concentrations, especially after 24 hours of exposure to this biochemical. After 48 and 72 hours of exposure, viability remained at 5% at the two highest concentrations, though it increased after this time at the 100 mM concentration. However, apparent toxicity of the 50 mM *trans-β*-ocimene increased after 48 hours, but decreased slightly after 72 hours (though higher than after 24 hours).

Viability results showed, as expected, the reverse trend, decreasing with the increasing concentrations of *trans -β*-ocimene (Table 3.14 and Figure 3.143).

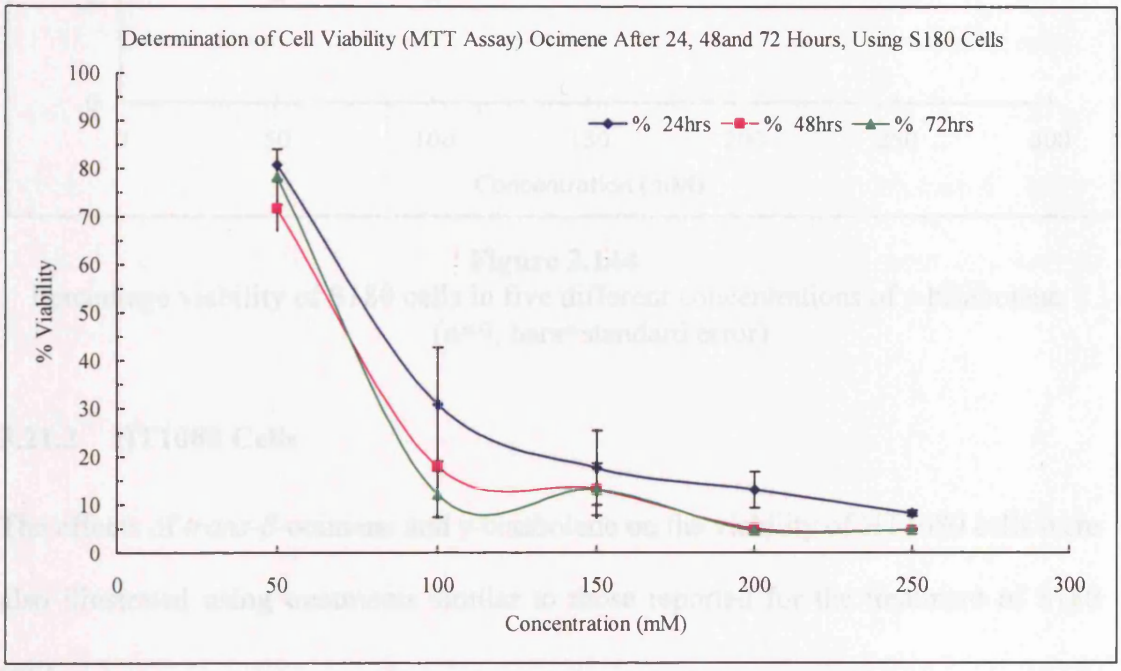


Figure 3.143
Percentage viability of S180 cells in five different concentrations of *trans-β*-ocimene (n=9, bars=standard error).

B. *γ*-Bisabolene

In contrast to exposure to *trans-β*-ocimene, exposure of S180 cells to *γ*-bisabolene resulted in lower percentage viability at lower concentrations. Lowest percentage

viabilities were obtained after exposure to 50 and 100 mM γ-bisabolene (Figure 3.144). However, after 72 hours of exposure to γ-bisabolene, percentage viabilities were higher in all concentrations than at 48 hours of exposure to this biochemical, especially at the higher concentrations (P<0.001).

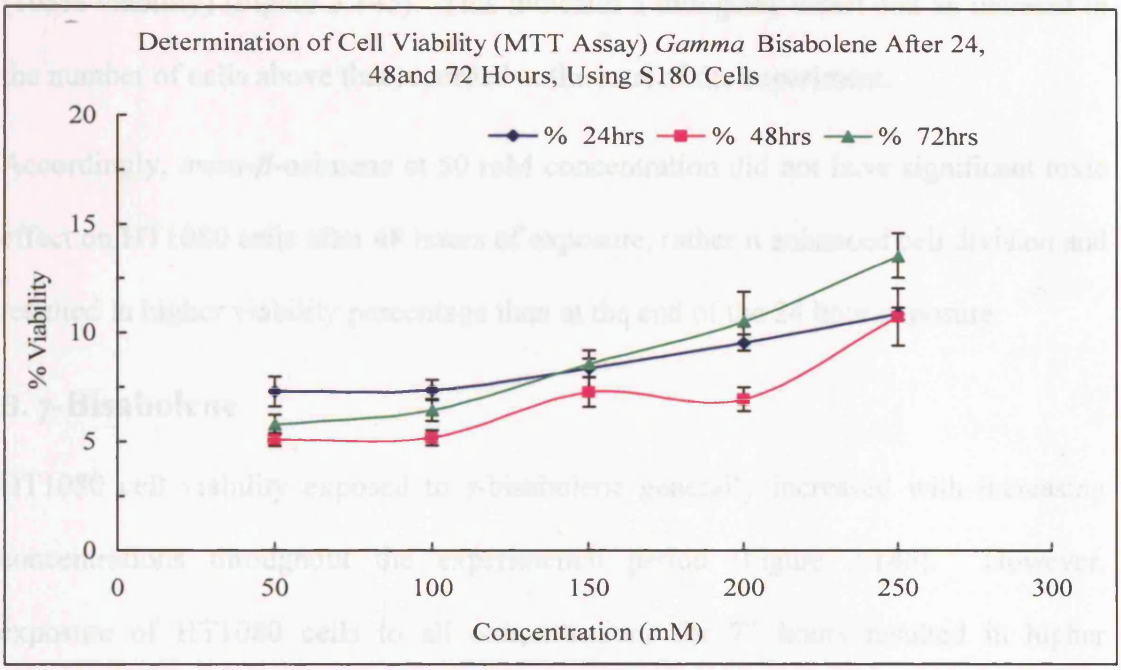


Figure 3.144
Percentage viability of S180 cells in five different concentrations of γ-bisabolene. (n=9, bars=standard error)

3.21.2 HT1080 Cells

The effects of *trans*-β-ocimene and γ-bisabolene on the viability of HT1080 cells were also illustrated using treatments similar to those reported for the treatment of S180 cells.

A. *Trans*-β-Ocimene

HT1080 cell viability in medium containing *trans*-β-ocimene decreased with increasing concentration after 24 hours of exposure (Figure 3.145). Viability also decreased after 48 hours of exposure, except at the lowest concentration (50 mM),

where it seems that *trans-β*-ocimene at this concentration was not toxic to these cells. After 72 hours of exposure, viability at the two highest concentrations (200 and 250 mM) remained at the 48 hours level, but increased at the three lowest concentrations, and even active growth was noted at the 50 mM *trans-β*-ocimene concentration (105% viability) (Figure 3.145). This indicates a mitogenic effect and an increase in the number of cells above that recorded at the start of the experiment.

Accordingly, *trans-β*-ocimene at 50 mM concentration did not have significant toxic effect on HT1080 cells after 48 hours of exposure, rather it enhanced cell division and resulted in higher viability percentage than at the end of the 24 hour exposure.

B. γ-Bisabolene

HT1080 cell viability exposed to γ-bisabolene generally increased with increasing concentrations throughout the experimental period (Figure 3.146). However, exposure of HT1080 cells to all concentrations for 72 hours resulted in higher viabilities than after 48 hours, though still lower than after the 24 hour exposure to this biochemical ($P<0.001$).

3.22 Summary

Results of the MTT investigation are summarised in Tables 3.14-3.17. It can be concluded that both of *trans-β*-ocimene and γ-bisabolene are active against both S180 and HT1080 cell lines at the five different concentrations and times tested. However, the apparent toxicity of *trans-β*-ocimene is different when applied at low concentrations, and is less effective than γ-bisabolene, which induces apoptosis earlier. Hence, γ-bisabolene is more effective than *trans-β*-ocimene. However, it seems that even the lower γ-bisabolene concentrations are very active in killing cells at the lower concentrations of 5 mM for both S180 and HT1080.

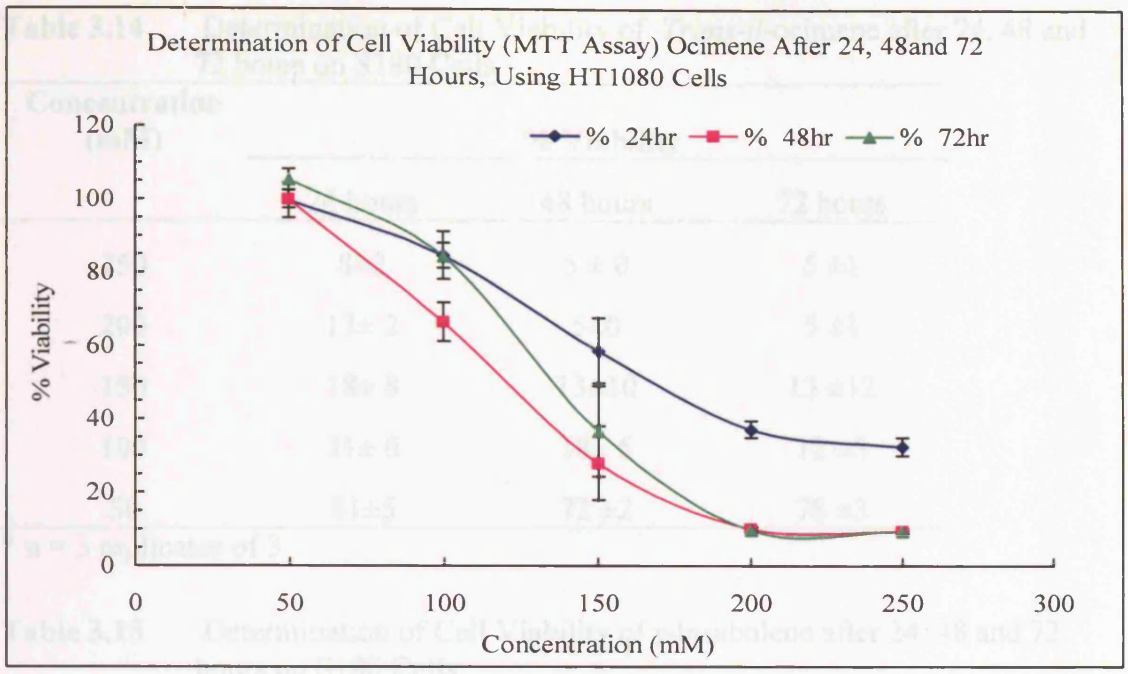


Figure 3.145
Percentage viability of HT1080 cells in five different concentrations of *trans-β*-ocimene. (n=9, bars=standard error)

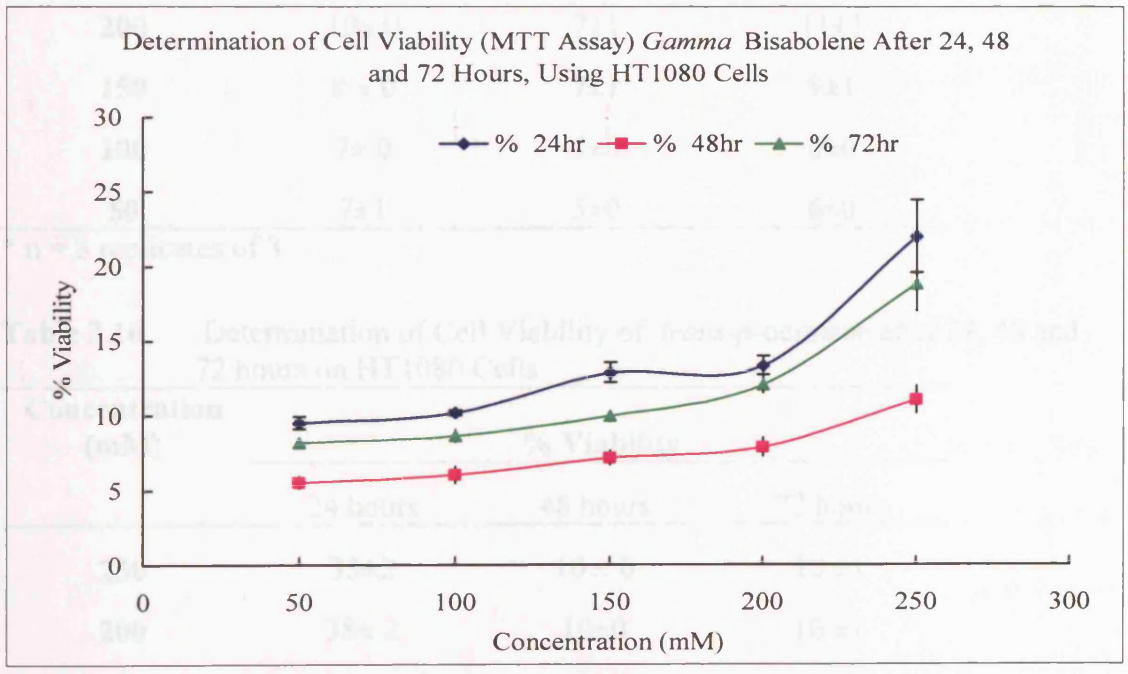


Figure 3.146
Percentage viability of HT1080 cells in five different concentrations of *γ*-bisabolene. (n=9, bars=standard error)

Table 3.14 Determination of Cell Viability of *Trans-β*-ocimene after 24, 48 and 72 hours on S180 Cells

Concentration (mM)	% Viability		
	24 hours	48 hours	72 hours
250	8±2	5 ± 0	5 ±1
200	13± 2	5±0	5 ±1
150	18± 8	13±10	13 ±12
100	31± 6	18± 5	12 ±3
50	81±5	72 ±2	78 ±3

* n = 3 replicates of 3

Table 3.15 Determination of Cell Viability of *γ*-bisabolene after 24, 48 and 72 hours on S180 Cells

Concentration (mM)	% Viability		
	24 hours	48 hours	72 hours
250	11± 0	12±1	14±1
200	10± 0	7±1	11±1
150	8 ± 0	7±1	9±1
100	7± 0	5±0	6±0
50	7±1	5±0	6±0

* n = 3 replicates of 3

Table 3.16 Determination of Cell Viability of *trans-β*-ocimene after 24, 48 and 72 hours on HT1080 Cells

Concentration (mM)	% Viability		
	24 hours	48 hours	72 hours
250	33±2	10 ± 0	10 ±1
200	38± 2	10±0	10 ±1
150	57± 8	29±10	36 ±12
100	84± 6	67 ± 5	85 ±3
50	99±5	100 ±2	105 ±3

* n = 3 replicates of 3

Table 3.17 Determination of Cell Viability of γ-bisabolene after 24, 48 and 72 hours on HT1080 Cells

Concentration (mM)	% Viability		
	24 hours	48 hours	72 hours
250	22±2	11±1	17 ±2
200	13± 1	8±1	12±1
150	13± 1	7 ±0	10 ±0
100	10± 0	6± 1	9 ±0
50	10± 0	6 ± 0	8 ±0

* n = 3 replicates of 3

3.23 Time Course Assay

For time course assay S180 cells and HT1080 cells were treated with *trans-β*-ocimene and γ-bisabolene, using the following concentrations:

A. *Trans-β*-ocimene : S180 cells were treated with two concentrations of 65 and 95 mM *trans-β*-ocimene for 3, 6, 9, and 12 hours. HT1080 cells were also treated for the same periods of time, but using two concentrations of 100 and 150 mM *trans-β*-ocimene.

B. γ-bisabolene: Both S180 cells and HT1080 cells were treated each with two concentrations of γ-bisabolene: 10 and 20 mM.

A. S180 cells

Exposure of S180 cells to 65 mM *trans-β*-ocimene was not effective in bringing about significant cytotoxicity to these cells (Figure 3.147). Viability ranged between 98.6% and 78.6% after 3 and 12 hours of exposure, respectively. In contrast, exposure of these cells to 95 mM *trans-β*-ocimene resulted in a very low percentage viability, ranging between 82.5% and 4.7% after 3 and 12 hours of exposure, respectively. Apparent cytotoxicity to S180 cells exposed to γ-bisabolene was lower at 10 mM concentration than at 20 mM concentration after 3 and 6 hours. However, after 6 and

12 hours, percentage viability of these cells exposed to both concentrations were very similar (Figure 3.148)

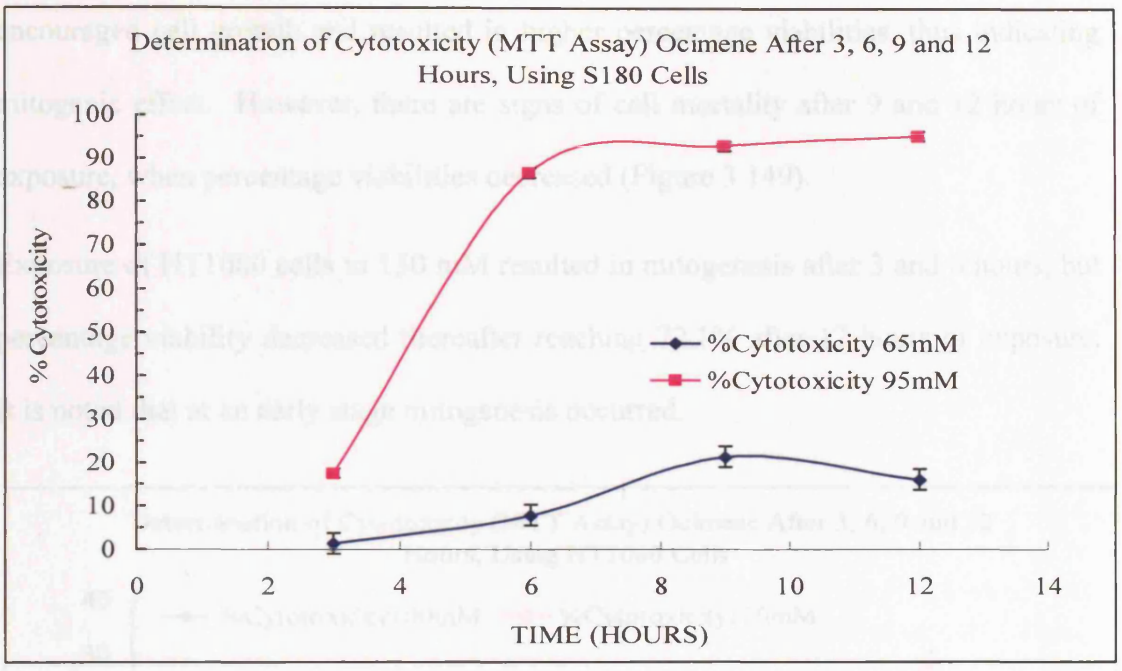


Figure 3.147
Percentage cytotoxicity of S180 cells in two different concentrations of *trans-β*-ocimene. (n=9, bars=standard error)

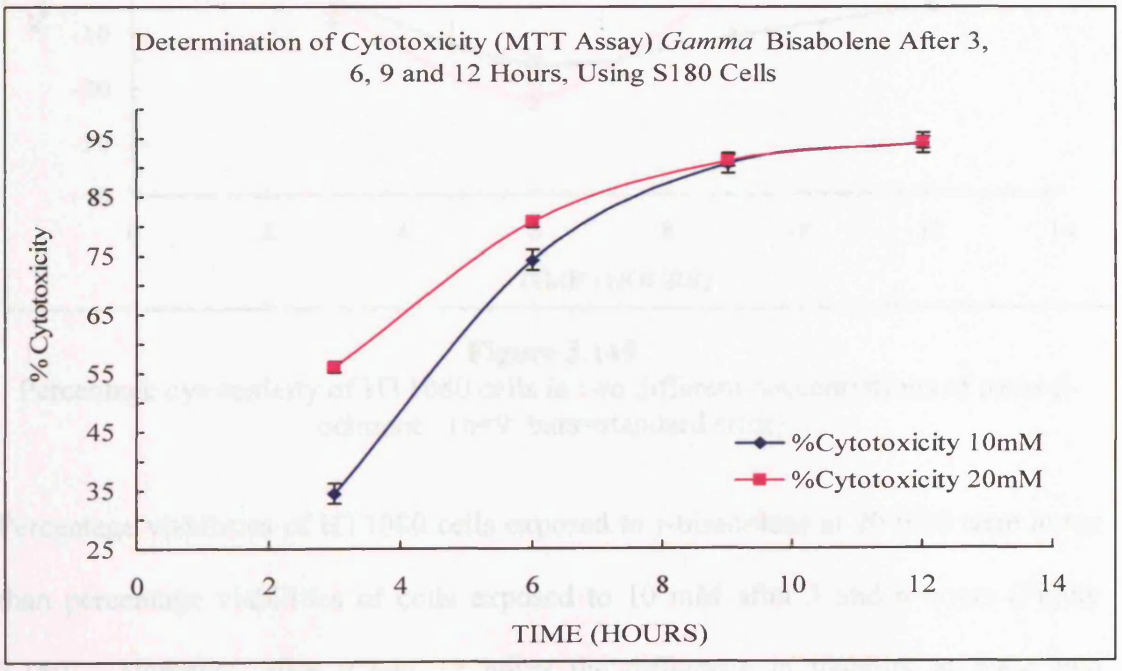


Figure 3.148
Percentage cytotoxicity of S180 cells in two different concentrations of *γ*-bisabolene. Note increase in cytotoxicity with time. (n=9, bars=standard error)

B. HT1080 Cells

Trans-β-ocimene at 100 mM was not active against HT1080 cells; rather it encouraged cell growth and resulted in higher percentage viabilities, thus indicating mitogenic effect. However, there are signs of cell mortality after 9 and 12 hours of exposure, when percentage viabilities decreased (Figure 3.149).

Exposure of HT1080 cells to 150 mM resulted in mitogenesis after 3 and 6 hours, but percentage viability decreased thereafter reaching 72.1% after 12 hours of exposure. It is noted that at an early stage mitogenesis occurred.

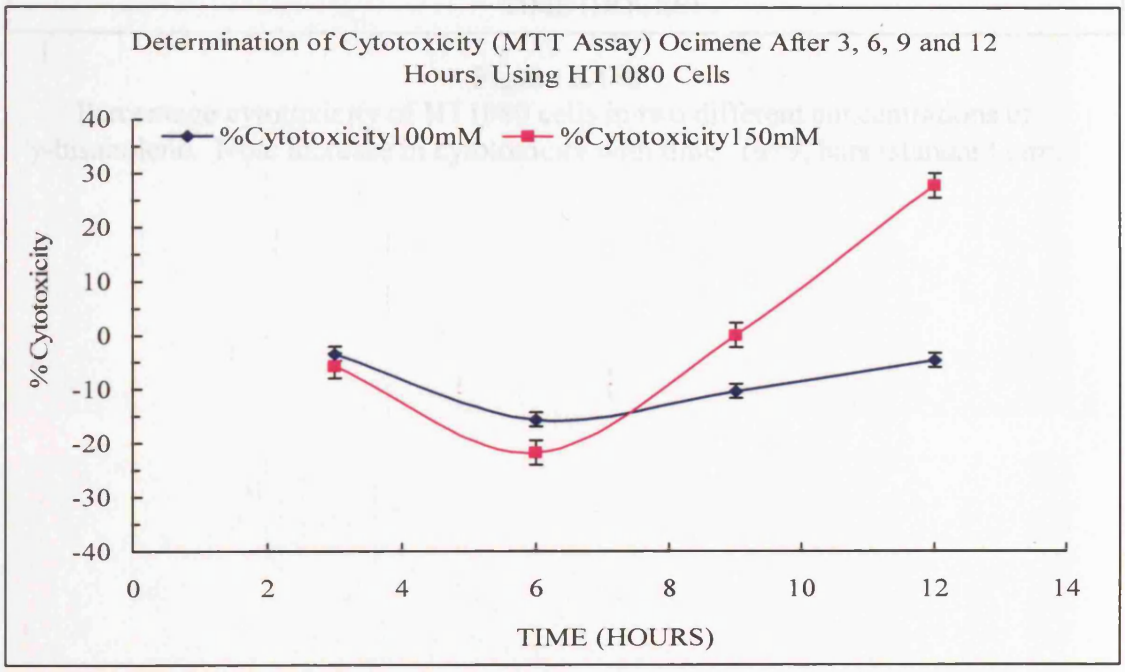


Figure 3.149
Percentage cytotoxicity of HT1080 cells in two different concentrations of *trans-β*-ocimene. (n=9, bars=standard error)

Percentage viabilities of HT1080 cells exposed to γ-bisabolene at 20 mM were lower than percentage viabilities of cells exposed to 10 mM after 3 and 6 hours (Figure 3.150). However, after 9 and 12 hours the difference in viability at these two concentrations was not significant.

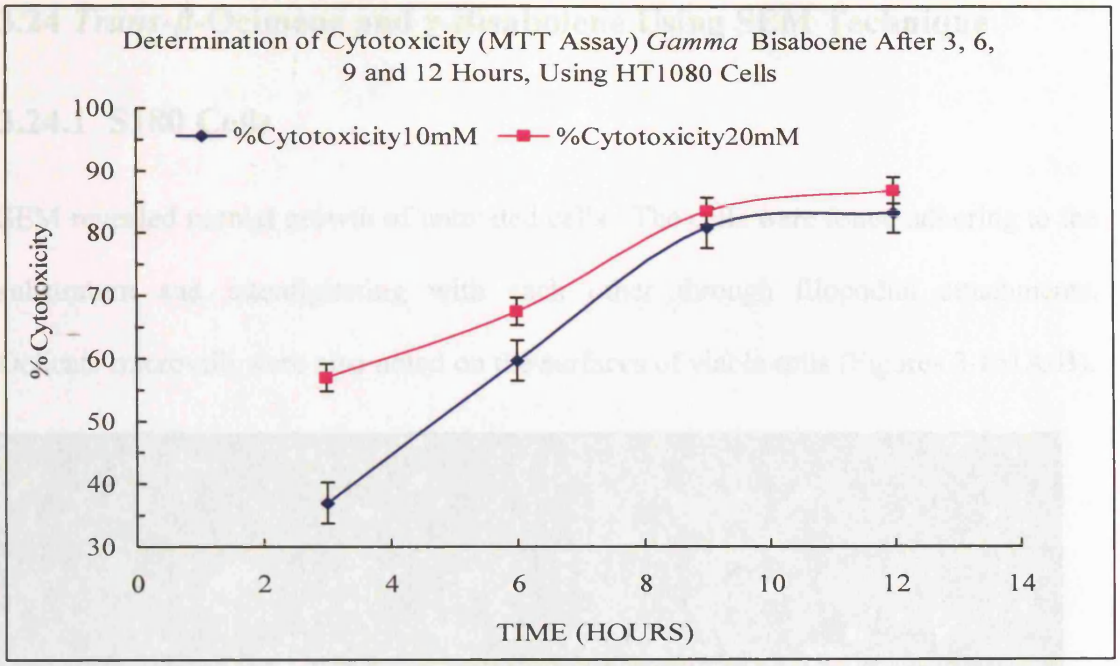


Figure 3.150

Percentage cytotoxicity of HT1080 cells in two different concentrations of γ-bisabolene. Note increase in cytotoxicity with time. (n=9, bars=standard error)

3.24 *Trans-β*-Ocimene and γ-Bisabolene Using SEM Technique

3.24.1 S180 Cells

SEM revealed normal growth of untreated cells. The cells were found adhering to the substratum and interdigitating with each other through filopodial attachments. Delicate microvilli were also noted on the surfaces of viable cells (Figures 3.151A-B).

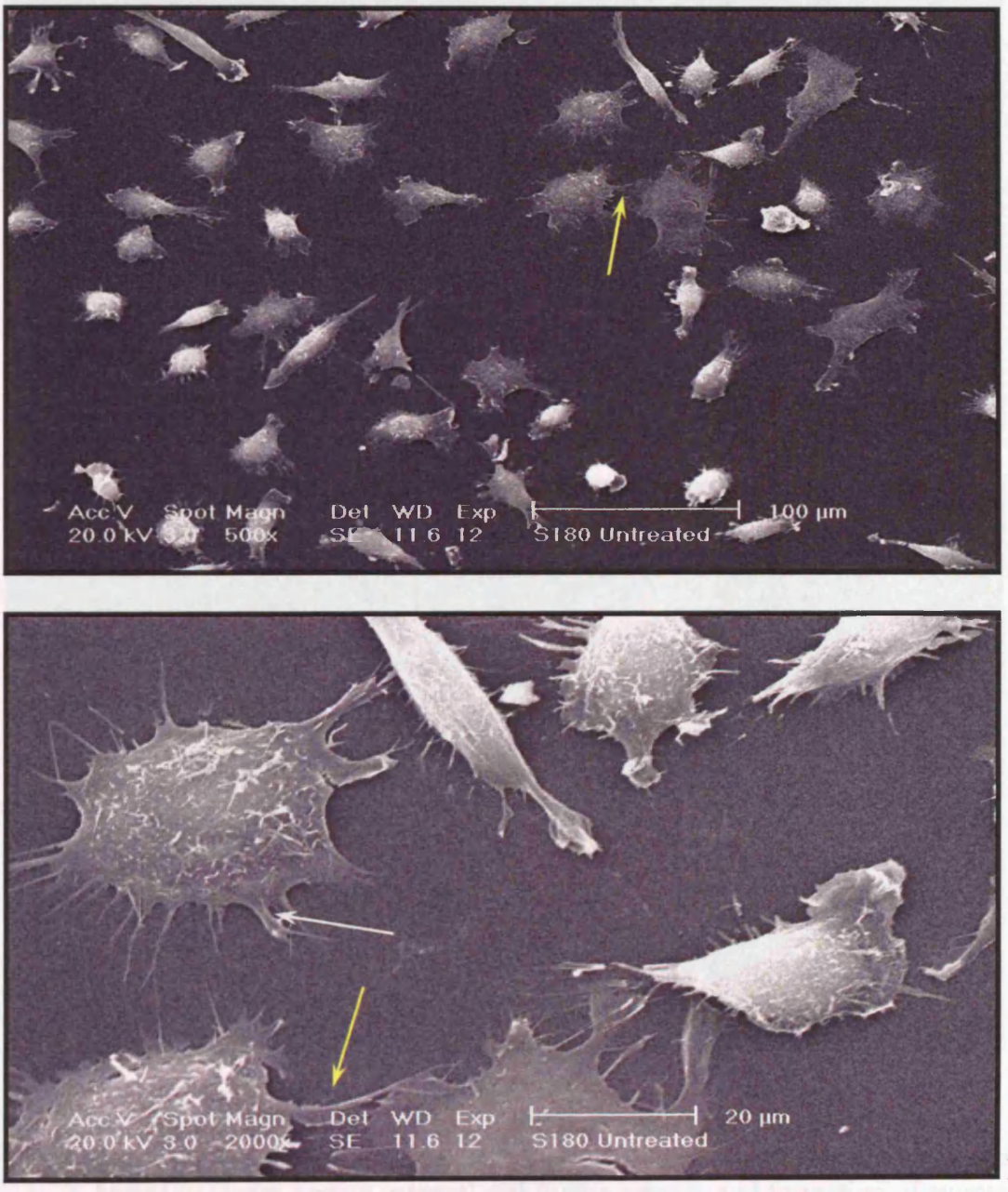
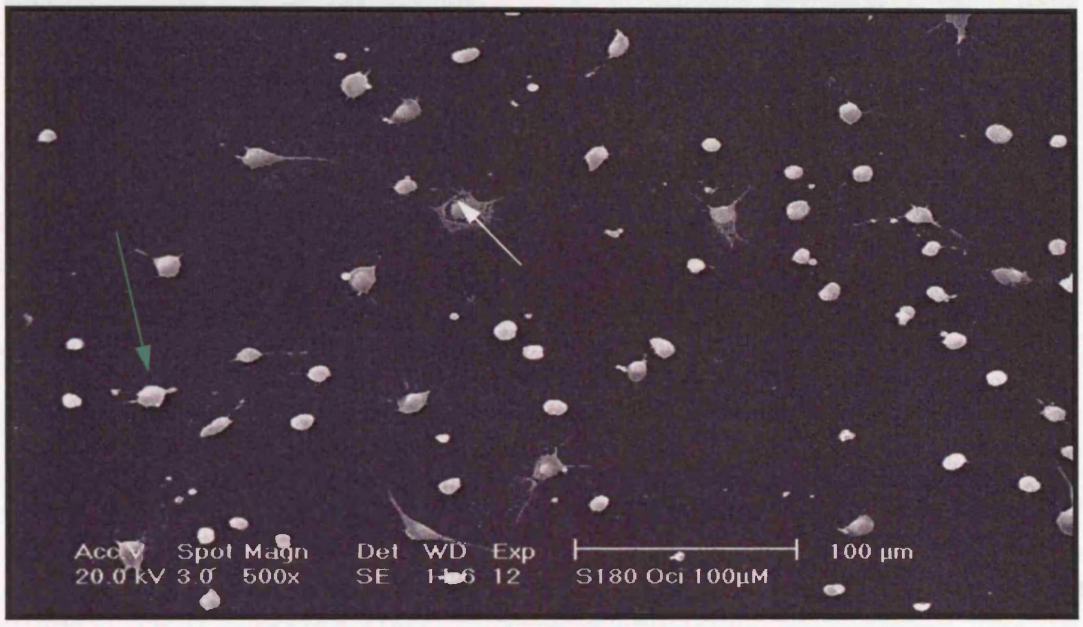
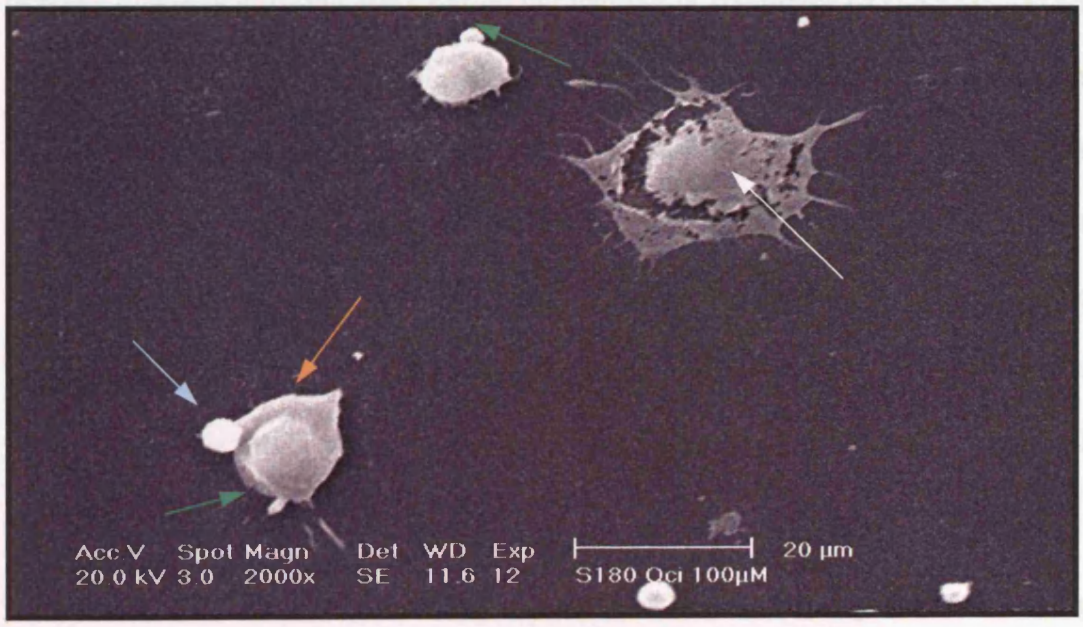


Figure 3.151A-B
SEM images of untreated S180 cells. Cells show surface microvilli (white arrow) and filopodial attachments (yellow arrow).

S180 cells exposed to for 12 hours to *trans-β*-ocimene at a concentration of 100 mM resulted in symptoms of apoptosis, including signs of blebbing, the formation of apoptotic cells and apoptotic bodies, fragmentation, and loss of microvilli and filopodial attachments (Figures 3.152A-C).



A



B

Figure 3.152A-B

SEM images of S180 cells treated for 12 hours with 100 mM *trans-β*-ocimene.

(A) Showing loss of most microvilli, loss of filopodial attachments, membrane blebbing (green arrow), normal cell (white arrow), and formation of apoptotic cells and apoptotic bodies.

(B) Showing loss of most microvilli, loss of filopodial attachments, and formation of apoptotic bodies (turquoise arrow), and a normal cell (white arrow).

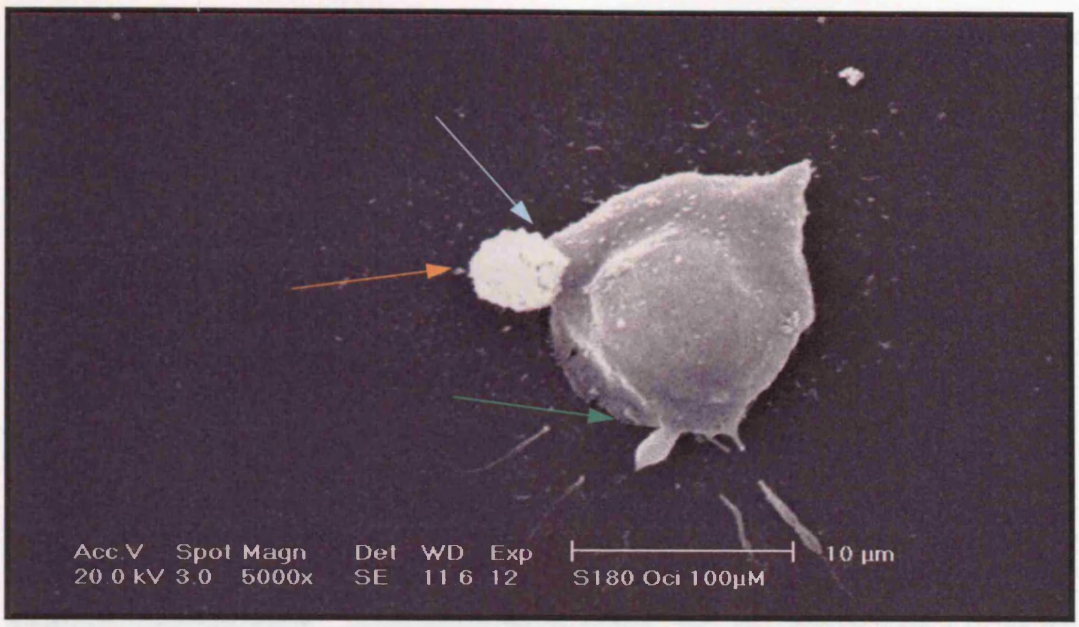


Figure 3.152C
SEM image showing an apoptotic cell (orange arrow), loss of microvilli, loss of filopodial attachments, an apoptotic body (turquoise arrow) membrane blebbing (green arrow).

Exposure of S180 cells for 12 hours to γ-bisabolene was not as effective as exposure to *trans-*β-ocimene for the same period in resulting in symptoms of apoptosis (Figures 3.153 A-C). The majority of cells showed normal growth similar to that of untreated cells. These cells were found adhering to the substratum and interdigitating with each other through filopodial attachments. Delicate microvilli were also noted on the surfaces of viable cells. Fewer than half of the cells showed signs of apoptosis, such as the formation of apoptotic cells and apoptotic bodies, and blebbing of outer membrane. This might be attributed to the low bisabolene concentration used in this experiment (5 mM), which seems not to have the cytotoxic effect of S180 cells.

γ-bisabolene at 50 mM 'killed' most of the S180 cells. Changes at 5 mM might suggest early necrosis rather than cytotoxicity.

Hence it can be said that *trans-β*-ocimene (at LC_{50} concentration, 100 mM) was more effective in bringing about apoptosis in S180 cells than *γ*-bisabolene (at LC_{50} concentration, 5 mM).

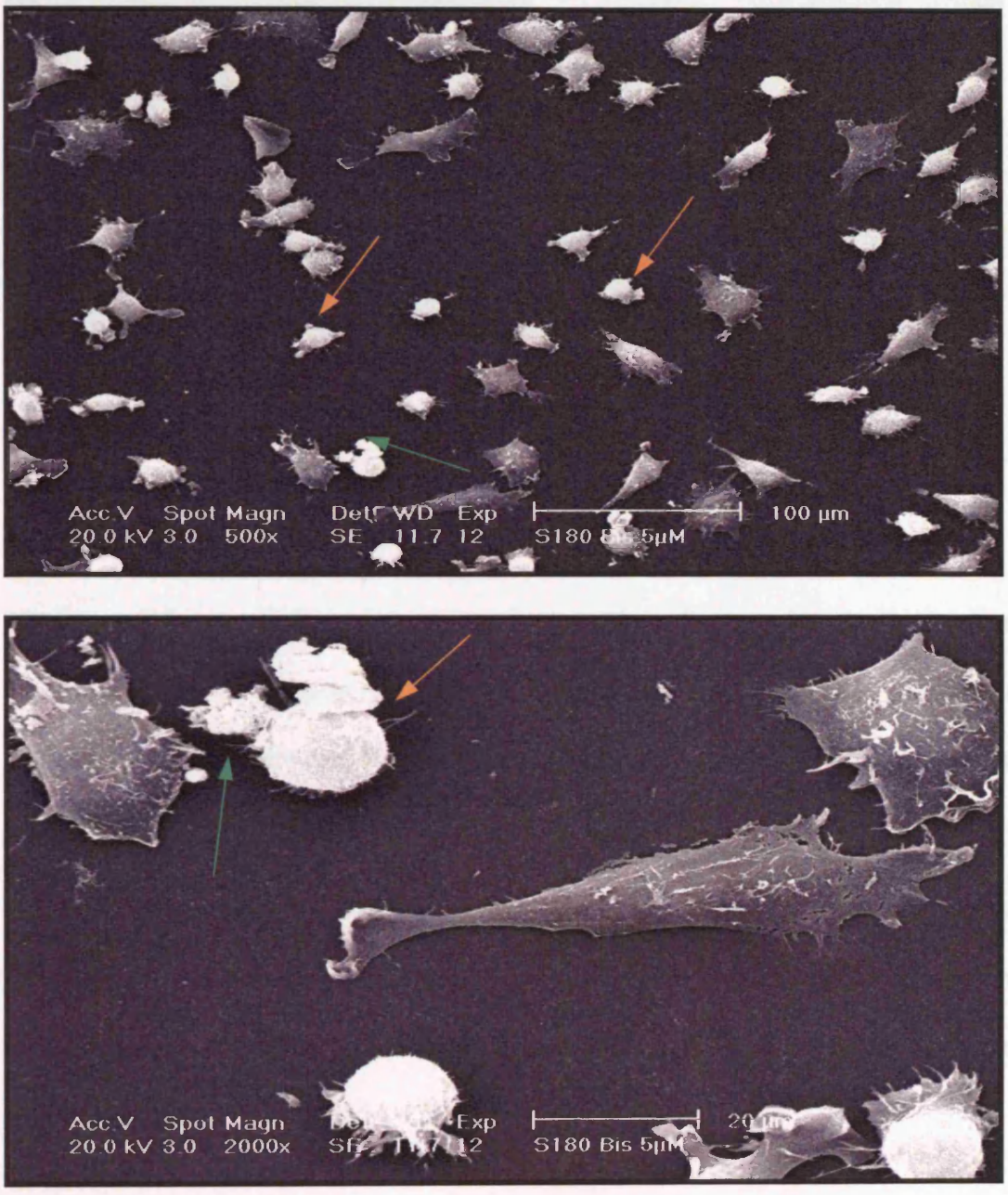
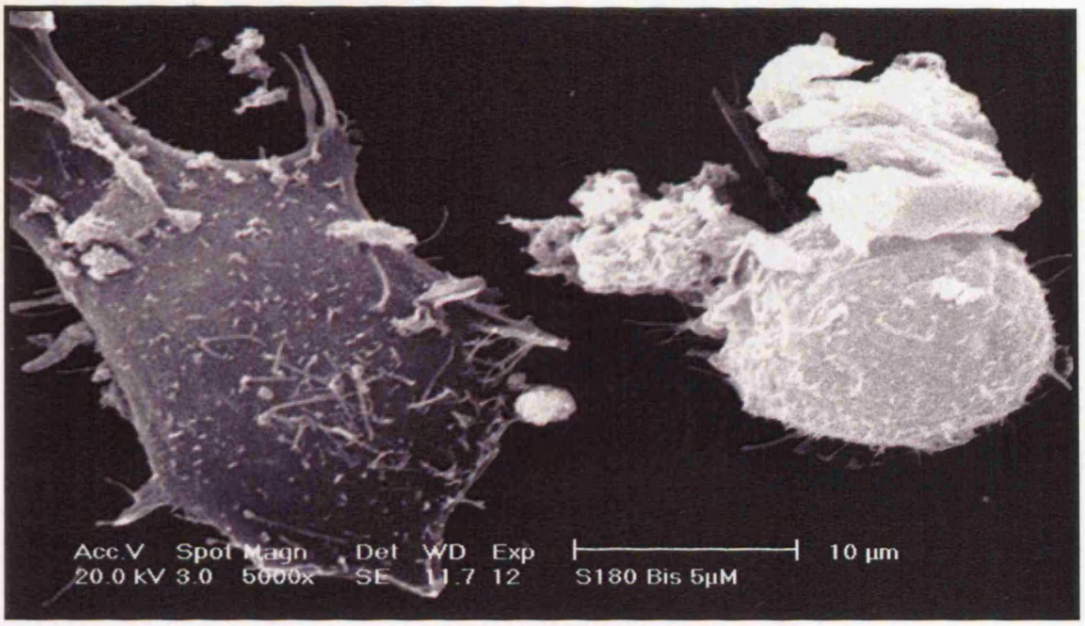


Figure 3.153A-B

SEM images of S180 cells treated for 12 hours with 5 mM *γ*-bisabolene.

(A) Showing fewer apoptotic cells (orange arrows) than normal cells. Only apoptotic cells show loss of most microvilli, loss of filopodial attachments, and membrane blebbing (green arrow).

(B) Showing both normal and apoptotic S180 cells.



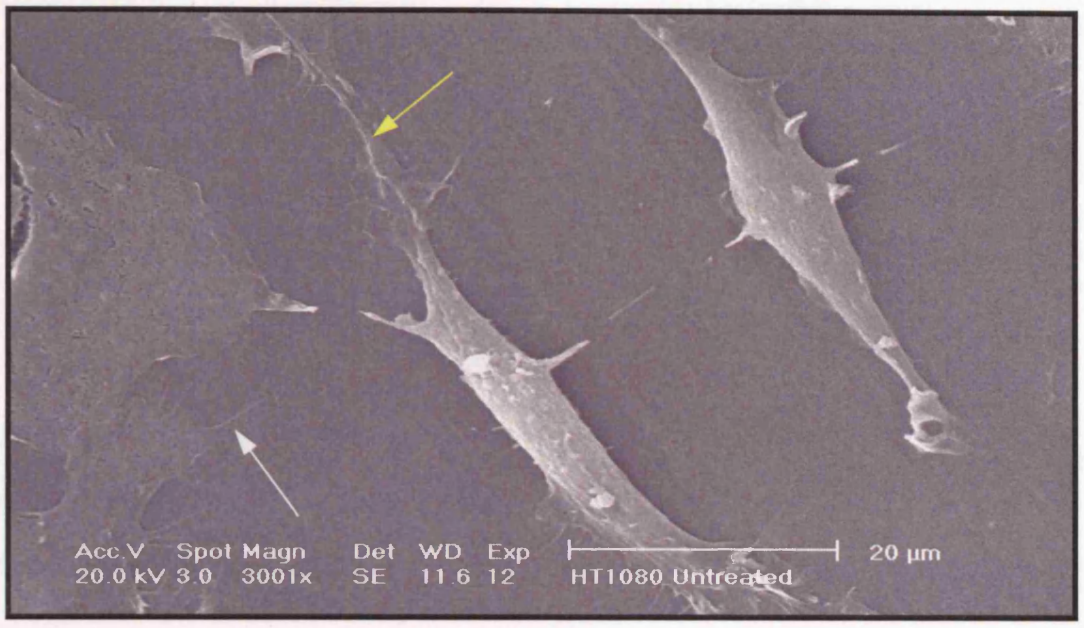
C

Figure 3.153C
Enlarged view of Figure 3B left top corner, showing normal S180 cell and an apoptotic S180 cell.

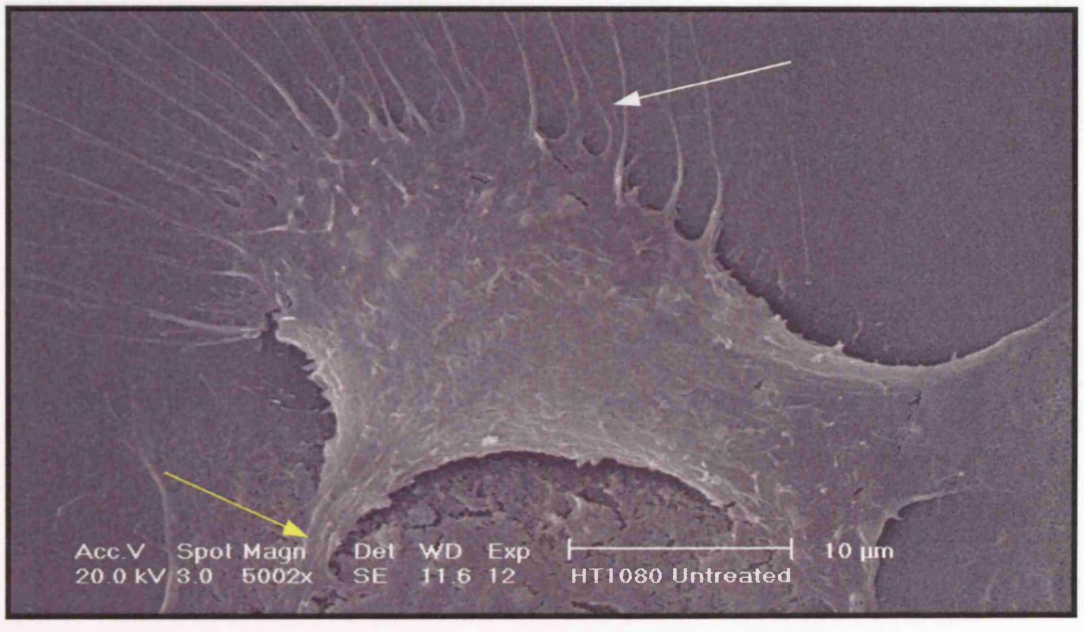
3.24.2 HT1080 Cells

SEM revealed normal growth of untreated cells. The cells were found adhering to the substratum and interdigitating with each other through filopodial attachments. Delicate microvilli were also found radiating from the surfaces of viable cells (Figure 3.154A-B).

HT1080 cells exposed to *trans-β*-ocimene (LC_{50} concentration, 100 mM) showed visible signs of apoptosis, including loss of microvilli and filopodial attachments, blebbing of outer surface membrane, and the formation of apoptotic cells and apoptotic bodies (Figure 3.155A-C).



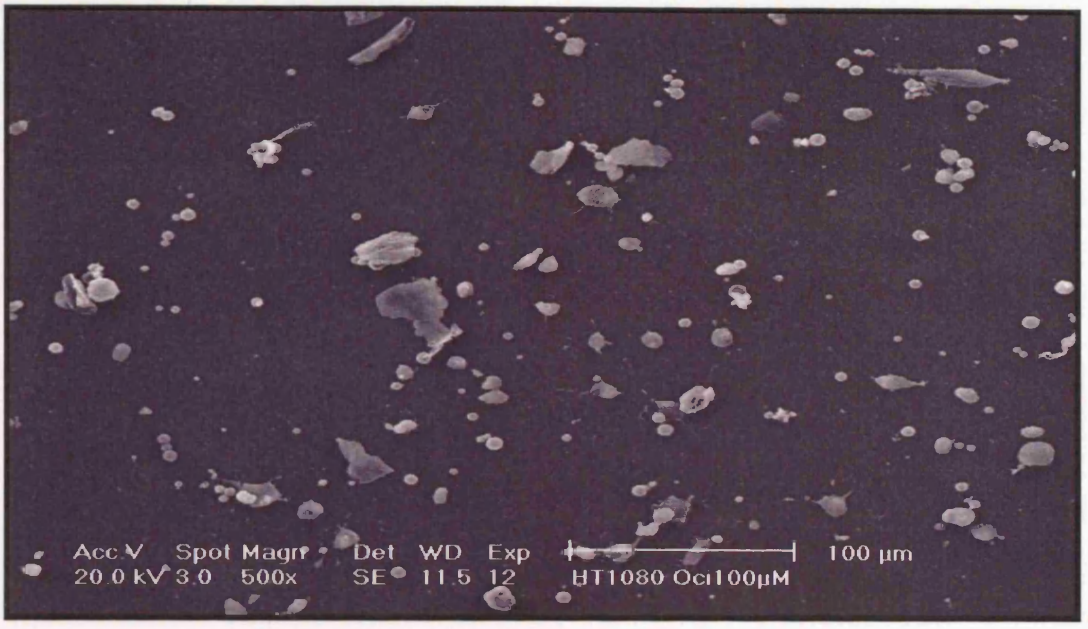
A



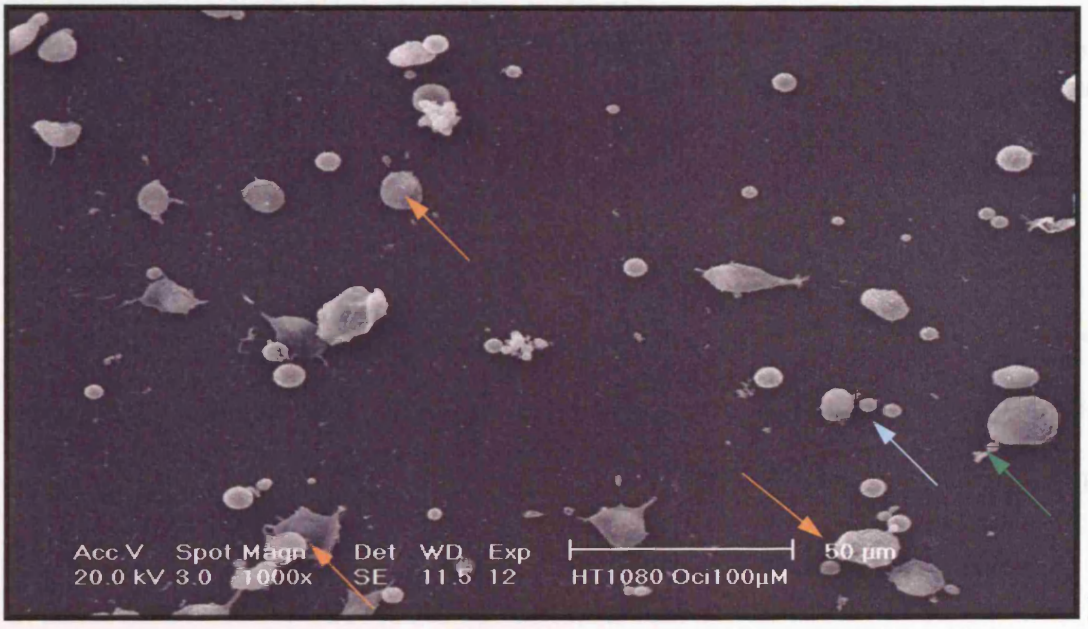
B

Figure 3.154A-B

SEM images of untreated HT1080 cells. Cells show surface microvilli (white arrow) and filopodial attachments (yellow arrow).



A



B

Figure 3.155A-B

SEM images of HT1080 cells treated for 12 hours with 100 mM *trans-β*-ocimene. (A&B) Showing formation of apoptotic cells (orange arrow) and apoptotic bodies (green arrow), and membrane blebbing (turquoise arrow).

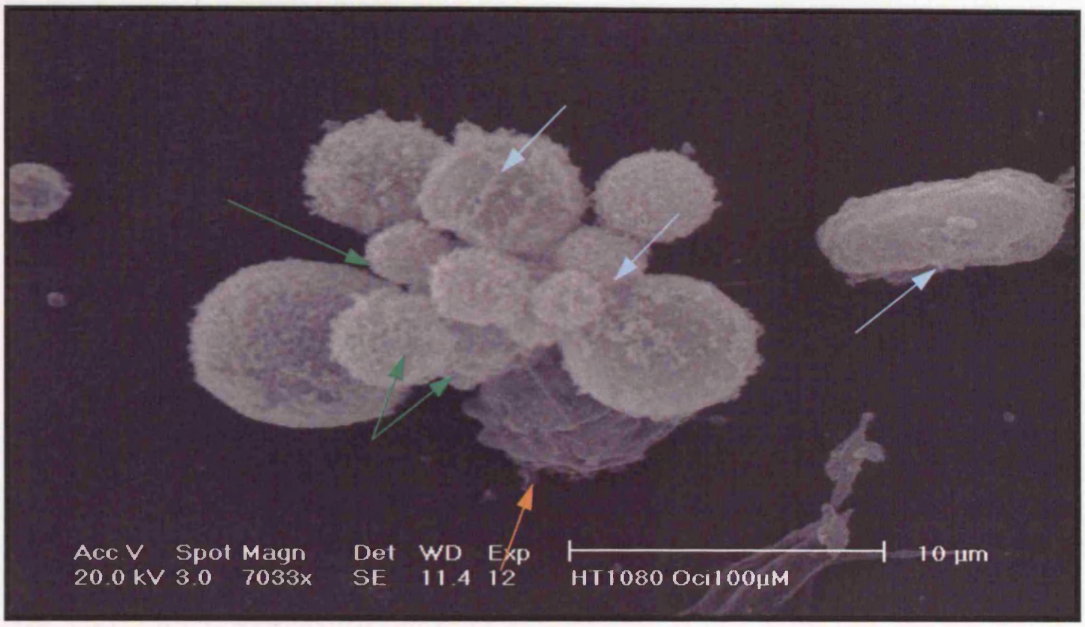
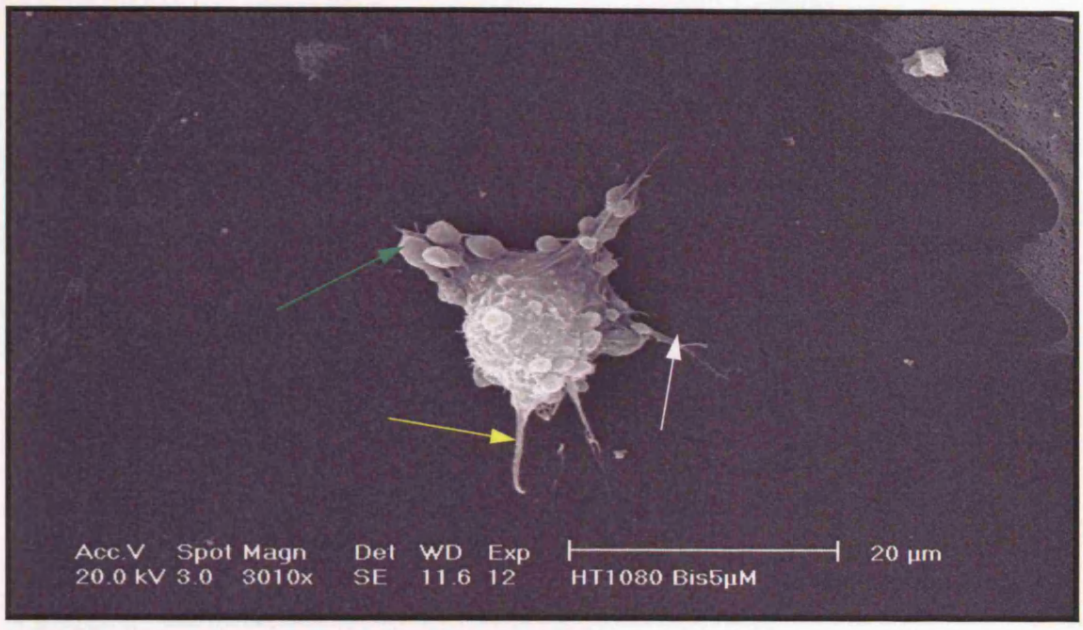


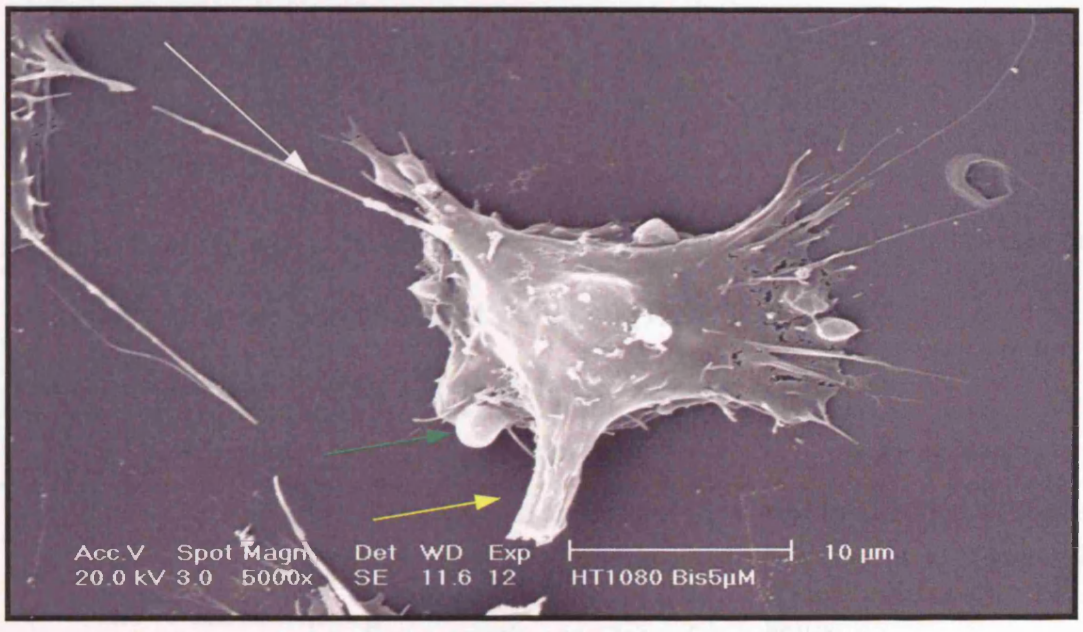
Figure 3.155C
SEM images of HT1080 cells treated for 12 hours with 100 mM *trans-β*-ocimene showing early apoptotic cell (orange arrow), apoptotic bodies (turquoise arrow) and blebbing of outer membrane (green arrow).

HT1080 cells treated for 12 hours with 5mM *γ*-bisabolene showed some signs of apoptosis, such as blebbing of the outer membrane (Figure 3.156A-C). However, these cells retained their microvilli and filopodial attachments, thus indicating possible early necrosis at the low bisabolene concentration (5 mM) used in this experiment.

Accordingly, treatment of HT1080 cells with *trans-β*-ocimene (100 mM) induced more apoptosis than in cells treated with *γ*-bisabolene (5 mM).



A



B

Figure 3.156A-B

SEM images of HT1080 cells treated for 12 hours with 5 mM γ-bisabolene. (A&B) Showing some signs of apoptosis (membrane blebbing - green arrow). Microvilli and filopodial attachment still visible.

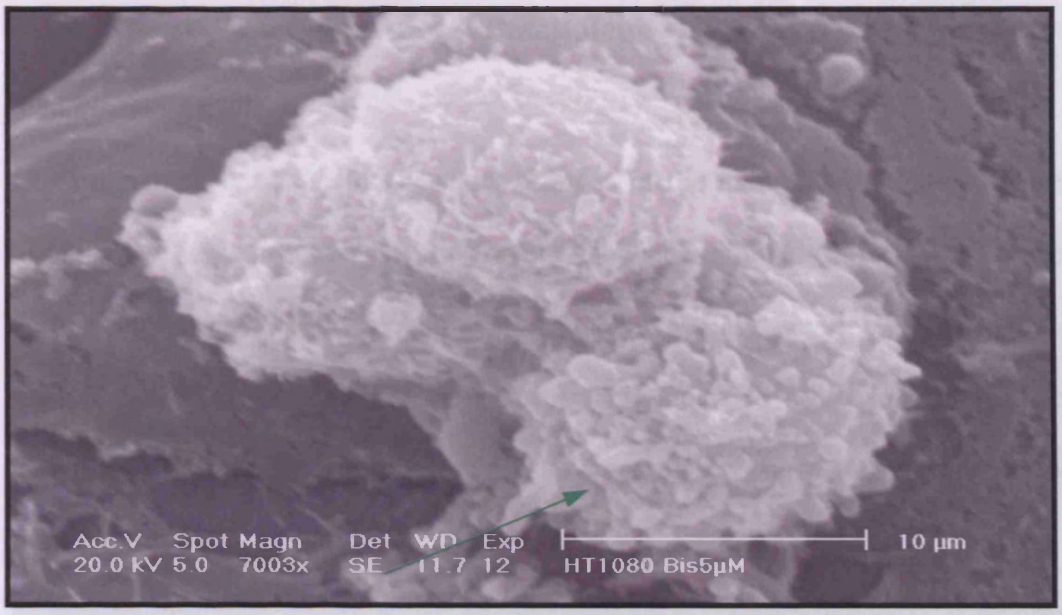


Figure 3.156C
(C) Showing some early signs of apoptosis (blebbing - green arrow)

3.25 Summary

The effects of *trans-β*-ocimene and *γ*-bisabolene on the S180 and HT1080 cell lines used in this study can be summarised as follows (Table 3.18).

Table 3.18 Summary of effects of *trans-β*-ocimene and *γ*-bisabolene

	Untreated	***Trans-β*-ocimene**	***γ*-bisabolene**
S180	None	▪ Symptoms of apoptosis ▪ Formation of apoptotic bodies ▪ Fragmentation and loss of microvilli and filopodial attachments ▪ Hence, high apoptosis	▪ Not as effective as ocimene ▪ Majority of cells showed normal growth after 12 hours. ▪ Less than half of cells showed signs of apoptosis ▪ Changes suggest early necrosis, not cytotoxicity ▪ Hence, low apoptosis
HT1080	None	▪ Visible signs of apoptosis ▪ Loss of microvilli and filopodial attachments ▪ Formation of apoptotic cells and bodies ▪ Blebbing ▪ Hence, high apoptosis	▪ Some signs of apoptosis ▪ Retention of microvilli and filopodial attachments ▪ Possible early necrosis ▪ Blebbing ▪ Hence, low apoptosis

3.26 *Trans-β*-Ocimene and γ-Bisabolene Using Annexin V Assay

3.26.1 S180 Cells

Control untreated S180 did not show changes in the membrane phosphatidylserine asymmetry. Accordingly, they were not labelled with Annexin V-FITC (Figure 3.157). Only a few cells showed positive Annexin V-FITC staining, appearing bright apple green on the cell membrane surface. This indicates a very low percentage of apoptosis. No control cells showed signs of necrosis, as none was stained red with propidium iodide.

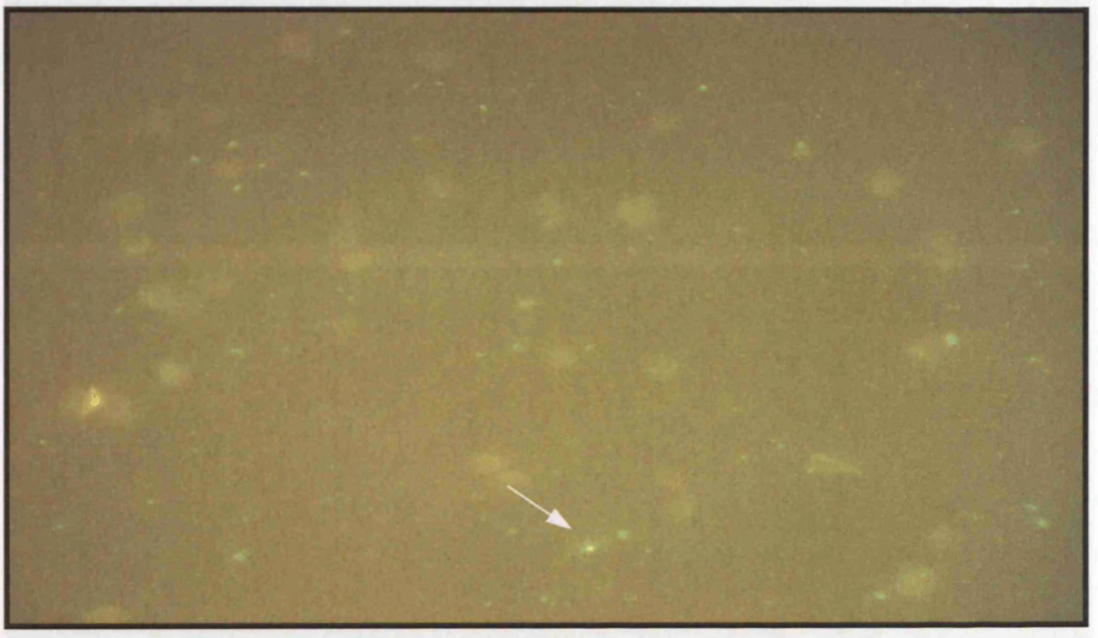
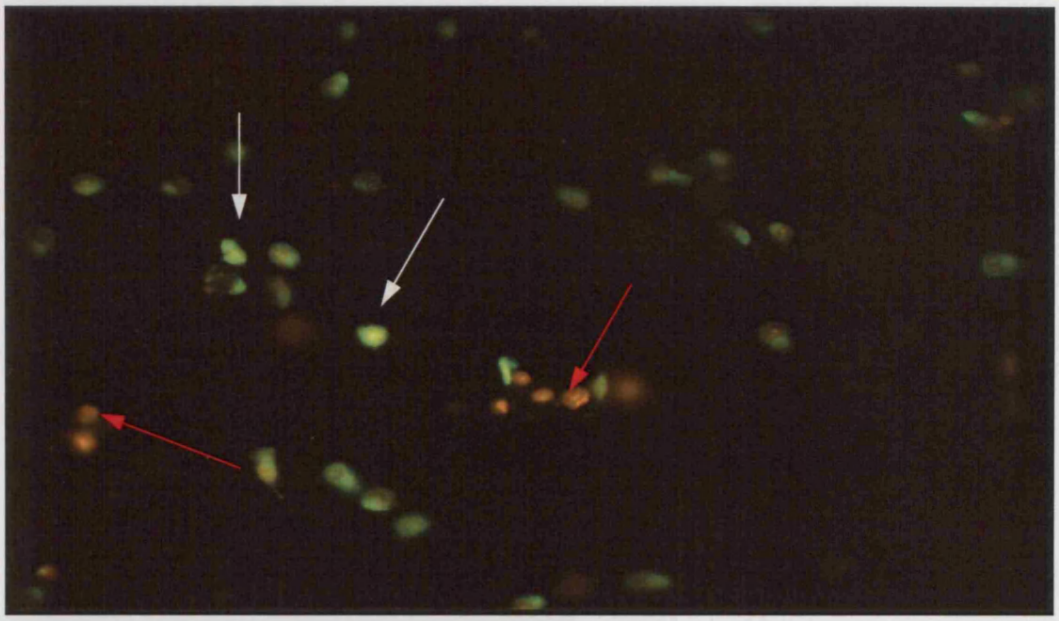


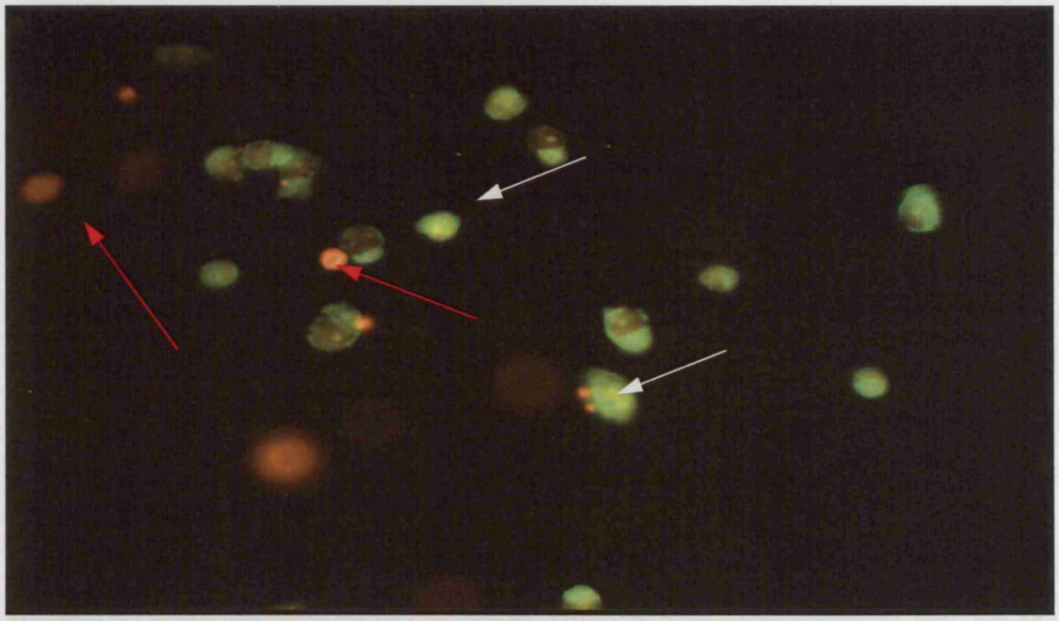
Figure 3.157
Fluorescent microscope images of untreated (control) S180 cells after treatment with fluorescein isothiocyanate (FITC) and propidium iodide (PI) (X75).
A few late apoptotic cells could be seen stained with FITC appearing bright apple green on cell membrane surface (white arrows)

S180 cells grown in culture containing 100 mM *trans-β*-ocimene for 12 hours (Figures 3.158 A-B), hence undergoing induced apoptosis, showed positive results for changes in membrane phosphatidylserine asymmetry. The majority of these cells stained green with Annexin V-FITC, showing positive labelling on the cell surface and within the cytoplasm. A number of cells, however, stained red with PI, possibly

indicating secondary necrosis after the death of apoptotic cells and the disintegration of plasma membrane, which allowed the penetration of PI into the dying or dead cells, staining these cells red.



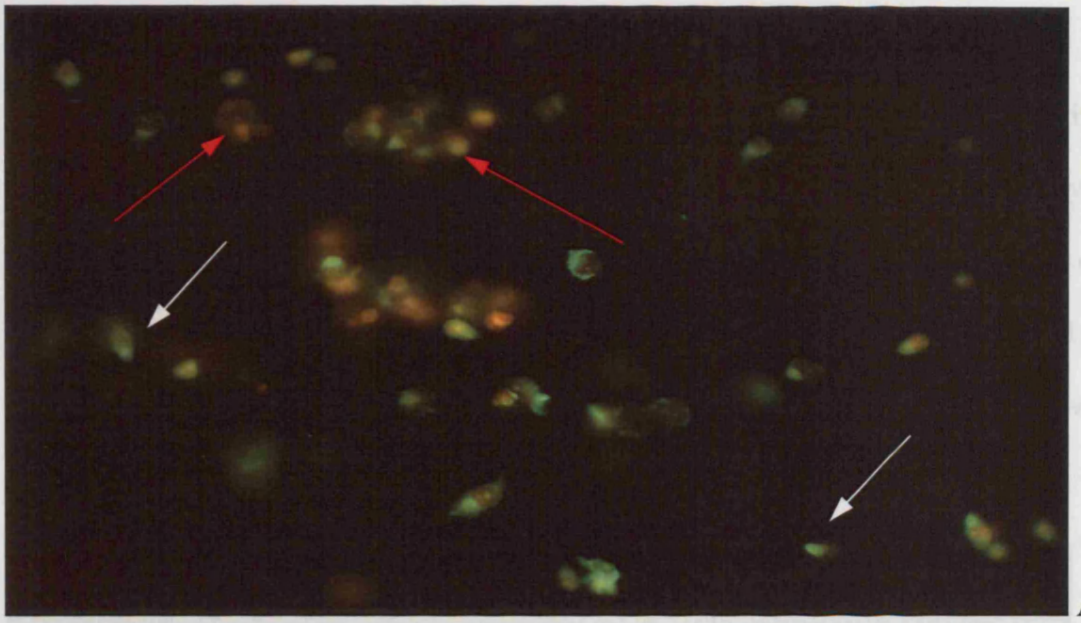
A



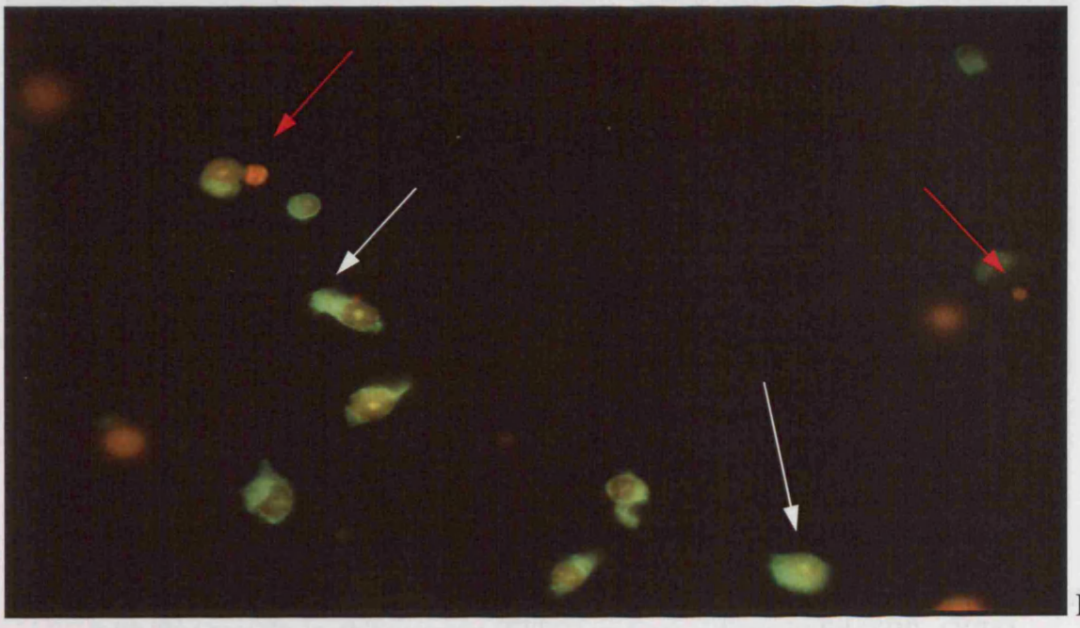
B

Figure 3.158
Fluorescent microscope images of S180 cells treated with 100 mM *trans-β*-ocimene for 12 hrs after treatment with fluorescein isothiocyanate (FITC) and propidium iodide (PI).
(A) Mag.X75. Apoptotic cells (white arrows) show even Annexin V distribution on the cell membrane. A large number of cells show signs of secondary necrosis (red stain with PI) (red Arrows).
(B) Mag.X120. Apoptotic cells (white arrows) and many showing signs of secondary necrosis (red arrows).

S180 cells grown in culture containing 5 mM γ-bisabolene for 12 hours (Figure 3.159A-B) also showed positive results for changes in membrane phosphatidylserine asymmetry.



A



B

Figure 3.159
Fluorescent microscope images of S180 cells treated with 5 mM γ-bisabolene for 12 hours after treatment with (FITC) and propidium iodide (PI).
(A) Mag.75X. Apoptotic cells (white arrows) show even Annexin V distribution on the cell membrane. A large number of cells show signs of secondary necrosis (red stain with PI) (red Arrows).
(B) Mag.120X. Apoptotic cells (white arrows) and many showing signs of secondary necrosis (red arrows)

Most of these cells stained green with Annexin V-FITC, showing positive labelling on the cell surface and within the cytoplasm. However, a number of S180 cells also stained red with PI, possibly indicating secondary necrosis.

3.26.2 HT1080 Cells

Control untreated HT1080 did not show changes in the membrane phosphatidylserine asymmetry. They were not labelled with Annexin V-FITC (Figure 3.160). Only a few cells showed positive Annexin V-FITC staining, appearing bright apple green on the cell membrane surface. Thus a very low number of apoptotic cells were found to occur in control cultures. No control cells showed signs of necrosis, and none was stained red with propidium iodide.

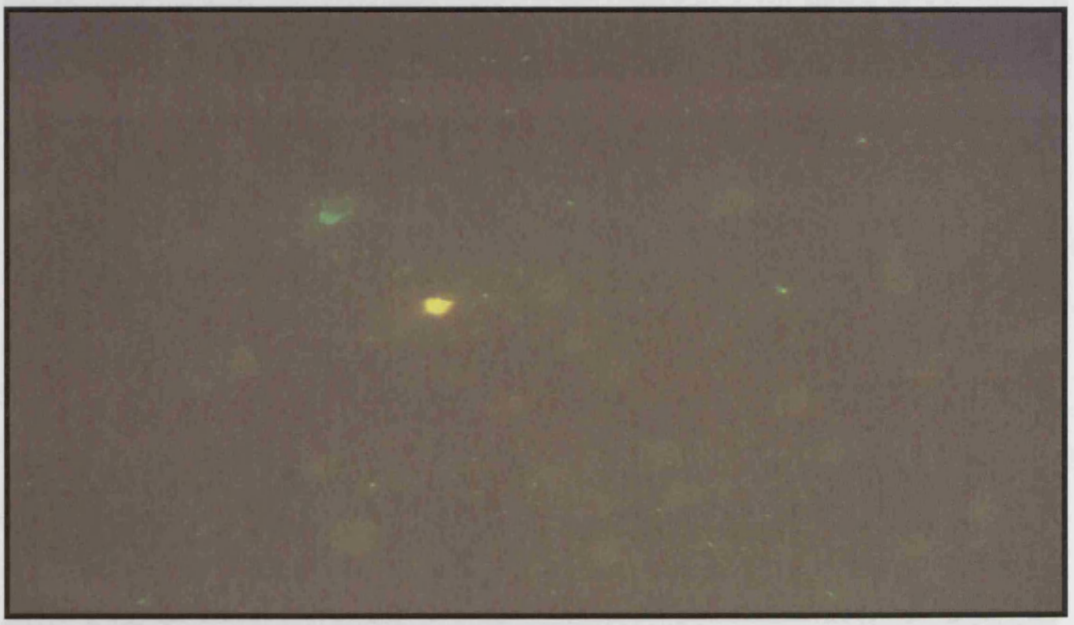


Figure 3.160
Fluorescent microscope images of untreated (control) HT1080 cells after treatment with fluorescein isothiocyanate (FITC) and propidium iodide (PI). (X75).
A few late apoptotic cells could be seen stained with FITC, appearing bright apple green on cell membrane surface (white arrows)

HT1080 cells grown in culture containing 100 mM *trans-β-o*cimene for 12 hours (Figures 3.161A-B), hence undergoing induced apoptosis, showed positive results for changes in membrane phosphatidylserine asymmetry. Cells stained green with

Annexin V-FITC, showing positive labelling on the cell surface and within the cytoplasm. Many cells stained red (positive test for PI), possibly indicating secondary necrosis. Some cells showed blebbings on the outer membrane.

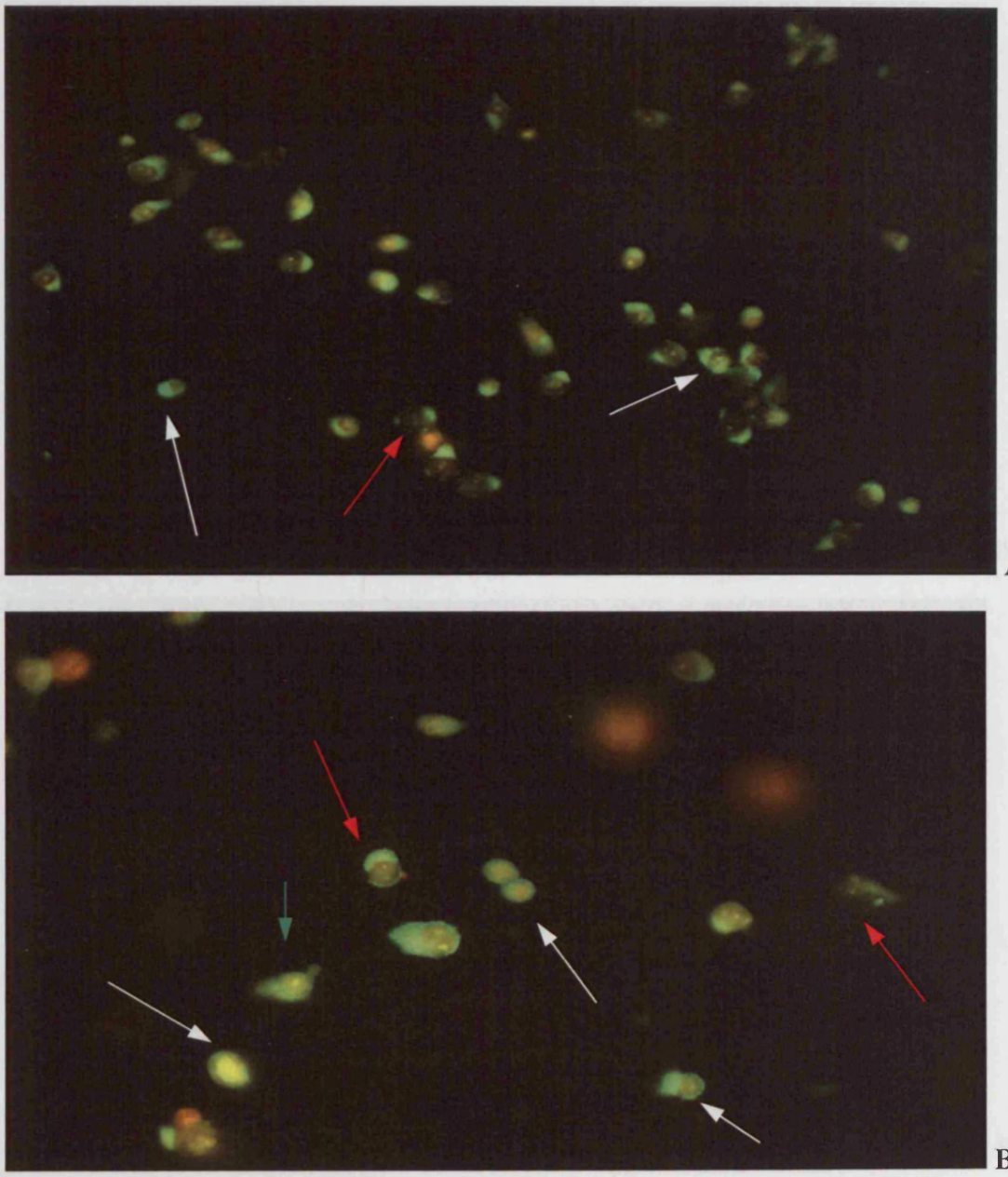


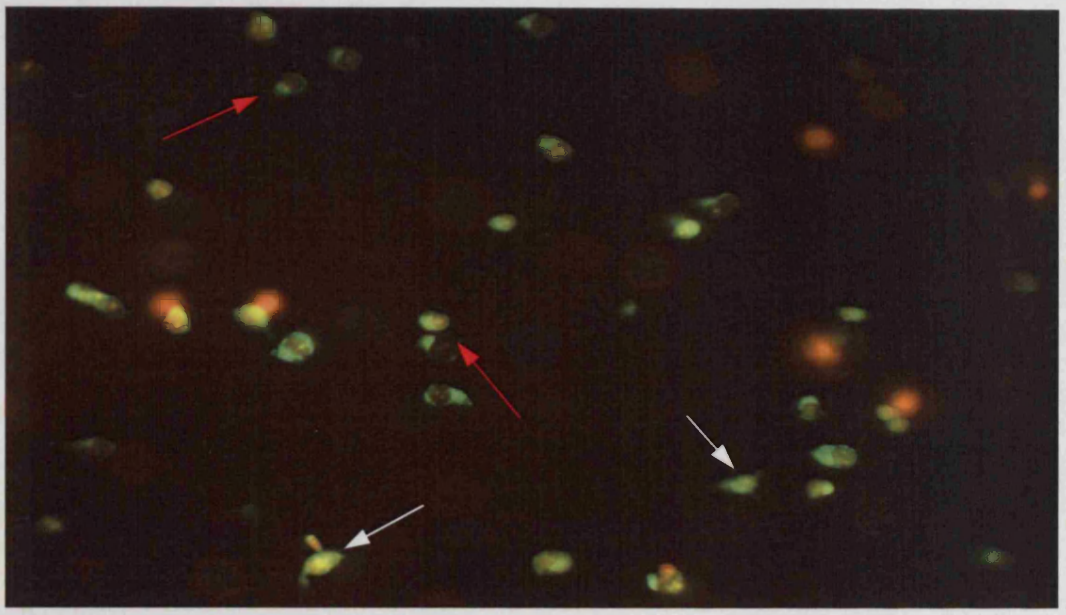
Figure 3.161

Fluorescent microscope images of HT1080 cells treated with 100 mM *trans-β*-ocimene for 12 hours after treatment with FITC and PI.

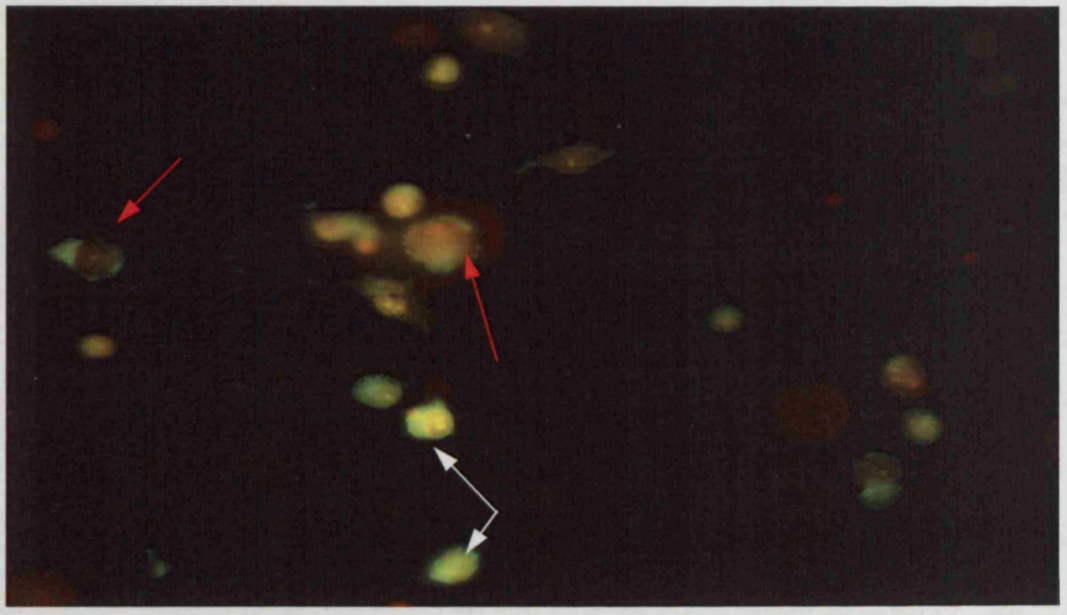
(A) Mag.75X. Apoptotic cells (white arrows) show Annexin V distribution on the cell membrane. Several cells show signs of secondary necrosis (red stain with PI) (red arrows).

(B) Mag.120X. Apoptotic cells (white arrows) and several showing signs of secondary necrosis (red arrows)

HT1080 cells grown in culture containing 5 mM γ-biasbolene for 12 hours (Figure 3.162A-B) also showed positive results for changes in membrane phosphatidylserine asymmetry.



A



B

Figure 3.162
Fluorescent microscope images of HT1080 cells treated with 5 mM γ-bisabolene for 12 hours after treatment with FITC and PI.
(A) Mag.75X. Apoptotic cells (white arrows) show Annexin V distribution on the cell membrane. Several cells show signs of secondary necrosis (red stain with PI) (red arrows).
(B) Mag.120X. Apoptotic cells (white arrows) and several showing signs of secondary necrosis (red arrows).

Cells stained green with Annexin V-FITC, showing positive labelling on the cell surface and within the cytoplasm. Many cells also stained red (positive for PI), indicating possible necrosis or secondary necrosis.

3.27 Summary

The effects of *trans*-β-ocimene and γ-bisabolene on S180 and HT1080 cells can be illustrated as follows, in Table 3.19.

Table 3.19 Summary of effects of *trans-β*-ocimene and γ-bisabolene

	Untreated	***Trans-β*-Ocimene (100 mM)**	**γ-Bisabolene (5 mM)**
S180	▪ No changes ▪ A few cells showed changes in phosphatidyl-serine asymmetry ▪ Very low percentage of apoptosis ▪ No necrosis	▪ Changes occurred (green yellow fluorescence). ▪ Positive changes in phosphatidylserine asymmetry ▪ Some cells showed positive test with PI (red fluorescence). ▪ Secondary necrosis after death of apoptotic cells and disintegration of plasma membrane	▪ Changes occurred (green yellow fluorescence). ▪ Positive changes in phosphatidylserine asymmetry ▪ Some cells showed positive test with PI (red fluorescence). ▪ Secondary necrosis after death of apoptotic cells and disintegration of plasma membrane
HT1080	▪ No changes ▪ A few cells showed changes in phosphatidyl-serine asymmetry ▪ Very low percentage of apoptosis ▪ No necrosis	▪ Changes occurred ▪ Positive changes in phosphatidylserine asymmetry (green yellow fluorescence) ▪ Some cells showed positive test with PI (red fluorescence). ▪ Secondary necrosis ▪ Some cells with blebbings	▪ Changes occurred ▪ Positive changes in phosphatidylserine asymmetry (green yellow fluorescence). ▪ Some cells showed positive test with PI (red fluorescence). ▪ Necrosis/secondary necrosis

Results above clearly illustrated that γ-bisabolene at 5 mM concentration was as effective as *Trans-β-o*cimene at 100 mM concentration.

Chapter Four

General Discussion

4.1 - Introduction

4.1.1 Modes of Cell Death

The end-point of apoptosis and necrosis is similar (Bowen, 1999), and hence it may not always be easy to differentiate between them, although some apparently diagnostic tools have been developed.

From a comparative perspective, apoptosis leads to cell condensation and shrinkage, whereas necrosis leads to cellular swelling. Other distinctions include apoptotic cells losing water, whilst necrosis leads to gaining water. Membrane pumps continue functioning during apoptosis, but fail early on in necrosis. Necrotic cells experience an increasing acidosis leading to dense pyknotic nuclei. In apoptosis, a dilation of the nuclear envelope occurs, leading to nuclear blebbing, and the blebs tend to fill up with marginating chromatin.

During the later stages, the lysosomes of necrotic cells fragment, and hydrolytic enzymes are released which further lyse the cells, leading to a complete breakdown of subcellular compartmentalisation. The necrotic cell finally bursts, often eliciting an inflammatory response. In contrast, apoptotic cells usually undertake a discreet but florid fragmentation into membrane-bound spheres or apoptotic bodies which are rapidly phagocytosed by neighbouring cells or phagocytes, which elicits an immune response (Bowen, 1999). Notwithstanding the priority of the above descriptions, three types of cell death have been envisaged (Clarke, 1990):

Type 1 Cell death, which accords with classical apoptosis, and is also described in morphological terms as Type 1 cell death.

Type 2 Cell death, or programmed cell death, is characterised by an increasing level of autophagotic activity. The dying cell fills up with active secondary lysosomes. There is no clear chromatin margination, and the nucleus survives until it is finally extruded from the cells or becomes autolysed. This type of programmed cell death usually involves changes in the subcellular distribution of hydrolases.

Type 3 Cell death, which can be induced by withdrawal of nerve growth factor, and as such, could be thought of as an atrophic cell death. Morphologically, it is characterised by dilation of the endoplasmic reticulum, Golgi and nuclear envelope, leading to extensive vacuolation. There is no chromatin margination, as seen in apoptosis.

Recetly, Lockshin and Zakeri (1996) have referred to apoptosis as Type 1 cell death, and non-apoptotic programmed cell death as Type 2. A vascular non-apoptotic form of cell death has been described in human breast cancer cells (Amin *et al.*, 2000). This appears to occur where caspase activity is inhibited, and thus the DNA cannot become fragmented. Such cells are Annexin V positive, but negative in terms of TUNEL assays. The cells also appear to swell and to be vacuolated in a similar way to that found with bisabolene treatment. It is possible that bisabolene therefore induces Type 2 cell death, and may inhibit caspase activity.

4.1.2 Myrrh and Cell Death

The three cell lines used in this study, namely murine S180 sarcoma, HT1080 human fibrosarcoma, and normal chick fibroblast, were selected because they proved to be an excellent model for the investigation of the cell death-inducing effects of molmol and

haddi plant extracts. The cells showed a high sensitivity to the cytotoxic activity of these extracts.

S180 cells are highly malignant, slightly immunogenic, and are used extensively as an experimental tumour model in chemotherapy screening studies.

The HT1080 cells, on the other hand, offer the advantage of producing high titres of retroviral vector resistant to inactivation by human serum, enabling their use in human therapy. Reducing the viral sequences has also reduced the risk of producing replication component retroviruses. Accordingly, in the future, this line cell offers great potential for the development of human gene therapy.

The normal chick fibroblast cells were used as a control to compare the effects of the molmol and haddi plant extracts on the S180 cells and HT1080 cells with their effects on available normal cells.

Myrrh, extracted from the stem of *Commiphira molmol* (family Burseraceae), has been used extensively for therapeutic applications, as well as in food and perfume. The U.S. Food and Drug Administration (21 Code of Federal Registration – CFR 172.510) approved it for food use, and the Flavor Extract Manufacturers' Association (FEMA) has given it generally recognised as safe (GRAS) status as a flavour ingredient (No. 2765) (Hall and Oser, 1965; Ford *et al.* (1992). It is therefore largely non-toxic.

Myrrh has been traditionally used as a treatment for many ailments for thousands of years. For example, the Sumerians and the Greeks used it to treat 'worms' (Mitchie & Cooper, 1991), and by the Chinese to relieve pain and swelling due to traumatic injury (Lee and Lam, 1993). The Somalis used it to treat stomach complaints, diarrhoea and

wounds (Cleason *et al.*, 1991). In modern times, myrrh has been used as a drug, and tested for its efficacy in various applications. It has, for example, been used for the therapy of aphthous ulcer (Pesko, 1990), and for the reduction of cholesterol and triglycerides (Michie & Cooper; Jain, 1994).

Myrrh has also been found to have anti-carcinogenic potential (Al-Harbi *et al.*, 1994), and gastric antiulcer and cytoprotective potentials (Al-Harbi *et al.*, 1997). Qureshi *et al.* (1993) evaluated the genotoxic, cytotoxic, and anti-tumour properties of myrrh, and found that the cytotoxic and anti-tumour activity of *C.molmol* was equivalent to those of the standard cytotoxic drug cyclophosphamide. They concluded that its use in cancer therapy seems to be appropriate, though they recommended further investigation.

Myrrh has been reported to be effective in the treatment of schistosomiasis (Massoud *et al.*, 1998). Massoud *et al.* (2001) administered a myrrh formulation, consisting of 8 parts of resin and 3.5 parts of volatile oil, extracted from myrrh, to seven patients passing *Fasciola* eggs in their stools. Patients were administered the formulation at a dose of 12 mg/kg/day for six successive days, in the morning, on an empty stomach. Patients were then followed-up for three months, and the therapy proved effective, with pronounced improvement of the general condition and amelioration of all symptoms and signs. Massoud *et al.* (2001) also noted a marked decrease in the number of eggs passed at the end of the six-day treatment. They also found that eggs were not detected in the faeces three weeks after the treatment, and after three months' follow-up period. Massoud *et al.* (2001) also did not notice any signs of toxicity or adverse effects, concluding that, despite the small number of patients involved in the study, myrrh has proven to be an effective

drug in patients who experienced marked improvement in the clinical and laboratory results.

Sheir *et al.* (2001) studied the effects of an antischistosomal therapy derived from myrrh in an attempt to find an alternative drug to praziquantel, the drug of choice to treat schistosomiasis, since resistance to this drug has been demonstrated. They involved 204 patients with schistosomiasis and 20 healthy volunteers (control). The drug derived from myrrh was administered at a dose of 10 mg/kg of body weight/day for three days, and induced a cure rate of 91.7%. Re-treatment of patients who did not respond with the 10 mg/kg/day treatment for six days resulted in a cure rate of 76.5%, increasing the overall cure rate to 98.09%. They concluded that myrrh has been shown to be a safe and effective anti-schistosomal drug at the dose indicated above for six days. Twenty cases provided biopsy specimens six months after the treatment, but none of them showed living eggs.

The Egyptian Pharmaceutical Company (Pharco) has launched a new antihelmith drug, Mirazid, derived from myrrh, as a treatment for schistosomiasis (Pharco Pharmaceuticals, 2003).

More recently, El Baz *et al.* (2003) studied the clinical and parasitological efficacy of Mirazid in treatment of schistosomiasis haematobium in Egypt, and found that Mirazid, administered in a dose of two capsules (600 mg) on an empty stomach before breakfast, for six consecutive days, proved to be very effective and safe in the treatment of schistosomiasis haematobium. Botros *et al.* (2004), however, have published a detailed paper demonstrating a lack of evidence for an antischistosomal activity of Myrrh in experimental animals.

The discussion above clearly shows that myrrh has been, and is still used as a therapy for a wide range of pathological cases, and that human patients tolerate treatment with Myrrh very well. The findings of the present study showed the efficacy of extracts obtained from *C. molmol* (myrrh) and *C. guidotii* (haddi) in treating human cancer cell lines (S180 and HT1080 cell lines), as discussed in the following sections.

4.2 Growth Assays

Untreated S180 cells were shown to increase exponentially, attaining their highest concentration by day 6 from the start of the experiment. On day 7, the cells reached a plateau but started declining in number on day 8, when the growth curve experiment was stopped. S180 cells were seeded initially on day 0 at 4 x 10^4 cells/ml. On day 6, the cell numbers increased sharply to more than 230 x 10^4, and on day 8, the cell numbers dropped to more than 150 x 10^4. Growth was somewhat slow during the first three days, but showed an increase on day 4. This increase in the cell numbers in a proliferating cell line such as the S180 cell line can be considered as an index of normal behaviour (Al-Hazzaa, 2000; Amin, 2001).

The addition of each of the ethanolic molmol and hexane haddi extracts to S180 cultures on day 4 of growth (when cell concentrations in the control showed a definite increase) induced a rapid decline on day 5, reaching its lowest on day 8. The results showed that both plant extracts had significant effect on the growth of this cell line only 24 hours after the culture exposure to these extracts, which resulted in sharp decline in cell numbers compared to the growth in control cultures. This may, at this experimental stage, suggest that the extracts of both plants were either cytostatic or were effective in inducing apoptosis and death of the S180 cells. This decline in cell number also suggests that the

two extracts may have anticancerous and cytotoxic activities. The anticancerous and cytotoxic activities of molmol have been demonstrated against several cancerous cell lines (for example, Tariq *et al.*, 1985; Qureshi *et al.*, 1993; Al-Harbi *et al.*, 1994, 1997; Rao *et al.*, 2001; Tipton *et al.*, 2003).

The study results also showed that hexane haddi extract was more effective against S180 than ethanolic molmol extract in reducing growth of S180 cells.

Growth assays using HT1080 showed that these cells proliferate exponentially, reaching their maximum concentration on day 12, rather than day 6, as was the case for S180. After day 12, growth declined abruptly. HT1080 cells were grown in their medium, which was then changed with a new medium containing each of ethanolic molmol and hexane haddi extracts, each being added on day 7 of the growth period, and the cells declined in numbers for the following seven days. In the case of HT1080, ethanolic extracted molmol was more effective than hexane extracted haddi in inducing cell death.

4.3 MTT Viability Assay

The S180, HT1080 and normal fibroblast cell lines were treated with different concentrations of molmol and haddi extracts over a period of 24, 48 and 72 hours before MTT solution was added to the growth media to measure the mean optical density (and hence estimate percentage toxicity), and also to establish percentage viability. Two series of dilutions for each of the ethanolic molmol, ethyl acetate molmol, ethanolic, haddi, hexane molmol, hexane haddi, and ethyl acetate haddi extracts were used in the MTT viability assay. Furthermore, Flavex molmol and essential oil haddi (Aldrich) were also used, both of which are commercial products.

4.3.1 Effects of Molmol and Haddi Extracts on S180 cells

Molmol extract inhibited the growth of S180 cells in cultures in a dose and time-dependent fashion, whilst in the control cultures, all the cells were viable throughout the experimental period. However, the treatment of S180 cells with a different series of molmol extract resulted in the death of cells, which reacted upon the addition of MTT reagent. Dead cells were those cells not producing formazan product. The results suggest that molmol at the lower concentration (83.3 μg/ml) was a growth promoter and that mitogenesis has occurred, resulting in increasing the number of viable cells above that of the control (100%). With further increase in the molmol concentration, there were fewer viable S180 cells observed. Results also showed that increasing the incubation period also reduced the viability of S180, especially at the highest molmol concentrations, and after 48 and 72 hours of exposure to the molmol extract. This substantiates the notion that growth inhibition was both dose- and time-dependent. It also suggests that mitosis and apoptosis may be closely linked, since low concentrations increase mitosis whilst high concentrations induce apoptosis. Recently, Zhao *et al.* (2003) found that boswellic acid acetate (BC-4), isolated from the resin of *Boswellia carteri* Birdw (family Burseraceae like that of *Commiphora*), inhibited the growth of B16F10 mouse melanoma cells in culture in a dose- and time-dependent fashion. Shao *et al.* (1998) reported the alcoholic extract of the resin (Boswellic acids) had an inhibitory activity against human leukaemia HL-60 cells in culture. They isolated four major triterpene acids (β-boswellic acid, 3-*O*-acetyl-β-boswellic acid, 11-keto-β-boswellic acid, and 3-*O*-acetyl-11-keto-β-boswellic acid) from the gum of *B. serrata* and examined them for their *in vitro* antitumour activity. They found that these acids inhibited the synthesis

of DNA, RNA and protein in human leukaemia HL-60 cells in a dose-dependent manner, with IC_{50} values ranging between 0.6 and 7.1 mM. They also found that 3-*O*-acetyl-11-keto-β-boswellic acid induced the most pronounced inhibitory effects of DNA, RNA and protein synthesis, with IC_{50} values of 0.6, 0.5, and 4.1 mM, respectively. The effect of this acid on DNA was found to be irreversible, and it significantly inhibited the cellular growth of HL-60 cells, but did not affect cell viability. The effect of ethanolic molmol extract at all concentrations seemed to be irreversible, since cell viability declined throughout the experimental periods used in the present study. As indicated above, the anticancerous and cytotoxic activities of molmol have been demonstrated against several cancerous cell lines (for example, Tariq *et al.*, 1985; Qureshi *et al.*, 1993; Al-Harbi *et al.*, 1994, 1997; Rao *et al.*, 2001; Tipton *et al.*, 2003), and the results of the MTT assay further substantiate these reports. At the higher series of concentration, the viability of S180 declined substantially with time.

Hexane molmol extract seemed to be less effective in treating S180 cells, in which case percentage cell viability was less than that reported above for ethanolic molmol extract. In fact, mitogenesis was noted at the lower concentration, even after 72 hours of exposure.

The ethyl acetate molmol extract resulted in a lower percentage viability of the S180 cells than did ethanolic molmol extract, using similar concentrations; but on the whole, its action was similar.

Treatment of S180 cells with Flavex molmol extract indicated that this commercially available extract had the least toxic effect on these cells, and the percentage viability was generally the highest among the four types of extracts used.

The effects of haddi extracts on the viability of S180 cells were also dose and time-dependent, though the effects varied according to the nature of the extractants used. Ethanolic haddi extract was not found to be effective for the treatment of S180. Treating these cells with this extract resulted in higher percentage viability than in the case of ethanolic molmol extract.

However, the results of the second series (concentrations ranging between 266.45 and 666.6 μg/ml) were rather contradictory, possibly due to the different experimental batches of the plant extract.

Hexane haddi extract was superior to ethanolic haddi extract, even at the lower end of the concentration spectrum. On the whole, its action was dose and time-dependent. Mitogenesis was only found at the lowest concentration (6.65 μ/ml), after 72 hours of exposure. Hexane haddi extract, though it performed better that the ethanolic haddi extract, did not perform as effectively against S180 cells as did the ethanolic molmol extract.

Ethyl acetate haddi extract also performed better against S180 cells than the ethanolic haddi extract, although higher concentrations were required to be effective (466μg/ml).

The essential oil (Aldrich) haddi extract performed poorly against S180 cells, compared to the hexane haddi extract.

4.3.2 Effects of Molmol and Haddi Extracts on HT1080 Cells

Molmol and haddi extracts also inhibited the growth of HT1080 cells in cultures in a dose and time-dependent fashion. Ethanolic molmol extract at the lower concentration levels was not toxic to HT1080 cells, and mitogenesis was noticed to occur, at the 0.65-83.33

μg/ml range, evidenced by the increase in the number of cells that was higher than in the control. However, there were fewer viable cells at the highest concentration (666.6 μg/ml) used in the second series, possibly due to different batches of the plant extracts used. The major impact of this extract seemed dependent on using high to very high concentration levels.

Hexane haddi extract seemed to perform better in terms of apparent cytotoxicity at the highest concentration level of 666.6 μg/ml and for all exposure times, whereas its effect was either very negligible, or mitogenic, at concentrations between 0.65 and 333.3 μg/ml. Hexane haddi extract was also not toxic to HT1080 cells, unlike ethanolic molmol extract, producing a contradictory toxic effect on this cell line. Flavex molmol was also not very toxic to HT1080 cells, where very high percentage viabilities were recorded, even at the high concentration range.

The study carried out by Qureshi *et al.* (1993) reported results obtained in Ehrlich ascites carcinoma cell-bearing mice revealed the cytotoxic and antitumour activity of Myrrh was equivalent to that of the standard cytotoxic drug cyclophosphamide, and that on the basis of the nonmutagenic, antioxidative, and cytotoxic potential of *Commiphora*, its use in cancer therapy seemed to be appropriate, and suggested further investigations. The results of the present study also indicate the cytotoxic activity of this plant extract on the S180 and HT1080 cell lines *in vitro*. In addition, Al-Harbi *et al.* (1993) found that treatment of *C. molmol* (250 and 500 mg/kg/day) was cytotoxic in Ehrlich solid tumour, and that the antitumour potential of *C. molmol* was comparable to cyclophosphamide.

More recently, Tipton *et al.* (2003) investigated the *in vitro* cytotoxic and anti-inflammatory effects of myrrh oil on human gingival fibroblasts and epithelial cells, and

reported that fibroblasts resident in the gingival tissue can participate in local inflammation through the production of cytokines, and that their results suggested that myrrh oil may reduce fibroblast production of such proinflammatory cytokines, and therefore reduce the participation of these cells in gingival inflammation associated with gingivitis and periodontitis.

Ethanolic haddi extract also showed dose and time-dependent effects on HT1080 cells. This extract was not toxic to HT1080 cells at the lower concentration range of 0.65 to 333.3 μg/ml, but was toxic at the 666 μg/ml concentration, and for all exposure times tested. A similar effect was noted at the 266.6 and 366.6 μg/ml concentrations, where even mitogenesis occurred after 24 hours of exposure.

Hexane haddi extract was more effective against HT1080 cells than the ethanolic extract. Cell viability was very low at 333.3 and 666.6 μg/ml concentration levels, at all exposure times. Mitogenesis occurred at the lowest concentration levels (0.65 and 10.42 μg/ml), thus indicating that these concentrations were not toxic to HT1080 cells; rather, they were growth promoters. In the second series, a similar trend was noted in which all concentrations (266.6 to 666.6 μg/ml) were toxic, though to varying degrees, depending upon time of exposure. Using high hexane haddi extract concentrations substantiated this pattern, in which percentage viability declined with concentration and exposure time.

Ethyl acetate haddi extract was not toxic to HT1080 at the lower concentration range, where mitogenesis occurred after 72 hours of exposure.

Essential oil haddi (Aldrich) extract was also not very toxic to HT1080 cells, and a high percentage viability persisted throughout the various concentration levels and exposure

times. Only by subjecting HT1080 cells to very high concentrations of this extract was cytotoxicity increased. However, even at this high concentration range, exposure for 72 hours to 600.17 and 900.26 μg/ml was less toxic than exposure for 24 and 48 hours.

Differences in the bioactivity of the hexane haddi extract and its essential oil (Aldrich) are probably due to the selectivity of compounds extracted. The solvent hexane specifically extracts only nonpolar compounds, whereas for the essential oil, steam distillation extracts both nonpolar and semipolar compounds, as they condense with the volatile steam vapours. According to Mr. Ahmed Ali (School of Biosciences, personal communication), although the gc-ms profile for both the hexane and essential oil is broadly the same, there are major unidentified compounds present in the essential oil which do not occur in large amounts in the hexane extract. The presence of these unidentified compounds could be responsible for reducing the cytoxic properties of the essential oil.

4.3.3 Effects of Molmol and Haddi Extracts on Chick Fibroblast Cells

Ethanolic molmol extract was toxic to normal fibroblast cells, especially at the higher range of concentrations, and after exposure for 48 and 72 hours. This indicates that the action of this extract was dose and time-dependent.

Hexane molmol extract seemed to have mitogenic effects at all concentrations (266.45-666.6 μg/ml) after 24 hours of exposure, but cytotoxicity appeared with increasing concentration and exposure time.

Ethyl acetate molmol extract was toxic to normal fibroblast cells only at the higher concentration range (> 366μg/ml) and longer exposure time.

Flavex molmol was not at all toxic at all concentrations and for various exposure times; rather, it was mitogenic as a whole. This is further evidence of the differing quality and nature of this commercial extract.

Ethanolic haddi extract at a concentration range of 266.5 and 666.6 µg/ml was not toxic to normal fibroblast cells after 24 hours of exposure at the three lowest concentrations; rather, its addition resulted in mitogenesis, and its toxicity at the higher concentration range was far lower than at the other exposure times. Hexane haddi extract was not effective against these cells, especially at the lower concentration range, where mitogenesis occurred after 48 and 72 hours of exposure. Ethyl acetate haddi extract was also not effective against normal fibroblast, resulting in mitogenesis in most of the treatments, and very low toxicity at the higher concentration levels. Aldrich haddi also proved ineffective against these cells. Only at very high concentrations was it toxic to the normal fibroblast cells. This differential impact on normal fibroblasts is encouraging in terms of exploring the therapeutic use of myrrh extracts.

4.4 Differential Effects of Molmol and Haddi Extracts

Molmol and haddi extracts seemed to have differential effects on the cell lines used in the present study. The experiments above clearly indicated that ethanolic molmol extract was more effective against S180 cells than against HT1080 cells after 72 hours of exposure, but it was not very effective against normal chick fibroblasts, which showed mitogenesis at the lower range of concentration. Ethanol haddi extract, on the other hand, was not toxic to S180 cells, but was more toxic to HT1080 cells and normal fibroblast cells.

Hexane molmol extract, on the other hand, showed a different pattern of cytotoxicity effect. It was more effective against HT1080 cells than against S180 cells at the lower concentrations, but showed mitogenesis at the higher range of concentration. Nonetheless, it was toxic to S180 and normal fibroblast cells at the higher concentration levels. In contrast, hexane haddi extract concentrations were much more toxic to S180 cells than to HT1080 and normal fibroblast cells.

Ethyl acetate molmol extract was also more toxic to S180 cells than to HT1080 and normal fibroblast cells, at the lowest concentration level of 266.45 μg/ml, but all cell lines exhibited recovery signs, indicating that mitogenesis had occurred even at the higher concentration range. Ethyl acetate haddi extract had mitogenic effects on HT1080 at lower concentrations, but was toxic at higher concentration ranges. In contrast, it was highly toxic to S180 cells at the lower concentrations, but unusually induced mitogenesis at the higher concentration levels, where cell numbers increased consistently.

4.5 Differential Effects of Molmol and Haddi Extrcts on S180, HT1080 and Normal Fibroblast Cells

Ethanolic molmol extract was more active against S180 than hexane molmol extract. However, as for ethyl acetate molmol extract, S180 cells viability increased with the increasing concentration of the treatments.

Ethanolic molmol extract was also more active against HT1080 cells, particularly at higher concentration levels, than hexane molmol and ethyl acetate molmol extracts. In the case of those two extracts, mitogenesis occurred at higher concentrations.

Ethanolic and hexane molmol extract showed similar patterns of increasing cell death in normal fibroblast cells with increasing concentrations, though ethyl acetate molmol extract was not very effective against these cells at all concentrations.

Hexane haddi extract was more toxic to S180 than ethyl acetate haddi extract, the latter induced only low mortality.

Ethyl acetate haddi extract was active against S180 cells, at the slightly higher concentration range (466μg/ml), but not against normal fibroblast cells, even at the highest concentration (666μg/ml).

Hexane haddi extract was also more active against HT1080 cells than ethanolic haddi and ethyl acetate haddi extracts. In all cases, cell death was dose and time-dependent.

Ethanolic haddi extract produced more cell death than the hexane extract in normal fibroblasts at all concentrations. Treatment of the normal fibroblasts with hexane haddi extract resulted in mitogenesis at lower concentrations, but was slightly more effective at the higher concentration levels.

4.6 Morphological Changes in Treated Cells and Interpretations of Apoptosis

Apoptosis can be identified using routine histological staining methods, such as the haematoxylin and eosin (H&E) staining technique. Bowen (1981) reported that histological assessments of cells and tissues is mainly based on very delicate membrane and pH changes which result in changes in membrane permeability and give rise to various staining patterns.

One of the characteristics of classical apoptosis is the presence of darkly stained hyperchromatic nuclei, known as pyknotic nuclei. During apoptosis, the nuclear chromatin generally marginates, clumps and aggregates at the nuclear membrane, a process followed by dilation of the nuclear membrane. In late apoptotic cells, the pyknotic chromatin becomes progressively less visible and is dispersed or lost. Bowen *et al.* (1991) reported nuclear pyknosis in cells undergoing necrosis, although the necrotic cells do not show characteristic chromatin margination.

Sandritter & Riede (1975) stated that the first changes leading to necrosis include degenerative swelling, caused by failure of the sodium pump, and ATP activity, which in turn causes inflow of charged ions such as sodium, calcium and water, and outflow of potassium ions into the extracellular space. This leads to changes in the cell membrane permeability, which at the light microscopy level could be reflected in the cell's reaction to dyes. Other plasma membrane changes during apoptosis are related to membrane bending and induction of blebbing and fragmentation. Furthermore, the apoptotic cells and fragments tend to lose rather than gain water, which might lead to changes in the pH and surface permeability of the cells. Such changes are reflected through reactions to histological stains (Bowen, 1981). Ballard has illustrated that apoptotic cell nuclei are intensively chromophilic and could be easily identified in iron/haematoxylin stained sections. This hyperchromasia of pyknotic nuclei is caused by chromatin condensation and also by exposure to negative charge phosphate groups. Sandritter & Riede (1975), for instance, found that pyknotic nuclei bind more eosin, due to the increase in free basic histone groups.

The haematoxylin-eosin staining results achieved in this work showed that all three cell lines exhibited their normal appearance when grown in cultures without any treatment with the molmol and haddi extracts. Untreated S180 cells, for example, were rounded or polygonal, and formed a confluent monolayer of cells that were attached to the substratum by their pseudopodial attachments. All cells had healthy nuclei and stained uniformly.

Untreated HT1080 cells were epithelial-like, with a polygonal appearance, also forming a confluent layer and attached to the substratum. Nuclei appeared healthy, and some cells showed mitotic activity. Pseudopodia were evident, connecting cells with each other.

Untreated normal fibroblast cells were also epithelial-like, with polygonal morphology. The cells formed a confluent monolayer, remaining attached to the substratum by their pseudopodial attachments. The nuclei appeared rounded or ellipsoidal.

Treatment of these cells with ethanolic molmol or hexane haddi extracts brought about changes in the morphology of some of these cells and their nuclei. Apoptotic changes started after 6 hours of treatment, and advanced as treatment time increased. For instance, treatment of S180 cells with ethanolic molmol extract for 6 hours brought about visible changes that are characteristic of apoptosis, as described above. Apoptotic cells became pyknotic, apoptotic bodies were formed, and the cell membrane broke up. Pyknosis, a regular feature of apoptosis, can be seen as a nucleus that is darkly stained. The earliest detectable change is often chromatin clumping that has been shown to consist of low molecular fragments (Wyllie, 1980; Wyllie & Morris, 1982), and expansion of the outer nuclear envelope. Fragments are produced through the cleavage of nuclear chromatin by calcium- and magnesium-dependent endonuclease which is present in the

nucleus (Cohen & Duke, 1984). Formation of apoptotic bodies is also one of the major morphological characteristics seen during the process of apoptosis. Bowen (1993) indicated that a considerable amount of membrane bending takes place during the formation of spherical bodies, which is accompanied by localised changes in calcium concentration. Pseudopodial attachment disappeared and apoptotic cells assumed various spherical morphologies. Bowen (1993) reported that specialised areas of membrane such as microvilli and junctional complexes are lost during apoptosis. Treatment for 12 hours brought about all these changes, in addition to the appearance of vacuoles in the cytoplasm of the apoptotic cells, especially in the endoplasmic reticulum. Bowen (1993) maintained that whilst the cytoplasm contained within apoptotic fragments appears normal, early vacuolar changes have been observed within apoptotic thymocytes. Robertson *et al.* (1978) reported vacuolation and dilation of the nuclear envelope, endoplasmic reticulum and Golgi in these cells. In this investigation, after 24 hours of treatment, changes became more evident, and the cells changed their morphology, becoming elongated or ellipsoidal. Recently, Zhao *et al.* (2003) also found that the treatment of melanoma culture with boswellic acid (BC-4) caused dramatic changes in cell morphology, with cells becoming elongated, whereas untreated cells remained rounded-up or in clumps.

As for HT1080 cells, the earliest changes during apoptosis were visible within six hours of treatment with ethanolic molmol. While some cells had pyknotic nuclei, others had their nuclear material dispersed, and apoptotic bodies mainly consisted of nuclear fragments. Some other cells appeared to have broken membranes, and the cytoplasm appeared disintegrated, or had become vacuolated. After 12 hours of treatment, these

changes persisted, and apoptotic bodies, consisting mainly of nuclear fragments and cytoplasm fragments, were formed. Some cells showed condensed cytoplasm, some without pyknotic nuclei, whereas others showed cytoplasm disintegration. After 24 hours of treatment, the cytoplasm disintegrated in some cells, accompanied by the formation of vacuoles, and blebs on the outer cytoplasmic membrane. However, free apoptotic bodies were not found.

Ethanolic molmol extract did not seem to have a direct effect on the normal fibroblasts. After 6 hours of treatment, all cells remained intact and unchanged, containing normal nuclei. Even after 12 hours, the majority of cells had normal nuclei, and only a few were apoptotic, with the formation of apoptotic bodies. The majority of cells were not affected, even after 24 hours, a few were apoptotic, and most of the non-apoptotic cells kept their pseudopodial attachments.

It can thus be said that molmol extract targeted cancerous cells and induced apoptosis, whereas at particular concentrations it did not target or induce cell death in the non-cancerous normal fibroblast cells.

With regard to hexane haddi extract, the earliest changes in S180 cells were visible within six hours of treatment. Cytoplasm became condensed, eventually breaking up into several membrane-bound apoptotic bodies, with smooth surfaces, consisting mainly of nuclear fragments. Apoptotic cells had pyknotic nuclei, and the cytoplasm in some cells formed blebs on the outer cell membrane, whereas in others it disintegrated and became vacuolated. After 12 and 24 hours of treatment, these changes became more evident, with some cells being highly vacuolated, whereas others had condensed cytoplasm. This

might suggest the incidence of a non-apoptotic programmed cell death (Amin *et al.*, 2000).

As for HT1080 cells, changes were visible within 6 hours of treatment with hexane haddi. Treated cells showed pyknotic nuclei, and apoptotic bodies consisted mainly of nuclear fragments. Some apoptotic cells showed cytoplasmic disintegration and the formation of vacuoles, some with blebbing. After 12 hours of treatment, these changes persisted and became more evident, with some cells showing disintegrated nuclear material. Almost all HT1080 cells were affected after a 24 hour treatment.

Normal fibroblast cells after 6 hours of treatment with hexane haddi extract were not affected, and only after a further 6 hours of treatment some cells became apoptotic. These changes persisted after 24 hours of treatment.

It can be argued that while hexane haddi extract was effective in inducing apoptosis in S180 cells and HT1080, it also induced apoptosis in normal fibroblasts at low rate, but little more than ethanolic molmol extract did.

S180 cells and HT1080 cells grown in control media showed very low percentages of apoptotic cells. However, treating them with ethanolic extracted molmol and hexane haddi extracts resulted in substantially higher numbers of apoptotic cells. This pattern of the occurrence of apoptosis reflects the possible suitability of both extracts in treating cancer cells. Hexane extracted haddi resulted in higher frequencies of apoptosis among normal fibroblast cells than did ethanolic molmol extract. This substantiates the conclusion that ethanolic molmol extract is more suitable than the hexane haddi extract, since the former selectively targets cancer cells better than the latter, which also results in higher frequencies of apoptosis in the non-cancerous cells.

Results also showed that higher frequencies of apoptosis are obtained with increasing concentrations of these extracts and increasing exposure time. This substantiates the finding that the action of these extracts is dose and time-dependent.

4.7 DNA Fragmentation Assay and Interpretation of Programmed Cell Death

Detection of *in situ* DNA fragmentation is currently one of the most frequently used techniques to detect apoptosis. According to Enari *et al.* (1998), the fragmentation of DNA is induced by caspase-mediated cleavage of an inhibitor (ICAD) and ensuing activation of caspase-activated DNase (CAD), which subsequently leads to the characteristic internucleosomal DNA double strand breaks. Kerr *et al.* (1972), Wyllie *et al.* (1980) and Arends *et al.* (1990) regarded DNA fragmentation into multiples of 180-200 base pairs (BP) as a feature of programmed cell death. However, it appears that large fragments in the range of 50-150 kbp are also generated (Bortner *et al.*, 1995). Oberhammer *et al.* (1993) and Zakeri *et al.* (1993) reported forms of programmed cell death that lack the prototypical internucleosomal pattern of DNA fragmentation. In addition, necrotic cell death is accompanied by late random DNA fragmentation through the release of lysosomal DNase, though this concept has been challenged. Techniques detecting fragmentation may therefore not be specific for apoptosis, but also detect DNA damage in a variety of cell injuries, including single strand breaks. It may therefore be the scale of double DNA strand breaks that is the basis for the relative specificity of *in situ* DNA fragmentation techniques for apoptotic cell death (Ansari *et al.*, 1993).

Only HT1080 cells and normal fibroblast cells were used in this experiment. Untreated HT1080 and normal fibroblast cells grown in the control medium showed normal cells, with intact nuclear material (see Section 4.5), and no DNA fragmentation.

It is clear however that some cells die in a programmed or genetically induced manner, without resulting in DNA fragmentation. This has been seen especially where kinases or caspases have been inhibited (Amin *et al.*, 2000). The form of cell death produced is aberrant since there is no cell shrinkage, rather a vacuolation. These cells however are Annexin V positive and thus do enter programmed cell death.

A. Ethanolic Extracted Molmol (234 µg/ml)

Treatment of HT1080 cells with ethanolic molmol extract for 3 hours was not adequate to produce signs of DNA strands breaking. It was only after 6 and 12 hours of treatment that some cells showed signs of disintegration, detected by blebbing of the outer membrane of the treated cells. DNA fragmentation and cytoplasmic disintegration were further accentuated after 24 hours of treatment with this extract. Complete disintegration of HT1080 cells and further disintegration of the DNA material was noted at the end of the experimental period (48 hours). Thus, apoptosis is a synthetic process and requires some hours to produce an end point.

Normal fibroblast cells treated with the ethanolic molmol extract for up to 12 hours was not sufficient to bring about apoptotic changes. It was only after 24 hours treatment that some signs of fragmentation of DNA material were detected, along with the formation of a few membrane blebs on the outer cell membrane. Treatment for 48 hours did not result in any further disintegration of the DNA material. HT1080 cells treated with hexane haddi extract for 3 hours did not show any sign of DNA strand breaks.

B. Hexane Extracted Haddi (201 μg/ml)

Treatment of the cells with this extract for 3 hours did not result in any apoptotic changes. Treatment for 6 and 12 hours, however, resulted in the production of clear signs of disintegration, such as the formation of blebs. Further signs of apoptotic disintegration were noted after 24 hours, but complete cellular disintegration and more DNA disintegration was observed at the end of the experimental period (48 hours). Normal fibroblast cells treated for up to 48 hours did not show signs of disintegration and DNA fragmentation. Hence, it can be said that this extract could not induce apoptosis in these normal cells, but targeted cancerous cells (HT1080).

4.8 Demonstration of Apoptotic Cells in Culture Using TUNEL Technique

Both HT1080 cells and normal fibroblast cells grown in control culture showed only a slight incidence of apoptotic cells. Incubating HT1080 cells in a medium containing either 666 μg/ml or 234 μg/ml ethanolic molmol extract resulted in developing more apoptotic cells with time, though lower percentages of apoptotic cells were observed using the weaker extract. In contrast, normal fibroblast cells grown in a medium containing 234 μg/ml ethanolic molmol extract, showed only a slight occurrence of apoptosis compared to that using HT1080 cells.

HT1080 cells grown in a medium containing either 666 μg/ml or 201 μg/ml hexane haddi extract showed a considerable increase in the number of apoptotic cells, especially using the stronger haddi solution, and in both cases, there were increases with time of exposure. Normal fibroblast cells grown in a medium containing 201 μg/ml hexane haddi extract

showed only a slight increase in the frequency of apoptotic cells compared to the control and HT1080 cells results.

4.9 Scanning Electron Microscopy

The morphological changes that occur during apoptosis can be conveniently analysed using scanning electron microscopy (SEM). Bowen *et al.* (1981) indicated that fine structural changes in cells observed at electron microscopy level can reflect underlying biochemical changes. Plasma membrane and cytomembrane changes can provide reliable and early signs as to the probability of cell death.

SEM is widely employed to study and time events in apoptotic cell cultures. Cells experiencing apoptosis primarily round off, and lose contact with the neighbouring cells as they condense. Then, the cytoplasm becomes convoluted and blebs outwards, eventually giving rise to apoptotic bodies. Bowen *et al.* (1993) reported a substantial degree of membrane bending taking place during apoptotic body formation, accompanied by localised changes in the calcium concentration. The results of the present study clearly showed these changes in the cell lines used, in comparison with control cultures.

The results showed that cells treated with ethanolic molmol and hexane haddi extracts demonstrated symptoms of classical apoptosis such as shrinkage, blebbing, fragmentation, and loss of microvilli and filopodial attachments, compared to untreated cells which did not show such symptoms.

SEM micrographs showed that cells grown in control cultures had normal growth patterns. For example, the S180 cells were found adhering to the substratum, and interdigitating with each other through filopodial attachments. Delicate microvilli were

also noted on the surfaces of viable cells. Exposure to either ethanolic molmol or hexane haddi extracts resulted in symptoms of apoptosis, including signs of fragmentation (blebbing) and membrane pitting, formation of apoptotic cells and apoptotic bodies, and loss of most microvilli and filopodial attachments.

HT1080 cells exposed to either extract also resulted in symptoms of apoptosis, including the formation of apoptotic bodies, blebbing, and loss of most filopodial attachments

The results also showed normal fibroblast cells were not affected by treatment with both extracts.

4.10 Transmission Electron Microscopy

The effects of ethanolic molmol extract at two concentration levels (234 and 666 μg/ml) and hexane haddi extract, also at two concentration levels (201 and 666 μg/ml), on HT1080 cells were investigated using transmission electron microscopy. Results showed that treating these cells with these two extracts at the two concentration levels induced apoptosis, and in the case of ethanolic molmol extract, the following apoptotic changes: formation of apoptotic cells, blebbing, condensation of chromatin alongside nuclear membrane (chromatin margination), formation of apoptotic bodies, cytoplasm vacuolation, loss of filopodia, swollen mitochondria, formation of cytoplasmic debris, and damaged cell membranes occurred. These results were similar to those produced treating these cells with ethanolic molmol extract.

Treating HT1080 cells with hexane haddi extract resulted in the same apoptotic changes as above, in addition to some other changes. These include extensive damage to cells, necrosis, disintegration of cell membranes, formation of apoptotic cells, blebbing,

formation of apoptotic bodies, chromatin margination, loss of filopodia, cytoplasmic vacuolation, swollen mitochondria, formation of cell debris, and phagocytosis.

4.11 Annexin V Assay to Detect Apoptotic Plasma Membrane Changes

Control, live, untreated S180 and HT1080 cells did not show changes in membrane phosphatidylserine asymmetry. However, when they were grown in media containing either ethanolic molmol or hexane haddi extract, they showed the characteristic symptoms of apoptosis, described earlier in this chapter.

For example, S180 cells grown in culture containing 666 μg/ml ethanolic molmol extract showed signs of induced apoptosis and positive results for changes in membrane phosphatidylserine asymmetry. The majority of these cells appeared green, and only a few appeared red with PI. It is possible that secondary necrosis after the death of apoptotic cells had occurred, leading to changes in permeability of the plasma membrane that allowed the penetration of propidium iodide into the cells. When grown in cultures containing 234 μg/ml (LC_{50}) ethanolic molmol extract, they also showed signs of apoptosis, that is, positive results for changes in membrane phosphatidylserine asymmetry.

HT1080 cells grown in culture containing either 666 μg/ml or 234 μg/ml ethanolic molmol extract also showed positive results for changes in membrane phosphatidylserine asymmetry. However, none of these cells in both treatments showed signs of secondary necrosis.

This may indicate that apoptosis and death of S180 were faster than in the case of HT1080, resulting in secondary necrosis in the former rather than the latter.

S180 cells grown in culture containing either 666 μg/ml or 201 μg/ml hexane haddi extract showed positive results for changes in membrane phosphatidylserine asymmetry. The majority of these cells appeared green, and only some showed possible secondary necrosis.

HT1080 cells grown in culture containing either 666 μg/ml or 201 μg/ml hexane haddi extract also showed positive results for changes in membrane phosphatidylserine asymmetry. Cells appeared green with Annexin V FITC, showing positive labelling on the cell surface, but none showed positive for necrosis at the stronger concentration level. However, a number of cells (around 2-3% of the total number of cells) treated with the weaker concentration level showed PI uptake suggesting secondary necrosis.

4.12 *Trans-β*-Ocimene and γ-Bisabolene as Inducers of Cell Death

Both molmol and haddi extracts consist of a large number of biochemical constituents. Two of these components, *trans-β*-ocimene and γ-bisabolene were found as major peaks in haddi plant extracts (Mr. Ahmed Ali, Personal communication, 2003). Accordingly, it was decided to run a viability test, SEM, and an Annexin V test to investigate their effects on both cancer cell lines, and to test whether or not they induce apoptosis in both.

4.12.1 MTT Viability Assay

Cytotoxicity of *trans-β*-ocimene to S180 cells increased with increasing concentrations, as well as increasing time of exposure. However, toxicity of the 50 mM *trans*-β-ocimene increased after 48 hours, but decreased slightly after 72 hours, though higher than after 24 hours of exposure, possibly indicating mitogenesis.

In contrast to exposure to *trans*-β-ocimene, exposure of S180 cells to γ-bisabolene resulted in higher percentage toxicity at lower concentrations (50 and 100 g/ml γ-bisabolene), though toxicity percentages declined in all concentrations at the end of the 72 hour exposure, especially at the higher concentrations. This pattern of cytotoxicity may indicate that *trans*-β-ocimene performed better than γ-bisabolone in inducing apoptosis in S180 cells.

The apparent cytotoxicity of *trans*-β-ocimene to HT1080 cells also increased with increasing concentration and increasing time of exposure, except at the lowest concentration (50 mM), where it seems that *trans*-β-ocimene was not lethal.

Trans-β-ocimene at 50 mM concentration did not have significant toxic effect on HT1080 cells after 48 hours of exposure; rather, it induced mitogenesis and resulted in a higher percentage viability than at the end of the 24 hour exposure.

The apparent cytotoxicity of γ-bisabolene to HT1080 cells generally increased with decreasing concentration throughout the experimental period, but extending exposure to all concentrations for 72 hours resulted in lower cytotoxicity than after 48 hours, a further indication of mitogenesis during this period. Hence, it can be said that *trans*-β-ocimene performed better than γ-bisabolene in inducing apoptosis and cell death in S180 than in HT1080 cells.

The results of the time course assay showed that exposing S180 cells to a higher concentration of *trans*-β-ocimene or γ-bisabolene resulted in higher cytotoxicity than using lower concentrations. The apparent cytotoxicity of *trans*-β-ocimene to HT1080 cells was also higher at the higher concentration level; in fact, mitogenesis was induced at the lower concentration.

The apparent cytotoxicity of γ-bisabolene to HT1080 cells was generally higher at the higher concentration than at the lower. Thus, it can be said that the apoptotic effect of these two biochemicals is, in general, dose and time-dependent action.

γ-bisabolene is synthesised from farnesyl pyrophosphate. Farnesyl pyrophosphate is an intermediate in the biosynthetic reaction of cholesterol and non-syterol isoprenoids (Hamada *et al.*, 2002). Bisabolene is a sesquiterpene, or an isoprenoid similar to farnesol. Furanosesquiterpenes have been extracted from the resin of *C. sphaerocarpa*, along with many other constituents (Dekebo *et al.*, 2002). Farnesylpyridinium, an analogue of isoprenoid farnesol, was found to initially induce morphological changes similar to those of typical apoptosis in human leukaemia HL-60 cells (Hamada *et al.*, 2002). Farnesol (and also geranylgeraniol) was also found to induce actin-based cytoskeleton disorganisation, growth inhibition, and apoptosis (Miquel *et al.*, 1996). Miquel *et al.* (1996) quoted two groups of researchers (Haug *et al.*, 1994; Ohizumi *et al.*, 1995) who stated that exposure of cells to free isoprenoids such as farnesol and geranylgeraniol resulted in the inhibition of cell proliferation. Ocimene is also an isoprenoid found in fruits, vegetables and herbs. It was reported by Tatman & Mo (2002) that the IC_{50} value of ocimene reflecting its relative tumour-suppressive potency is 250μmol/l, which is far less than the concentrations used in the present work. [The IC_{50} value is the concentration of an isoprenoid required to suppress the net 48 hour increase in the B16 melanoma population by 50% (Tatman and Mo, 2002)].

Other biochemicals extracted from plants have also been used to induce apoptosis in cancer cells lines. For example, aspirin (in the past, extracted from the bark and leaves of black willow - *Salix nigra*-trees), acetylsalicylic acid, is known, has been found to exert

anti-carcinogenic effects in humans. Aspirin has been most intensively used in studies of large bowel cancer, where most studies (for example, Kune *et al.*, 1988; Rosenberg *et al.*, 1991; Thun *et al.*, 1991, 1993; Suh and Petcelli; 1993; Greenberg *et al.*, 1993; Logan *et al.*, 1993; Giovannucci *et al.*, 1994; Muscat *et al.*, 1994; Peleg *et al.*, 1994; Schreĭnemachers and Eversen, 1994), though not all (for example, Paginini-Hill *et al.*, 1989, 1991; Paginini-Hill, 1995), have reported an inverse association to the incidence of cancer. An essential finding relating to aspirin is that the effect seems to require continued aspirin use (Baron, 1995). Infrequent or previous use of aspirin has generally not been associated with a reduced risk (Kune *et al.*, 1988; Rosenberg *et al.*, 1991; Thun *et al.*, 1991, 1993; Greenberg *et al.*, 1993; Logan *et al.*, 1993; Giovannucci *et al.*, 1994; Schreinemahers & Everson, 1994). This pattern, according to Baron (1995), parallels the experimental literature on the subject, where cancer in laboratory animals increases rapidly after exposure to aspirin is stopped (Baron & Greenberg, 1991).

Piqué *et al.* (2000) cited a number of studies showing that aspirin, as well as other non-steroidal anti-inflammatory drugs (NSAIDs) induce apoptosis in several cell types, including human rectal tumour cell lines (Piazza *et al.*, 1995; Shiff *et al.*, 1996; Elder *et al.*, 1996; Castaño *et al.*, 1999), fibroblasts (Lu *et al.*, 1995; Schwenger *et al.*, 1997), B-cell chromic lymphocytic leukaemia cells (Bellosillo *et al.*, 1998), and myeloid leukaemia cell lines (Klampfer *et al.*, 1999). Piqué *et al.* (2000) also reported that administration of NSAIDs induces apoptosis in colon cancer cells *in vivo* (Pasricha *et al.*, 1995; Boolbol *et al.*, 1996). Piqué *et al.* (2000) concluded that their results demonstrate that aspirin-induced apoptosis involved release of cytochrome *c* from mitochondria, preceding caspase activation and loss of mitochondrial transmembrane potential ($\Delta\Psi_m$),

and recommended that the elucidation of the mechanisms involved in the induction of cytochrome *c* release by aspirin warrants further investigation.

Stark *et al.* (2001) indicated that their experimental data demonstrate that aspirin induces degradation of IκBα and, consequently, nuclear translocation of p50/p65 NF-κB complexes in colon cancer cells. They concluded that this nuclear translocation is predominantly responsible for the ability of aspirin to induce apoptosis. They also indicated that the NF-κB and death response to aspirin appear relatively cell-specific, as they were evident in numerous colon cancer cell lines but not in cell lines of non-colonic origin.

4.12.2 Scanning Electron Microscopy

S180 cells and HT1080 grown in control cultures showed normal growth (see Section 4.8).

Exposure of S180 cells to *trans*-β-ocimene induced apoptosis, with the formation of blebbing apoptotic bodies, fragmentation, and loss of microvilli and filopodial attachments. This is similar to the effects of both ethanolic molmol and hexane haddi extracts (see Section 4.8).

Exposure of S180 cells to γ-bisabolene was not as effective as exposure to *trans*-β-ocimene in inducing symptoms of apoptosis. In fact, only about 50% of cells showed signs of apoptosis. This might be attributed to the low bisabolene concentration used in this experiment (5 mM), which seems not to have cytotoxic effect on S180 cells. Bisabolene at 50 mM 'killed' most of the S180 cells. Changes obtained after 5 mM might suggest early stages of necrosis. Accordingly, *trans*-β-ocimene (at LC_{50}

concentration, 100 mM) was more effective in bringing about apoptosis in S180 cells than γ-bisabolene (at LC_{50} concentration, 5 mM), though this effect can be attributed to the big differences in the concentrations of the two biochemicals used.

HT1080 cells exposed to *trans*-β-ocimene (LC_{50} concentration, 100 mM) also showed visible signs of apoptosis, as did HT1080 cells treated for 12 hours with 5mM γ-bisabolene.

4.12.3 Annexin V Assay

Untreated S180 and HT1080cells showed normal growth similar to that shown in Section 4.10.

S180 cells grown in culture containing 100 mM ocimene showed positive results for changes in membrane phosphatidylserine asymmetry. Cells appeared apple green with Annexin V-FITC, indicating positive labelling on the cell surface and within the cytoplasm. However, some cells showed signs of possible secondary necrosis after the death of apoptotic cells and the disintegration of plasma membrane. S180 cells grown in culture containing 5 mM γ-bisabolene also showed positive results for changes in membrane phosphatidylserine asymmetry, but a small number showed signs of possible secondary necrosis. This indicates that ocimene performed better than bisabolene in inducing apoptosis and secondary necrosis.

Results of HT1080 cells exposed to these two biochemicals are similar to those described above, except that more secondary necrosis can be detected for both biochemicals.

4.13 Induction of Programmed Cell Death and its Interpretation

The data show that bisabolene induces a higher level of cell death than ocimene. It is not entirely clear, however, exactly what forms this cell death may take. It is clear from popidium iodide distribution that some necrosis results. Part of this may be due, of course, to secondary necrosis of previously apoptotic cells. Another possibility is that a Type 2 cell death, as defined by Clarke (1990), is taking place. Bisabolene treatment may thus induce some classical apoptosis (Type 1 cell death), and a non-apoptotic programmed cell death (Type 2), sometimes called a vacuolar cell death, as well as classical necrosis in due course.

Such a spectrum would lead us to a possible re-classification of cell death along the following lines (see Figures 4.1 A-C).

A.

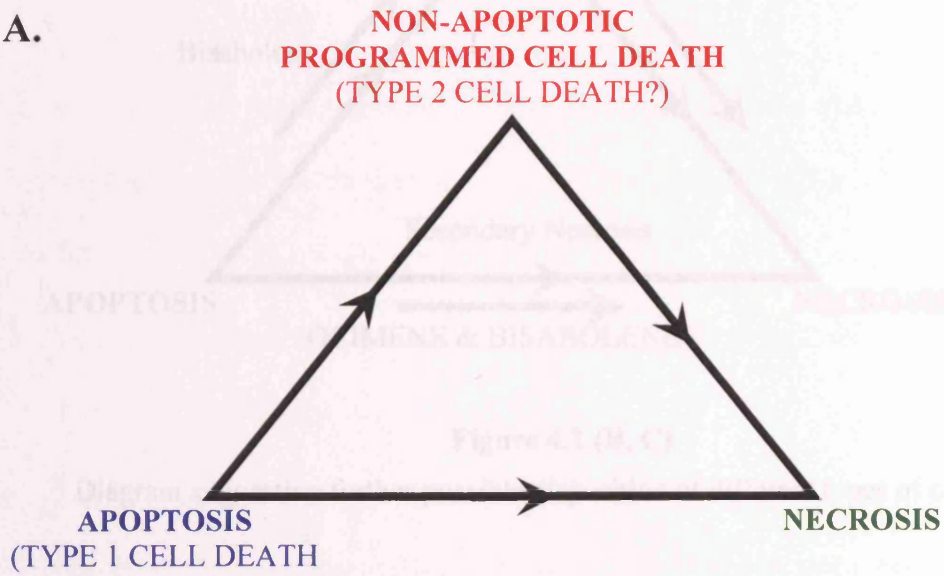
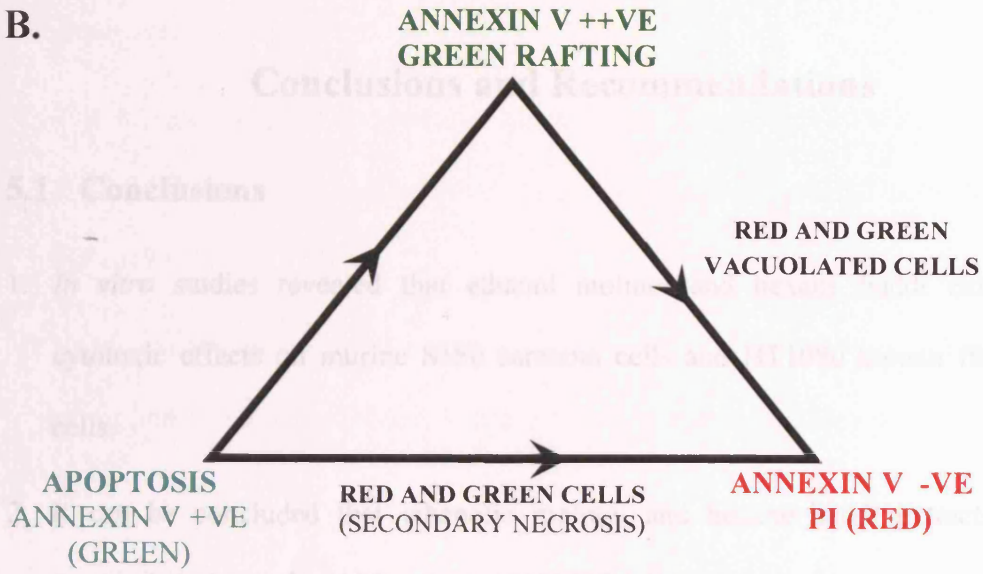


Figure 4.1 (A)

Diagram suggesting possible disposition of different types of cell death

B.



C.

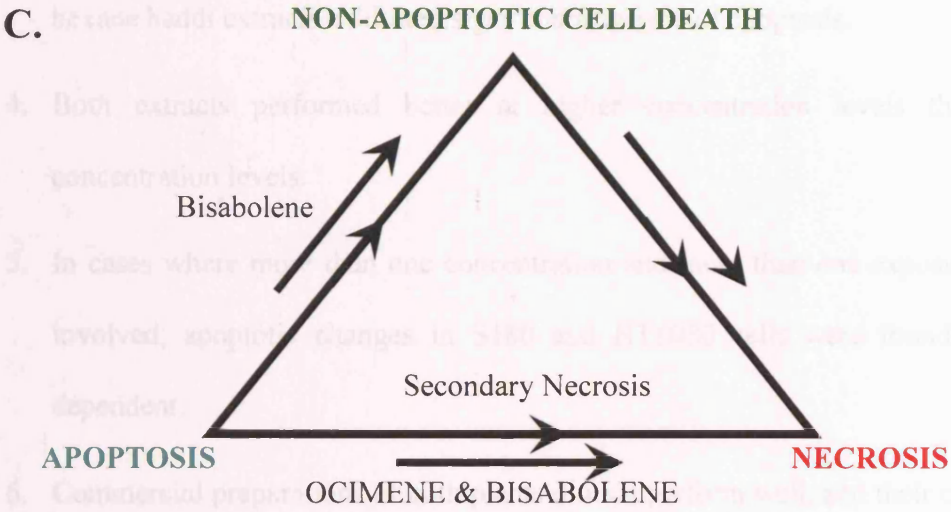


Figure 4.1 (B, C)

Diagram suggesting further possible disposition of different types of cell death

Chapter Five

Conclusions and Recommendations

5.1 Conclusions

1. *In vitro* studies revealed that ethanol molmol and hexane haddi extracts have cytotoxic effects on murine S180 sarcoma cells and HT1080 human fibrosarcoma cells.
2. It can be concluded that ethanolic molmol and hexane haddi extracts are both apoptosis inducers in S180 cells and HT1080 cells.
3. Morphological analysis revealed that cancer cells subjected to ethanolic molmol and hexane haddi extracts exhibited signs characteristic of apoptosis.
4. Both extracts performed better at higher concentration levels than at lower concentration levels.
5. In cases where more than one concentration and more than one exposure time were involved, apoptotic changes in S180 and HT1080 cells were found to be time-dependent.
6. Commercial preparations of both plants did not perform well, and their cytotoxicity to these cells is questionable.
7. Molmol extracts at particular concentrations targeted cancer cells but not normal fibroblast cells, whereas haddi extracts targeted both cancer and normal cells.

8. In the TUNEL experiment, it can be said that the apoptotic changes are a time-dependent function.

9. The Annexin V assay showed that apoptosis and death of S180 cells was faster than that of HT1080 cells, and resulted in secondary necrosis in S180 cells rather than in HT1080 cells.

10. SEM results using *trans*-β-ocimene and γ-bisabolene are similar to those obtained using haddi extracts. Thus, it is likely that both chemicals are major constituents of haddi.

11. *Trans*-β-ocimine (at LC_{50} concentration, 100 mM) was more effective in bringing about apoptosis in S180 cells and HT1080 cells than γ-bisaboline (at LC_{50} concentration, 5 mM).

12. *Trans*-β-ocimene performed better than γ-*b*isabolene in inducing apoptosis in S180 than in HT1080.

5.2 Recommendations

1. The extracts should be tested in parallel against a wider range of human tumour cell lines.

2. More components should be isolated from haddi and molmol extracts and tested separately.

3. Further *in vitro* and *in vivo* investigations of molmol, haddi and their constituent biochemicals should be undertaken to identify the best preparation of constituents that would have maximum cytotoxic effects.

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Appendices

Appendix I

Summaries of S180

Table 1.1 Summary of S180 Ethanolic Molmol after 24 hours (see Figure 3.9)

	Concentration (µg/ml)			
	0.65	**10.45**	**83.33**	**333.30**
10.45	P=0.0013*	-	-	-
83.33	P=0.0006*	P=0.0036*	-	-
333.30	P=0.0004*	P=0.0004*	P=0.0023*	-
666.60	P=0.0007*	P=0.0023*	P=0.0062*	P=0.0341*

*Significant Differences.

Table 1.2 Summary of S180 Ethanolic Molmol after 48 hours (see Figure 3.9)

	Concentration (µg/ml)			
	0.65	**10.45**	**83.33**	**333.30**
10.45	N.S.**	-	-	-
83.33	P=0.0004*	P=0.0009*	-	-
333.30	P=0.0004*	P=0.0004*	P=0.0004*	-
666.60	P=0.0004*	P=0.0004*	P=0.0004*	P=0.0004*

*Significant Differences.
**Non-significant Differences

Table 1.3 Summary of S180 Ethanolic Molmol after 72 hours (see Figure 3.9)

	Concentration (µg/ml)			
	0.65	**10.45**	**83.33**	**333.30**
10.45	N.S.**	-	-	-
83.33	P=0.0004*	P=0.0004*	-	-
333.30	P=0.0004*	P=0.0004*	P=0.0004*	-
666.60	P=0.0004*	P=0.0004*	P=0.0004*	P=0.0004*

*Significant Differences.
** Non-significant Differences

Table 2.1 Summary of S180 Ethanolic Molmol after 24 hours (see Figure 3.10)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	P=0.0004*	-	-	-
466.24	P=0.0004*	P=0.0007*	-	-
566.10	P=0.0004*	P=0.0041*	N.S.**	-
666.60	P=0.0004*	P=0.0023*	N.S.**	N.S**

*Significant Differences.
**Insignificant Differences.

Table 2.2 Summary of S180 Ethanolic Molmol after 48 hours (see Figure 3.10)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	P=0.0081*	-	-	-
466.24	P=0.0027*	N.S.**	-	-
566.10	P=0.0047*	N.S.**	N.S.**	-
666.60	P=0.0041*	N.S.**	N.S.**	N.S.**

*Significant Differences.
**Insignificant Differences.

Table 2.3 Summary of S180 Ethanolic Molmol after 72 hours (see Figure 3.10)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	P=0.0081*	-	-	-
466.24	P=0.0134*	N.S.**	-	-
566.10	N.S.**	N.S.**	N.S.**	-
666.60	N.S.**	N.S.**	N.S.**	N.S.**

*Significant Differences.
**Insignificant Differences.

Table 3.1 Summary of S180 Hexane Molmol after 24 hours (see Figure 3.11)

	Concentration (µg/ml)			
	0.65	**10.45**	**83.33**	**333.30**
10.45	N.S.**	-	-	-
83.33	N.S.**	N.S.**	-	-
333.30	P=0.0047*	P=0.0047*	P=0.0217*	-
666.60	P=0.0027*	P=0.0004*	P=0.0004*	P=0.0036*

*Significant Differences.
**Insignificant Differences

Table 3.2 Summary of S180 Hexane Molmol after 48 hours (see Figure 3.11)

	Concentration (µg/ml)			
	0.65	**10.45**	**83.33**	**333.30**
10.45	N.S.**	-	-	-
83.33	P=0.0004*	P=0.0004*	-	-
333.30	P=0.0004*	P=0.0004*	P=0.0004*	-
666.60	P=0.0004*	P=0.0004*	P=0.0004*	P=0.0006*

*Significant Differences.
**Insignificant Differences

Table 3.3 Summary of S180 Hexane Molmol after 72 hours (see Figure 3.11)

	Concentration (µg/ml)			
	0.65	**10.45**	**83.33**	**333.30**
10.45	P=0.0004*	-	-	-
83.33	P=0.0004*	P=0.0004*	-	-
333.30	P=0.0004*	P=0.0004*	P=0.0134*	-
666.60	P=0.0004*	P=0.0004*	P=0.0047*	P=0.0008*

*Significant Differences.
**Insignificant Differences

Table 4.1 Summary of S180 Hexane Molmol after 24 hours (see Figure 3.12)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	N.S.**	-	-	-
466.24	N.S.**	N.S.**	-	-
566.10	P=0.0341*	P=0.0134*	N.S.**	-
666.60	N.S..**	N.S.**	N.S.**	N.S.**

*Significant Differences.
**Insignificant Differences.

Table 4.2 Summary of S180 Hexane Molmol after 48 hours (see Figure 3.12)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	N.S.**	-	-	-
466.24	N.S.**	N.S.**	-	-
566.10	N.S.**	N.S.**	N.S.**	-
666.60	N.S.**	N.S.**	N.S.**	N.S.**

**Insignificant Differences.

Table 4.3 Summary of S180 Hexane Molmol after 72 hours (see Figure 3.12)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	N.S.**	-	-	-
466.24	N.S.**	N.S.**	-	-
566.10	P=0.0273*	P=0.0423*	N.S.**	-
666.60	P=0.0036*	P=0.0062*	P=0.0134*	N.S.**

*Significant Differences.
**Insignificant Differences.

Table 5.1 Summary of S180 Ethyl Acetate Molmol after 24 hours (see Figure 3.13)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	P=0.0004*	-	-	-
466.24	P=0.0004*	P=0.0004*	-	-
566.10	P=0.0004*	P=0.0062*	N.S.**	-
666.60	P=0.0004*	P=0.0015*	N.S.**	N.S.**

*Significant Differences.
**Insignificant Differences.

Table 5.2 Summary of S180 Ethyl Acetate Molmol after 48 hours (see Figure 3.13)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	P=0.0023*	-	-	-
466.24	P=0.0047*	N.S.**	-	-
566.10	P=0.0134*	N.S.**	N.S.**	-
666.60	P=0.0062*	N.S.**	N.S.**	N.S.**

*Significant Differences.
**Insignificant Differences.

Table 5.3 Summary of S180 Ethyl Acetate Molmol after 72 hours (see Figure 3.13)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	P=0.0004*	-	-	-
466.24	P=0.0011*	N.S.**	-	-
566.10	P=0.0134*	N.S.**	N.S.**	-
666.60	P=0.0134*	N.S.**	N.S.**	N.S.**

*Significant Differences.
**Insignificant Differences.

Table 6.1 Summary of S180 Flavex Molmol after 24 hours (see Figure 3.14)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	N.S.**	-	-	-
466.24	N.S.**	N.S.**	-	-
566.10	N.S.**	N.S.**	N.S.**	-
666.60	N.S.**	N.S.**	N.S.**	N.S.**

**Insignificant Differences.

Table 6.2 Summary of S180 Flavex Molmol after 48 hours (see Figure 3.14)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	N.S.**	-	-	-
466.24	N.S.**	N.S.**	-	-
566.10	P=0.0423*	P=0.0171*	N.S.**	-
666.60	N.S.**	P=0.0273	N.S.**	N.S.**

*Significant Differences.
**Insignificant Differences.

Table 6.3 Summary of S180 Flavex Molmol after 72 hours (see Figure 3.14)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	N.S.**	-	-	-
466.24	P=0.0015*	P=0.0273*	-	-
566.10	P=0.0004*	P=0.0027*	N.S.**	-
666.60	P=0.0008*	P=0.0062*	N.S.**	N.S.**

*Significant Differences.
**Insignificant Differences.

Table 7.1 Summary of S180 Ethanolic Haddi after 24 hours (see Figure 3.15)

	Concentration (µg/ml)			
	0.65	**10.45**	**83.33**	**333.30**
10.45	N.S.**	-	-	-
83.33	P=0.0217*	N.S.**	-	-
333.30	P=0.0134*	N.S.**	N.S.**	-
666.60	P=0.0027*	N.S.**	N.S.**	N.S.**

*Significant Differences.
**Insignificant Differences.

Table 7.2 Summary of S180 Ethanolic Haddi after 48 hours (see Figure 3.15)

	Concentration (µg/ml)			
	0.65	**10.45**	**83.33**	**333.30**
10.45	P=0.0006*	-	-	-
83.33	N.S.**	N.S.**	-	-
333.30	P=0.0011*	N.S.**	P=0.0134*	-
666.60	P=0.0004*	P=0.0004*	P=0.0004*	P=0.0027*

*Significant Differences.
**Insignificant Differences.

Table 7.3 Summary of S180 Ethanolic Haddi after 72 hours (see Figure 3.15)

	Concentration (µg/ml)			
	0.65	**10.45**	**83.33**	**333.30**
10.45	P=0.0081*	-	-	-
83.33	P=0.0047*	N.S.**	-	-
333.30	N.S.**	P=0.0217*	P=0.0134*	-
666.60	P=0.0004*	P=0.0004*	P=0.0004*	N.S.**

*Significant Differences.
**Insignificant Differences.

Table 8.1 Summary of S180 Ethanolic Haddi after 24 hours (see Figure 3.16)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	N.S.**	-	-	-
466.24	N.S.**	N.S.**	-	-
566.10	P=0.0171*	N.S.**	N.S.**	-
666.60	P=0.0011*	P=0.0104*	P=0.0015*	P=0.0134*

*Significant Differences.
**Insignificant Differences.

Table 8.2 Summary of S180 Ethanolic Haddi after 48 hours (see Figure 3.16)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	P=0.0423*	-	-	-
466.24	P=0.0273*	N.S.**	-	-
566.10	P=0.0004*	P=0.0217*	N.S.**	-
666.60	P=0.0004*	P=0.0027*	N.S.**	N.S.**

*Significant Differences.
**Insignificant Differences.

Table 8.3 Summary of S180 Ethanolic Haddi after 72 hours (see Figure 3.16)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	P=0.0027*	-	-	-
466.24	P=0.0004*	P=0.0004*	-	-
566.10	P=0.0004*	P=0.0004*	P=0.0104*	-
666.60	P=0.0004*	P=0.0004*	P=0.0134*	P=0.0008*

*Significant Differences.
**Insignificant Differences.

Table 9.1 Summary of S180 Hexane Haddi after 24 hours (see Figure 3.17)

	Concentration (µg/ml)			
	0.65	**10.45**	**83.33**	**333.30**
10.45	P=0.0217*	-	-	-
83.33	P=0.0004*	P=0.0006*	-	-
333.30	P=0.0006*	P=0.0008*	P=0.0062*	-
666.60	P=0.0006*	P=0.0008*	P=0.0062*	N.S.**

*Significant Differences.
**Insignificant Differences.

Table 9.2 Summary of S180 Hexane Haddi after 48 hours (see Figure 3.17)

	Concentration (µg/ml)			
	0.65	**10.45**	**83.33**	**333.30**
10.45	P=0.0104*	-	-	-
83.33	P=0.0004*	P=0.0004*	-	-
333.30	P=0.0004*	P=0.0004*	P=0.0004*	-
666.60	P=0.0004*	P=0.0004*	P=0.0004*	N.S.**

*Significant Differences.
**Insignificant Differences.

Table 9.3 Summary of S180 Hexane Haddi after 72 hours (see Figure 3.17)

	Concentration (µg/ml)			
	0.65	**10.45**	**83.33**	**333.30**
10.45	N.S.**	-	-	-
83.33	P=0.0047*	P=0.0047*	-	-
333.30	P=0.0004*	P=0.0004*	P=0.0004*	-
666.60	P=0.0004*	P=0.0004*	P=0.0004*	N.S.**

*Significant Differences.
**Insignificant Differences.

Table 10.1 Summary of S180 Hexane Haddi after 24 hours (see Figure 3.18)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	P=0.0004*	-	-	-
466.24	P=0.0006*	N.S.**	-	-
566.10	P=0.0004*	P=0.0104*	N.S.**	-
666.60	P=0.0004*	N.S.**	N.S.**	N.S.**

*Significant Differences.
**Insignificant Differences.

Table 10.2 Summary of S180 Hexane Haddi after 48 hours (see Figure 3.18)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	N.S.**	-	-	-
466.24	P=0.0104*	N.S.**	-	-
566.10	P=0.0011*	P=0.0171*	N.S.**	-
666.60	P=0.0006*	P=0.0062*	N.S.**	N.S.**

*Significant Differences.
**Insignificant Differences.

Table 10.3 Summary of S180 Hexane Haddi after 72 hours (see Figure 3.18)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	N.S.**	-	-	-
466.24	P=0.0217*	N.S.**	-	-
566.10	P=0.0015*	N.S.**	N.S.**	-
666.60	P=0.0004*	N.S.**	N.S.**	N.S.**

*Significant Differences.
**Insignificant Differences.

Table 11.1 Summary of S180 Ethyl Acetate Haddi after 24 hours (see Figure 3.19)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	N.S.**	-	-	-
466.24	N.S.**	N.S.**	-	-
566.10	N.S.**	N.S.**	N.S.**	-
666.60	N.S.**	N.S.**	N.S.**	N.S.**

**Insignificant Differences.

Table 11.2 Summary of S180 Ethyl Acetate Haddi after 48 hours (see Figure 3.19)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	N.S.**	-	-	-
466.24	P=0.0341*	N.S.**	-	-
566.10	P=0.0217*	P=0.0273*	N.S.**	-
666.60	P=0.0027*	P=0.0036*	N.S.**	N.S.**

*Significant Differences.
**Insignificant Differences.

Table 11.3 Summary of S180 Ethyl Acetate Haddi after 72 hours (see Figure 3.19)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	N.S.**	-	-	-
466.24	P=0.0004*	P=0.0011*	-	-
566.10	P=0.0011*	P=0.0081*	N.S.**	-
666.60	P=0.0008*	P=0.0036*	N.S.**	N.S.**

*Significant Differences.
**Insignificant Differences.

Table 12.1 Summary of S180 Essential Oil Aldrich Haddi after 24 hours (see Figure 3.20)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	P=0.0217*	-	-	-
466.24	P=0.0004*	P=0.0006*	-	-
566.10	P=0.0006*	P=0.0008*	P=0.0062*	-
666.60	P=0.0006*	P=0.0008*	P=0.0062*	N.S.**

*Significant Differences.
**Insignificant Differences.

Table 12.2 Summary of S180 Essential Oil Aldrich Haddi after 48 hours (see Figure 3.20)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	P=0.0104*	-	-	-
466.24	P=0.0004*	P=0.0004*	-	-
566.10	P=0.0004*	P=0.0004*	P=0.0004*	-
666.60	P=0.0004*	P=0.0004*	P=0.0004*	N.S.**

*Significant Differences.
**Insignificant Differences.

Table 12.3 Summary of S180 Essential Oil Aldrich Haddi after 72 hours (see Figure 3.20

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	N.S.**	-	-	-
466.24	P=0.0047*	P=0.0047*	-	-
566.10	P=0.0004*	P=0.0004*	P=0.0004*	-
666.60	P=0.0004*	P=0.0004*	P=0.0004*	P=0.0004*

*Significant Differences.
**Insignificant Differences.

Appendix II

Summaries of HT 1080

Table 1.1 Summary of HT1080 Ethanolic Molmol after 24 hours (see Figure 3.21)

	Concentration (µg/ml)			
	0.65	**10.45**	**83.33**	**333.30**
10.45	N.S.**	-	-	-
83.33	P=0.0193*	P=0.0104*	-	-
333.30	P=0.0004*	P=0.0004*	P=0.0004*	-
666.60	P=0.0004*	P=0.0004*	P=0.0004*	P=0.0004*

*Significant Differences.
**Insignificant Differences

Table 1.2 Summary of HT1080 Ethanolic Molmol after 48 hours (see Figure 3.21)

	Concentration (µg/ml)			
	0.65	**10.45**	**83.33**	**333.30**
10.45	N.S.**	-	-	-
83.33	N.S.**	N.S.**	-	-
333.30	P=0.0004*	P=0.0004*	P=0.0004*	-
666.60	P=0.0004*	P=0.0004*	P=0.0004*	P=0.0004*

*Significant Differences.
**Insignificant Differences

Table 1.3 Summary of HT1080 Ethanolic Molmol after 72 hours (see Figure 3.21)

	Concentration (µg/ml)			
	0.65	**10.45**	**83.33**	**333.30**
10.45	N.S.**	-	-	-
83.33	N.S.**	N.S.**	-	-
333.30	P=0.0004*	P=0.0004*	P=0.0004*	-
666.60	P=0.0004*	P=0.0004*	P=0.0004*	N.S.**

*Significant Differences.
**Insignificant Differences

Table 2.1 Summary of HT1080 Ethanolic Molmol after 24 hours (see Figure 3.22)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	N.S.**	-	-	-
466.24	P=0.0011*	N.S.**	-	-
566.10	P=0.0004*	P=0.0171*	N.S.**	-
666.60	P=0.0004*	P=0.0015*	N.S.**	N.S.**

*Significant Differences.
**Insignificant Differences.

Table 2.2 Summary of HT1080 Ethanolic Molmol after 48 hours (see Figure 3.22)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	N.S.**	-	-	-
466.24	N.S.**	N.S.**	-	-
566.10	N.S.**	N.S.**	N.S.**	-
666.60	N.S.**	N.S.**	N.S.**	N.S.**

**Insignificant Differences.

Table 2.3 Summary of HT1080 Ethanolic Molmol after 72 hours (see Figure 3.22)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	N.S.**	-	-	-
466.24	P=0.0171*	N.S.**	-	-
566.10	N.S.**	N.S.**	N.S.**	-
666.60	N.S.**	N.S.**	N.S.**	N.S.**

*Significant Differences.
**Insignificant Differences

Table 3.1 Summary of HT1080 Ethanolic Molmol after 24 hours (see Figure 3.23)

	Concentration (µg/ml)			
	366.68	**391.12**	**416.72**	**441.34**
391.12	P=0.0020*	-	-	-
416.72	P=0.0004*	P=0.0004*	-	-
441.34	P=0.0004*	P=0.0004*	P=0.0004*	-
466.62	P=0.0004*	P=0.0004*	P=0.0004*	P=0.0004*

*Significant Differences.

Table 3.2 Summary of HT1080 Ethanolic Molmol after 48 hours (see Figure 3.23)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	P=0.0027*	-	-	-
466.24	P=0.0006*	N.S.**	-	-
566.10	P=0.0104*	N.S.**	N.S.**	-
666.60	P=0.0341*	N.S.**	N.S.**	N.S.**

**Insignificant Differences.

Table 3.3 Summary of HT1080 Ethanolic Molmol after 72 hours (see Figure 3.23)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	P=0.0004*	-	-	-
466.24	P=0.0015*	P=0.0134*	-	-
566.10	P=0.0015*	P=0.0081*	N.S.**	-
666.60	P=0.0031*	P=0.0036*	N.S.**	N.S.**

*Significant Differences.
**Insignificant Differences

Table 4.1 Summary of HT1080 Ethanolic Molmol after 12 hours (see Figure 3.24)

	Concentration (µg/ml)			
	65	**130**	**260**	**520**
130	N.S.**	-	-	-
260	N.S.**	N.S.**	-	-
520	N.S.**	N.S.**	N.S.**	-
1040	N.S.**	N.S.**	N.S.**	N.S.**

*Significant Differences.

Table 4.2 Summary of HT1080 Ethanolic Molmol after 24 hours (see Figure 3.24)

	Concentration (µg/ml)			
	65	**130**	**260**	**520**
130	N.S.**	-	-	-
260	N.S.**	N.S.**	-	-
520	N.S.**	N.S.**	N.S.**	-
1040	N.S.**	N.S.**	N.S.**	N.S.**

**Insignificant Differences.

Table 4.3 Summary of HT1080 Ethanolic Molmol after 48 hours (see Figure 3.24)

	Concentration (µg/ml)			
	65	**130**	**260**	**520**
130	N.S.**	-	-	-
260	N.S.**	N.S.**	-	-
520	N.S.**	N.S.**	N.S.**	-
1040	N.S.**	N.S.**	N.S.**	N.S.**

**Insignificant Differences

Table 4.4 Summary of HT1080 Ethanolic Molmol after 72 hours (see Figure 3.24)

	Concentration (µg/ml)			
	65	**130**	**260**	**520**
130	N.S.**	-	-	-
260	N.S.**	N.S.**	-	-
520	N.S.**	N.S.**	N.S.**	-
1040	N.S.**	N.S.**	N.S.**	N.S.**

**Insignificant Differences

Table 5.1 Summary of HT1080 Hexane Molmol after 24 hours (see Figure 3.25)

	Concentration (µg/ml)			
	0.65	**10.45**	**83.33**	**333.30**
10.45	P=0.0217*	-	-	-
83.33	P=0.0004*	P=0.0020*	-	-
333.30	P=0.0004*	P=0.0011*	N.S.**	-
666.60	P=0.0004*	P=0.0004*	P=0.0004*	P=0.0004*

*Significant Differences.
**Insignificant Differences

Table 5.2 Summary of HT1080 Hexane Molmol after 48 hours (see Figure 3.25)

	Concentration (µg/ml)			
	0.65	**10.45**	**83.33**	**333.30**
10.45	N.S.**	-	-	-
83.33	P=0.0171*	P=0.0423*	-	-
333.30	P=0.0217*	P=0.0423*	N.S.**	-
666.60	P=0.0004*	P=0.0004*	P=0.0004*	P=0.0004*

**Insignificant Differences.

Table 5.3 Summary of HT1080 Hexane Molmol after 72 hours (see Figure 3.25)

	Concentration (µg/ml)			
	0.65	**10.45**	**83.33**	**333.30**
10.45	N.S.**	-	-	-
83.33	P=0.0273*	P=0.0423*	-	-
333.30	P=0.0081*	P=0.0071*	N.S.**	-
666.60	P=0.0004*	P=0.0004*	P=0.0004*	P=0.0004*

*Significant Differences.
**Insignificant Differences

Table 6.1 Summary of HT1080 Hexane Molmol after 24 hours (see Figure 3.26)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	N.S.**	-	-	-
466.24	N.S.**	N.S.**	-	-
566.10	P=0.0273*	N.S.**	N.S.**	-
666.60	N.S.**	N.S.**	P=0.0273*	P=0.0047*

*Significant Differences.
**Insignificant Differences.

Table 6.2 Summary of HT1080 Hexane Molmol after 48 hours (see Figure 3.26)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	N.S.**	-	-	-
466.24	N.S.**	N.S.**	-	-
566.10	N.S.**	N.S.**	N.S.**	-
666.60	P=0.0423*	P=0.0341*	P=0.0171*	P=0.0217*

*Significant Differences.
**Insignificant Differences.

Table 6.3 Summary of HT1080 Hexane Molmol after 72 hours (see Figure 3.26)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	N.S.**	-	-	-
466.24	N.S.**	N.S.**	-	-
566.10	N.S.**	N.S.**	N.S.**	-
666.60	P=0.0273*	P=0.0004*	N.S.**	N.S.**

*Significant Differences.
**Insignificant Differences.

Table 7.1 Summary of HT1080 Ethyl Acetate Molmol after 24 hour (see Figure 3.27)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	N.S.**	-	-	-
466.24	P=0.0006*	P=0.0171*	-	-
566.10	P=0.0004*	P=0.0062*	N.S.**	-
666.60	P=0.0004*	P=0.0006*	N.S.**	N.S.**

*Significant Differences.
**Insignificant Differences.

Table 7.2 Summary of HT1080 Ethyl Acetate Molmol after 48 hour (see Figure 3.27)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	N.S.**	-	-	-
466.24	N.S.**	N.S.**	-	-
566.10	N.S.**	N.S.**	N.S.**	-
666.60	N.S.**	N.S.**	N.S.**	N.S.**

**Insignificant Differences.

Table 7.3 Summary of HT1080 Ethyl Acetate Molmol after 72 hour (see Figure 3.27)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	P=0.0273*	-	-	-
466.24	N.S.**	N.S.**	-	-
566.10	N.S.**	P=0.0134*	N.S.**	-
666.60	N.S.**	P=0.0273*	N.S.**	N.S.**

*Significant Differences.
**Insignificant Differences

Table 8.1 Summary of HT1080 Flavex Molmol after 24 hours (see Figure 3.28)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	N.S.**	-	-	-
466.24	N.S.**	N.S.**	-	-
566.10	N.S.**	N.S.**	N.S.**	-
666.60	N.S.**	N.S.**	N.S.**	N.S.**

**Insignificant Differences.

Table 8.2 Summary of HT1080 Flavex Molmol after 48 hours (see Figure 3.28)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	N.S.**	-	-	-
466.24	N.S.**	N.S.**	-	-
566.10	N.S.**	N.S.**	N.S.**	-
666.60	N.S.**	N.S.**	N.S.**	N.S.**

**Insignificant Differences.

Table 8.3 Summary of HT1080 Flavex Molmol after 72 hours (see Figure 3.28)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	N.S.**	-	-	-
466.24	N.S.**	N.S.**	-	-
566.10	P=0.0273*	P=0.0217*	N.S.**	-
666.60	P=0.0171*	P=0.0171*	N.S.**	N.S.**

*Significant Differences.
**Insignificant Differences

Table 9.1 Summary of HT1080 Ethanolic Haddi after 24 hours (see Figure 3.29)

	Concentration (µg/ml)			
	0.65	**10.45**	**83.33**	**333.30**
10.45	N.S.**	-	-	-
83.33	P=0.0171*	N.S.**	-	-
333.30	N.S.**	N.S.**	N.S.**	-
666.60	P=0.0004*	P=0.0004*	P=0.0004*	P=0.0004*

*Significant Differences.
**Insignificant Differences.

Table 9.2 Summary of HT1080 Ethanolic Haddi after 48 hours (see Figure 3.29)

	Concentration (µg/ml)			
	0.65	**10.45**	**83.33**	**333.30**
10.45	N.S.**	-	-	-
83.33	P=0.0027*	P=0.0008*	-	-
333.30	P=0.0423*	N.S.**	P=0.0004*	-
666.60	P=0.0004*	P=0.0004*	P=0.0004*	P=0.0004*

*Significant Differences.
**Insignificant Differences.

Table 9.3 Summary of HT1080 Ethanolic Haddi after 72 hours (see Figure 3.29)

	Concentration (µg/ml)			
	0.65	**10.45**	**83.33**	**333.30**
10.45	N.S.**	-	-	-
83.33	N.S.**	N.S.**	-	-
333.30	P=0.0047*	P=0.0062*	P=0.0081*	-
666.60	P=0.0004*	P=0.0004*	P=0.0004*	P=0.0004*

*Significant Differences.
**Insignificant Differences.

Table 10.1 Summary of HT1080 Ethanolic Haddi after 24 hours (see Figure 3.30)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	N.S.**	-	-	-
466.24	N.S.**	N.S.**	-	-
566.10	N.S.**	N.S.**	N.S.**	-
666.60	N.S.**	N.S.**	N.S.**	N.S.**

**Insignificant Differences.

Table 10.2 Summary of HT1080 Ethanolic Haddi after 48 hours (see Figure 3.30)

	Concentration (µg/ml)			
366.00	N.S.**	-	-	-
466.24	N.S.**	N.S.**	-	-
566.10	N.S.**	N.S.**	N.S.**	-
666.60	N.S.**	N.S.**	N.S.**	N.S.**

**Insignificant Differences.

Table 10.3 Summary of HT1080 Ethanolic Haddi after 72 hours (see Figure 3.30)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	N.S.**	-	-	-
466.24	N.S.**	N.S.**	-	-
566.10	N.S.**	N.S.**	N.S.**	-
666.60	P=0.0273*	N.S.**	N.S.**	N.S.**

*Significant Differences.
**Insignificant Differences.

Table 11.1 Summary of HT1080 Hexane Haddi after 24 hours (see Figure 3.31)

	Concentration (µg/ml)			
	0.65	**10.45**	**83.33**	**333.30**
10.45	N.S.**	-	-	-
83.33	P=0.0004*	P=0.0004*	-	-
333.30	P=0.0004*	P=0.0004*	P=0.0004*	-
666.60	P=0.0004*	P=0.0004*	P=0.0004*	P=0.0193*

*Significant Differences.
**Insignificant Differences.

Table 11.2 Summary of HT1080 Hexane Haddi after 48 hours (see Figure 3.31)

	Concentration (µg/ml)			
	0.65	**10.45**	**83.33**	**333.30**
10.45	P=0.0104*	-	-	-
83.33	P=0.0004*	P=0.0004*	-	-
333.30	P=0.0004*	P=0.0004*	P=0.0004*	-
666.60	P=0.0004*	P=0.0004*	P=0.0004*	N.S.**

*Significant Differences.
**Insignificant Differences.

Table 11.3 Summary of HT1080 Hexane Haddi after 72 hours (see Figure 3.31)

	Concentration (µg/ml)			
	0.65	**10.45**	**83.33**	**333.30**
10.45	N.S.**	-	-	-
83.33	P=0.0047*	P=0.0047*	-	-
333.30	P=0.0004*	P=0.0004*	P=0.0004*	-
666.60	P=0.0004*	P=0.0006*	P=0.0006*	N.S.**

*Significant Differences.
**Insignificant Differences.

Table 12.1 Summary of HT1080 Hexane Haddi after 24 hours (see Figure 3.32)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	N.S.**	-	-	-
466.24	P=0.0217*	N.S.**	-	-
566.10	P=0.0341*	N.S.**	N.S.**	-
666.60	N.S.**	N.S.**	N.S.**	N.S.**

*Significant Differences.
**Insignificant Differences.

Table 12.2 Summary of HT1080 Hexane Haddi after 48 hours (see Figure 3.32)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	N.S.**	-	-	-
466.24	N.S.**	N.S.**	-	-
566.10	P=0.0217*	N.S.**	N.S.**	-
666.60	P=0.0171*	N.S.**	N.S.**	N.S.**

*Significant Differences.
**Insignificant Differences.

Table 12.3 Summary of HT1080 Hexane Haddi after 72 hours (see Figure 3.32)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	N.S.**	-	-	-
466.24	N.S.**	N.S.**	-	-
566.10	P=0.0104*	P=0.0062*	N.S.**	-
666.60	P=0.0081*	P=0.0217*	N.S.**	N.S.**

*Significant Differences.
**Insignificant Differences.

Table 13.1 Summary of HT1080 Hexane Haddi after 24 hours (see Figure 3.33)

	Concentration (µg/ml)			
	533.71	**733.24**	**932.4**	**1133.22**
733.24	P=0.0004*	-	-	-
932.4	P=0.0004*	P=0.0004*	-	-
1133.22	P=0.0004*	P=0.0004*	P=0.0004*	-
1333.2	P=0.0004*	P=0.0004*	P=0.0004*	P=0.0004*

*Significant Differences.

Table 13.2 Summary of HT1080 Hexane Haddi after 48 hours (see Figure 3.33)

	Concentration (µg/ml)			
	533.71	**733.24**	**932.4**	**1133.22**
733.24	P=0.0004*	-	-	-
932.4	P=0.0004*	P=0.0004*	-	-
1133.22	P=0.0004*	P=0.0004*	N.S.**	-
1333.2	P=0.0004*	P=0.0004*	N.S.**	N.S.**

*Significant Differences.
**Insignificant Differences.

Table 13.3 Summary of HT1080 Hexane Haddi after 72 hours (see Figure 3.33)

	Concentration (µg/ml)			
	533.71	**733.24**	**932.4**	**1133.22**
733.24	N.S.**	-	-	-
932.4	N.S.**	N.S.**	-	-
1133.22	N.S.**	N.S.**	N.S.**	-
1333.2	N.S.**	N.S.**	N.S.**	N.S.**

**Insignificant Differences.

Table 14.1 Summary of HT1080 Ethyl Acetate Haddi after 24 hours (see Figure 3.34)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	N.S.**	-	-	-
466.24	N.S.**	N.S.**	-	-
566.10	N.S.**	N.S.**	N.S.**	-
666.60	N.S.**	N.S.**	N.S.**	N.S.**

**Insignificant Differences.

Table 14.2 Summary of HT1080 Ethyl Acetate Haddi after 48 hours (see Figure 3.34)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	N.S.**	-	-	-
466.24	N.S.**	N.S.**	-	-
566.10	N.S.**	N.S.**	N.S.**	-
666.60	N.S.**	N.S.**	N.S.**	N.S.**

**Insignificant Differences.

Table 14.3 Summary of HT1080 Ethyl Acetate Haddi after 72 hours (see Figure 3.34)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	N.S.**	-	-	-
466.24	N.S.**	N.S.**	-	-
566.10	P=0.0171*	N.S.**	N.S.**	-
666.60	P=0.0004*	P=0.0006*	N.S.**	N.S.**

*Significant Differences.
**Insignificant Differences.

Table 15.1 Summary of HT1080 Essential Oil Aldrich Haddi after 24 hours (see Figure 3.35)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	N.S.**	-	-	-
466.24	P=0.0171*	N.S.**	-	-
566.10	P=0.0423*	P=0.0423*	N.S.**	-
666.60	P=0.0171*	P=0.0171*	N.S.**	N.S.**

*Significant Differences.
**Insignificant Differences.

Table 15.2 Summary of HT1080 Essential Oil Aldrich Haddi after 48 hours (see Figure 3.35)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	N.S.**	-	-	-
466.24	N.S.**	N.S.**	-	-
566.10	N.S.**	N.S.**	N.S.**	-
666.60	N.S.**	N.S.**	N.S.**	N.S.**

**Insignificant Differences

Table 15.3 Summary of HT1080 Essential Oil Aldrich Haddi after 72 hours (see Figure 3.35

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	N.S.**	-	-	-
466.24	N.S.**	N.S.**	-	-
566.10	P=0.0423*	N.S.**	N.S.**	-
666.60	N.S.**	N.S.**	N.S.**	N.S.**

*Significant Differences.
**Insignificant Differences

Table 16.1 Summary of HT1080 Essential Oil Aldrich Haddi after 24 hours (see Figure 3.36)

	Concentration (µg/ml)			
	600.17	**900.26**	**1199.23**	**1500.97**
900.26	P=0.0341*	-	-	-
1199.23	P=0.0004*	P=0.0341*	-	-
1500.97	P=0.0004*	P=0.0027*	N.S.**	-
1799.82	P=0.0004*	N.S.**	N.S.**	N.S.**

*Significant Differences.
**Insignificant Differences.

Table 16.2 Summary of HT1080 Essential Oil Aldrich Haddi after 48 hours (see Figure 3.36)

	Concentration (µg/ml)			
	600.17	**900.26**	**1199.23**	**1500.97**
900.26	N.S.**	-	-	-
1199.23	P=0.0036*	P=0.0011*	-	-
1500.97	P=0.0036*	P=0.0036*	N.S.**	-
1799.82	P=0.0008*	P=0.0004*	N.S.**	N.S.**

*Significant Differences.
**Insignificant Differences.

Table 16.3 Summary of HT1080 Essential Oil Aldrich Haddi after 72 hours (see Figure 3.36

	Concentration (µg/ml)			
	600.17	**900.26**	**1199.23**	**1500.97**
900.26	P=0.0081	-	-	-
1199.23	P=0.0004	P=0.0004	-	-
1500.97	P=0.0004	P=0.0004	N.S.**	-
1799.82	P=0.0004	P=0.0004	N.S.**	N.S.**

*Significant Differences.
**Insignificant Differences.

Appendix III

Summaries of Normal Fibroblast Cells

Table 1.1 Summary of Normal Fibroblast Ethanolic Molmol after 24 hours (see Figure 3.37)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	N.S.**	-	-	-
466.24	N.S.**	N.S.**	-	-
566.10	N.S.**	N.S.**	N.S.**	-
666.60	P=0.0004*	N.S.**	P=0.0004*	P=0.0015*

*Significant Differences.
**Insignificant Differences.

Table 1.2 Summary of Normal Fibroblast Ethanolic Molmol after 48 hours (see Figure 3.37)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	N.S.**	-	-	-
466.24	P=0.0217*	N.S.**	-	-
566.10	P=0.0273*	P=0.0273*	P=0.0423*	-
666.60	P=0.0004*	P=0.0004*	P=0.0004*	P=0.0011*

*Significant Differences.
**Insignificant Differences.

Table 1.3 Summary of Normal Fibroblast Ethanolic Molmol after 72 hours (see Figure 3.37)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	N.S.**	-	-	-
466.24	P=0.0423*	N.S.**	-	-
566.10	N.S.**	N.S.**	N.S.**	-
666.60	P=0.0004*	P=0.0004*	P=0.0004*	P=0.0273*

*Significant Differences.
**Insignificant Differences.

Table 2.1 Summary of Normal Fibroblast Ethanolic Molmol after 24 hours (see Figure 3.38)

	Concentration (µg/ml)			
	366.68	**391.12**	**416.72**	**441.34**
391.12	P=0.0011*	-	-	-
416.72	P=0.0004*	P=0.0004*	-	-
441.34	P=0.0004*	P=0.0004*	P=0.0004*	-
466.62	P=0.0004*	P=0.0004*	P=0.0004*	P=0.0004*

*Significant Differences.

Table 2.2 Summary of Normal Fibroblast Ethanolic Molmol after 48 hours (see Figure 3.38)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	P=0.0004*	-	-	-
466.24	P=0.0004*	P=0.0004*	-	-
566.10	P=0.0004*	P=0.0004*	P=0.0011*	-
666.60	P=0.0004*	P=0.0004*	P=0.0004*	P=0.0006*

*Significant Differences.

Table 2.3 Summary of Normal Fibroblast Ethanolic Molmol after 72 hours (see Figure 3.38)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	N.S.**	-	-	-
466.24	N.S.**	N.S.**	-	-
566.10	N.S.**	N.S.**	N.S.**	-
666.60	P=0.0273*	P=0.0273*	N.S.**	N.S.**

*Significant Differences.
**Insignificant Differences

Table 3.1 Summary of Normal Fibroblast Hexane Molmol after 24 hours (see Figure 3.39)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	N.S.**	-	-	-
466.24	N.S.**	N.S.**	-	-
566.10	N.S.**	N.S.**	N.S.**	-
666.60	N.S.**	N.S.**	N.S.**	N.S.**

**Insignificant Differences.

Table 3.2 Summary of Normal Fibroblast Hexane Molmol after 48 hours (see Figure 3.39)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	N.S.**	-	-	-
466.24	N.S.**	N.S.**	-	-
566.10	N.S.**	N.S.**	N.S.**	-
666.60	P=0.0004*	P=0.0004*	P=0.0062*	P=0.0431*

*Significant Differences.
**Insignificant Differences.

Table 3.3 Summary of Normal Fibroblast Hexane Molmol after 72 hours (see Figure 3.39)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	N.S.**	-	-	-
466.24	N.S.**	N.S.**	-	-
566.10	P=0.0273*	N.S.**	N.S.**	-
666.60	P=0.0004*	P=0.0004*	P=0.0004*	N.S.**

*Significant Differences.
**Insignificant Differences.

Table 4.1 Summary of Normal Fibroblast Ethyl Acetate Molmol after 24 hours (see Figure 3.40)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	P=0.0423*	-	-	-
466.24	P=0.0062*	P=0.0423*	-	-
566.10	N.S.**	N.S.**	N.S.**	-
666.60	P=0.0004*	P=0.0004*	P=0.0004*	P=0.0081*

*Significant Differences.
**Insignificant Differences.

Table 4.2 Summary of Normal Fibroblast Ethyl Acetate Molmol after 48 hours (see Figure 3.40)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	P=0.0020*	-	-	-
466.24	P=0.0104*	N.S.**	-	-
566.10	P=0.0004*	P=0.0273*	N.S.**	-
666.60	P=0.0004*	P=0.0027*	N.S.**	N.S.**

*Significant Differences.
**Insignificant Differences.

Table 4.3 Summary of Normal Fibroblast Ethyl Acetate Molmol after 72 hours (see Figure 3.40)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	P=0.0020*	-	-	-
466.24	P=0.0062*	N.S.**	-	-
566.10	P=0.0004*	P=0.0104*	N.S.**	-
666.60	P=0.0004*	P=0.0004*	N.S.**	N.S.**

*Significant Differences.
**Insignificant Differences.

Table 5.1 Summary of Normal Fibroblast Flavex Molmol after 24 hours (see Figure 3.41)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	N.S.**	-	-	-
466.24	N.S.**	N.S.**	-	-
566.10	N.S.**	N.S.**	N.S.**	-
666.60	N.S.**	N.S.**	N.S.**	N.S.**

**Insignificant Differences.

Table 5.2 Summary of Normal Fibroblast Flavex Molmol after 48 hours (see Figure 3.41)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	N.S.**	-	-	-
466.24	N.S.**	N.S.**	-	-
566.10	N.S.**	N.S.**	N.S.**	-
666.60	N.S.**	N.S.**	N.S.**	N.S.**

**Insignificant Differences.

Table 5.3 Summary of Normal Fibroblast Flavex Molmol after 72 hours (see Figure 3.41)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	N.S.**	-	-	-
466.24	N.S.**	N.S.**	-	-
566.10	N.S.**	N.S.**	N.S.**	-
666.60	N.S.**	N.S.**	N.S.**	N.S.**

**Insignificant Differences.

Table 6.1 Summary of Normal Fibroblast Ethanolic Haddi after 24 hours (see Figure 3.42)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	N.S.**	-	-	
466.24	N.S.**	P=0.0273*	-	
566.10	N.S.**	N.S.**	N.S.**	
666.60	P=0.0008*	P=0.0004*	P=0.0047*	N.S.**

*Significant Differences.
**Insignificant Differences.

Table 6.2 Summary of Normal Fibroblast Ethanolic Haddi after 48 hours (see Figure 3.42)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	P=0.0273*	-	-	-
466.24	N.S.**	N.S.**	-	-
566.10	N.S.**	N.S.**	N.S.**	-
666.60	P=0.0004*	P=0.0004*	P=0.0062*	N.S.**

*Significant Differences.
**Insignificant Differences.

Table 6.3 Summary of Normal Fibroblast Ethanolic Haddi after 72 hours (see Figure 3.42)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	N.S.**	-	-	-
466.24	N.S.**	N.S.**	-	-
566.10	P=0.0020*	P=0.0134*	N.S.**	-
666.60	P=0.0004*	P=0.0004*	P=0.0047*	P=0.0047*

*Significant Differences.
**Insignificant Differences.

Table 7.1 Summary of Normal Fibroblast Hexane Haddi after 24 hours (see Figure 3.43)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	N.S.**	-	-	-
466.24	N.S.**	N.S.**	-	-
566.10	N.S.**	N.S.**	N.S.**	-
666.60	N.S.**	N.S.**	N.S.**	N.S.**

**Insignificant Differences.

Table 7.2 Summary of Normal Fibroblast Hexane Haddi after 48 hours (see Figure 3.43)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	N.S.**	-	-	-
466.24	N.S.**	N.S.**	-	-
566.10	N.S.**	N.S.**	N.S.**	-
666.60	P=0.0036*	P=0.0341*	N.S.**	N.S.**

*Significant Differences.
**Insignificant Differences.

Table 7.3 Summary of Normal Fibroblast Hexane Haddi after 72 hours (see Figure 3.43)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	N.S.**		-	-
466.24	N.S.**	N.S.**	-	-
566.10	N.S.**	N.S.**	N.S.**	-
666.60	P=0.0020*	P=0.0062*	N.S.**	N.S.**

*Significant Differences.
**Insignificant Differences.

Table 8.1 Summary of Normal Fibroblast Ethyl Acetate Haddi after 24 hours (see Figure 3.44)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	N.S.**	-	-	-
466.24	N.S.**	N.S.**	-	-
566.10	N.S.**	N.S.**	N.S.**	-
666.60	N.S.**	N.S.**	N.S.**	N.S.**

**Insignificant Differences.

Table 8.2 Summary of Normal Fibroblast Ethyl Acetate Haddi after 48 hours (see Figure 3.44)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	N.S.**			
466.24	N.S.**	N.S.**		
566.10	N.S.**	N.S.**	N.S.**	
666.60	N.S.**	N.S.**	P=0.0134*	N.S.**

*Significant Differences.
**Insignificant Differences.

Table 8.3 Summary of Normal Fibroblast Ethyl Acetate Haddi after 72 hours (see Figure 3.44)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	N.S.**	-	-	-
466.24	N.S.**	N.S.**	-	-
566.10	N.S.**	N.S.**	N.S.**	-
666.60	P=0.0273*	P=0.0081*	N.S.**	N.S.**

*Significant Differences.
**Insignificant Differences.

Table 9.1 Summary of Normal Fibroblast Essential Oil Aldrich Haddi after 24 hours (see Figure 3.45)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	N.S.**	-	-	-
466.24	N.S.**	N.S.**	-	-
566.10	N.S.**	N.S.**	N.S.**	-
666.60	N.S.**	N.S.**	N.S.**	N.S.**

**Insignificant Differences.

Table 9.2 Summary of Normal Fibroblast Essential Oil Aldrich Haddi after 48 hours (see Figure 3.45)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	N.S.**	-	-	-
466.24	N.S.**	N.S.**	-	-
566.10	N.S.**	N.S.**	N.S.**	-
666.60	N.S.**	N.S.**	N.S.**	N.S.**

**Insignificant Differences.

Table 9.3 Summary of Normal Fibroblast Essential Oil Aldrich Haddi after 72 hours (see Figure 3.45)

	Concentration (µg/ml)			
	266.50	**366.00**	**466.24**	**566.10**
366.00	N.S.**	-	-	-
466.24	N.S.**	N.S.**	-	-
566.10	N.S.**	N.S.**	N.S.**	-
666.60	N.S.**	N.S.**	N.S.**	N.S.**

**Insignificant Differences.

Table 10.1 Summary of Normal Fibroblast Essential Oil Aldrich Haddi after 24 hours (see Figure 3.46)

	Concentration (µg/ml)			
	533.71	**733.24**	**932.4**	**1133.22**
733.24	N.S.**	-	-	-
932.4	N.S.**	N.S.**	-	-
1133.22	P=0.0036*	P=0.0062*	N.S.**	-
1333.2	P=0.0015*	P=0.0015*	P=0.0171*	N.S.**

*Significant Differences.
**Insignificant Differences.

Table 10.2 Summary of Normal Fibroblast Essential Oil Aldrich Haddi after 48 hours (see Figure 3.46)

	Concentration (µg/ml)			
	533.71	**733.24**	**932.4**	**1133.22**
733.24	N.S.**	-	-	-
932.4	N.S.**	P=0.0423*	-	-
1133.22	P=0.0008*	P=0.0004*	P=0.0047*	-
1333.2	P=0.0008*	P=0.0004*	P=0.0062*	N.S.**

*Significant Differences.
**Insignificant Differences.

Table 10.3 Summary of Normal Fibroblast Essential Oil Aldrich Haddi after 72 hours (see Figure 3.46)

	Concentration (µg/ml)			
	533.71	**733.24**	**932.4**	**1133.22**
733.24	P=0.0004*	-	-	
932.4	P=0.0004*	P=0.0004*	-	
1133.22	P=0.0004*	P=0.0004*	P=0.0062*	
1333.2	P=0.0004*	P=0.0004*	P=0.0004*	P=0.0171*

*Significant Differences.

Appendix IV

Differential effect of extracts on cell lines viability

Table 1.1 Comparison of differential effect of Ethanolic Molmol Extract on viability of different cell lines after 72 hours (see Figure 3.47)

	Cell Lines	
Cell Lines	**S180**	**HT1080**
HT1080	N.S.**	-
Normal Fibroblast	N.S.**	N.S.**

**Insignificant Differences.

Table 1.2 Comparison of differential effect of Hexane Molmol Extract on viability of different cell lines after 72 hours (see Figure 3.48)

	Cell Lines	
Cell Lines	**S180**	**HT1080**
HT1080	N.S.**	-
Normal Fibroblast	N.S.**	N.S.**

**Insignificant Differences.

Table 1.3 Comparison of differential effect of Ethyl Acetate Molmol Extract on viability of different cell lines after 72 hours (see Figure 3.49)

	Cell Lines	
Cell Lines	**S180**	**HT1080**
HT1080	P=0.0122*	-
Normal Fibroblast	P=0.0216*	N.S.**

*Significant Differences.
**Insignificant Differences.

Table 2.1 Comparison of differential effect of Ethanolic Haddi Extract on viability of different cell lines after 72 hours (see Figure 3.50)

	Cell Lines	
Cell Lines	**S180**	**HT1080**
HT1080	N.S.**	-
Normal Fibroblast	N.S.**	N.S.**

**Insignificant Differences.

Table 2.2 Comparison of differential effect of Hexane Haddi Extract on viability of different cell lines after 72 hours (see Figure 3.51)

	Cell Lines	
Cell Lines	**S180**	**HT1080**
HT1080	N.S.**	-
Normal Fibroblast	P=0.0367*	N.S.**

*Significant Differences.
**Insignificant Differences.

Table 2.3 Comparison of differential effect of Ethyl Acetate Haddi Extract on viability of different cell lines after 72 hours (see Figure 3.52)

	Cell Lines	
Cell Lines	**S180**	**HT1080**
HT1080	N.S.**	-
Normal Fibroblast	P=0.0122*	N.S.**

*Significant Differences.
**Insignificant Differences.

Table 3.1 Comparison of differential effect of different Molmol Extracts on viability of S180 cell line after 72 hours (see Figure 3.53)

Molmol Extract	Ethanolic	Hexane	Ethyl Acetate
Hexane	N.S.**	-	-
Ethyl Acetate	N.S.**	N.S.**	-
Flavex	P=0.0374*	N.S.**	P=0.0374*

*Significant Differences.
**Insignificant Differences.

Table 3.2 Comparison of differential effect of different Molmol Extracts on viability of HT1080 cell line after 72 hours (see Figure 3.54)

Molmol Extract	Ethanolic	Hexane	Ethyl Acetate
Hexane	N.S.**	-	-
Ethyl Acetate	N.S.**	N.S.**	-
Flavex	P=0.0250*	P=0.0250*	P=0.0250*

*Significant Differences.
**Insignificant Differences.

Table 3.3 Comparison of differential effect of different Molmol Extracts on viability of normal fibroblast cell line after 72 hours (see Figure 3.55)

Molmol Extract	Ethanolic	Hexane	Ethyl Acetate
Hexane	N.S.**	-	-
Ethyl Acetate	N.S.**	N.S.**	-
Flavex	N.S.**	P=0.0104*	N.S.**

*Significant Differences.
**Insignificant Differences.

Table 4.1 Comparison of differential effect of different Haddi Extracts on viability of S180 cell line after 72 hours (see Figure 3.56)

Molmol Extract	Ethanolic	Hexane	Ethyl Acetate
Hexane	N.S.**	-	-
Ethyl Acetate	N.S.**	N.S.**	-
Flavex	N.S.**	N.S.**	N.S**

**Insignificant Differences.

Table 4.2 Comparison of differential effect of different Haddi Extracts on viability of HT1080 cell line after 72 hours (see Figure 3.57)

Molmol Extract	Ethanolic	Hexane	Ethyl Acetate
Hexane	N.S.**	-	-
Ethyl Acetate	N.S.**	N.S.**	-
Flavex	N.S.**	N.S.**	N.S.**

**Insignificant Differences.

Table 4.3 Comparison of differential effect of different Haddi Extracts on viability of normal fibroblast cell line after 72 hours (see Figure 3.58)

Molmol Extract	Ethanolic	Hexane	Ethyl Acetate
Hexane	N.S.**	-	-
Ethyl Acetate	P=0.0163*	N.S.**	-
Flavex	P=0.0065*	N.S.**	N.S.**

*Significant Differences.
**Insignificant Differences.