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Suppression of some cell lines using ethanolic root extract of *Alpinia galanga* L. Willed roots *in vitro*

A Thesis

Submitted to the College of Science Al-Nahrain University as a Partial Fulfillment of the Requirements for the Degree of Master of Science in Biotechnology

By

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Supervisors Certification

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Summary

The roots of greater galanga are used as aphrodisiac in traditional medicine. However, their use has not been reported experimentally on scientific bases. Therefore, this study was designed to investigate the effect of ethanolic extract of *A. galanga* (L.) Willed. roots on some cell lines and to diagnose this effect.

The search for novel anticancer drugs is continuing. Agents that can eliminate the cancerous cells but don't affect the normal cells may have a therapeutic advantage for the elimination of cancer cells. This work includes a preliminary study of the effect of *A. galanga* ethanolic crude extracts on four malignant human cell lines and one normal cell line.

Ethanolic extract was prepared from dried roots of *A. galanga* to determine its anti tumor activity on four studied cell lines. Tested cell lines included human epidermoid larynx carcinoma (HEp-2), human rhabdomyosarcoma (RD), human glioblastoma multiform ($AMGM_5$), murine mammary adenocarcinoma (AMN_3) and normal rat embryo fibroblasts (REF).

The ethanolic crude extract of greater galangal roots showed a time and dose - dependent effect on viability of HEp-2 cells. The highest three concentrations, (60, 125 and 250) μg/ml, showed both time and dose - dependent effects, while the highest concentration (250 μg/ml) gave the highest decrease in AMN_3 cells viability (0.021) with inhibition reached (98.8%) after 72 hours. The ethanolic crude extract of greater galangal roots reduced the viability of RD cells gradually with time. The highest concentration (250 μg/ml) gave the highest decrease in $AMGM_5$ cells viability (0.028) with inhibition reached (98.2%) after 72 hours. The ethanolic crude extract of greater galangal roots reduced the viability of REF cells very slightly after 72 hours.

It is concluded that the AMN_3 cell line was the most sensitive to the inhibitory effects of ethanolic crude extract of greater galangal roots while the $AMGM_5$ cell line was the less sensitive to the inhibitory effects of the ethanolic crude extracts of greater galangal roots.

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List of Abbreviations

Abbreviation	Full name
ACA	1,S-1-Acetoxychavicol Acetate
AIDS	Acquired Immune Deficiency Syndrome
$AMGM_5$	Ahmed-Majeed-Glioblastoma Multiform-2005
AMN_3	Ahmed-Mohammed-Nahi-2003
CD_4	Cluster of Differentiation 4
CD_8	Cluster of Differentiation 8
CDK	Cycline Dependent Kinases
COX-2	Cyclooxygenase-2
CRC	Colorectal Cancer cell line
DSBs	DNA Double-Strand Breaks
DTP	Developmental Therapeutics Program
ELISA	Enzyme-Linked ImmunoSorbent Assay
FAA	Flavone Acetic Acid
FBS	Fetal Bovine Serum
GBM	Glioblastoma Multiform
GC	Gas Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
GST	Glutathione-s-Transferases Enzyme
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
Hep-2	Human Epidermoid Larynx carcinoma
ICCMGR	Iraqi Center for Cancer and Medical Genetics Research
IL1	Interleukin -1
IL12	Interleukin -12
IL6	Interleukin – 6
MDR	Mediating Multidrug Resistance

Abbreviation	Full name
MEM	Minimum Essential Medium
MPF	Maturation Promoting Factor
NCI	National Cancer Institute
NHEKs	Normal Human Epidermal Keratinocytes
PBS	Phosphate Buffer Saline
RD	Rhabdomyosarcoma
REF	Rat Embryo Fibroblast
RNA	Ribonucleic acid
RPMI-1640	Rosswell Park Memorial Institute medium
SFM	Serum Free Medium
TNFα	Tumor Necrosis Factor Alpha
U.V.	Ultraviolet
WHO	The World Health Organization

Chapter One

Introduction and Literature Review

1.Introduction and Literature Review

1.1-Introduction

Cancer is a group of diseases characterized by uncontrolled growth and spread of abnormal cells. It is caused by both external and internal factors. These casual factors may act together or in sequence to initiate or promote carcinogenesis (American Cancer Society, 2005).

Conventional cancer therapy is based on surgery, radiotherapy, chemotherapy or a combination of them. Generally, surgery and radiotherapy are preferred in localized tumors and chemotherapy when cancer cells are spread through the body (Hellman, 1997; Rosenberg, 1997; Lopez-Lazaro, 2002). The introduction of cancer chemotherapy in the 5^{th} and 6^{th} decades of the last century has resulted in the development of curative therapeutic interventions for patients with several types of solid tumors and hematopoietic neoplasms. However important obstacles were encountered in the use of the chemotherapy that included toxicity to the normal tissues of the body and the presence of mutation that confer resistance to these chemotherapeutic agents (De Vita, 1997; Lehne, 2001).

Plants were used as a source of medical treatments since the beginning of human existence on the earth, and had been a source of medicine in general before the eighteenth century. The preparation of medicines was developed and appeared as extracts, concentrated formulations and dyes until the late nineteenth century and early twentieth century, which witnessed the beginning of the scientific revolution in the modern pharmaceutical industry. This improved human health and restored the reliance on traditional medicine in most areas

except those hard-to-reaches, or in areas where people abandoned their customs and ancestral social beliefs especially poor areas (Delgado and Remers, 1991).

Therefore, cancer patients as well as many physicians are beginning to request natural products for treatment due to their multiple effects in treating the disease, relieving patent's symptoms' in addition to improved safety and lower cost (Katz, 2002). Natural products have long been a fertile source of cancer cure, as cancer is projected to become the major cause of death in this century (Mukherjee *et al*, 2001).

Most of the therapies involved the use of plant extracts or their active components (Craig, 1999). At least 250,000 species of plants do exist, out of which more than one thousand plants have been found to possess significant anticancer properties (Mukherjee *et al*., 2001).

Currently, one key component of a comprehensive preclinical was screening and drug development program at the National Cancer Institute (NCI) is the cell line screen (Takimoto, 2003). Crude plant extracts from *Withania somnifera* (Iraq) (Al-Atby, 2001), *Cyperus rotundus* L. (Iraq) (Al Hilli, 2004), *Dioscorea membranacea* (Thailand) , *Platycodon grandiflorum* A. DC. (Korea), and *Entanda abyssinica* (Tanzania) (Lee *et al;* 2004) had shown significant cytotoxic effects against malignant cell lines *in vitro*.

The plant *A. galanga*, which belongs to ginger family and locally called greater galangal; is from the basic spices that are widely used in the East (South-East Asian countries) as food flavoring product and for treating various chronic diseases as part of the folk Asian medicine (Purseglove, 1981; Charles *et al*., 1992).

Accordingly, the present work was designed to:

1. Study whether the ethanolic crude extract of *A. galanga* has any cytotoxic effect on malignant cell lines or not.
2. Determine the safety of this extract by exposing normal cell lines to the crude extract of *A. galanga*.

1.2-Literature Review

1.2.1-Cancer

1.2.1.1-Definitions

Neoplasia is the process of tumor growth. The terms tumor and neoplasm are formally synonymous in pathology. The notable British pathologist, Rupert Willis, defined a tumor as "an abnormal mass of tissue, the growth of which exceeds and is uncoordinated with that of the normal tissues, and persists in the same aggressive manner after cessation of the stimuli which evoked the change" (Wyllie, 1992).

Tumors are either benign or malignant (Junqueira *et al*., 1998). A tumor is said to be benign when its microscopic and gross characteristics are considered relatively innocent, implying that it will remain localized, it cannot spread to other sites, and it is generally amenable to local surgical removal; the patient generally survives. Malignant, as applied to a neoplasm, implies that the lesion can invade and destroy adjacent structures and spread to distant sites (metastasize) to cause death. Malignant tumors are collectively referred to as cancers (Kumar *et al.,* 2003).

Most cancers fall into one of three main groups: carcinomas, sarcomas, and leukemias or lymphomas. Carcinomas, which include approximately 90% of human cancers, are malignancies of epithelial cells.

Sarcomas, which are rare in humans, are solid tumors of connective tissues, such as muscle, bone, cartilage, and fibrous tissue. Leukemias and Lymphomas, which account for approximately 8% of malignancies, arise from

the blood-forming cells and from cells of the immune system, respectively (Cooper, 1997).

1.2.1.2-Characteristics of Benign and Malignant Tumors

A- Benign Tumors

Benign neoplasms are composed of well-differentiated cells that closely resemble their normal counterparts. Most benign tumors grow slowly. A benign neoplasm remains localized at its site of origin. It does not have the capacity to infiltrate, invade, or metastasize to distant sites (Kumar *et al*., 2003), e.g. benign prostatic hyperplasia (Guess, 1994) and rhabdomyoma (Enzinger and Weiss, 1983).

B- Malignant Tumors

Hanahan and Weinberg (2000) suggested that the vast catalog of cancer cell genotypes was a manifestation of six essential alterations in cell physiology that collectively dictated malignant growth: self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. This multiplicity of defenses might explain why cancer is relatively rare during an average human lifetime.

1.2.1.3-Cancer Incidence

In the United States of America, about 1,372,910 cancer cases were diagnosed in 2005. In addition, about 570,280 Americans were expected to die of cancer, more than 1,500 people a day.

Cancer is the second leading cause of death in the US, exceeded only by heart disease. In the US, cancer causes 1 of every 4 deaths (American Cancer Society, 2005). It ranked first in the causes of deaths in Japan since 1981.

1.2.1.4-Risk Factors

Some of the main causative factors of cancer include:

A-Certain chemical factors

Such as tobacco, ethanolic beverages, benzene, ethylene oxide, arsenic, cadmium and nickel compounds (Yuspa and Shields, 1997).

- **Tobacco and ethanolic beverages**

Vineis *et al*. (2005) conducted a large prospective study that lasted over seven years of follow up a total of 500,000 people. This extensive study confirmed that environmental tobacco smoke was a risk factor for lung cancer and other respiratory diseases, particularly in ex-smokers.

Carcinogen-DNA addicts formed by exposure to tobacco smoke had a key role in the initiation of various types of cancer including cancers of the lung, oral and nasal cavities, esophagus, larynx, pharynx, pancreas, liver, kidney, stomach, urinary tract and cervix (Phillips, 2002).

Results obtained by Barba *et al*. (2004) suggested that alcohol intake distribution across lifetime might play an important role in prostate cancer etiology. Terry *et al*. (2003) suggested relationships of smoking and alcohol with *p53* early in the adenoma to carcinoma sequence.

Epidemiological evidence indicated that avoidance of smoking, increased consumption of fruits and vegetables, and control of infections would have a

major effect on reducing rates of cancer. Other factors include avoidance of intense sun exposure, increases in physical activity, and reduction of alcohol consumption and possibly red meat (Ames *et al*., 1995).

B- Certain physical factors

Ionizing radiations and ultraviolet rays and mineral fibers (Hall, 1997).

- **Ionizing radiation**

DNA double-strand breaks (DSBs) are induced by ionizing radiations and as a result, they generate chromosomal aberrations (Lips and Kaina, 2001).

The tumorigenic effect of gamma rays was proportional to the dose used for induction of liver, pituitary, ovarian and lung tumors; whereas the tumorigenic effect for bone tumors was proportional to the square of the dose. A significant increase in incidence was also found for gastrointestinal tumors, kidney tumors, adrenal tumors and hemangiomas of spleen (Sasaki and Fukuda, 1999).

X-rays induce chromosome-type aberrations when cells are treated in the G1 phase of the cell cycle, whereas they induce chromatid-type aberrations in the G2 phase of the cycle. However, if cells are treated in the S phase, induction of both types of aberrations is possible (Nath and Krishna, 1997).

Because DNA damage occurs spontaneously and as a result to ubiquitous environmental agents, most organisms possess some capacity to repair their DNA and DNA is the only macromolecule which is repaired by cells. We can divide "repair" mechanisms into 3 categories:

- Damage reversal--simplest; enzymatic action restores normal structure without breaking backbone.

- Damage removal--involves cutting out and replacing a damaged or inappropriate base or section of nucleotides.
- Damage tolerance--not truly repair but a way of coping with damage so that life can go on (Internet 1).

C- Certain biological factors

Some of the biological factors leading to cancer include.

- **Hepatitis B and Hepatitis C viruses (HBV and HCV)**

HBV is a member of the Hepadnaviridae family (*hepa*-tropic DNA viruses), while HCV is a member of the Flaviviridae family. They cause hepatocellular carcinoma.

It is thought that HBV caused tumors by expression of a viral gene (the X gene) that encoded for the X protein. The X protein deregulates checking point controls and thus participates in the selection of cells that are genetically unstable, some of which would accumulate unrepaired transforming mutations (Senn and Schneider, 1995; Lee *et al*., 1999).

Alternatively, cancers might develop simply because of continual cell proliferation resulting from chronic damage to the liver (Cooper, 1997; Kumar, 2003).

- **Hormonal factors**

Based on hypotheses (Henderson et al., 1997) that neoplasia is the consequence of excessive hormonal stimulation of a particular target organ, the normal growth and function of which is under the control of one or more steroid or polypeptide hormones. The role of hormones in this process is to drive cell

division on. The rate of cell division per unit of stimulation can be altered by mutations in DNA repair or cell cycle control genes, such as:

- Estrogens as: Initiators: Sex steroid metabolites can induce DNA adducts. (Han & Liehr. 1994, Ho & Roy. 1994) (Internet 2)
- Estrogens as promoters: Anything that enhances proliferation enhances tumorigenesis. (Cavalieri et al. 1997,Devanesan et al. 1999) (Internet 2).

D- Genes associated with cancer

Key genes involved, in neoplastic cell development include oncogenes, tumor suppressor genes and-DNA repair genes (Cooper *et al*., 1997).

1.2.1.5-Cancer Therapy

A- Conventional therapy

Conventional cancer therapy is based on surgery, radiotherapy, chemotherapy or combinations of them (DeVita, 1997; Hellman, 1997; Rosenberg, 1997). Generally, surgery and radiotherapy are preferred in localized tumors and chemotherapy when cancer cells are spread through the body (Lopez-Lazaro, 2002).

I- Radiotherapy

Radiation therapy is a medical specialty in which ionizing radiations are used to treat patients with cancer or other diseases (Parker, 2000).

Radiations may be electromagnetic (x-rays, gamma rays) or corpuscular (electrons, protons, heavy ions, neutrons, alpha particles) (Svensson and Mijnheer, 1995). The most common objective of radiation oncology is the loco-

regional eradication of cancer with preservation of the structure and function of normal tissues. Such a treatment is potentially curative for about half of patients and palliative for the others (Steel, 1995).

However, every effective therapy may generate undesirable and even dangerous side effects. In radiotherapy these could be early radiation-induced reactions (*e.g*. anorexia, nausea, esophagitis, diarrhea, and erythema) or late radiation-induced reactions (*e.g*. myelopathy, necrosis of bone, bowel stenosis, fibrosis of lung, and renal damage with loss of function, pericardial and myocardial damage) (Parker, 2000).

II- Chemotherapy

Classes of chemotherapeutic agents (also known as antineoplastic or anticancer agents) include (Lehne, 2001):

- **Alkylating agents;** it works by three different mechanisms all of which achieve the same end result - disruption of DNA function and cell death.
  - In the first mechanism an alkylating agent attaches alkyl groups to DNA bases. This alteration results in the DNA being fragmented by repair enzymes in their attempts to replace the alkylated bases. Alkylated bases prevent DNA synthesis and RNA transcription from the affected DNA.
  - A second mechanism by which alkylating agents cause DNA damage is the formation of cross-bridges, bonds between atoms in the DNA. In this process, two bases are linked together by an alkylating agent that has two DNA binding sites. Bridges can be formed within a single molecule of DNA or a cross-bridge may connect two different DNA

molecules. Cross-linking prevents DNA from being separated for synthesis or transcription.

- The third mechanism of action of alkylating agents is the induction of mispairing of the nucleotides leading to mutations. In a normal DNA double helix, A always pairs with (is across from) T and G always pairs with C. Alkylated G bases may erroneously pair with Ts. If this altered pairing is not corrected it may lead to a permanent mutation (Interne 3).

- **Antimetabolites**; they interfere with DNA and RNA synthesis by substrating or mimicking natural metabolites, causing wrong information to be given to the cell (e.g. methotrexat and 5-fluorouracil).

- **Antitumor antibiotics;** which act on inhibition or destruction of either DNA or RNA synthesis such as: Doxorubicin an anthracycline antibiotic that exerts its effects on cancer cells via two different mechanisms:

  - Intercalation: in its role as an intercalating agent the drug wedges between the bases of DNA and blocks DNA synthesis and transcription.

  - Enzyme inhibition: the drug inhibits the activity of an enzyme, topoisomerase type II. This leads to breaks in the genomic DNA. Both of these mechanisms result in DNA disruption that ultimately can lead to the death of the cell (Internet 4).

- **Mitotic Inhibitors (vinka alkaloids and taxoids)**; vinka alkaloids (e.g. vinblastine and vindesine) crystallize microtubular spindle proteins during metaphase and cause cell death, Vinblastine prevents the cancer cells from undergoing mitosis. It does this by inhibiting the formation of spindle

fibers. Spindle fibers are responsible the alignment of chromosomes and the separation of the chromosomes in anaphase. Vinblastine blocks the tubulin monomers from forming microtubules. Without proper microtubule formation cell division is not possible. Like all of the vinca alkaloids, this drug also affects cell division in normal cells, explaining many of the side effects seen. While taxoids (e.g. paclitaxel) promote formation of stable microtubule bundles, thereby inhibiting cell division (internet 5).

- **Hormones**; which block synthesis of RNA and new proteins, and alter cell metabolism by changing the hormonal environment of the cell. Such as the "anti-estrogen therapy." The goal of therapy is to starve the breast cancer cells of the hormone they thrive on, which is estrogen. Estrogen and progesterone travel through the bloodstream and find their matching receptor sites on both healthy cells and cancer cells. Receptors are very specialized protein molecules that sit on the outside or inside the cells in your body. They act like an on–off switch for a particular activity in the cell. If the right substance comes along that fits into the receptor—like a key fitting into a lock—the switch is turned on and a particular activity in the cell begins. Many breast cancers are hormone-dependent—which means that estrogen and progesterone stimulate their growth by "turning on" hormone receptors in the cancer cells. Without these hormones, the cancer cells are not stimulated to grow. They wither, and eventually they may die.

Estrogen and progesterone play roles in the development of certain breast cancers:

- Estrogen is a very important "key" for the estrogen-receptor (ER) sites throughout the body AND on some breast cancer cells.
- Progesterone receptors (PR) can also be involved in turning on breast cancer cell growth.

When a cancer shows few or no estrogen receptors (when it is "ER negative,") hormonal therapy is usually not effective. But if there are progesterone receptors, hormonal therapy may sometimes be helpful anyway. Women whose cancers are PR-positive but ER-negative have about a 10% chance of responding to hormonal therapy (internet 6).

However, not all of these agents spare normal cells from their devastating action (Lopez-Lazaro, 2002). Another major problem in cancer chemotherapy is development of resistance to the agents used; therefore, chemotherapeutic drugs are sometimes used in combination in order to improve their effectiveness (Murray, 1996, DeVita 1997).

This multi drug resistance is attributed to some extent to a protein called P-glycoprotein, which is present normally in the plasma membrane of some cells of kidney and the gut where it plays a role in the excretion of potentially toxic compounds from cells of these organs. This protein acts as an energy-dependent efflux pump expelling a variety of drugs and thus mediating multidrug resistance (MDR). The amount of P-glycoprotein increased in resistant cultured cells and in resistant tumor cells *in vivo*; increased in vivo amount correlates with poor

prognosis. When transfected into cells, P-glycoprotein can confer multidrug resistance (Murray, 1996).

B- Biologic therapy

I- Immunotherapy

Immunotherapy is cancer treatment that produces antitumor effects primarily through the action of natural host defense mechanisms or the administration of natural mammalian substances. Most applications of biologic therapy for cancer have attempted to stimulate immune defense mechanisms (Rosenberg, 1997).

Recently, Gentschev *et al.* (2005) described the development of a vaccine based on an attenuated *Salmonella enterica* serovar Typhimurium *aroA* strain. The vaccine strain significantly reduced tumor growth in two transgenic mouse models of induced lung adenomas. Repeated attempts are being made to induce specific antibodies against cancer cells. Yang and Baltimore (2005) developed a method to genetically program mouse hematopoietic stem cells to develop into functional CD8 or CD4 T cells of defined specificity *in vivo*. This effective method represents a unique direction for T cell immunotherapy. It combines stem cell therapy, gene therapy, and immunotherapy to guide the host to develop, *in vivo*, a large population of antigen-specific T cells and thus could be called "instructive immunotherapy" and could be developed for controlling the growth of human tumors and attacking established pathogens.

Berzofsky *et al*. (2004) examined the fundamental immunologic advances and the novel vaccine strategies arising from these advances, as well as the early clinical trials studying new approaches to treat or prevent cancer. Smith-Jones

(2004) reviewed the recent advances in the treatment of metastatic prostate cancer with radiolabeled antibodies with a specific emphasis on 177Lu-huJ591, a labeled monoclonal antibody currently under clinical evaluation.

This treatment involves the use of antibodies to target cancer cells. While antibodies are naturally occurring proteins in our bodies, the antibodies used in the treatment of cancer have been manufactured for use as drugs. The antibodies may work by several different mechanisms, either depriving the cancer cells of necessary signals or causing the direct death of the cells. Because of their specificity, antibodies may be thought of as a type of specific inhibitor.

II- Gene therapy

Gene therapy is a therapeutic technique in which a functioning gene is inserted into a cell to correct a metabolic abnormality or to introduce a new function (Rosenberg, 1997).

C- Alternative methods of cancer therapy

Several cancer patients seek therapy for their illness in unproven or alternative therapies (Vickers and Zollman, 1999a, b, c). These methods vary greatly; from dietary approaches (changing their diets, taking multivitamins, shark cartilage, vitamin injections, mistletoe injections, herbal medicine, Chinese herbs, miatake mushrooms), to seeking alternative therapists (nutritionist, acupuncturist, psychotherapist or Chinese herbalist), or using some form of alternative healing methods (support groups, visualization, positive thinking, yoga, acupuncture, cold swimming, prayer,Hypnosis or psychic healing) (Jacobs, 1997; Vickers and Zollman, 1999a,b,c; Costa Rosa, 2004; Jennen and Uhlenbruck, 2004; Shen *et al*., 2004).

1.2.1.6-Use of Cell Lines in Anticancer Drug Development

Use of *in vitro* assay systems for the screening of potential anticancer agents have been common practice almost since the beginning of cancer chemotherapy in 1964 (Wilson, 2000). The last decade has seen an enormous trend towards isolated cellular systems, primary cells in culture and cell lines. These systems provide the desirable complexity of structurally and functionally intact cells combined with excellent experimental accessibility. They offer the unique possibility to elucidate interactions with vital cellular functions such as metabolism, intercellular communication, signal transduction, growth and death that were formerly difficult to address (Gebhardt, 2000).

An increasing pressure is being put for a more comprehensive adoption of *in vitro* testing in safety evaluation of chemicals for cancer chemotherapy. The impetus for this originates partly from financial considerations, since *in vitro* testing has considerable economic advantages over *in vivo* testing. There is also an increasing realization of the limitations of animal models in relation to human metabolism, as more and more metabolic differences between species come to be identified. Finally yet importantly, there is the moral pressure to reduce animal experimentation (Wilson, 2000). The main disadvantages of this technique are the good level of expertise required, instability of continuous cell lines resulting from their unstable aneuploid chromosomal constitution, and loss of differentiation properties of the cultured cells, which requires stable markers for characterization (Freshney, 2000).

Currently, the cell line screen is a key component of a comprehensive *in vitro* and *in vivo* preclinical screening and drug development program that is overseen by the National Cancer Institute's (NCI's) Developmental Therapeutics

Program (DTP) in the Division of Cancer Treatment and Diagnosis (Takimoto, 2003). Voskoglou-Nomikos *et al*. (2003) looked at the value of three preclinical cancer models, the *in vitro* human cell lines, the human xenograft, and the murine allograft, to examine whether they are reliable in predicting clinical utility. They concluded that under the right framework and when panels are used, the *in vitro* cell line and human xenograft models may be useful in predicting the phase II clinical trial performance of cancer drugs. Murine allograft models, as used in this analysis, seemed of limited utility.

Odds were defined by Yaseen (1990) as he managed to establish the hard-to-establish human colorectal cancer (CRC) cell line, NYT27 cell line, and studied the *in vitro* karyotypic evolution of this cell line along with molecular studies at several passage levels. The first murine plasmacytoma cell line in Iraq, SU.99, was established by Abdul-Majeed (2000) and was found to be sensitive to the ethanolic extract of *Withania somnifera* (Al-Atby, 2001). Al-Shamery (2003) managed to establish two murine cell lines in Iraq: AMN_3, the first transplantable mammary adenocarcinoma tumor cell line in Iraq adapted to grow *in vivo* in BALB/C mice, and AMN_3, the first *in vitro* continuous murine mammary adenocarcinoma tumor cell line in Iraq.

Chemosesitivity testing of tumor cells has become faster, relatively safer and much easier due to the development of colorimetric assays for accurate proportional measurement of living cells. These assays could be carried out entirely in 96-well microtiter plates and read on a scanning multiwell spectrophotometer (ELISA reader) with no radioisotopes used (Mosmann, 1983; Cole, 1986; Mahony *et al*., 1989; Betancur-Galvis *et al*., 2002; Lee *et al*., 2004).

However, clonogenic assay remains the best measure of the integrity of cell reproduction (Smit *et al*., 1995).

Cultures derived from embryonic tissue proliferate better than those from the adult. This reflects the lower level of specialization and higher proliferative potential in the embryo. Mesodermally derived cells (fibroblasts, endothelium, and myoblasts) are overall easier to culture than epithelium, neurons, or endocrine tissue. Freshly isolated cultures are known as primary cultures until they are subcultured. They are more representative of the cell types in the tissue from which they were derived and in the expression of tissue-specific properties (Freshney, 2000b).

1.2.1.7-Use of Plant Extracts and Phytochemicals

Natural compounds had practical advantages with regard to availability, suitability for oral application, regularity approval and mechanisms of action (Tsuda *et al*., 2004). By producing a variety of secondary metabolites, plants defend themselves from invasion by other creatures by a system called "multichemical defense". As epidemiological data suggest, daily intake of these naturally occurring chemicals confers prevention of chronic diseases like cancer (Matsumoto *et al*., 1997).

In China, over half of the Chinese population still uses traditional herbal prescriptions, particularly when Western medicines do not produce the desired result. Most hospitals in China, whether modern or traditional, are equipped with a traditional herbal apothecary to distribute Chinese herbs upon request. Patients in China seem to as well as those in the West, indicating that the traditional Chinese medicine can give results as effective as Western medicine (Huang,

1999). The popularity of herbal medications as adjunctive therapy for acute and chronic medical problems has grown significantly in the past several years. US herb sales in 1996 were expected to reach or exceed $12 billion (Spaulding-Albright, 1997).

Herbs or their extracts contain different phytochemicals with biological activity that can provide therapeutic effects. Research interest has focused on herbs that possess hypolipidemic, antiplatelet, antitumor, or immune-stimulating properties that may be useful adjuncts in helping reduce the risk of cardiovascular disease and cancer (Craig, 1999; Abuharfeil *et at*., 2000). Among the phytochemicals with antitumor or cytotoxic activity are polyphenolic compounds, flavonoids, lignans, saponins, tannins, terpenoids, sesquiterpene lactones, quassinoids, triterpene glucosides, colchicine derivatives and quinone derivatives (Bas Bueno-de-Mesquita *et at*., 1997; Craig, 1997; Okuda, 1997; Watanabe *et at*., 1997; Craig, 1999; Lee, 1999).

Ethanolic crude extracts from the roots and rhizomes of *Cyperus rotundus* L. were tested for cytotoxicity against several cell lines included human larynx epidermoid carcinoma (HEp-2), human rhabdomyosarcoma (RD), human glioblastoma multiform ($AMGM_5$), murine mammary adenocarcinoma (AMN_3) and normal rat embryo fibroblasts (REF). Ethanolic extracts showed a significant cytotoxic activity against the five malignant cell lines, with AMN_3 and RD cells being the most sensitive and $AMGM_5$ the most resistant of the five cell lines. This suggested that the active compounds in these extracts might have some specificity in their toxicity towards cancer cells rather than normal cells (Al Hilli, 2004). Sa'eed (2004) showed that terpenoids and polyphenols from green and black tea had cytotoxic specificity on HEp-2, RD, and AMN_3.

1.2.1.8-Classification of Greater Galangal

The scientific name of the greater galangal *A. galanga* (L.) Willed. (Burtt and Smith, 1972) is as follows: -

Kingdom:	Plantae
Subkingdom:	Tracheobionta
Division:	Spermatophyta
Subdivision:	Magnoliophyta
Class:	Liliopsida
Subclass:	Zingiberidae
Order:	Zingiberales
Family:	Zingiberaceace
Genus:	*Alpinia*
Species:	*galanga*

A-Local names of greater galangal

The common name of greater galangal was derived from the Arabic word (Khalanjan: خولنجان), which may have misrepresented from the Chinese word Liang-tang, meaning mild ginger (Stuart, 1987; Ravindran and Balachandran, 2004).

The greater galangal has several local names such as: galangal (which means spice, a herbal root of this plant, which is grown in China), Siamese ginger, Laos ginger, Galanga, Garingal galingale, Galangal major, Java galangal and Kaempferia. These names are also known according to the world's countries (Stuart, 1987; Ravindran and Balachandran, 2004), including for example: -

Arab countries:	Khalanjan
English:	Galangal
China:	Koling-ching
France:	Grand galangal
Germany:	Galanga
India:	Kulinjan
Indonesia:	Laos
Italy:	Galanga
Malaysia:	Languas
Spain:	Galang
Thailand:	Khaa

B- Traditional use of galangal

There are two major classes of galangal, the first one is greater galangal (*A. galanga*) and it is found in Malaysia and Indonesia. The second is lesser galangal (*A. officinarum*) and it is found in China. Greater galangal is cultivated in Java, and it is used frequently as flavor and spice for food in Indonesia, Malaysia and South-East Asia. While lesser galangal cultivated in China and specially grows in the South-East regions, and also is grown in India and the rest of South-East Asia. Despite the limited use of both in Europe, they are among the most famous export and in large quantities, as spices, stimulant and sexual treatment. Galangal was defined by ancient Indians and the West since the middle Ages. Both possess stimulant characteristics and have proved by the Arabs when given to the horse to stimulate vitality, and by the Tatars when used with the tea. While in the East, galangal is used in the manufacture of perfumes, cosmetics and beverages brewing (Rosengarten, 1969; Perry and Metzger, 1980).

C- Description of greater galangal

Greater galangal is tropical herbal plant of the ginger family, grows up to the height of about 2 meters, and its leaves are long and wide 50 x 9 cm. Flowers are green-white in color and their edges are narrow dark red in color and their fruit is similar to red berries fruit (figure 2-1), while the roots are orange-brown in color (Lust, 1984).

D- Greater galangal in the traditional medicine

Greater galangal resembles the ginger *Zingiber officinale* in terms of appearance and uses, ginger is one of the famous popular spices in the world and its production is estimated at around 100,000 tons per year, China contributed approximately 80% of this production (Langner *et al*., 1997).

Ginger has many uses in the traditional medicine and has a similar pharmacological effects of many of the plants within the ginger family (containing complex mixture of effective components) as a factor in the anti-inflammatory musculoskeletal diseases, and it has been used in the Chinese medicine for more than 2500 years (Awang, 1992). There are also other medical uses, such as sexual stimulant, nausea, vomiting, motion sickness, urinary tract infections and various gastrointestinal disorders (Stewart *et al*., 1991; Afreen *et al*., 1995).

According to this, the medical use of the greater galangal is widely spread, because it possesses the same pharmaceutical characteristics of ginger (Anonymous , 1962a).

It is also recommended in Asia in the treatment of nauseas, fever and incontinence of urine (Perry and Metzger, 1980).

1.2.1.9-Greater Galangal Roots

Figure (1-1) Vegetative and flowering parts of *A. galanga* (Anonymous , 2005b, Spice World Directory).

A- Description of the greater galangal roots

The branched pieces of roots ranged from 1.5 to 3 inches in length, and seldom more than 0.75 inch thick. They are cut while fresh, and the pieces are usually cylindrical, marked at short intervals by narrow, whitish, somewhat raised rings, which are the scars left by former leaves. They are dark reddish-brown externally, and the section shows a dark centre surrounded by a wider, paler layer which becomes darker in drying (figure 2-2). Their odor is aromatic, and their taste pungent and spicy. They are tough and difficult to break, the fracture being granular, with small, ligneous fibres interspersed throughout one side (Lust, 1984).

B- Natural products of greater galangal roots

One of the most important components of the greater galangal roots is the essential oils, which possess a strong smell of spice flavor (Anonymous, 1962a).

The extracted oil from roots is characterized by its pale yellow color with distinctive smell. When analyzing 100 g of the fresh roots, it is found that it contains the following: 14 g moisture, 9 g total ash, 49 g dissolved material (in 80% ethanol), 19 g dissolved material (in water), 9 g total sugar, 16 g protein and 0.2-1.5% essential oil (dry weight). The soft stem of the greater galangal contains about 0.1% of the extracted oil that is characterized by strong and distinctive spice smell. Initial studies show that the essential oil consists of 48% methyl cinnamate, 20 - 30% 1, 8-cineole, camphor and D-pinene (Ravindran and Balachandran, 2004).

Figure (1-2) Greater galangal roots (Anonymous, 2005b, Spice World Directory).

The roots also contain tannin, starch, chlorine, sulphur, phosphorus and manganese in the ashes of this plant without any flavor (Sastry, 1960). The roots contain volatile oil (0.5-1.0%), resin, galangol, kaempferid, galangin (which is a dioxyflavanol compound) and alpinin.

Several studies on the chemical composition of the extracted essential oils from the greater galangal roots in some countries found that these oils have differences in their chemical composition between these different types of plants. For example, the oil extracted from the roots of the Indonesian plant consists mostly of aromatic oils monoterpenoids with pinenes (18.6%) and 1, 8 cineole (47.3%) as major compounds (De Pooter *et al*., 1985). While the oil of the roots of the Malaysian plant is characterized by more aromatic oils with (E)-β-

farnesene (18.2%) and β-bisabonlene (16.2%) as major compounds (Scheffer *et al*., 1981).

In another study, Jantan *et al*., (2004) mentioned that the oil of the roots is rich in 1,8-cineole compound (40.5%), with the presence of other compounds with similar quantities of the volatile oil, such as β-bisabonlene (8.4%), (Z, E)-farnesol (3.8%) and (E)-β-farnesene (3.2%).

In the Indian medicine, it was found that the compound myrecene (94.5%) is the major compound in the roots (Charles *et al*., 1992). In another similar study, Raina *et al*., (2002) analyzed the volatile oil of the greater galangal roots at the bottom of the Himalayas in India through Gas Chromatography (GC) and Gas Chromatography-Mass Spectrometry (GC-MS) and found that the major contents of the volatile oil in the roots were 1, 8-cineole (39.4%) and β-pinene (11.9%).

According to this, Jirovetz *et al*., (2003) concluded that the comparative investigation of the various oils in the greater galangal roots from southern India varies quantitatively rather than qualitatively, to include more than 115 volatile oil of the Indian galangal. These essential oils are rich in aromatic compounds and Phenyl propane derivatives.

C-Traditional use of the greater galangal roots

I- Greater galangal roots in cooking and food

The powder of the greater galangal roots in Asia is considered among the essential spices and food flavoring that is widely used in local food and meals in Malaysia, Thailand, Indonesia and China, it is also used as an alternative of ginger to give flavor to foods (Anonymous, 1962a; Perry and Metzger, 1980).

In Indonesia, it is only used in popular dishes like Indian spices, and in Europe it has been used since the Middle Ages. Galangal is used as similarly of ginger in fish cooking consistently in the preparation of fish meals and shellfish to give flavor in cooked fish. Laos's powder is more important in terms of the broad use in various popular meals such as soup, chicken, meat and vegetable spices (Heal and Allsop, 1983).

II- Greater galangal roots in the traditional medicine

Greater galangal roots were used in the traditional Asian medicine for many purposes such as, assistance for digestion and for appetite in China, or as antifungal and anti-itching in Thailand, and also used for sexual value, nervous debility, breathing problems and arthritis in India (Purseglove, 1981).

It was found that the essential oil of the greater galangal roots has anti-muscle cramps characteristics and antibacterial effects for both gram negative and positive bacteria such as *Streptococci* and *Staphylococci* (Anonymous, 1962a). Also the essential oil of the greater galangal roots is used for the problems of respiratory infections, particularly whooping cough in children (Nadkarni, 1976).

D- Pharmacological effects of the greater galangal roots

I- Anti-tumor activity

The normal cell cycle consists of an ordered set of events, resulting in the production of two daughter cells, it consists of:

- G1 = growth and preparation of the chromosomes for replication;

- S = synthesis of DNA [see DNA Replication] and duplication of the centrosome;
- G2 = preparation for
- M = mitosis.

When a cell is in any phase of the cell cycle other than mitosis, it is often said to be in interphase, not all cells proceed through the stages of the cell cycle at the same rate. Embryonic cells divide very rapidly, while mature cells might divide rarely, or in response to signals such as wounding, or not at all. This regulation requires a number of control mechanisms (figure 1-3) (Norbury and Nurse, 1992).

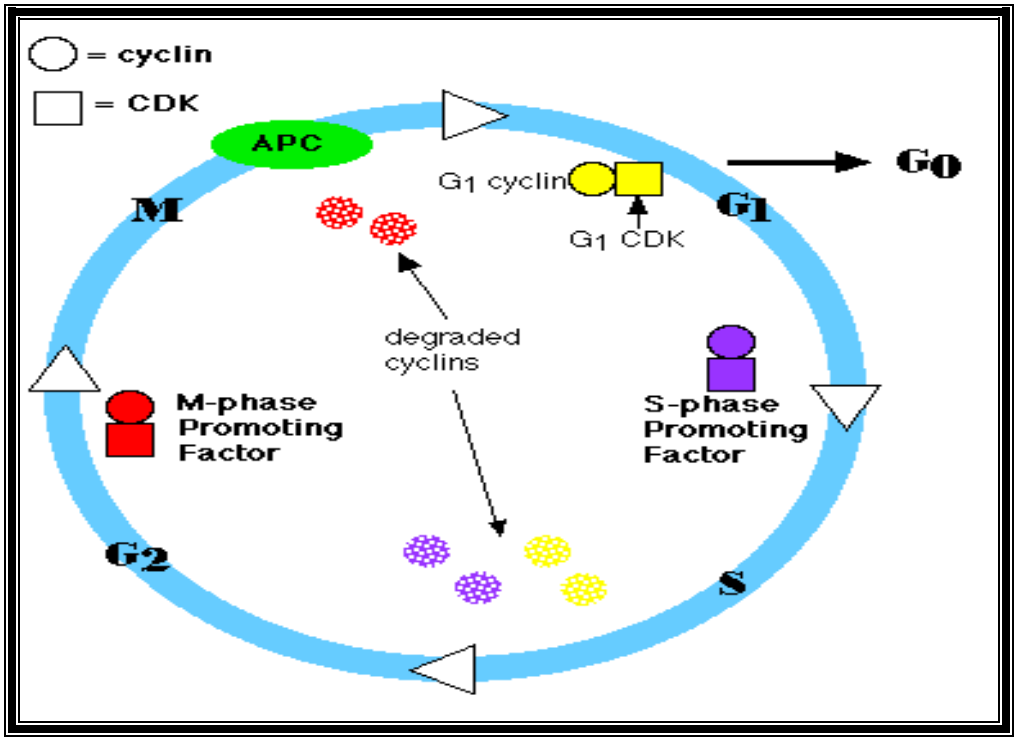

Figure (1-3) Eukaryotic normal cell cycle (Norbury and Nurse, 1992).

Transitions out of gap phases (G1, G2) are regulated by cyclins and cyclin dependent kinases (CDK). Cyclins are only present at certain times during the cell cycle. MPF (Maturation Promoting Factor) includes the CDK and cyclins that triggers progression through the cell cycle. Growth factors can also stimulate cell division. Growth factors serve as signals that tell the cell to move through the cell cycle and in order to divide (Norbury and Nurse, 1992).

While cancers are diseases in which there is a defect in the regulation of the cell cycle. Cancer cells are rapidly dividing cells that are no longer controlled by the mechanisms listed above. Cancer cells can form tumors due to this unchecked growth. Cancers are caused by genetic mutations. Sometimes these mutations are found in the germ line, and result in inherited cancers or a predisposition to cancer. Most often these mutations are found in somatic cells. Somatic mutations accumulate over our lifetime. Three classes of genes are involved in cancer: oncogenes, tumor suppressor genes, and DNA repair genes (figure 1-4). Proto-oncogenes (unmutated oncogenes) stimulate cell division in a regulated manner. Proto-oncogenes include growth factors, growth factor receptors, and cyclins. Oncogenes are mutated forms of these genes that result in unregulated stimulation of cell division. Tumor suppressor genes prevent cell division. Mutations in tumor suppressor genes result in the loss of this prevention of cell division. DNA repair genes promote repair of mutations that occur during the cell cycle. Loss of DNA repair genes results in the accumulation of many mutations within a cell (Sherr, 2000; Hoeijmakers, 2001).

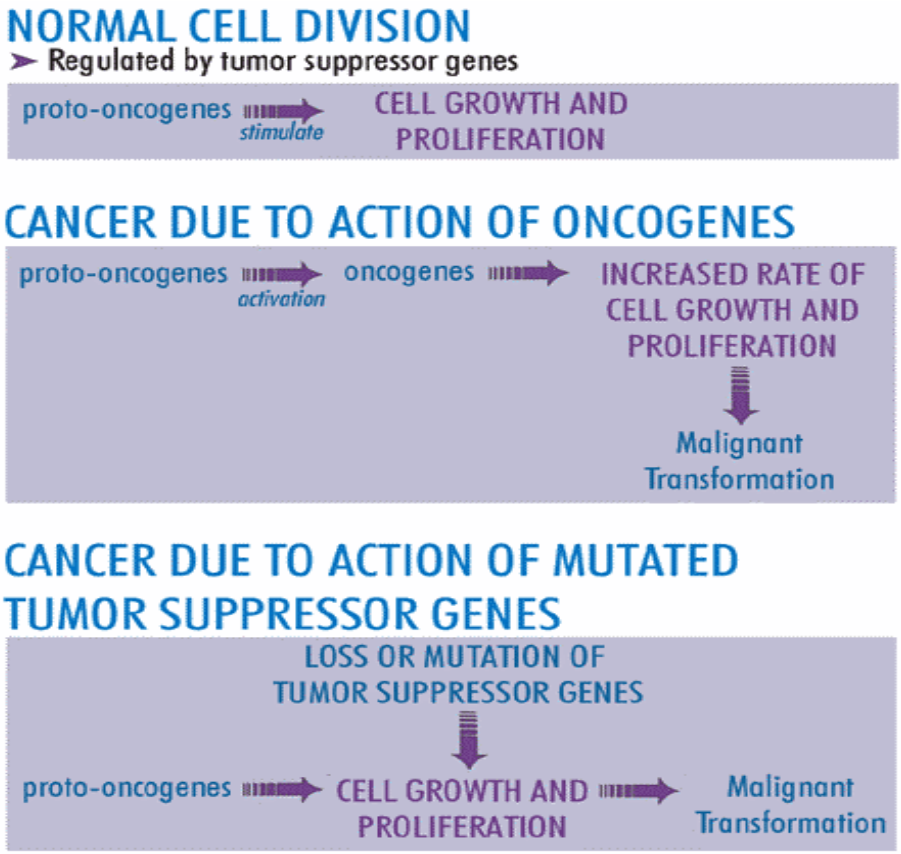

Figure (1-4) The effect of proto-oncogenes, oncogenes, and tumor suppressor genes on cell division (Sherr, 2000; Hoeijmakers, 2001)

Plant roots of the ginger family in general were used in Malaysian traditional medicine for cancer treatment. The plants of this family exert inhibitory effect against tumors (Vimala *et al*., 1999). One of these is the greater galangal roots which are used in Thailand for treatment of stomach cancer (Sirirugsa, 1998).

It was found that the (ACA) compound has anti-tumor activity through inhibition of chemically induced tumor formation (Kondo *et al*., 1993; Ohnishi *et al*., 1996). Also (ACA) inhibit Xanthine oxidase enzyme, which gives high oxidation ions and found in large quantities in certain brain tumors (Noro *et al*.,

1998). As shown by studies on cancer in rodents the compound (ACA) showed protective effects on chemically induced cancer in mouse skin (Murakami *et al*., 1996), mouth (Ohnishi *et al*., 1996) and colon (Tanaka *et al*., 1997) in rats.

On the other hand, studies showed the ginger stimulates glutathione-s-transferases (GST) enzymes, which are a group of enzymes known as cellular detoxification enzymes that may reduce the occurrence of cancer. It was observed that there was a close correlation between the induction of these enzymes and inhibition of some carcinogens (Krishnaswamy and Polasa, 2001; Ganguly *et al*., 2003). Laboratory tests also showed that the extract from both ginger and greater galangal inhibit the production of tumor necrosis factor alpha (TNFα) through inhibition of gene expression in human OA synoviocytes and Chondrocytes (Frondosa *et al*., 2000).

II- Immunological activity

Several studies have shown that the therapeutic activity of the various plants extracts come through overlapping with the host's immune system (Wanger and Proksch, 1984; Franz, 1989; Wanger, 1990). Polysaccharides in these extracts may contribute to this medical use (Franz, 1989).

A recent study of AIDS patients showed that some water and ethanolic extract of medical plants such as the greater galangal roots has inhibitory effect against HIV-1 protease (Tewtrakul *et al*., 2003). This enzyme is one of the most important enzymes in the major functional life cycle of the virus (which is the main objective in the development of antiretroviral (HIV-1), which plays a key role in the process of maturation and virus infection) (Kohl *et al*., 1988).

III- Anti-inflammatory activity

Herbs treatment or alternative medicine is used in the treatment of various inflammatory disorders, in that the majority of natural compounds found in plants possess great activity as anti-oxidation and anti-inflammation (Surh *et al*., 2001).

The anti-inflammatory properties of the different compounds come from the inhibition of nitric oxide, prostaglandins and cytokines production, such as Interleukin (IL) -1, IL-6 and IL-12 and Interferon-y (Lin and Lin, 1997). Greater galangal has anti-inflammatory activity (Nakamura *et al*., 1998), because it contains (ACA) compound which effectively inhibits the production of nitric oxide (Ohata *et al*., 1998), prostaglandins production (Rhizoma, 1990) and inhibits phagocytosis and protein kinase C enzyme activity (Watanabe *et al*., 1995).

The greater galangal and ginger together have inhibitory effects against cyclooxygenase-2 (COX-2) enzyme. This enzyme is responsible for the formation of inflammatory prostaglandins (Lehne, 1998).

IV- Other influences

Other impacts of the greater galangal are shown in the treatment of diabetes in Asia (Nadkarni, 1976). The effect of the powder, water and ethanolic extracts of the greater galangal roots on the level of blood sugar in rabbits was studied and it was noted that the oral dose of the greater galangal roots (adopted on the duration) causes a decrease in the level of blood sugar when giving doses by 3 - 4 g/kg of body weight without showing any side effects or toxicity even

when using acute high dose of 8 g/kg of body weight for 7 days (Khan *et al*., 2000).

In a similar study, it was found that rabbits treated with acute doses (2, 3 and 4 g/kg of body weight) of greater galangal roots powder for 24 hours showed a reduction in the level of blood sugar regardless of whether the extract is water or ethanolic (Akhtar *et al*., 2002).

Several studies suggested that the mechanisms associated with the effects of greater galangal extracts are possession of anti-oxidative, anti-fungal and anti-microbial characteristics. It was also found that the greater galangal extract has great benefit in preventing oxidation of fats and the spread of microbes in meat stock (Cheah and Gan, 2000).

Greater galangal was used with other anti-fungal herbs in the treatment of intestinal candidasis, as studies indicated that the greater galangal is highly effective against *Candida albicans*. It was found that the effectiveness of this comes from the diterpene-1 compound which is isolated from the greater galangal, and leads to depression of unsaturated fatty acids and analyzes fungal protoplast (Jantan *et al*., 2003).

Chapter Two

Materials and Methods

2-Materials and Methods

2.1-Materials

2.1.1-Apparatus used

Instruments used in this study and their manufacturers are listed in Table 2-1.

Table 2-1.Instruments used in this study and their manufacturers.

Instrument	Company Name	Origin
Electrical Blender	National MX-T76N, Deluxe Super	(Japan)
Sensitive Balance	METTLER TOLEDO, PG503-S	(Switzerland)
Stirrer	Ika Rh	(Germany)
Incubator	NBS CO_2, Model CO- 20	(U.S.A)
Magnetic Stirrer	Gallenkamp	(U.K)
Micropipette	Eppendorf	(Germany)
Oven	Chilipson	(U.K)
Plate bottles for tissue culture (Falcons)	Falcon	(U.S.A)
Water bath	Percisterm	(Germany)
Siliconized tube	Beliver Industrial	(U.K)
Centrifuge	Universal 16A	(U.K)
ELISA	Quick fit	(Germany)
Compound Light microscope	Olympus CK40	(Japan)
Freezer	National	(Japan)
Refrigerator	National	(Japan)

2.1.2-Solutions and Chemicals

Solutions and chemicals used in this study are listed in Table 2-2

Table 2-2.Solutions and chemicals used in this study and their origin of industry.

Chemical	Company Name	Origin
Ethanol Alcohol 96%	BDH	(U.K)
Fetal Bovine serum	GIBCO	(Auckland N.Z)
Benzyl penicillin, sodium salt	Samarra drug factory	(Iraq)
Streptomycin	GMBH	(Germany)
RPMI-1640 Media	US Biological	(U.S.A)
Crystal violet	BDH chemical	(U.K)
Sodium Bicarbonate, Analar	BDH chemical	(U.K)
Absolute Methanol	BDH chemical	(U.K)
Formaldehyde 37%	Sigma	(U.S.A)
Trypsin- Versene	GIBCO	(Auckland N.Z)
Benzyl Penicillin, sodium salt	Samarra Drug Factory	(Iraq)
HEPES buffer	Flow Laboratories	(Scotland)
Minimum Essential Medium (MEM)	Gibco	(Scotland)

2.1.3-Preparation of stock solutions for tissue culture

All solutions were prepared in the Iraqi Center for Cancer and Medical Genetics Research labs (ICCMGR) according to Freshney (2000b).

A- Antibiotics solutions

Benzyl penicillin, sodium salt (1000000 I.U) and streptomycin (1g) were each dissolved in 5ml of distilled water (DS) and stored at -20 °C.

From each of these stocks, 0.5 ml was added to one liter of the culture medium under preparation.

B- Sodium bicarbonate

Sodium Bicarbonate solution, 4.4% (w/v) was prepared; a quantity of 4.4 g of sodium bicarbonate was dissolved in 100 ml D.W. The solution was autoclaved at 121 C° for 15 minutes and stored at 4 C° using refrigerator.

C- PBS (pH 7.2) Phosphate buffer saline

Phosphate Buffer Saline (PBS) was prepared which composed of:

1. Sodium chloride (NaCl) …….8 g.
2. Potassium chloride (KCl) …..0.2 g.
3. Disodium hydrogen phosphate (Na_2HPO_4)…… 0.9 g.
4. Potassium dihydrogen phosphate (KH_2PO_4) .....0.2 g.
5. D.W. 1000 ml.

After dissolving of all components, the solution was autoclaved at 121 °C for 15 minutes and then stored at 4 °C, prior to any usage, PBS was warmed to 37 °C.

D- Trypsin -Versene solution

Trypsin- Versene Solution was prepared by mixing 100 ml of Trypsin-Versene powder, 0.10 ml of Na_2HCO_3, 0.20 ml of Ampicillin and 0.07 ml of Streptomycin, mixed in sterilized cabinet using magnetic bar, the volume was completed to 100 ml with D.W, the pH was adjusted to (7) and the solution was stored at 4 °C after usage.

E- Crystal violet

A concentration of 1% was prepared by dissolving 1 g form the Crystal Violet powder in 100 ml PBS, then filtered and stored at room temperature.

F- Fetal bovine serum

Fetal Bovine Serum was prepared from GIBCO (Auckland N.Z) and stored at -20 °C.

G- Growth media

I- Preparation of (RPMI)-1640 medium

(RPMI)-1640 was prepared as follows:

RPMI-1640 medium powder (with HEPES buffer and L-glutamine) 10.4 g, as the powder was dissolved in approximately 600 ml of double distilled water (DDW) and the other components were added:

a) Fetal Bovine Serum (FBS) …..100 ml.

b) Sodium bicarbonate (4.4%)…..5-10 to obtain final pH of 7.6.

c) Benzyl penicillin solution ….0.5 ml.

d) Streptomycin ……0.5 ml.

The volume was completed to one liter with (DDW) and was ultra filtered using Seitz filter. Filtration was repeated using 0.22 µm filter unit.

II- Preparation of maintenance media, Serum Free Medium (SFM)

SFM is RPMI-1640 (prepared as described before (G-I)) without including FBS, i.e. media without serum.

III- Preparation of minimum essential medium (MEM)

Minimum Essential Medium (MEM) was kindly provided by ICCMGR and was prepared as follows:

Amount of 11g of MEM powder (with L-glutamine) was dissolved in approximately 600 ml of (DDW) and then the other components were added as follows:

a) HEPES buffer (1M) ……...10 ml.

b) FBS …….....100 ml.

c) Sodium bicarbonate (4.4%) …5-10 ml to obtain a final pH of 7.6.

d) Benzyl penicillin solution…….0.5 ml.

e) Streptomycin solution ……0.5 ml.

The volume was completed to one liter with (DDW) and was ultra filtered using Seitz filter. Filtration was repeated using 0.22 µm filter unit.

H- Preparation of cell lines

I- Human epidermoid larynx carcinoma (Hep-2)

Human Larynx Epidermoid Carcinoma (HEp-2) Cell Line was kindly provided by (ICCMGR). This human cell line had originally come from a 57-year old man with a primary tumor of the larynx. Then it was implanted in irradiated and cortisone treated rat. After growth in the rat, the tumor was excised and implanted as an *in vitro* tissue culture. HEp-2 cells grew rapidly; doubling cell number occurred within in 2-3 days and was shown to be extremely resistant to ultraviolet rays (Moore *et al*., 1955).

Passages 219-220 were used through this study. The Hep-2 grew on RPMI-1640 (which was prepared as mentioned before (G-I). After the growth of Confluent Monolayer the cells were subcultured. This cell line was incubated for two days and it can be used after the confluent monolayer growth.

II- Ahmed-Mohammed-Nahi-2003 (AMN_3)

It is mammary adenocarcinoma for female mouse type (Balb/c) which was infected with *in vivo* spontaneous mammary adenocarcinoma.

This Cell Line was prepared in 2003, passage 178-179 of $AMGM_5$ cell line was used on this study and the cells were grown and maintained in RPMI-2 1640 media.

III- Rat Embryo Fibroblast (REF)

REF cell line was established and kindly provided by Dr. A. Al-Shamery from ICCMGR. Cells of this normal murine cell line were mixture of fibroblastic and epithelial cells with normal chromosomal picture. Passage

67-68 of this cell line was used in this study and the cell line was maintained using RPMI-1640

IV- Rhabdomyosarcoma (RD) cell line

Rhabdomyosarcoma (RD) cell line was kindly provided by ICCMGR. This human cell line was derived from a biopsy specimen obtained from a pelvic rhabdomyosarcoma of a 7- year- old Caucasian girl (McAllister *et al*., 1969)

Passage 224-225 of RD cell line was used through this study and RPMI-1640 was used in maintaining the cells.

V- Ahmed-Majeed-Glioblastoma Multiforme-2005 ($AMGM_5$) Cell Line

Ahmed- Majeed-Glioblastoma- Multiforme-2005 ($AMGM_5$) Cell Line was kindly provided by Dr. Ahmed Al-Shamery from ICCMGR. This human cell line was taken from a human cerebral glioblastoma multiform (GBM) obtained from 72- year- old Iraqi male who underwent surgery for intracranial tumor.

This cell line was incubated for Five days, it was grown on RPMI-1640 and passage 15- 16 of $AMGM_5$ cell line was used in this study.

2.1.7-Maintenance and subculturing of cell lines

Following the protocol described by Yaseen (1990), cell line used in this study was subcultured when monolayer were confluent. The growth medium was decanted off and the cell sheet washed twice with PBS. Two to three ml of trypsin – Versene was added to the cell sheet and the flask rocked gently. After approximately 30 seconds most of the trypsin was poured off and the cells incubated at 30 °C until they had detached from the flask. Cells

were further dispensed by pipetting in growth medium and then redistributed at the required concentration into culture flasks and reincubated at 37 °C.

2.1.8-Preparation of the medical plant

The dry *A. galangal* roots were bought from Kut market (200 Km south from Baghdad). The plant was classified by Botany Department/Natural Herbarium in Abu Ghraib, according to the certificate of state commission of testifying and certifying seeds, No. 1437, 8/11/2004.

Roots were cleaned from any blemishes, defects and blots, cut into small pieces grinded to powder using Electrical Blender. The powder was preserved using sealed glass tubes and stored in freezer at 4 °C.

2.1.9-Preparation of ethanolic crude extract of *A. galanga* roots

The ethanolic extract of *A. galanga* was prepared according to Qureshi *et al. (*1992), Al-Ataby (2001):

- A quantity of 25 g from *A. galanga* powder was weighed using sensitive balanced placed in one liter flask.
- A liquot of 125 ml from ethanol 70% was added to the powder.
- The contents were mixed using magnetic stirrer.
- The mixture was left to stirrer on a magnetic stirrer for 72 hours at room temperature.
- The mixture was filtered through Whatman No.1 filter paper at the end of the 72 hours period .
- Filtrate was gently poured into pre-weighed glass petri dishes and placed in the incubator for seven days at 40 °C until dryness.

- Dried extracts were scrubbed off the petri dishes, placed in labeled, tightly sealed plastic tubes and stored in the freezer at -20 °C

2.1.10-Preparation of the stock solution

The stock was prepared by dissolving 0.01 mg from *A galanga* root powder (prepared as described before in 2.1.9) in 0.2 mg DEMSO and then 9.8 ml of RPMI (free serum) was added to prepare 1mg/ml concentration.

The solution was filtered with 0.22 μm Millipore unit and incubated for 24 hours at 37 °C.

2.1.11-Cell line preparation for cytotoxicity study

Seeding was carried out under septic conditions; confluent monolyers were treated (as described before in 2.1.7). Afterwards, 200 μl of cells 10^3 cells in each well in growth medium were added to each well of sterile 96-well microtitration plate. The plates were sealed with a self-adhesive film, lid placed on and incubated at 37 °C.

When cells are in exponential growth, *i.e.* after lag phase, the medium was removed and serial dilutions of ethanolic extract (250, 125, 60, 30, 15 and 7 μg/ml) were added to the wells. Three replicates were used for each concentration of either extract. The last two columns were used as control (cells treated with RPMI-1640 free media). The same exposure was repeated for three 96-well microtitration plate according to the three incubation periods (24, 48 and 72 hours at 37 °C).

2.1.12-Staining

After the incubation (as mentioned before 24, 48 and 72 hours), microtitration plates were stained with freshly prepared crystal violet for half an hour, washed with distilled water (crystal violet and the distilled water

should be warm to avoid cell shrinkage), left to dry at room temperature and examined under (100X). The percentage of the living cells can be measured through Elisa.

2.1.13-Statistical analysis

The values of the investigated parameters were given in terms of mean ± standard error, while differences between means were assessed by analysis of variance (ANOVA) and Duncan test, using the computer program SPSS version 7.5. The differences was considered significant when the probability value was equal or less than 0.05. (Perez Serrano *et al*., 1997).

The percentage of inhibition; also known as rate of inhibition of cell growth (Guo *et al.*, 2003) or percentage of toxicity (Betancur-Galvis *et al.* 2002,) was calculated as (Chiang *et. al.*, 2003):

Inhibition (%) = (Optical density of test wells/Optical density of control wells)*100

Chapter Three

Results and Discussion

3. Results and Discussion

3.1. Effect of ethanolic crude extract of greater galangal roots on HEp-2 cell line

The ethanolic extract of greater galangal roots showed a time - dependent effect on the viability of HEp-2 cells. The highest three concentrations, (60, 125 and 250) µg/ml, showed both time and concentration - dependent effects (figure 3-1).

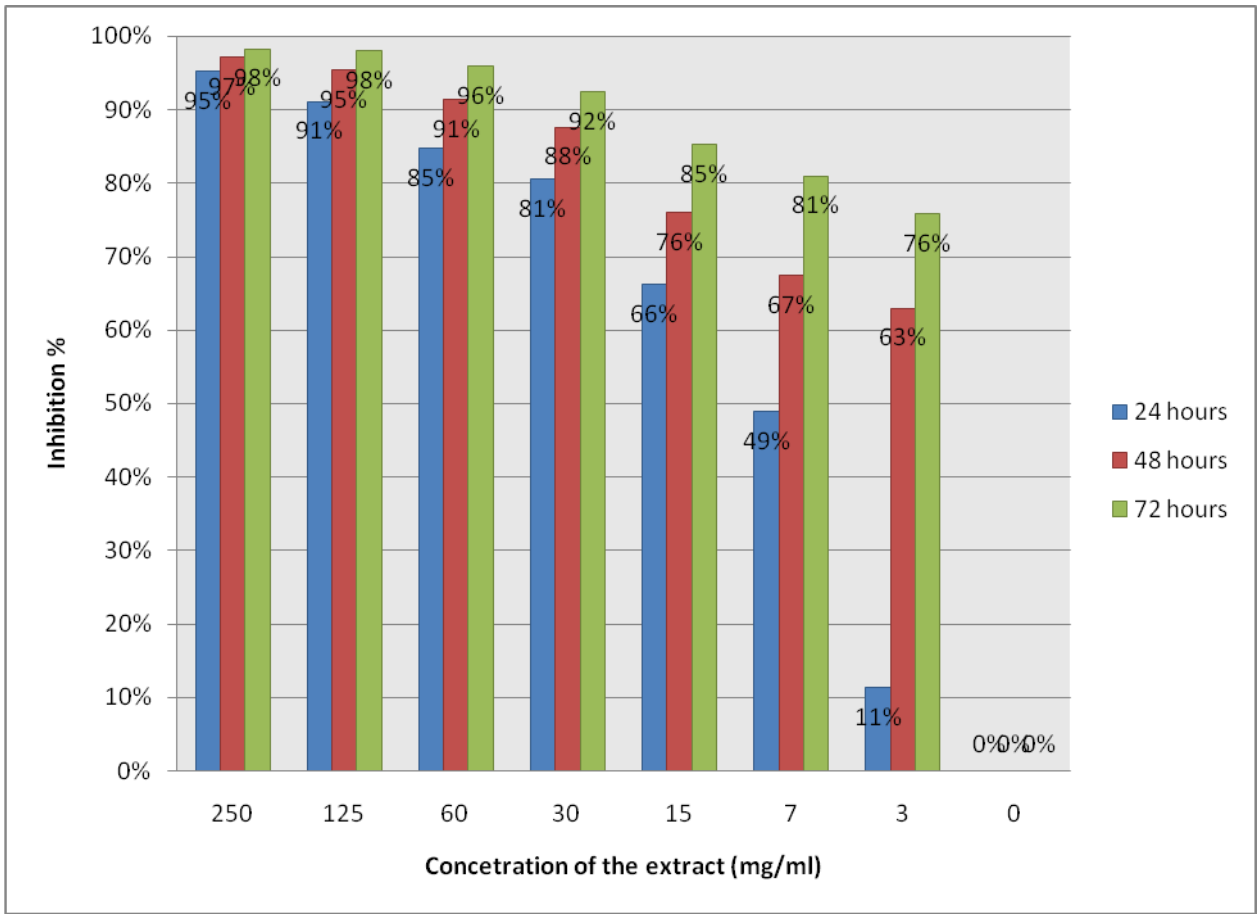

Figure (3-1) Effect of the ethanolic crude extract of greater galangal roots on inhibition of HEp-2 cells (n=3).

Significance between controls and samples was determined using student's t-test. A P value ≤ 0.05 was considered statistically significant.

Cell viability reached as low as (0.085, 0.047 and 0.019) after treatment with (250 µg/ml) for 24, 48 and 72 hours respectively. This gave very high percentages of inhibition (95.2, 97.2 and 98.3) % respectively (figure 3-1).

Treatment with (125, 60, 30, 15, 7 and 3) µg/ml for 72 hours caused significant reduction in cell viability ($P \leq 0.05$) which reached (0.032, 0.068, 0.126, 0.246, 0.318 and 0.405) respectively. This represented (98, 95.9, 92.4, 85.3, 81 and 75.8) % inhibition respectively.

Plants contain several different families of natural products among which are compounds with weak estrogenic or antiestrogenic activity towards mammals. These compounds, termed phytoestrogens, include certain isoflavonoids, flavonoids, saponins and lignans (Dixon, 2004).

Chemical and pharmaceutical studies of the greater galangal roots documented that one of the components of the essential oil is 1,S-1-acetoxychavicol acetate (ACA), which owns various biological effects such as anti-tumor (Itokawa *et al*., 1987; Moffatt *et al*., 2000; Zheng *et al*., 2002), anti-inflammatory (Nakamura *et al*., 1998), anti-fungal (Janssen and Scheffer, 1985; Jantan *et al*., 2003), anti-oxidant and it has inhibitory effect on Xanthine oxidase enzyme (Kubota *et al*., 2001).

ACA was first isolated from the rhizomes of *A. galanga* and found to prevent the growth of various fungi (Janssen &Scheffer, 1985). It was also reported that ACA showed antituberculosis and anti-allergy activity (Palittapongarnpim *et al*., 2002; Matsuda *et al*., 2003; Yoshikawa *et al*., 2004). Furthermore, numerous studies have demonstrated that ACA suppresses the development of many tumors, such as skin cancer, oral cancer, colon cancer, liver cancer, bile-duct cancer and oesophageal cancer *in vivo* (Murakami *et al*., 1996; Ohnishi *et al*., 1996; Tanaka *et al*., 1997 a, b;

Kobayashi *et al.*, 1998; Kawabata *et al*., 2000; Miyauchi *et al.*, 2000), but the mechanism is less well understood.

The growth inhibition in the present study also may be contributed to DNA damage by inhibiting the catalytic activity of topoisomerase II (Muller *et. al.*, 1999). The results were also agreed with the study of *in vitro* which was carried out by (Whelan and Ryan, 2003). They mentioned that the cell proliferation of Hep-2 cell line reduced in a dose and time – dependent manner after exposure to *Psoralea corylifolia* containing as major constituents DNA polymerase and topoisomerase II inhibitors that inhibit DNA replication enzyme (Sung *et al.*, 1998).

Accordingly cell line showed a decrease at higher concentrations on third day of exposure, time and concentration dependent.

3.2. Effect of ethanolic crude extract of greater galangal roots on AMN_3 cell line

Figure (3-2) showed that the highest concentration of ethanolic crude extract of greater galangal roots (250 µg/ml) gave the maximum decrease in AMN_3 cells viability (0.021) with inhibition (98.8%) after 72 hours. Also the other concentrations (125, 60, 30, 15, 7 and 3) µg/ml showed a significant ($P \leq 0.05$) decrease in AMN_3 cells viability (0.043, 0.075, 0.135, 0.254, 0.325 and 0.410 respectively). This represented (97.5, 95.7, 92.3, 85.6, 81.6 and 76.8) % inhibition respectively (figure 3-2).

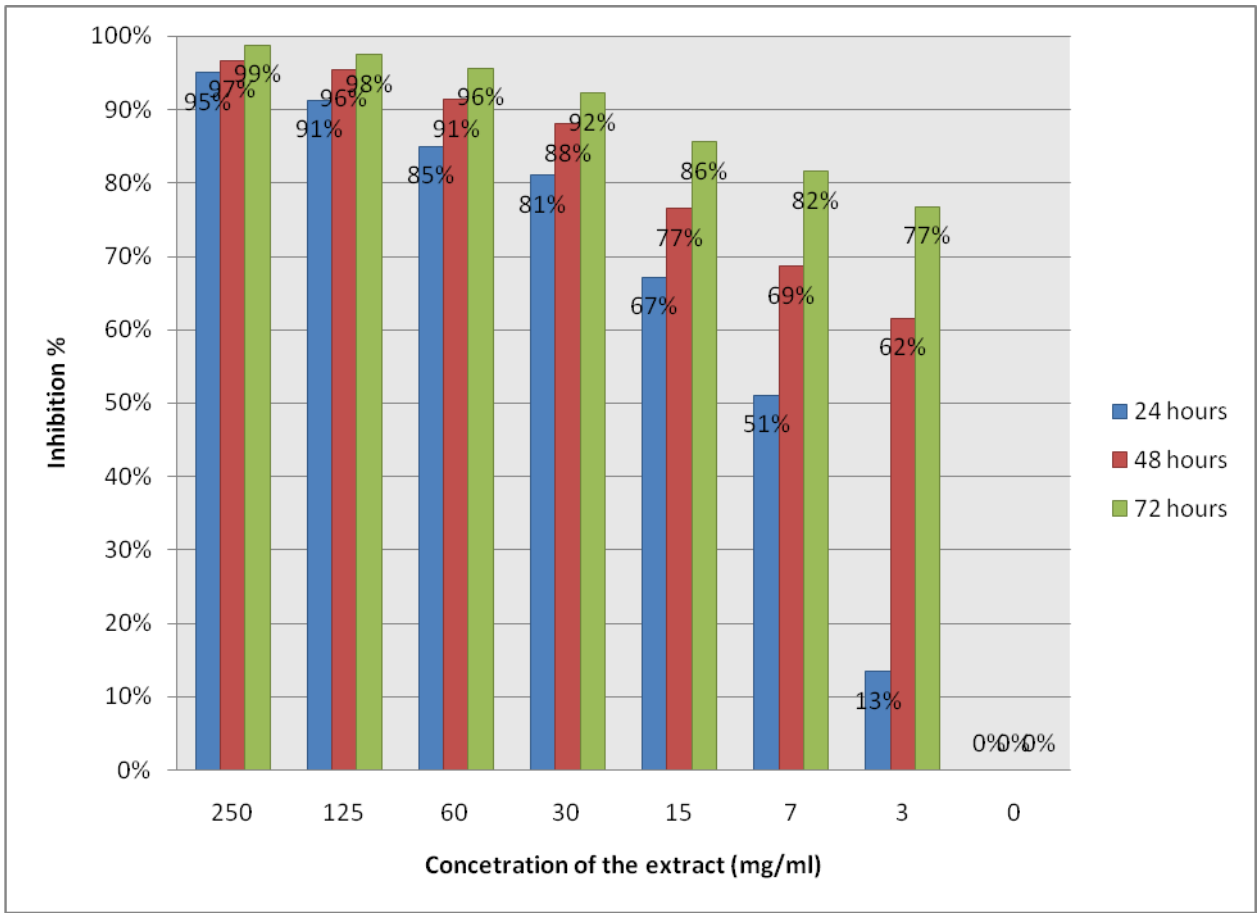

Figure (3-2) Effect of the ethanolic crude extract of greater galangal roots on inhibition of AMN_3 cells (n=3).

The ethanolic extract of greater galangal roots showed significant ($p \leq 0.05$) inhibitory effect on AMN_3 cells. In addition the extract demonstrated time and dose-dependent inhibitory effect.

Sukardiman and his colleagues (2000) found that the flavonoid pinostrobin inhibited DNA topoisomerase I activity resulting in the cleavage of DNA and thus cytotoxicity towards cell culture of human mammary carcinoma.

The sensitivity of mammary gland adenocarcinoma (AMN_3) may be due to the natural isoflavonoids, phytoestrogen which inhibit the tyrosine-kinase activity of growth factor receptors and oncogenes products, as well as the *in vitro* growth of some tumor cell lines (Pagliacci *et al.*, 1994).

It was found after the comparison between the AMN_3 and Hep-2 cell lines, that the AMN_3 cell lines were more sensitive than the Hep-2 to the ethanolic crude extract of *A. galanga* and that because of the differences of its receptors on the cancer cell membranes which differ according to the kind and origin of the current cancer cells, as mentioned by (Sa'eed *et al.*, 2004) in his study on the effect of green and black tea extracts on different cell lines.

A. galanga ethanolic crude extract activity on apoptosis, may be due to the huge number of the active compounds affect the cancer cells, as more than one kind of the active compounds can be found that work together the cancer cell membrane receptors which are responsible for the apoptosis (Sa'eed *et al.*, 2004).

3.3. Effect of ethanolic crude extract of greater galangal roots on RD cell line

The ethanolic crude extract of greater galangal roots reduced the viability of RD cells gradually with time (figure 3-3). The highest concentration, (250 µg/ml) produced the lowest percentage of cell viability thus reaching (0.022) after 72 hours (percentage of inhibition 98.7 %, figure 3-3).

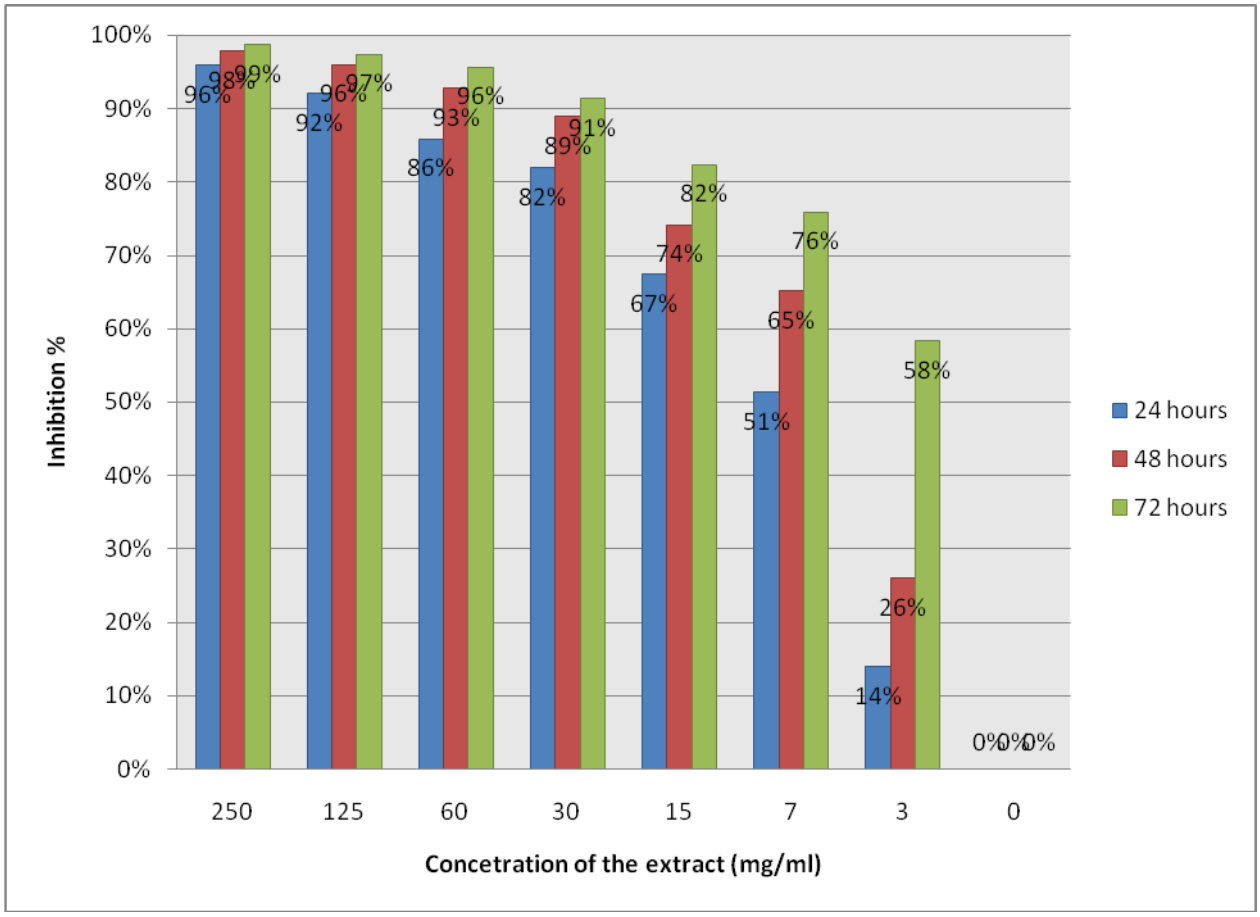

Figure (3-3) Effect of the ethanolic crude extract of greater galangal roots on inhibition of RD cells (n=3).

All other concentrations significantly ($P \leq 0.05$) reduced cell viability after 72 hours of exposure. Cell viability reached (0.045, 0.076, 0.152, 0.311, 0.427 and 0.738) after exposure to (125, 60, 30, 15, 7 and 3) µg/ml

respectively. This represented significant ($p \leq 0.05$) inhibition percentages of (97.4, 95.7, 91.4, 82.4, 75.9 and 58.4) % respectively.

From these results, it was concluded that the ethanolic crude extract of *A. galanga* showed time-dependent inhibitory activity on RD cells. The most profound inhibitory effects were shown after 72 hours of exposure.

Compared with inhibition of HEp-2 cell, RD inhibition by ethanolic extract of greater galangal roots was higher. This reflects the sensitivity of RD cells compared with HEp-2 cells. Al Hilli (2004) studied the effect of crude extracts of *Cyperus rotundus* L. and showed that RD cells were more sensitive to hexane, aqueous and ethanolic extracts as compared with HEp-2 cells. Highest inhibition was recorded after 72 hours of treatment with any each the extracts. Exposure of HEp-2 and RD cells to green and black tea terpenoids and polyphenols also revealed that RD cells were more sensitive than HEp-2 cells (Sa'eed, 2004).

Panaro *et.al*. (1999) observed that flavone acetic acid (FAA), a synthetic flavonoid, worked as a colcemid-like effect on cytokinesis by causing accumulation of condensed C-metaphases, which are caused by colcemid and named accordingly.

3.4. Effect of ethanolic crude extract of greater galangal roots on $AMGM_5$ cell line

Figure (3-4) showed that the highest concentration (250 µg/ml) caused the highest decrease in $AMGM_5$ cells viability (0.028) with inhibition (98.2%) after 72 hours. Also the other concentrations (125, 60, 30, 15, 7 and 3) µg/ml showed significant ($P \leq 0.05$) decrease in $AMGM_5$ cells viability as (0.055, 0.082, 0.137, 0.274, 0.423 and 0.823) respectively. This represented (96.6, 94.9, 91.6, 83.3, 74.3 and 50) % inhibition respectively (figure 3-4).

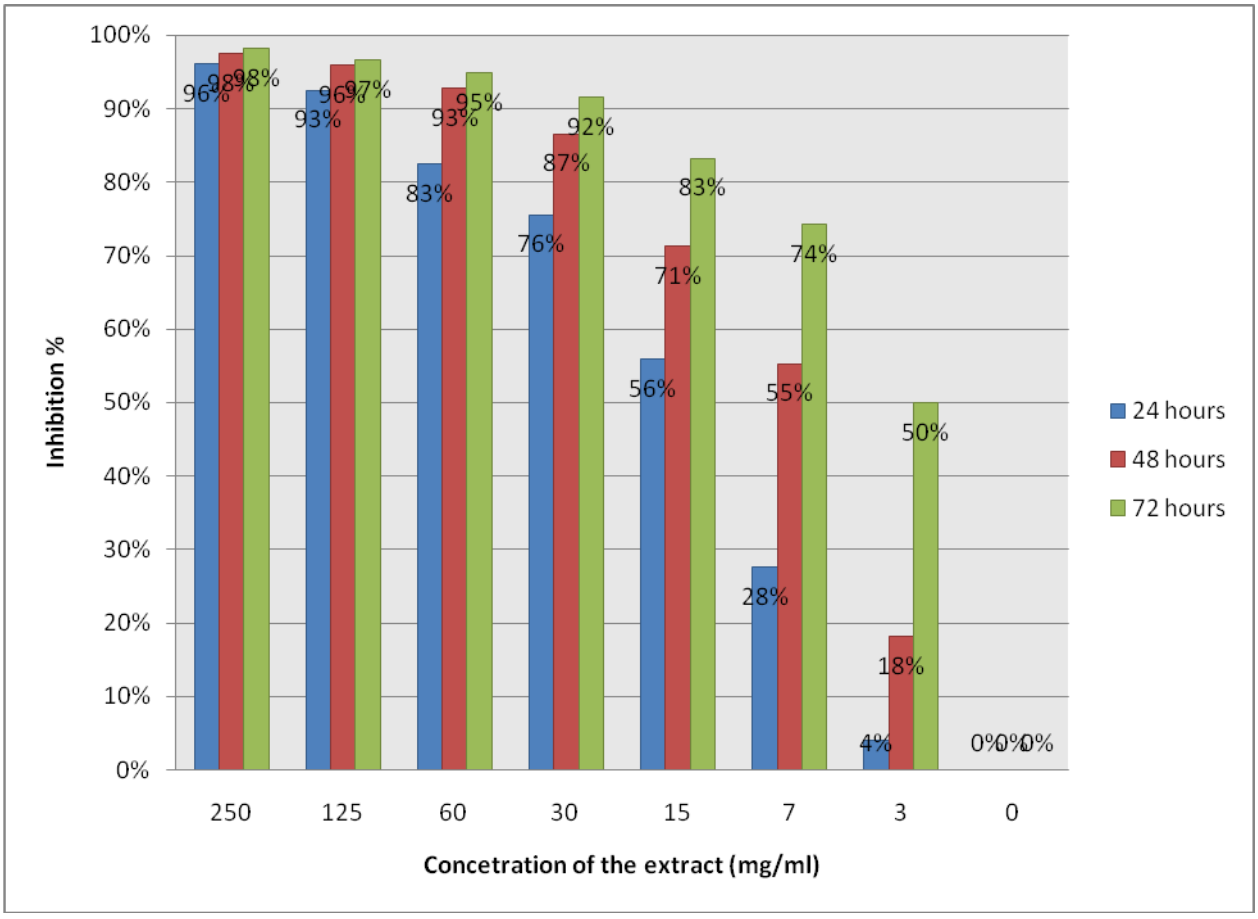

Figure (3-4) Effect of the ethanolic crude extract of greater galangal roots on inhibition of $AMGM_5$ cells (n=3).

From these results it was concluded that the extract showed time and concentration-dependent inhibitory effect. Glioblastoma is usually rapidly fatal. Glioblastomas are the most common and aggressive type of malignant glioma. They are characterized by rapid cell division, invasion into normal brain, and a high degree of vascularity (Stupp *et al*., 2005).

Tamoxifen had been shown to have cytotoxic activity against glioma cells at high doses. Tamoxifen caused dose-dependent growth inhibition in human glioma cell lines U87MG, U373MG and U138MG (Hui *et al*., 2004).

3.5. Effect of ethanolic crude extract of greater galangal roots on REF cell line

The ethanolic crude extract of greater galangal roots reduced the viability of REF cells slightly over time (figure 3-5). The highest concentration, (250 μg/ml) showed significant ($P \leq 0.05$) a slight decrease in cell viability reaching (1.753) after 72 hours (percentage of inhibition (4.364 %), figure (3-5).

All other concentrations slightly reduced cell viability after 72 hours of exposure. Cell viability reached (1.762, 1.775, 1.782, 1.790, 1.796 and 1.813) after the exposure to (125, 60, 30, 15, 7 and 3) μg/ml respectively. This represented inhibition percentages of (3.873, 3.164, 2.782, 2.345, 2 and 1) % respectively (Figure 3-5).

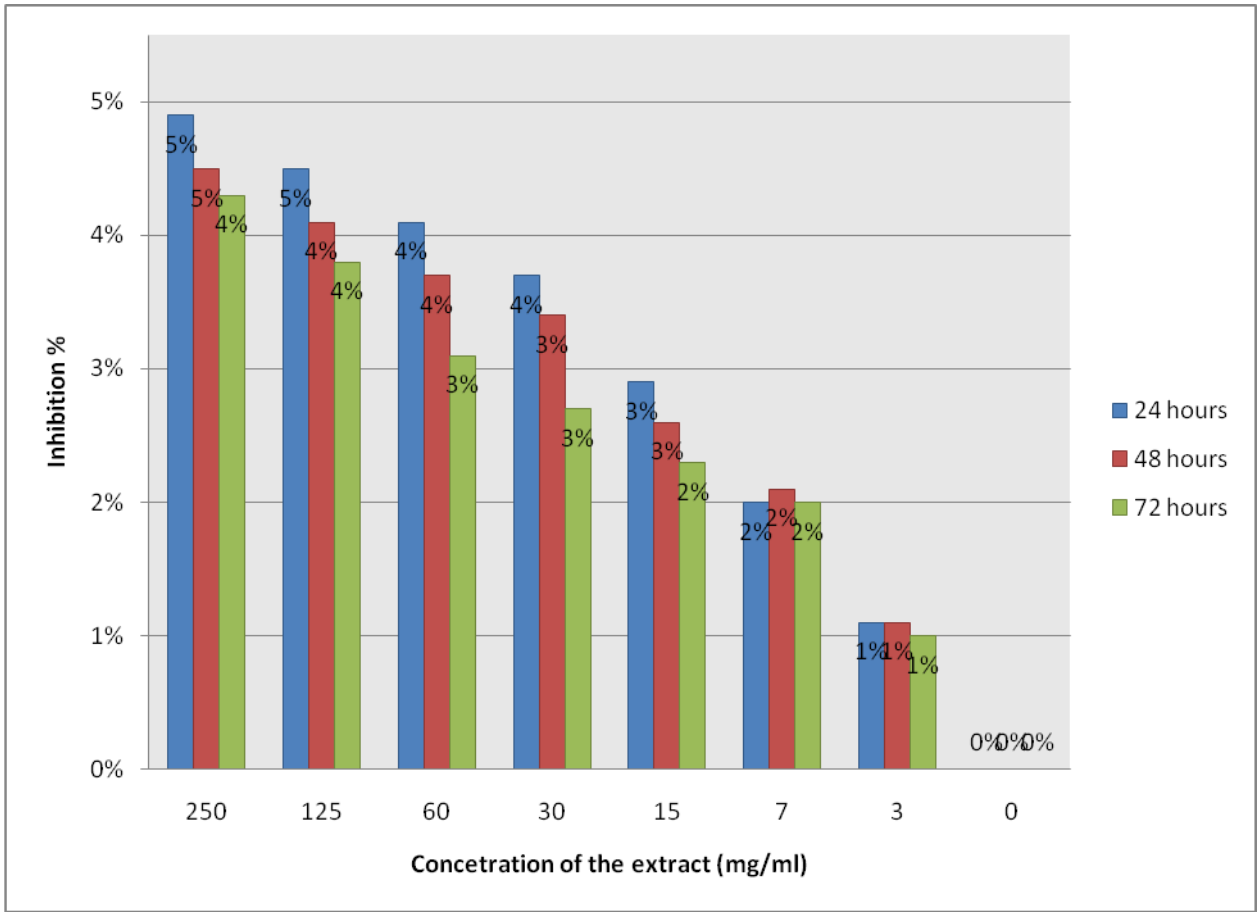

Figure (3-5) Effect of the ethanolic crude extract of greater galangal roots on inhibition of REF cells (n=3).

According to these results it was found that reduction in growth of REF cells even at the highest concentrations was less than that for the malignant cell lines used in this study. This could be considered as a further indication of the relative safety of ethanolic crude extracts of greater galangal roots towards normal cells. Beljanski (2000) reported that PB-100, a purified, well-defined extract of *Pao pereira* (Apocynaceae), selectively destroyed cancer cells; yet it did not inhibit normal (non-malignant) cell multiplication. He attributed the selectivity of this anticancer agent to its preferential binding to purine-rich nucleic acid stretches. However, prevention of entry of this agent into normal cell was not explained, though it could be due to the difference in membrane properties between cancer and normal cells.

Sanguinarine, derived from the root of *Sanguinaria canadendid*, was tested for antiproliferative and apoptotic potential against human epidermoid carcinoma (A431) cells and normal human epidermal keratinocytes (NHEKs). Sanguinarine treatment was found to result in a dose-dependent decrease in the viability of (A431) cells as well as (NHEKs) albeit at different levels because Sanguinarine-mediated loss of viability occurred at lower does and was much more pronounced in the (A431) carcinoma cells than in the normal keratinocytes. Sanguinarine treatment of (A431) cells resulted in didn't result in apoptosis of (NHEKs) even at the very high dose of 10 μM (Ahmed *et al*., 2000).

An ethanol extract of A. galanga was administered to mice. This treatment did not affect the number of micronucleated polychromatic erythrocytes (PCE) in bone marrow cells, alter the protein and nucleic acid contents in liver and testes, or cause mitodepression of bone marrow. Cyclophosphamide (CP) induced micronucleated PCE, and A. galanga treatment significantly reduced this effect without altering cytotoxicity. The minimum effective dose of A. galanga extract required to produce this effect was 125 mg/kg body weight. Biochemical changes caused by CP-treatment in the liver of treated animals were also significantly inhibited by *A. galanga* treatment (Qureshi *et al.*, 1994).

Referring to the safety of ethanolic crude extracts of greater galangal roots towards normal cells, it was used as food additives in cooking, traditional medicine (assist in for digestion, appetite, antifungal and anti aching), anti tumor activity, anti inflammatory activity and some immunological activities.

The table (3-1) shows the inhibition percentage of the ethanolic crude extract of greater galangal roots on the cell lines used in the study.

Table (3-1) Inhibition percentage of the ethanolic crude extract of greater galangal roots on studied cell lines.

Cell line	**Inhibition%**
AMN_3	98.8
RD	98.7
HEp-2	98.3
$AMGM_5$	98.2
REF	4.3

Conclusions and Recommendations

Conclusions

1. Ethanolic crude extract of greater galangal roots at higher concentrations exert a direct cytotoxic effect on studied malignant cell lines and safe towards the studied non- malignant cells.
2. Ethanolic crude extract of greater galangal has a potent cytotoxic activity in time and concentration manners.
3. AMN_3 cell line is the most sensitive to the inhibitory effects of ethanolic crude extract of greater galangal roots while $AMGM_5$ cell line is the least sensitive to the inhibitory effects of the ethanolic crude extracts of greater galangal roots.

Recommendations

1. Study the inhibitory effect of greater galangal on other cell lines *in vivo.*
2. Purification and characterization of the active compounds in this plant.
3. Study the molecular basis (metabolism and catabolism) of these active components on cancer cells.
4. Study the synergistic effect of aqueous and ethanolic extracts in combinations with other anticancer drugs *in vivo and in vitro.*

References

References

Abdul-Majeed, M.R. (2000). Induction and characterization of SU. 99 plasmacytoma cell line and its effect on mice immune response. pH.D thesis, Dept. of Biotechnology, Al-Nahrain University.

Abuharfeil, N. M., Maraqa, A. and Von Kleist, S. (2000). Augmentation of natural killer cell activity *in vitro* against tumor cells by wild plants Jordan. J. Ethnopharmacol., 71: 55-63.

Afreen, Z., Owen, H. and Plimmer, J. I. (1995). A double - blind randomizes controlled trial of ginger for the prevention of post operation nausea and vomiting. Anaesth. Intensive Care, 23: 449-452.

Ahmed, N., Gupta, S., Husain, M. M., Heiskanen, K. M. and Mukhtar, H. (2000). Differential antiproliferative and apoptotic response of sanguinarine for cancer cells versus normal cells. Clin. Cancer. Res., 6: 1524-1528.

Akhtar, M. S.; Khan, M.A. and Malik, M. T. (2002). Hypoglycemic activity of *Alpinia galangal* rhizome and its extracts in rabbits. Fitoterapia, 73: 623-628.

Al-Atby, S. M. H. (2001). Effect of Crude Alcoholic Extract of *Withania somnifera* Dun on Growth of Cancer Cell Line *in vitro* and on Some Physiological Parameters in Mice, pH.D. Thesis, college of Veterinary Medicine, University of Baghdad, Baghdad, Iraq.

Al Hilli, Z. A. (2004). Effect of *Cyperus rotundus* L. Crude Extracts on Cancer Cell Lines. M.Sc. Thesis, College of Science, University of Baghdad.

Al-Shamery, A. M. H. (2003). The Study of Newcastle Disease Virus Effect in the Treatment of Transplanted Tumors in Mice. M.Sc. Thesis, College of Veterinary Medicine, University of Baghdad.

American Cancer Society, (2005). Cancer Facts and Figures. Americ. Cancer Soc.., Atlanta.

Ames, B. N., Gold, L. S. and Willett, W. C. (1995). The Causes and prevention of Cancer. Proc. Natl. Acad. Sci. USA, 92: 5258-5265.

Anonymous (a), (1962). Wealth of India (Raw Materials). CSIR: New Delhi, A1: 195-199.

Anonymous (b), (2005). Spice world directory Spice Description and Uses. Accessed 10/20/2005. http://www.harvesfields.ca/. (Internet).

Awang, A. (1992). Ginger. Canadian Pharmaceut. Plant, 5: 309-311.

Barba, M., McCann, S. E., Schunemann, H. J., Stranges, S., Fuhrman, B., De Placido, S., Carruba, G., Freudenheim, J. L., Trevisan, M., Russell, M., Nochajski, T. and Muti, P. (2004). Lifetime Total and Beverage Specific-Alcohol Intake and Prostate Cancer Risk: A Case-Control Study. Nutr. J., 3: 23-30.

Bas, Buero-de-Mesquita, H., ocke, M. C. and Kromhout, D. (1997). Epidemiology of Vegetables and Fruits in Cancer Prevention. In: H. ohigashi, T. Osawa, J. Terao, S. Watanabe, T. Yoshikawa (Eds.), Food Factors for Cancer Prevention. Sringer-Verlag, Tokyo. Pp: 14-20.

Beljanski, M. (2000). The anticancer agent PB-100 selectivity active malignant cell inhibits multiplication of sixteen malignant cell lines, even multidrug resistant. Genet. Mol. Bio., 23: 224-235.

Berzofsky, J. A., Terabe, M., Oh, S. K., Belyakov, I. M., Ahlers, J. D., Janik, J. E. and Morris, J. C. (2004). Progress on New Vaccine Strategies for the Immunotherapy and prevention of Cancer. Clin. Invest., 113(11): 1515-1525.

Betancur-Galvis, l. A., Morals, G. E., Forero, J. E. and Roldan, J. (2002). Cytotoxic and Antiviral Activity of Colomian Medicinal Plant Extract of the *Euphorbia* genus. Mem. Inst. Oswaldo Cruz, Rio de Janeiro, 97(4): 541-546.

Burtt, B. L. and Smith, R. M. (1972). Key species in the taxonomic history of Zingiberaceace. Notes Roy. Bot. Gard, Edinburgh, 31: 177-227.

Charles, D. J., Simon, J. E. and Singh, N. K. (1992). The essential oil of *Alpinia galanga*. Willd. J. Essent. Oil Res., 11: 719-723.

Cheah, P. B. and Gan, S. P. (2000). Antioxidative/Antimicrobial effects of galangal and alpha tocopherol in minced beef. J. Food Prot., 63: 404-407.

Chiang, L.-C; Chiang, W.; Chang, M.-Y. and Lin, C.-C. (2003). In Vitro cytotoxic, Antiviral and Immunomodulatory effects of Plantago major and American Journal of Chines Medicine, 2: 225-234.

Cole. S. P. C. (1986). Rapid Chemosensitivity Testing of Human Lung Tumor Cells Using the MTT Assay. Cancer Chemother. Pharmacol., 17: 259-263.

Cooper, E.L. (1997). Complementary on CAM and NK cells by Kazuyoshi Takada and Ko Okumura. eCAM., 1:29-34.

Costa Rosa, L. F. B. P. (2004). Exercise as a Time- Conditioning Effecter in Chronic Disease: a Complementary Treatments Strategy. eCAM, 1(1): 63-70.

Craig, W. J. (1999). Health-Promoting Properties of Common Herbs. Am. J. Clin. Nutr., 70(1): 491S-499S.

Craig, W. J. (1997). Photochemical: Guardians of Our Health. Am. Diet. Assoc., 97(10): S199-S204.

Delgado, J. N. and Remers, W.A. (1991): Wilson and Gisvold Text Book of Organic Medicinal and pharmaceutical Chemistry. J.B. Lippincott Company, Philadelphia**.**

De Pooter, H. L., Nor Omar, M., Coolsate, B. A. and Schamp, N. M. (1985). The essential oil of greater galangal (*Alpinia galanga*) from Malaysia. Phytochemistry, 24: 93-96.

De Vita Jr., V. T. (1997). Principles of Cancer Management: Chemotherapy. In: V. T. DeVita, Jr., S. Hellman, S. A. Rosenberg (Eds), Cancer: principles and Practice on Oncology. 5th ed., Lippincott-Raven Publishers, Philadelphia. Pp: 333-347.

Dixon, R. A. (2004). Phytoestrogens. Annu. Rev. plant Physiol. Plant Mol. Biol., 55: 225-261.

Enzinger, F. M. and Weiss, S. W. (1983). Soft Tissue Tumors. C. V. Mosby Company, St. Louis. Pp: 325-337.

Franz, G. (1989). Polysaccharides in pharmacy: current application and future concepts. Planta Med., 55: 493-497.

Freshney, R. I. (2000). Application of Cell Cultures to Toxicology. Cell Biol. Toxicol., 17: 213-230.

Freshney, R. I. (2000) (a). Culture of Animal Cells: A Manual of Basic Technique. B 4th ed. Wiley-Liss, New York. Pp: 329-344.

Freshney , R. I. (2000) (b). Introduction to Basic Principles. In: J. R. W. Masters (ED.), Animal Cell Culture: A Practical Approach. Oxford University Press, Oxford. Pp: 1-18.

Frondosa, C. G.; Frazier, C., Folotsky, A., Hungerford, D. S. and Weidner, M. S. (2000). Inhibition of chondrocyte and synoviocyte TNFα expression by hydroxyl-alcoxy-phenyl compounds (HAPC). Trans. Orthop. Soc. P: 1038.

Ganguly, N. K., Medappa, N. and Srivastava, V. R. (2003). Ginger: its role in xenobiotic metabolism. ICMR Bulletin, 33(6): 57-58.

Gebhardt, R. (2000). *In vitro* Screening of Plant Extract and phytopharmaceuticals: Novel Approaches for the Elucidation of Active Compounds and their Mechanisms. Planta Med., 66: 99-105.

Gentschev, I., Fensterle, J., Schmidt, A., Potapenko, T., Troppmair, J., Goebel, W., Rapp, U. R. (2005). Use of a Recombinant *Salmonella* enteric serovar Typhimurium Strain Expressing C-Raf for Protection against C-Raf induced Lung Adenoma in Mice. BMC Cancer, 5: 15-23.

Guess, H. A. (1994). The Epidemiology and Natural History of Benign Prostatic Hyperplasia. In: G. D. Chisholm (Ed.), Handbook on Benign Prostatic Hyperplasia, Raven Press, New York. Pp: 1-18.

Guo, J. M., Kang, G. Z., Xiao, B. X., Liu, D. H. and Zhang, S. (2004). Effect of Daidzein on Cell Growth, Cell Cycle, and Telomerase Activity of Human Cervical Cancer *in vitro*. Int. J. Gynecol. Cancer, 14: 882-888.

Hall, E. J. (1997). Etiology of Cancer: Physical Factors. In: V. T. DeVita, Jr., S. Hellman, S. A. Rosenberg (Eds.) Cancer: Principles and Practice of Oncology. 5th ed., Lippincott-Raven Publishers, Philadelphia. P: 203-218.

Hanahan, D. and Weinberg, R. A. (2000). The Hallmarks of Cancer. Cell, 100: 57-70.

Heal, C. and Allsop. M. (1983). Cooking with Spices. David and Charles, Vermont, USA.

Hellman, S. (1997). Principles of Cancer Management: Radiation Therapy. In: V. T. DeVita, Jr., S. Hellman, S. A. Rosenberg (EDs.), Cancer: Principles and Practice of Oncology. 5th ed., Lippincott- Reven Publishers, Philadelphia. Pp: 307-332.

Henderson, B. E.; Bernstein, L. and Ross, L. (1997). Etiology of Cancer: Chemical Factors. In: V. T. DeVita, Jr., S. Hellman and S. A. Rosenberg

(EDs.), Cancer: Principles and Practice of Oncology. 5th ed., Lippincott-Reven Publishers, Philadelphia. Pp: 219-229.

Hoeijmakers, J.H., Genome maintenance mechanisms for preventing cancer. Nature, 2001.

Huang, K. C. (1999). The Pharmacology of Chinese Herps. 2nd ed. CRC Press, Boca Raton, USA.

Hui, Y. – G., Yun, Y. and Won, J. (2004). Rosmarinic Acid Induced p56 lck-Dependent Apoptosis in Jurkat and Peripheral T Cells via Mitochondrial Pathway Independent from Fas/Fas Ligand Interaction. J Immunol., 172: 79-87.

Iraqi Cancer Board, (2000). Results of the Iraqi Cancer Registry. Iraqi Cancer Registry Center, Ministry of Health, Baghdad, Iraq.

Itokawa, H., Mortia, H., Sumitomo, T., Totsuka, N. and Takeya, K. (1987). Antitumor principles from *Alpinia galanga*. Planta Med., 53: 32-33.

Jacobs, J. J. (1997). Unproven Alternative Methods of Cancer treatment. In: V. T. DeVita, Jr., S. Hellman and S. A. Rosenberg (EDs.), Cancer: Principles and Practice of Oncology. 5th ed., Lippincott- Reven Publishers, Philadelphia, 71: 395-399.

Janssen, A. M. and Scheffer, J. J. C. (1985). Acetoxychavicol acetate, an antifungal component of *Alpinia galanga*. Planta Med., 6: 507-511.

Jantan, B. I., Yassin, M. S. M.; Chin, B. C., Chen, L. L. and Sim, N. L. (2003). Antifungal activity of the essential oils of nine *Zingiberaceace* species. Pharmacol. Biol., 5(41): 392-397.

Jantan, I. B., Ahmed, F. B. and Ahmed, A. S. (2004). Constituents of the rhizome and seed oils of greater galangal *Alpinia galanga* (L.) Willd. From Malaysia, J. Essent. Oil Res., 16: 172-176.

Jennen, C. and Uhlenbruck, G. (2004). Exercise and Life-Satisfactory-Fitness: Complementary Strategies in the Prevention and Rehabilitation of Illnesses. eCAM, 1(2): 157- 165.

Jirovetz, L., Buchbauer, G., Shafi, M. P., and leela, N. K. (2003). Analysis of the essential oils of leaves, stem, rhizomes and roots of the plant *Alpinia galanga* from southern India. Acta Pharm., 53: 73-81.

Junqueira, L. C., Carneiro, J. and Kelley, R. O. (1998). Basic Histology. 9th ed. Appleton and Lange, Norwalk, California. P: 61.

Katz, A.E. (2002). Flavonoid and botanical approaches to prostate health. The journal of Alternative and Complementary Medicine, 8: 813-821.

Kawabata, K.; Tanaka, T.; Yamamoto, T.; Ushida, J.; Hara, A.; Murakami, A.; Koshimizu, K.; Ohigashi, H.; Stoner, S.D. and Mori, H. (2000). Suppression of N-nitrosomethylbenzylamine-induced rat esophageal tumorigenesis by dietary feeding of 1'-acetoxychavicol acetate. Jpn. J. Cancer Res., 91:148-155.

Kawabata, K., Tanaka, T., Yamamoto, T. & 7 other authors (2000). Suppression of N-nitrosomethylbenzylamine-induced rat esophageal tumorigenesis by dietary feeding of 19-acetoxychavicol acetate. Jpn J Cancer Res 91, 148–155. Kobayashi, Y., Nakae, D., Akai, H. & 9 other authors (1998).

Khan, M. M. A., Ara, I., Qureshi, I. A., Latif. M. S. H, Akhtar, M. S., Malik, T. M. and Hasan, M. (2000). Hypoglycemic effect of *Alpinia galanga*

rhizome on normal and alloxan-treated diabetic rabbits. J. Rawal. Med. Coll. Dec., 4(1-2): 3-10.

Kohl, N. E.; Emini, E. A. and Schlief, W. A. (1988). Active human immunodeficiency virus protease is required for viral infectivity. Porc. Natl. Acad. Sci., 85: 4686- 4690.

Kondo, A., Ohigashi, H., Murakami, A., Suratwadee, J. and Koahimitzu, K. (1993). 1'-Acetoxychavicol acetate as a potent inhibitor of tumor promoter-induced Epstein - Barr virus activation from *Languas galanga*, traditional Thai Condiment. Biosci. Biotechnol. Biochem., 57: 1344-1345.

Krishnaswamy, K. and Polasa, K. (2001). Non- nutrient and cancer prevention. ICMR Bull., Vol. 31: 1-2.

Kubota, K., Ueda, Y., Yasuda, M. and Masuda, A. (2001). Occurrence and antioxidative activity of 1'-acetoxychavicol acetate and its related compounds in the rhizomes of *Alpinia galanga* during cooking. Pec. Publ. R. Soc. Chem., 274: 601-607.

Kumar, V., Cotran, R. S. and Robbins, S. L. (2003). Robbins Basic Pathology. 7th ed. W. B. Sauanders Co., Philadelphia. Pp: 165-210.

Langner, E. S., griefenberg, S. and Gruenwald, J. I. (1997). Gimger. A medicinal plant with a history. Balance Z. Prax. Wiss Kpmplementarer Ther., 1: 5-16.

Lee, J. – Y. Hwang, W. – I. and Lin, S. – T. (2004). Antioxidant and Anticancer Activities of Organic Extracts from *Platycodon gradiflorum* A. De Candolle Roots. J. Ethnopharmacol., 93: 409- 415.

Lee, T. – H., Elledge. S. J. and Butel, J. S. (1999). Hepatitis B Virus X protein Interacts with a probable Cellular DNA Repair Protein. J. Virol., 69(2): 1107-1114.

Lehne, R. A. (1998). Other NSAIDs that inhibit cyclooxygenase-1 and cyclooxygenase-2. In: Pharmacology for Nursing Care. 3rd ed., Philadelphia, Pa: W. B. Saunders. Pp: 700-704.

Lehne, R. A. (2001). Pharmacology for Nursing Care. 4th ed. W. B. Saunders Company, Philadelphia. Pp: 1101-1140.

Lin, Y. L. and Lin J. K. (1997). Epigallocatechin-3-gallate blocks the induction of nitric oxide synthase by down - regulating Lipopolysaccharide-induced activity oftranscription factor nuclear factor- kB. Mol. Pharmacol., 52: 465-472.

Lips, J. and Kaina, B. (2001). DNA Double-Strand breaks Trigger Apoptosis in p53-deficient Fibroblasts. Carcinogenesis, 22(4): 579-585.

Lopez-Lazaro, M. (2002). Flavonoids as Anticancer Agents: Structure-Activity Relationship Study. Curr. Med. Chem.-Anti Cancer Agents, 2: 691-714.

Lust, j. (1984). The Herp Book. Bantam Books, New York, USA.

Mahony, D. E., Gilliatt, E., Dawson, S. Stockdale, E. and Lee, S. H. S. (1989). Vero Cell Assay for Rapid Detection of Clostridiumigashi, T. Osawa, J. Terao, S. Watanabe and T. Yoshikawa (Eds.) perfringens Enterotoxin. Appl. Environ. Microbial., 55(9): 2141- 2143.

Matsuda, H., Morikawa, T., Managi, H. & Yoshikawa, M. (2003). Antiallergic principles from A. galanga: structural requirements of phenylpropanoids for inhibition of degranulation and release of TNF-a and IL-4 in RBL-2H3 cells. Bioorg Med Chem Lett 13, 3197–3202.

Matsumoto, T., Matsumoto, A., ToKuda, H. and Nishino, H. (1997). Antitumor Promoters from Plants Used as Condiments or Rarnishes. In: H.

Ohigashi, T. Osawa, J. Terao, S. Watanabe and T. Yoshikawa (Eds.), Food Factors for Cancer Prevention. Springer-Verlag, Tokyo. P: 265-269.

McAllister, R. M., Melnyk, J., Finklestein, J. Z., Adams, E. C. and Gardner, M. B. (1969). Cultivation *in vitro* of Cells Derived from a Human Rhabdomyosarcoma. Cancer, 24(3): 520-526.

Miyauchi, M., Nishikawa, A., Furukawa, F., Nakamura, H., Son, H.-Y., Murakami, A., Koshimizu, K., Ohigashi, H. & Hirose, M. (2000). Inhibitory effects of 19-acetoxychavicol acetate on N-nitrosobis(2- oxopropyl)-amine-induced initiation of cholangiocarcinogenesis in Syrian hamsters. Jpn J Cancer Res 91, 477–481.

Moffatt J., Hashimoto, M, Kojima, A., Pare Kennedy, D., Murakami, A., Koshimizu, K., Ohigashi, H. and Matsui-Yuasa' I. (2000). Apoptosis induced by 1'-acetoxychavicol acetate in Ehrlich ascites tumor cells is associated with modulating of polyamine metabolism and caspase-3 activation. Carcinogenesis, 21(12): 2151-2157.

Moore, A.E; Sabachewosky, L. and Toolan, H.W. (1955). Culture Characteristics of Four Permanent Lines of Human Cancer Cells. Cancer Res., 15(8): 598-605.

Mosmann, T. (1983). Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxicity Assays. J. Immunol. Methods, 65: 55-63.

Mukherjee, A., Basu S., Sarkar N and Ghosh A. Advances in cancer therapy with plant based natural products. Current Medicinal Chemistry. (2001). 8: 1467-1486.

Muller, S. O., Schmitt M., Dekant W.,Stooper H., Schlatter J., Schreier P., Lutz W. K. (1999). Occurrence of emodin chrysophanol and physcion in

vegetables herbs and liquors. Genotoxicity ant- genotoxicity of the anthraquinones and of the whole plants. Food Chem. Toxicol. 37: 481-491.

Murakami, A., Ohura, Y., Nakamura, Y., Koshimizu, K. and Ohigashi, H. (1996). 1'-Acetoxychavicol acetate, a superoxide anion generation inhibitor, potently inhibits tumor promotion by 12-O-tetradecanoylphorbol-13-acetate in ICR mouse skin. Oncology, 53: 386-391.

Murakami, A., Koshimizu, K., Ohigashi, H. & Hirose, M. (2000). Inhibitory effects of 19-acetoxychavicol acetate on N-nitrosobis(2- oxopropyl)-amine-induced initiation of cholangiocarcinogenesis in Syrian hamsters. Jpn J Cancer Res 91, 477–481.

Murray, R. K. (1996). Cancer, Cancer Genes, and growth Factors. In: R. K. Murry, D. K. Granner, P. A. Mayes, V. W. Rodwell (Eds.), Harper's Biochemistry. 24th ed. Appleton and Lange, Stanford. Pp: 757-778.

Nadkarni, K. M. (1976). The Indian Material Medic, 3rd .Vol.1/ Popular Prakahan, Bombay.

Nakamura, Y, Murakami, A., Ohto, Y., Torikai, K., Tanaka, T. and Ohigashi, H. (1998). Suppression of tumor promoter-induced oxidative stress and inflammatory responses in mouse skin by a superoxide generation inhibitor 1'-acetoxychavicol acetate. Cancer Res., 58: 4832-4839.

Nath, J. and Krishna, G. (1997). Fundamental and Applied Genetic Toxicology. In: C. R. Craig and R. E. Stitzel (Eds.). Modern Pharmacology with Clinical Applications. 5th ed. Little Brown and Company, Boston. Pp: 69-78.

Norbury, C. and P. Nurse (1992). Animal cell cycles and their control. Annu Rev Biochem.

Noro, T., SekiyaKuroyanagi, M., Ueno, A. and Fukushima, S. (1998). Inhibitors of xanthine oxidase from *Alpinia galanga*. Chem. Pharm. Bull., 36: 244-248.

Ohata, T., Fukuda K., Murakami, A., Ohigashi H. Sugimura, T. and Wakabayashi, K. (1998). Inhibition by 1'-acetoxychavicol acetate of Lipopolysaccharide- and interferon-gamma-induced nitric oxide production through suppression of inducible nitric oxide synthase gene expression in RAW264 cells. Carcinogenesis, 19: 1007-1012.

Ohnishi, M., Tanaka, T. and Makita, H. (1996). Chemopreventive effect of a xanthine oxidase inhibitor, 1'-acetoxychavicol acetate, on rat oral carcinogenesis. Jon. J. Cancer Res., 87: 349-356.

Ohnishi, M., Tanaka, T., Makita, H. & 7 other authors (1996). Chemopreventive effect of a xanthine oxidase inhibitor, 19-acetoxychavicol acetate, on rat oral carcinogenesis. Jpn J Cancer Res 87, 349–356.

Okuda, T. (1997). Structure-Activity Relationship of Antioxidant and Antitumor Polyphenols. In: H. Ohigashi, T. Osawa, J. Terao, S. Watanabe, T. Yoshikawa (Eds.), Food Factors for Cancer Prevention. Springer-Verlag, Tokyo. Pp: 280-285.

Pagliacci, M., Smacchia, M, Migliorati, Grignani, F., Riccardi, C. Nicoletti, I. (1994). Growth-inhibitory effects of the natural Phytoestrogens genistein in MCF- 7 human breast cancer cells. Eur J. Cancer, 30 A (11): 1675-1682.

Palittapongarnpim, P., Kirdmanee, C., Kittakoop, P. & Rukseree, K. (2002). 19-Acetoxychavicol Acetate for Tuberculosis Treatment. US Patent Application no. 2002192262.

Panaro, N. J., Popescu, N. C., Harris, S. R. and thorgerisson, U. P. (1999). Flavone Acetic Acid Induces a G2/M Cell Cycle Arrest in Mammary Carcinoma Cells. Br. J. Cancer, 80(12): 1905-1911.

Perez-Serrano, J.; Denegri, G,; Casado, N. and Fodriguez-Cabaeiro (1997) *In vivo* effect of oral albendazole and albendazole sulphoxide on development of secondary echinococcosij in mice. Int. J. for Parasitology, 27: 1341-1345.

Parker, R. G. (2000). Radiation Oncology. In: Manual of Clinical Oncology. 4th ed. D. A. Casciato and B. B. Lowits (Eds.). Lippincott Williams and Wilkins, Philadelphia. Pp: 43-47.

Perry, L. and Metzger, J. (1980). Medical Plant of East and Southeast Asia. MIT Press, Cambridge, MA, USA. Pp: 436-444.

Phillips, D. H. (2002). Smoking-Related DNA and Protein Adducts in Human Tissues. Carcinogenesis, 23(12): 1979-2004.

Purseglove, J. W. (1981): *Spices*, Vol. 2, Longman Inc., New York, P. 543.

Qureshi, S; Shah, A. H. and Ageel, A.M. (1992): Toxicity studies on *Alpinia galanga* and *Curcuma long*, Planta med., 85(2): 124-127

Raina, V. K., Srivasta, S. K. and Symasuunder, K. V. (2002). The essential oil of greater galangal *Alpinia galangal* (L.) Wild from the lower Himalayan region of India, Flavor. Frager. J., 17: 358-360.

Ravindran, P. N. and Balachandran, I. (2004). Underutilized medicinal spices. Spices Indi, VII. 12: 2-4.

Rhizoma galanga, (1990). Koln: Bundesanzeiger [Federal German Legal Gazette]. Commission S monograph no. Pp: 173.

Rosenberg, S. A. (1997). Principles of Cancer Management: Surgical Oncology. In: V. T. DeVita, Jr., S. Hellman, S. A. Rosenberg (Eds.), Cancer:

Principles and Practice of Oncology. 5th ed., Lippincott-Raven Publishers, Philadelphia. Pp: 295-306.

Rosengarten, Jr. F. (1969). The Book of Spices. Livingston Publishing Co., Penn., USA.

Sa'eed, O. F. (2004). The Effect of Green and Black Tea Extracts on Different Cell Lines *in vitro*. M. Sc. Thesis, College of Pharmacy, University of Mosul, Mosul, Iraq.

Sasaki, S. and Fukuda, N. (1999). Dose-Response Relationship for Induction of Solid Tumors in Female B6C3F1 Mice Irradiated Neonatally with a Single Dose of Gamma Rays. J. Radiate. Res., 40: 229-241.

Sastry, M. S. (1960). Chemical examination of *Alpinia galanga* (L.) Willd. Ind. J. Pharm, 23(3): 76.

Scheffer, J. J. C.; Gani, A. and Baertheim- Svendsen (1981). Monoterpenes of the essential rhizomal oil of *Alpinia galanga* (L.) Willd. Sci. Pharm., 49: 337-346.

Senn, J. and Schneider, R. J. (1995). Hepatitis B Virus HBx Protein Deregulates Cell Cycle Checkpoint Controls. Proc. Natl. Acad. Sci. USA, 92: 11215-11219.

Shen, J., Andersen, R., Albert, P. S., Wenger, N., Glaspy, J., Cole, M. and Shekelle, P. (2004). Use of Complementary/Alternative Therapies by Women with Advanced- Stage Breast Cancer. BMC Complement. Alternative Med., 2: 8-13.

Sherr, C.J. (2000). The Pezcoller lecture: cancer cell cycles revisited. Cancer Res.

Sirirugsa, P. (1998). Thai Zingiberaceace: species diversity and their uses. Pure Appl. Chem., Vol. 70 (II). URT: http://www.iupac.org/symposia/phuket97/sirirugosa.html1999IUPAC. (Internet).

Smit, H. F., Woerdebag, H. J., Singh, R. H,; Meulenbeld, G. J., Labadie, R. P. and Zwaving, J. H. (1995). Ayurvedic Herbal Drugs with Poddible Cytostatic Activity. J. Ethnopharmacol., 47: 75-84.

Smith-Jones, P. M. (2004). Radioimmunotherapy of prostate cancer. Q. J. Nucl. Med. Mol. Imaging, 48: 297-304.

Spaulding-Albright, N. (1997). A Review of Some Herbal and Related Products Commonly Used in Cancer Patients. J. Am. Diet. Assoc., 97(10): S208-S215.

Steel, G. G. (1995). The Biological Basis of Radiotherapy. In: Oxford Textbook of Oncology. M. Peckham, H. Pinedo and U. Veronesi (Eds.). Oxford University press, Oxford. Pp: 668-680.

Stewart, J. J., Wood M. J. and Wood C. D. (1991). Effect of ginger on motion sickness susceptibility and gastric function. Pharmacology, 42: 111-120.

Stuart, M. (1987), The Encyclopedia of Herbs and Herbalism Macdonald and Co, Turin, Italy.

Stupp, R., Mason, W. P., Van Den Bent, M. J., Weller, M., Fisher, B., Taphoorn, M. J. B., Belanger, K., Brandes, A. A., Marosi, C., Bogdahn, U., Curschmann, J., Janzer, R. C., Ludwin, S. K., Gorlia, T., Allgeier, A., Lacombe, D., Cairncross, J. G., Eisenhauer, E. and Mirimanoff, R. O. (2005). Radiotherapy Plus Concomitant and Adjuvant Temozolomide for Glioblastoma. N. Engl. J. Med., 352(10): 987-996.

Sukardiman, Darwanto, A., Tanjung, M. and Darmadi, M. O. (2000). Cytotoxic Mechanisms of Flavonoid from Temu Kunci (*Kaempferia Pandurata*) in Cell Culture of Human Mammary Carcinoma. Clin. Hemorheol. Microcircul. 23: 185-190.

Sung, N. J., Woo S.H., Cassady, J. M. Snapka, R. M. (1998). DNA polymerase and topoisomerase II inhibitors from Psoralea corylifolia. J. Natur. Products 61: 362-366.

Surh, Y. J., Chun, K. S., Cha, H. H., Keum, Y. S., Park, K. K. and Lee, S. S. (2001). Molecular Mechanisms underlying chemopreventive activities of anti-inflammatory phytochemicals: down- regulation of COX-2 and iNOS through suppression of NF-kB activation. Mutat. Res. 480-481: 243-268.

Svensson, H. and Mijnheer, B. J. (1995). Current Practice of Radiotherapy: Physical Basis. In: M. Peckham, H. Pinedo and U. Veronesi (Eds.), Oxford Tectbook of Oncology. Oxford University Press, Oxford. Pp: 680-702.

Takimoto, C. H. (2003). Anticancer Drug Development at the US National Cancer Institute. Cancer Chemother. Pharmacol., 52(1): S29-S33.

Tanaka, T., Makita, H., Kawamori, T., Kawamori, T., Kawabata, K., Mori, H., Murakami, A., Satph, K., Hara, A., Ohigashi, H. and Koshimizu, K. (1997). A xanthine oxidase inhibitor 1'-acetoxychavicol acetste inhibits azoxymethane-induced colonic aberrant crypt foci in rats. Carcinogenesis, 18: 1113-1118.

Tanaka, T., Kawabata, K., Kakumoto, M. & 8 other authors (1997a). Chemoprevention of azoxymethane-induced rat colon carcinogenesis by a xanthine oxidase inhibitor, 19-acetoxychavicol acetate. Jpn J Cancer Res 88, 821–830. Tanaka, T., Makita, H., Kawamori, T. & 7 other authors (1997b). A

xanthine oxidase inhibitor 19-acetoxychavicol acetate inhibits azoxymethane-induced colonic aberrant crypt foci in rats. Carcinogenesis 18, 1113–1118.

Terry, M. B., Neugut, A. I., Mansukhani, M., Waye, J., Harpaz, N. and Hibshoosh, H. (2003). Tobacco, Alcohol andp53 Overexpression in Early Colorectal Neoplasia. BMC Cancer, 3: 29-37.

Tewtrakul, S., Subhadhirasakul, S. and Kummee, S. (2003). HIV-1 protease inhibitory effects of medicinal plants used as self medication by AIDS patients. J. Sci. Technol., 25(2): 239-243.

Tsuda, H., Ohshima, Y., Nomoto, H., Fujita, K., Matsudo, E., Iigo, M., Takasuka, N. and Moore, M. A. (2004). Cancer Prevention by Natural Compounds. Drug Metab. Pharmacokin., 19(4): 245-263.

Vimala, S., Norhanom, A. W. and Yadav, M. (1999). Anti-tumor promoter activity in Malaysian ginger rhizoma used in traditional medicine. Br. J. Cancer, 80(1-2): 110-116

Vineis, P., Airoldi, L., Veglia, F., Olgiati, L., Pastorelli, R., Autrup, H., Dunning, A., Garte, S., Gormally, E., Hainaut, P., Malaveille, C., Matullo, G., Peluso, M., Overvad, K., Tjonneland, A., Clavel-Chapelon, F., Boeing, H., Krogh, V., Palli, D., Panico, S., Tumino, R., Bueno-De-Mesquita, B., Peeters, P., Berglund, G., Hallmans. G., Saracci, R. and Riboli, E. (2005). Environmental Tobacco Smoke and Risk of respiratory Cancer and Chronic Obstructive Pulmonary Disease in Former Smokers and Never Smokers in the EPIC Prospective Study. BMJ, 330: 277-281.

Vickers, A. and Zollman, C. (1999) (a). ABC of Complementary Medicine: Acupuncture. BMJ, 319: 973-976.

Vickers, A. and Zollman, C. (1999) (b). ABC of Complementary Medicine: Acupuncture. BMJ, 319: 1346-1349.

Vickers , A. and Zollman, C. (1999) (c). ABC of Complementary Medicine: Acupuncture. BMJ, 319: 1419-1422.

Wanger, H. (1990). Search for plant derived natural products with Immunostimulatory activity. Pure Appl. Chem., 2: 1217-1222.

Wanger, H. and Proksch, A. (1984). Immunostimulatory drugs of fungi and higher plants. In: Economic and Medicinal Plant Research. Vol. 1, Academic Press, New York, Pp: 113-153.

Watanabe, N., Kataoka, T., Tajika, T., Uramoto, M., and Nagai, K. (1995). 1'-Acetoxychavicol acetate as an inhibitor of phagocytosis of macrophages. Biosci Biotechnol. Biochem., 59: 1566-1567.

Watanabe, S., Kimira, M. and Subue, T. (1997). Diet and Cancer: Epidemiological Apptoaches. In: H. Ohigashi, T. Osawa, J. Terao, S. Watanabe, T. Yoshikawa (Eds.). Food Factors for Cancer Prevention. Springer-Verlag, Tokyo. Pp: 3-8.

Whelan, I.C. and Ryan, M.E. (2003). Ethanolic extracts of Euphorbia and other ethanobotancial species as inhibitors of human tumor cell growth Phytomedicine. 10: 53-58.

Wilson, A. P. (2000). Cytotoxicity and Viability Assays. In: J. R. W. Masters (Ed.), Animal Cell Culture: A Practice Approach. Oxford University Press, Oxford. Pp: 175-219.

Wyllie, A. D. (1992). Growth and Neoplasia. In: Muir's Textbook of Pathology. 13th ed. R. N. M. MacSween and K. Whaley (Eds.). Arnold, London. Pp: 355-410.

Yang, L. and Baltimore, D. (2005). Long-Term *in vivo* Provision of Antigen-Specific T Cell Immunity by Programming Hematopoietic Stem. PNAS, 102(12): 4518-4523.

Yaseen, N. Y. (1990). Cytogenetic Study on Human Colorectal Cancer cells. Ph. D. Thesis, University of Sheffield Medical School, Sheffield, U.K.

Yoshikawa, M., Matsuda, H. & Morikawa, T. (2004). Antiallergy agent obtained from *A. galanga* and method for production thereof. Jpn Kokai Tokkyo Koho patent no. 2004189669 (in Japanese).

Yuspa, S. H. and Shields, P. G. (1997). Etiology of Cancer: Chemical Factors. In: V. T. DeVita, Jr., S. Hellman, S. A. Rosenberg (Eds.), Cancer: Principles and Practice of Oncology. 5th ed., Lippincott-Raven Publishers, Philadelphia, Pp: 185-202.

Zheng, Q., Hirose, Y., Yoshimi, N., Murakami, A., Koshimizu, K, Ohigashi, H., Sakata, K., Matsumoto, Y., Sayama, Y. and Mori, H. (2002). Further investigation of the modifying effect of various chemopreventive agents on apoptosis and cell proliferation in human colon cancer cells. J. Cancer Res. Clin. Oncol., 128: 539-546.

Internet References

1- http://www-personal.ksu.edu/~bethmont/mutdes.html#repair, Bootsma and J. H. J. Hoeijmakers, "Engagement with transcription", Nature 363: 114-115, 1993.
"DNA repair comes into its own", Science 266: 728-730, 1994

2- http://cred.mdanderson.org/surp/seminars/Hormones_cancer.pdf

3- http://www.cancerquest.org/index.cfm?page=486

4- http://www.cancerquest.org/index.cfm?page=494&lang=english

5- http://www.cancerquest.org/index.cfm?page=522

6- http://www.breastcancer.org/treatment/hormonal/what_is_it/hormone_role.jsp

Appendices

Appendices

I. HEp-2

Effect of the alcoholic crude extract of greater galangal roots on viability of HEp-2 cells.

O.D. at 492 nm								
Day	**250µl**	**125µl**	**60 µl**	**30 µl**	**15 µl**	**7 µl**	**3 µl**	**Control**
1	0.085	0.158	0.271	0.345	0.603	0.911	1.583	1.786
2	0.047	0.078	0.146	0.211	0.410	0.558	0.632	1.712
3	0.019	0.032	0.068	0.126	0.246	0.318	0.405	1.678

Effect of the alcoholic crude extract of greater galangal roots on inhibition of HEp-2 cells.

Inhibition %								
Day	**250µl**	**125µl**	**60 µl**	**30 µl**	**15 µl**	**7 µl**	**3 µl**	**Control**
1	95.2%	91.1%	84.8%	80.6%	66.2%	48.9%	11.3%	0%
2	97.2%	95.4%	91.4%	87.6%	76%	67.4%	63%	0%
3	98.3%	98%	95.9%	92.4%	85.3%	81%	75.8%	0%

II. AMN_3

Effect of the alcoholic crude extract of greater galangal roots on viability of AMN_3 cells.

O.D. at 492 nm								
Day	**250µl**	**125µl**	**60 µl**	**30 µl**	**15 µl**	**7 µl**	**3 µl**	**Control**
1	0.092	0.164	0.283	0.358	0.621	0.926	1.641	1.895
2	0.058	0.081	0.154	0.213	0.423	0.564	0.691	1.802
3	0.021	0.043	0.075	0.135	0.254	0.325	0.410	1.772

Effect of the alcoholic crude extract of greater galangal roots inhibition of AMN_3 cells.

Inhibition %								
Day	**250µl**	**125µl**	**60 µl**	**30 µl**	**15 µl**	**7 µl**	**3 µl**	**Control**
1	95.1%	91.3%	85%	81.1%	67.2%	51.1%	13.4%	0%
2	96.7%	95.5%	91.4%	88.1%	76.5%	68.7%	61.6%	0%
3	98.8%	97.5%	95.7%	92.3%	85.6%	81.6%	76.8%	0%

III. RD

Effect of the alcoholic crude extract of greater galangal roots on viability of RD cells.

O.D. at 492 nm								
Day	**250µl**	**125µl**	**60 µl**	**30 µl**	**15 µl**	**7 µl**	**3 µl**	**Control**
1	0.076	0.148	0.264	0.337	0.612	0.914	1.628	1.882
2	0.038	0.072	0.135	0.203	0.473	0.637	1.357	1.835
3	0.022	0.045	0.076	0.152	0.311	0.427	0.738	1.775

Effect of the alcoholic crude extract of greater galangal roots on inhibition of RD cells.

Inhibition %								
Day	**250µl**	**125µl**	**60 µl**	**30 µl**	**15 µl**	**7 µl**	**3 µl**	**Control**
1	95.9%	92.1%	85.9%	82%	67.4%	51.4%	13.9%	0%
2	97.9%	96%	92.8%	88.9%	74.2%	65.2%	26%	0%
3	98.7%	97.4%	95.7%	91.4%	82.4%	75.9%	58.4%	0%

IV. $AMGM_5$

Effect of the alcoholic crude extract of greater galangal roots on viability of $AMGM_5$ cells.

O.D. at 492 nm								
Day	**250µl**	**125µl**	**60 µl**	**30 µl**	**15 µl**	**7 µl**	**3 µl**	**Control**
1	0.067	0.128	0.301	0.421	0.762	1.251	1.657	1.728
2	0.042	0.068	0.120	0.225	0.482	0.753	1.379	1.685
3	0.028	0.055	0.082	0.137	0.274	0.423	0.823	1.647

Effect of the alcoholic crude extract of greater galangal roots on inhibition of $AMGM_5$ cells.

Inhibition %								
Day	**250µl**	**125µl**	**60 µl**	**30 µl**	**15 µl**	**7 µl**	**3 µl**	**Control**
1	96.1%	92.5%	82.5%	75.6%	55.9%	27.6%	4.1%	0%
2	97.5%	95.9%	92.8%	86.6%	71.3%	55.3%	18.1%	0%
3	98.2%	96.6%	94.9%	91.6%	83.3%	74.3%	50%	0%

V. REF

Effect of the alcoholic crude extract of greater galangal roots on viability of REF cells.

O.D. at 492 nm								
Day	**250µl**	**125µl**	**60 µl**	**30 µl**	**15 µl**	**7 µl**	**3 µl**	**Control**
1	1.773	1.781	1.788	1.795	1.810	1.827	1.843	1.865
2	1.766	1.775	1.781	1.788	1.802	1.812	1.829	1.851
3	1.753	1.762	1.775	1.782	1.790	1.796	1.813	1.833

Effect of the alcoholic crude extract of greater galangal roots on inhibition of REF cells.

Inhibition %								
Day	**250µl**	**125µl**	**60 µl**	**30 µl**	**15 µl**	**7 µl**	**3 µl**	**Control**
1	4.9%	4.5%	4.1%	3.7%	2.9%	2%	1.1%	0%
2	4.5%	4.1%	3.7%	3.4%	2.6%	2.1%	1.1%	0%
3	4.3%	3.8%	3.1%	2.7%	2.3%	2%	1%	0%

VI. Effect of the alcoholic extract of greater galangal roots on viability of HEp-2 cells , AMN3 cells , RD cells , AMGM5cells , REF cells.

O.D. at 492 nm (μ±SD)								
Groups/con.	**250μl**	**125μl**	**60 μl**	**30 μl**	**15 μl**	**7 μl**	**3 μl**	**Control**
HEp-2 cells	A,a 0.050+0.033	A,a 0.089+0.063	A,a 0.161+0.102	A,a 0.227+0.110	AB,a 0.419+0.178	AB,a 0.595+0.298	B,a 0.873+0.625	C,ab 1.725+0.055
AMN3 cells	A,a 0.057+0.035	A,a 0.096+0.061	A,a 0.170+0.105	A,a 0.235+0.113	AB,a 0.432+0.183	AB,a 0.605+0.302	B,a 0.914+0.645	C,a 1.823+0.064
RD cells	A,a 0.045+0.027	A,a 0.088+0.053	A,a 0.158+0.096	A,a 0.230+0.095	AB,a 0.465+0.150	B,a 0.659+0.244	C,a 1.241+0.456	D,a 1.830+0.053
AMGM5cells	A,a 0.045+0.019	A,a 0.083+0.038	A,a 0.167+0.11	A,a 0.261+0.145	AB,a 0.506+0.244	B,a 0.809+0.416	BC,a 1.286+0.0405	C,b 1.686+0.040
REF cells	A,b 1.764+0.010	AB,b 1.772+0.009	AB,b 1.781+0.0065	B,b 1.788+0.0065	BC,b 1.800+0.010	C,b 1.811+0.015	CD,a 1.828+0.015	D,a 1.84+0.016

Different capital letters in the same column: significant difference (P≤0.05) between means.
Different small letters in the same rows: significant difference (P≤0.05) between means.

VII. Effect of the alcoholic extract of greater galangal roots on inhibition of HEp-2 cells , AMN3 cells , RD cells , AMGM5cells , REF cells.

Inhibition %(μ±SD)								
Day	**250μl**	**125μl**	**60 μl**	**30 μl**	**15 μl**	**7 μl**	**3 μl**	**Control**
HEp-2 cells	A,a 96.90+1.57	A,a 94.83+3.48	AB,a 90.70+5.58	AB,a 86.87+5.93	AB,a 75.83+9.55	B,a 65.77+16.11	B,a 50.03+34.15	C,a 0.00+0.00
AMN3 cells	A,a 96.87+1.86	A,a 94.77+3.16	AB,a 90.70+5.38	AB,a 87.17+5.66	AB,a 76.43+9.20	BC,a 67.13+15.31	C,a 50.60+33.10	D,a 0.00+0.00
RD cells	A,a 97.50+1.44	A,a 95.17+2.75	A,a 91.47+5.03	A,a 87.43+4.87	AB,a 74.67+7.51	B,a 64.17+12.28	C,a 32.77+23.01	D,a 0.00+0.00
AMGM5cells	A,a 97.27+1.07	A,a 95.00+2.19	A,a 90.07+6.64	A,a 84.60+8.19	AB,a 70.17+13.74	B,a 52.40+23.48	C,a 24.07+23.52	C,a 0.00+0.00
REF cells	A,b 4.56+0.30	AB,b 4.13+0.35	BC,b 3.63+0.50	C,b 3.26+0.51	D,b 2.60+0.30	E,b 2.03+0.05	F,a 1.06+0.05	G,a 0.00+0.00

Different capital letters in the same column: significant difference ($P \leq 0.05$) between means.
Different small letters in the same rows: significant difference ($P \leq 0.05$) between means.

وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية العلوم
قسم التقانة الأحيائية

تثبيط نمو بعض الخطوط الخلوية

باستعمال المستخلص الكحولي الخام لجذور نبات الخوانجان الكبير

(Alpinia galanga (L.) Willed)

رسالة

مقدمة الى كلية العلوم - جامعة النهرين وهي جزء من متطلبات نيل درجة ماجستير علوم في التقانة الأحيائية

من قبل

علا موسى فاضل الخفاجي

بكالوريس تقانة احيائية – جامعة النهرين 2006

كانون الثاني **محرم**

2009 **1429**

بسم ٱللَّهِ ٱلرحمَن ٱلرَّحِيم

قُل كُلٌ يَعمَلُ علَى شَاكِلَتِهِ فَرَبّكُم اعَلمُ بِمَن هُوَ اَهدّى سَبيلاً ۞ وَيَسئَلُونّك عَنِ الرُّوحِ قُلِ الرُّوحُ مِن أمرِ رَبِّي وَمَا أُوتِيتُم مِن العِلمِ اِلاّ قَلِيلاً ۞

صَدَقَ ٱللَّهُ ٱلعَظِيم

سورة الاسراء
الاية (٨٤ ـ ٨٥)

الأهداء

الى الشجرة الطيبه التي أصلها ثابت في الأرض وفرعها في السماء..

والدتي الحبيبة

الى الشمس التي نستمد منها البقاء....

والدي الغالي

الى أفلاكي اللامعه في السماء....

أخواني

الى رفيق الدرب الذي يمدني بالضياء .....

زوجي

اليكم يا كل أكواني أهدي جهدي المتواضع

علا

الخلاصة

مع إستمرار البحث عن أدوية جديدة لعلاج مرض السرطان تبرز المواد التي تقضي على الخلايا السرطانية دون التأثير على الخلايا الطبيعية بوصفها علاجات محتملة للتخلص من هذه الخلايا الخبيثة، ويمثل هذا الجهد العلمي دراسة أولية لمعرفة تأثيرات المستخلص الخام لنبات الخولنجان *Alpinia galanga* على أربعة خطوط من الخلايا السرطانية وخط الخلايا الطبيعية.

حضر المستخلص بالكحول 70%(الأثيلي) من الجذور المجففة لنبات الخولنجان، وشملت الخطوط الخلوية المدروسة خط الحنجرة البشري (Hep-2) وخط خلايا سرطان العضلات المخططة البشري (RD) وخط خلايا سرطان الأرون الدبقية البشري ($AMGM_5$) وخط خلايا سرطان الثدي للفأر (AMN_3) و خط لخلايا الجرذ الجينية مولدة الألياف (REF).

أظهر المستخلص الكحولي تأثيراً مثبطاً يعتمد على مدة التعريض وتركيز المستخلص على خطوط الخلايا السرطانية. حيث أن أعلى ثلاث تراكيز (60 و 125 و 250) مغ \ مل على خط الخلايا السرطاني HEp-2 أظهرت تأثيرا مثبطا يعتمد على مدة التعريض والتركيز بينما أعطى أعلى تركيز(250 مغ \ مل) أعلى انخفاض في الخلايا الحيوية AMN3 ، (0.021) مع تثبيط وصل (98.8 ٪) بعد 72 ساعة.

أثر المستخلص الأثيلي لجذور نبات الخولنجان على خط الخلايا RD تدريجيا مع الوقت، بينما أعطى المستخلص أعلى تأثير على الخلايا الحيوية عند أعلى تركيز(250 مغ \ مل) على خط الخلايا AMGM5، (0.028) ووصلت نسبة التثبيط إلى 98.2% بعد 72 ساعة.

وسجل أثر تثبيطي أقل للمستخلص على الخلايا REF بعد 72 ساعة مقارنتا مع خطوط الخلايا السرطانية بما فيها الخلايا الأكثر مقاومة وهي خلايا AMGM5.

يستنتج من دراسة تأثير المستخلص الأثيلي لجذور نبات الخولنجان أن خط خلايا AMN3 هو الأكثر حساسية للمستخلص بينما خط خلايا AMGM5 كان اقل حساسية للمستخلص الأثيلي.