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Cytogenetic and Cytotoxic Studies on the Effect of Phytoinvestigated Active Compounds of *Hyoscyamus niger* (*in vivo* and *ex vivo*)

A Dissertation

Submitted to the College of Science, Al-Nahrain University as a Partial fulfillment of the requirements for the Degree of Doctorate of Philosophy of Science in Biotechnology

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Summary

In this study, different extracts of *Hyoscyamus niger* were detected using different chemical methods. Alkaloids were represented the major compound in thus plant, this, it selected for further study. Genetic effects of alkaloidal extract on mice were determined using several parameters such as mitotic index (MI); micronuclei assay (MN) and chromosomal aberrations (CAs). The results showed increasing mitotic index and decreasing the spontaneous frequency of chromosomal aberrations and micronuclei. On the other hand, the free radicals scavenging activity of seven fractions of alkaloidal extract were evaluated by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay. Only one fraction of alkaloidal extract exhibited moderate free radical scavenging activity in comparison with the positive and negative controls. The cytotoxic activity of different concentrations of alkaloidal extract of *H. niger* on cancer cell lines (Hep-2, AMN-3, RD) estimated at various period of incubation (24, 48, or 72hrs).The effect of this extract was time and concentration-dependant. Remarkably, alkaloidal extract at 1000 µg\ml achieved a significant cytotoxicity on Hep-2 after 72 hrs of incubation period. Furthermore, viability assay was used to assess the cytotoxic effects of this extract on other types of cell lines (PC-3, HepG2, A549 and WRL68), obtained from Malaysia centre for natural product research and drug discovery(MCNPRD), university of Malaya, It was noticed that low decrease in cell viability of cell lines used above. In addition, the apoptotic activity of this extract on cancer cells A549 and PC-3 were examined by using multiparameter 3 kits; it was observed a changing in nuclear size, morphology, and cell membrane permeability, mitochondrial membrane potential and releasing of cytochrome C.

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List of abbreviations

Abbreviation	Meaning
A549	Human lung adenocarcinoma
AMN-3	Ahmed-Mohamed-Nahi2003
CA	Chromosomal aberration
DMSO	Dimethyl sulphaoxide
DNA	Deoxyribonucleic acid
DPPH	2,2- diphenyl-1-picrylhydrazyl
ELISA	Enzyme Linked Immunosorbent Assay
EMEM	Eagal minimum essential medium
G	Gram
H .niger	*Hyoscyamus niger*
Hep-2	Human Epidermoid Larynx Carcinoma
HepG2	Human hepatocellular carcinoma
HPLC	High performance liquid chromatography
MEF	Mouse embryonic fibroblast
MI	Mitotic index
MN	Micronucleus assay
MTT	(3-Dimethyl -2-yl)-2,5- diphenyltetrazolium bromide
PBS	Phosphate buffer saline
PC-3	Human prostatic carcinoma
PCE	Polychromatic erythrocytes
RD	Human rhabdomyosarcoma
RPMI	Rosswell park memorial institute
WRL	Human hepatic cell
WHO	World Health Organization

1.1 Introduction

Plants play an important role in the medical practices of many, if not all, peoples. Thus plants are used not only in the diagnostic process, but also in the physical and ritual purification procedures that commonly precede ceremonies and in the act of healing itself (Moerman, 1996).

Folk use of herbal remedies is familiar to all of us in some form or another. This is because herbal remedies are learned by being passed down from generation to generation. (Al-Khazraji, 1991).

The World Health Organization (WHO) has shown that about 80% of the world's population still relies on traditional medicine. This is of interest to a natural product chemist for many reasons. There is the possibility that herb used in the traditional medicine is harmful to the patient, in which case the treatment may do more harm than good. Conversely, there is the possibility that the herbs used are not effective at all. That may not be of concern for minor ailments, but in more serious cases an ineffective treatment could result in the death of the patient. Hopefully, however, the herbs used are effective. If that is the case then investigation of that remedy could be of benefit to the remaining 20% of the world's population (Farnsworth *et al.*, 1985).

Natural products still play a very important role in the medicine of the remaining 20% of the world's population. Thus percentage increases to over 60% that used natural products as anti-infective and anticancer compounds (Cragg *et al*., 1997).

Many herbs have a long history of use of claimed health benefits. However, herbal supplements and botanicals have potent

pharmacological activity and consequently, contribute to potential adverse effects and drug interactions (NCCAM, 2005).

Over 60% of currently used anti-cancer agents are derived in one way or another from natural sources, including plants, marine organisms and microorganisms (Newman *et al*., 2003).

Hartwell (1982) list more than 3000 plant species that have reportedly been used in the treatment of cancer; Graham *et al*. (2000) had added another 350 species to Hartwell's list.

Plants have been a major source of highly effective conventional drugs for the treatment of many forms of cancer, and while the actual compounds isolated from the plant frequently may not serve as the drug, they provide leads for the development of potential novel agents. The search for anti-cancer agents from plants started earliest in the 1950s with the discovery and development of the vinca alkaloids, vintblastine and vincristine, and the isolation of the cytotoxic podophyllotoxins. However, Cragg and Newman (2005) indicated that no new plant-derived clinical anti-cancer agents have, as yet, reached the stage of general use, but a number of agents are in preclinical development. As new technologies are developed, some of the agents, which failed earlier clinical studies, are now stimulating renewed interest. The ability to attach agents to carrier molecules directed to specific tumors, shows promise for effectively targeting highly cytotoxic natural products to the tumors while avoiding their toxic side-effect on normal healthy tissues.

The goal in the search for new anticancer drugs is to find drug that act via a specific mode of action. In this manner it is hoped that the cancer cells can be targeted and little or no damage to noncancerous cells are very similar. Due to the deadly nature of cancer, the FDA has allowed

drugs that are less than completely specific to be approved. Despite the potential far side effects, these drugs are considered the most successful means to treat cancer (Nicolau *et al*., 1999).

H. niger belongs to Solanaceae family commonly known as Henbane or Hogsbean in English and Sakran in Arabic. *H. niger* is traditionally used in medicine for its use in stomach cramps, heavy coughs, neuralgia and manic psychosis. The plant also said to possess anti-spasmodic, sedative and analgesic properties (Duke, 1985).

The seeds of *H. niger* are used in anthelmintic, antitumor and febrifuge, they are also found to be useful in the treatment of stomach/intestinal pain due to worm infection, toothache, infection of pulmonary regions and tumors (Tsewang, 1994).

H. species are rich sources of tropane alkaloids, mainly hyoscyamine and scopolamine, which are widely used for their mydriatic, antispasmodic, anticholinergic, analgesic and sedative properties (Supria, 1998).

H. niger also produces non-alkaloidal secondary metabolites like withanolides, flavonoids, lignans, coumarinoligins, saporins, glycerides, glycosides and phenolics (Lunga *et al*., 2008).

The aims of the study are proposed for:

1. Preparation of alkaloidal extract and detection of some active compounds using chemical detection.

2. Further purification of alkaloidal extract using analytical and preparative HPLC.

3. *In vivo* study the cytogenetic effects of alkaloidal extract on mouse bone morrow cells by using different parameter (Mitotic index, micronucleus test and chromosomal aberrations).
4. Study the free radical scavenging activity of the alkaloidal extract.
5. *In vitro* study the cytotoxic activity of alkaloidal extract on cancer and normal cell lines.
6. Effect of the alkaloidal extract on the apoptosis activity by using high content screening assay (Multi parameter cytotoxicity kit).

1.2. Literature review

1.2.1. History of medicinal plants

Plants have the ability to produce tens of thousands of highly complex secondary metabolites to assist their survival in the environment, many of which protect the plant from predators. Man has exploited these compounds of self-defense as sources of medicinal agents (Mohanasundaram, 2001).

There are distinct chemical substances derived from plants that are considered as important drugs currently in use in one or more countries in the world. Several of the drugs sold today are simple synthetics modifications or copies of the naturally obtained substances (Lesile, 2000).

The earliest known medical document is a 4000 year old Sumerian clay tablet that recorded plant remedies for various illnesses. By the time of the ancient Egyptian civilization, a great wealth of information already existed on medicinal plants. Among the many remedies prescribed were mandrake for pain relief, and garlic for the treatment of heart and circulatory disorders (Kong *et al*., 2003).

The therapeutic utilization of plants is part of universal human culture, and products derived from plants are frequently used for the treatment or prevention of diseases. Plants, vegetables, herbs and spices used in folk and traditional medicine are one of the sources of cancer drug discovery and development. A major group of these products include pigments, vitamins, phenols, flavonoids, tannins and alkaloids (Halmurat *et al*., 2008).

Medicinal plant represents a diverse group of herbs spread throughout the world with a high content of bioactive compounds possessing a variety of biological activities (Capek *et al*., 2009).

So medicinal plants display diverse pharmacological activities (e.g. antimicrobial, adaptive, stimulatory, and sedative properties). They are used as cholinergic, hypertensive, antiulcer, anticancer, spasmolytic, analgesic and analeptic medications. An advantage of medicinal plants is to provide patients with a complex of natural compounds, have smoother action and are better tolerated than synthetic drugs, and produce few allergic reactions. They do not accumulate and therefore can be administered for longtime. Medicinal plant and phytopreparations are used for therapy and prevention of various human diseases (Lavkova *et al.,* 2001).

1.2.2. Classification of *Hyoscyamus niger*

This plant was classified according to (Schultes *et al.*, 1976) as below:

Kingdom: *Plantae*

Division: *Magnoliophyta*

Class: *Magnoliopsida*

Order: *Solanales*

Family: *Solanaceae*

Genus: *Hyoscyamus*

Species: *Hyoscyamus niger*

The Solanaceae, commonly known as the night shade family, consists of about 96 genera with 3000 species. This family includes a wide variety of economical, agricultural and pharmaceutical important plants (Wink, 2003).

H. niger, a natural source of hyoscyamine, hyoscine and atropine. The common names of *Hyoscyamus niger* are common henbane, devils eye, Sakran, hogs been, Jupiter bean and banj (Drager, 2002).

1.2.2.1. Description

H. niger is slightly sticky, hairy annual or biennial plant. A rosette of basal leaves grows in the first year, followed in the second year by an erect simple or slightly branched stem, up to 80 cm tall and covered with sticky hairs. The alternate leaves are oval, deeply toothed and lobed-pinnate to in dent. The lower leaves are stalked, the upper semi-enclosing. Sessile flowers grow from the axils of the upper leaves and have a bell-shaped, glandularly, markedly veined calyx running into five sharply pointed tips, a funnel –shaped, externally hairy corolla with violet veins and a reddish –violet mouth. The fruit capsules contain up to five hundred grayish –brown seeds (Chevallier, 1996).

Figure 1-1: Picture of *Hyoscyamus niger* (Graham and Johnson, 2004).

1.2.2.2. Active constituents

Phytochemical studies of *H. niger* revealed the presence of hyoscine (scopolamine), hyoscyamine, anisodine, anisodamine, aesculetin, coumarin, kaempferol, quercetin, rutin, cuscohygrine, chlorogenic acid, linoliec acid, myristic acid, oleoic acid, stearic acid, pyridine, trimethylamine, B-sitosterol, grossamide, cannabisin D and G daucosterol, vanillic acid, calystegines and with anolides (Gilarii *et al*.,2008) .

H. muticus contains up to 1.3% and not less than 0.8% of total alkaloids calculated as hyoscyamine. Other alkaloids are hyoscine 0.02%, tigloidine, cuscohygrine, hygrine, apohyoscine, atropine, nor hyoscine, noratropine and apoatropine (Saker and Ashal, 1995).

Hyosgerin, a new optically active coumarinoligan, has been isolated along with three other coumarinoligan, venkatasin, cleomiscosin A and cleomiscosin B, from the seeds of *H.niger* and also this plant is one of the known sources of alkaloids , has also shown the presence of tyramine derivatives, with anolides , lignanamides and flavonoids(Begum *et al.*,2006).

The chief constituent of *H.niger* (henbane) leaves is the alkaloid hyoscyamine, together with smaller quantities of atropine and hyoscine also known as scopolamine. Other constituents of henbane are a glucosidal bitter principle called hyoscytricin, choline, mucilage, calcium oxalate and potassium nitrate. The chief constituent of the seeds is 0.5 % to 0.6% of alkaloid, consisting of hyoscyamine, with a small proportion of hyoscine. The seeds also contain about 20% of fixed oil (Morishita, 1991).

1.2.2.3. Medicinal uses

H.niger has a very long history of use as a medicinal herb; it is used extensively as a sedative and pain killer and is specifically used for pain

affecting the urinary tract, especially when due to kidney stones. Its sedative and antispasmodic effect makes it available treatment for the symptoms of Parkinson's disease, relieving tremer and rigidity during the early stages of the disease. This species is the form generally considered test for external use, while the white henbane (*H. albus*) is considered the most appropriate for internal use. All parts of the plant especially the leaves and the seeds can be used. They are anodyne, antispasmodic, mildly diuretic, hallucinogenic, hypnotic, mydriatic, narcotic and sedative (Matsuda *et al*., 1991).

Hyoscine and hyoscyamine previously identified as the anticholinergic constituents in *H. niger* are considered responsible for its antispasmodic effect.However, it is generally believed that the plant contains multiple compounds acting on different sites. *H. niger* also used as spasmolytic, antidiarrhoeal, antisecretary and urinary bladder relaxant (Gilani *et al*., 2008).

The ancient Egyptians inhaled the vapour of heated *Hyoscyamus muticus* which contains antimuscarinic alkaloid; scopolamine for the treatment of asthma like conditions (Peter, 2006).

The effects of alkaloids present in *H.muticus* include stimulation of the central nervous system simultaneous depression of the peripheral nerves for parasympathomimetic, spasmolytic, anticholinergic, narcotic and anesthetic properties (Ibrahim *et al*., 2009).

Also *H. muticus* relieves painful spasmodic conditions of non -striated muscles, characteristics of lead colic and irritation of the bladder. It is used to allay nervous irritation of hysteria. Smoke of cigarettes is effective against asthma. It is used in toothache, cough and for treatment of some forms of fever (Jain and De Filipps, 1991).

Hyoscyamus species is used mainly for its antispasmodic effect on the digestive and urinary tracts, and to counteract griping due to purgatives. Hyoscyamine and hyoscine cause decrease in sweat, salivary, gastric and bronchial secretions, decrease the tone and motility muscle in the digestive and urinary tracts and increase the pulse rate. The alkaloids hyoscine is used very widely as preoperative medications and a travel sickness preventative (Chevallier, 1996).

Henbane is a powerful brain relaxant; it inhibits the release of acetylcholine as a neurotransmitter, a similar action to that of *Belladonna.* Henbane has also been used against rabies, delirium associated with fevers, bronchitis, renal colic coughs (Harrison and Bartels, 2006). Methanol extract of *H. niger* possess the anticonvulsant activity against picrotoxin induced seizures in mice (Heidari *et al.,* 2009).

1.2.3. Cytogenetic analyses

Cytogenetic is a branch of genetics that is concerned with the study of the structure and function of the cell, especially the chromosome. Cytogenetic analyses are essential to the diagnosis and treatment of different forms of cancer by analysis of blood or bone marrow cells that reveals the organization of chromosomes (Chauhan *et al.,* 2007).

1.2.3.1. Mitotic index (MI)

The Mitotic index is a reliable predictor of cell proliferation in the tissue. The mitotic index assay is used to characterize proliferating cells and to identify the compounds that inhibit mitotic progression resulting in a decrease in the MI of that population. It is defined as the ratio between the number of cells in mitosis and their total number of the cells. It is the

number of cells containing visible chromosomes divided by the total number of cells in the field of view. If colchicines or other colchicines-derivative medications (colcemid) you can arrest the cell cycle at this point leaving the chromosomes in their visible form. Colchicines disrupt the microtubule formation which is necessary for the spindle fibers to separate the chromosomes during anaphase (Fahad and Salim, 2009).

Mitotic abnormalities often arise directly from defects of centrosome and /or mitotic spindles, which then induce prolonged mitotic arrest or delayed mitotic exit and trigger induction of apoptosis (Mollinedo and Gajab, 2003). Depression of the mitotic index is usually consequence of a reduced rate of cell proliferation (Galloway *et al*., 1994).

1.2.3.2. Micronucleus assay (MN)

Micronuclei are cytoplasmic chromatin –containing bodies formed when acentric chromosomes fragments or chromosomes lag during anaphase and fail to become incorporated into daughter cell nuclei during cell division–because genetic damage that results in chromosome breaks, structurally abnormal chromosome or spindle abnormalities leads to micronucleus formation, the incidence of micronuclei serves as an index of these types of damage (Armstrong and Galloway, 1994).

Micronuclei originate from chromosome fragments or whole chromosomes that are not included in the main daughter nuclei during nuclear division. Thus micronucleus provide a measure of both chromosome breakage and chromosome loss and it has been shown to be at least as sensitive an indicator of chromosome damage as classical metaphase chromosome analysis. The key advantage of the MN assay is the relative ease of scoring and the statistical power obtained from scoring larger

numbers of cells than are typically used for metaphase analysis, when kinetochore or centromere detection methods are used it is possible to distinguish between MN caused by chromosome breakage and MN caused by chromosomal segregation (Fenech, 1997).

An important steps in the evolution of the MN assay was the recognition that it could provide reliable data if the scoring of MN was limited to those cells that had completed one nuclear division , which allow MN to form (Fenech *et al.,* 1999).

Thus, the micronucleus assay has been widely used to measure genotoxicity, both *in vivo* and *in vitro*. This assay performed appropriately to detects clastogenicity due to chromosome breakage, and also a neugenicity, due to chromosome lagging resulting from dysfunction of mitotic apparatus (Jorge *et al*., 2003).

Positive results in the micronucleus test indicate that substances are micronuclei inducer, which are the result of chromosomal damage or damage to the mitotic apparatus in the erythroblasts of the test species. Negative results indicate that, under the test conditions, the test substance does not produce chromosomal or spindle damage leading to the formation of micronuclei in the immature erythrocytes of the test species (Schlegel and Mac- Gregor, 1982).

1.2.3.3. **Chromosomal aberrations**

Any change in the structure or number of any of the chromosomes of a given species. In humans, a number of physical disabilities and disorders are directly associated with aberrations of both the autosomes and the sex chromosomes. The incidence of most chromosomal disorders is significantly higher than that of single-gene disorders (Fechheimer, 1968).

Abnormalities can be detected before birth by means of aminocentesis, or after birth, but many are probably never observed because they cause death and disposal of the fetus. The abnormalities are either of number or of composition of the individual chromosomes. The cause of these abnormalities is not known. Their importance is that many of them are linked with structural or functional defects of the animal body. The type of chromosomal structural alterations produced by physical and chemical agents depend on the lesions induced in the DNA and therefore, upon chemical structure of the genotoxic substance. Structural chromosome aberrations result from the breakage and rearrangement of whole chromosomes into abnormal forms. They are most efficiently induced by those substances that directly break the back bone of DNA significantly distort the DNA helix. Structural aberrations can be classified as either unstable or stable, indicating their ability to persist in dividing cell populations (Carrano, 1986).

Many types of structural rearrangement of chromosomes have been identified in neoplastic cells, such as deletion, duplication, inversion, insertion and translocation. Such rearrangement gives rise to loss, gain, and relocation of genetic material. In addition, numerical aberrations, giving rise to loss or gain of entire chromosomes, are common (Anders, 1996).

It is probable (Cornforth and Bedford, 1993) that most chromosome aberration result from illegitimate reunion (mis rejoining) of free ends from different DNA double-strand breaks. Accepting this picture, one finds that aberration formation is influenced by proximity effects, proximity effects can be inferred by analyzing aberration yields as a function of aberration type, of radiation quality, or of dose (Sachs *et al*., 1997).

According to (Meera and Nagarjuna, 2010) all chemicals that produce DNA damage leading to mutation or cancer are described as genotoxic. One of the best ways to minimize the effect of mutagens and carcinogens is to identify the anticlastogenes and antimutagenes (substances which suppress or inhibit the process of mutagenesis by acting directly on the mechanism of cell) and desmutagens (substances which somehow. destroy or inactivate, partially or fully the mutagens, thereby affecting less cell population) in our diets and increasing their use.

1.2.4. Antioxidants

Antioxidants are the first line of defense against free radicals damage, and are critical for maintaining optimum health and well being. The need for antioxidants becomes even more critical with increased exposure to free radicals, pollution, cigarette smoke, drugs, illness, stress and even exercise can increase free radical, because so many factors can contribute to oxidative stress individual. Antioxidants are capable of stabilizing or deactivating free radicals before they attack cells. Antioxidants are absolutely critical for maintaining optimal cellular and systemic health and well-being (Mark, 1998).

The ability to utilize oxygen has provided humans with benefit of metabolizing fats, proteins and carbohydrates for energy; however, it does not come without cost. Oxygen is a highly reactive atom that is capable of becoming part of potentially damaging molecules commonly called free radicals. Free radicals are capable of attacking the healthy cells of the body, causing them to lose their structure and function. (Maxwell, 1959)

To protect the cells and organ systems of the body against reactive oxygen species, humans have evolved a highly sophisticated and complex

antioxidant protection system. It involves variety of components, both endogenous and exogenous in origin, that function interactively and synergistically to neutralize free radicals. These components include:-

1. Nutrient –derived antioxidants like ascorbic acid (vitamin c), vitamin E , carotenoids and other low molecular weight compounds such as glutathione and lipoic acid .
2. Antioxidant enzymes examples Superoxide dismutase, glutathione peroxidase, and glutathione reductase, which catalyze free radicals quenching reactions.
3. Metal binding proteins, such as ferritin, lactoferrin, albumin that sequester free iron copper ions that are capable of catalyzing oxidative reactions.
4. Numerous other antioxidant phytonutrients present in awide variety of plant foods (Sies and Stahl, 1995).

Antioxidants that have traditionally been used to inhibit oxidation in food also quench dreaded free radicals and stop oxidation chains *in vivo*, so they have become viewed by many as nature answer to environmental and physiological stress, aging , atherosclerosis and cancer. Plant-derived antioxidants have been shown to single and triplet oxygen quenchers, free radicals scavengers, peroxide decomposers, enzyme inhibitors, and synergists (Sulekha *et al*., 2009).

Activity of several plant constituents, beyond the vitamins in the form of crude extract or isolated compound has been put widely into consideration (Cesqaini *et al.,* 2003).

The antioxidants activity of methanolic extracts of many plants such as *Achillea withelnsii, Berteris crataegina, Hyoscyamus niger, Mentha*

pulegium reached to (0.41 <IC_{50} <1.64 µg) comparable to α-tocopherol which was used as the positive control (Effat *et al*., 2004).

The aqueous extract of *Hyoscyamus reticulatus* aerial parts exhibited significant antioxidant scavenging properties and inhibitory effect on xanthine oxidase activity (Mohammad e*t al*., 2010).

The free radical-scavenging activity of natural compound has also been confirmed by using several methodologies (Kumar and Kumar, 2009), and one of these is examining their ability to bleach the stable radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH) *in vitro* .This assay provides information on the reactivity of test compounds with a stable free radical, because of its odd electron DPPH that gives a strong absorption band at 517 nm in visible spectroscopy (deep violet color). As this electron becomes paired off in the presence of a free radical scavenger, the absorption vanishes, and the resulting decolorization is stoichiometric with respect to the number of electrons taken up (Moreira *et al.,* 2008).

1.2.5. Apoptosis

Apoptosis or programmed cell death is a series of genetically controlled events that result in the removal of unwanted cells. Apoptosis is an important method of cellular control and any disruption of this process leads to abnormal growth cancer. Apoptotic cell showed very characteristics morphology as well as specific molecular features. Induction of apoptosis in cancer cells or malignant tissues has recognized an inefficient strategy for cancer chemotherapy. Apoptosis also seems to be reliable marker for the evaluation of potential agent for cancer prevention. Characterization of apoptosis mainly derives from morphological and ultra structural observation. A wide variety of natural compounds appear to possess

significant cytotoxic as well as chemopreventive activity. Many of these agents act via apoptosis (Amit *et al*., 2001).

Intracellular and plasma membrane structural modification have been widely recognized as crucial factors involved in cell injury and death. Changes in nuclear morphology and in organelle structure as well as specific phenomena at the cell surface level, namely surface smoothing and surface blatting, are often considered as marker associated with cell pathology (Walter, 1993).

It is generally accepted that cell death can either be the consequence of a passive, degenerative process, or the consequence of an active process. The former type of cell death represents necrosis, the latter apoptosis (Maroco *et al.,* 1996).

Apoptosis is characterized by a series of typical morphological features, such as shrinkage of cell, cytoplasmic vacuolization, condensation, margination then fragmentation of the nuclear chromatin to membrane-bound apoptosis bodies and rapid phagocytosis by neighboring cells (Saraste and Pulkki, 2000).

Events common to cells undergoing apoptosis are the profound cytoplasmic boiling the condensation of chromatin, and the endocleolytic cleavage of DNA these events are likely to represent discrete steps in the cell destruction process. Although there are increasing numbers of reports that cell death can occur without DNA destruction (Eileen, 1993).

Apoptosis could play a role in autoimmune disease under two different guises. First, controlled regulation of cell death is a normal part of T-cell selection.

Second, cell death could represent a lymphocyte-independent mechanism causing cells in certain organs to die prematurely (Wmkuhtreiber *et al*., 2003).

1.2.6. High-content screening assay

High-content screening (HCS) is defined as multiplexed function on all screening based on imaging multiple targets in the physiologic context of intact cells by extraction of multicolor fluorescence information. Today, HCS is broadly established to support throughout the pharmaceutical research and drug discovery chain. HCS is applied to identify biomarkers, exploring cytotoxicity and genotoxicity and tracking of cellular process applying living cells to support basic research as well as pharmaceutical research (Kerstin and Eberhard, 2007).

High content screening technology offers a major opportunity to improve the drug discovery and development process. HCS enables the evaluation of multiple biochemical and morphological parameters in cellular systems and facilitates characterization of the sub cellular distribution of fluorescent signals with labeled reagents (Couliano *et al*., 2003).

High Content Screening is the application of automated microscopy and image analysis to both drug discovery and cell biology. This technique has grown from an interesting proposition, to a useful technology, and onto a valuable utility over the last decade (Joseph, 2009). Historically, HCS has its origins in drug discovery, initially providing novel secondary assays formats, selectivity screens, and cytotoxicity profiling using the multi-parameter and individual cell attributes of the approach. “High content, in context, and with

correlation" describes the data coming from the current HCS platforms. All HCS assays can measure a cell's physiological responses to stimulate, whether it is environmental or chemical. From relatively simple measure of acute cytotoxicity such as cell counting and cell rounding, to more specific measures of organelle health (Dykens *et al*., 2008).

The potential of HCS to enable more complex toxicity assays is becoming increasingly recognized as independent approaches. Several parameters, such as cell number, nuclear size and area, mitochondrial membrane potential, intracellular levels, or membrane permeability are determined, offering increased specificity and selectivity for toxic events. HCS assay was applied to HepG2 human hepatoma cells cultured in 96 – well plates and loaded with four fluorescent dyes for calcium, mitochondrial membrane potential, and DNA content to determine nuclear area and cell number, and plasma membrane permeability (Fowler *et al*., 2006).

Mitochondria play a key role in many variants of apoptosis. They are the target for the action of specialized pro- and anti- apoptotic protein. The main response of mitochondria to apoptotic stimuli in the release of a number of soluble proteins such as cytochrome C (Shchepina *et al*., 2002).

Cytochrome C is a small heme protein that resides in the mitochondrial intermembrane space. It is primarily known for its function as a key participant in oxidative phosphorylation and ATP synthesis. However, it also plays a key role in regulating apoptosis. When a cell detects an apoptosis stimulus, such as DNA damage or metabolic stress, the intrinsic apoptosis pathway is triggered, and mitochondrial

cytochrome C is released into cystosol, triggering a series of events leading to apoptosis cell death (OW *et al*., 2008).

In recent years, extensive research has focused on screening for chemical compounds, small molecules and peptides that could target the mitochondria. As a key regular of apoptosis, cytochrome C release is widely used as a measurement in drug discovery approaches targeting apoptosis (Kagan *et al.*, 2009).

HCS cytochrome C assay provides a complete solution for identifying and quantifying the mitochondrial presence and cytoplamic release of cytochrome c via cellular imaging. The reagents in this assay have been specifically optimized for HCS applications (Andre *et al*., 2000).

2. Materials and Methods

2.1. Materials

2.1.1. Equipments and Apparatus

The following equipments and apparatus were used throughout this study:

Apparatus	**Company and origin**
Analytical Balance	Sartorious (Germany)
Autoclave	SES little Sister (England)
Centrifuge	Beckman (England)
Incubator	Memmert (Germany)
Elisa reader	Organon Teknika
Laminar flow hood	Heraeus (Germany)
Rotary Evaporator	Buchi (Switzerland)
Soxhlet	Electrothermal (England)
Microscope	Motic (Japan)
pH meter	Radiometer (Denmark)
Spectrophotometer	Cecil (France)
Water bath	Gallen Kamp (England)

2.1.2. Chemical Materials

Chemicals	**Company**
Methanol	BDH (England)
Petroleum ether	BDH
Crystal violate	BDH
Chloroform	BDH
Trypsin – versin	US Biological (USA)
Giemsa stain	BDH
RPMI – 1640	Sigma (UK)
Ammonia	BDH
Colchicine	BDH
Bovin serum albumin	Sigma
Glacial acetic acid	Fluka (Switzerland)
Ferric chloride	BDH
Dimethyl Sulphaoxide (DMSO)	Sigma
HCl	Fluka
KCl	Fluka
Mitomycin C (MMC)	Kyowa (Japan)
Penicillin	Sigma
Trypan Blue stain	Farmacia fine chemical Uppsala (Sweden)

2.2. Methods

2.2.1. *Hyoscyamus niger*

The plant was collected in August 2008 from Hasarost Mountain at the height of 1550 meters. The aerial part of the plant was air dried at room temperature and grinded into powder form for the experimental study. This plant was kindly identified as *H .niger* (figure 2.1) by Professor Dr. Ali Al Mosawy, the herbarium. College of Science, Biology department, University of Baghdad.

Figure (2 .1) Aerial part of *Hyoscyamus niger* plant.

2.3. Extraction of alkaloids from *H. niger*

A quantity of 50 grams of dried and powdered aerial parts of *H. niger* plant was extracted with methanol in a soxhlet apparatus for 2 hrs. Methanol extract was evaporated in a rotary evaporator at 50ºC to dryness. The pH of the residue was adjusted to 3 with 1% HCl and then filtered. The filtrate was extracted 3 times with 20ml of diethyl ether. Alkaloids were extracted 4 times with 50ml of chloroform

from the alkaliqueous solution (pH10) using ammonia. The chloroform extract contain primary, secondary and tertiary alkaloids as shown in the figure (3-2) (Richard, 1998 and Murat *et al.,* 2002).

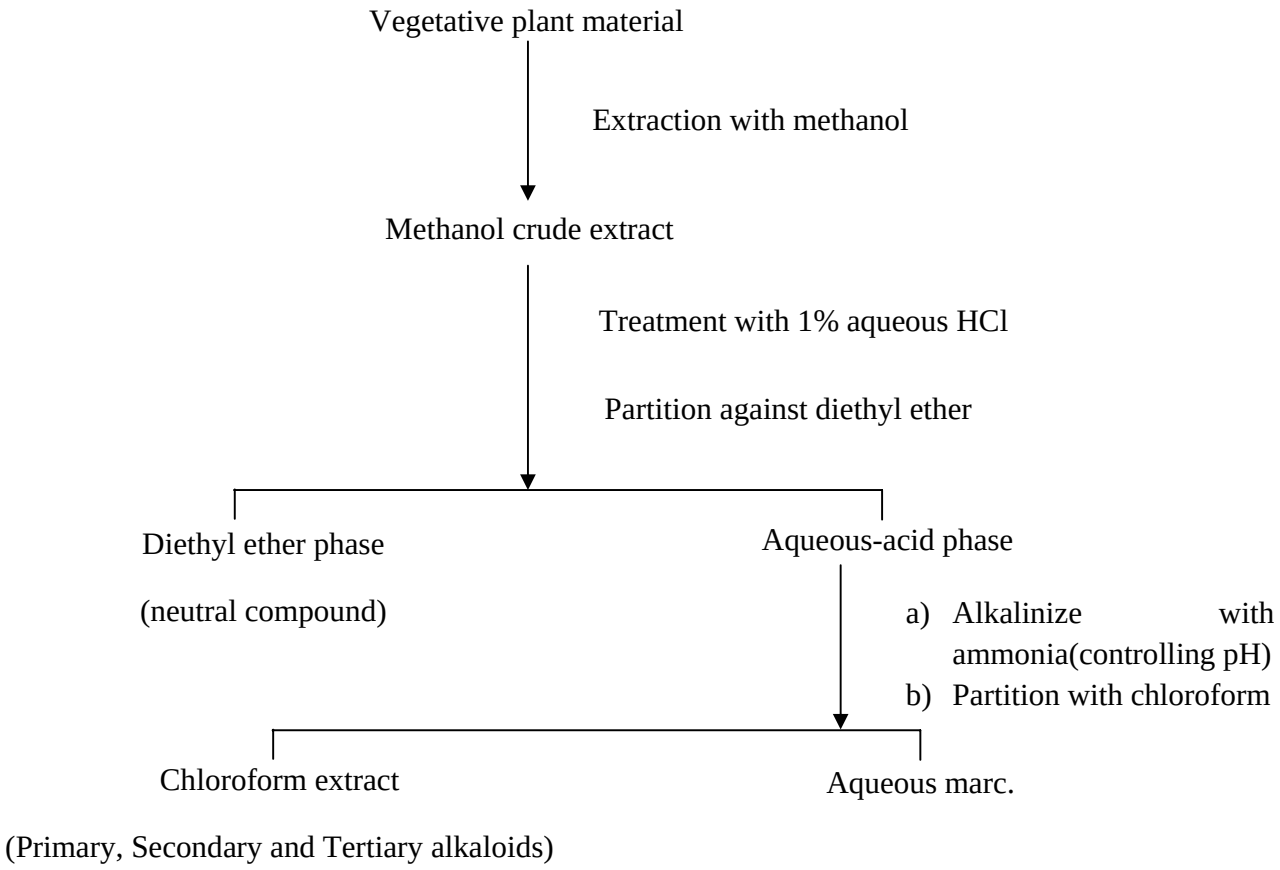


Figure (2.2): Extraction of alkaloids from *H. niger*

2.4. Preparation of chemicals and solutions

2.4.1. Colchicine (1mg/ ml)

Colchicine was prepared by dissolving 1mg of colchicine tablet in 1ml of normal saline to be used for mice injection. Each animal was intrapritionaily (IP) injected with 0.25ml of this solution (Allen *et al*., 1977).

2.4.2. Potassium chloride (KCl) (hypotonic solution)

A concentration of 0.075M was prepared by dissolving 5.75g of KCl salt in 1000ml distilled water. The solution was sterilized by autoclaving and stored at 4ºC until use (Patton, 1967).

2.4.3. Mayer's reagent

This reagent was prepared as follows:

Solution A: Mercuric chloride (1.35g) was dissolved in 60ml D.W.

Solution B: Potassium iodide (2.5g) was dissolved in 20ml D.W.

The solutions A and B were mixed and the volume completed to 100ml by D.W. (Sousel *et al.*, 1999).

2.4.4.. Benedict reagent

This reagent was prepared by dissolving 137g of sodium citrate and 100g of sodium bicarbonate in 800ml of distilled water. The mixture was filtered (Whatman filter paper No.3), and then copper sulphate solution (17.3g in 100ml D.W.) was added and the volume was made-up to 1000ml D.W. (Mojat *et al*., 2003).

2.4.5. Ferric chloride solution (1%)

This solution was prepared by dissolving 1g of ferric chloride in 100ml of D.W. (Collee *et al.*, 1996).

2.4.6. Antibiotic solutions

Two antibiotics used were penicillin and streptomycin. The penicillin (1000000 IU) was dissolved in 10ml of sterilized distilled water, while one gram of

streptomycin was dissolved in 10ml of sterilized distilled water. Both solutions were sterilized through Millipore filter(0.22µm) and stored at -20ºC after dividing them into aliquots each contains 1ml in a sterile vial (Freshney, 2000).

2.4.7. Crystal violets stain

It was prepared by mixing 5 mg of crystal violet, 200ml methanol and 50ml formaldehyde (37%).

2.4.8. Rosswell Park Memorial Institute (RPMI)- 1640 Medium (Gibco, USA)

RPMI-1640 medium (with HEPES buffer and L-glutamine) was prepared by dissolving 10.4g in approximately 600ml of double distilled water and then the other components were added:

Sodium bicarbonate powder (2.2g).

Penicillin (100000 IU/ml).

Streptomycin 100mg/ml.

Fetal serum (FBS) 100ml.

The volume was completed to one liter with double distilled water and the medium was sterilized using Millipore filter (0.22µm Millipore filter).

2.4.9. Fixative solution

This solution was freshly prepared by mixing 3 volumes of absolute methanol with 1 volume of glacial acetic acid (Allen *et al*., 1977).

2.4.10. Human plasma

Human AB plasma was supplied by the National Blood Transfusion in Baghdad. The plasma was transferred to the laboratory in an ice box. In the laboratory, the plasma was divided into aliquots (5ml) in sterile test tube. The tubes were placed in a water bath (56ºC) for 30 minutes to inactivate the complement, and then stored at -20ºC until use in the micronucleus assay (Schmid, 1976).

2.4.11. Giemsa stain

Giemsa stock solution was prepared by dissolving 2g of giemsa stain in 100ml of methanol. The solution was then kept in a dark bottle. When staining the slide, 1ml of giemsa stock solution was taken and added to 4ml of freshly prepared Sorenson's buffer solution.

2.4.12. Sorenson's buffer solution

It was prepared by dissolving 7.08g of Na_2HPO_4 with 7.74g of KH_2PO_4 in 1L of distilled water and the pH was adjusted to 7 and then stored in a refrigerator until use (Al-Rikabi, 1997).

2.4.13. Trypsin /Versin solution

It was prepared by dissolving 2g of trypsin/versin powder in 100ml of distilled water and the pH was adjusted to 7.0 using sodium bicarbonate solution. Then it was sterilized by filtration (Millipore filter; 0.22µm) and kept at 4ºC (Freshney, 2000).

2.4.14. Preparation of {(3 – Dimethylthiazol – 2 – yl) – 2, 5 – Diphenyltetrazolium bromide} (MTT) dye

A quantity of 50 mg/ ml {(3 – Dimethylthiazol – 2 – yl) – 2, 5 – Diphenyltetrazolium bromide} (MTT) dye was used as a final concentration (Freshney, 2005). The solution was filtered through 0.22 µm syringe filter to remove any blue formazan product as recommended by the Denizot and Lang (1986), and then stored in sterile, dark, screw-capped bottles at 4ºC.

2.4.15. Preparation of solution used in multiparameter cytotoxicity kit

1. 1X wash buffer	Aliquot of 20 ml 10X wash buffer was added to 180 ml distilled water. Store buffer at 4ºC for up to 7 days.
2. Fixation solution	Aliquot of 3ml of 16% paraformaldehyde solution was added to 9ml of 1X Wash Buffer just before use.
3. 1X Permeabilization Buffer	Aliquot of 1.5 ml of 10X Permeabilization Buffer was added to 13.5 ml of the 1X Wash Buffer. Store this buffer at 4ºC for up to 7 days.
4. 1X blocking buffer	Aliquot of 5 ml of Blocking Buffer was added to 44 ml of 1X Wash Buffer. Store this buffer at 4ºC for up to 7 days.
5. Primary antibody solution	Aliquot of 15 µl of the cytochrome c Primary Antibody was added to 6 ml of 1X Blocking Buffer. Prepare solution just before each assay.
6. Secondary antibody staining solution	Aliquot of 0.6 µl of Hoechst Dye and 12 µl of the DyLight 649 Goat Anti-Mouse were added to 6 ml of 1X Blocking Buffer. Prepare solution just before each assay.

7. Live cell staining solution	Aliquot of 117 µl of DMSO was added to the Mitochondrial Membrane Potential Dye to make a 1mM stock solution. Just before use, add 2.1 µl of Permeability Dye and 21 µl of Mitochondrial Membrane Potential Dye to 6ml complete medium pre-warmed to 37ºC.

2.5. Detection of some active compounds

2.5.1. Detection of tannins

A quantity of ten grams of the aerial part of plant was mixed with 50ml distilled water in a magnetic stirrer. The mixture was boiled in boiling water bath for few minutes. Then filtered and the filterate was treated with few drops of 1% ferric chloride solution. The development of greenish-blue precipitate indicates a positive result. (Shihata, 1951).

2.5.2. Detection on flavonoids

Ethanol extract of the plant was partitioned with petroleum ether; the aqueous layer was mixed with ammonia solution. The appearance of yellow color indicates as positive result (Harborn, 1973).

2.5.3. Detection of glycosides

Few drops of HCl were added to the test tube containing 1ml of the plant extract, and then the tube was transferred to the boiling water bath for 2 minutes and after that, 2ml of Benedict reagent was added. The appearance of red color indicates of glycosides (Evans, 1999).

2.5.4. Detection of alkaloids

One of the plant extract was added to the tube containing 2ml of Mayer's reagent. The appearance of a white residue after shaking the tube was considered as an indicator of the presence of alkaloids (Sousek, 1999).

2.6. Quantitive and qualitative analysis of *H. niger* extract

2.6.1. HPLC analysis of *H. niger* extract

HPLC analysis was done to detect the most active compound alkaloids (hyoscine) by using hyoscine standard as reference. HPLC analysis was done using column: HAISIL100C185U, 9.6mm X 25 cm; the mobile phase acetonitril 80 ml and deionized water 20 ml, and the flow rate was 0.9 ml/min at 259 nm.

2.6.2. Advance analytical HPLC

Analysis of plant extract was performed on Shimadzu UFLC system equipped with a PDA UV detector and Ion Trap TOF mass spectrometer. Programmable fluorescence detector with column; waters Xbridge 50×2.1 mm 2.5µm.Mobile phase: H2O (0.1% formic acid): MeCN (0.1% formic acid). Flow rate: 0.50mL ⁄ min. The column temperature was 40 c. The gradient 10-100% MeCN over 7 minutes. Different wave lengths were used (190, 200, 254. 400, 450 nm).

2.6.3. HPLC – Purification

To separate crude extract preparative HPLC was used. Fractionation was performed using a Gilson preparative HPLC equipped with a GX – 281 liquid handlers and 156 UV detectors. Column used water Novapak C18 – 25 X 100 mm,

6 mm and RPC18 19X50, 5 µm solvents: Water CO-140 formic acid, Acetonitrile (0.1% formic acid).A quantity of 500 mg of crude extract was dissolved in 3 ml of methanol; this sample was then filtered through filter (0.45 µm).Fractions were obtained from preparative HPLC purification of the sample – Novak 25X100 mm column and eluting with a gradient from 30% MeCN (0.1% formic acid), 100% water (0.1% formic acid) t0 100% MeCN (0.1% formic acid) over 45 minutes at a flow rate of 12 ml/min. UV detection: 200 nm and 400 nm.Fraction collector was done with Gilson FC204 with microtiter plate flow split using passive splitter: that obtain 7 fractions.

2.7. Cytotoxicity assay of plant extract on mice

2.7.1. Laboratory animals

Balb/C mice were obtained from Biotechnology department, college of science. Al-Nahrin University. Their age ranged between (8-12) weeks and weighting (23-25g). They were divided into groups; each group was put in a separate plastic cage. The cages were put in a room with optimal temperature.

2.7.2. Cytogenetic experiments

This experiment was carried out to assess the cytogenetic analysis of plant extract at three different concentrations (200, 300 or 400) mg/kg.

The animals were divided into:

Group 1: As negative control (3 mice) treated with 0.1ml of normal saline.

Group 2: As positive control (3 mice) treated with 0.1ml of mitomycin C.

Groups 3, 4 and 5: The animals were treated with three doses (200, 300 or 400 mg/kg) respectively, of crude alkaloidal extract (9 mice).

The plant extract was injected intraperitioneally as a single dose (0.1ml) per a day for 7 days, and then the mice were sacrificed at day 8 for laboratory assessment.

2.7.2.1. Mitotic index and chromosome preparation from somatic cell of the mice bone marrow

The experiment was done according to Evan's *et al*, (1964) as follows:

1. Animals were injected with 0.25ml of colchicine with concentration of 1mg/ml intraperitianially for 2 hours before sacrificing the animals.
2. Animals were sacrificed by cervical dislocation and then dissected to obtain femur.
3. Femur was cut from both ends, and its cellular contents were collected in a test tube using normal saline (5ml), with aid of a disposable insulin syringe (1ml).
4. Cell suspension was gently pipette using Pasteur pipette, and the tube was centrifuged (2000rpm) for 10 minutes.
5. Supernatant was discarded, and the cell deposit was suspended in 5ml of a warm (37ºC) hypotonic solution (KCl 0.075M). Then the tube was incubated in a water bath (37ºC) for 30 minutes with a gentle shaking every 5 minutes.
6. The tube was centrifuged at 2000rpm for 10 minutes and the supernatant was discarded.
7. Fixative solution (5ml) was added as drop-wise to the cell deposit with a gentle and continuous mixing to make a homogenous cell suspension. Then the tube was incubated in the refrigerator (4ºC) for 30 minutes.
8. The tube was centrifuged (2000 rpm) for 10 minutes, and the last step was repeated two times.

9. Cell deposit was well-suspended in 2ml of the fixative and 4-5 drops of the cell suspension were dropped on a clean slide from a height of about two feet.
10. The slide was air-dried at room temperature, and then it was stained with Gimesa stain for 15 minutes and rinsed with distilled water.
11. The slide was examined under oil emersion lens (100 x) and at least 1000 cells were examined for mitotic index.

    The percentage of divided cells was recorded using the equation

$$\textit{Mitotic index (\%)} = \frac{\textit{Number of divided cells}}{\textit{Total Count (1000)}} x100$$

12. In the case of chromosomal aberration assay, the prepared slides were examined under the oil immersion lens for 100 divided cells per mice and the cells should beat the metaphase stage of the mitotic division where the chromosomal aberrations were clear and the percentage of this aberration could be estimated.

2.7.2.2. Micronucleus test in mice bone marrow cells

The experiment was done according to Schmid (1979) as follows:

1. The femur bone cleaned from tissues and muscles, and then gapped from the middle with a forceps in a vertical position over the edge of a test tube by a sterile syringe.
2. Aliquot of 1ml of fetal bovine serum (heat inactivated) was injected so as to wash and drop the bone narrow in test tube.
3. The test tubes were centrifuged at speed of 1000 rpm (5 minutes).

4. Supernatant was removed, and one drop from the pellet was taken to make a smear on a clean slide. The slides were kept at room temperature for (24 hrs.).
5. The slides were fixed with absolute methanol for (5 min.) then stained with Giemsa stain for (15 min.), then washed with D.W. and left to dry.
6. The slide was examined under oil immersion lens (100x), and at least 1000 poly chromatic erythrocytes (PCE) were examined for the presence of micronucleus formation.

The micronucleus index was obtained using the following equation:

$$\textit{Micronucleus index (\%)} = \frac{\textit{Number of micronuclei}}{\textit{Total count of (PCE 1000)}} \; x\; 100$$

2.8. Measurement of radical scavenging activity

This is the most widely reported method for screening of antioxidant activity of many plant drugs. DPPH (2, 2 diphenyl 1, picrylhydrazyl) assay method is based on the reduction of methanol solution of colored free radical DPPH by free radical scavenger. The antioxidant activity of plant extract was determined by measuring their abilities to reduce the stable nitrogen-centered DPPH radial *in vitro*. First prepared stock solution of plant extract 1mg/ml that dissolved in 10% DMSO + 90% methanol from each fraction of extract and also prepared positive control ascorbic acid at concentration 9mg/ml also dissolved in 10% DMSO + 90% methanol and negative control only 10% DMSO + 90% methanol was prepared.

Aliquot of 50 ml of DPPH solution (130 ml methanol) was added to 20 ml of each diluted sample. The mixture was vortexed and the resulting solution was allowed to develop for 30 min. in the dark at ambient

temperature.The absorbance caused by the DPPH radical at 517 nm was determined by Unicam UV500 spectrophotometer for the control and each test sample. The antioxidant activity, expressed as percentage inhibition of the radical absorption (Maarit *et al*., 2002).

$$\text{Inhibition (\%)} = \left[\frac{\text{Control Absorbance} - \text{Sample Absorbance}}{\text{Control absorbance}}\right]$$

2.9. Cell lines used in the study

All cell lines were provided by Iraqi Center for Cancer and Medical Genetic Researches, Al Mustansirya University and the Biotechnology Research Center, Al Nahrain University.

Types of Cell Lines		Source
1. Human Rhabdomyosarcoma (RD)		Biotechnology Research Center
2. Human epidermoid larynx carcinoma (Hep-2)		Iraqi Center for Cancer and Medical Genetic Research
3. Ahmed-Mohamed-Nahi2003 (AMN-3)		Iraqi Center for Cancer and Medical Genetic Research
4. Mouse embryonic fibroblasts (MEF)		Iraqi Center for Cancer and Medical Genetic Research

The percentage of growth inhibition was calculated according to an equation presented by Phuangsab *et al*., (2003).

$$\textit{Growth inhibition (\%)} = \left[\frac{\textit{Control Absorbance} - \textit{Treated Absorbance}}{\textit{Control Absorbance}}\right] x\ 100$$

2.9.1. Rhabdomyosarcoma (RD)

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). Passages of RD cell line were used throughout this study and RPMI medium was used in propagation and maintenance.

2.9.2. Human epidermoid larynx carcinoma (Hep-2)

This human cell line originally came from a 57-year-old man with a primary tumor of the Larynx. It was implanted in irradiated and cortisone treated rat. After growth in the rat, the tumor was excised and implanted as in vitro tissue culture. HEP-2 cells growth rapidly, doubling themselves in 2-3 days and were shown to be extremely resistant to ultra violet rays (Moore *et al*., 1955). Passages 235 were used throughout this study and RPMI-1640 medium was used in maintaining the cells.

2.9.3. Ahmed-Mohamed-Nahi2003 (AMN-3):

This animal cell line is murine mammory adenocarcinoma was derived from a spontaneous mammary adenocarcinoma of female BALB/c mice (Al Shammary, 2003). This cell line was established and propagated in the Iraqi Center for Cancer and Medical Genetic Research. Passages 186 of AMN-3 cell line were used throughout this study and the cells were maintained using RPMI-1640 medium.

2.9.4. Mouse Embryonic Fibroblast (MEF):

Mouse embryonic fibroblast cells were used as a feeder layer for the culture of mouse embryonic stem cells to maintain them as plural potent stem cells (David, 2000).These normal cells are known to have a limited life span *in vitro*, and after a certain number of divisions the cells pass into a “crisis” stage and die.

The same cells survived and acquired the ability to unlimited divisions, forming so-called stable or immortalized cell lines. These cells were prepared and employed in the present study as a normal cell line (Matise *et al.,* 2000). The passages two of the cell line was used in this study and the cells were maintained using RPMI-1640 medium.

2.9.5. Cell Line Procedure

This was applied according to the method adopted by Abdul-Majeed (2000).

1. All cell lines used in this study (RD, Hep-2, AMN-3 and normal cell lines MEF) were cultured in culture bottles (falcons) then stored at -20ºC.
2. The cultured cell lines were washed with phosphate buffer satin (PBS). The trypsine-versine solution was added with gentle shake, then the final mixture was poured into another culture bottles (sub-culturing) and incubated at 37ºC for 1 minute.
3. Counting of viable cells was carried out using trypan-blue dye (0.04%). Dead cells take up the dye and appear blue under microscope while living cells exclude the dye and appear white.
4. Cytotoxicity assay of plant extract was done using crystal-violet cytotoxic assay. The plant extract was dissolved in dimethyl sulfoxide, then five concentrations (125, 250, 500, 750 and 1000) µg/ml of plant extract were prepared.
5. Aliquot of 0.2 ml of cultured cells (Hep-2, AMN-3, RD and MEF) were transported to 96-well micro plates, so each well contained 10^5 cells, followed by addition of 0.2 ml of prepared concentration of plant extract, leaving some wells contained cultured cells but without any treatment by plant extract to be considered as control.

6. Cells were incubated for 24, 48 and 72 hrs and then washed with PBS followed by addition of crystal-violet solution (0.1ml) to each well re-incubated at (37ºC) for 2 hrs.
7. After incubation, the contents of the plate were discarded and the cells were washed with water to remove the excess stain, then dried at room temperature for a few minutes.
8. The plate was read by micro ELISA reader at an optimal density at (492nm).

2.10. Viability assay using MTT assay

2.10.1. Cytotoxic assay of extract on cell lines

Another four cell lines were used for cytotoxic assay of plant extract, these cell lines were:

- Human lung adenocarcinoma (A549) cells.
- Human prostatic carcinoma (PC-3) cells.
- Human hepatocellular carcinoma (Hep G2)
- Human hepatic cell (WRL- 68) (Normal Cell Line)

All lines were obtained from center for natural product research and drug discovery, University of Malaya.

2.10.2. Cell line procedure

Cytotoxic assay was prepared under aseptic conditions according to Freshney (2001). Plant extracts were prepared for microtitrtion assay by dissolving 0.01g of the extract in 10 ml of solvent (0.1 ml DMSO + 9.9 ml DDW), the stock was prepared at a concentration 1000 µg/ml then all solutions were filtered by 0.22 µm Millipore filter. The extract was ready to be used as stocks. Five concentrations were made (1000, 500, 250,125, 62.5µg/ml).

The cells were supplemented as a monolayer attached cells in falcon culture flasks (25ml) containing RPMI-1640 medium. The cells were washed with PBS, and then 1ml of trypsine-versine solution was added with a gentle shaking until the cells were detached from the flask surface. The same procedure of cytotoxicity can be used except using MTT solution dye that was added to each well and incubated at 37ºC for 3 hours. At the end of last incubation period the dye was removed from the plate and the wells washed with warm PBS twice, then 0.2 ml of DMSO was added to each well to dissolve the MTT-formazan crystals. Finally the plate became ready for reading by ELISA reader at 570 nm. The inhibitory concentration was calculated as the drug concentration that is required to reduce absorption to half of the control. The equation was:

$$\text{Inhibition (\%)} = \frac{Mean\ of\ Test}{Mean\ of\ Control} \text{ X } 100$$

2.11. High content screening assay (multiparameter cytotoxicity kit)

Cell-based high content screening (HCS) assays enable quantitative measurement of multiple parameters related to cytotoxicity. Multi parameter cytotoxicity Kit enables simultaneous measurements in the same cell of six independent parameters that monitor cell health, including cell loss, nuclear size and morphological changes, mitochondrial membrane potential changes, cytochrome c release and changes in cell permeability.

2.11.1. Cell Preparation

This protocol was used for A549 cells and PC-3 cells, the routine culture of cells, EMEM medium that used was containing the following supplements: 10% fetal bovine serum, 1 µm sodium pyruvate, 1 X non-essential aminoacids, 100

units⁄ml penicillin and 100 mg/ml streptomycin C eagal minimum essential medium (EMEM) complete medium. The cells were split when they reach 90% confluence at a dilution of 1:4. Cells were harvested by trypsinization, diluted into EMEM complete medium and cell density was determined. Cells were diluted to 7.5 X 10^4 cells/ml in EMEM complete medium. Aliquot of 100 ml of the cell suspension were added per well of a 96 – well micro plate. Cells were incubated over night at 37ºC in 5% CO_2 before treatment with plant extract. (Zhang, 1999).

2.11.2. Procedure

Solution of valinomycin (5X) used as positive control (120 µm) and four concentrations of plant extract (1000, 250, 125, 62.5 µg/ml) were prepared.

1. Aliquot of 50 µl of Live Cell Staining Solution was added to each well.
2. Cells were incubated at 37ºC for 30 minutes.
3. The medium was gently aspirated and the staining solution and add 100µl/well of fixation solution and incubate plate for 20 minutes at room temperature.
4. The fixation solution was gently aspirated and added 100µl/well of 1X wash buffer.
5. Wash buffer was removed and 100µl/well of 1X Permeabilization buffer was added and incubated for 10 minutes at room temperature protected from light.
6. Permeabilization buffer was aspirated and washed plate twice with 100µl/well of 1X wash buffer.
7. Wash buffer was aspirated and add 100µl/well of 1X blocking buffer and incubated for 15 minutes at room temperature.

8. Blocking buffer was aspirated and added 50µl/well of Primary Antibody Solution. Incubated for 60 minutes protected from light at room temperature.
9. Primary antibody solution was aspirated and washed plate three times with 100µl/well of secondary antibody / staining solution. Incubated for 60 minutes protected from light at room temperature.
10. Secondary antibody / staining solution were aspirated and washed plate three times with 100µl/well of 1X Wash Buffer.
11. Aliquot of100µl/well of 1X Wash Buffer was added.
12. The plate was sealed and evaluated on the Array Scan HCS Reader.
13. Store sealed plates in dark at 4ºC. (Shiau-chuen *et al*., 2011).

For best results, plates were evaluated within 24 hrs after completion of the assay.

2.12. Statistical analysis

Statistical Analysis System- SAS (2004) was used to analyze the data (to effect of concentration and time in percentage of inhibition) in difference lines of cell. The Least Significant Difference (LSD) test was used to compare between means (percentage) in this study.

3.1. Detection of Some Active Compounds in *Hyoscyamus niger* Extract:

In this study the chemical properties of aqueous extract of *Hyoscyamus niger* were determined. The chemical analysis showed that this extract contained several compounds (Table 3.1).Alkaloids were detected by Mayer's reagent, it showed white precipitation, also this extract contained tannins, it was detected by adding FeCl2, a greenish blue precipitation appeared. As well as, flavonoids found in aqueous extract of this plant it revealed a yellow color after treated with ammonia. Whereas treating extract with Benedict reagent showed negative results that mean no glycosides were in this extract.

Indeed, *Hyoscyamus* species are rich sources of tropane alkaloids, mainly hyoscyamine and scopolamine (Atefeh *et al.*, 2009).The quantity of total alkaloids in aerial parts of *H. niger was* detected form 324.09 mg. (Fazel *et al*., 2008).

Recently Sajeli (2010) found that the non-alkaloidal constituents of *H.niger* seeds included lignans, coumarinolignans, with anolides, lignanamides, glycerides, saponins and flavonoids.

Table (3.1): Some active compounds in *Hyoscyamus niger* extract

Chemical Compounds of Aqueous Extract	Reagents used for detection	Result
Alkaloids	Mayer's reagent	White ppt.
Tannins	$FeCl_2$	Greenish blue
Flavonoids	Ammonia	Yellow color
Glycoside	Benedict reagent	No result

3.2. Quantitative Analysis of *Hyoscyamus niger* Extract by HPLC

HPLC analysis was done to detect hyoscine, the most important active compounds alkaloid found in the plant, using hyoscine standard as a reference.

Figure (3-1) revealed a major peak with different retention times and semi identical to the retention time of hyoscine standard.

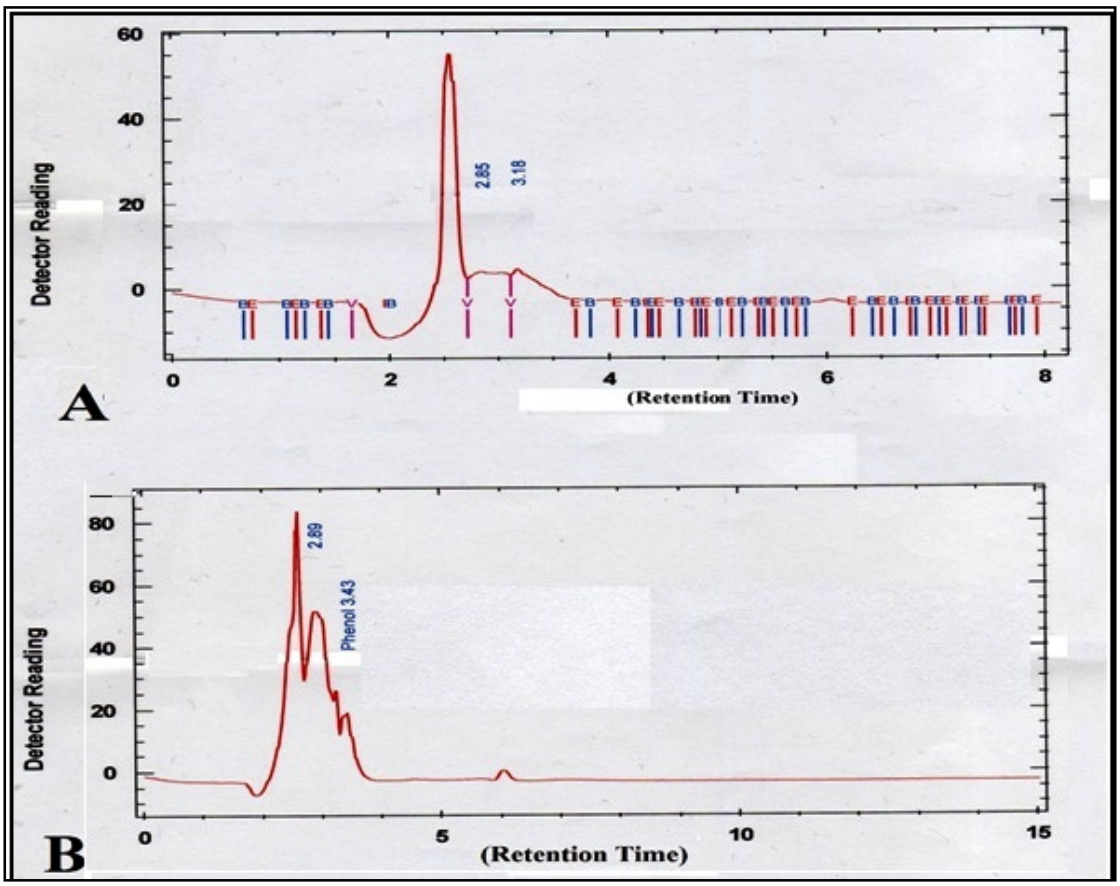


Figure (3 – 1) HPLC Analysis of Hyoscine Standard (A) and *H. niger* extract (B)

Then, the crude alkaloidal extract was analyzed by analytical HPLC using different waves length (190 nm, 200 nm, 254 nm, 400 nm and 450 nm) to determine the suitable wave length by which the fraction displayed a high amount of this extract as shown in the figure (3.2).The results indicated the main peaks obtained at two wave lengths, 190nm and 400nm.

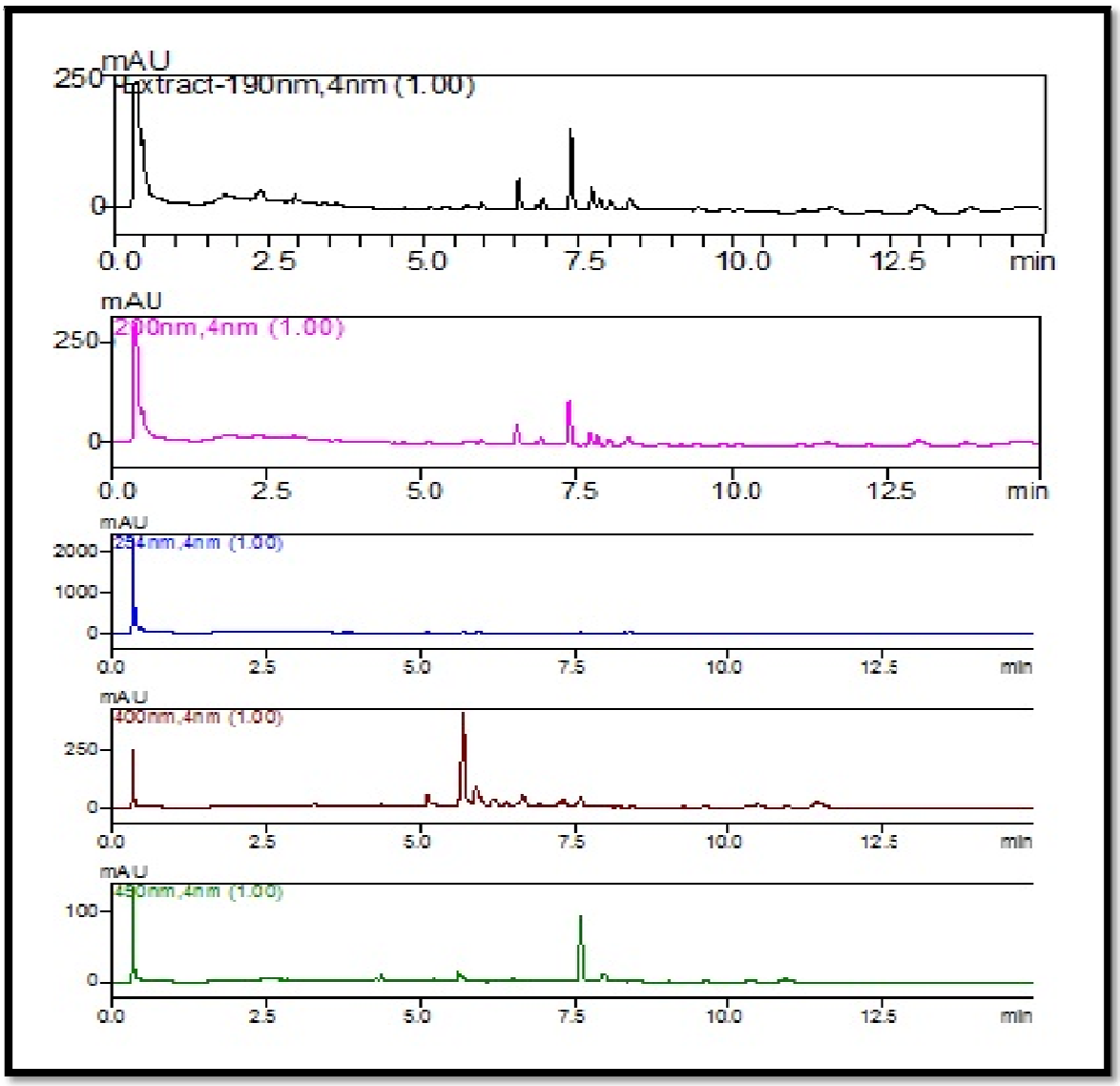


Figure (3 – 2) HPLC Analysis of *H. niger* extract using a different wave lengths

According to the above result, the crude extract was also analyzed using preparative HPLC at wave length ranged from (200 nm – 400 nm).

Seven fractions collected from the preparative HPLC correspond to the positive peaks detected. These fractions were collected according to the dry mass

(Table 3-2). The fraction C exhibited the lowest dry mass, it was 0.1 mg. While fraction G showed a high amount of dry mass, it formed 2.0mg (as shown in the appendix). In a previous study, the preparative HPLC can be used in pharmaceutical development for troubleshooting purposes or as part of a systematic scale-up process. The importance of preparative HPLC as a positive tool has been increased. For example, a chromatographic separation could remove impurities of a different polarity or reduce the content of one enantiomer in a racemic mixture. In both cases, crystallization might be used to prepare the pure product (Lorenz *et al.*, 2001).

Table (3 - 2) Types of fraction and their dry mass isolated from preparative HPLC

Symbol of Fraction	Fractions	Dry Mass (mg)
A	110 – 112	1.0
B	120 – 121	1.2
C	134	0.1
D	151 – 152	1.4
E	162	0.3
F	164	0.3
G	174 – 175	2.0

3.3. Cytogenetic effects of *H.niger* extracts *in vivo:*

3.3.1. Effects of *H.niger* on Mitotic index:

Mice were treated with different concentrations of alkaloidal extract of *H. niger* for 7 days. Table(3- 3) showed non significant differences ($p< 0.05$) in mitotic index between mice treated with low concentration of alkaloidal extract(200 mg\kg) and non treated group, the mitotic index was 2.84 and 2.60 respectively. Whereas increasing the concentration of this extract to 300 and 400 mg\ kg demonstrated a significant effect on mitotic index; it was 3.08 and 4.1, respectively in comparison with the negative group.

Table (3-3): Mitotic index of bone marrow cells in mice treated with different concentrations of *Hyoscyamus niger* extracts

Groups		Dose (mg/kg)	Mean ± SE (%)* of mitotic index
Negative control group		0	B 3.60 ±0.16
Positive control group Mitomycin-C		2	A 0.67 ±0.18
Groups treated with various	First dose	200	B 2.84 ±0.13
	Second dose	300	B 3.08 ±0.24
	Third dose	400	B 4.1 ± 0.27

significant difference ($P \leq 0.05$) between the means of the mitotic index in the same column

Remarkably, the positive control with dose 2mg/kg mitomycin C result in the reduction of mitotic index in mice bone marrow cells(0.67%), this may be related to the protein required for mitosis which were not produced at the same quantities or due to the defect occurred in the mitotic spindle composition during cell division (Shiraishi, 1978).Decreased mitotic index reflects inhibition of cell cycle progression or loss of proliferative capacity (Marucia *et al*., 2000). The depressed of the mitotic index is usually a consequence of a reduced rate of cell proliferation (Galloway *et al*, 1994, Hagelstrom *et al.*, 1995).

The mitotic index assay is used to characterize proliferating cells and to identify compounds that inhibit or induce mitotic progression. Compounds that inhibit mitotic progression result in an increase in the mitotic index of a population (Fahmy and Abdulla, 2001).

The reduction of mitotic index by alkaloids extracted from *Peganum barmale* was observed in bone marrow cells treated with high concentration, this reduction occurred due to decrease in number of cells moving into prophase from G2. The decrease in mitotic index or inhibition of the DNA synthesis might be caused by decreasing ATP levels (Modallal *et al.*, 2008).

Plesca *et al*., (2007) explained this decreasing could be due to either cell cycle block in GI-S phase, suppressing DNA synthesis or blocking in G2-M phase preventing the cell from entering mitosis.

3.3.2. Effects of *Hyoscyamus niger* extract on Micronucleus (MN):

Mice bone marrow nuclei micronuclei (MN) assay holds a key position in all schemes for detecting carcinogens and mutagen (Helen and John, 1994).

Micronucleus frequencies of polychromatic erythrocytes of negative control group were 8.65%. The percentages of micronuclei were increased to 15.15 % after treatment with 2mg/kg mitomycin C as a positive control agent. This result was significantly different ($P \leq 0.05$) from the negative control.

Whereas, mice treated with *Hyoscyamus niger* extract revealed reduction in MN frequency, and this reduction was depending on the concentration of the extract as seen in the table (3-4). A significant decrease in MN (5.40, 4.14, and 2.95 %) was observed after seven days of treatment with extract at concentration 200, 300, and 400 mg/kg respectively when compared with negative and positive control groups.

Table (3-4): Micronucleus index of bone marrow cells in albino mice treated with alkaloidal extract.

Treatment Group	Dose (mg/kg)	Mean ± SE (%)*
Negative control	0	A 8.65 ± 0.52
Positive control (Mitomycin-C)	2	B 15.15 ± 0.62
First dose	200	C 5.40 ± 0.76
Second dose	300	C 4.14 ± 0.37
Third dose	400	C 2.95 ± 0.29

*Significant difference ($P \leq 0.05$) between the means of the micronuleus in the same column

Micronuclei are separated form of nuclei and they present as acentric fragments or lagging chromosomes that fail to incorporate into either of the daughter nuclei during telophase of the mitotic cells. In addition, the micronuclei frequency in mice bone marrow is a very sensitive index of damage produced by either ionizing radiation or chemical mutagen (Vieira *et al*., 2010).

In previous study, Miller and Adler (1990) pointed that 5 – 15 % of the micronuclei were induced by clastogen (Mitomycin C). Micronucleus induction by methotrexate may be resulted from chromosomal damage or damage to the apparatus in the erythroblast of the mouse bone marrow (Armstrong and Galloway, 1993).

Several reports described alkaloids as being mutagens and carcinogens (Wang and Peng, 1996; Fu *et al*., 2004). Nevertheless, there are also some reports described these alkaloids as non-mutagenic and even anti-mutagenic alkaloids (Proud-lock *et al*., 2004).

The appearance of micronuclei may be produced as a result of the formation of laggards and chromosome fragments, which may be surrounded by a nuclear membrane (EL-Bayoumi *et al.*, 1979). No reports on the effect of *H. niger* extract on mice bone marrow cells have been found.

3.3.3. Effects of *Hyoscyamus niger* extract on Chromosomal Aberrations (CAs):

The spontaneous frequency of chromosomal aberration in mice bone marrow cells was 18.0% which represents a negative control (Table 3-5).while the positive group treated with mytomycin-C showed increasing in CAs frequencies (chromosome break, chromatid break, acentric fragment, dicentric fragment and ring chromosome) it recorded 101.0% after 7 days. High significant differences appeared between negative and positive control group.

On the other hand, mice treated with various concentrations of *H.niger* extract were affected chromosomes structure. Notable, the two concentrations 200 and 300 mg⁄kg caused significant chromosomal aberrations such as chromosome

breaks and dicentric fragments. The total number of affected cells was 26.00 and 21.14 per 100 cells. Whereas, using high concentration of extract, 400 mg\kg, reduced the structural chromosome abnormalities in mice treated with this concentration; it achieved 13.33 per 100 cells. (Figure 3-4).

Indeed, decreasing in the frequencies of CAs may be time dependant, which might be due to activation of repair mechanisms and ablation of cell cycle arrest. (Salam *et al.*, 2011).

The Methanolic extract of different plants have activity for decreasing the spontaneous frequency of CAs which occur as a result of metabolic changes inside the body or as a result of presence of undetected environmental and genetic mutagen (Shubber, 1981).

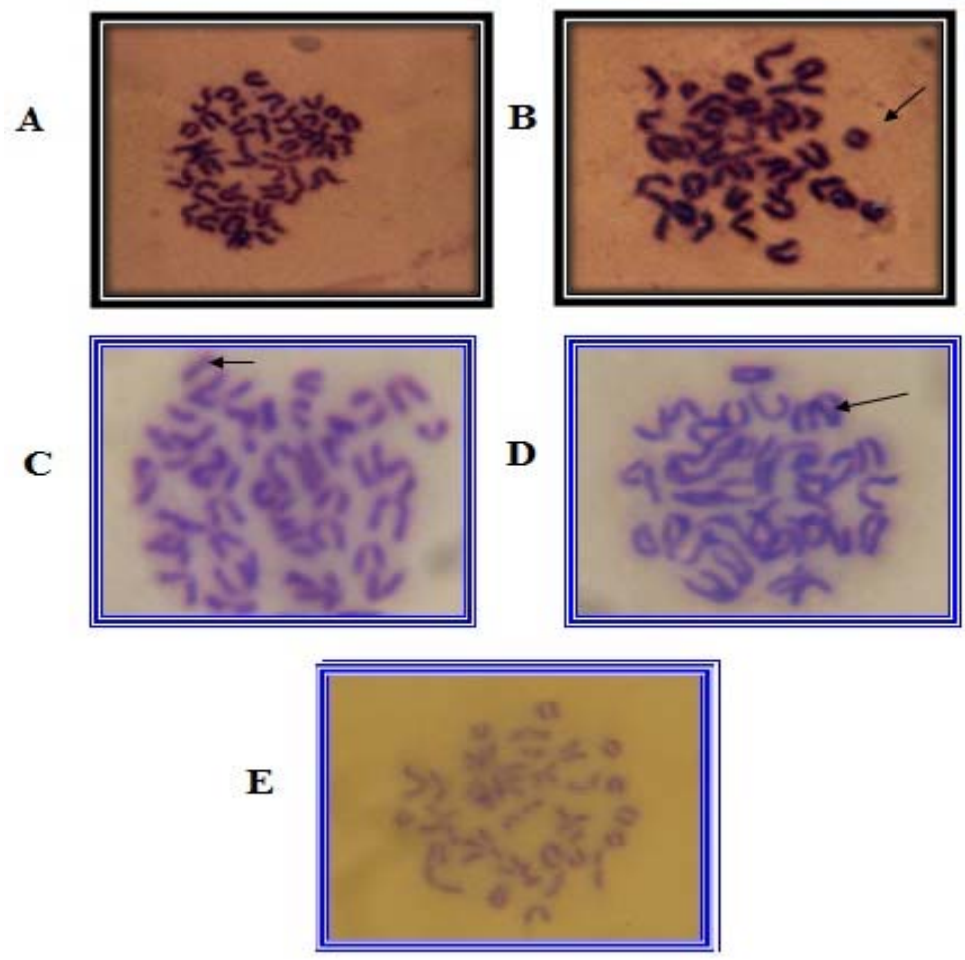


Figure (3- 3) Structure chromosome aberrations in mice treated with extract included: A; normal chromosome; B: ring chromosome; C: acentric chromosome; D: dicentric chromosome E: chromosome break.

Table (3-5): Chromosomal aberration of bone marrow cells in albino mice treated with *Hyoscyamus niger* extract

Chromosomal aberration / Groups	Chromosome break	Chromatid break/100 cells	Acentric fragment/ 100 cells	Ring chromosome/ 100 cells	Dicentric fragment/ 100 cells	Total No./100 cells
Negative control (PBS)	A 5.33+1.08	A 3.22+0.94	A 6.00+2.01	A 0.0+0.0	A 4.00+1.10	A 18.00+5.72
Positive control (Mitomycin C)	B 14.0+4.76	B 24.56+7.42	B 29.53+8.45	B 12.00+3.86	B 22.81+7.92	B 101.0+14.12
Extract 200mg/kg	C 9.24+2.44	A 4.11+0.84	AB 4.02+1.15	C 3.11+1.04	C 6.33+2.81	C 26.00+6.31
Extract 300mg/kg	C 7.51+2.03	A 4.00+1.38	B 3.33+0.94	A 0.0+0.0	C 7.00+1.75	C 21.14+6.39
Extract 400mg/kg	A 4.41+1.85	A 3.33+1.03	B 2.00+0.84	A 0.0+0.0	A 4.33+1.25	A 13.33+3.72

Significant difference ($P \leq 0.05$) between the means of the mitotic index in the same column

Generally abnormal mitotic mechanisms may result in numerical or structural aberration in the daughter cells. Numerical aberration can be caused either by loss of chromosomes at metaphase/anaphase or by multipolar divisions associated with abnormal number or structure of centrosomes. Structural rearrangements have been associated with the chromosomal breakage that can be initiated by telomeric dysfunction, giving rise to unstable dicentric or ring chromosomes, this large number of chromosomal changes frequently seen in malignant solid tumors (Agapova *et al.,* 1996).

3.4. Free Radical Scavenging Activity:

The effect of the extract fractions on scavenging the free radicals was determined, and given as a percentage of antiradical activity (ARA) for seven fractions.

Table (3-6) and figure (3-4) showed that fraction G had a significant influence on scavenging free radicals in comparison with other fractions obtained from alkaloidal extract of this plant, and in comparison with positive and negative controls; the antioxidant activity (%) reached to 39.62 %.

Table (3 – 6) antiradical activity (%) of seven fractions of *H. niger* extract beside positive control (aa)

Fractions	Antradical activity(%) ±SE*
A	C 6.36±0.69
B	C 3.75±0.07
C	B 32.01±2.14
D	C 1.46±0.02
E	C 1.67±0.06
F	C 3.02±2.22
G	B 39.62±2.37
Positive Control (aa)	A 78.31±3.75
LSD value	13.74

*Different capital letters: Significant difference ($p< 0.05$) between means of column.

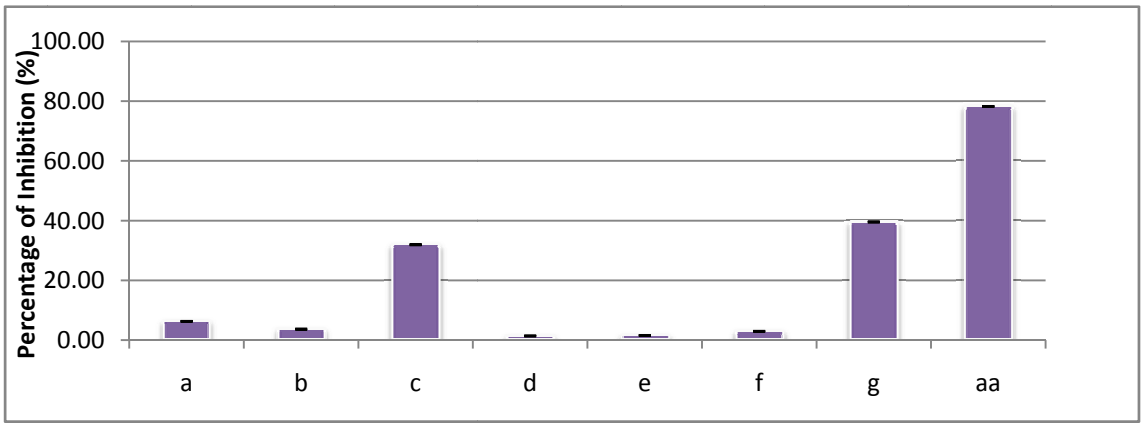


Figure (3 – 4) Antiradical Activity of *H. niger* extract

Different concentrations of the fraction G (1000, 500, 250, 125, 62.5, 31.3 µg/ml) was taken and assayed for scavenging free radicals. Table (3-7) showed significant differences in their activity.

Table (3–7) Antiradical activity (%) of different concentrations of fraction G.

Concentrations of G Fraction(µg/ml)	Antiradical activity (%) Mean+SE*
1000	A 1.5150±0.02
500	A 1.7650±0.23
250	B -2.4450±0.09
125	B -1.1800± 0.02
62.5	B -1.1800±0.11
31.3	B -2.2750±0.13
LSD value	0.748

* Different capital letters: Significant difference ($p < 0.05$) between means of column.

Generally, there are numerous reports stating that the risk of degenerative diseases is diminished in people consuming large quantities of vegetables and fruits. At the same time it should be taken into account that the antioxidant defense system of the human body is composed of different antioxidant compounds. The quality and antioxidant capacity of vegetables have also been recognized as effective supplement (Vinson *et al*., 1998).

It should be noted that the antioxidative performance depend not only on the extraction method, but also on the quality of the original plant, its geographic origin, the harvesting date, its storage and the processing prior to extraction (Armstrong, 1998).

The potential antioxidant protective effects of natural compounds on affected tissues are topics of high current interest. Initially it is necessary to

investigate *in vitro* antioxidant properties of any natural product or drug to consider it as an antioxidant substance, followed by evaluation of its antioxidant function in biological systems (Anand and Shrihari, 2010).

The inhibitory effect of methanolic extract of *H.niger* on linoleic acid peroxidation is more than 70% inhibition, using 4mg of plant extract residue (Effect *et al.*, 2004).

Flavonoids compounds in *H. squarrosus* showed nitric oxide scavenging activity this may help to arrest the chain of reactions initiated by excess generation of nitric oxide that are detrimental to human health. The compounds such as flavonoids, which contain hydroxyl functional groups, are responsible for antioxidant effect in plants (Cook and Samman, 1996).

Seyd (2009) investigated the antioxidant activity of fruits of *H.squarious* I.C.50 for DPPH radical, scavenging activity was 0.325 mg/ml and also the leaves extract can serve as an electron donor for terminating the radical chain reaction. So reduction in absorbance is due to the pairing of the odd electron of the radical indicating the ability of the compounds to scavenge free radicals (Ganiyat et *al.*, 2010).

3.5. Cytotoxicity of Alkaloidal Extract on Tumor Cells

Five concentrations (1000, 500, 250, 125, 62.5 µg/ml) of crude alkaloidal extract of *H. niger* were evaluated for their cytotoxicity as percentage of growth inhibition (PGI) on growth of cancer cell lines (AMN-3, Hep-2, RD) and normal cell line (MEF) after three periods of incubation (24, 48 and 72 hours).After 24 hrs. of incubation, the alkaloidal extract of *H. niger* at all concentrations used in the experiment showed significant reduction ($p < 0.05$) on all cell lines (MEF, Hep-2, AMN-3, RD) as shown in the table (3- 8). The first two concentration (1000, 500 µg/ ml) recorded a percentage of growth inhibition (84.40, 82 %) respectively in Hep-2 cell line, while another three concentration (250, 125, 62.5) decrease the PGI range of (30, 21.85, to 20%).

While in RD cell line the PGI was 43.22 % in the first conc. (1000 µg/ml) and then reduce in PGI with decrease concentration to reach 23.66 % in the five conc. (62.5 µg/ml). The maximum inhabitation of AMN-3 cell line reached to 33% at the conc. (1000µg/ml). Whereas, no effect where notice on MEF normal cell line at all concentrations of alkaloidal extract of *H. niger.*

Table (3-8): Growth inhibition of alkaloidal extract on the cell lines after 24 hrs.

Concentration (µg / ml)	Inhibition (%)± S.E.*				LSD Value
	AMN-3	MEF	Hep-2	RD	
1000	33.00 ± 2.06	18.30 ± 0.96	84.40 ± 4.29	43.22 ± 2.37	15.83
500	9.52 ± 0.74	13.30±0.72	82.00 ± 4.17	41.96 ± 2.24	13.69
250	5.95 ± 0.35	13.30 ± 0.71	30.00 ± 1.98	39.21 ± 2.19	9.51
125	- 30.95 ± 1.98	11.60±0.59	20.00 ± 0.87	30.60 ± 1.96	16.38
62.5	- 16.67 ± 0.73	8.30±0.47	21.58 ± 1.14	23.66 ± 1.28	11.57

* Significant difference ($p< 0.05$) between means of column.

After 48 hrs. of incubation approximated values of PGI were reduced in the five concentration for all cell lines, the first three concentrations (1000, 500, 250 µg/ml) reduced a PGI rang of (86, 84.8 and 84 %) respectively in Hep-2 cell line. Also the PGI of first three concentration of alkaloidal extract reached to (46, 43.82 43.82 %) respectively in AMN-3 cell line. While no PGI was notice in RD cell line when treated with five concentrations and also in MEF cells showed no growth inhibition (Table 3-9).

Table (3-9): Growth inhibition of alkaloidal extract on the cell lines after 48 hrs.

Concentration (µg / ml)	Inhibition (%) ±S.E*				LSD Value
	AMN-3	MEF	Hep-2	RD	
1000	46.00 ± 2.43	2.08 ± 0.06	86.00 ± 4.61	15.89 ± 0.72	17.41
500	43.82 ± 2.37	4.17 ± 0.08	84.80 ± 4.52	5.51 ± 0.09	16.83
250	43.82 ± 2.37	2.08 ± 0.06	84.00 ± 4.25	- 5.30 ± 0.07	19.02
125	31.46 ± 2.05	2.10 ± 0.07	48.70 ± 2.73	- 8.61 ± 0.21	13.58
62.5	30.34 ± 1.94	2.08 ± 0.06	42.70 ± 2.46	- 31.13 ± 2.06	16.37

* Significant difference ($p< 0.05$) between means of column.

Five concentration of alkaloidal extract (1000, 500, 250, 125, 62.5 µg/ml) were effective in reducing the growth inhabitation of Hep-2 cell line which reached to (83, 83, 75, 64.4, 50 %) respectively after 72 hrs. of incubation.

While alkaloidal extract showed significant difference in the growth of RD cell line, PGI reached to 46 % when treated with (1000µg/ml) of the alkaloidal extract where as no growth inhabitation were noticed in the AMN-3 and MEF cell lines in all conc. of alkaloidal extract as shown in table (3-10).

Table (3-10): Growth inhibition of alkaloidal extract on cell lines after 72 hrs.

Concentration (µg / ml)	Inhibition (%) ± S.E*				LSD Value
	AMN-3	MEF	Hep-2	RD	
1000	28.79 ± 1.58	- 3.92 ± 0.05	83.00 ± 4.12	46.00 ± 2.59	13.26
500	- 9.09 ± 0.67	9.43 ± 0.75	83.00 ± 4.12	30.94 ± 1.96	14.54
250	- 3.03 ± 0.06	- 3.92 ± 0.05	75.00 ± 3.68	29.32 ± 1.68	11.79
125	-15.15 ± 0.83	- 23.53 ± 1.48	69.40 ± 3.51	21.82 ± 1.14	16.36
62.5	- 33.33 ± 2.17	- 3.92 ± 0.05	50.00 ± 2.78	15.31 ± 0.68	9.44

* Significant difference ($p < 0.05$) between means of column.

In comparison, the alkaloidal extract was effective in reducing the growth inhabitation % in Hep- 2 cell line during 72 hrs of incubation, which showed all five concentrations were inhibited Hep-2 cells as compared when treated after 24, 48 hrs. of incubation as shown in the figure (3-5) no significant difference in the growth inhibition in two conc. (1000, 500 µg/ml) of alkaloidal extract in three period times of incubation. (Table 3-11)

Table (3-11): Growth inhibition of alkaloidal extract on Hep-2 cell line in three periods time of incubation

Concentration (µg / ml)	Inhibition (%) means±S.E*			LSD Value
	24	48	72	
1000	Aa 84.40 ± 4.29	Aa 86.00 ± 4.61	Aa 83.00 ± 4.12	7.36 NS
500	Aa 82.00 ± 4.17	Aa 84.80 ± 4.52	Aa 83.00 ± 4.12	6.94 NS
250	Ab 30.00 ± 1.98	Ba 84.00 ± 4.25	Cab 75.00 ± 3.68	10.41
125	Ab	Bb	Cb	

	20.00 ± 0.87	48.70 ± 2.73	69.40 ± 3.51	8.57
62.5	Ab 21.58 ± 1.14	Bb 42.70 ± 2.46	Cc 50.00 ± 2.78	7.19
LSD Value	12.47	11.87	8.26	---

* Different capital letters: Significant difference ($p< 0.05$) between means of column.
Different small letters: Significant difference ($p<0.05$) between means of rows.
NS:non- significant.

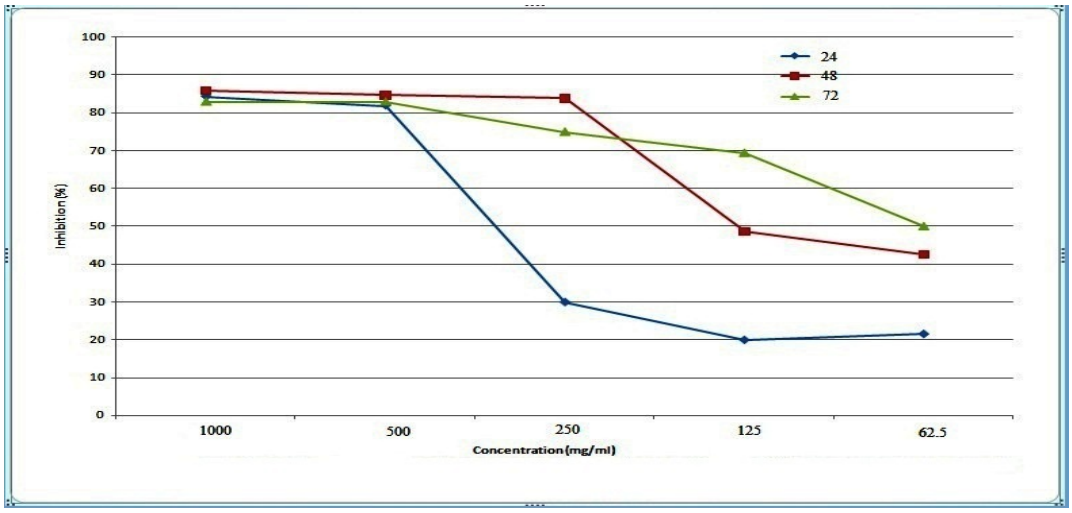


Figure (3- 5): Growth inhibition of alkaloidal extract on Hep-2 cell line in three periods of incubation.

While the best growth inhibition of RD cell line where treated with alkaloidal extract of *H. niger* shown after 24 and 72 hrs. as compared after 48 hrs. of incubation which reached to 43.22, 46% respectively as shown in the table (3-12) and figure (3-6).

Table (3-12): Growth inhibition of alkaloidal extract on RD cell line in three periods of incubation.

Concentration (µg / ml)	Inhibition (%)*			LSD Value
	24	48	72	
1000	Aa 43.22 ± 2.37	Ba 15.89 ± 0.72	Aa 46.00 ± 2.59	6.41
500	Aa 41.96 ± 2.24	Ba 5.51 ± 0.09	Cb 30.94 ± 1.96	7.83
250	Aa 39.21 ± 2.19	Bb - 5.30 ± 0.07	Ab 29.32 ± 1.68	11.26
125	Ab 30.60 ± 1.96	Bb - 8.61 ± 0.21	Abc 21.82 ± 1.14	9.63
62.5	Ac	Bb	Ac	

	23.66 ± 1.28	- 31.13 ± 2.06	15.31 ± 0.68	12.92
LSD Value	6.18	10.43	8.51	---

* Different capital letters: Significant difference ($p< 0.05$) between means of column.
Different small letters: Significant difference ($p<0.05$) between means of rows.

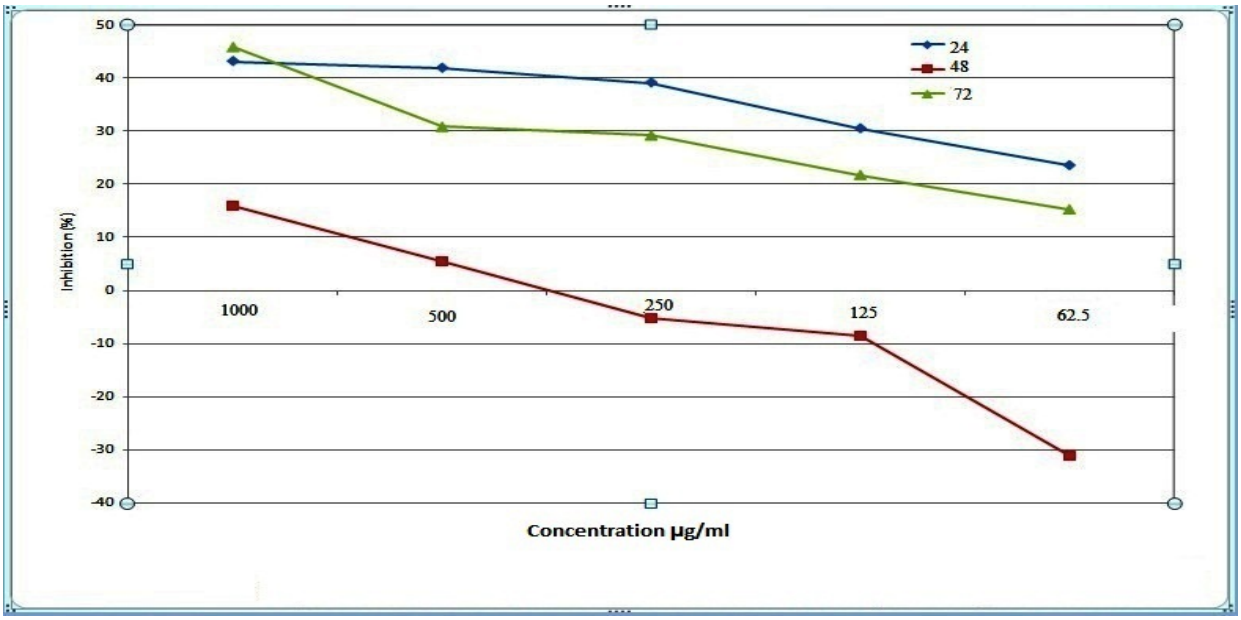


Figure (3-6): Growth inhibition of alkaloidal extract on RD cell line in three periods time of incubation.

Where as the inhibition percentage in AMN-3 cell line obtained after 48 hrs. of incubation reached to 46% when treated with (1000 µg/ ml) concentration of alkaloidal extract, as compared with 24 and 72 hrs.(table 3-13 and figure 3-7).

Table (3- 13): Growth inhibition of alkaloidal extract on AMN-3 cell line in three period of incubation.

Concentration (µg / ml)	Time (hour)			LSD Value
	24	48	72	
1000	Aa 33.00 ± 2.06	Ba 46.00 ± 2.43	Aa 28.79 ± 1.58	6.253
500	Ab 9.52 ± 0.74	Ba 43.82 ± 2.37	Cb - 9.09 ± 0.67	12.58
250	Ab 5.95 ± 0.35	Ba 43.82 ± 2.37	Cb - 3.03 ± 0.06	10.92
125	Ac - 30.95 ± 1.98	Bb 31.46 ± 2.05	Ab - 15.15 ± 0.83	16.44

62.5	Ac - 16.67 ± 0.73	Bb 30.34 ± 1.94	Ab - 33.33 ± 2.17	14.91
LSD Value	11.26	7.02	9.83	---

*Different capital letters: Significant difference ($p< 0.05$) between means of column.
Different small letters: Significant difference ($p<0.05$) between means of rows.

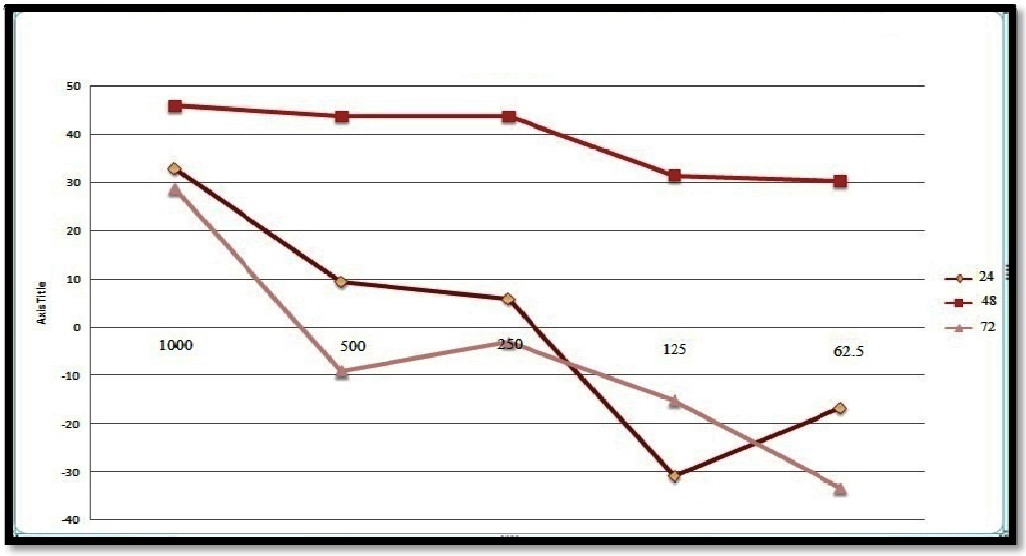


Figure (3-7): growth inhibition of alkaloidal extract on AMN-3 cell line in three periods of incubation

No growth inhibition on MEF normal cell line when treated with five concentration of alkaloidal extract in all period of times as shown in the table (3-14) and figure (3-8).

Table (3-14): Growth inhibition of alkaloidal extract on MEF cell line in three periods of incubation.

Concentration (µg / ml)	Inhibition (%)*			LSD Value
	24	48	72	
1000	Aa 18.30 ± 0.96	Ba 2.08 ± 0.06	Ca - 3.92 ± 0.05	4.19
500	Aa 13.30 ± 0.72	Ba 4.17 ± 0.08	Ab 9.43 ± 0.75	3.62
250	Aab 13.30 ± 0.71	Ba 2.08 ± 0.06	Ba - 3.92 ± 0.05	5.98
125	Aab	Ba	Cc	

	11.30 ± 0.59	2.10 ± 0.07	- 23.53 ± 1.48	7.16
62.5	Ab 8.30 ± 0.47	Ba 2.08 ± 0.06	Ba - 3.92 ± 0.05	6.54
LSD Value	6.44	3.25 NS	8.30	---

*Different capital letters: Significant difference ($p< 0.05$) between means of column.
Different small letters: Significant difference ($p<0.05$) between means of rows.

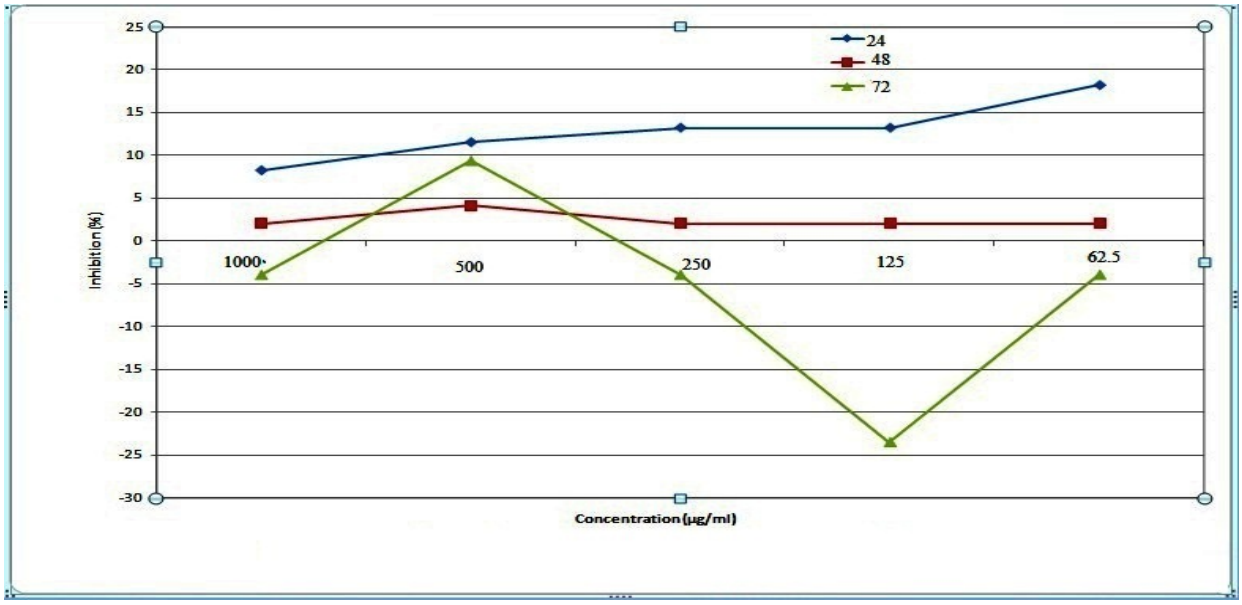


Figure (3-8): Growth inhibition of alkaloidal extract on MEF cell line in three periods of incubation.

3.6. Cytotoxicity of alkaloidal extract on tumor cells using MTT assay:

The cytotoxicity of alkaloidal extract at various concentrations (1000, 500, 250, 125, 62.5 µg/ml) was examined on different cell lines,WRL68, A549, PC-3, HepG2 for 24 hours, the viability of the cells was determined by using MTT assay, which is based on the ability of live cells to cleave the tetrazoliam ring and convert it to formazan crystals. Table (3-15) showed the significant decrease ($p\leq 0.05$) of *H. niger* on cell viability of cancer cell lines (PC-3, HepG2, and A549) when treated with 1000 µg/ml.

Table (3-15): Growth inhibition of alkaloidal extract on different cell lines

Concentration (µg / ml)	Inhibition (%) ± S.E*				LSD Value
	WRL	A549	PC-3	Hep-G2	

1000	Ba 27.63 ± 1.78	Aa 39.22 ± 2.15	Ca 15.27 ± 0.68	BCa 21.49 ± 1.07	6.42
500	Bb 8.92 ± 0.64	Ab 22.73 ± 1.29	Bb 6.03 ± 0.11	Bb 3.57 ± 0.08	5.38
250	Bb 6.07 ± 0.13	Abc 15.04 ± 0.67	Bb 3.62 ± 0.07	Bb 3.03 ± 0.03	5.02
125	Bb 4.69 ± 0.08	Ac 10.49 ± 0.42	Bc 1.61 ± 0.02	Bb 2.68 ± 0.03	4.73
62.5	Bb 3.11 ± 0.04	Ac 10.43 ± 0.38	Bc 0.00 ± 0.0	Bb 1.26 ± 0.02	4.29
LSD Value	7.53	9.66	4.37	5.26	---

*Different capital letters: Significant difference ($p < 0.05$) between means of column.
Different small letters: Significant difference ($p<0.05$) between means of rows

Bioactive components present in plants can prevent carcinogenesis by blocking metabolic activation, increasing detoxification, or providing alternative targets for electrophonic metabolites (Milner, 2001 and Keum *et al*., 2004).

The tumor inhibitors of plant origin depend upon the type of cancer cells and plant species as well as the extract used. Most chemo preventive compounds and their analogs or derivatives are initially of plant origin and inhibit spontaneous and chemical mutagenesis in a variety of *in vitro* and *in vivo* test systems (Xifeng *et al*, 2007). Recently, Bassem *et al*., (2010) studied the anticancer activity of *Hyoscyamus desertorum* on HepG2 cells, which showed the percentage of inhibition of cell proliferation is 97%.

Dried powders of *Hyoscyamus* seeds showed no cytotoxicity *in vitro* to normal skin fibroblast when the cells were cultured by either a direct or indirect culture method. In fact, culture of the fibroblast at 10% showed a slight increase in cell proliferation (Sirlkhatim, 2011).

3.7. High Content Screening Assay (Multiparmeter Cytotoxicity 3 kit) of *Hyoscyamus niger* extract *in vitro*

Two cancer cell lines A549 and PC-3 were treated with plant extract using high-content screening assays to enable quantitative measurement of multiple parameters related to cytotoxicity. This kit enabled simultaneous measurements in

the same cell of independent parameters that monitor cell health, including cell loss, nuclear size and morphological changes, mitochondrial membrane potential changes, cytochrome c release and change in cell permeability by using different dyes.

1- Changes in nuclear size and morphology:

Following plant extract take, cells often undergo either necrosis, or apoptosis, accompanied by changes in nuclear size and/or morphology. Hoechst dye, one of the dyes used in this assay, labels DNA and emits a blue fluorescence. The Hoechst dye enables monitoring of cell loss, nuclear morphology changes and DNA content that are caused by plant extract.

Plant concentrations showed significant differences when treated with A549 cell line. The concentration 1000 µg/ml of alkaloidal extract was showed remarkably changes in nuclear morphology and size when compared with positive control (Valinomycin) and negative control (untreated cell) (Figure 3-9), resulted in cell loss, nuclear condensation and increased total nuclear intensity.

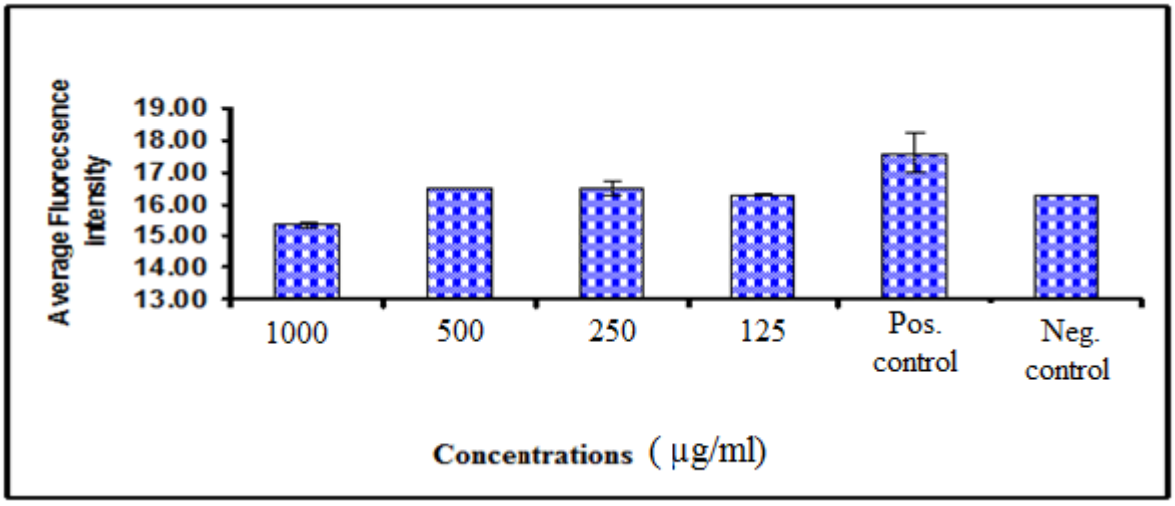


Figure (3-9) Effect of various concentrations of alkaloidal extract on nuclear morphology of cancer cell line A549.

Also the alkaloidal extract affected in the nuclear morphology of PC-3 which show significant differences when treated with four concentration of alkaloidal extract as compared with positive control as shown in the figure (3-10).

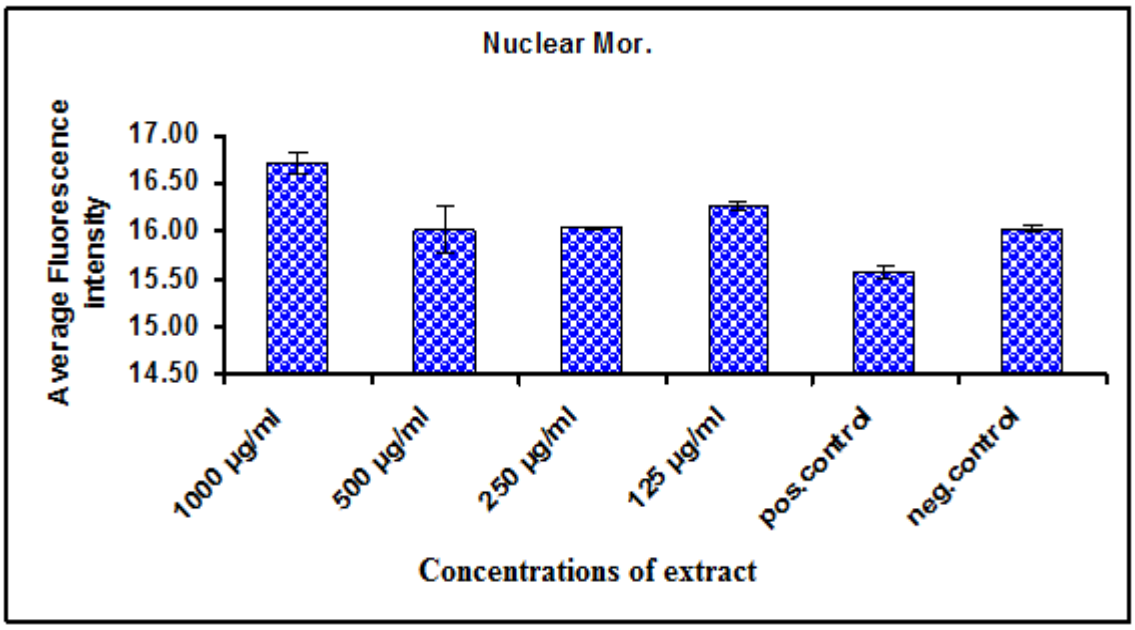
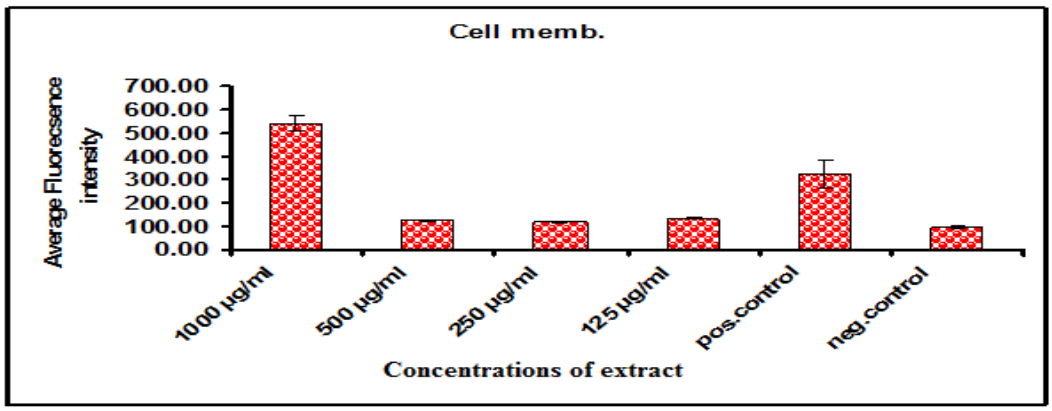


Figure (3 -10) Effect of various concentrations of alkaloidal extract on nuclear morphology of cancer cell line PC-3

2- Cell membrane permeability: the cell membrane maintains cellular homeostasis by holding the varied constituents of the cell together, providing a specialized environment different from its extra cellular surroundings, and providing a mechanism for the controlled exchange of its nutrients with its surroundings (Manjo, 1995).

The concentrations of alkaloidal extract can affect cell membrane integrity leading to the cell becoming permeable, eventually causing cell death. Healthy cells are nearly changed from impermeable to the permeability dyes; however, after compromising the cell membrane's permeability, the nucleus appeared with a



bright green fluorescence. 1000µg\ml was the maximum concentration of plant extract that affected cell membrane permeability of two cancer cell lines PC-3 and A549 in comparison with positive and negative controlsas shown in the figure (3-11 and 3-12).

Figure (3-11) Effect of various concentrations of alkaloidal extract on cell membrane of cancer cell line A549

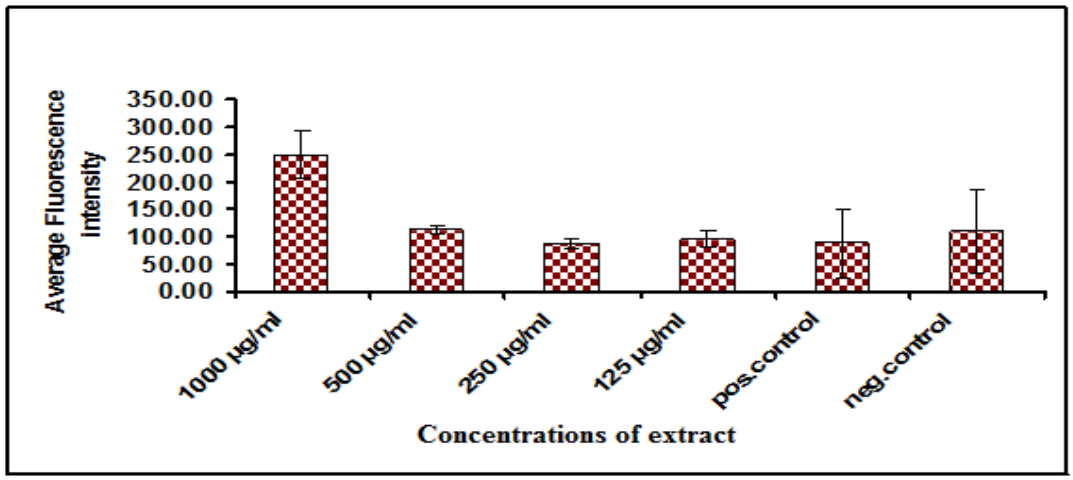
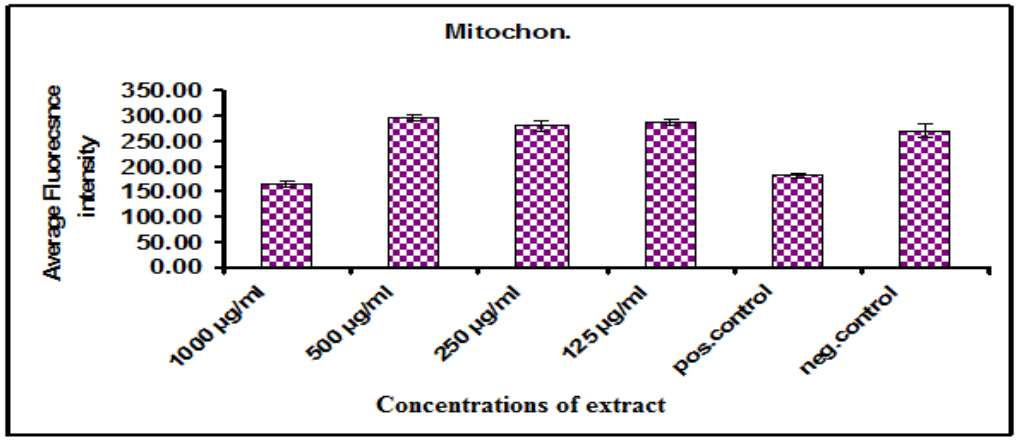


Figure (3 -12) Effect of various concentrations of *H. niger* extract on cell membrane of cancer cell line PC-3

3- Changes in Mitochondrial membrane potential:

Changes in mitochondria play a central role in apoptosis. Mitochondria can proliferate during stimulation of apoptosis leading to an increase in total mitochondrial mass. The mitochondrial membrane potential dye accumulates in healthy mitochondria, caused by its transmembrane potential, and is absent from depolarized mitochondria that result from a cytotoxic compound (Green, 1998).

Crude alkaloidal extract of *H. niger* affected the mitochondrial membrane of the cancer cell line at maximum concentration 1000 µg/ml which lead to release of



cytochrome C as compared with positive and negative control (Figure 3-13 and 3-14).

Figure (3 -13) Effect of different concentrations of *H. niger* extract on mitochondrial cell line A549

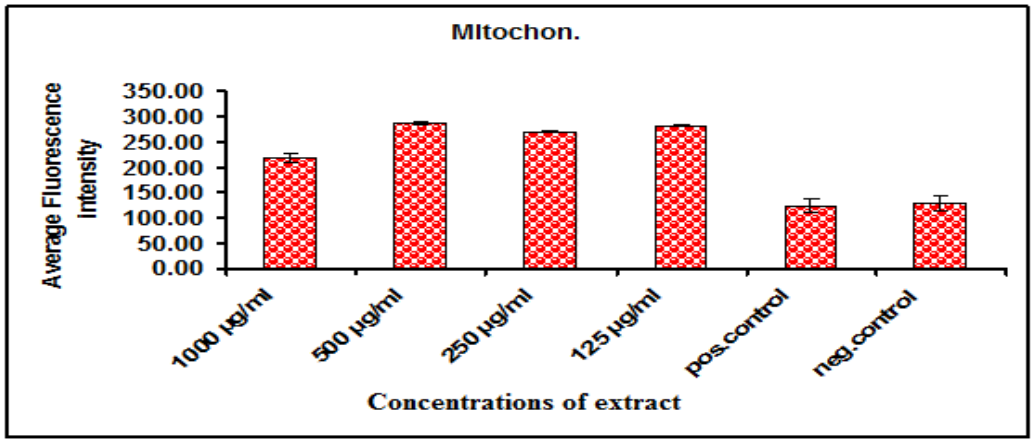


Figure (3 - 14) Effect of different concentrations of *H. niger* extract on mitochondrial cell line PC-3

Screening potential drugs for toxicity is an essential aspect of the drug discovery process. Cytotoxicity is a complex process affecting multiple parameters and pathways. After plant extract take, cells often undergo either apoptosis or necrosis accompanies by changes in nuclear morphology, cell permeability and mitochondrial function, resulting in loss of mitochondrial membrane potential and release of cytochrome C from mitochondria as shown in the figure (3-15) and (3-16).

During the past decades, killing of tumors through the induction of apoptosis has been recognized as a novel strategy for the identification of anticancer drugs. Apoptosis can be triggered by activation of the death receptor (extrinsic) and mitochondrial (intrinsic) pathways (Maneesha *et al*., 2011).

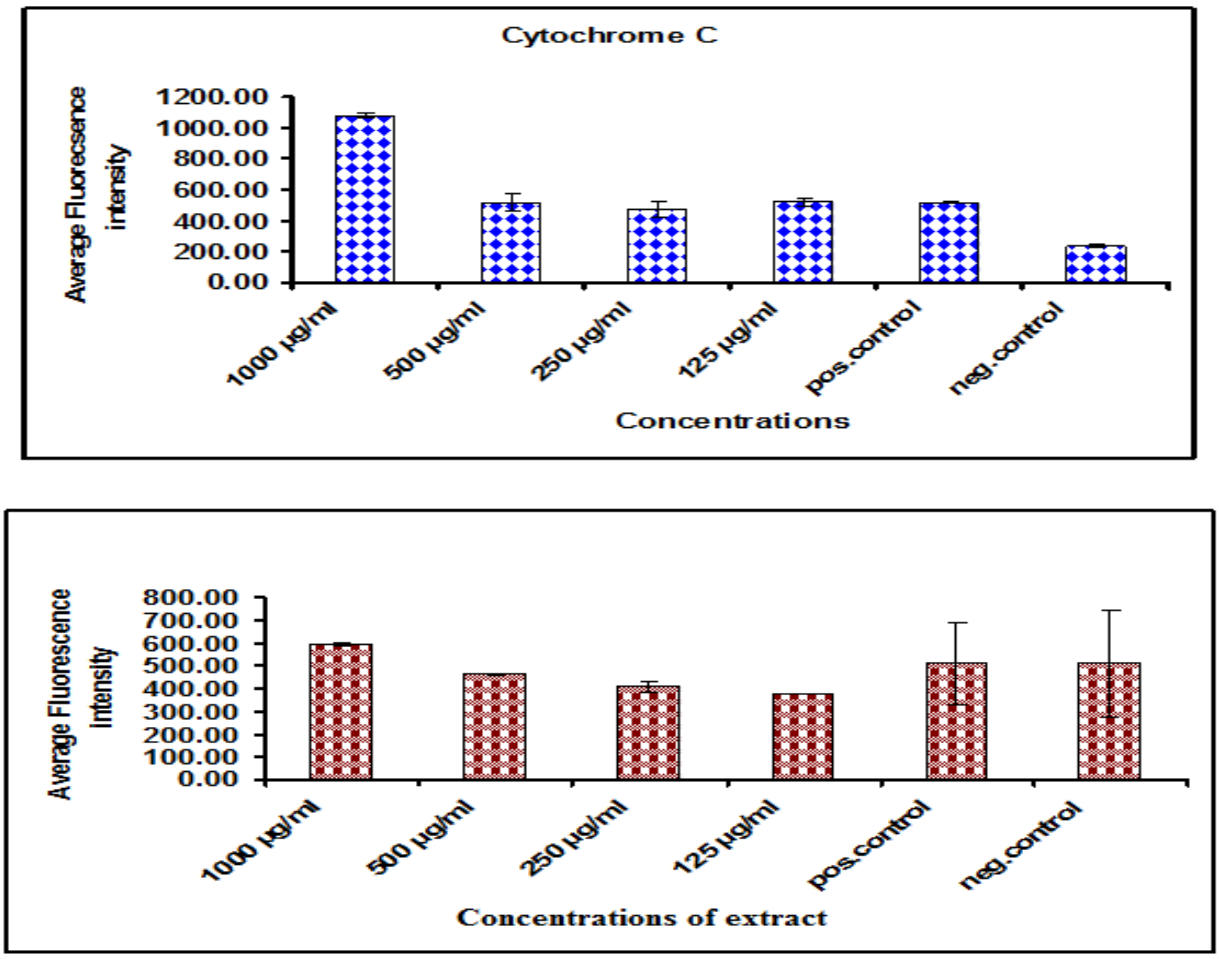


Figure (3 - 15) Effect of different concentrations of alkaloidal extract on cytochrome C cancer cell line A549

Elemene is a novel anticancer drug, which is extracted from the ginger plant, which triggered apoptosis in non-small-cell lung cancer cells through a mitochondrial release of the cytochrome C-mediated apoptotic pathways. In addition 6-gingerol has two types of antitumor effects: 1) direct cancer cell growth suppression and 2) inhibition of the blood supply of the tumor via angiogenesis (Brown *et al*, 2009).

A number of plant extracts were found to induce loss of cell viability without inducing apoptosis. Furthermore, some extracts induced apoptosis with a minimal

amount of loss of cell viability. This phenomenon might be due to differences in sensitivity of the cell lines. In addition, apoptosis might be induced at late time-points after exposure, but cells still remaining viable (Bassem, et al 2010).

Apoptosis is an attractive screening endpoint in anticancer drug discovery. First, this form of cell death is induced by many clinically used anticancer agents. Secondly, it seems logical that by screening for apoptosis, agents that are cytotoxic by unspecific mechanisms such as membrane destabilization will be excluded. In cell cultures, apoptosis cells will eventually disintegrate and may escape detection if cells are analyzed at late-time-points. (Kola and Landis, 2004).

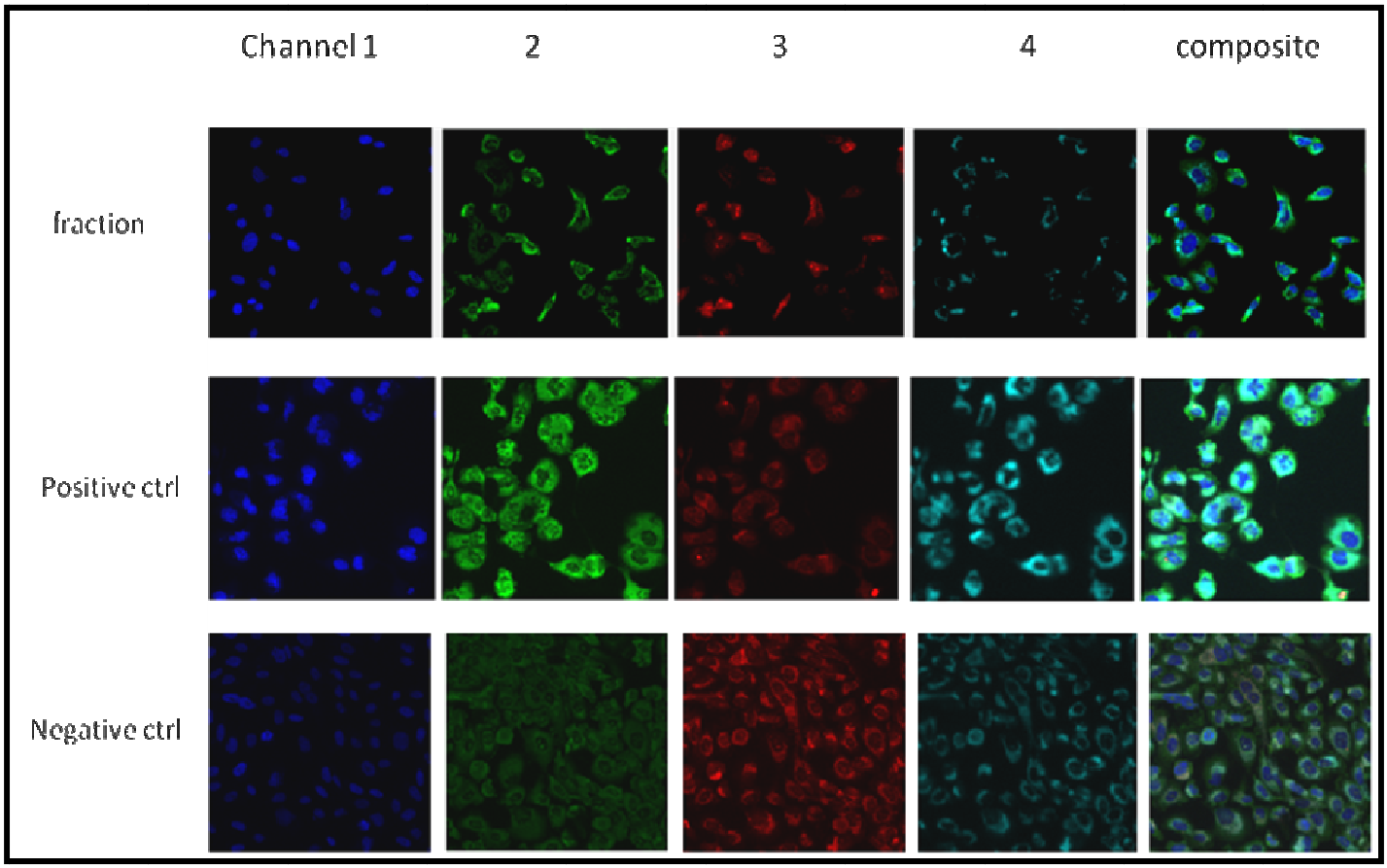


Figure (3-17) Effects of alkaloidal extract on nuclear size (1), cell membrane permeability (2), mitochondrial membrane potential (3), cytochrome C (4) of the A549 cell line.

Channel 1: Hoechst dye

Channel 2: Cell membrane permeability staining

Channel 3: Mitochondrial membrane potential staining

Channel 4: Cytochrome c staining

Channel 5: Composite Image

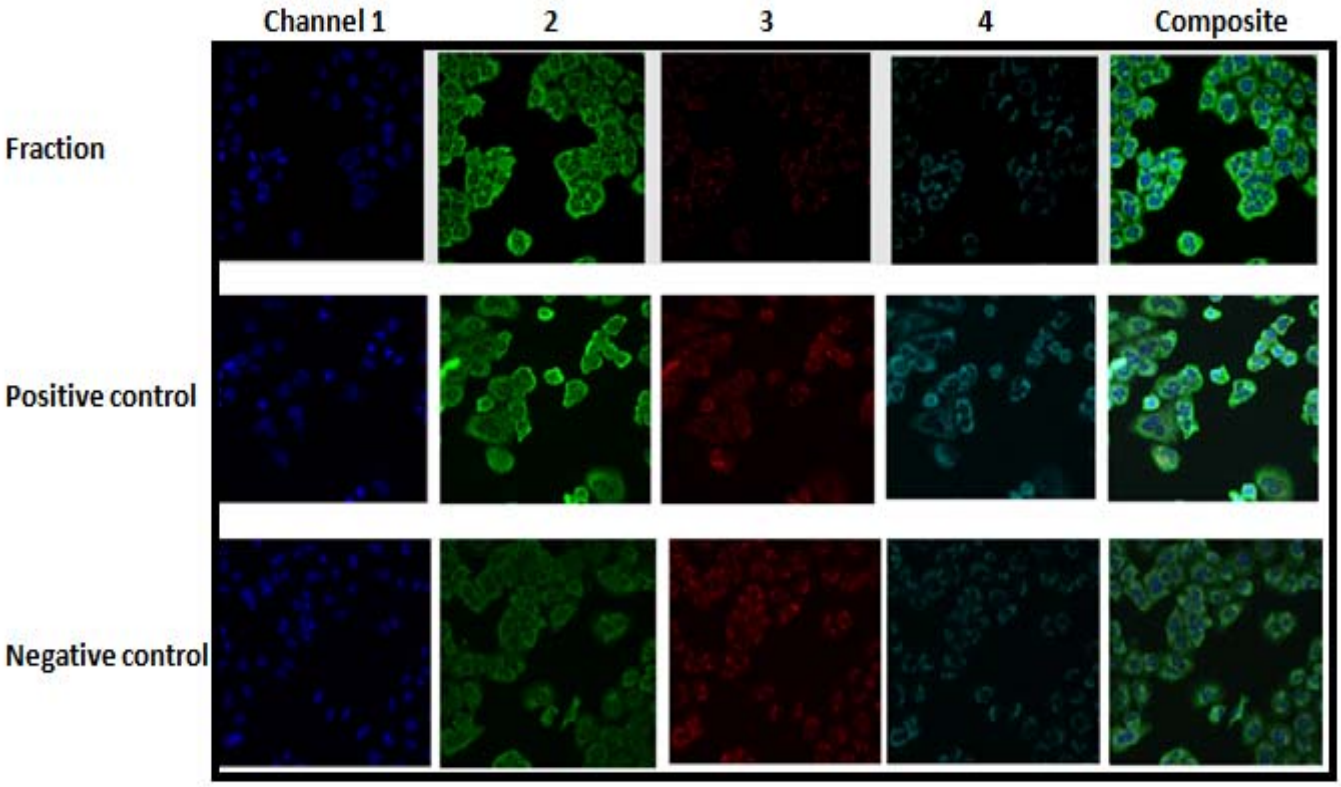


Figure (3-18) Effects of alkaloidal extract on nuclear size (1), cell membrane permeability (2), mitochondrial membrane potential (3), cytochrome C (4) of the A549 cell line.

Channel 1: Hoechst dye

Channel 2: Cell membrane permeability staining

Channel 3: Mitochondrial membrane potential staining

Channel 4: Cytochrome c staining

Channel 5: Composite Image

- Agapova, L.S.; Ilyinskaya, G.; Turovets, N.; Ivanov, A. and Kopnin, B. (1996). Chromosome changes caused by alteration of P53 expression. Mutation Research. 354 (1): 129 – 138.
- AL- Shamary, A.M. (2003). The study of the Newcastle disease virus effect in the treatment of transplanted tumors in mice. MSC thesis. College of veterinary medicine, University of Baghdad.
- AL-Khazraji, S.M. (1991). Biopharmacological study of *Artemisia herba Alba*, M.Sc. Thesis, College of Pharmacy. University of Baghdad. Iraq.
- Allen, J.W.; Shuler, C.F.; Mendes, R.W. and Latt, S.A. (1977). A simplified technique for *in vivo* analysis of sister chromatid exchanges using 5- bromodeoxy uridine tablets. Cell Genet. 18: 231 – 237.
- Allen, R.T. (1997). Morphological and biochemical characterization and analysis of apoptosis. J. Pharmacol.,Toxicol.Methods, 37: 215 – 228.
- AL-Mohammed, N.T.; AL-Rawi, K.M.; Younis, M.A.; and AL-Morani, W.K. (1986). Principles of Statistics. J. AL-Mosul University. Iraq.(in Arabic)
- AL-Rikabi, A.G. (1997). Cytogenetic and reproductive study of cobbelt effect on the working in industrial Battery in Iraq. Ph.D. thesis. College of science - AL-Mustensiria University.
- Amit, K. T.; Madhumitam, R. and Bhattacharya, R. K. (2001). Natural products as inducer of apoptosis. Current Science, 80, (11), p.1387-1394.
- Anand, B. and Shrihari, M. (2010). Antioxidant and Immunomodulatory activity of the alkaloidal fraction of *Cissampelos pareira*. Sci. Pharm. 78: 21 – 31.
- Anders, E. (1996). Oxidation of hydroxy benzotriazole by laccase and lignin pexoxidase. Science, 274:2119-2121.
- Andre, N.; Braguer, D.; Brasseur, G.; Goncalves, A.; Lemesle-Meunier, D.; Guise, S.; and Jordan, M. A. (2000). Pactitaxel induces release of cytochrome c from mitochondria isolated from human neuroblastoma cells. Cancer Res. 60:5349-5353.
- Armstrong, D. (1998). Free radical and antioxidant protocols. Methods in molecular biology, vol. 108. Humana Press., NJ, pvii.

- Armstrong, M.J. and Galloway, S.M. (1994). Micronuclei induced in peripheral blood of mu-PIM-2 transgenic mice by chronic oral treatment with 2-Acetyl aminofluorene or benzene but not with diethylnitorosamine or 1, 2-Dichloroethane. Mutation Research, 302: 61 – 70.
- Atefeh, B.; Fatemeh, S.; and Ali, S. (2009). Determination of Hyoscyamine and Scopolamine in four *Hyoscyamus* species from Iran. Iranian J. of Pharmaceutical Research 8 (1): 65 – 70.
- Bassem, S.E.; Walid, F.; Khaled, M.; Salwa, M. and May, E. (2010). Screening of natural products for therapeutic activity against solid tumors. Indian J. of Exper. Bio. 48:258 – 284.
- Begum, M.; Roderich, S.; and Teigo, A. (2006). Hyosgerin, a new optically active coumarinolignan, from the seeds of *Hyoscyamus niger*. Chemical pharmacology Bull. 54(4): 538-541.
- Belijianski, M. (2002). The anticancer agent PB.100 selectivity active malignant cell line inhibits multiplication of sixteen malignant cell lines. Genetic Mol. Bio., 23:224 – 235.
- Brown, A.C.; Shah, C.; Liu, J.; Pham, J.T.; Zhang, J.G. and Jadus, M.R. (2009). Ginger's (*Zingiber officinale*): inhibition of rat colonic adenocarcinoma cell proliferation. Phytotherapy Res., 23 (5): 640 – 645.
- Capek, P.; Machova, E. and Turjan, J. (2009). Scavenging and antioxidant activities of immune modulating polysaccharides isolated from *Salvia officinalis*. International J. of Bio.macromolecules, 44:75-80.
- Carrano, A.V. (1986). Sister chromatide exchange induction and persistence in peripheral blood and spleen lymphocyte of mice. Environmental Mutagenesis, 8 (3):345-355.
- Cesquini. M.; Torsoni, M.A.; Stoppa, G.R. and Ogo, S.H.(2003). The role of flavonoids in induced oxidative damage in red blood cells. Biomed. Pharmaco.Therapy, 57:124-129.
- Chauhan, L.K. Kumar, M. Paul, B.N. Goel, S.k. and Gupta, SK. (2007). Cytogenetic effects of commercial formulations of deltamethrin and/or isoproturon on human peripheral lymphocytes and mouse bone marrow cells. Environ. Mol. Mutagen. 48 (8):636-643.

- Chevallier, A. (1996). The Encyclopedia of Medical Plants, Dorling Kinderseley. London. P. 250-251.
- Collee, J. G; Fraser, A.G.; Mariman, B.P. and Simmons, A. (1996).Macki and MacCartney practical Medical Microbiology (eds.). Churchill Linvingston (14th) Ed.
- Cook, N.C. and Samman, S. (1996). Flavonoids chemistry, metabolism, cardioprotective effects and dietary sources. Nutr. Biochem. 7:66-76.
- Cornforth, M. N. and Bedford, J. S. (1993). Ionizing radiation damage and its early development in chromosome. Advances in Radiation Biology. 17: 423-496.
- Couliano, K.A. Haskins, J.R.; and Taylor, D.L. (2003). Advances in high content screening for drug discovery. Assay Drug Develop. Technol.1: 565-577.
- Cragg, G.M. and Newman, D.J. (2005). Plants as a source of anti cancer agents. J. of Ethno-pharmacology, 100:72 – 79.
- Cragg, G.M.; Newman, D.J.; and Snader, K.M. (1997). Natural products in drug discovery and development. J. Natl. Prod., 60:52-60.
- David, A. F. (2000). Mouse Embryo Fibroblast (MEF) Feeder Cell Preparation. ISMB. 23:23 – 27.
- David, E. E. and Timothy, L. (2009). Plant-derived compounds as antioxidants for health- Are they all really antioxidants? Functional J. Plant Science and Biotechnology. 5(4): 1-12.
- Denizot, F. and Lang, R. (1986). Rapid colorimetric assay for cell growth and survival: modification of the tetrazolum dye procedure giving improved sensitivity and reliability. J. immune. Methods, 89:271-277.
- Drager, B.J. (2002). Analysis of tropane and related alkaloids. Chromatogr. 978:1-35.
- Duke, J.A. (1985). Handbook of Medicinal Herbs. Press, Boca Raton, 297-300.
- Dykens, J.A.; Jamieson, J.D.; Marroquin, L.D.; Nadanaciva S.; XuJ.J.; Dunn, M.C et al. (2008). *In Vitro* assessment of mitochondrial dysfunction

and cytotoxicity of nefazodone, trazodone, and buspirone. Toxicol Sci., 103: 335-345.

- Effat, S.; Gholamreza, A.; Anahita, D.; Atefeh, N.; and Hassan, F. (2004). Antioxidation activity of sixty plants from Iran. Iranian J. of Pharmaceutical Research. 3: 55 – 59.
- Eileen, W. (1993). Death-defying acts: a meeting review on apoptosis. Genes and Development. 7: 2277 – 2284.
- El-Bayoumi, A.S.; Kabarity, A. and Habib, A. (1979). Cytological effects of Papaverine hydrochloride on root tips of *Allium cepa*. Cytologia, 44: 745 – 755.
- Elisabeth, L.; Carine, L.; Veronique, G.; Nathalie, B.; Jean-Luc, D. and Nancy, C. (2006). Performance of the *in vitro* micronucleus test compared to the mouse Lymphoma assay and *in vitro* chromosome aberration assay. Int. J. Cancer, 118, 2470-2478.
- Evans, E.P.; Breckon, G.; and Ford, C.E. (1964). An air drying method for meiotic preparation from mammalian testers. Cytogenetics, 3:284 – 294.
- Fahad, A.Q. and Salim (2009). Mutagenic effects of sodium azide and its application in crop improvement. World Applied Sciences J. 6(12):1589-1601.
- Fahmy, M.A. and Abdulla, E.F. (2001) .Cytogenetic effects induced by the natural pyrethrins and the synthetic lambda cytobaloibrin in mice *in vivo*. Cytologia, 66:139 – 149.
- Fazel, S.; Hamidreza, M.; Rouhollah, G.; and Mohammadreza, V.; (2008). Spectrophotometric determination of total alkaloids in some Iranian medical plants. Thai. J. Pharm. Sci., 32: 17 – 20.
- Fechheimer, N. S. (1968). Consequences of chromosomal aberration in mammals, J. Animal Science, 27:27-50.
- Fenech, M. (1997). The advantages and disadvantages of the cytokinesis-block micronucleus method, Mutation Res., 392:11-18.
- Fenech, M.; Jimmy, C.; Julei, T.; and Shauna B. (1999). Necrosis, apoptosis, cytostasis and DNA damage in human lymphocytes measured simultaneously with in the cytokinesis- block Micro nucleus assay. Mutagenesis, 14(6):605-612.

❖Finch, M.; Chang, W.P.; Kirsch-Volders, M.; Holland, N.; Bonassi, S.; Zeiger, E. (2003): HUMAN project: detailed description of the scoring criteria for the cytokinesis–block micronucleus assay using isolated human lymphocyte cultures. Mutation Research .534:65-75.

- ❖ Folkman, J. (2008). Tumor angiogenesis In: Bast, R.C.; Kufe, D.W.; Pollock, R.F.; Weichsellbom, R.R.; Holland, J.F.; Ferri, E. and Ganster, T.E. (eds.). Cancer Medicine (5th Ed.). BC. Decher Inc. Canada.22-23.
- ❖ Fowler, A.; Seifert, N. Aeker, V.; Woehrle, T.; Kilpert, C. and De-Saizieu, A. (2006). A non radioactive high-throughout/high-content assay for measurement of the human serotonin reuptake transporter function *in vitro*. J. Biomol. Screen. 11(8):1027-1034.
- ❖ Fransworth, N.R.; Akerele, D.; Bingel, A.S.; Soejarto, D.D.; and Guo, Z. (1985). Medicinal Plants in Therapy. J. Bull. 63, 965 – 981.
- ❖ Freshney, R.I. (2000). Culture of animal cell: A manual for Basic Technique. Wiley liss, A John Wiley and Sons, Inc. Publications, New York.
- ❖ Freshney, R.I. (2001). Application of cell culture to toxicology. Cell Biol. Toxicol., 17:213-230.
- ❖ Freshney, R.I. (2005). Culture of animal cells: A manual for Basic Technique. Wiley-liss, A John Wiley and Son, Inc. Publications, New York.
- ❖ Fu, P.P.; Xia, P.; G. and Chou, M.W. (2004). Pyurolizidine alkaloids-genotoxicity, metabolism enzymes, metabolic activation, and mechanisms. Drug Metab. Rev., 31(1) 1 – 55.
- ❖ Galloway, S.M.; Aardema, M.J.; Ishidate, M.; Ivett, J.L.; Kirklqnd, D.J.; Morita, T.; Mossesso, P. and Sofunl, T. (1994). Report from working group on *in vitro* tests for chromosomal aberrations. Mutat. Res., 312:241 – 261.
- ❖ Ganiyat, K.O.; James, M.; Yunus, R. and Tiwalade, A. (2010). Antioxidant and Anticonvulant alkaloids in *Crinum ornatum* Bulb extract. World J. of Chemistry, 5(1):26-31.
- ❖ Gilani, A.H.; Khan, A.U.; Raoof, M.; Ghayur, M.N. and Siddiqui, B.S. (2008). Gastrointestinal, selective airways and urinary bladder relaxant effects of *Hyoscyamus niger* are mediated through dual blockade of

muscarinic receptors and Ca^{+2} channels. Fundamental and Clinical Pharmacology. 22:87-99.

- Gilarii, A.S.; Verma, S.; Sahai, M.; Schneider, K.; and Sussmuth, (2008). Hyoscyamal, a new tetrahydrofurano lignin from *Hyoscyamus niger* Linn. Nat. Prod. Res. 23(7): 595-600.
- Gragg, G.M.; Newman, D.J.; Sander, K.M. (1997). Natural products in drug discovery and development. J. Nat. Prod. 60: 52 – 60.
- Graham, J. and Johnson, W.S. (2004). Managing black henbane. Univ. of Nevada Coop. Fact Sheet FS-04-10.
- Graham, J.G.; Quinn, M.L.; Fabricant, D.S. and Farnsworth, N.R. (2000). Plants used against cancer – an extension of the work of Jonathan Hartwell. J. of Ethno pharmacology, 73:347 – 377.
- Hagelstrom, A.H.; Gorla, N.B.; and Larripa, I.B. (1995). Chromosomal damage in workers occupationally exposed to chronic low level ionizing radiation. Toxicol. Lett., 76:113 –117.
- Halmurat, U.; Abdiryim, Y.; Isabelle, B., Anwar, U. and Nicholas, M. (2008). Inhibition of cell growth and cellular protein, DNA and RNA synthesis in human hepatoma cells by ethanol extract of abnormal *Savda Munziq* of traditional Uighur medicine. CAM advance access published, 15.p:1-9.
- Harborne, J.B. (1973). Phytochemical methods. Science paper Backs, Chapman and Hall, New York.
- Harrison, A. P. and Bortels, E. M. (2006). A modern appraisal of ancient Etruscan herbal practices. American J. of pharmacology and toxicology, 1(1):21-24.
- Hartwell. J.L. (1982). Plants used against cancer. Quarterman, Lawrence. M.A.
- Heidari, M. R.; Heidari, M.; Eimani, G. and Sepehri, G. (2009). Effect of methanolic extract of *Hyoscyamus niger* on the seizure induced by picrotoxcin in mice. J. Pharmacology Science, 22(3):308-312.
- Helen, F.; and John, B. (1994). The human micronucleus project – an international collaborative study on the use of the micronucleus technique for measuring DNA damage in humans. Mutation Research. 428:271-283.

- Ibrahim, A.I.; AbdEL K.; Mustafa, G. and Nower, A. (2009). Alkaloid production and organgenesis from callus of *Hyoscyamus muticus*. In vitro. J. of Applied Sciences Research. 5:88-92.
- Jain, S.K.; and De Filipps, R.A. (1991). Medicinal Plants of India. 1-2. - Reference Pubi. Inc. Michigan. 310-314.
- Jorge, I.; Gonzalez, B.; Amadeu, C.; Ricard, M.; and Jorge, Z. (2003). The mutagenic potential of the furyl ethylene derivative 2-Furyl-1nitroethene in the mouse bone morrow micronucleus test. Toxicological Science, 72:359-362.
- Jorge, S.B.; Melo, M.B.; Costa, F.; and Sonata, M. (2003). Screening for mutations in human alpha-globin genes by nonradioactive single-strand, conformation. Braz.J.Med.Bio.Res., 36 (11):47-49.
- Joseph, M. Z. (2009). Application of High content screening in life science research. Combinatorial chemistry and High throughput screening; 12:870-876.
- Kagan, V. E.; Bayir, A.; Bayir, H.; Stoyanovsky, D.; Borisenko, G. G.; Tyurina, Y. Y.; Wipf, P. and Belikovo, N. A. (2009). Mitochondria-targeted disruptors and inhibitors of cytochrome c/cardiolipin peroxidase complexes. Mol. Nutr. Res; 53:104-114.
- Kakazu, N.; Bar-Am, I.; Hada, S.; Ago, H. and Abe, T. (2003). A new chromosome banding technique, spectral colour for full characterization of chromosomal abnormalities. Genes Chromosomes Cancer, 37: 412 – 416.
- Kerstin, K. and Eberhard, K. (2007). Cell-based high content screening of small-molecule libraries. Current opinion in Chemical Biology, 11:503-510.
- Keum, Y.; Jeong, W.; Kong, A. (2004). Chemoprevention by isothiocyanates and their underlying molecular signaling mechanisms. Mutation Research 555:191 – 202.
- Kola, I. and Landis, J. (2004). Can the pharmaceutical industry reduce attrition rates? Nat. Rev. Drug Discovery. 3:711-715.
- Kong, J.; Ngoh-Khang, G.; CHia, L.S.and CHia, T.F. (2003). Recent advances in traditional plant drugs and orchids. Actapharmacology Science, 24(1):7-21.
- Kumar, S. and Kumar, D. (2009). Antioxidants and free radical scavenging activities of edible weeds. Amarican J. Fundamental 9:1174-1190.

- Lavkova, M. Y.; Buzuk, G.N.; Sokolowa, S. M. and Kliment, N. I. (2001). Chemical features of medical plants. Applied biochemistry and Microbiology. 37(3):229-237.
- Leslie, T. (2000). Plant based drugs and medicines. Phytochemistry, 17: 1875–1877.
- Lorenz, H.; Sheehan, P.; and Seidel-Morgenstern, A (2001). Use of herbal medicine in primary care patients with mood and anxiety disorders, J. Chromatography. 908. 201 – 214.
- Lunga, I. P.; Kintia, S. S.; Bassarella, S.; and Pizzat, C. (2008). Steroidal saponins from the seeds of *Hyoscyamus niger*. Chem. J. Moldovo General Ind. Ecol. Chem. 3:89 – 93.
- Lynn, H.; Rainer, K. S.; Mariel, V. and Michael, N. C. (2002). Radiation-induced chromosome aberration: insights gained from biophysical modeling. Bio. Assays, 24:714-723.
- Ma, C.Y.; Liu, W.K.; and Che, C.T. (2002). Lignanamides and non-alkaloidal components of *Hyosyamus niger* seeds. J. Natural Product, 65:206 – 209.
- Maarit, O.; Anna, P.; Kari, H.; Raimo, H.; and Marja-Liisa, R. (2002). Extraction of sage (*Salvia officinalis*) by pressurized hot water and conventional methods: antioxidant activity of the extracts. Eur. Food Res. Technol. 215:158 – 163.
- Maneesha, P.; Sarita, S.; Sarawati, S.; and Agrawal, S. (2011). Antiproliferative effects of *Datura innoxia* extract in cervical HeLa cell line. J. of Pharmacy Research 4(4):1124 – 1126.
- Manjo, G. (1995).Apoptosis, oncosis, and cecrosis. An over view of cell death. Am.J. Pathol.146:3-15.
- Mark, P. (1998). Antioxidants. Clinical nutrition insights, (31) 1-4.
- Maroco, D.R. (1996). Apoptotic pathways. the roads to ruin. Cell, 94:695-698.
- Maroco, G.; Evans, C.A. and Whetton, A.D.(1996).Induction of apoptosis: new targets for cancer chemotherapy. Cancer Biol., 3:417-427.
- Marucia, B.B.; Talukder, G. and Shorama, A. (2000). Effects of culture media on spontaneous residence of mitotic index, chromosomal aberration,

micronucleus counts, sister chromatid exchange and cell cycle. Cytobios., 67:71-75.

- Matise, M.P.; Auerbach, W. and Joyner, A.L. (2000). Mouse embryonic stem cell culture. Mol. Bio., 16:80-83.
- Matsuda, J.; Okabe, S.; Hashimoto, T. and Yamaday. (1991). Cultured roots of *Hyoscyamus niger*. J-Biology-Chemistry. 25(15):460-464.
- Maxwell, S.R. (1995). Prospects for the use of antioxidant therapies. Drugs, 49:12-16.
- Mcallister, R.M.; Melnyk, J.; Finklestein, J.Z.; Adams, E.C. and Gardner, M.B. (1969). Cultivation *in vitro* of cells derived from human Rhabdomyosarcoma. Cancer, 24:520-526.
- Meera, S. and Nagarjuna, G. C. (2010). Antimutagenic activity of aqueous extract of *Momordica charantia.* International J. for Biotechnology and Molecular Biology Research. 1(4):42-46.
- Miller, S.E. and Peng, S.M. (1990). Micronuclei a biomarker for chemoprevention trials: results of a randomized study in oral pre-malignancy. Int. J. Cancer, 59: 457– 459.
- Milner, J.A.; (2001). A historical perspective on garlic and cancer. J. Nutr. 131:1027 – 1031.
- Modallal, N.; Salim, A.; and Alession, P. (2008). Cytogenetic effect of *Arum maculatum* extract on the bone marrow cell of mice. Caryologia. 61(4):383 – 387.
- Moerman, D.F. (1996). An analysis of the food and drug plants of native North America. J. Ethonopharmacol. 52:1 – 22.
- Mohammad, K. M.; Ihab, M. A.; Khaled, T.; Ala, I.; Afaf, A.; Mohammad, H.; Hatim, S. A. and Yasser, B. (2010). Antioxidant, antihyperuricemic and xanthine oxidase inhibitory activities of *Hyoscyamus reticulatus*. Pharmaceutical biology, 10(0):1-8.
- Mohanasan, D.J. (2001). Screening of medical plants. Indian Journal of Pharmacology, 33:460-462.
- Mojat, A.; Fraser, A.G.; Marimon, B.P.; and Simons, A. (2003). The encyclopedia of medicinal plants dorling Kindersley. ISBN, 9: 402-406.

- Mollinedo, F.; and Gajate, C. (2003). Microtubules, microtubule-interfering agents and apoptosis. Apoptosis, 8:413-450.
- Moore, A.E.; Satachewesky, L. and Toolen, H.W. (1955). Culture characteristics of four permanent lines of human cancer cells. Cancer Res., 15:598-605.
- Moreria, L.; Dias, L. G.; Pereria, J. A.; Estevinho, L. (2008). Antioxidant properties – Food Chem. Toxicol., 46:3482-3485.
- Morishita, D.W. (1991). Dalmatian toadflax, yellow toadflax, black henbane: importance, distribution, and control. Iranian J. Pharmaceutical Research, 2:14.
- Moteki, H.; Hibasmai, H.; Yamadsa, Y.; Katsuzaki, H.; Imai, and Komiya, T. (2002). Specific induction of apoptosis 1, 8-cineole in two human leukemia cell lines. Oncology Reports, 9:757 – 760.
- Mukherajee, A.K.; Basu, S.; Sarkar, N. and Ghosh, A.C. (2001). Advances in cancer therapy with plant based natural products. Current Medicinal Chemistry. 8:1467 – 1486.
- Newman, D.J; Cragg, G.M.; and Snader, K.M. (2003). Natural products as sources of new drugs over the period 1981 – 2002. J. of Natural Products 66:1022 – 1037.
- Nicolau, K.C.; Hepworth, D.; King, N.P. and Finlay, M.R. (1999). Chemistry, biology and medicine of Selected Tubulin Polymerizing Agents. Pure Appl. Chem. 71, 889 – 997.
- OW, Y. P.; Green, D. R.; Hao, Z.; and Mark, T. W. (2008). Cytochrome c: functions beyond respiration. Mat. Rev. Mol. cell Biol., 9:532-542.
- Patton, J.L. (1967). Chromosome studies of certain pocket genus perogenathus (Rodentia: Heteromyidae). J. Mamm., 48: 27 – 37.
- Peter, J.B. (2006). Drugs for asthma. British J. of pharmacology. 147:297-303.
- Phuangsab, A.; Lorence, R.M.; Reichard, K.W.; Peeples, M.E; and Walters, R.J.(2001). Newcastle disease virus therapy of human tumor zenografti antitumor effects of local or systemic administration. Cancer Lett. , 172:72-36.

- Plesca, D.; Meredith, E.; Crosby, M.E.; Gupta, D. and Almasan, A. (2007). E2F4 Function in G2: Maintaining G2 arrest to prevent Mitotic entry with damaged DNA. Cell cycle. 6(10): 1147 – 1152.
- Proudlock, R.; Thompson, C. and Longstaff, E. (2004). Evaluation of 10 aliphatic halogenated hydrocarbons in the mouse bone marrow micronucleus test. Mutagenesis, 14(2):207-215.
- Richard, J.P. (1998). Natural products isolation. The Benlamin cummings Bublishing company, Inc. Menlopank , USA.: 585-614.
- Sajeli, A. (2010). Bioactive non-alkaloidal secondary metabolites of *Hyoscyamus niger* seeds. Res. J. of Seed Science, 3 (4):210 – 217.
- Saker, M.M. and Ashal, H.A. (1995). Stimulating effects of salt stress on alkaloid production of cultured *Hyoscyamus* cells. Fitoterpia. (4): 360-362.
- Salam, H.D.; Doli, T. and Sarbani, G. (2011). Areca nut, Sadagura extract alone and in combination in inducing genotoxicity in the mouse bone marrow cells *in vivo*. Biological and Environmental Sciences, 7(1): 43 – 49.
- Saraste, A. and Pulkki, K. (2000). Morphologic and biochemical hall marks of apoptosis. Cardiovascul. Res., 45:528-537.
- Schlegel, R.; and MacGregor, J.T. (1982). The persistence of micronuclei in peripheral blood erythrocytes: detection of chronic chromosome breakage in mice. Mut. Res. 104:367-369.
- Schmid, W. (1976). The micronucleus test for cytogenetic analysis. Chemical Mutagens, Principles and Methods for their Detection. 4. Hollaender A, (Ed.) Plenum New York and London. Pp. 31 – 53.
- Schmid, W. (1979). The cell micronucleus test for cytogenetic analysis. In:Holaender, A. (Ed.) Chemical mutagenes principles and methods for their detection. Vol. 4, Plenum press, New York and London.
- Schultes, R.E. (1976). Plantae Mexicanae II. The Identification of Teonanacatle, a Narcotic Basiomycetes of the Aztecs. Bot. Mus.Leaflets of Harvard vol.7 (3):37-54.
- Seyed, M.N. (2009). Free radical scavenging ability of methanolic extract of *Hyoscyamus squarrosus* leaves. Pharmacology online. 2:796 – 802.
- Shchepina, L. A.; Papova, E.N.; Pletjushkina, O. Y.; and Chernyak, B. V. (2002). Respiration and mitochondrial membrane potential are not required

for apoptosis and anti-apoptotic action of BCl-2 in HeLa cells. Cancer Res., 14:660-666.

- Shihata, I.M. (1951). A pharmacological study of *anagalis arvensis*, M.D. Vet. Thesis Cairo University, P43.
- Shiraishi, P.S.(1978).Cell cycle kinetics by BrdU-Hoechst flow cytometry: an alternative to the differential metaphase labelling technique. Cell Tissue Kinetics, 18: 551-562.
- Shubber, E.K. (1987). Sister-chromatid exchanges in lymphocytes from patients with schistosoma hematobium. Mutation Research, 180:93-9.
- Sies, H. and Stahl, W. (1995). Vitamin E and C, beta carotene, and other carotenoids as antioxidants. Am. J. Clim. Nutr. 62:13-21.
- Sirelkhatim, B.E. (2011). Cytotoxicity and biological activity of selected Sudanese medicinal plants. Research J. of Medicinal Plant. 5 (3):201 – 229.
- Sousek, J.; Guedon, D.; Adam, T.; Bochorakova, H. and Simanek, V. (1999). Alkaloids and organic acid content of eight *Fumaria* species. Phytochem. Anal., 10:6 – 11.
- Stuppia, L.; Gobbi, P.; Zamai, L.; Palka; G., Votale, M.; and Falcieri, E. (1996). Morphometric and functional study of apoptotic cell chromatin. Cell Death Diff. 3:397 – 401.
- Sulekha, M.; Satish, Y., Sunitam, Y. and Rajesh, K. N. (2009). Antioxidants: A review. J. of Chemical and Pharmaceutical Research, 1(1):102-104.
- Supria, K.B. (1998). Hand book of medicinal plants. Poiter. Publishers, India Tox.sci. Oxford Journal. 7:447-455.
- Tsewang, J.T. ;(1994). Tibetan Medicinal Plants. 1st Ed., Tibetan Medical Publications, India.
- Tucek, S. (1995). Allosteric modulation of muscarinic acetyl choline receptors. Trends phormacol.Sci.16-205-212.
- Valentine, I.; Philippe, M.; Lhuguenot, J.; and Chagnon, M. (2001). Uridine uptake inhibition as cytotoxicity test for a human hepatoma cell line (HepG2 cells). Toxicology, 158: 127 – 39.
- Vierra, P.M; Santos, S.C.; and Chen, L. (2010). Assessment of mutagenicity and Chen, L. (2010). Assessment of mutagenicity and cytotoxicity of

Solanum paniculatum extracts using *in vivo* micronucleus test in mice. Braz. J. Biol. 70(3):601 – 606.

- Vinson, J.A.; Hao, Y.; Su, X. and Zubi K, L. (1998). Phenol antioxidant quantity and quality in foods: vegetables. J. Agric. Food Chem. 46: 3630 – 3634.
- Wagner, E.T.; and Drager, U.C. (2002). Retinoic acid synthesis in the postnatal mouse brain marks distinct developmental stages and functional systems. Cerebral Cortex, 12: 1244-1253.
- Walter, R. (1993).Options: the alternative cancer therapy book. Avery publishing group. Garden city Park. PP: 9-13.
- Wang, C.; and Peng, C. (1996). Cytotoxicity and mutagenicity of some extracts of *Nerium oleander* leaves in cultured mammalian cell. J. Egyot. Ger. Soc. Zool., 30: 1-11.
- Wink, M. (2003). Evaluation of secondary metabolites from an ecological and molecular phylogenetic perspective. Phytochemistry, 64 (1).3-19.
- Wmkuhtreiber,K**.;** Hayashi, T.; Dale, E.A. and Faustman, D. L. (2003). Central role of defective apoptosis in autoimmunity. J. of Molecular Endocrinology. 31:373-399.
- Xifeng, W.U.; Jian, G.; and Spitz, M. (2007). Mutagen sensitivity; Genetic predisposition factor for cancer. Cancer Research, 67:3493 – 3495.

Chapter Four: Conclusions and Recommendations

4.1 Conclusions

1- *Hyoscyamus niger* contained different active compounds including alkaloids, flavonoids, tannins, lignins and The absence of glycosides. The chemical properties of alkaloidal extract were further investigated using HPLC.

2- Alkaloidal extract showed cytotoxic activity; it reduced the spontaneous frequency of CAs, MN, and increased the mitotic index in mice bone marrow cells. In addition, this extract exhibited cytotoxic activity against cancer cell line Hep-2 in all periods of time, it had moderate effect on RD and AMN-3 and no effect on normal cell lines using crystal-violate stain.

3- The antioxidant activity of alkaloidal extract showed moderate activity.

4- The cytotoxicity of alkaloidal extract using MTT assay showed significant differences in cell viability.

5- Alkaloidal extract of *Hyoscyamus niger* induced apoptosis activity rather than cytotoxic activity against cancer cell lines (A549, PC-3) by using high-content screening system.

4.2. **Recommendations**

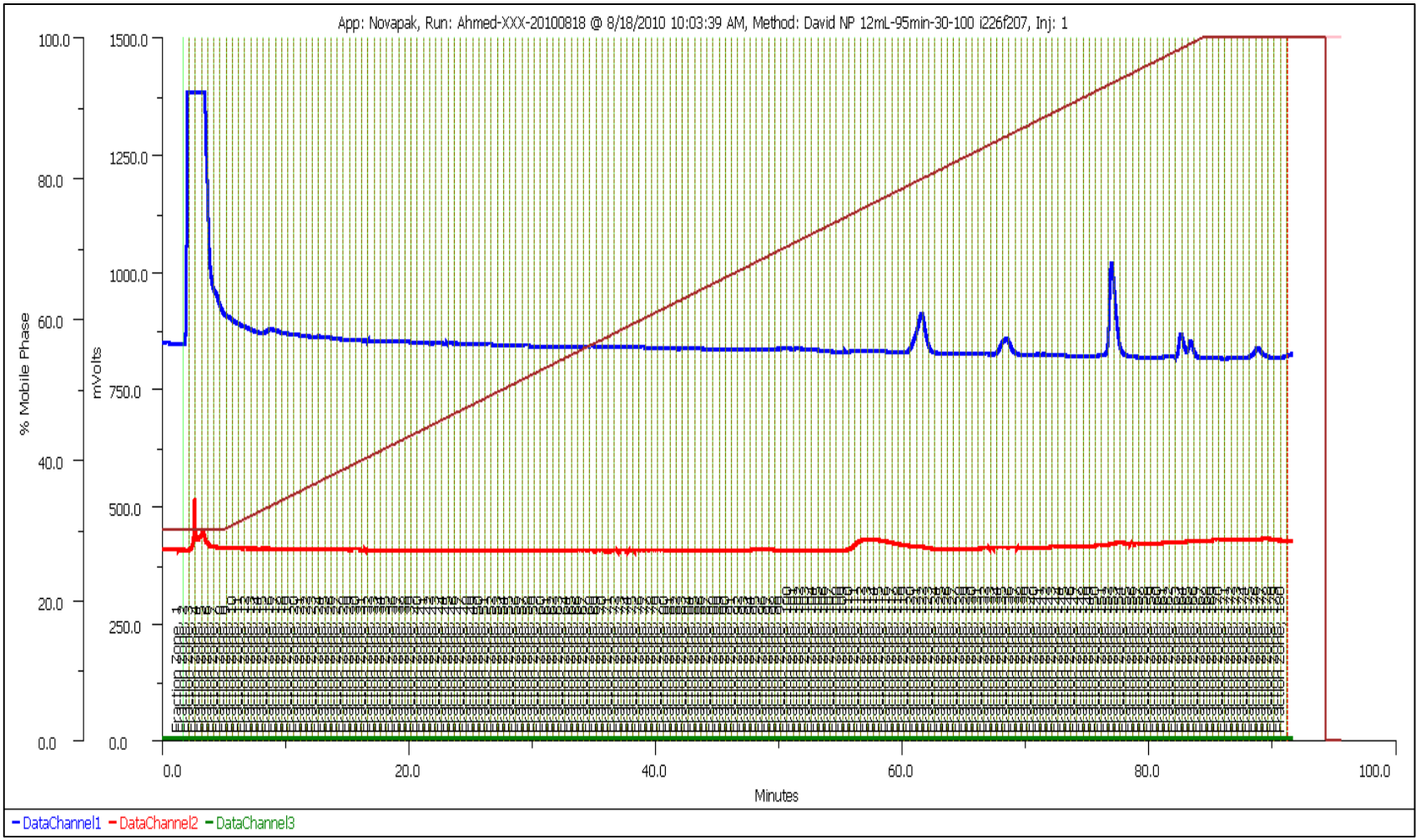
1- Studying the immunological and histopathological effects of *Hyoscyamus niger in vitro* and *in vivo*.

2- Advanced methodologies are needed to study the anti-tumor effects such as commet assay, DNA fragmentation, immunohistochemical and *in situ* hybridization.

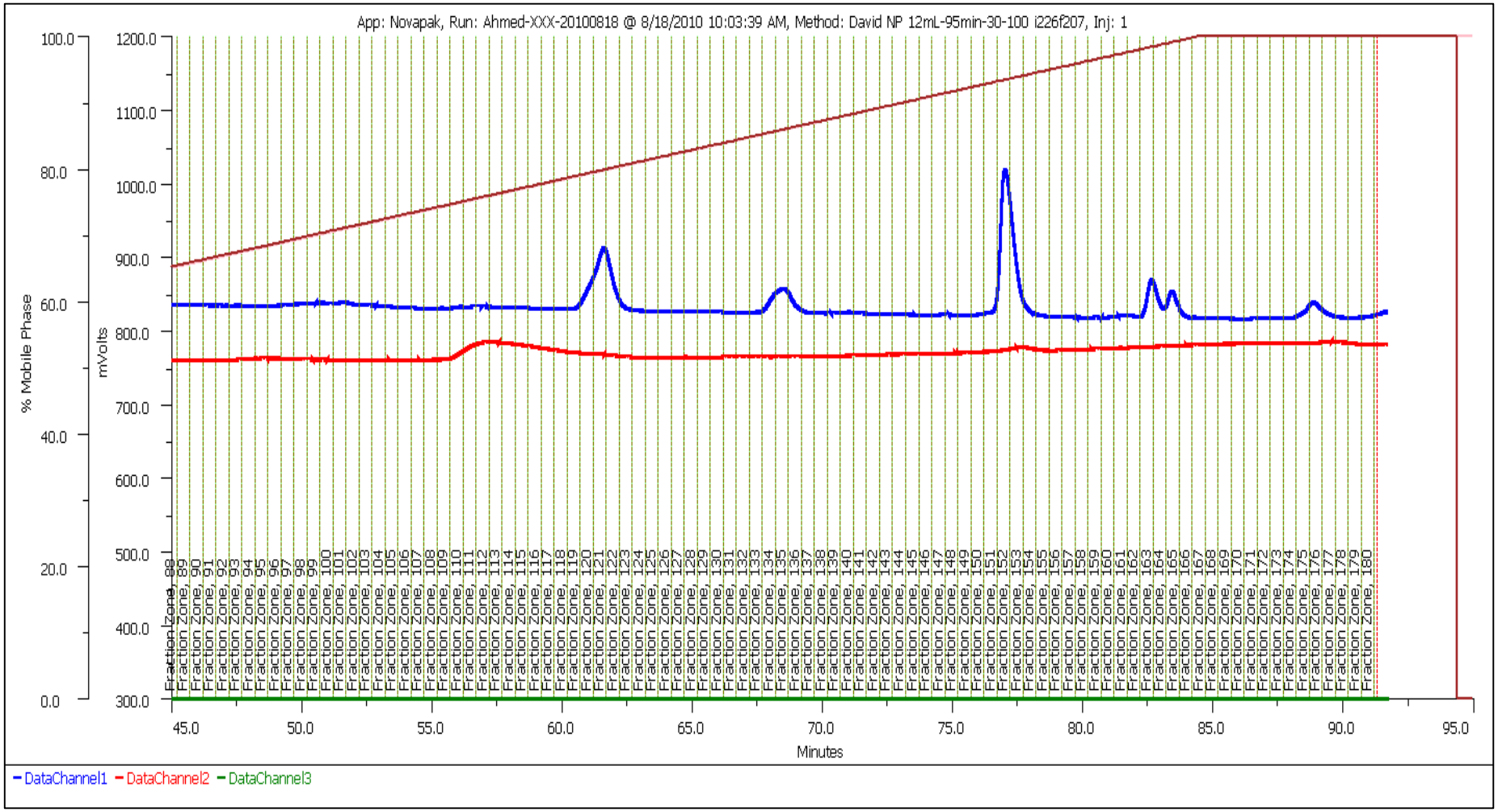
3- Study the antimicrobial activity of *H. niger* active compounds.

4- Study the molecular effects of *H. niger* extract on cancer cells.

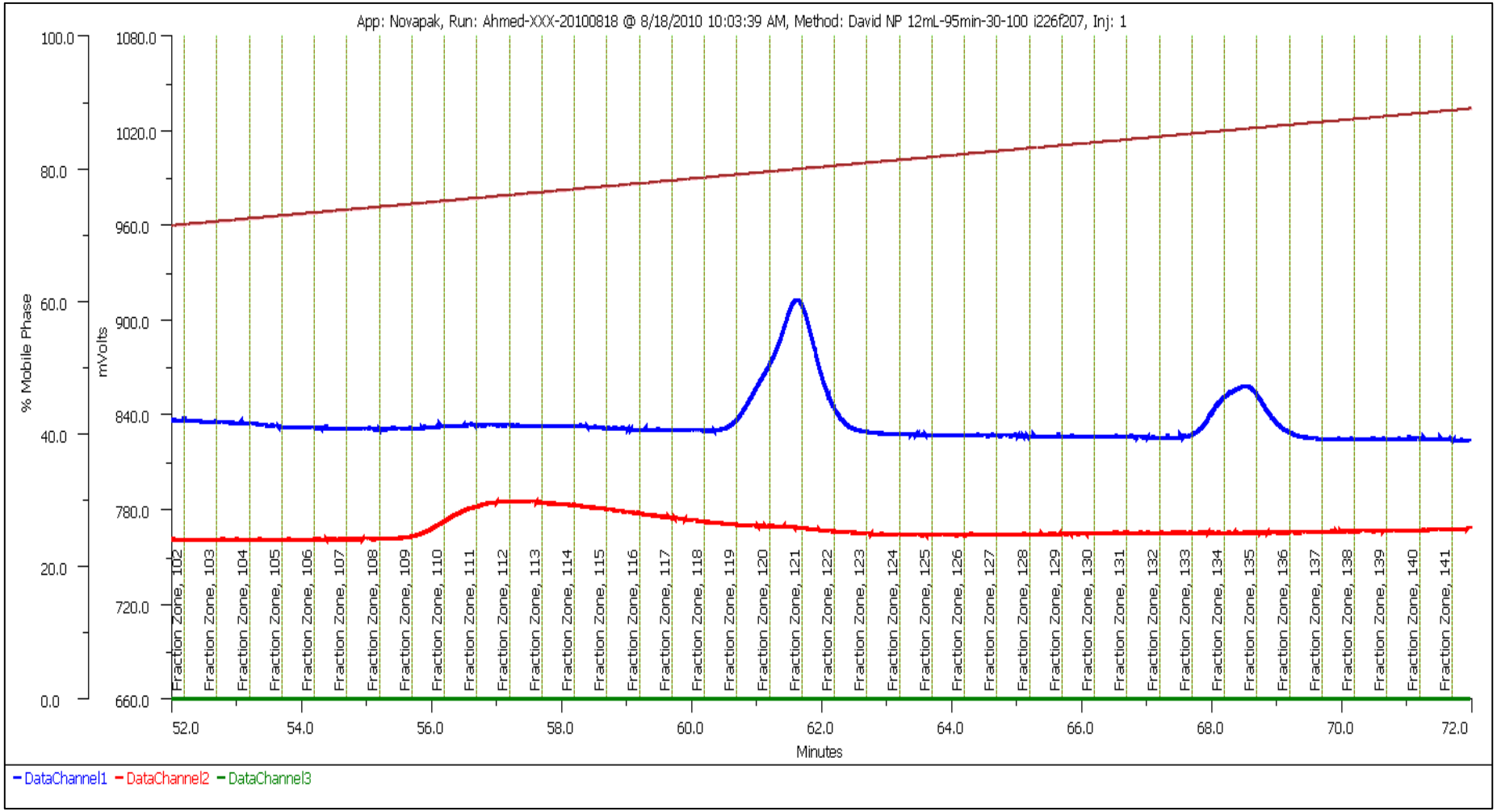
Appendix (4): Fraction four of alkaloidal extract isolated by HPLC range from 290-350



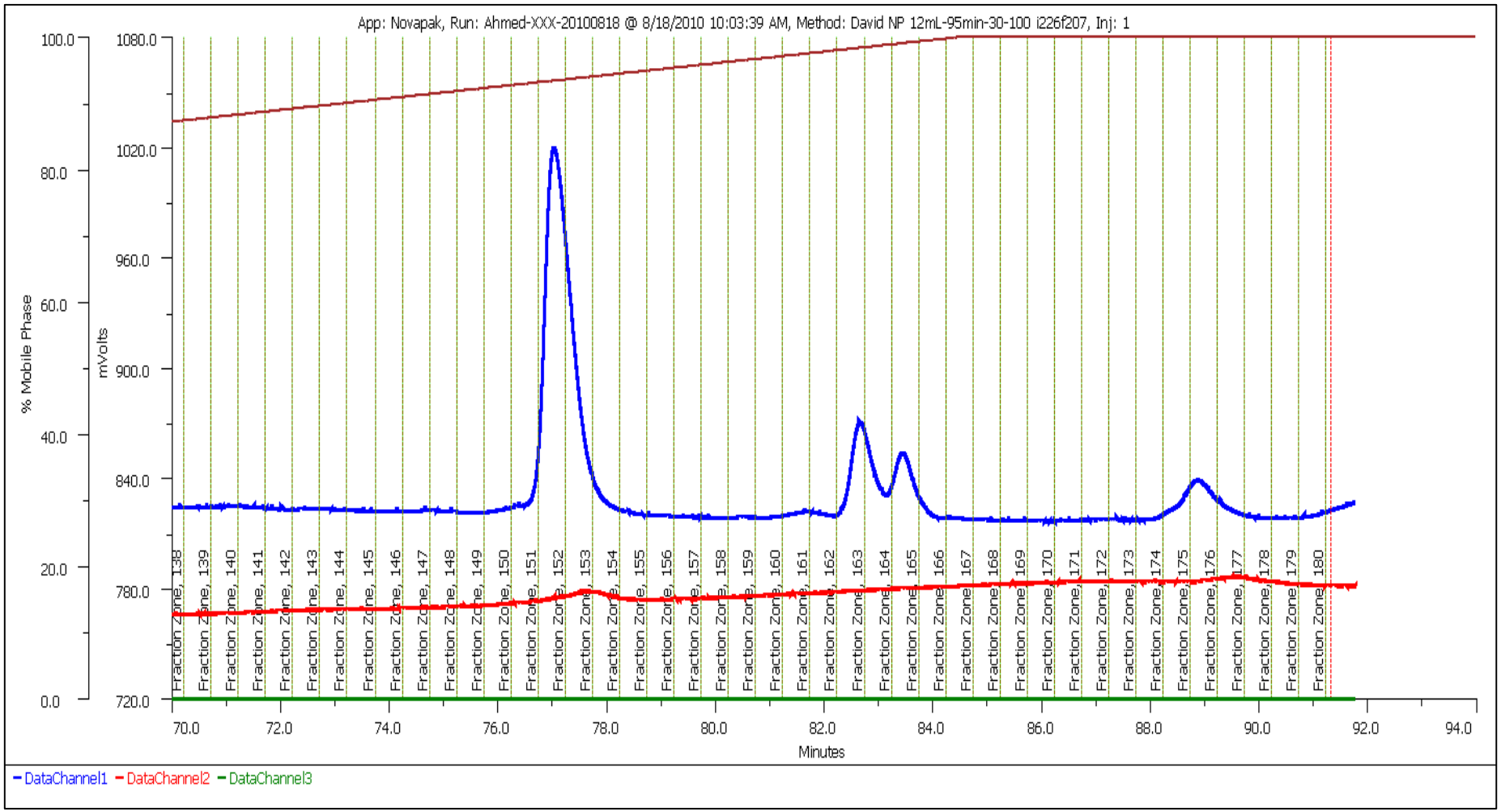
Appendix (5): Fraction five of alkaloidal extract isolated by HPLC range from 88-180.



Appendix (6): Fraction seven of alkaloidal extract isolated by HPLC range from 102-141



Appendix (7): Fraction seven of alkaloidal extract isolated by HPLC range from 138-180.



خلاصة

تهدف الدراسة الحالية للكشف عن مركبات الفعالة لنبات البنج بوساطة التحليل الكيميائي لوحظ ان المستخلص القلويدي يمثل المركب الرئيسي لنبات البنج لذلك استخدم هذا المستخلص في هذه الدراسة. تم اجراء دراسة وراثية خلوية للمستخلص القلويدي لهذا النبات بأستخدام معاملات وراثية مثل الانقسام الخيطي ووجود النيكلوتيدات الصغيرة وتشوهات الكروموسومية على الفئران المختبرية.

واظهرت النتيجة فعلا مؤثرا على معامل الانقسام والذي ارتفع ارتفاعا معنويا، وتكون النوى الصغرى وتشوهات الكروموسومية واللذين انخفضا معنويا.

كما تضمنت الدراسة اجراء تقييم لفعالية المستخلص القلويدي في كسح الجذور الحرة بأستخدام فحص كسح الجذور الحرة واظهرت النتائج في قابلية جزء واحد من المستخلص على كسح هذه الجذور DPPH (2.2- dipheny-1-picrylhydrozyl).

ان الفعالية السمية الخلوية لمستخلص القلويدي باستخدام عدة تراكيز لوحظت لثلاث انواع من خطوط الخلوية السرطانية (Hep-2, RD,AMN-3) وخط خلوي طبيعي (MEF)، وبعد حضنها لفترات زمنية مختلفة(٢٤,٤٨، و٧٢) ساعة لوحض ان هذا المستخلص يعتمد على الوقت والتركيز.

على النحو الاخر وجد ان كل التراكيزالمستخدمة هي افضل في تأثيرها على الخط الخلوي Hep-2.

وكذلك تضمنت هذه الدراسة على اختبار الفعالية الحيوية لمعرفة تأثير المستخلص القلويدي على خطوط خلوية اخرى المأخوذة من المركز الانتاج الطبيعي و اكتشاف الدواء (Hep G2, PC-3, A549)، وخط خلوي طبيعي WRL68 وبعد٢٤ ساعة من فترة الحضن لوحظ وجود نقصان بسيط ومعنوي في تأثير المستخلص على الخلايا.

لذلك تم اجراء دراسة على تأثير المستخلص على خطين من الخلايا السرطانية (PC-3,A549) بأستخدام نظام الفرز عالي المحتوى (مجموعة متعددة المعالم) في تأثيره على حجم النواة وشكلها وعلى نفاذية غشاء الخلية وعلى عمل المايتوكوندريا وطرحها للسيتوكروم ولوحظ انه يؤثر على حجم الخلية وعلى نفاذية الغشاء الخلوي وكذلك يؤثر على المايتوكندريا في طرحها للسايتوكروم.

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة النهرين

كلية العلوم

دراسة وراثية خلوية و سمية حول تأثير المركبات الفعالة لنبات البنج داخل وخارج الجسم الحي

أطروحة

مقدمة إلى مجلس كلية العلوم / جامعة النهرين

وهي جزء من متطلبات نيل درجة دكتوراه فلسفة في التقانة الأحيائية

من قبل

أسماء عبيد أسماعيل

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تشرين الثاني ٢٠١١

ذي الحجة ١٤٣٢