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Al-Nahrain University
College of Sciences
Biotechnology Department

Inhibitory Effects of *Nigella sativa* Oil and Honey on the Genotoxicity of Tamoxifen in Mice

A thesis

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By

Ayat Monem Ali Al- Sudany

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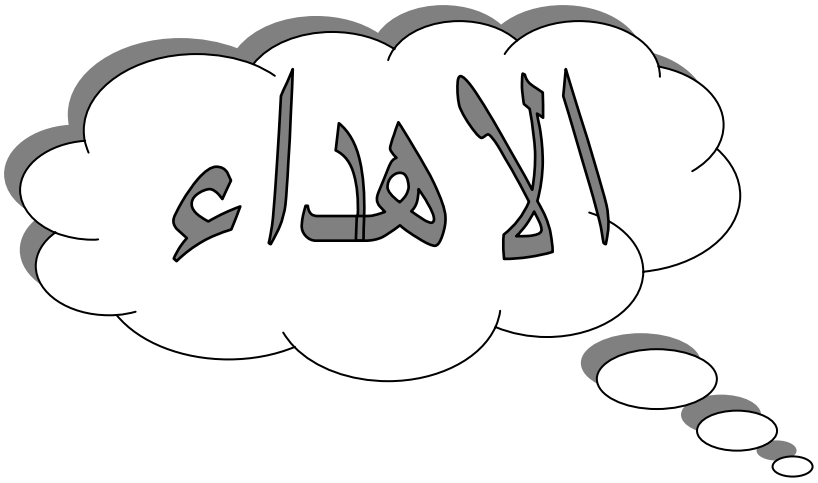

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روح المصطفى واله

رمز العزة و الشموخ ..... والدي

شلال العطاء الذي لاينضب ..... والدتي

انساب جسدي ... مثال الوفاء ... اخواتي واخي

انساب روحي ...رفاق الدرب... اصدقائي

كل من جاد علي بالعلم و المعرفة...

كل من احب ...

اهدي ثمرة جهدي هذا

آيات

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Ayat

Supervisor Certification

We certify that this thesis was prepared under our supervision in the College of Science, Al-Nahrain University as a partial requirement for the degree of Master of Science in Biotechnology.

Signature:	Signature:
Supervisor:	Supervisor:
Dr.Khulood W.Al-Samarraei	**Dr.Esmail K. Shubber**
Scientific Degree:	Scientific Degree:
Assist Professor	Professor
Date:	Date:

In review of the available recommendations, I forward this thesis for debate by the examining committee.

Signature:

Name: **Dr. Nabeel Kh. Al-Ani**

Scientific Degree: Assistant professor.

Title: Head of Biotechnology Department.

Date:

Committee Certification

We, the examining committee, certify that we have read this thesis and examined the student in its contents and that, according to our opinion, is accepted as a thesis for the degree of Master of Science in Biotechnology.

Signature:

Name: **Dr. Nahi Y. Yaseen**

Scientific Degree: Professor

Date:

(Chairman)

Signature:

Name: **Dr. Ali H. Idhia**

Scientific Degree:

Assist Professor

Date:

(Member)

Signature:

Name: **Dr. Kadhum E. Muhamad**

Scientific Degree:

Assist Professor

Date:

(Member)

I hereby certify upon the decision of the examining committee

Signature:

Name: **Dr. Laith A. Z. Al- Ani**

Scientific Degree: Assistant Professor

Title: Dean of College of Science

Date:

Summary

The present study was designed to shed light on the cytogenetic effects of tamoxifen in laboratory females' mice (*in vivo*), and in human blood lymphocytes (*in vitro*). It was also aimed to investigate the role of black seed oil and honey in reducing these effects in mice.

The cytogenetic effects of the drug were investigated after one day, three and five days of treatment with two doses, low dose (0.4 mg/kg) and high dose (0.8 mg/kg).

An interaction between black seed oil, honey and the drug (high dose) was carried out through two types of treatments (before and after drug treatment), to determine the activity of black seed oil and honey in preventing or reducing the drug effects.

The following results were obtained:

1- Tamoxifen revealed clear devastating effects, which were pronounced in reducing mitotic activity and increasing chromosomal aberrations and micronuclei in mouse bone marrow and human blood lymphocytes (*in vitro*). These effects were proportional with the number of doses, a phenomenon which suggested that the drug has an accumulated genotoxic effect.

2- Black seed oil and honey had no genotoxic effects on the bone marrow in albino mice.

3- Black seed oil and honey showed highly performance in protecting the bone marrow cells in mice against toxic tamoxifen effect by increasing the MI and decreasing the abnormal chromosomes and micronuclei when the treatment was before the drug, so black seed oil and honey were classified as desmutagen compounds in the first order. They had the ability to repair the tamoxifen destroyed cells, by increasing MI , repairing CA and decreasing MN in bone marrow cells in mice when treated after the drug , therefore , black seed oil and honey classified as bioantimutagens against tamoxifen in the second order.

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List of Abbreviations

Code	Word
AHH	Arylhydrocarbon Hydroxylase
AIDS	Aquired Immune Deficiency Syndrome
BI	Blastogenic Index
BUdR	Bromodeoxy Uridine
CA	Chromosomal Aberration
CAPE	Caffeic Acid Phenylthyl Ester
CCP	Cell Cycle Progression
CHO	Chinese Hamster Ovary
CP	Cyclophosphamide
CP	Cisplatin
DAPI	Diamino Phenyl Indole
DLA	Daltons Lymphoma Acsites
DMSO	Dimethyle Sulfoxide
DNA	Deoxyribonucleic Acid
D.W.	Distilled Water
EAC	Ehrlich Ascites Carcinoma
GST	Glotathion S-Transferase

IL	Interleukine
I.P	Intra Peritoneal
LDLs	Low Density Lipoproteins
M	Molar
MI	Mitotic Index
Min	Minute
MMC	Mitomycine-C
MN	Micronucleus
MNU	Methylnitrosourea
MTX	Methotrexate
NK	Natural Killer Cell
PCE	Polychromatic Erythrocytes
PHA	Phytohaemagglutinine
RI	Replicative Index
r.p.m	Rate Per Minute
SCE	Sister Chromatid Exchange
SE	Standard Error
SOD	Super Oxide Dismutase
SSC	Sodium Citrate-sodium Carbonate
TAM	Tamoxifen
TNF	Tumor Necrosis Factor
UPW	Ultra Purified Water
UV	Ultra violet

Chapter one

Introduction

1.1: Introduction

Tamoxifen , the most widely used hormonal therapy, works by blocking estrogen receptors on breast cancer cells. It is used to halt, slow or prevent tumor growth. Studies have shown that women who take tamoxifen for five years reduce their annual risk of a recurrence of breast cancer by 50% . Taking the drug for more than five years offers no additional advantage, and it increases the risk of toxic effects (Brown et al.1999).

Nigella sativa is found in North Africa, as well as some parts of East. The oil of black seed is so beneficial due to its content of over a hundred components such as aromatic oils, trace elements, proteins and carbohydrates (El – Zawahry, 1997). It contains 58% of essential fatty acids including omega 6 and omega 3. These fatty acids are necessary for the forming of prostaglandin E1 which balances and strengthens the immune system giving it the power to prevent infections and allergies and control chronic illnesses. Also healthy cells are protected from viruses thus inhibiting tumors. Black seed oil also contains about 0.5-1.5 % volatile oils including nigellone and thymoquinone which are responsible for its anti-histaminic, anti- oxidant, anti- infective and bronchodilating effect (Randhawa and Al-Ghamdi , 2002).

Black seed have a unique effect on physiology and can reduce the side effects of cancer treatment, while at the same time increasing their effectiveness (Nair, 1991).It contains flavonoids and phenols, which have free radicals scavenging and antimutagenic activities (Smajima *et al*, 1995).

For thousands of years, honey has been used in just about every culture for the healing of wounds, burns, rashes and ulcers of every kind. This consistent historical documentation led modern scientists of the last few decades to study the properties and therapeutic effects of honey. It contains numerous therapeutic compounds, including essential oils, flavonoids, terpenes and polyphenols (Hassanein, 1989).

Honey is known to be rich in both enzymatic and non-enzymatic antioxidants, including catalase, ascorbic acid, flavonoids and alkaloids. A unique flavonoid, pinocembrin, is present in high quantities in honey. Other flavonoids found in honey are pinobanksin, chrysin, galangin, quercetin, luteolin and kaempferol (Molan, 1998).

Honey is known to be good for the digestive system. It has a warming effect on the body and relieves constipation. It provides slow, continuous energy and is a great way to beat fatigue. It promotes brisk mental efficiency, heals fractured bones by supplying calcium, is good for colds and anemic conditions and heals bronchial infections. Honey also has anti-bacterial properties and can be used for dressing wounds and burns to help keep them sterile and promote healing.

The aims of the study:

1- Studying the cytogenetic effects of tamoxifen by using (mitotic index, chromosomal aberrations and micronucleus assays) in mouse bone marrow cells (*in vivo*) and in human blood lymphocytes (*in vitro*).

2- Studying the cytogenetic effects of black seed oil and honey in mouse bone marrow cells.

3- Studying the ability of black seed oil and honey in reducing the genotoxic effects produced by tamoxifen treatment.

Chapter two

Literature Review

2.1: Breast cancer

Breast cancer is clearly a hormone-dependent disease. More specifically, it is dependent on the female hormone, estrogen. This means that many breast cancers (specifically, the ones referred to as estrogen receptor or ER positive) have estrogen binders (receptors). Consequently, estrogens can stimulate the growth of these tumors. Men do get breast cancer, but only at 1% the rate of women (Armstrong, 2000).

Breast cancer is considered a heterogenous disease, meaning that it is a different disease in different women, a different disease in different age groups and has different cell populations within the tumor itself. Generally, breast cancer is a much more aggressive disease in younger women. Autopsy studies show that 2% of the population has undiagnosed breast cancer at the time of death. Older women typically have much less aggressive disease than younger women (Armstrong, 2000).

The risk of breast cancer rises with age, family history of breast cancer, early menarche or late menopause, age which the first child is

born, not having children at all, obesity oral contraceptives and possibly hormone replacement therapy. High fat diets may also increase the risks of getting breast cancer. Moderate drinking of alcohol has been linked to breast cancer in several studies (Rhodes, 2002).

2.1.1: Types of Breast Cancer Treatment

Breast cancer treatments are local or systemic.

Local treatments are used to remove, destroy or control the cancer cells in a specific area, such as the breast. They include:

1. Surgery:

Either mastectomy or lumpectomy also called breast conserving therapy or (partial mastectomy) with or without lymph node removal.

2. Radiation therapy .

Systemic treatments are used to destroy or control cancer cells all over the body. They include:

1-Chemotherapy:

Which uses drugs to kill cancer cells. Side effects can include nausea, hair loss, early menopause, hot flashes, fatigue and temporarily lowered blood counts.

2-Hormone therapy:

Including tamoxifen, and the aromatase inhibitors arimidex, aromasin and femara. Hormone therapy uses drugs to prevent hormones, especially estrogen, from promoting the growth of breast cancer cells that may remain after breast cancer surgery. Side effects can include hot flashes and vaginal dryness.

3-Biological Therapy:

Such as herceptin, works by using the body's immune system to destroy cancer cells. Herceptin targets breast cancer cells that have high levels of a protein called HER2.

Systemic therapy can be given after local treatment (adjuvant therapy) or before (neoadjuvant therapy). Adjuvant therapy is used after local treatments to kill any cancer cells that remain in the body and may be in other parts of the body.

(Goldhirsch, 2001).

2.2: Hormonal therapy

The female hormones estrogen and progesterone play a role in the growth of breast cells. To make use of these hormones, breast cells normally have special receptors that permit the hormones to connect to the cells. Breast cancer cells that also have such receptors are known as "hormone-receptor positive." Reducing the amount of these hormones available to the cancer cells, the goal of hormonal therapy, may cause them to cease growing or die (Hays et al., 2003).

After cancerous cells are surgically removed, they are tested for hormone receptors. If the tumor is receptor positive, hormonal therapy may lower the risk that cancer will recur in breast tissue or other parts of the body by either preventing the hormones from acting on the cell or lowering the amounts of hormones in the body. Patients with

receptor-positive tumors usually experience fewer local recurrences and longer overall survival than those with receptor-negative tumors. Hormonal therapy has little effect on cancers that are not receptor positive (Hortobagyi, 1998).

2.3:Tamoxifen

Tamoxifen is a drug in pill form, taken orally (20-40 mg/day) interferes with the activity of estrogen. Tamoxifen has been used to treat both early and advanced stages breast cancer. It has been used for nearly 20 years to treat patients with advanced breast cancer. More recently, it also being used as adjuvant, or additional, therapy following primary treatment for early stage breast cancer (Hozumi et al., 1998; Umemoto et al., 2000; Goss et al., 2003).The chemical structure of TAM is shown in figure (2.1).

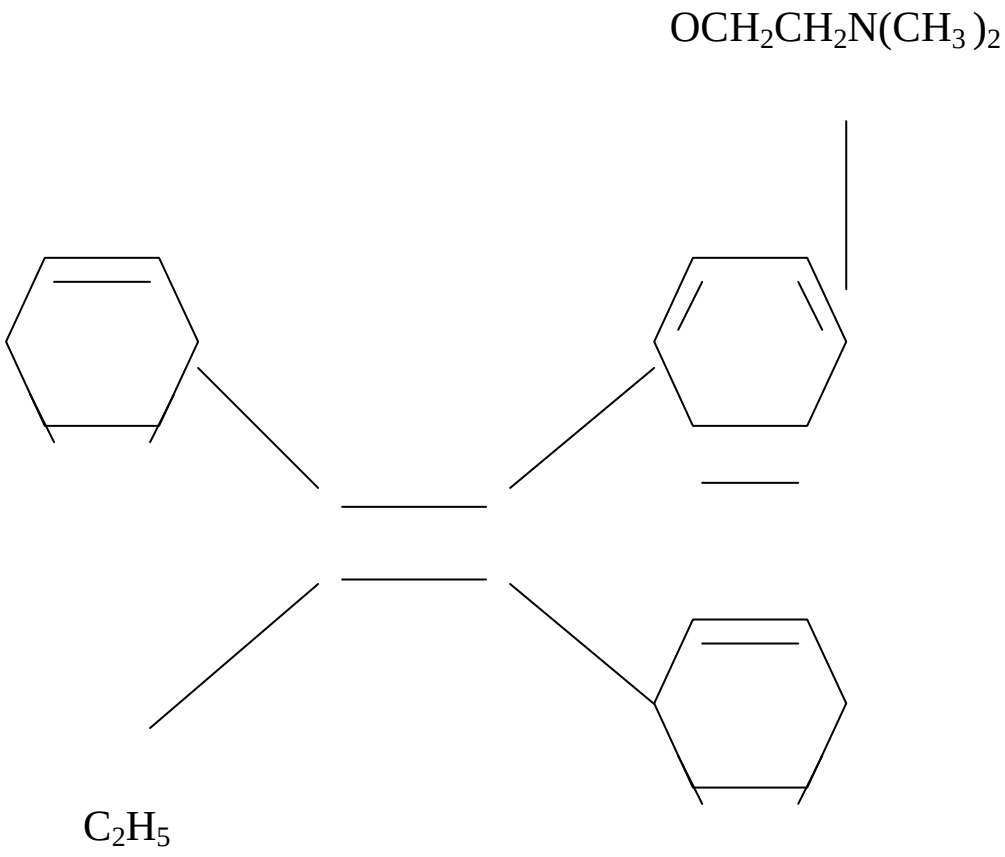


Figure (2.1): Chemical Structure of tamoxifen

(Z)-2-4-(1,2-Diphenyl-1-butenyl) phenoxy – N,N-dimethylethamin)

(Wolf and Jordan, 1992)

2.3.1:Uses of Tamoxifen

Tamoxifen was used for palliative therapy of breast cancer in postmenopausal women, who are considered poor candidates for surgery, and is also used to treat female infertility (Goss et al., 2003).

A high proportion (40 – 60 %) of all women who undergo potentially curative surgery for breast cancer now receive adjuvant TAM therapy for a period of 2-5 years (IARC, 1996). Tamoxifen is also used to reduce the risk of breast cancer in women who are at high risk for developing the disease (FDA, 1998). Tamoxifen has been tested as a possible treatment for hepatocellular carcinoma, stomach carcinoma, renal cell carcinoma, melanoma, pancreatic cell carcinoma, cervical carcinoma, ovarian carcinoma and other tumors; however it is not widely used for these treatments. TAM worldwide use was estimated at more than 7 million patients / year by the mid 1990s (IARC, 1996).

A study of Goss and his partner (2003) found that taking TAM for five years significantly reduces both breast cancer recurrence (42 %) and mortality (22%) for all women. Results found that

premenopausal women, not just post menopausal women , and those whose breast cancer has spread to the local lymph gland benefit substantially from TAM therapy(Internet , 2003 a).

2.3.2:Mechanisms of breast cancer treatment with tamoxifen . Some breast cancer cells are “estrogen – sensitive “. In other words, estrogen binds to these cells and stimulates them to grow and divided .TAM prevent the binding of estrogen. These stop the cells from growing and prevent or delay breast cancer recurrence. TAM is also known to work through growth factors and the immune system and may provide some benefit even in patients whose tumors are not estrogen sensitive (Internet, 2003 a).

As a treatment for breast cancer, the drug slow or stops the growth of cancer cells that are already present in the body. As adjuvant therapy, TAM has been shown to prevent the original breast cancer from returning and also prevent the development of new cancers in the opposite breast (Internet , 2003 b).

2.3.3:Side effects of tamoxifen

The most common side effects are hot flashed, nausea, vomiting, depression and eye problems. The drugs can also cause vaginal bleeding and discharge, skin rashed, transient leucopoenia and thrombocytopenia. Increased bone and tumor pain may occur. Infrequent side effects are anorexia and hypercalacemia. TAM may increase the risk of deep vein thrombosis and blood clots in the lung (Internet , 2003 c). It may induce menopause in a women who are close to menopause (Internet , 2003 a).

2.3.4:Absorption and distribution of tamoxifen

Following a single oral dose of 20 mg TAM, an average peak plasma concentration of 40 ng/ml occurred approximately 5 hours after dosing.

The decline in plasma concentrations of TAM is biphasic with a terminal elimination half-life of about 5 to 7 days. The average peak plasma concentration of N-desmethyl tamoxifen is 15 ng/ml. Chronic administration of 10 mg TAM given twice daily for three months to

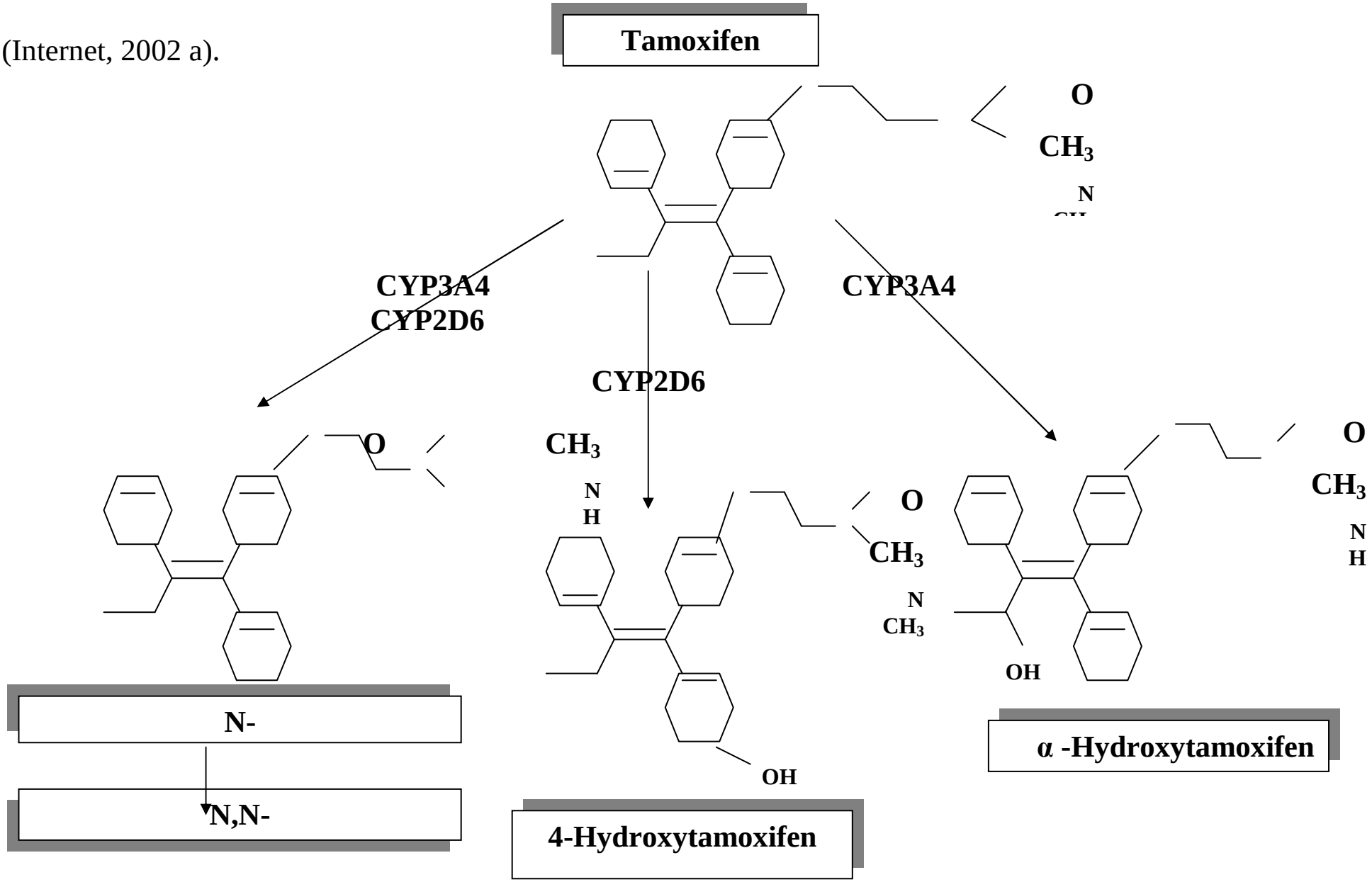
patients results in average steady-state plasma concentrations of 120 ng/ml for TAM and 336 ng/ml for N-des-methyl tamoxifen are achieved in about 8 weeks, suggesting a half-life of approximately 14 days for this metabolite (Internet , 2002 a).

2.3.5:Metabolism of Tamoxifen

TAM is extensively metabolized after oral administration. N-desmethyl tamoxifen is the major metabolite found in patients' plasma. The biological activity of N-desmethyl tamoxifen appears to be similar to that of TAM. 4-hydroxy tamoxifen and a side chain primary alcohol derivative of TAM have been identified as minor metabolites in plasma. TAM is a substrate of cytochrome P-450, 3A, 2C9 and 2D6, and an inhibitor of P-glycoprotein (Internet, 2002 a).

Figure (2.2): Metabolism of tamoxifen

(Internet, 2002 a).



2.3.6: Excretion of tamoxifen

Studies in women receiving 20 mg of 14C TAM have shown that approximately 65 % of the administrated dose was excreted from the body over a period of 2 weeks with fecal excretion as the primary route of elimination. The drug is excreted mainly as polar conjugates, with unchanged drug and un conjugated metabolites accounting for less than 30 % of the total fecal radioactivity (Internet , 2002 a).

2.3.7: Bioactivation of tamoxifen and reaction with DNA

One of the suggested pathways leading to the potential toxicity of TAM involves its oxidative metabolism to 4-hydroxytamoxifen, which may be further oxidized to an electrophilic quinone methide. Alternatively, TAM could undergo O-dealkylation to give cis/trans-1, 2-diphenyl-1-(4-hydroxyphenyl) –but-1-ene, which is commonly known as metabolite E. Because of its structural similarity to 4-hydroxytamoxifen, metabolite E could also be biotransformed to a

quinone methide , which has the potential to alkylate DNA and may contribute to the genotoxic effect of TAM (Fan and Bolton ,2001).

Unlike TAM, metabolite E is estrogenic. Although estrogens have been linked to the development of hormone-dependent cancer, there is an interest in these compounds acting as chemical carcinogens by binding to cellular macromolecules. Metabolite E has been shown to alkylate DNA, a reaction mechanism likely involving the formation of a quinone methide intermediate (Fan and Bolton, 2001).

2.3.8: Tamoxifen and the risk for developing uterine cancer

Many experts believe that TAM may increase the risk of uterine cancer (Soural *et al.*, 1997; Fan and Bolton, 2001; Hirsimaki *et al.*, 2002). There have been some reports of uterine cancer in women taking TAM. However, the incidence is less than 1% in women taking doses of 20 mg daily. Most cancer specialists believe that the benefits of TAM out weight the risk of developing uterine cancer (Internet, 2003 a).

2.3.9: Mutagenicity and carcinogenicity of tamoxifen

In general, many anticancer drugs including TAM are shown to be mutagenic and carcinogenic due to their ability to chemically modify DNA. Among such anti-cancer drugs, the treatment period of TAM is exceptionally long (3-5 years). Therefore, careful evaluation of safety for chronic use of TAM is required (Umemoto et al., 2000).

In rats, TAM is a potent hepatocarcinogen in both males and females. Also, when administrated to neonatal rats, uterine adenocarcinomas were induced along with a lower frequency of squamous cell carcinomas of the vagina/cervix (Styles et al., 1994 and Phillips, 2001). Consistent with tumorigenesis are findings of hepatic cell aneuploidy and mitotic spindle disruption as well as DNA adduct formation in rat liver and kidney (Divi et al., 1999).

In mice, however, liver is not the target tissue for carcinogenesis . Instead, tumors of the testes are induced in males, and of the ovaries in females (Phillips, 2001); and also produced proliferative lesions in oviduct and uterus, followed by uterine carcinoma (Divi et al., 1999). When administered transplacentally to

mice, TAM causes a high incidence of hyperplasia in the reproductive tract, and a lower incidence of tumors in offspring (Phillips, 2001).

Studies on the carcinogenicity of TAM have continued with investigations of which rat organs are capable of activating the drug to DNA–binding products. Only the liver, where TAM is a potent carcinogen, has this ability. Importantly, TAM-DNA adducts were not detected in rat uterus or human endometrium , where TAM is also carcinogenic. This suggests that TAM has carcinogenic activity as a genotoxin and as non-genotoxin, depending on the tissue and species (Phillips et al., 1999).

Initiation of tumors in the rat is the result of metabolic activation of TAM by CYP enzymes to an electrophile(s) that binds irreversibly to DNA. This is not related to the estrogen receptor status of the tissue (Umemoto et al., 2000; Phillips, 2001).

4-hydroxytamoxifen exhibits potent DNA binding activity both in primary cultures of rat hepatocytes and in rat liver in vivo. The DNA adduct patterns were indistinguishable from those formed by the parent compound, providing strong evidence that this metabolite is an

intermediate in the pathway of activation of TAM to DNA – binding products (Phillips et al., 1999).

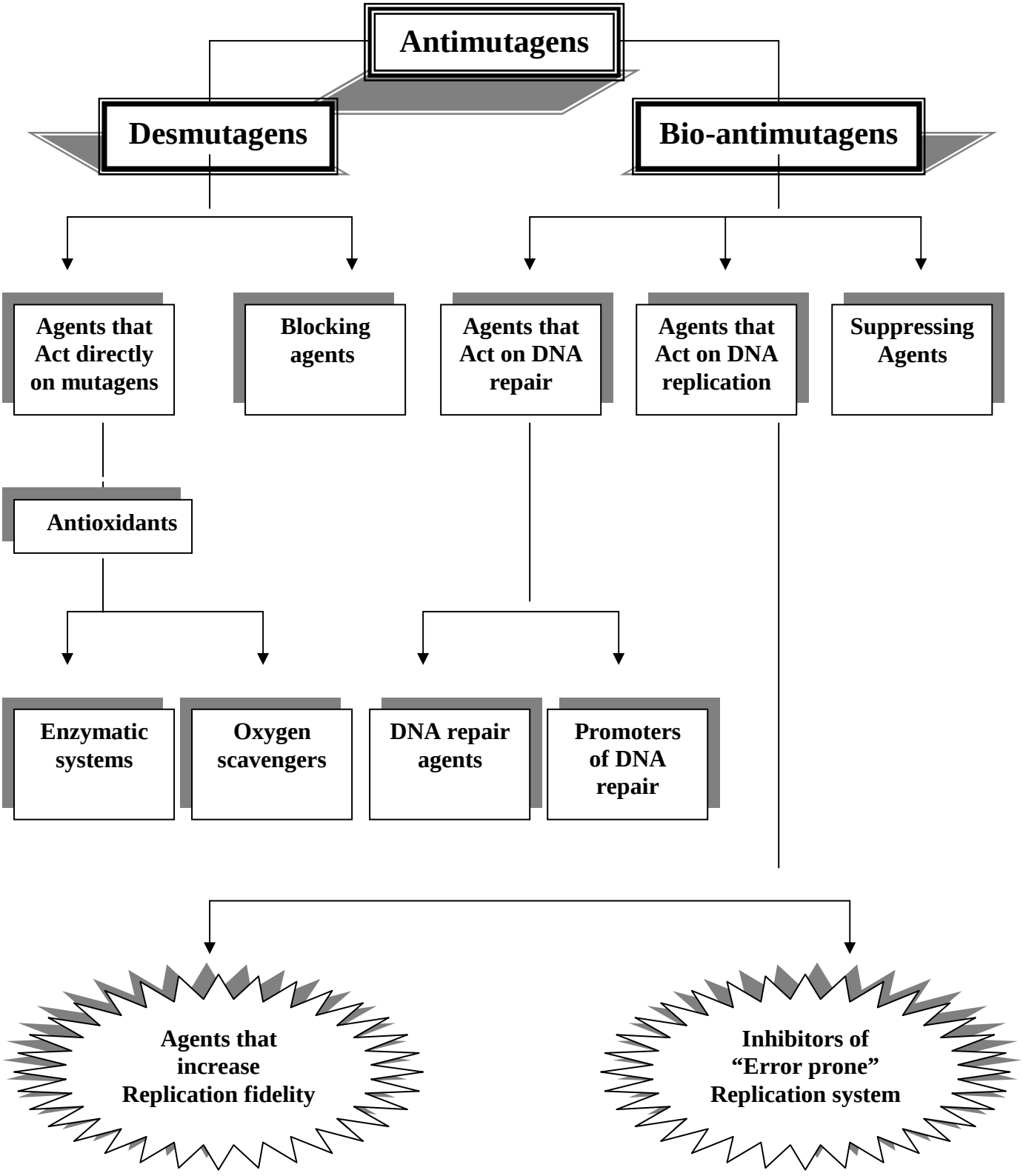
Available data indicate that the receptor-mediated mechanisms involved in the carcinogenic actions of TAM are operative in humans. Genotoxic mechanisms may also be operative in people, but preliminary studies suggest that they are quantitatively less than in rodents (Internet, 2001 a).Tamoxifen has been shown to be genotoxic in several studies. It induces unscheduled DNA synthesis in rat hepatocytes (Hirsimaki et al., 2002).

Although TAM is not mutagenic in bacteria (Salmonella typhimurium), it is positive for micronuclei formation in human cells in vitro (Otto et al.,1996).Tamoxifen caused the formation of micronuclei in human lymphoblastoma cell line (White, 1999; Phillips ,2001) and in MCL-5 cells in vivo , it increases aneuploidy and chromosomal aberration in the liver of rats (Sargent et al.,1996 ; Phillips ,2001 ; Hirsimaki et al.,2002) and mutations in the lac I reporter gene in the liver of transgenic rats (Phillips , 2001). Tamoxifen also induce chromosomal aberration and micronuclei in bone marrow

(Hirsimaki et al., 2002). Further more, tumors induced in rat liver by TAM were found to contain mutations in the P53 gene (Phillips, 2001).One preliminary report suggested that mutations at P53 in endometrial tumors associated with TAM therapy in women had a similar spectrum of mutations to those found in the livers of TAM-treated rats. This might infer a genotoxic mechanism of TAM associated with tumor formation (White, 1999).

2.4:Anti-mutagens

Anti-mutagens could be divided as: desmutagens and bioantimutagens (Bronzetti, 1997) and their mechanisms of action are presented in figure (2.3).



(Bronzetti, 1997)

Figure (2.3): Mechanism of action of anti-mutagens

2.5: *Nigella sativa* (The black seed)

Black seed has history of 3000 years. This makes it one of the safest plant extracts for human consumption. It is an adaptogen, which means an agent causing adaptive reactions. It increases the resistance of the human body, protecting it against various diseases. Clinical trails have shown that black seed oil is considered to be one of the greatest healing herbs of all times (El-Kadi and Kandil , 1986).

Black seed in its complete natural form, acts on the principle of assisting the bodies own natural healing process in overcoming illness or maintaining health. It works on the part or system of the body affected without disturbing its natural balance elsewhere (El-Kadi and Kandil , 1986).

Black seed has different names depending on where in the world been used. In India it is referred to as Kalonji or Kalwanji , whilst in Arabia it is commonly known as Al-Habba Al- Sauda or Habbat-ul Barakah . Other names include the Black cumin, Blessed seed, and Nigella sativa (Randhawa and Al-Ghamdi, 2002).

2.5.1: Black seed description

Black seeds (Nigella sativa) belong to the botanical family Rununculaceae and commonly grown in Europe, Middle East, and Western Asia (Randhawa and Al-Ghamdi, 2002). The black seed is a herbaceous plant, 15-60 cm high, the leaves are 2.5 – 5 cm long, and pale bluish purple or white flowers. The seeds are ovate, tiny and hairy, being no more than 3 mm in length, and those seeds are originated from common fennel flower plant. The plant has divided foliage ; the flowers grow opposite each other in pairs on the either side of the stem . Its lower leaves are small, while the upper leaves are long of about 6-10 cm (Mukherji , 1953 ; Townseed ,1980).

Black seed reproduced with itself and forms a fruit capsule which consists of many white trigonal seeds .Once the fruit capsule has matured, it opens up and the seeds contained within are exposed to air become black in color.

2.5.2: Black-seed constituents

The specific seed constituents that have been identified and investigated include:

- ❖ Fixed oils
- ❖ Saponins
- ❖ Volatile or essential oils
- ❖ Alkaloids
- ❖ Amino acids
- ❖ Trace elements (calcium, iron, sodium, potassium, and crude fiber).

The fixed oil constitutes 37 % of the seed and may be subdivided into triglycerides and sterols.

A-Triglycerides:

Which are a storage form of fatty acids, are further subdivided into:

1- Saturated fatty acids :

Myristic	0.16 %
Palmatic	12.08 %
Stearic	3.11 %

2- Unsaturated fatty acids :

Which are essential in the diet of man as they can not be synthesized in the body.

- ❖ Oleic 24.64 %
- ❖ Linoleic 56.12 %
- ❖ Linolenic 0.70 %
- ❖ Eicosadienoic 2.53 %

B- Sterols:

The second component of fixed oils is the sterols. Sterols are steroids containing 27 or more carbon atoms with an OH group. A remarkable number of 23 sterols have been identified in the seed (Bhikha, 1990).

2.5.3: Nutritional value of black seed

Black seed is rich in nutritional values: monosaccharides in the form of glucose, rhamnose, xylose and arabinose. The black seed contains a non-starch polysaccharide component which is a useful

source of dietary fiber. It is rich in fatty acids, particularly the unsaturated and essential fatty acids. Essential fatty acids can not be manufactured by the body alone, and therefore, we acquire these from food.

Fifteen amino acids make up the protein content of the black seed, including eight of the nine essential amino acids. Essential amino acids can not be synthesized within our body in sufficient quantities and are thus required from our diet.

Black seed contains arginine , which is essential for infant growth. Chemical analysis has further revealed that the black seed contains caroten, which is converted by the liver into vitamin A, the vitamin known for its anti- cancer activity.

The black seed is also a source of calcium, iron, sodium and potassium. These elements main function is to act as essential cofactors in various enzyme functions (Kumara and Huat , 2001).

Black seed also contain limonene, which is being investigated for use as a treatment for some types of cancer (Vigushin et al., 1998).

Black seed also contain thymoquinone , which is the major component of the essential oil and also present in the fixed oil .Thyoquinone pharmacological action include protection against nephrotoxicity and hepatotoxicity induced by either disease or chemicals. It would appear that the beneficial effects of the use of thyoquinone might be related to their cytoprotective and antioxidant actions, and to their effect on some mediators of inflammation (Giorgio, 1994).

Black seed contain linoleic acids, which play a major role in protection against mutagens by blocking metabolic activation with enzyme and/or in trapping mutagen molecules (Giorgio, 1994).

Black seed contain alkaloids, the main type is diterpene alkaloid called nigellamines .In pharmacology experiments, the isolated alkaloids have been shown to lower cholesterol (Kumara and Huat, 2001).

2.5.4: History of black seed uses:

Black seed has been used historically in Egypt and Middle East for more than 3000 years. It has used as well as in parts of Asia and Africa and is now well known in the USA and Europe. Ibn sina, the author of the Canon of Medicine, one of the most famous books in the history of medicine, recommends black seed stimulates the metabolism and to recover from dispiritedness and lethargy .The earliest written reference on black seed is found in the Old Treatment. It is most famous for the saying of the holy prophet Muhammad (SWS), “Hold on to use of the black seed, for it has a remedy for every illness except death”. The wording “Hold on to” indicates a long term use (Internet, 2002 b).

Since 1959, there have been over 200 different studies at universities and laboratories. One of the largest experimental studies so for proved that black seed oil had enormous success in tumor therapy with out the negative side effects of common chemotherapy. It was found that black seed increased the growth rate of bone marrow cells by a staggering 250 % and it inhibited tumor growth by 50%. It stimulated immune cells and raised the interferon

production which protects cells from the cell-destroying effects. Black seed also has an effect in lowering the blood sugar level which is essential for the treatment of diabetes (Internet 2002 b).

2.5.5:Biological and pharmacological activities of black seed

Black seed has antibacterial, antiyeast, antihyperglycemic, antihypercholestrolemic, bronchodilator, anti-tumor, antitoxic, antispasmoidic , haemostatic , analgesic , hypotensive , diuretic , hypoglycemic , antifungal , anti-inflammatory , antiarthritic , antiseptic and antiviral activities .

It is also have the following activities:

- ❖ Dermatological activity: treat acne and eczema.
- ❖ Urinary tract activity: treat kidney stone.
- ❖ Gastrointestinal tract activity: treat nausea, diarrhea, flatulence, colic, constipation and hemorrhoids.
- ❖ Circulatory system activity: improves efficiency of the heart.
- ❖ Nematocidal activity: effective against tapeworms.
- ❖ Respiratory tract activity: treat short breath.

(Bhikha , 1990).

2.5.6: Black seed and immunity

Studies begun just over a decade ago suggest that black seed can play an important role to enhance human immunity, particularly in immunocompromise patients (Internet 2004 b).

It was established by E1-Kadi and Kandil (1986) that four weeks after administration of black seed to volunteers, the majority of complete lymphocyte count displayed a 72 % increase in T-helper cells to T-suppressor cells ratio, as well as an increase in natural killer cells (NK) functional activity. These findings may be of great practical significance since a natural immune enhancer like black seed could play an important role in treatment of cancer, AIDS, and other disease conditions associated with immune deficiency state.

Haq and his partner (1999) revealed that black seed can enhance the production of Interleukin-3 by human lymphocytes when cultured with pooled allogenic cells or without any added stimulator and has an effect on macrophages as well .When cells

treated with black seed proteins, produced greater amounts of cytokines ,specifically interleukin –1-B and tumor necrosis factor alpha.

2.5.7:Anti-tumor and antimutagenic activities of black seed:

Black seed is considered a biological response modifier (compound that have a unique effect on physiological and can reduce the side effects of cancer treatment, while at the same time increasing their effectiveness). Nair and his team (1991) showed that extracts from the seeds are toxic to cancer cells, and in mice , prevent blood cell toxicity caused by the anti-cancer cisplatin.

The active compounds of black seed are the volatile oils thymoquinone and dithymoquinone, both of which inhibit tumor cells in laboratory experiments – even tumor cells resistant to anti-cancer drug (Worthern et al., 1998).

A recent cell study showed that when incubated with black seed extract, cancer cells were unable to produce fibroblast growth

factor and the protein collagenase, both necessary for blood–vessel growth into the tumor (Medenica et al., 1997).

Another recent experiment indicates that thymoquinone may also prevent some toxic side effects of cancer treatments .Scientists from King Saudi University found that mice pretreated with thymoquinone were protected from carbon tetrachloride-induced liver toxicity. Carbon tetrachloride is a toxin that in small amounts can kill by causing the liver and kidney to atrophy. Thymoquinone also demonstrated antioxidant activity, which may be how it protects the liver (Nagi et al., 1999).

Black seed contains flavonoids, which have antimutagenic and free-radicals scavenging activities (Samajima *et al.*,1995), and also have the ability to reduce DNA-breakages (Miski *et al.*,1983) and induce the activity of GST enzyme, which have antitoxygenic and anticarcinogenic activities (Ketterer,1988). Black seeds also contain carotenoids, which considered anticarcinogenic agents.

Phenols also founded in black seed, which have anti mutagenic activity by blocking the metabolic activation of the mutagens and scavenging the free radicals produced from mutagen metabolism. Phenols can also reduce the DNA-adduct formation by its binding to

the target sites in the DNA to prevent the binding of the mutagen (Raj *et al.*, 1983).

In other study, 33% reduction in the tumor mass has been observed in experimental soft tissue sarcoma induced by methylcolanthrene after treatment with black seed extract at the dose of 100 mg/kg body weight for 30 days (Salomi et al., 1991).

Using an active principle of fatty acids derived from black seed , studies with Swiss albino mice showed that this active principle could completely inhibit the development of a common type of cancer cells called Ehrlich Ascites Carcinoma (EAC), and Daltons Lymphoma Ascites (DLA) cells. Mice which had received the EAC cells and black seed remained normal without any tumor formation illustrating that the active principle was 100% effective in preventing EAC tumor development. Results on mice who received DLA cells and black seed showed that the active principle had inhibited tumor development by 50% less compared to mice not given the active principle. The study concluded that the active principle isolated from black seed is a potent anti-tumor agent , and

the constituent long chain fatty acid may be the main active component (Salomi et al.,1992).

Salomi and his team (1991) showed that topical application of black seed extract inhibited two-stage initiation / promotion of skin carcinogenesis in mice . A dose of 100 mg/kg body weight of black seed extract delayed the onset of papilloma formation.

In a study of the protective effect of black seed on the oxidative stress and carcinogenesis induced by methylnitrosourea (MNU) in Sprague Dawely rats, it was shown that black seed given orally protected against MNU – induced oxidative stress and carcinogenesis by 80%. This result showed that supplementation of diet with black seed has a protective effect against MNU-induced oxidative stress, inflammatory response and carcinogenesis (Mabrouk et al., 2002).

Black seed and its oil inhibit the adverse cytogenetic and enzymatic effects of gamma rays on albino mice (Al-Azawi , 1999).

Black seed extract and thymoquinone were considered as protective agents against the chromosomal aberrations induced in

mice bone marrow and spleen as a result of schistosomiasis (Aboul-Ela, 2002).

Black seed oil acted as an antiparasitic agent with efficacy of 88% against infection with Cryptosporidium parvum in mice (Al-Azawi, 2003).

2.6: Honey

Honey has been known for its healing properties for thousands of years. The ancient Greeks used it, and so have many other people through the ages. Even up to the Second World War, honey was being used for its antibacterial properties in treating wounds (Hassanein, 1989).

Scientists after having done decades of research have agreed that honey is safe and has no allergic or side effects. Fructose present in the honey encourages the blood absorb water without sodium for the intestine, a desirable effect that may save us from a lot of health problems (Hassanein, 1989).

Honey is composed of:

- ❖ Many vitamins: especially of the B-groups, like the B-complex, Vitamin C, D and E.
- ❖ A wide range of minerals: such as calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium and zinc.
- ❖ Acids & Enzymes: invertase, amylase, glucose oxidase, catalase, acetic and at least 8 other organic acids, proline and at least 18 other free amino acids.
- ❖ Carbohydrates: fructose, glucose, maltose, sucrose, turanose, isomaltose, maltulose, erlose, Theanderose and panose
- ❖ Antioxidants: pinocembrin, pinobanksin, chrysin and galagin

(Molan, 1998).

2.6.1: Uses of honey for treatments and healing

Honey was used for the treatment of diarrhea , stomach and intestine infections , ulcers , cough , skin wounds , burns , weakness and iron deficiency , etc (Hassanein,1989).

Efem (1988) reported clinical observations on the healing with honey of 59 cases of wounds and skin ulcers that had not been healing

for 1 – 24 months with conventional treatment. The wounds were found to become sterile and odorless in 1 week , pus and gangrenous tissue separating by themselves painlessly. Swelling and exudation of lymph subsided rapidly and there was rapid development of new tissue to repair wounds. The honey caused no adverse reactions.

Salem (1981) reported a clinical trial in which 45 patients with dyspepsia were given no medication other than 30 ml of honey before meals 3 times daily. After treatment with honey the number of patients passing blood (from peptic ulcers) in their faeces had decreased from 37 to 4; the number of patients with dyspepsia had decreased from 41 to 8; the number of patients with gastritis or duodenitis seen on endoscopy had decreased from 24 to 15; the number of patients with a duodenal ulcer seen on endoscopy had decreased from 7 to 2 .

Emarah (1982) reported that treating with honey 102 patients with a variety of opthalmological disorders not responding to conventional treatment, such as keratitis , conjunctivitis and blepharitis . The honey was applied under the lower eyelid as eye ointment would

be applied. Improvement was seen in 85% of the cases, with no deterioration seen in any of the other 15%.

2.6.2: Hony and the immune system

Honey may clear infection through stimulating the body's immune system to fight infection. It has been reported that honey stimulates B-lymphocytes and T-lymphocytes in cell culture to multiply, and activates neutrophils (Abuharfeil *et al,* 1999). It has also been reported that honey stimulates monocytes in cell culture to release the cytokines TNF-a, IL-1 and IL-6, the cell "messengers" that activate the many facets of the immune response to infection. In addition to its stimulation of these leucocytes, honey provides a supply of glucose which is essential for the ‘respiratory burst’ in macrophages that produces hydrogen peroxide, the dominant component of their bacteria-destroying activity. Furthermore it provides substrates for glycolysis, which is the major mechanism for energy production in the macrophages, and thus allows them to function in damaged tissues and exudates where the oxygen supply is often poor. The acidity of honey may also assist in the bacteria-destroying action of macrophages, as an acid pH inside the phagocytotic vacuole is involved in killing ingested bacteria (Tonks *et al*, 2001).

2.6.2: Antibacterial activity of honey

The antibacterial activity of honey was first recognized in 1892 , by Van Ketel (Dusmann , 1979).The studies carried out on this science have been reviewed by Molan (1992a , 1992b) , It has been found that mostly the activity is due to hydrogen peroxide produced enzymically in honey , but there have been some reports of minor additional antibacterial components.

Honey contains specific enzymes as well as other compounds such as terseness that may be responsible for honeys ability to exert antimicrobial effects against such organisms as staphylococcus aureus, E.coli, and candida albicans (Hassanein, 1989)

Perhaps most remarkable is the effect of honey on Helicobacter pylori, the bacterium now known to cause gastric ulcer. Within three days , honey stopped the growth of H.pylori colonies obtained from patients (Challem ,1995).

Honey also reduces the duration of diarrhea in patients infected by Salmonella, Shigella and E.coli (Badawy et al., 2004).

2.6.3: Antioxidant activity of honey

Honey has been found to have significant antioxidant content, measured as the capacity of honey to scavenge free radicals. The antioxidant activity of honey also been demonstrated as inhibition of chemiluminescence in a xanthine-xanthine oxidase-luminol system that works via generation of superoxide radicals. This antioxidant activity may be at least partly what is responsible for the anti-inflammatory action of honey, as oxygen free radicals are involved in various aspects of inflammation, such as further recruitment of leucocytes that initiate further inflammation. But even if the antioxidants in honey do not directly suppress the inflammatory process they can be expected, by scavenging free radicals, to reduce the amount of damage that would otherwise have resulted from these (Gheldof et al, 2001).

As well as scavenging free radicals to neutralize them after they have been formed, honey has the potential to exert an antioxidant action by a completely different mechanism, inhibition the formation of free radicals in the first place. The superoxide that is first formed in inflammation is relatively un-reactive, and is converted to hydrogen

peroxide which is much less reactive , but from this is generated the extremely reactive peroxide radical. This formation of the oxidant peroxide radical is catalyzed by metal ions such as iron and copper, and sequestering of these metal ions in complexes with organic molecules is an important antioxidant defense system. Flavonoids and other polyphenols, common constituents of honey, will do this (Internet, 2001 b).

Antioxidants identified in honey included phenolic compounds , ascorbic acid and the enzymes glucose oxidase , catalase and peroxidase .The antioxidant power of honey appeared chiefly due to their phenolic composition. Dark-colored honey was especially good at removing free radicals (Internet, 2005). In addition, many honeys provide small amounts of vitamin antioxidants such as vitamin C (Schramm and Keen, 2004).

One published study says that honey slows the oxidation of low-density lipoproteins (LDLs), a process that leads to atherosclerotic plaque deposition (Internet, 2003 d).

2.6.4: Antimutagenic and anticarcinogenic activities of honey

Antioxidants often have antimutagenic properties. Antimutagenic compounds interfere with or reduce the effect of harmful changes in cells in the body. Research in the university of Illinois shows that honey is antimutagenic, offering yet another reason to use it as an ingredient and in the diet (Internet, 2000).

Honey exert a radioprotective activity of mouse somatic and germ cells against gamma irradiated mice (0.1- 400 Gray/ whole body) (Al-Bedairi, 2002).

A study at the University of California showed that antimutagenic effect of honeys against Trip-p-1 free radicals have been implicated in aging and many diseases such as cancer and heart disease (Wang and Engeseth, 2002).

Turkish researchers found that honey seemed to block tumors from taking hold in the animals. The investigator made incisions in the animals' necks and injected them with tumor cells. Half of the mice had honey smeared in the incisions before and after the tumor cell

injections. Only 25% developed tumors, compared with 100% in the honey-less mice (Internet, 2004 c).

Studies by Gribel and Pashinskii (1990) indicated that honey possessed moderate antitumor and pronounced antimetastatic effects in five different strains of rat and mouse tumors. Further more ,honey potentiated the antitumor activity of chemotherapeutic drugs such as 5-flurouracil and cyclophosphamide.

Caffeic acid phenylthyl ester (CAPE) is a phenolic antioxidant derived from honey. Studies suggest that CAPE has anti-cancer properties. These caffeic acids prevented the formation of pre-cancerous tissue in rats after they were exposed to cancer causing chemicals (Rao and Valhalla, 1993).

In a study of Croager (2004), tumors were generated in the mice via the injection of cancer cells, and honey was given to different mice before, at the same time, or after wards. It was found that giving honey orally appeared to inhibit the development of tumors when it was given before the injection of cancer cells although if given afterwards, it appeared to fuel the development of secondary cancers.

The flavonoids pincocembrin and pinostrobin are antioxidants found in honey , apparently are potent inducers of certain enzymes that deactivate carcinogens , known as mammalian phase –2 detoxification enzymes , they help to destroy the reaction centers of carcinogens or assist in their elimination from the body (Internet , 2004 a).

The way in which the honey works to combat the tumors is not clear, but the authors suggest the chemicals cause apoptosis (cell suicide) or necrosis of the cancerous cells , or that they exert directly toxic or immunomodulatory effects. They may also reduce harmful oxyradicals in cells or body fluids. These results suggest the benefits of potential clinical trials using honey, combined with chemotherapeutic agents (Orsolic, 2004).

2.7: Cytogenetic analysis

Cytogenetic Analysis is a widely employed indication system for the evaluation of physically, chemically and biologically induced mutations. It allows for the objective evaluation of the genetic material damages and is a method that permits direct image analysis for the chromosome damage (Nakashi and Schneider, 1979).

Cytogenetic analyses have been carried out using in vivo and in vitro systems. These have been proved to be good and reliable for mutagen-carcinogen exposure and chromosomal aberration detection (Nakashi and Schneider, 1979 ; Gebhart, 1981). Chicken embryos were the first to be utilized in in vivo (in ovo) analysis (Bloom and Hsu , 1975) but later mice became the animals most frequently used due to their fast reproduction ,small size ,easy handling and represent a typical mammalian system (Tice et al.,1989 ; Haung et al.,1990). Rabbits have also been used, but less frequently (Stetka and Wolff, 1976).

A variety of somatic and germ tissues have been analyzed in *in vivo* studies including spermatogonial cells (Allen and Latt,1976),bone marrow cells (Karm *et al.*,1975) and spleen cells (Nath *et al.*,1988).

Concerning *in vivo* and *in vitro* analysis, there are obvious advantages of each approach for the screening of compounds. *In vivo* analysis simulate human *in vivo* exposure and can detect compounds, which require metabolic activation and utilize normal cell population .While *in vitro* analysis; or the adding of a compound to a culture media; will guarantee an instantaneous, uniform delivery to the cells, also the delivery of the compound will not be affected by the catabolic and excretory mechanisms operative in the intact organisms (Schneider and Lewis, 1982).

As a result of these differences between *in vivo* and *in vitro* analysis ,many chemical agents were very potent inducers for the same

abnormalities *in vitro* , where as other chemical agents were very potent inducers of chromosomal abnormalities *in vitro* only , with regard to the fact that the replication kinetics are similar *in vivo* and *in vitro* (Shubber , 1981).

2.7.1: Mitotic index (MI)

Proliferating cells go through a regular cycle of events, the mitotic cell cycle, in which the genetic material is duplicated and divided equally between two daughters. This is brought absent by the duplication of each chromosome to form two closely adjacent sister chromatids, which separate from each other to become two daughter chromosomes. These along with the other chromosome of each set, are then packaged into two genetically identical daughter nuclei. The molecular mechanisms underlying the cell cycle are highly conserved in all organisms with nucleus eukaryotes (Eva Therman, 2001).

Mitotic index (MI) was determined as a ratio of mitotic cells to inter phase nuclei in 1000 cells.

$$MI = \frac{\text{number of dividing cells}}{\text{(number of dividing cells + number of non-dividing cells)}} \times 100$$

(King *et al.*, 1982; Shubber and Al-Allak, 1986).

2.7.2: Chromosomal aberrations (CAs)

The increasing variety of chemicals, radiations and other physical agents we are exposed to nowadays has stimulated the development of many rapid, reliable assay for the detection of the mutagenicity or carcinogenicity of such agents. One of these methods is the chromosome aberration assay (Lambert et al. 1978; Ardito et al., 1980).

This test serves to detect structural chromosome aberrations, as may be induced via DNA breaks by various types of mutagens .Such DNA breaks may either rejoin, such that the chromosome is restored to its original states, rejoin incorrectly or not rejoin at all. These last two cases may be observable on microscopic preparations of metaphase cells. However, many of these gross changes probably will not allow cell survival after division, but they serve as indicators for the inductions of smaller, not readily observable changes, which do allow cell survival but many have deleterious consequences for the organism (Moutschen, 1985). The test is most often done on human peripheral blood lymphocytes, but established cell line like Chinese hamster ovary (CHO) cells may also be used. As peripheral lymphocytes are in the resting Go stage of the cell cycle they have to be stimulated to divide by a specific antigen, like phytohaemagglutinin to obtain a sufficient number of mitotic cells, a spindle inhibitor like colchicines

may be added shortly before fixation to block cells in (pro) metaphase. An exogenous metabolisation system, like a liver microsome fractions, can also be added (Winter *et al.*, 1998).

Shubber and his partners in their study confirmed that C 57 black mice showed spontaneous frequencies of chromosomal aberrations and sister chromatid exchanges in bone marrow cells which increased due to the effect of carcinogenesis, which causes cytogenetic damage (Shubber *et al.*,1985).

Other studies also done by Shubber and his partners showed that chromosomal aberrations and SCE could be resulted from the effect of DNA damaging agents that cause DNA strand breaks in the treated animal (Shubber and Al-Shaikhly, 1988; Shubber and Salih, 1988).

Another study showed that the unbalance in the concentrations of the two classes, DNA-damaging agents and antimutagenic compounds, may lead to genetic damage in the cells of the treated animals (Shubber and Juma,1999).

Jensen and Nyfros(1979) confirmed that the cytogentic examination of the bone marrow cells must be included in the study of the possible chromosome damaging effect of chemical agents *in vivo* because the bone marrow cells are very sensitive to chemicals , so they represent a good indicator for the effect of chemical oncogens and mutagens.

Chemotherapeutic drugs (as chemical agents) have been extensively studied as damaging agents for the DNA by using

cytogenetic analysis. Some phenomena were observed by the physicians and scientists on some cancer patients suffering from primary tumors or (cancer at stage I), these patients were completely cured by chemotherapy, but after some time the physicians realized the returning of the tumors in the same organs .One of the acceptable proved mechanisms was the (late effects) of chemotherapy used in the treatment of the first tumors, the late effects were explained by certain DNA damage and chromosomal abnormalities (Aronson *et. al*.,1982).

There are many cytogenetic changes that may lead to cancer development including deletions, translocations and inversions. Deletions often resulting in loss of tumor suppressor gene , while translocations and inversions can be divided into those consistently found in certain tumor types (specific)and those observed only in the tumor from one patients (idiopathic) (Rabbitts ,1994).

2.7.3: Micronucleus (MN)

Micronuclei are cytoplasmic chromatin masses with the appearance of small nuclei that arise from chromosomes lagging at anaphase or from acentric chromosomal fragments.

As an alternative to classical metaphase analysis, the frequency of the occurrence of micronuclei in treated cells provides a comparatively rapid and sensitive indication of both chromosomal aberrations and chromosomal loss that lead to numerical chromosome anomalies.

The first serious attempt to use micronuclei as a monitor of cytogenetic damage appears to be that reported by Evans *et al.*(1959); they used the micronucleus frequency to measure the cytogenetic damage induced in root-tips by fast neutrons and X-rays in the presence and absence of oxygen. It was found that all chromatid ,chromosome, and chromatid breaks, as well as asymmetrical and incomplete symmetrical exchange , will give rise to acentric fragments at mitosis, and that these fragments are frequently excluded from the daughter nuclei and appear in the following interphase as micronuclei.

Subsequently, Schroder (1966, 1970) recommended the use of bone-marrow smears to detect *in vivo* damage from chemical mutagens and demonstrated the occurrence of micronuclei in bone-marrow cells in connection with cytogenetic damage. Frohberg and Bauer (1973) recommended scoring micronuclei along with other nuclear anomalies, this nuclear anomaly test was utilized in early attempts to substitute direct observation of bone-marrow smears for metaphase cytogenetic analysis (Frohberg and Bauer, 1973)

Beginning about 1970, Schmid and coworkers and Heddle initiated studies to determine which parameters might serve as the most useful indicators of cytogenetic damage in bone-marrow *in vivo* (Schmid and Staiger ,1969; Boller and Schmid,1970; Matter and Schmid ,1971; Matter *et al.*,1973; Heddle,1973).This work led to the conclusion that the incidence of micronucleated polychromatic erythrocytes (PCE)was a particularly useful index of *in vivo* bone marrow cytogenetic damage (Von Ledebur and Schmid,1973).Other

had also begun to explore the use of micronuclei in cytogenetic studies of mutagens (Deig *et al.*, 1962; Koller and Casarini,1966; Smoliar,1969; Joshi *et al.*,1970; Muller *et al.*,1972 ; Roberts and Sturrok,1973) but the work of Von Ledebur and Schmid is of particular historical importance because it led directly to the development of the simple *in vivo* test based on the identification of micronuclei in PCEs of mouse bone marrow . This assay is now in such wide spread use that it is commonly reffered to as “the micronucleus test” (Schmid , 1976).

The increasing use of various micronucleus assays since 1973 undoubtedly stems mainly from the primary advantages of speed and simplicity. Heddle (1973) pointed out that scoring for micronuclei was considerably more than 10-fold faster than metaphase scoring at a similar power of test.

2.7.4: Sister chromatid exchange (SCE)

SCEs are determined as reciprocal exchanges of chromatid arms in a cell which have replicated in the presence of 5-bormodeoxyuridine (BUdR) which differentially labels sister chromatids by its incorporation into DNA on the bases of thymidine replacement during two cycles of DNA synthesis (Latt, 1974).These exchanges which are generally detected in cytological preparations of

metaphase chromosomes, presumably involve DNA breakage and reunion.

After years of intensive researches, the biological significance and the mechanism of SCEs induction is known, although they give a certain indication for some types of DNA damages. Much of the work has concentrated on the relationships between SCE induction and other genetic changes involving the processes of mutation, carcinogenesis, chromosome breakage, mitotic recombination, and DNA repair mechanisms. They are an “S-dependent” event induced in replication, their induction may be due to perturbation or lesions in the DNA helix (Ockey, 1983).The frequency of SCEs was found to be positively correlated to the levels of DNA damage (Perry and Woiff, 1974), the specific locus mutation, the high incidence of lung cancer in man and the spontaneous leukemia in rats (Carrano *et al.*, 1978).

In recent years, more attention has been devoted to the study of SCE levels in relation to health and diseases. SCE frequency was reported to be significantly higher in lymphocytes of patients with Blooms syndromes (Chaganti *et al.*, 1974), acute lymphoblastic leukemia (Otter *et al.*, 1979), chronic myeloid leukemia (Shirishi and Sandberg, 1980) and in patients with schistosomiasis (Shubber, 1987; Shubber *et al.*, 1991 ; Juma *et al.*,1999).

The good qualitative correlation of SCE induction with mutagenicity in mammalian cells (Carrano *et al.*,1978) and with carcinogenicity in laboratories approximately 90%(Latt *et al.*,

1984),that finding supports the use of SCE analysis as a measure of genetic toxicity.SCE can be measured in a wide variety of target cells *in vivo* and *in vitro*.

SCE can be used as a predictor of carcinogenic potential (Latt *et al.*,1984),some of the mutagenic and carcinogenic agents increased SCE yields at 10-100 fold lower concentrations than those required to induce other genetically events . Moderate increases in SCEs did not; however, appear to produce any serious perturbations in the cells. These findings suggest that an SCE test would be of great value in discovering even weak genotoxic agents in the environment (Ockey, 1983).

The mutagenic and carcinogenic agents have the ability to increase the frequency of SCE *in vivo* , especially that interact with metabolism and DNA repair system or the drug that cross-link with DNA,like cisplastin (CP)(Deen *et al.*,1989), mitomycin-C (MMC) drug (Evan and Vijayalaxmi ,1981), ionized irradiation (Shubber and Al-Shaikly , 1989),in addition to a number of flavinoides like querecetin and kaempferol , and some plant extracts like sumac (Al-Ani and Al-Asuad, 1997).

Many halogenated pyrimidine analogues have been assessed for the labeling of DNA including bromodeoxy uridine , iododeoxyuridine,chlorodeoxyuridine ,bromodeoxycytidine, chlorodeoxycytidine , and iododeoxycytidine (Dufrain , 1974). When incorporated into DNA or chromatin, BUdR can quench the fluorescence of DNA binding dyes such as 33258 Hoechst (Craig –

Holmes and Shaw, 1976), acridine orange (Kato, 1974; Perry and Wolff, 1974). And under appropriate conditions 4,6-di-amidino-2-phenylindole (DAPI)(Lin and Alfi, 1976). Fluorescence methods for sister chromatid staining do not provide permanent preparations, and rapid fading of stained specimens make even initial photomicroscopy difficult. Giemsa methods (Ikushima and Wolff, 1974) can easily utilize BUdR – sensitive restain for the photo-sensitization methods, have thus, replaced fluorescence techniques for routine cytogenetic analysis.

2.7.5: Cell cycle progression (CCP)

The BrdUrd-Giemsa technique is a useful method for identifying, with accuracy, the percentage of cells which have gone through one, two or three divisions in vitro and in vivo (Bianchi and Lezana , 1976). In vitro CCP was found to be affected by the medium, BrdUrd levels, and colchicine (Shubber and Al-Allak, 1986). The nomenclature utilized for the evaluation of CCP pattern, according to the number of cell cycles was:

1- First cell division (M1):

These groups of cells have no incorporated BrdUrd or are able to incorporate BrdUrd during a single DNA replication phase. The chromosomes of this phase all appear bright under the light microscope.

2- Second cell division (M2):

This group contains cells, which incorporated BrdUrd during two “S” phases and display atypical differential staining of sister chromatids (One dull and one bright).

3- Third cell division (M3):

These metaphases incorporated BrdUrd during three “S” phases and contain BrdUrd-substituted DNA in both sister chromatids.

(Becher et al., 1984)

2.7.6: Replicative index (RI)

Cell cycle progression (CCP) may be defined using a parameter that considers, at the same time, the number of M1, M2 and M3 metaphase cells. Many indicators were considered, but the most suitable was the RI. This index is formulated using the following equation:

$$RI = \frac{(1x\ \%M1) + (2x\%M2) + (3x\%M3)}{100}$$

(Lamberti et al., 1983).

Chapter three

Materials & Methods

3.1: Materials

3.1.1: Equipments and apparatus

The following equipments and apparatus were used throughout the study:

Apparatus	**Company**
Autoclave	Webeco Gmbh (Germany)
Centrifuge	Beckman (England)
Cold incubator	Memmert (Germany)
Digital camera	Smartek (China)
Electric balance	Sartorius (Germany)
Microscope	Motic (Japan)
pH-Meter	Radiometer (Denmark)
Vortex Mixer	Griffin (England)
Water bath	Gallenkamp (England)

3.1.2: Chemical materials

The following chemical materials were used in this study:

Materials	Company
Colchicine	BDH (England) Ibn Hayan (Syria)
Glycerin	Fluka (Switzerland)
Giemsa stain	Fluka (Switzerland)
Glacial acetic acid	Fluka (Switzerland)
Fetal calf serum (FCS)	Sigma (USA)
Human serum	Biotest pharma (Germany)
Hoechst stain	BDH (England)
Bromodeoxyuridine	BDH (England)
Tri sodium citrate DPX	Sigma (USA)
KCl KH**2**PO**4**	Fluka (Switzerland)
Methanol	Fluka (Switzerland)
Na**2**HPO**4**	Fluka (Switzerland)
HCl NaCl	Fluka (Switzerland)
RPMI	Sigma (USA)
Tamoxifen	Hexal (Germany)

Sodium bicarbonate	BDH (England)
Heparin	Sigma (USA)
DMSO	Sigma (USA)
Penicillin	Sigma (USA)
Streptomycin	Sigma (USA)
PHA	Ministry of Science and Technology (Iraq)

3.2: Chemical preparations

1- Colchicine (Ibn Hayan / Syrian)

Colchicin was prepared by dissolving one tablet (0.5 mg) of colchicine in (0.5 ml) of PBS to be used for mice injection. Each animal was injected with (0.25 ml) of this solution in the intaperitoneal membrane (IP).

2- Colchicine (BDH)

One gram of colchicine was dissolved in (20 ml) of sterile D.W. to make a stock solution. This solution was stored at (-20ºC) until used for human blood culture.

3- Phosphate buffer saline (PBS)

This solution was prepared by dissolving the following chemicals in (1000ml) of D.W.:

Sodium chloride (NaCl) 8gm

Potassium chloride (KCl) 0.2gm

Sodium phosphate hydrate (Na_2HPO_4) 1.15 gm

Potassium phosphate dihydrate (KH_2PO_4) 0.2gm

The pH was adjusted to (7.2).The solution was sterilized by autoclaving and stored at (4º C).

4- Potassium chloride (KCl) (hypotonic solution)

A concentration of (0.075 M) was prepared by dissolving (5.75 gm) of KCl salt in (1000ml) of D.W. The solution was sterilized by autoclaving and stored at (4º C).

5- Fixative solution

This solution was freshly prepared by mixing 3 parts of absolute methanol with 1 part of glacial acetic acid.

6- Sodium bicarbonate (NaHCO3)

Sodium bicarbonate (4.4 gm) was dissolved in (100ml) sterile D.W. This was stored at (4º C) until use.

7- Giemsa stain

Giemsa stock solution was prepared by dissolving (1 gm) of Giemsa powder in (33ml) glycerine in water bath at (60º C) for 2 hours with continuous shaking , then left for 30 min. at room temperature , then add (66ml) of absolute methanol with continuous shaking. The solution was kept in dark bottle at room temperature.

For slide staining, Giemsa solution was prepared as follow:

Giemsa stain stock	1ml
Absolute methanol	1.25 ml
Sodium bicarbonate solution	0.5 ml
D.W	40ml

8- Bromodeoxyuridine (BrdUrd)

5-bromo-2-deoxyuridine powder (50 mg) was suspended in (37.5 ml) of D.W .The solution was sterilized by filtration through a 0.22µm sterile filter, distributed in sterile tubes with final concentration (1.33 mg/ml) and stored at (-20ºC) until use.

9- RPMI-1640 medium

This medium contained the following:

RPMI-1640 medium base	10 gm
Fetal calf serum (heat inactivated)	15 %
Penicillin	1000 iu
Streptomycin	100 mg
Heps	1 %
Sodium pyrovate	1 %
Sodium bicarbonate	1 %
BrdUrd	1 %

The volume was completed with sterile D.W. to (1000 ml), and the pH was fixed to 7.2 and sterilized by filtration using 0.22µm size filter. Then (2 ml) of the medium was transferred into sterile test tubes and kept at (4◦C) until use.

3.3: Laboratory animals

Albino Swiss female mice (100) were obtained from biotechnology research center. Their age ranged between (8-12) weeks and weighting (23-27) gm. They were divided into 8 groups, each group was put in a separated plastic cage. The cages were put in a room with temperature (23-25ºC).

The animals were given water and fed with a suitable quantity of water and complete diet, which was locally made of the following materials:

Product	**Percentage%**
Crushed barley	24.50
Crushed wheat	30.00
Crushed yellow corn	22.50
Soya bean	15.20
NaCl	0.45
Calce stone	0.20
Animal protein	7.15

3.4: Preparation of tamoxifen

Tamoxifen (20 mg/tablet) was obtained from (Hexal company).One tablet of tamoxifen was dissolved in (1ml) of sterile PBS to make a stock solution, and from this solution, two concentrations of (0.01mg/ml) and (0.02 mg/ml) which equivalent to (20 mg/mouse) and (40 mg/mouse) were prepared to be used in mouse studies. While in human blood culture studies, one tablet of tamoxifen was dissolved in (1ml) of DMSO to make a stock solution. Different concentrations (0.5, 1, 5, 10, 50, 100, 500 and 1000 μg/ml) were prepared from this stock solution, and then sterilized by filtration and kept at (4º C) until being used.

3.5: Source of black seed oil

The oil used in this experiment was obtained from black seeds cultivated and identified in Iraq. This oil was extracted by the use of mechanical press machine at the Agriculture and Biological Research Center (Former IAEC). 110 ml of oil /kg of black seed were obtained.

3.6: Source of honey

The honey used in this experiment was obtained from Al-Yaqdhan apiary in Koffa, their honey bees were feed on the flowers of alfa-alfa.

3.7: Administration of laboratory animals

3.7.1: Tamoxifen

The animals in this experiment were treated with cumulative doses of tamoxifen in a short time. The main aim of this experiment is to evaluate the acute treatment effect of tamoxifen by applying cytogenetic analysis and examining the inhibition in mitotic activity and the induction of micronucleus and chromosomal aberrations in normal bone marrow cells. It also aims to select the most suitable duration of tamoxifen treatment with the highest side effects to be used in the next experiments.

Tamoxifen dose was chosen according to the therapeutic doses given for women breast cancer.

Three groups of mice were used for this experiment and treated as follow:

Group I: Negative control (4 mice)
Treated with (0.1 ml) PBS.

Group II: Low-dose tamoxifen treatment (12 mice)
Treated with (0.1 ml) of tamoxifen (0.4 mg/kg)

Group III: High-dose tamoxifen treatment (12 mice)
Treated with (0.1 ml) of tamoxifen (0.8 mg/kg)

The tamoxifen was given orally for 5 successive days, and then the mice were sacrificed after one day, three and five days post treatment along with the first group of control mice. Bone marrow

samples were taken and cytogenetic analyses were carried out as described later (see 3.9.1).

3.7.2: Black-seed oil and honey

The aim of this experiment was to evaluate the cytogenetic effects of black seed oil treatment and honey treatment by applying cytogenetic analysis (MI, CA, and MN) in normal bone marrow cells of mice.

Three groups of mice were used for this experiment and treated as follows:

Group I: negative control (4 mice)

Treated with (0.2 ml) PBS.

Group II: black- seed treatment (12 mice)

Treated with (0.2ml) of black seed oil /day.

Group III: Honey treatment (12 mice)

Treated with (0.2 ml) of honey / day.

The doses were given orally for 5 successive days, and then the mice were sacrificed after one day, three and five days post treatment along with the first group of control mice. Bone marrow samples were taken and cytogenetic analyses were carried out as described later (see 3.9.1).

3.8: The Interaction studies

3.8.1: The interaction between tamoxifen and black-seed oil

3.8.1.1: Pre-drug treatment with black seed oil

This group of mice was divided into three subgroups, as follow:

Group I: negative control (4 mice)

Treated with (0.2 ml) PBS.

Group II: positive control (4 mice)

Treated with (0.1 ml) of tamoxifen (0.8 mg/kg) for five successive days.

Group III: Pre-treatment group (4mice)

Treated with (0.2 ml) of black seed oil for five successive days, then treated with (0.1ml) of tamoxifen (0.8 mg/kg) for five successive days.

The mice in these groups were sacrificed, and bone marrow samples were taken for cytogenetic analysis (MI, CA, and MN).

3.8.1.2: Post-drug treatment with black seed oil

This group of mice was divided into three subgroups, as follows:

Group I: negative control (4 mice)

Treated with (0.2 ml) PBS.

Group II: positive control (4 mice)

Treated with (0.1 ml) of tamoxifen (0.8 mg/kg) for five successive days.

Group III: Post-treatment group (4mice)

Treated with (0.1ml) of tamoxifen (0.8 mg/kg) for five successive days then treated with (0.2 ml) of black-seed oil for five successive days.

These groups of mice were sacrificed, and bone marrow samples were taken for cytogenetic analysis (MI, CA, and MN).

3.8.2: The interaction between tamoxifen and honey

3.8.2.1: Pre-drug treatment with honey

This group of mice was divided into three subgroups, as follows:

Group I: negative control (4 mice)

Treated with (0.2 ml) PBS.

Group II: positive control (4 mice)

Treated with (0.1 ml) of tamoxifen (0.8 mg/kg) for five successive days.

Group III: Pre-treatment group (4mice)

Treated with (0.2 ml) of honey for five successive days ,then treated with (0.1ml) of tamoxifen (0.8 mg/kg) for five successive days .

3.8.2.2: Post-drug treatment with honey

This group of mice was divided into three subgroups, as follows:

Group I: negative control (4 mice)

Treated with (0.2 ml) PBS.

Group II: positive control (4 mice)

Treated with (0.1 ml) of tamoxifen (0.8 mg/kg) for five successive days.

Group III: Post-treatment group (4mice)

Treated with (0.1 ml) of tamoxifen (0.8 mg/kg) for five successive days, then treated with (0.2ml) of honey for five successive days. The mice of these groups were sacrificed, and bone marrow samples were taken for cytogenetic analysis (MI, CA, and MN).

3.9: Cytogenetic experiments

3.9.1: Chromosome preparation from somatic cells of the mouse bone marrow

The experiment was done according to (Allen *et al.*, 1977) as follows:

1- The animals was injected with (0.25 ml) of colchicin with concentration of (1mg/ml) intraperitoneally (I.P.) 2 hours before sacrificing the animals.

2- The animals were sacrificed by cervical dislocation.

3- The animal was fixed on her ventral side on the anatomy plate and the abdominal side of the animal and its thigh region was swabbed with 70 % ethanol.

4- The femur bone was taken and cleaned from the other tissues and muscles, then gapped from the middle with a forceps in a vertical position over the edge of a test tube by a sterile syringe , (5 ml) of PBS was injected so as to wash and drop the bone marrow in the test tube.

5- The test tubes were taken and put in the centrifuge at speed of 2000 rpm for (10 min.).

6-The supernatant was removed and (5ml) of potassium chloride (KCL) was added as a hypotonic solution at (0.075 M), then the tubes were put in water bath at (37º C) with shaking from time to time.

7- The tubes were centrifuged at 2000 rpm for (10 min).

8-The supernatant was removed and the fixative solution was added (as drops) on the inside wall of the test tube with continuous shaking, the volume was fixed to (5 ml) and the contents were shaken well.

9- The tubes were kept at (4 º C) for (30 min) to fix the cells.

10- The tubes were centrifuged at 2000 rpm for (10 min).

The process was repeated for 3 times and the cells were suspended in 2 ml of the fixative solution.

11- By a Pasteur pipette, few drops from the tube were dropped vertically on the chilled slide from a height of 3 feet at a rate of (4-5)

drops to give the chance for the chromosomes to spread well. Later, the slides were kept to dry.

12- The slides were stained with Giemsa stain and left for (15 min) and then washed with D.W.

13-Two slides for each animal were prepared for cytogenetic assays.

3.9.2: Micronucleus test in mouse bone marrow cells

The experiment was done according to (Schmid, 1979) as follow: -

1-The femur bone cleaned from tissues and muscles, then gapped from the middle with a forceps in a vertical position over the edge of a test tube by a sterile syringe, (1 ml) of human serum (heat inactivated) was injected so as to wash and drop the bone marrow in the test tube.

2-The test tubes were centrifuged at speed of 1000 rpm (5 min).

3-The 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 hours).

4-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.

5-Two slides for each animal were prepared for micronucleus test.

3.9.3: Cytogenetic analysis of human blood lymphocytes

(Shubber, 1987)

1-Human blood was collected in a heparin coated syringe.

2- Peripheral blood (0.25 ml) was added into test tube containing (2 ml) of culture medium (RPMI-1640).

3- PHA (0.25 ml) was added. The components were mixed very well and transferred to (37º C) incubator.

4-After (24) hours of incubation ,different concentrations of tamoxifen (0.5, 1, 5, 10, 50, 100, 500 and 1000 µg/ml)were added to each test tube (0.1 ml) .Also (0.1 ml) of PBS was added and this considered to be a negative control.

5- Test tubes were put back in the incubator at (37ºC), and shake gently each (24 hours) one try at least. The incubation period was completed to (72 hours).

6-Colchicine (0.1 mg/ml) was added to each tube 1/2 hours before harvesting the cells, and gently shaked each (10 min.).

7-The test tubes were centrifuged at speed of 2000 rpm for (10 min.).

8-The supernatant was removed and (5ml) of potassium chloride (KCL)(0.075 M) was added as a hypotonic solution, then the test tubes were left for (30 min.) in the water bath at (37ºC) and the tubes were shake from time to time.

9-The tubes were centrifuged at 2000 rpm for (10 min.).

10-The supernatant was removed and the fixative solution was added as drops on the inside wall of the test tube with the continuous shaking, and then, the volume was fixed to (5 ml), and the contents were shake well.

11-The tubes were kept at (4ºC) for (30 min.) to fix the cells.

12-The tubes were transferred into the centrifuge at 2000 rpm for(10 min.).The process was repeated for 3 times and then, the cells were suspended in (2 ml) of the fixative solution.

13- By a Pasteur pipette, few drops from the tube were dropped vertically on the chilled grease-free slide from a height of 3 feet at a rate (4-5) drops to give the chance for the chromosomes to spread well. Later, the slides were kept to dry at room temperature.

14- The slides were stained with Giemsa stain and left for (15 min.), then washed with D.W.

15- Two slides for each concentration were prepared for cytogenetic assays.

Other slides for each concentration were stained with Hoechst stain (33258) for the analysis of cell cycle progression and sister chromatid exchange.

3.9.4: Hoechst (33258) staining

(Ian-Freshney, 2000).

1- The slides were immersed in a coplin jar Hoechst 33258 at a concentration of (20 μg / ml) for (10 min.).

2- The slides were transferred to a slide rack, and drop (500 μl) of 2x SSC.

3- The slides were covered with a 22 – mm x 50 – mm cover slips, and the edges were sealed with a temporary seal, such as cow gum, to prevent evaporation.

4- The covered slides in the slide rack (cover slip facing downwards) were placed on a short-wave UV box. Maintain a distance of approximately (4 cm) between the slides and the UV source. The longer the pale chromatid will become, expose the slides for about (24 – 60 min.).

5- The cover slips were removed from the slides, and the slides were washed three times in ultra purified water (UPW), 5 min. per wash. The slide holder was covered with aluminum foil.

6- The slides were air dried in the dark.

7- The slides were stained in a coplin jar containing 3.5 % Giemsa solution in PBS buffer (PH 6.8) for (3-5 min.).

8- The slides were carefully rinsed in tap water, and drained using a paper tissue.

9- The slides were air dried on the bench for (1 hour), and dipped into xylene, 4 drops of DPX mountant were dropped onto the slide and a 22- mm x 50 –mm cover slip was lowered, expressing any air bubbles with tissue.

10- The slides were air dried in a fume hood overnight.

3.9.5: Micronucleus test in human blood lymphocytes culture

(Cited by Al-Adami, 2000)

1-Human blood was collected in a heparin coated syringe.

2- Peripheral blood (0.25 ml) was added into test tube containing (2 ml) of culture medium (RPMI-1640).

3- PHA (0.25 ml) was added. The components were mixed very well and transferred to (37º C) incubator.

4-After (24) hours of incubation, (0.1ml) of different concentrations of tamoxifen (0.5, 1, 5, 10, 50, 100, 500 and 1000 μg/ml) were added to each test tube. Also (0.1 ml) of PBS was added and this considered to be a negative control.

5- The test tubes were put back in the incubator at (37ºC), and gently shaken each (24 hours) one try at least. The incubation period was completed to (72 hours).

6-The test tubes were centrifuged at speed of 800 rpm for (5min.).

7-The supernatant was removed and (5 ml) of potassium chloride (KCL) (0.1M) was added as a hypotonic solution, then the test tubes were left for (30 min) in water bath at (37ºC) and the tubes were shake from time to time.

8-The tubes were centrifuged at 800 rpm for (5min.).

9-The supernatant was removed and the fixative solution was added as drops on the inside wall of the test tube with continuous shaking, and then, the volume was fixed to (5 ml), and the contents were shaken well.

10-The tubes were kept at (4ºc) for (30 min.) to fix the cells.

11-The tubes were transferred into the centrifuge at 800 rpm for (5 min).The process was repeated 3 times and after that, the supernatant was discarded and one drop of the pellet was smeared on a clean slide and left to dry at room temperature.

12- The slides were stained with Giemsa stain and left for (15 min), then washed with D.W.

13- Two slides for each concentration were prepared for micronucleus assay.

3.10: Cytogenetic analysis test

3.10.1: Mitotic index (MI) assay

The slides were examined under high dry power (40 x) of light microscope, and (1000) of divided and non divided cells were counted and the percentage rate was calculated for only the divided ones according to the following equation:-

$$\mathbf{MI} = \frac{\textbf{no. of the divided cells}}{\textbf{total number of the cells (1000)}} \times 100$$

3.10.2: Blastogenic index (BI) assay

The slides were examined under high dry power (40%) of light microscope, and (1000) cells were counted to calculate the percentage rate of the blast cells according to the following equation: -

$$BI = \frac{\text{No. of the blast cells}}{\text{Total no. of the cells (1000)}} \times 100$$

3.10.3: Chromosomal aberration (CA) assay

. The prepared slides were examined under the oil immersion lens for 100 divided cells per each animal or blood lymphocyte culture, and the cells should be at the metaphase stage of the mitotic division where the chromosomal aberrations were clear and the percentage of these aberrations could be estimated.

3.10.4: Replicative index (RI) assay

The replicative index (RI) was determined by counting the number of cells at the first, second and the third metaphase in (100) cells at metaphase, the RI was calculated according to the following equation: -

$$RI = \frac{(1xM1\%) + (2xM2\%) + (3xM3\%)}{100}$$

3.10.5: Sister chromatid exchange (SCE) Assay

Sister chromatid exchanges were counted in 25 well spread second metaphases, which contained 20 pairs of chromosomes at least.

3.10.6: Micronucleus test (MN)

The number of MN in (2000) cells of polychromatic Erythrocytes (PCE) was scored under the oil immersion lens, and the percentage of MN was calculated.

3.11: Determination of the protective value of black- seed oil and honey

The protective value of black- seed oil and honey was calculated according to the following equation:-

$$\text{Protective value} = \frac{A-C}{A-B} \times 100$$

A= (+) ve control (treatment with tamoxifen only).

B= (-) ve control (treated with PBS only).

C= interaction group (treated with tamoxifen and black- seed oil, or with tamoxifen and honey).

(Rawat *et al*., 1997)

3.12: Statistical analyses

A one-way analysis of variance was performed to test whether group variance was significant or not, the comparison between groups were used analyses of variance test (ANOVA) (Al-Mohammed *et al*., 1986).

Chapter four

Results & Discussion

Results and discussion

4.1: Cytogenetic effects of tamoxifen

4.1.1: Cytogenetic effects of tamoxifen on mouse bone marrow cells

4.1.1.1: Tamoxifen effect on mitotic index (MI)

Under normal experimental conditions, albino mice had a mitotic index of (5.94%) in their bone marrow cells (table 4.1, 4.2). This was considered as a negative control.

Table (4.1) shows that low-dose of TAM caused a significant reduction ($p<0.05$) in MI (4.51%, 4.19% and 2.86%) after one day, three and five days of treatment, respectively. Table (4.2) shows that high dose of TAM caused a significant reduction ($p<0.05$) in MI (4.18%, 3.51% and 2.6%) after one day, three and five days of treatment, respectively in comparison with negative control.

These results indicate that TAM with its low and high doses resulted in the reduction of mitotic index in mouse bone marrow cells and this reduction depends on the dose and duration of TAM treatment. This may be related to the proteins required for mitosis which were not produced at the same quantities, or the code was not reached the cell to induce it to proliferate, or the drug may cause the death of bone marrow cells (Turner *et al*., 1988), or the mitotic activity of the cell which affected with TAM could not repaired, or due to defect occurred in the mitotic spindle composition during cell division (Shiraishi, 1978).

These results are in agreement with the results of Syhood (2000), who found that low and high doses of TAM caused reduction in MI of mouse bone marrow cells.

Many researchers pointed to the accumulative effect of TAM (Rutavirust *et al*., 1995), which increases the cytotoxic effect on bone marrow cells, and this was reflected on the MI.

There are other chemotherapeutic drugs that also cause mitotic index inhibition, like cyclophosphamide (CP) (Al-Fayadh, 2000 and Hasan, 2002), methotrexate (MTX) (Al-Amiry, 1999 and Al-Kadumi, 1999) and mitomycin-c (MMC) (Littlefield *et al*., 1980).

Table (4.1): Cytogenetic effects of low dose tamoxifen on mouse bone marrow cells *in vivo*

Treatment Period	Mitotic Index % μ±SE	Micro-nucleous % μ±SE	Chromosomal Aberration % μ ±SE							
			Chromatid Break	Chromatid Gap	Deletion	Dicentric	Ring	Chromosome Break	Chromosome Gap	Total CA
0 Day (Negative Control)	5.940 ± 0.134 A	2.253 ± 0.114 A	0.196 ± 0.012 A	0.451 ± 0.015 A	0.427 ± 0.011 A	0.265 ± 0.015 A	0.230 ± 0.003 A	0.027 ± 0.003 A	0.132 ± 0.003 A	1.734 ± 0.04 A
1 Day	4.51 ±0.063 B	3.00 ± 0.046 B	0.340 ± 0.005 B	0.940 ± 0.005 B	0.500 ± 0.06 AB	0.735 ± 0.06 B	0.430 ± 0.03 B	0.260 ± 0.015 B	0.745 ± 0.003 B	3.95 ± 0.09 B
3 Days	4.19 ± 0.22 B	4.69 ± 0.034 C	0.415 ± 0.008 C	0.835 ± 0.02 B	0.630 ± 0.001 BC	0.800 ±0.03 B	0.440 ± 0.01 B	0.310 ± 0.005 B	0.935 ± 0.003 C	4.415 ±0.08 C
5 Days	2.86 ±0.005 C	7.06 ±0.45 D	0.490 ±0.025 D	1.280 ±0.09 C	0.75 ± 0.035 C	1.09 ±0.05 B	0.600 ±0.005 C	0.275 ± 0.035 B	0.780 ± 0.02 D	5.035 ± 0.28 D

Table (4.2): Cytogenetic effects of high dose tamoxifen on mouse bone marrow cells *in vivo*

Treatment Period	Mitotic Index % μ±SE	Micro-nucleous % μ±SE	Chromosomal Aberration % μ ±SE							
			Chromatid Break	Chromatid Gap	Deletion	Dicentric	Ring	Chromosome Break	Chromosome Gap	Total
0 Day (Negative Control)	5.940 ± 0.134 A	2.253 ± 0.114 A	0.196 ± 0.004 A	0.451 ± 0.011 A	0.427 ± 0.014 A	0.265 ± 0.014 A	0.23 ± 0.011 A	0.027 ± 0.004 A	0.132 ± 0.054 A	1.734 ± 0.044 A
1 Day	4.18 ± 0.06 B	3.590 ±0.300 B	0.645 ± 0.002 B	1.395 ±0.002 B	0.925 ± 0.02 B	0.315 ± 0.002 B	0.68 ±0.017 B	0.315 ± 0.002 B	0.93 ± 0.011 B	5.565 ±0.05 B
3 Days	3.51 ±0.265 C	6.805 ± 0.051 C	0.99 ± 0.006 C	1.975 ± 0.002 C	1.325 ± 0.002 C	0.865 ± 0.002 C	0.73 ±0.055 B	0.365 ±0.002 C	1.235 ± 0.03 C	7.485 ± 0.06 C
5 Days	2.60 ±0.083 D	9.660 ±0.023 D	1.52 ±0.023 D	2.38 ± 0.011 D	1.545 ±0.031 D	1.25 ± 0.005 D	0.915 ± 0.02 C	0.385 ± 0.008 D	1.255 ± 0.02 C	9.250 ±0.005 D

4.1.1.2: Tamoxifen effect on chromosomal aberrations (CAs)

The spontaneous frequency of chromosomal aberrations in mouse bone marrow cells was (1.73%) which represented as negative control (table 4.1, 4.2).

The results of this experiment indicated that low dose of TAM can increase CAs frequencies (Chromatid break, chromatid gap, deletion, dicentric chromosome, ring chromosome, chromosome break and chromosome gap)(figure 4.1) which reached (3.95%, 4.41% and 5.03%) after one day, three and five days of TAM treatment, respectively. These results were significantly different ($p<0.05$) from the negative control and from each others.

High dose of TAM (table 4.2) also caused remarkable increase in CAs which reached (5.56%, 7.48% and 9.25%) After one day, three and five days of treatment, respectively. Those differences were significant ($p<0.05$) in comparison with negative control and with each others.

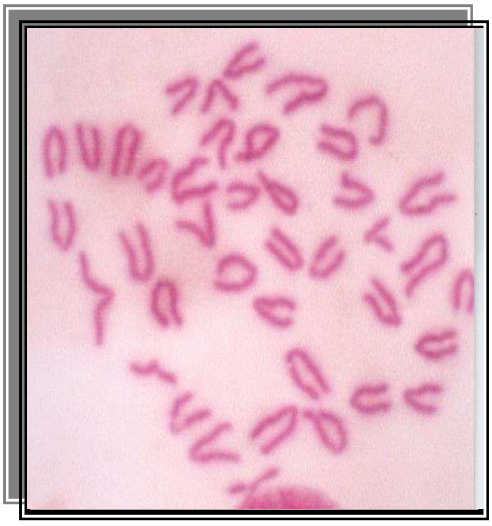
In this experiment the highest effect of TAM was noticed after treatment with the high dose for five days, this indicated that the effect of TAM was depending on the dose and treatment period.This may suggest that high doses of TAM may act on the repair systems inside the cells more than the low doses, as a result the cells were lost the ability to repair the damage of DNA molecules (Uzeily *et al*., 1993).

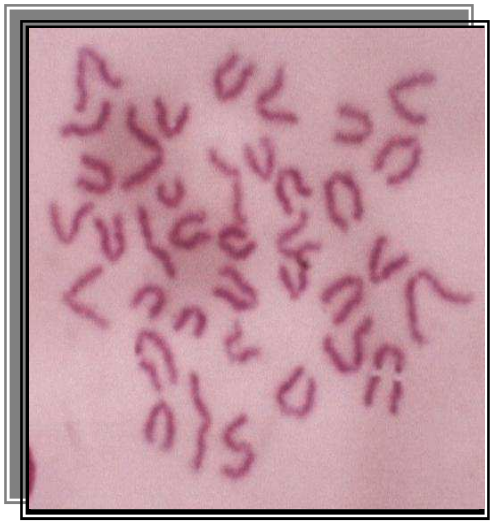
Tamoxifen or its metabolites may act as cytotoxic agents on bone marrow cells either by direct effect, or interferes and damages the chromosomal proteins (Weiss and Gurpid, 1988).

It was pointed that the percentage of chromatid breaks was higher than the percentage of chromosome breaks after treatment with TAM; this may be related to the differences in the repair systems for each change.

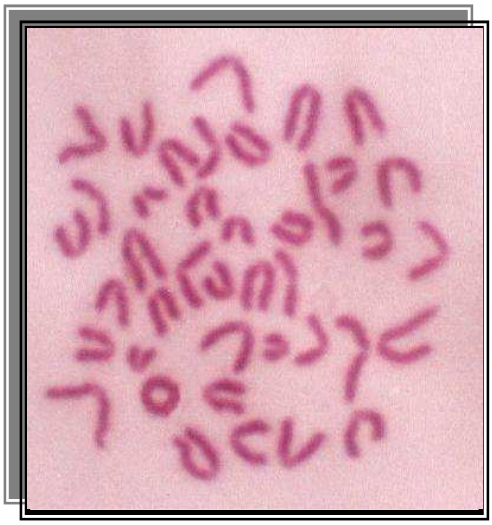
Gill (1980) indicated that the double-stranded DNA break will induce the production of ADP-ribose enzyme more than the single stranded DNA breaks. ADP-ribose will activate the repair of DNA break.

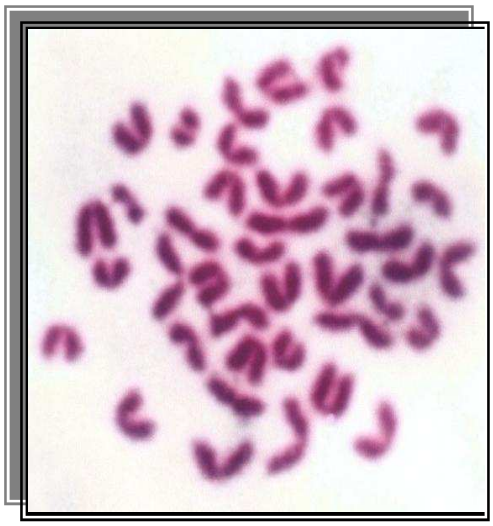
Tamoxifen has been shown to be genotoxic in several studies. It increased aneuploidy and CAs in the liver of rats (Sargent *et al*., 1996; Phillips, 2001; Hirsimake *et al*., 2002) and also induce CAs in bone marrow cells (Syhood, 2000; Hirsimake *et al*., 2002).





B **A**





C **D**

Figure(4.1):Cells in metaphase stage taken from mice treated with tamoxifen, showing: normal chromosomes(A),chromatid and chromosome gap(B),ring chromosome(C), and deletion(D) (2000 x).

4.1.1.3: Tamoxifen effect on micronucleus induction (MN)

Micronucleus frequencies of polychromatic erythrocytes from negative control mice was (2.25%) (table 4.1, figure 4.2). This percentage was increased to (3%, 4.69% and 7.06%) after one day, three and five days of low dose TAM treatment, respectively. These results are significantly different ($p<0.05$) from the negative control and from each other.

The results of high dose TAM effect on MN induction are represented in table (4.2).

MN percentage increased from (2.25%) in the negative control to (3.5%, 6.8% and 9.66%) after one day, three and five days of TAM treatment, respectively. These results are significantly different ($p<0.05$) from the negative control and from each others.

The previous results indicated that TAM can increase the MN percentage in mouse bone marrow cells, and this increase was depending on the dose and the duration of treatment.

Hirsimake (2002) mentioned that TAM induced the micronuclei in bone marrow cells.

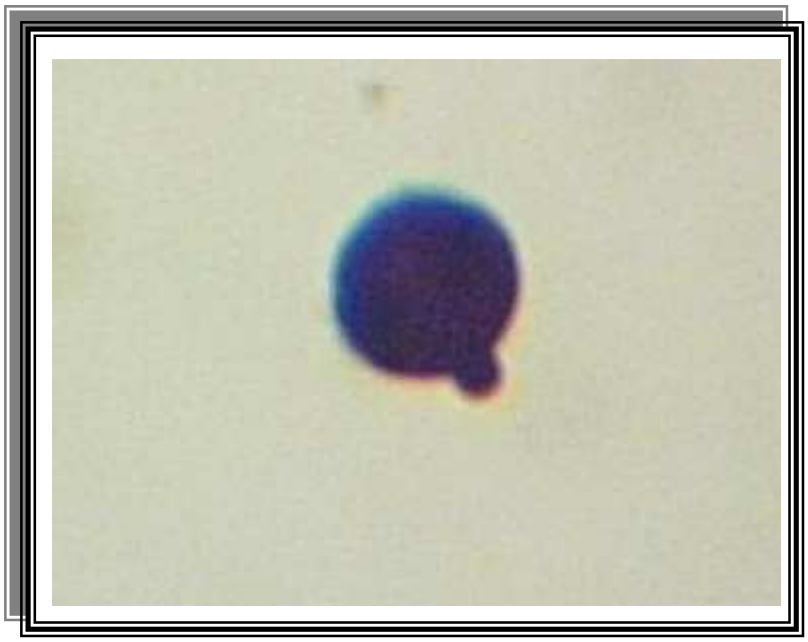


Figure (4.2):Micronucleated bone marrow cell from mouse treated with tamoxifen.(1000 x)

This experiment also found a positive high correlation between MN induction and CAs in TAM treated mice.

The correlation coefficient was (0.836) in low dose TAM treated mice, while in high dose TAM treated mice the correlation coefficient was (0.937). Table (4.3) represented the correlation coefficient between MN and each type of CAs in mouse bone marrow cells.

Table (4- 3): Correlation coefficient between micronuclei and types of chromosomal aberration in mouse bone marrow cells

*** low correlation **intermediate correlation ***high correlation ****very high correlation**

Micronucleus induction	Chromosomal Aberrations							
	Chromatid Break	Chromatid Gap	Deletion	Dicentric	Ring	Chromosome Break	Chromosome Gap	Total CA
TAM (Low dose)	0.925 ****	0.864 ***	0.991 ****	0.899 ***	0.909 ****	0.642 **	0.621 **	0.836 ***
TAM (High dose)	0.984 ****	0.943 ****	0.954 ****	0.992 ****	0.868 ***	0.785 ***	0.831 ***	0.937 ****
Black seed oil	0.605 **	0.976 ****	0.791 ***	0.745 ***	0.869 ***	0.403 **	0.386 *	0.827 ***
Honey	0.452 **	0.537 **	0.852 ***	0.983 ****	0.989 ****	0.990 ****	0.994 ****	0.980 ****

4.1.2: Cytogenetic effects of tamoxifen on human blood lymphocytes

The mitotic index of human blood cells from healthy individuals was (5.39%), as shown in table (4.4).

A gradual inhibition in MI was noticed after using different concentrations of TAM in human blood culture, this inhibition was dose dependent. The MI when using the concentration of (0.05µg/ml) was not significantly different ($p<0.05$) from the negative control, while the significant reduction was started from the concentration of (0.1 µg/ml) to (100 µg/ml). This reduction in MI indicated that TAM has a cytotoxic activity on human blood lymphocytes *in vitro*.

TAM also caused an increase in blastogenic index (BI) in human blood lymphocytes *in vitro*, as shown in table (4.4). The BI increased from (45.66%) in the negative control to (63.49%) in the concentration of (100 µg/ml). This increase was not significant ($p<0.05$) at the concentrations of (0.05, 0.1, 0.5, 1.0 and 5.0 µg/ml) while the significant increase was clear at the concentrations of (10, 50 and 100 µg/ml) when compared with negative control.

Other cytogenetic effects of TAM in human blood cells *in vitro* were the increase in the spontaneous frequencies of micronuclei (MN) and chromosomal aberrations (CAs), as shown in table (4.4).Those increases in MN and CAs were significant ($p<0.05$) in all concentrations of TAM as compared with negative control.

Spontaneous frequencies of chromaid break, chromatid gap, deletion and dicentric chromosomes were increased depending on the concentration of TAM in human blood culture, while ring chromosomes, chromosome break and chromosome gap were not observed in negative control culture, but these types of chromosomal aberrations were induced after the addition of TAM to the culture (figure 4.3).

Micronucleated cells were increased gradually until reached the concentrations of (10, 50 and 100 µg/ml) in which the MN percentages were not more increased, this may due to the cytotoxic effect of TAM which caused inhibition in cell division, so the micronuclei did not appear (Salamon *et al*., 1980) (figure 4.4).

The increase in the percentages of CAs and MN after the addition of TAM gave an indication that TAM had a genotoxic effects on human blood cells *in vitro*.

A very high correlation (0.952) was found between the CAs and MN induction in human blood culture (figure 4.5).

The correlation between MN induction and each type of CAs are shown in figure (4.6).

The effect of TAM on the replicative index (RI) in human blood culture was not clear, except when the concentrations of (50 and 100 µg/ml) were used, which caused a significant increase ($p<0.05$) in the RI as compared with the negative control, as shown in table (4.4).

Another effect of TAM was an increase in the spontaneous frequencies of sister chromatid exchange (SCE) (figure 4.7). It caused a significant increase ($p<0.05$) in SCE percentage in comparison with negative control, and this increase was concentration dependent, as shown in table (4.4).

Table (4.4): Cytogenetic effects of tamoxifen on human blood lymphocytes *in vitro*

Concentration of TAM (µg/ml)	Mitotic Index% µ±SE	Blast Index % µ±SE	Micronucleus% µ±SE
0 (Negative Control)	**5.39 ± 1.05** A	**45.66 ± 3.56** A	**1.26 ± 0.11** A
0.05	**4.17 ± 0.18** AB	**45.23 ± 1.11** A	**2.58 ± 0.10** B
0.1	**3.56 ± 0.13** B	**49.11 ± 0.54** AB	**3.64 ± 0.16** C
0.5	**3.29 ± 0.38** B	**49.00 ± 0.84** AB	**4.22 ± 0.15** D
1	**2.66 ± 0.30** BC	**49.70 ± 1.39** AB	**5.29 ± 0.28** E
5	**2.29 ± 0.24** C	**50.06 ± 0.50** AB	**5.49 ± 0.12** E
10	**1.68 ± 0.32** C	**54.05 ± 0.59** BC	**6.69 ± 0.15** F
50	**1.11 ± 0.08** C	**57.05 ± 1.67** CD	**6.77 ± 0.11** F
100	**0.87 ± 0.04** C	**63.49 ± 2.23** D	**6.51 ± 0.33** F

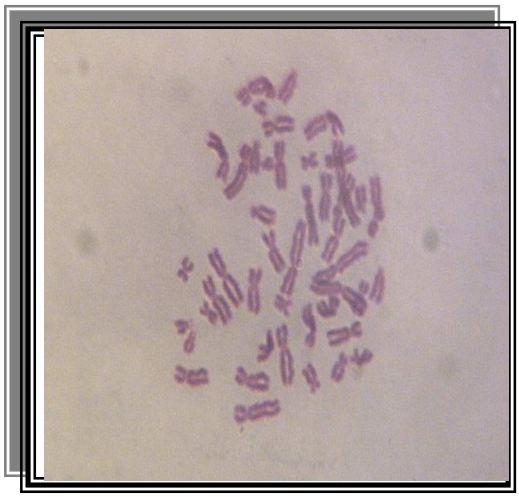
Table (4.4): (Continued)

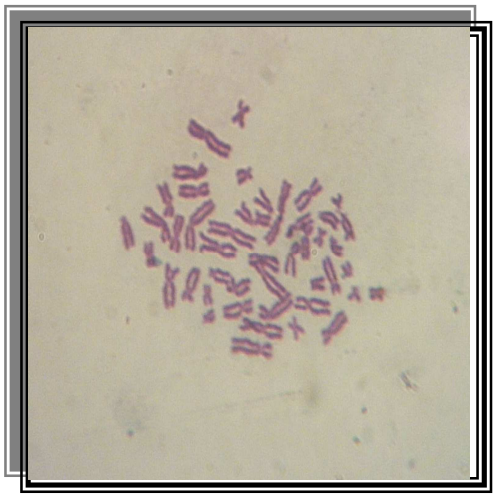
Concentration of TAM (µg/ml)	Chromosomal aberrations % µ±SE							
	Chromatid Break	Chromatid Gap	Deletion	Dicentric	Ring	Chromosome Break	Chromosome Gap	Total
0 (Negative Control)	0.19 ± 0.00 A	0.73 ± 0.01 A	0.49 ± 0.01 A	0.01 ± 0.00 A	0.00 A	0.00 A	0.00 A	1.44 ± 0.03 A
0.05	0.42 ± 0.0 B	1.49 ± 0.03 B	1.08 ± 0.01 B	0.32 ± 0.04 B	0.18 ± 0.01 B	0.10 ± 0.02 B	0.47 ± 0.02 B	3.97 ± 0.26 B
0.1	0.78 ± 0.0 C	1.61 ± 0.12 B	1.36 ± 0.14 C	0.32 ± 0.03 B	0.11 ± 0.01 B	0.11 ± 0.01 B	0.80 ± 0.03 C	5.52 ± 0.37 C
0.5	0.64 ± 0.00 D	1.95 ± 0.01 C	1.42 ± 0.11 C	0.36 ± 0.05 B	0.15 ± 0.01 B	0.15 ± 0.01 B	0.62 ± 0.08 B	5.86 ± 0.20 C
1	0.78 ± 0.01 C	1.89 ± 0.05 C	1.64 ± 0.04 D	0.56 ± 0.004 C	0.24 ± 0.01 B	0.24 ± 0.01 C	0.90 ± 0.02 CD	6.22 ± 0.06 C
5	0.97 ± 0.04 E	2.33 ± 0.10 D	1.58 ± 0.09 D	0.40 ± 0.03 B	0.25 ± 0.01 B	0.24 ± 0.01 C	1.03 ± 0.05 D	6.87 ± 0.16 D
10	1.08 ± 0.03 F	2.24 ± 0.12 D	1.65 ± 0.02 D	0.52 ± 0.01 C	0.48 ± 0.03 C	0.48 ± 0.03 D	1.21 ± 0.08 E	7.56 ± 0.16 E
50	0.89 ± 0.06 G	2.36 ± 0.07 D	1.84 ± 0.08 DE	0.73 ± 0.03 D	0.26 ± 0.02 C	0.26 ± 0.02 C	1.11 ± 0.02 D	8.61 ± 0.07 F
100	1.5 ± 0.02 H	2.93 ± 0.01 E	2.09 ± 0.06 D	0.91 ± 0.05 E	0.54 ± 0.02 D	0.454 ± 0.02 D	1.45 ± 0.09 F	9.82 ± 0.23 G

Table (4.4): (Continued)

Concentration of TAM (µg/ml)	Replicative Index % µ±SE	Cell Cycle Progression%(µ)			Sister Chromatid Exchange % µ±SE
		M1	M2	M3	
0 (Negative Control)	1.5 ± 0.17 A	41.33	33.33	25.35	3.54 ± 0.25 A
0.05	1.9 ± 0.03 AB	33.33	36.00	30.67	4.31 ± 0.22 B
0.1	2.01 ± 0.14 AB	33.00	30.00	56.00	4.41 ± 0.075 BC
0.5	2.26 ± 0.09 AB	31.22	27.39	41.39	4.96 ± 0.057 BC
1	2.14 ± 0.03 AB	29.00	28.00	43.00	5.04 + 0.045 BC
5	2.21 ± 0.05 AB	24.00	30.75	44.84	5.18 ± 0.048 BC
10	2.16 ± 0.04 AB	24.00	36.00	40.00	5.33 ± 0.021 BC
50	2.16 ± 0.01 B	32.00	20.00	48.00	5.43 ± 0.004 C
100	2.32 ± 0.00 B	22.00	24.00	45.00	5.55 ± 0.021 C

($p<0.05$)





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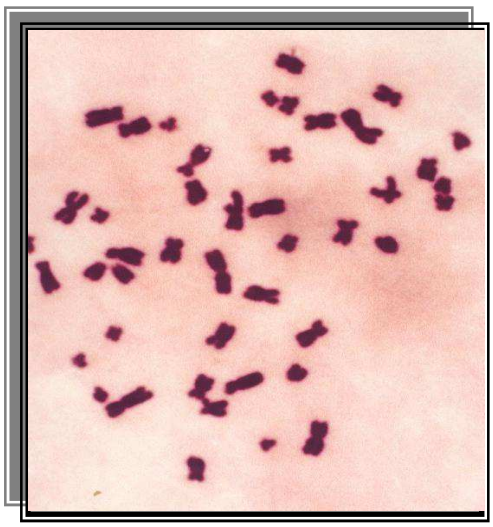
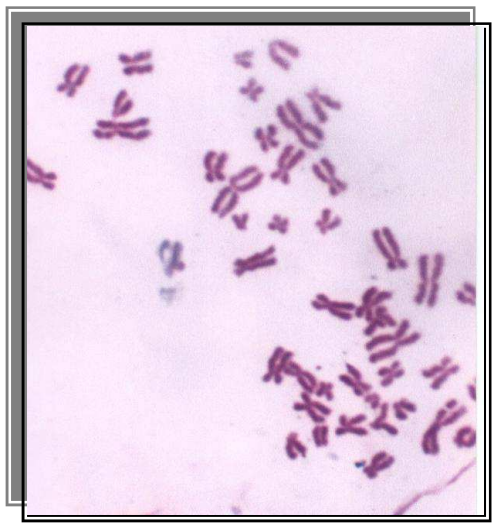




D

C

Figure(4.3):Cells at metaphase stage of human blood lymphocytes treated with tamoxifen *in vitro*, showing: normal human chromosomes(A),dicentric chromosome(B)(1000 x),deletion(C) and chromatid gap(D)(2000 x).

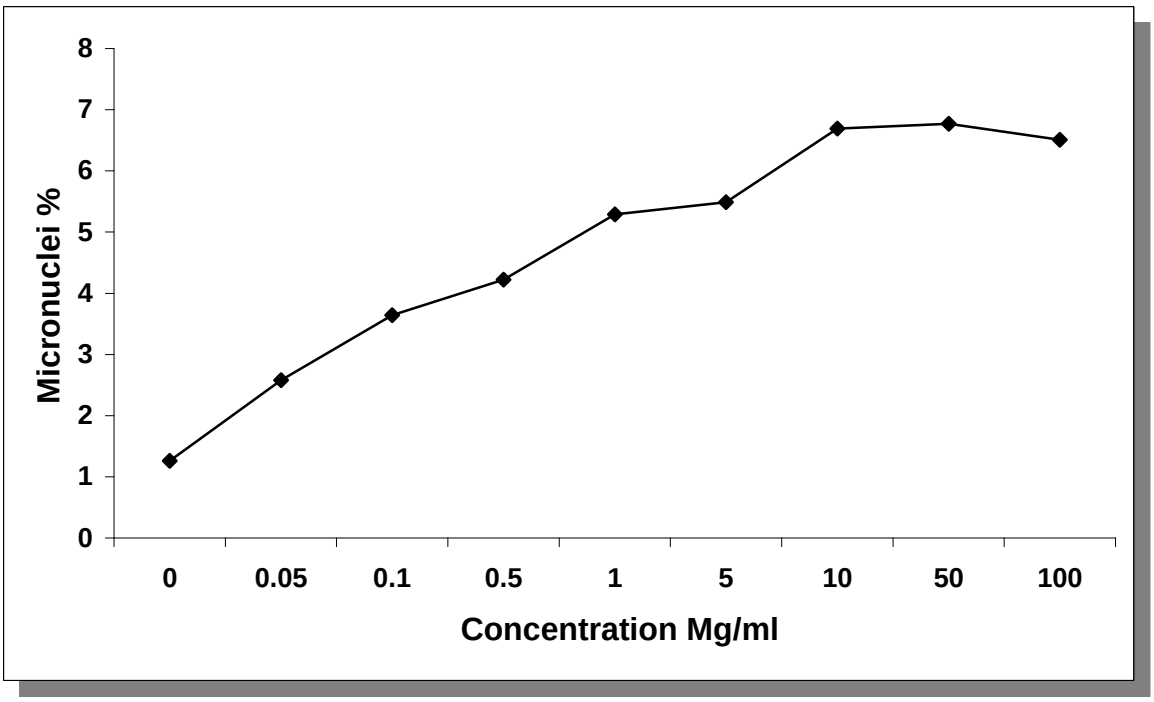


Figure (4-4): Correlation between TAM concentration & micronuclei in human blood lymphocytes = 0.57

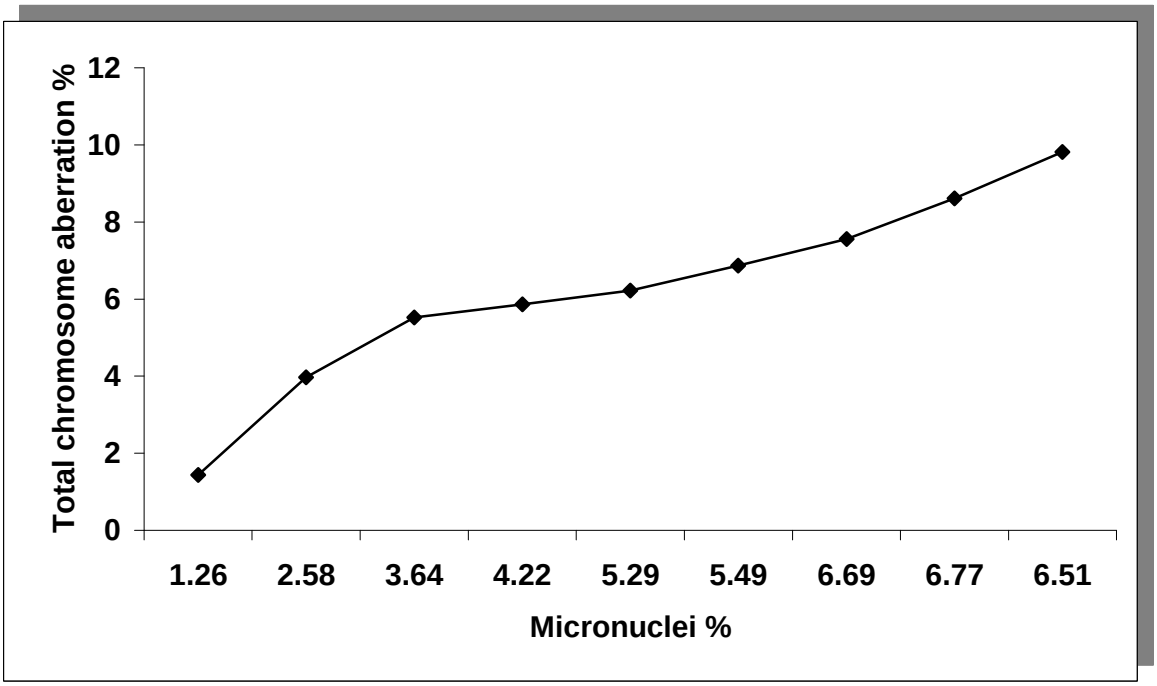
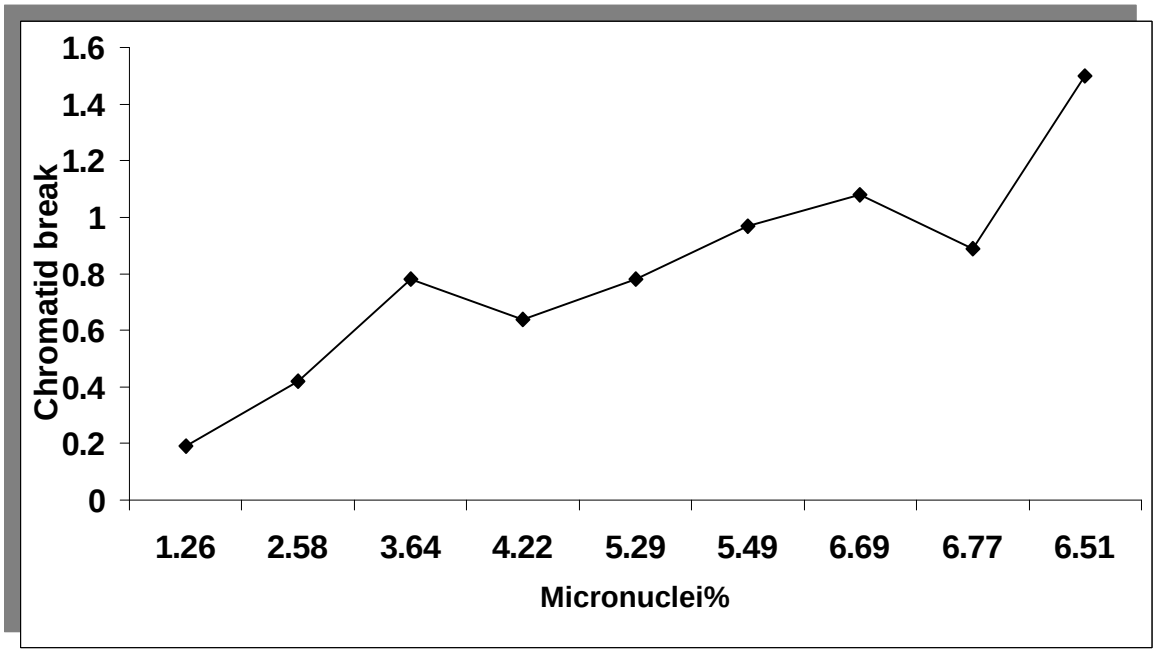
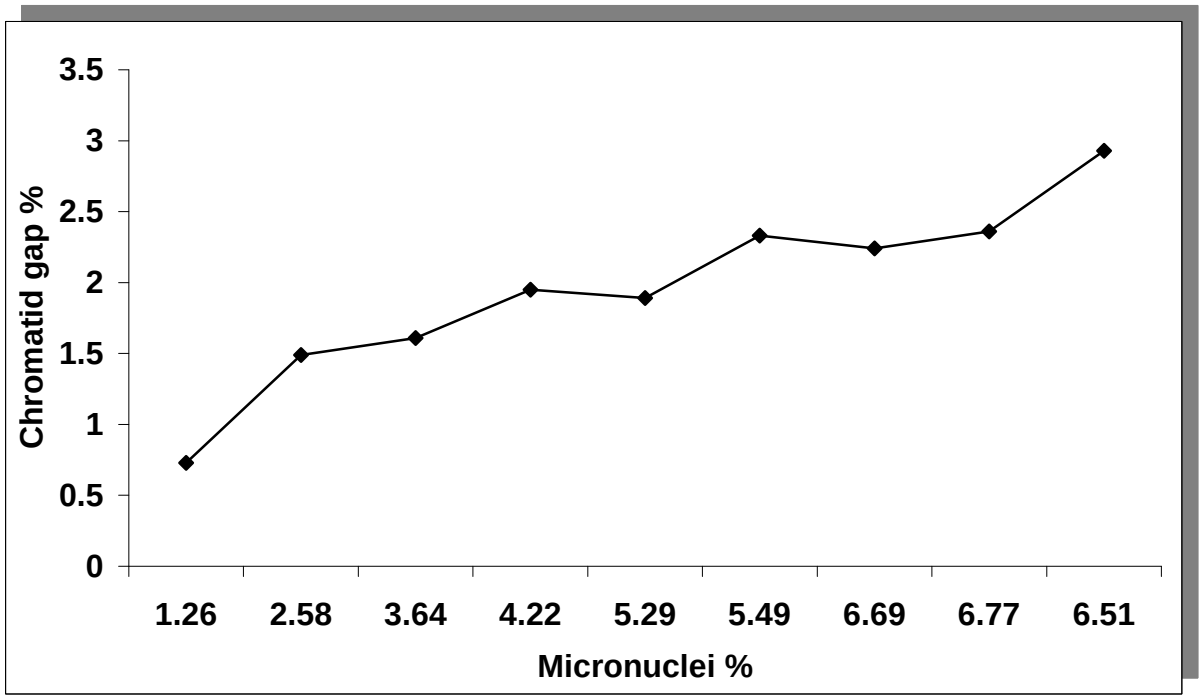


Figure (4-5): Correlation between micronuclei &total chromosome aberration in human blood lymphocytes = 0.95

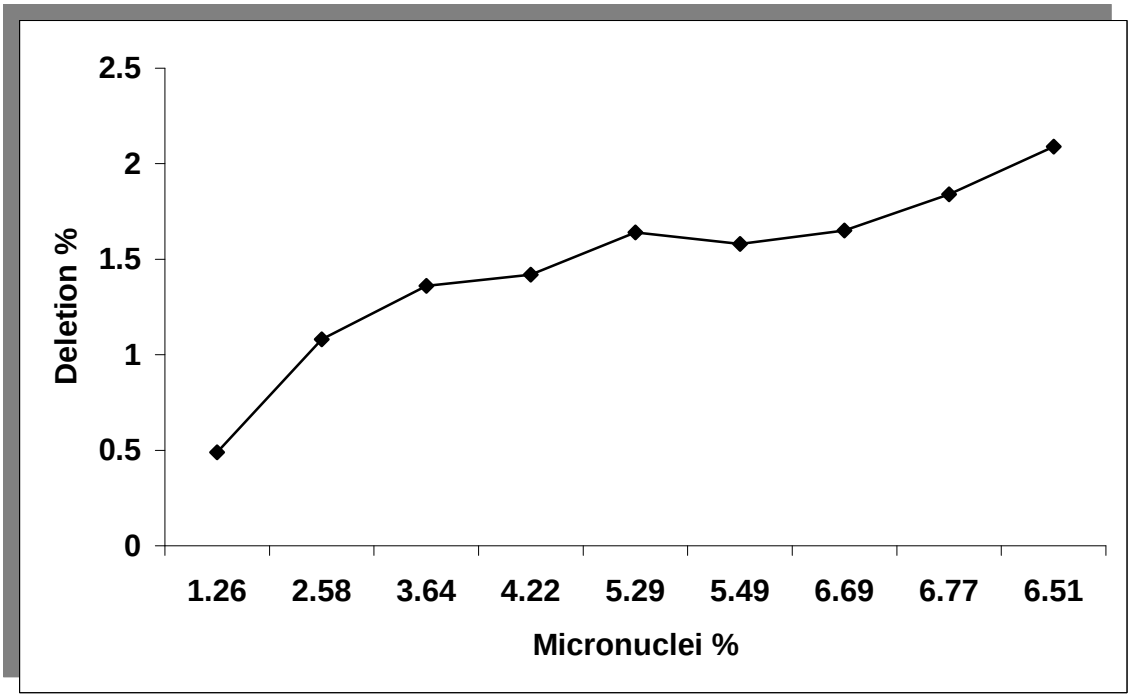


Correlation between micronuclei & chromatid break =0.872

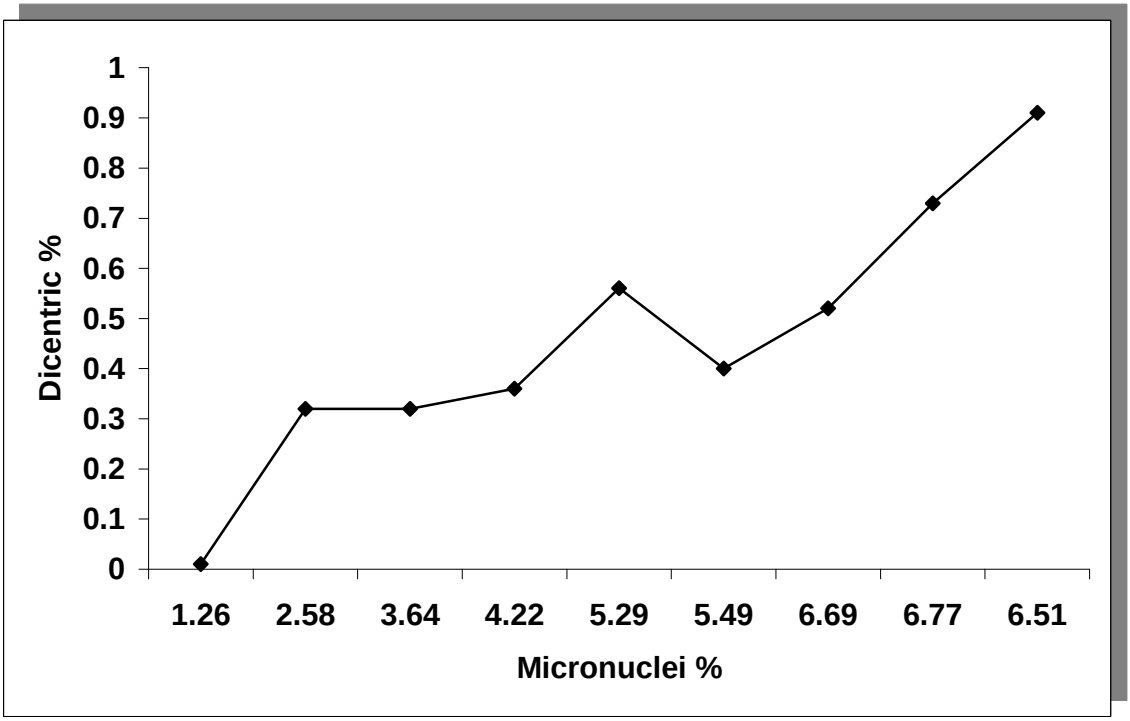


Correlation between micronuclei & chromatid gap=0.919

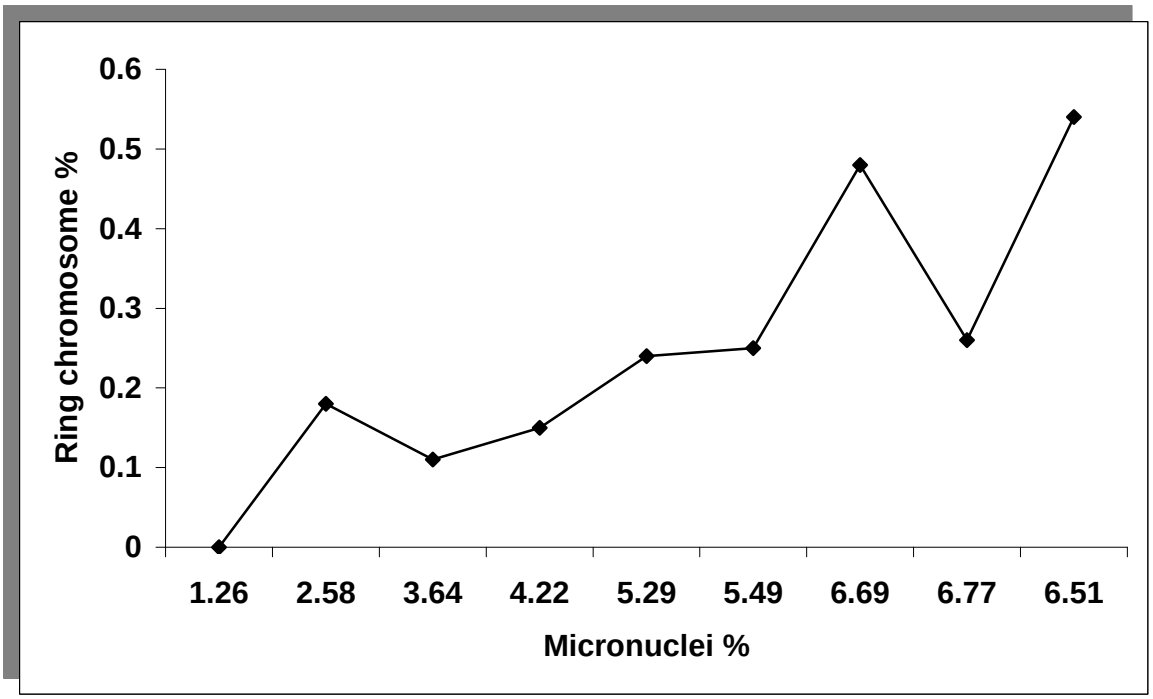
Figure(4.6):The correlation coefficients between micronucleus induction and each type of chromosomal aberrations in human blood lymphocytes



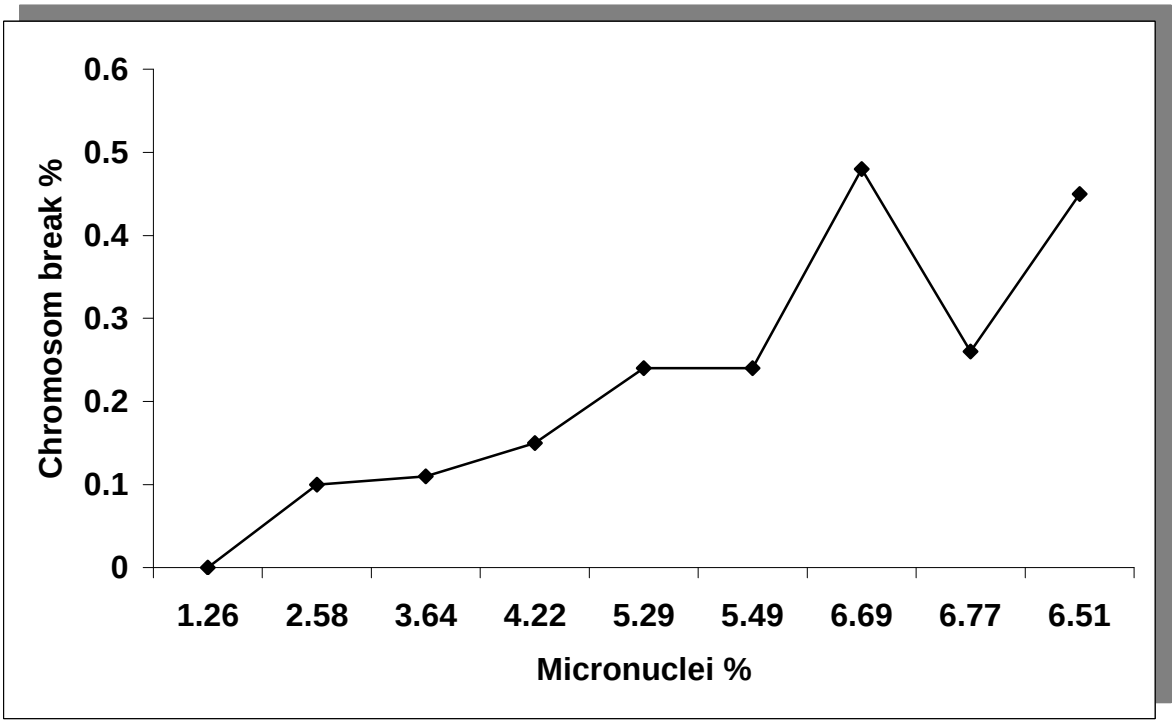
Correlation between micronuclei & deletion = 0.940



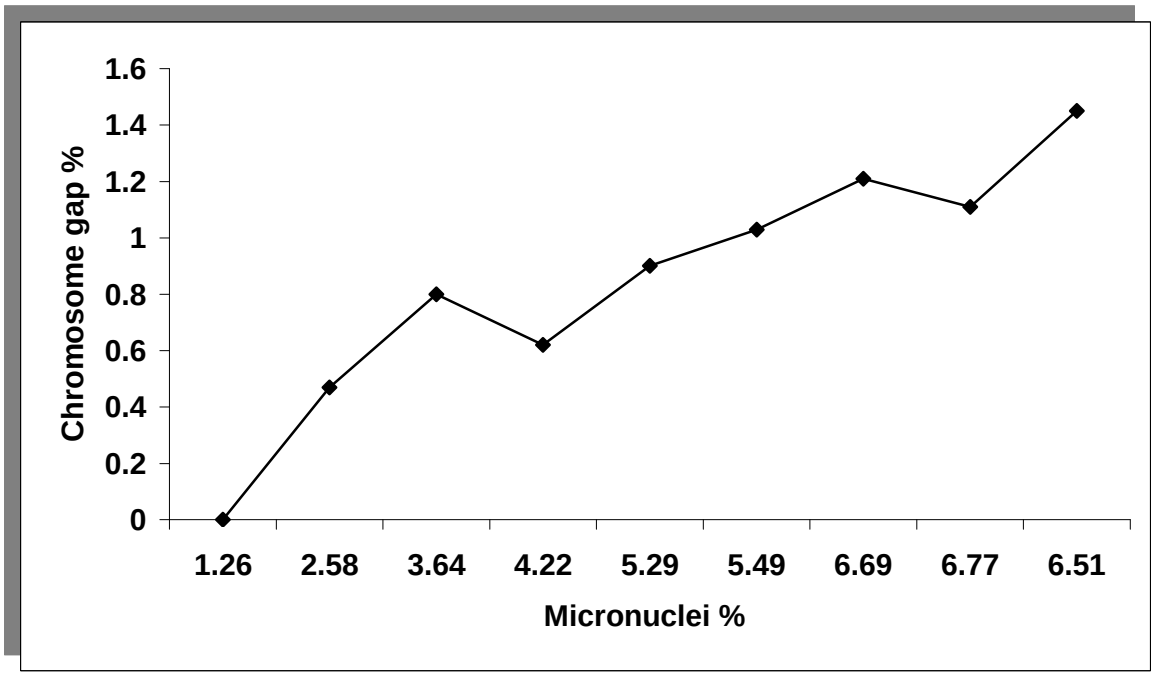
Correlation between micronuclei & dicentric= 0.875



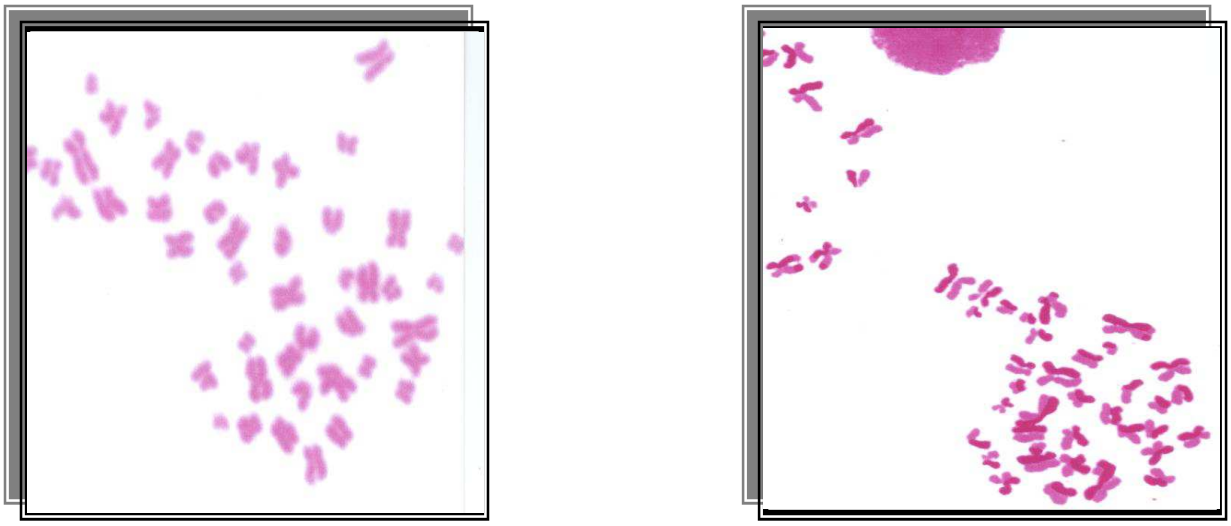
Correlation between micronuclei & ring chromosome= 0.829



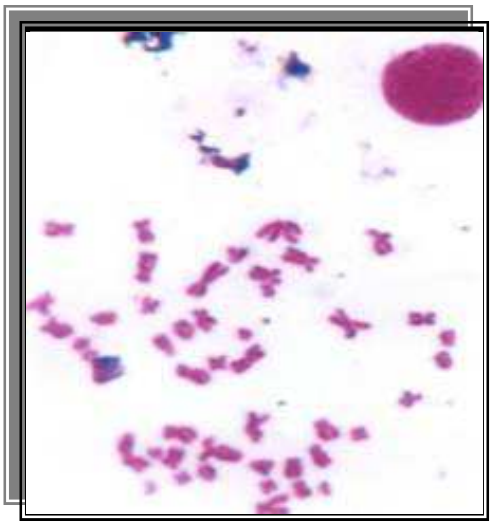
Correlation between micronuclei & chromosome break =0.892



Correlation between micronuclei & chromosome gap= 0.948



A **B**



C

Figure(4.7):Cells at metaphase stage of human blood lymphocytes stained with Hoechst stain, showing:M1 cell (A),M2 cell with sister chromatid exchanges (B) and M3 cell (2000 x).

4.2: Cytogenetic effects of black seed oil on mouse bone marrow cells

4.2.1: The effect of black seed oil on mitotic index (MI).

Treatment with black seed oil caused an increase in MI (table 4.5). The increase depended on the accumulative effect of oil. In comparison with negative control (5.94%), there was non-significant increase ($p<0.05$)|in MI after three days of treatment with oil (6.38%), only the treatment for five days was significantly different ($p<0.05$) and the MI reached (6.95%).

The black seed and its oil contained mitogenic agents in its constituents because of that, MI might increase after treatment with this oil. This result agreed with Haq and his team (1995) who indicated that black seed extract had a mitogenic activity and was able to induce cell division without any other mitogen.

These results agreed with the results of Hasan (2002) which indicated that the treatment of mice with aqueous and alcoholic black seed extracts caused an increase of MI in mouse bone marrow cells, and this increase depended on the extract dose.

Table (4.5):Cytogenetic effect of black seed oil on mouse bone marrow cells *in vivo*

Treatment Period	Mitotic Index % μ±SE	Micro-nucleous % μ±SE	Chromosomal Aberration % μ ±SE							
			Chromatid Break	Chromatid Gap	Deletion	Dicentric	Ring	Chromosome Break	Chromosome Gap	Total
0 Day (Negative Control)	5.940 ± 0.134 AB	2.253 ± 0.114 A	0.196 ± 0.004 A	0.451 ± 0.011 A	0.427 ± 0.014 A	0.265 ± 0.014 A	0.23 ± 0.011 A	0.027 ± 0.004 A	0.132 ± 0.054 A	1.734 ± 0.044 A
1 Day	5.76 ± 0.06 A	2.41 ± 0.15 A	0.14 ± 0.004 B	0.44 ± 0.002 A	0.37 ± 0.008 AB	0.22 ± 0.005 AC	0.205 ±0.002 A	0.00 B	0.02 ±0.004 B	1.405 ±0.002 B
3 Days	6.38 ± 0.21 AC	1.86 ±0.01 B	0.115 ± 0.002 B	0.36 ± 0.02 B	0.30 ± 0.02 BC	0.155 ± 0.002 C	0.130 ± 0.01 B	0.00 B	0.035 ± 0.002 B	1.10 ±0.055 C
5 Days	6.95 ± 0.04 C	1.49 ± 0.02 B	0.12 ± 0.017 B	0.30 ± 0.35 B	0.30 ± 0.25 C	0.17 ± 0.017 BC	0.130 ±0.004 B	0.00 B	0.02 ± 0.004 B	0.97 ±0.028 C

4.2.2: The effect of black seed oil on chromosomal aberrations (CAs)

Black seed oil had the ability to reduce the spontaneous CAs from (1.73%) in the negative control to (1.40%, 1.10% and 0.97%) after one day, three and five days of treatment, respectively (table 4.5). These results were significantly different ($p<0.05$) in comparison with control.

Black seed extracts and oil contain chemical compounds which may increase the activity of the detoxification enzymes, such as superoxide dismutase (SOD) and glutathione-S-transferase (GST) that scavenging free radicals from the cell, or may act to decrease the activity of arylhydrocarbon hydroxylase (AHH) enzyme which has the ability to activate mutagens inside the cells (Wang *et al*., 1989).

These results were in agreement with the results of Hasan (2002), which indicated that the aqueous and alcoholic extracts of black seed reduce the spontaneous CAs in mouse bone marrow cells.

4.2.3: The effect of black seed oil on micronucleus induction (MN)

The spontaneous frequency of MN in mouse bone marrow cells was (2.25%). Black seed oil reduced this frequency, and the reduction in MN was depending on the accumulative effect of the oil (table 4.5). The significant decrease ($p<0.05$) in MN was observed after three days (1.86%) and after five days (1.49%) when compared with the negative control.

These results agree with the results of Hasan (2002), which indicated that aqueous and alcoholic black seed extracts caused reduction in the spontaneous MN frequency in mouse bone marrow cells.

A high correlation was found between the induction of MN and CAs when the mice treated with black seed oil, and the correlation coefficient was (0.827). The correlation between MN induction and each type of CAs was shown in table (4.3).

4.3: Cytogenetic effects of honey on mouse bone marrow cells

4.3.1: The effect of honey on mitotic index (MI)

Table (4.6) displayed the effect of honey on MI in mouse bone marrow cells.

The results of this experiment showed that MI was decreased after one day of treatment with honey, but this decrease was non-significant ($p<0.05$) in comparison with control, while after three days of treatment, the MI was significantly increased ($p<0.05$) in comparison with control. The MI decreased after five days of treatment, but this decrease was also non-significant ($p<0.05$) in comparison with control.

These results indicated that honey may have a mitogenic activity due to its constituents, but this activity was not depends on the accumulative doses.

Table (4.6):Cytogenetic effects of honey on mouse bone marrow cells in vivo

Treatment Period	Mitotic Index % μ±SE	Micro-nucleous % μ±SE	Chromosomal Aberration % μ ±SE							
			Chromatid Break	Chromatid Gap	Deletion	Dicentric	Ring	Chromosome Break	Chromosome Gap	Total CA
0 Day (Negative Control)	5.940 ± 0.134 AB	2.253 ± 0.114 A	0.196 ± 0.004 A	0.451 ± 0.011 A	0.427 ± 0.014 A	0.265 ± 0.014 A	0.23 ± 0.011 A	0.027 ± 0.004 A	0.132 ± 0.054 A	1.734 ± 0.044 A
1 Day	5.65 ±0.145 A	1.71 ± 0.063 B	0.21 ± 0.005 A	0.48 ± 0.005 A	0.365 ±0.025 AB	0.02 ±0.023 B	0.02 ±0.044 B	0.00 B	0.01 ± 0.02 B	1.105 ± 0.01 B
3 Days	6.53 ± 0.02 B	1.64 ±0.06 B	0.175 ± 0.002 B	0.37 ± 0.011 B	0.28 ± 0.005 B	0.04 ± 0.014 B	0.03 ±0.006 B	0.00 B	0.01 ± 0.005 B	0.85 ± 0.06 C
5 Days	5.44 ±0.265 A	1.61 ± 0.054 B	0.16 ± 0.011 B	0.325 ±0.02 B	0.23 ± 0.028 AB	0.025 ± 0.002 B	0.015 ±0.002 B	0.00 B	0.005 ± 0.002 B	0.76 ± 0.06 C

4.3.2: The effect of honey on chromosomal aberrations (CAs)

The results of this experiment indicated that honey had the ability to reduce spontaneous frequencies of CAs in mouse bone marrow cells from (1.73%) in the negative control to (1.10%, 0.85% and 0.76%) after one day, three and five days of treatment, respectively, and this reduction was significant ($p<0.05$) in comparison with negative control (table 4.6).

The ability of honey to reduce the spontaneous CAs may be related to the detoxification enzymes in its constituents, such as SOD, or this ability may be considered as a result of reducing the AHH enzyme activity by honey. This enzyme (AHH) activates the mutagens inside the cell (Wang *et al*., 1989). Others considered that honey contains antioxidant agents which act as scavengers for the free radicals in the cell (Orsolic, 2004).

These results agreed with the results of Al-Bedairi (2002) which indicated that honey has no genotoxic properties on mouse bone marrow cells, but it could reduce the spontaneous CAs after treatment for three and seven days.

4.3.3: The effect of honey on micronucleus induction (MN)

As a result of honey treatment, the spontaneous frequency of MN decreased from (2.25%) in the negative control to (1.17%, 1.64% and 1.16%) after one day, three and five days of treatment, respectively.

These results were significantly different ($p<0.05$) from the negative control (table 4.6).

This experiment indicated that honey had the ability to reduce the spontaneous frequency of MN, and this reduction depends on the period of honey treatment, in which the highest reduction was seen after five days of treatment, but the differences between MN frequencies after one day, three and five days was not significant ($p<0.05$) when compared with each others.

The ability of honey to reduce MN frequency might due to its antioxidant and free-radical scavenging activities, and also due to its antimutagenic and detoxification activities (Wang *et al*., 1989).

There was very high correlation (0.980) between the reduction in MN frequency and CAs in mouse bone marrow after treatment with honey (table 4.3).

Table (4.3) also displayed the correlation coefficients between MN reduction and each type of CAs in mouse bone marrow cells.

4.4: Interaction between tamoxifen and black seed oil or honey

Two experiments were designed for this purpose, one of them between TAM and black seed oil, while the other between TAM and honey. These experiments were carried out to understand the

mechanism of action of the black seed oil and honey against the mutagenic effects of TAM (whether they have a prophylactic or treatment activity) by applying them before and after TAM treatment.

4.4.1: Interaction between tamoxifen and black seed oil

4.4.1.1: Pre-drug treatment with black seed oil

The results of this experiment are displayed in table (4.7).The MI of mice treated with black seed oil before the drug was increased to (4.32%), this was significantly different ($p<0.05$) from the positive control (2.60%) that treated with the drug only. So, the black seed oil provided (51.5%) protection against the inhibitory effect of TAM on MI (figure 4.8)

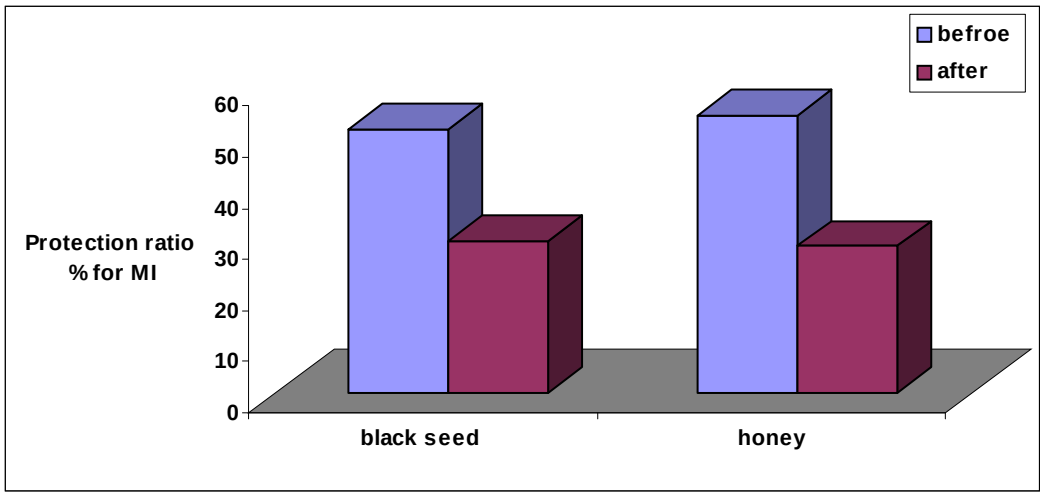
The percentage of CAs reduced significantly ($p<0.05$) when black seed oil used before TAM treatment, which reached (2.62%) in comparison with the positive control (9.25%). All types of CAs decreased significantly ($p<0.05$) when compared with the positive control. Pretreatment with black seed oil provided (88.03%) protection against the genotoxic effect of TAM (figure 4.8)

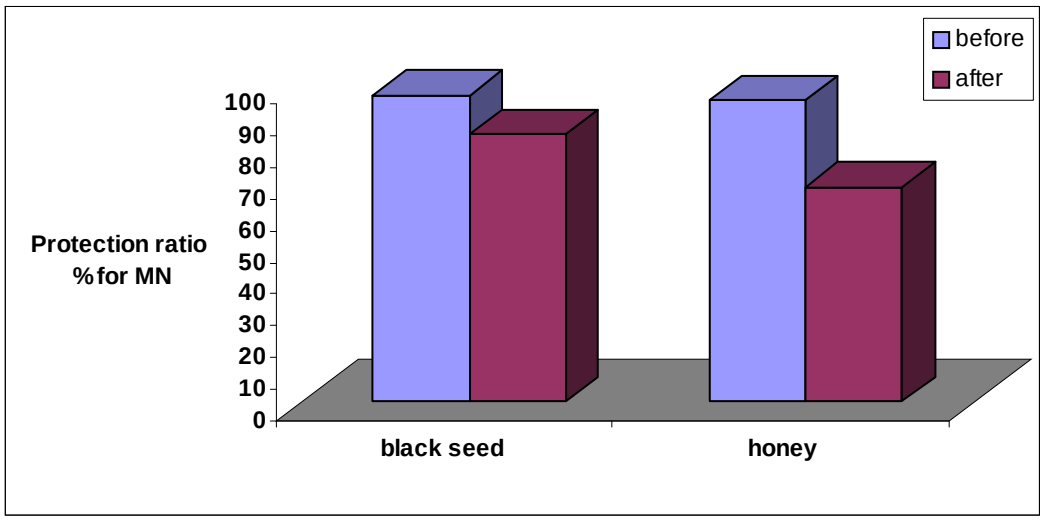
The percentage of micronucleated cells reduced to (2.46%) when black seed oil given to the mice before the drug. This result was significantly different ($p<0.05$) from the positive control (9.66%), and the protection ratio of the oil against the effect of TAM was (97.03%) (Figure 4.8)

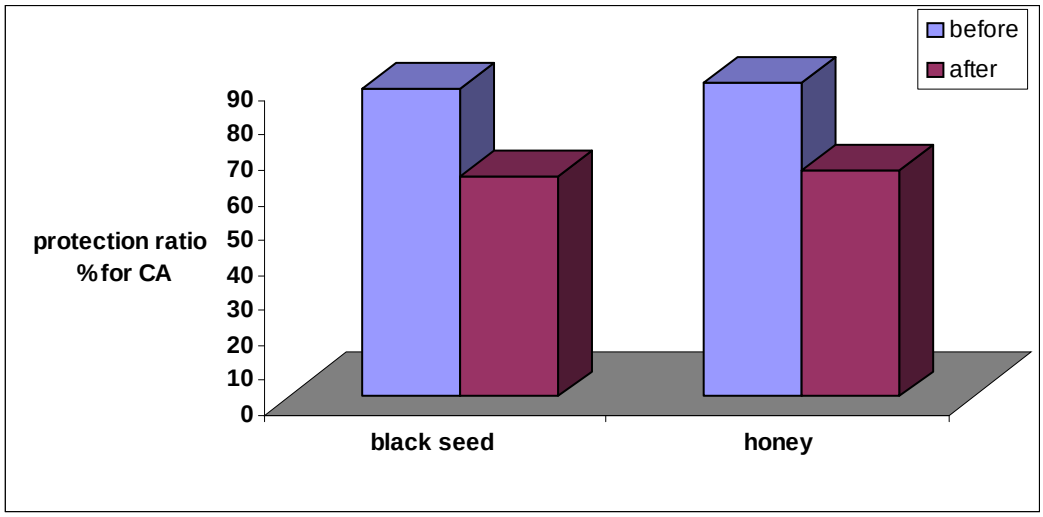
Table(4.7):Interaction between black seed oil and tamoxifen in vivo

Groups		Mitotic Index % μ±SE	Micro-nucleous % μ±SE	Chromosomal Aberration % μ±SE							
				Chromatid Break	Chromatid Gap	Deletion	Dicentric	Ring	Chromosome Break	Chromosome Gap	Total CA
Negatives Control		5.945 ± 0.134 A	2.252 ± 0.11 A	0.196 ± 0.004 A	0.451 ± 0.011 A	0.427 ± 0.011 A	0.265 ± 0.011 A	0.23 ± 0.001 A	0.027 ± 0.001 A	0.132 ± 0.004 A	1.734 ± 0.04 A
Positive Control(TAM)		2.605 ± 0.08 B	9.66 ± 0.023 B	1.22 ± 0.037 B	1.715 ± 0.008 B	1.06 ± 0.023 B	1.085 ± 0.03 B	1.17 ± 0.02 B	1.10 ± 0.011 B	1.94 ± 0.023 B	9.25 ± 0.005 B
Interaction	Before	4.32 ± 0.17 D	2.46 ± 0.02 A	0.26 ± 0.005 A	0.60 ± 0.057 A	0.525 ± 0.014 C	0.54 ± 0.011 C	0.29 ± 0.005 C	0.095 ± 0.002 C	0.315 ± 0.014 C	2.62 ± 0.05 C
	After	3.59 ± 0.06 C	3.38 ± 0.184 C	0.68 ± 0.011 C	1.22 ± 0.026 C	0.77 ± 0.008 D	0.73 ± 0.085 D	0.43 ± 0.002 D	0.085 ± 0.002 C	0.44 ± 0.002 D	4.515 ± 0.05 D

(P<0.05)







Figure(4.8):The protection ratios provided by black seed oil and honey when given before and after TAM treatment

It could be seen that black seed oil gave clear results as a protective agent against the genotoxic effect of TAM if it was given before TAM treatment. This might related to the chemical constituents of the black seed oil which may be linked with the drug or with its metabolites to form non-absorbable complexes (Negishi *et al*., 1994), or act to prevent activation of the drug by inhibiting cyt.P450 enzymes, or may inhibit the metabolic activation of the drug (Zhang *et al*., 1989), or may act as antioxidants or scavengers for the free radicals in the cell.

Black seed oil contains oleic acid and linoleic acid (Al-Ani, 1998).These acids play a major role in protection against mutagens by blocking metabolic activation through interaction with enzyme and / or in trapping mutagen molecules (Giorgio, 1994). It is also reported that black seed oil contains another kind of components such as the unusual eicosanoids (unsaturated fatty acids), which may contribute to its antioxidant activity (Houghton *et al*., 1995).

It was proved that the black seed shared in complete the gaps in the nucleotides by synthesizing it in the DNA strand because of its protein content. For this reason it might be played an important role in the repair system in the S-phase (Jones, 2002).

Our results are in agreement with Al-Azawi (1999) who indicated that pretreatment with black seed gave a protective effect against the genotoxicity of ionizing radiation.

These results are also in agreement with Hasan (2002) who indicated that pretreatment with aqueous and alcoholic extracts of

black seed gave a protective effect against the gentoxicity of cyclophosphamide (CP) and methotrexate (MTX) in mice.

4.4.1.2: Post-drug treatment with black seed oil

The results of this experiment are illustrated in table (4.7).Post drug treatment with black seed oil caused a significant increase ($p<0.05$) in MI (3.59%) when compared with the positive control (2.60%). So, the black seed oil provided (29.73%) protection from the effect of drug on MI (figure 4.8)

The percentage of CAs after treatment with black seed oil was (4.51%), which significantly different ($p<0.05$) from the positive control (9.25%). All of the CAs decreased significantly ($p<0.05$) when compared with the positive control.Post drug treatment with black seed oil provided (62.90%) protection from the effect of drug on CAs (figure 4.8)

The percentage of micronucleated cells was also reduced to (3.38%) after treatment with black seed oil, which significantly different ($p<0.05$) from the positive control (9.66%). The black seed oil provided (84.62%) protection from the effect of TAM on MN induction.

From these results, it was clear that post drug treatment with black seed oil may activate the suppressing agents (Ramel *et al*., 1986) or

activate the promoters of DNA repair (Kuroda and Hara, 1999), or may increase the error-free repair fidelity in the cell (Bronzetti, 1997).

Treatment with black seed oil before the drug provided protection ratios for MI, CAs and MN more than these ratios when it was given after drug treatment. So, black seed oil could be classified as “desmutagen” in the first order, and “bioantimutagen” in the second order.

Our results are in agreement with Al-Azawi (1999) who indicated that the use of black seed after inonizing radiation of mice provide treatment activity against the effect of radiation.

Those results are also agreed with Hasan (2002) who indicated that black seed oil might treat mouse bone marrow cells if it is given after treatment with cyclophosphamide and methotrexate.

4.4.2: Interaction between tamoxifen and honey

4.4.2.1: Pre-drug treatment with honey

The results of this treatment are shown in table (4.8). MI in bone marrow cells of mice treated with honey before the drug increased to (4.41%), which was significantly different ($p<0.05$) form the positive control (2.60%). Honey provided a (54.35%) protection form the effect of TAM on MI (Figure 4.8).

Table (4.8): Interaction between honey and tamoxifen *in vivo*

Groups		Mitotic Index % µ±SE	MN% µ±SE	Chromosomal aberration % µ±SE							
				Chromatid Break	Chromatid Gap	Deletion	Dicentric	Ring	Chromosome Break	Chromosome Gap	Total CA
Negative Control		5.945 ± 0.134 A	2.252 ± 0.11 A	0.196 ± 0.004 A	0.451 ± 0.011 A	0.427 ± 0.011 A	0.265 ± 0.011 A	0.23 ± 0.001 A	0.027 ± 0.001 A	0.132 ± 0.004 A	1.734 ± 0.04 A
Positive Control(TAM)		2.605 ± 0.08 B	9.66 ± 0.023 B	1.22 ± 0.037 B	1.715 ± 0.008 B	1.06 ± 0.023 B	1.085 ± 0.03 B	1.17 ± 0.02 B	1.10 ± 0.011 B	1.94 ± 0.023 B	9.25 ± 0.005 B
Interaction	Before	4.41 ± 0.193 D	2.75 ± 0.04 A	0.20 ± 0.023 A	0.595 ± 0.002 A	0.50 ± 0.018 C	0.47 ± 0.032 C	0.30 ± 0.023 C	0.08 ± 0.017 C	0.35 ± 0.011 C	2.50 ± 0.017 C
	After	3.56 ± 0.06 C	4.60 ± 0.147 C	0.66 ± 0.023 C	1.14 ± 0.046 C	0.84 ± 0.017 D	0.88 ± 0.032 D	0.44 ± 0.008 D	0.04 ± 0.005 A	0.43 ± 0.011 D	4.37 ± 0.016 D

(P<0.05)

The reduction in the genotoxic effects of TAM may be related to the chemical composition of honey, such as flavonoids and phenolic antioxidants that act to scavenge the free radicals and prevent the formation of precancerous cells after exposure to cancer-causing chemicals (Rao and Valhala, 1993), or induce detoxification enzymes that deactivate carcinogens by destroying the reaction centers of carcinogen, or assist in their elimination from the body (Internet, 2004 a).

Honey contains many polysaccharides, proteins, vitamins, enzymes and minerals in addition to pectins and tannins (White, 1979), which play a role in genetic mutation reduction. Polysaccharides act to block the DNA-adducts (Kim and Lee, 1997). Vitamin-C found in honey has the ability to reduce many of physical and chemical mutations (Deflora and Ramel, 1988). So it was considered a general antimutagen (Alekperov, 1982). Mita and his partner (1982) indicated that vitamin-C blocked the target sites in DNA and prevents the mutagen form reaction with DNA.

Honey may also protect the DNA methylase enzyme, which reduces the effect of mutagens (Cox *et al*., 1988).

These results agree with AL-Bedairi (2002) who found that honey could protect mouse bone marrow cells if used pre-gamma radiation exposure.

4.4.2.2: Post-drug treatment with honey

The results of this experiment are displayed in table (4.8).Post-drug treatment with honey increased the MI significantly ($p<0.05$) form (2.60%) in the positive control to (3.56%), and provided (28.83%) protection from the effect of TAM on MI (Figure 4.8).

CAs decreased significantly ($p<0.05$) in mice when treated with honey after the drug, from (9.25%) in the positive control to (4.37%). Post-drug treatment with honey provided (64.76%) protection from the effect of the drug on CAs (figure 4.8).

Micronucleus frequency reduced from (9.66%) in the positive control to (4.60%) in mice treated with honey after TAM. This reduction was significant ($p<0.05$). So, honey provided (68.02%) protection form the effect of the TAM on MN induction when used after drug treatment (Figure 4.8).

The treatment effect of honey from the genotoxic effects of TAM may be due to the increase in DNA replication fidelity (Shimoi *et al*., 1985; Bronzetti, 1997), or to the reduction in error-prone DNA repair systems (Obana *et al*., 1986).

From these results, it is indicated that protective effect of honey was more than the treatment effect of it, this suggested that honey considered as a desmutagen in the first order, and a bioantimutagen in the second order.

Our results were in agreement with Al-Bedairi (2002) who used honey pre and post-gamma ray exposure of mice, and found that honey could reduce the genotoxic effects of radiation in mouse bone marrow cells, and also indicated that pre-treatment with honey was more effective than post-treatment.

Chapter Five

Conclusions & Recommendations

5.1:Conclusions

1- Tamoxifen is shown to be genotoxic in mouse bone marrow cells (*in vivo*) and human blood lymphocytes (*in vitro*)

2- Tamoxifen can inhibit mitotic index, increase chromosomal aberrations and induce micronuclei in mice , and human blood lymphocytes, inhibit replicative index and increase sister chromatid exchange in human blood lymphocytes .

3- Black seed is a promising plant that could be used for reducing the genotoxicity caused by tamoxifen in mice.

4- Honey could be used for reducing the genotoxicity caused by tamoxifen in mice.

5- The pretreatment with black seed oil or honey is more effective than post-treatment in decreasing the genotoxic effects of tamoxifen.

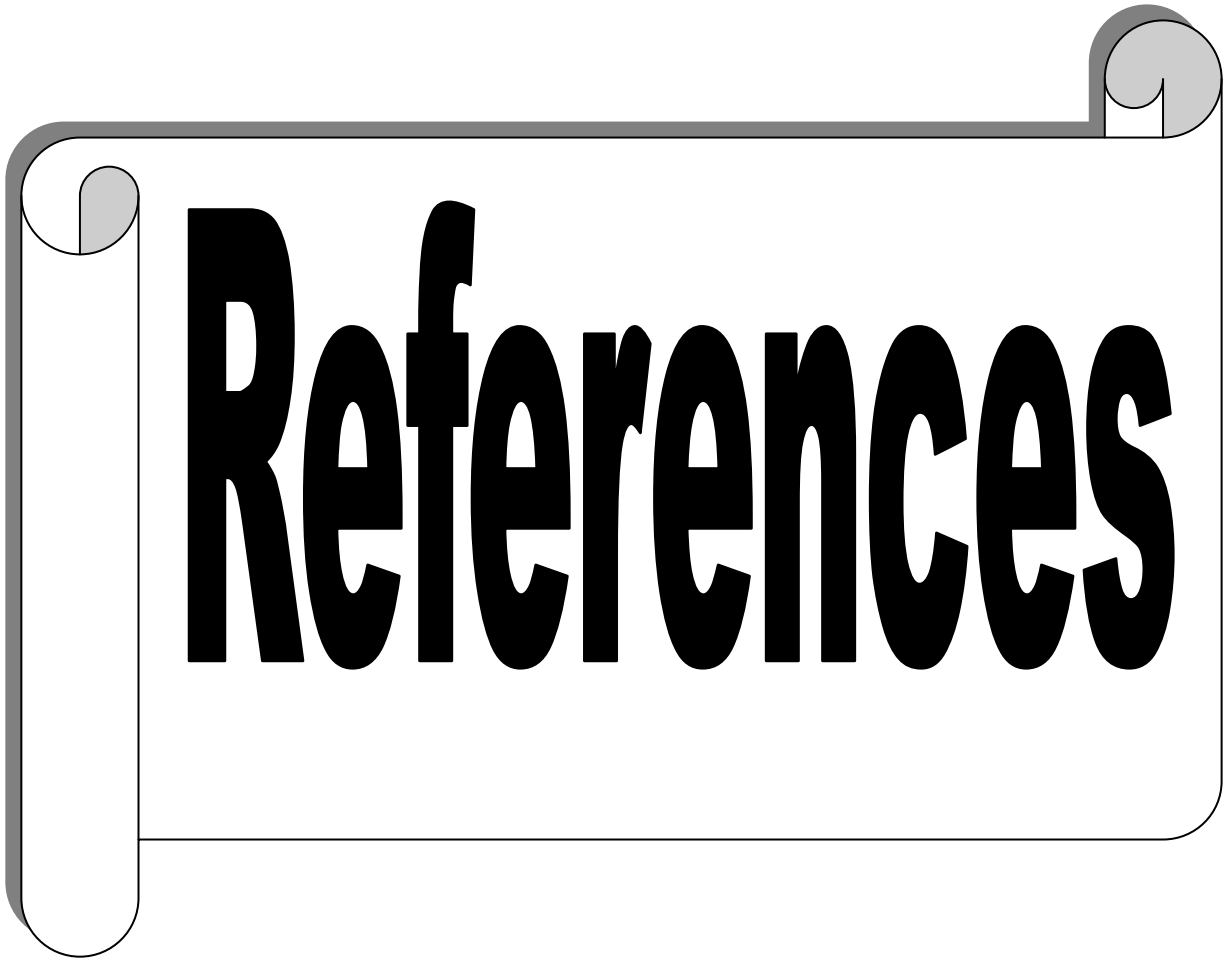
5.2:Recommendations

1- Further studies are needed on the effect of tamoxifen on the chromosomes of other organs such as liver, kidney, spleen and ovaries.

2- Further studies are required on the chromosomes of breast cancer patients treated with tamoxifen.

3- Using of black seed oil and honey for prevention or treatment of genotoxicity caused by tamoxifen treatment.

Chapter Six



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الخلاصة

لقد أجريت هذه الدراسة من اجل تسليط الضوء على تأثير عقار التاموكسفين في الصفات الوراثية الخلوية لخلايا نقي العظم في اناث الفئران المختبرية وخلايا الدم المحيطي للانسان (خارج الجسم) كما هدفت الدراسة ايضا الى اختبار كل من زيت الحبة السوداء و العسل في في الحد من هذه التأثيرات.

لقد أجريت دراسة التأثيرات الوراثية الخلوية للعقار بعد يوم واحد وبعد ثلاثة ايام وبعد خمسة ايام من التجريع وبجرعتين الاولى واطئة (0.4 ملغم/كغم) والثانية عالية (0.8 ملغم/كغم) .

اجري التداخل مابين زيت الحبة السوداء(٠,٢ مل) ، العسل(٠,٢ مل) وبين العقار (الجرعة العالية) من خلال نوعين من المعاملات (قبل وبعد المعاملة بالعقار)، لاجل اختبار فعالية كل من زيت الحبة السوداء و العسل في منع اوتقليل تأثير العقار.

وقد توصلت الدراسة الى النتائج الاتية :

- لقد اظهر العقار تأثيرات سلبية واضحة تمثلت بانخفاض معامل الانقسام الخيطي وزيادة في التغيرات الكروموسومية وزيادة تكون النوى الصغيرة في خلايا نقي العظم للفأر وخلايا الدم المحيطي للانسان. وقد ازدادت جميع هذه التغيرات بزيادة مدة التجريع مما يدل على ان للعقار تأثير سميا وراثيا تراكميا.
- عدم امتلاك زيت الحبة السوداء او العسل اي تأثيرات سامة على خلايا نقي العظم للفئران البيض.

- امتلاك كل من زيت الحبة السوداء و العسل كفاءة عالية في قدرة كل منهما على حماية نقي العظم للفئران من تأثيرات العقار السامة ، من خلال رفع قيمة معامل الانقسام الخيطي وتقليل نسبة التشوهات الكروموسومية ونسبة تكون النوى الصغيرة عند المعاملة قبل العقارلذلك فقد عدت من المثبطات المباشرة للعقار بالدرجة الاولى ، كما اظهرا كفاءة اصلاح عالية تجاه ماتم تدميره من الخلايا بفعل العقار ، فقد رفعا قيمة معامل الانقسام الخيطي بعد ما تم خفضه وكما عملا على اصلاح التشوهات الكروموسومية وخفض نسبة تكون النوى الصغيرة الناتجة بفعل تأثير العقار ، وعليه فقد عد زيت الحبة السوداء و العسل من المثبطات الحيوية لعقار التاموكسفين بالدرجة الثانية .

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية العلوم

التأثير التثبيطي لزيت الحبة السوداء والعسل على التأثير السمي الوراثي لعقار التاموكسفين في الفئران

رسالة

مقدمة الى كلية العلوم جامعة النهرين

وهي جزء من متطلبات نيل درجة ماجستير علوم في التقانة الاحيائية

من قبل

ايات منعم علي السوداني

بكلوريوس تقانة احيائية جامعة النهرين ٢٠٠١

تشرين الأول ٢٠٠٥