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Worood

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 K. 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:

Scientific Degree:

Date:

(Chairman)

Signature:

Name:

Scientific Degree:

Date:

(Member)

Signature:

Name:

Scientific Degree:

Date:

(Member)

I hereby certify upon the decision of the examining committee

Signature:

Name: **Dr.Laith Abdul Aziz Al- Ani**

Scientific Degree: Assistant Professor

Title: Dean of College of Science

Date:

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Chapter Two

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Chapter Three

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Results and Discussion

Conclusions and Recommendations

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5.1 Conclusions

1. The presence of active compounds in the root of *Glycyrrhiza glabra* like saponins and flavonoids.
2. Methotrexate is shown to be genotoxic in mouse bone marrow cells (*in vivo*), and human blood lymphocytes of healthy individuals and ALL patients (*iv vitro*).
3. *Glycyrrhiza glabra* aqueous extract antagonist to the genotoxic effects of methotrexate in mouse bone marrow cells .
4. Methotrexate induces the abnormalities in sperm morphology, while *Glycyrrhiza glabra* extract are useful in reduction the percentage of abnormalities caused by genotoxic drug (MTX).
5. In blood film test, the total number of white blood cells reduced as a result of methotrexate treatment, while the extract has the induction activity.
6. The pre treatment with extract is more effective than post treatment in decreasing the genotoxic effects of methotrexate.
7. Cytogenetic parameters (BI, MI, RI, SCE and CA) are appropriate in evaluation of the disease picture of ALL.

5.2 Recommendations

1. Evaluation of DNA metabolizing enzymes like dihydrofolate reductase in ALL cells.
2. Further studies are needed on the effect of methotrexate and *Glycyrrhiza* extract on sperm mobility.
3. Detection the effect of methotrexate and *Glycyrrhiza* extract on other immunological functions like phagocytosis.

1.1: Introduction

Herbal and traditional medicines have been used for thousands of years to improve human health, and they have been proven to have both medicinal and nutritional value (Eisenberg *et al*., 1993).

A variety of herbs and herbal extracts contain different phytochemicals, including the flavonoids, terpenoids, lignans, sulfides, polyphenolics, cartenoids, coumarins, saponins, and plant sterols with biological activity that can provide therapeutic effects like reducing high blood cholesterol concentrations, some protection against cancer, and stimulate the immune system (Farrell, 1985). Today herbal preparations are widely used for a host of common ailments and conditions, such as anxiety, arthritis, cold, coughs, constipation, fever, headaches, infections, intestinal disorders, stress, ulcers, and weakness (Tyler, 1994). Additionally the plants have served humans well as valuable components of seasonings, beverages, cosmetics, and dyes. The World Health Organization (WHO) estimated that 80% of the earth's inhabitants rely on traditional medicine for their primary health care needs, and most of this therapy involves the use of plant extracts or their active components like salicylic acid (a precursor of aspirin) obtained from willow tree bark (*Salix alba*) to help relieve fevers (Bruneton, 1995).

Several commonly used herbs have been identified by the National Cancer Institute (NCI) as possessing cancer – preventive properties, these herbs include members of the Alliums family; members of the Labiatae family; members of the Zingiberaceae family; licorice root; green tea and others (Caragay, 1992).

Leukaemia is a progressive, malignant disease of the blood forming organs, characterized by distorted proliferation and development of leukocytes and their precursors in the blood and bone marrow, which can be classified clinically on the basis of duration and character of disease into (a) acute leukaemia and (b) chronic leukaemia (Goldmen and Tarig, 1999).

Cytotoxic chemotherapeutic agents, commonly used in combination with radiation, are the standard therapy for acute lymphoblastic leukaemia and both of these two modalities are known to be mutagenic and carcinogenic (Andersone *et al.,* 1972), so the *Glycyrrhiza glabra* L was one of the plants that has been studied as antitumour plant, and this belong to isoliquiritigenin, one of the components in the root of the plant *G. glabra* is a member of flavonoids, which are known to have an anti-tumour activity *in vitro* and *in vivo* (Kanazawa *et al*., 2004). Other studies have shown that glycyrrhizin triggers an immune response against tumours (Suzuki *et al*., 1992). Also its components have proved to be effective over a wide spectrum of diseases from the simple sore

throat and cough to human immunodeficiency virus (HIV) infection (Hatano *et al*., 1991). But an over dose can result in hypertension, hypokalemia (low serum potassium), sodium retention, and edema (Snow and Joanne, 1996).

Aim of study :

The aim is to select the antimutagenic effects of *G.glabra in vivo and in vitro* by employing the following parameters:

1. Preparation of water extract from *G. glabra* roots for detection of some classes of active compounds in this extract.
2. Studying the cytogenetic effects of this extract by using (mitotic index and chromosomal aberration) in mouse bone marrow cells (*in vivo*) and in human blood lymphocytes (*in vitro*).
3. Study the efficacy of antileukaemia drug methotrexate (MTX) in the induction of cytogenetic damage *in vivo* in mouse and *in vitro* on human cells from normal and leukaemic patients.
4. Studying the ability of *Glycyrrhiza* aqueous extract in reducing the genotoxic effects of treatment with MTX.
5. Influence of *Glycyrrhiza* aqueous extract and MTX on sperm head and tail morphology in mouse.
6. The effect of *Glycyrrhiza* extract and MTX on total and blood differential count of leukocytes in mouse.

List of abbreviations

G.glabra	***Glycyrrhiza glabra***
5-BudR	**5-Bromodeoxyuridine**
ALL	**Acute lymphoblastic leukaemia**
AML	**Acute myeloid leukaemia**
BI	**Blast index**
CA	**Chromosomal aberration**
CCP	**Cell cycle progression**
CLL	**Chronic lymphocytic leukaemia**
CML	**Chronic myeloid leukaemia**
DHFR	**Dihydrofolate reductase**
DPX	**Distyrene plasticizer xylene**
FCS	**Fetal calf serum**
GA	**Glycyrrhetinic acid**
GL	**Glycyrrhizin**
HIV	**Human immunodeficiency virus**
KCL	**Potassium chloride**
MI	**Mitotic index**
MTX	**Methotrexate**
NaHCO3	**Sodium bicarbonate**
NaOH	**Sodium hydroxide**

NCI	**National Cancer Institute**
PBS	**Phosphate buffered saline**
PHA	**Phytohaemoaglutinine**
RI	**Replicative index**
SCE	**Sister chromatid exchange**
SSC	**Salt sodium citrate**
UPW	**Ultra purified water**
UV	**Ultraviolet**
WHO	**World Health Organization**
W.B.C.	**White blood cell**

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2.1*: Glycyrrhiza glabra*

Glycyrrhiza glabra or licorice is a perennial herb found in temperate regions, the stoloniferous root has been used as a medicinal and flavoring agent for over 3000 years (Snow and Joanne, 1996).

Glycyrrhiza belongs to Fabaceae family that includes more than 600 genus and 1300 species (Trease and Evans, 1983) and *Glycyrrhiza* genus includes about 14 species (Grieve, 1995).

The name *Glycyrrhiza* comes from Greek word meaning "sweet root". Glabra, the species name means "smooth", in reference to the smooth seed pods (Tyler *et al*., 1988).

In the Chinese book, 365 crude drugs are classified into three classes (upper: Plants with lowest side effects and non toxic for health care; middle: Plants that are non toxic or possess only weak toxicity; lower: toxic and only for clinical use), and the licorice is described as belonging to the upper class (Nomura and Fukai, 1998).

2.1.1: Description

The plant is a herbaceous perennial, it is 1 to 2 m high on a long sturdy primary taproot and this taproot is 15cm long and subdivides into 3 to 5 subsidiary roots that is 1.25m in length, and several horizontal woody stolons which may reach 8m, also the stems are sturdy, erect, and branched, while the leaves are

alternate, odd pinnate and 10 to 20cm long and they are in 3 to 8 pairs, the flowers are 1 to 1.5cm long that is bluish to pale violet, however, the fruit is a pod 1.5 to 2.5cm long and 4 to 6mm wide and usually has 3 to 5 brown, reniform seeds(Figure 2-1) (Gruenwald *et al*., 1998).

Figure2-1: *Glycyrrhiza glabra (Lange,1998)*

2.1.2: Distribution

The plant is widely distributed in Eurasia, including the Mediterranean region, India, East Central Asia and Western Siberia (Lange, 1998). The plant requires rich soils and grows in subtropical climates so it is indigenous to Turkey, Iraq, Spain, Greece and northern China (Davis and Morris, 1991). In Iraq, the plant is widely distributed in all regions from north to south (Townsend and Guest, 1974).

2.1.3: Chemical Components

Since licorice roots have been widely used for a variety of purposes, the chemical constituents were the subjects of many investigations (Nomura and Fukai, 1998). The chemical components of *Glycyrrhiza* can be described as the following:

a: Triterpenoid Saponins

The most prominent is the water – soluble triterpenoid glycoside glycyrrhizin (GL), the root content of this compound varies from 2%-4% depending upon growing conditions and this compound is responsible for the characteristic sweet taste of licorice (50 times sweeter than sucrose) as well as for most of the herb pharmacological activity (Tyler, 1994). Other saponins in *Glycyrrhiza* are, liquirtic acid, glycyrrhetol, glabrolide, and phytosterols (Hoffmann, 1996).

b.Phenolic compounds

1: Flavonoids and Isoflavonoids

The flavonoids in *Glycyrrhiza* are responsible for it is yellow colouring and they include liquirtin, isoliquirtin, liquirtigenin, licoflavonol, glabrone, and glysarin (Evans, 1989; Hoffmann, 1996), glabridin, glabrene, hispglabridin A, and hispglabridin B (Asada *et al*., 1998).

2: Coumarins

Coumarins are phenolic compounds, include liqoumarin,

umbelliferone, and herniarin (Tawata *et al*., 1990).

C: Volatile oils

More than 80 compounds were identified in *Glycyrrhiza* such as fenchone, linalool, and benzaldehyde (Hoffmann, 1996).

D: Sterols

Types of sterols are: Beta – sito sterol, stigmasterol, and dihydrostigmasterol (Tyler *et al*., 1988).

E: Nutritional Constituents

2 Vitamins: B_1, B_2, B_3, B_6, C, E, biotin, and folic acid (Beresford, 1999).

3 Minerals: Phosphorus, manganese, iodine, chromium, and zinc (Grieve, 1995).

4 Others: Lecithin, starch (2%-20%), sucrose, glucose, lignans, amino acids: Asparagines (1%-2%), and gums (Tyler *et al*., 1988).

2.1.4 Medicinal uses and pharmacological action

Recent studies have revealed that the healing properties of licorice components could be effective against wide spectrum of diseases like the effective treatment of peptic ulcers in many countries for hundreds of years and the antiulcer drug carbenoxolone, a succinate derivative of glycyrrhetinic acid (GA), has become the preferred form of licorice used to promote healing of ulcers (Gaudio and Carpino, 1993).

Alcohol extracts of *Glycyrrhiza* have displayed antimicrobial activity *in vitro* against *Staphylococcus aureus*, *Streptococcus mutans*, and *Mycobacterium* (Mowrey, 1986). Murray, (1995) showed that the majority of antimicrobial effects are due to isoflavonoid components (Particularly hispaglabridin, glabridin, glabriol, and 3-hydroxyglabrol). Also the glycyrrhizin was shown to protect thermally injured mice and mice infected with AIDS from *Candida albicanus* infection (Utsunomiya *et al*., 1999; Utsunomiya *et al*., 2000).

Colin-Jones, (1957) indicated that licorice has long been used to treat a variety of inflammatory conditions such as asthma, skin diseases (e.g., eczema), and even rheumatoid arthritis. This anti inflammatory action is due to flavonoid liquirtin, glycyrrhitinic acid and its derivatives (Cyong, 1982). A possible mechanism for these anti-inflammatory effects was offered by Shiki and Ishikawa, (1986) who showed that glycyrrhizin stabilizes lysosomes by inhibiting phospholipase A activity in the lysosomal membrane and this preventes the release of proteolytic enzymes and acid phosphatases from lysosomes into damaged tissue.

Since 1980 strong evidence has accumulated supporting the efficacy of several compounds in licorice in the treatment of many types of viral infections (Utsunomiya *et al*., 1997). Glycyrrhizin has been shown to have antiviral activity against the human

immunodeficiency virus (HIV) both *in vitro* and *in vivo* (Ito and Nakashima, 1987). In addition, Hatano and colleagues (1988) showed that several phenolic compounds isolated from licorice, especially licopyronocoumarin, inhibited the cytopathic activity of human HIV in cell culture. Other clinical and laboratory studies carried out mainly in Japan have demonstrated the efficacy of GL in the treatment of viral hepatitis types A, B and C (Crance and Levieque, 1994), and the mechanism of this antiviral effect appears to be both direct inhibitory action on viral replication and function and a stimulating effect on the host immune system to produce interferon, which has known antiviral effects (Eisenburg, 1992).

In genetic and tumor disease study, Suzuki and colleagues (1992) indicated that in animal and cell culture the GL and GA have inhibitory activity against certain tumours,and mice treated with GL developed a resistance to experimentally induced tumours most likely due to stimulation of host immune system and GL isolated from *G. radix* has an importance in treatment the patients with hepatocellular carcinoma and liver cirrhosis (Acharya *et al*., 1993; Abe *et al*., 1994; Arase *et al*., 1997). Other study by Chung and colleagues (2000) showed that GA isolated from *G. glabra* has inhibitory activity against human colon tumour *in vitro*, and it was found that 3, 4 dimethyl – 3 hydroxychalcone exert the strongest antineoplastic effect by inhibiting human gastric carcinoma cell

proliferation *in vitro* (Shibata, 1994). In the same line Juan and colleagues (1996) confirmed that herbal preparation which consists of eight different plants, one of them *Glycyrrhiza* showed significant cytotoxic and cytostatic activity on several tumour lines, including U937 histiomonocytic lymphoma, promyelocytic leukaemia HL60 and T cell leukaemia MOL T4 leukaemias.

Glycyrrhiza also has expectorant and suppressant action so chiefly used to treat the symptoms of common cold (Hoffmann, 1996).

Flavonoid components of *Glycyrrhiza* root exhibit antispasmodic action (Beresford, 1999). But excessive ingestion and prolonged use of licorice led to a hyperaldosteronism syndrome and was thought to be caused by GL and GA and the mechanism, however, turned out to involve the inhibition of 11-B-hydroxysteroid dehydrogenase enzyme (Homma *et al*., 1994; Lee *et al.*, 1996), which controls cortisol breakdown, the resulting high concentration of cortisol which also has a high affinity for aldosterone receptor in the kidney is responsible for stimulating sodium and water retention as well as for the excessive excretion of potassium (hypokalemia) (Heidemann and Kreuzfelder, 1983; Shintani *et al*., 1992), hypertension (Gomez-Sanchez and Gomez-Sanchez, 1992), and Pulmonary Oedema (Chamberlain and Abolnik, 1997).

2.2: Leukaemia

Leukaemia is a group of malignant disorders of the hemopoietic cells, characteristically associated with increased numbers of primitive white blood cells (blasts) in the bone marrow (Edward and Bouchier, 1999), with a progressive and fatal condition most often resulting in death from anemia, hemorrhage or intermittent infection (Boles *et al*., 1984).

The course of leukaemia may vary from few days or weeks to many years depending on the type of Leukaemia. The incidence of Leukaemia in all types in the population is approximately 10 per 100000 per year and males are affected more frequently than females in ratio about 3:2 in acute Leukaemia, 2:1 in chronic Leukaemia (Edward and Bouchier, 1999).

At the present time leukaemia in Iraq is considered as one of the most important causes of death especially after the war in 1991,for this reason large numbers of studies have been conducted and the genetic studies were one of these approaches that have been proved to be useful and vital in leukaemia researches (Martein *et al.,* 1999).In Iraq, Al-Bayatii and colleagues (1997) found that ALL reach a frequency of about 85% compared to other types of leukaemia. Other study demonstrated that the incidence of ALL in Iraq increased in the recent years especially in ages between (1-10)years (Mukheef,1999).

2.2.1: Causes of Leukaemia

The causes of leukaemia are unknown. It may results from the interaction of a number of factors:

1. Biological factors

A. Genetic factors

Specialists usually refer to genetic factors as factors that we borne with, those that involve our genes and chromosomes, for example children born with Down syndrome whose genetic make up is abnormal are prone to develop acute Leukaemia which are due to mutations in the DNA, chromosomal translocation (crossing over of parts of chromosomes to others) are common that led to disrupt specific genes that mediate cell division rate (Seppa, 2004). Moreover, genetic studies of several leukaemias have identified a small number of genes that must be mutated in order to trigger the development of leukaemia, in this respect the term oncogenes is introduced which refer to these mutated DNA sequences whose expression plays a fundamental role in the formation and growth of malignant tumours (Shovlin *et al.,* 1999*)*.Other genes that share in development of leukaemia was proto-oncogene that identified as normal cellular gene involved in normal cell proliferation, these genes have the potential to contribute to induction of malignant tumour when their structure or expression is altered, and the process that converts a normal proto-oncogene to a cancer

gene(oncogene) is activation ,this activation could occur as a result of many genetic mechanisms like mutation, and chromosomal rearrangements (Bishop,1983).

B.Infection

Viruses cause leukaemia in cats and other animal species, and a rare leukaemia-related cancer of lymphocytes of adults seen in Japan and Caribbean known to be due to a virus called retroviruses, are unusual in that they contain RNA rather than DNA and undergo replication in host cells by making a DNA copy which inserted in host genome (Johan, 2000).

2. Ionizing radiation

Ionizing radiation like gamma-ray was the first identified as agent associated with induction of leukaemia and this become apparent in the survivors of atomic bomb explosions in Hiroshima and Nagasaki (Henshaw *et al*., 1990).In addition, patients who received radiotherapy for malignant disease may have a chance to develop leukaemia (Goldmen and Tarig,1999).

3. Chemical factors

An unsubstantiated suggestion that benzene may be a leukaemogen was proposed in the late nineteenth century because of immunosuppressive effects for a rang of lymphoid and myloid malignancies (Rinsky and Young, 1981). Also the exposure to paints, herbicides, pesticides, and chemicals that are used in a

cytotoxic chemotherapy of various cancers may result in a gene mutation, which may result in leukaemia (Peter, 1997).

2.2.2: Types of leukaemia

Leukaemia is a cancer which affectes the blood forming system of the body including the lymphatic system and bone marrow ,Two different types were recognized; acute and chronic (the medical meaning of these terms is that acute diseases run a rapid course while chronic ones run a slow gradual course) and the difference between acute and chronic was obvious not only in the patients survival, acute leukaemia survived 3 months, where as those with chronic disorders would run a longer course but in the appearance of the leukaemia cells (John, 2000).

*** Acute leukaemia**

Acute leukaemia can be considered as a heterogeneous group of disorders in which the malignant clone arise from progenitors in the bone marrow or lymphatic system, resulting in an increased number of immature non functioning leukaemic cells, and the most common presentations are fever and/or infection which just does not seem to clear up indicating that the immune system is not functioning as well as it might, anemic symptoms of thrombocytopenia may be the reason for presentation of bleeding which does not stop, easy bruising, or purpuric rash, and pressure effects of a filled marrow may cause bone pain(Bain,1999) .Acute

lymphoblastic leukaemia (ALL) occurs when primitive blood forming cells called lymphoblast which normally mature to form lymphocytes proliferate without developing into normal blood cells and the lymphoblast crowed out healthy blood cells and frequently collect in lymph nods to cause swelling , ALL is the most commonly kinds of childhood cancer which accounting for 80% of leukaemia cases in children under 15 years old but in adults is rare only in persons over age of 50 (Al-Duliemy,2005) .While acute myloid leukaemia (AML) occurs when the primitive blood forming cells called myeloblast proliferate without developing into normal blood cells then the immature blast cells crowed the bone marrow, AML account 50% of leukaemia diagnosed in teenagers and 80% of all acute leukaemia cases in adult (Aguayo *et al*.,2002).

*** Chronic leukaemia**

Chronic leukaemia involves the over production of blood cells that appear to be mature but actually lacks the normal functions the mature blood cells have and it is usually has slower less dramatic course than acute leukaemia, there are 2 main groups of chronic leukaemia, chronic lymphoid leukaemia (CLL) which is characterized by production of too many apparently mature lymphocytes in the bone marrow which seems to be fully, CLL accounting 30% of leukaemia and it is rare in persons under 30

years old but its frequently increases with age between (60-70) years old, the second type is chronic myeloid leukaemia (CML) that characterized by over production of mature appearing but defective myloid cells to the point where almost no health cells remain and this type account for 20% to 30% of all adult leukaemia which most frequently affects people aged (25-60) years old (Al-Duliemy, 2005).

The mainstay of treatment of leukaemia is chemotherapy, sometimes with the addition of radiation therapy but because of the severity of some courses, bone marrow transplants are sometimes necessary, also the biological therapy-using the body immune system to fight cancer, blood transfusion (red blood cells, platelets) and medications (to prevent or treat damage to other systems of body caused by leukaemia treatment) (Seppa, 2004).

2.2.3: Genetic disorders in leukaemia

Much of recent cancer research in leukaeogenesis has revealed the presence of specific chromosomal abnormalities in most human leukaemia, these abnormalities serve to establish the precise location of the altered (Mutated) gene, chromosomal aberration could be quantitative (abnormalities of numbers) or qualitative (abnormalities of structures) (Le, 1997).

In CML the Philadelphia chromosome is an abnormal chromosome which usually results from reciprocal transfer of

genetic material between chromosome number 9 and 22, and two specific genes which are ABL gene that presents on chromosome 9 and BCR gene that present on chromosome 22 are involved in this translocation (t) (Rowley and Testa, 1982).

In AML the most chromosomal abnormalities restricted to AML and have very specific clinical correlations are t(8;21), t (15;17), t(9;11), t (8;16), inversion (16) and trisomy (4) (Le, 1997).

Two sub types of ALL are identified the B-Lineage and T-Lineage cells, in B-Lineage ALL the t(8;14) (q24;q32), t(2;8)(P12;q24), and t(8;22) (q24;q11) disrupt the c-myc oncogene while in T-lineage ALL the t(8;14) (q24;q11), t(10;14) (q24;q11),and t(11;14) (P13;q11) are specifically associated with T-cell neoplasia (Gibbons and Czepulkwski, 1992).

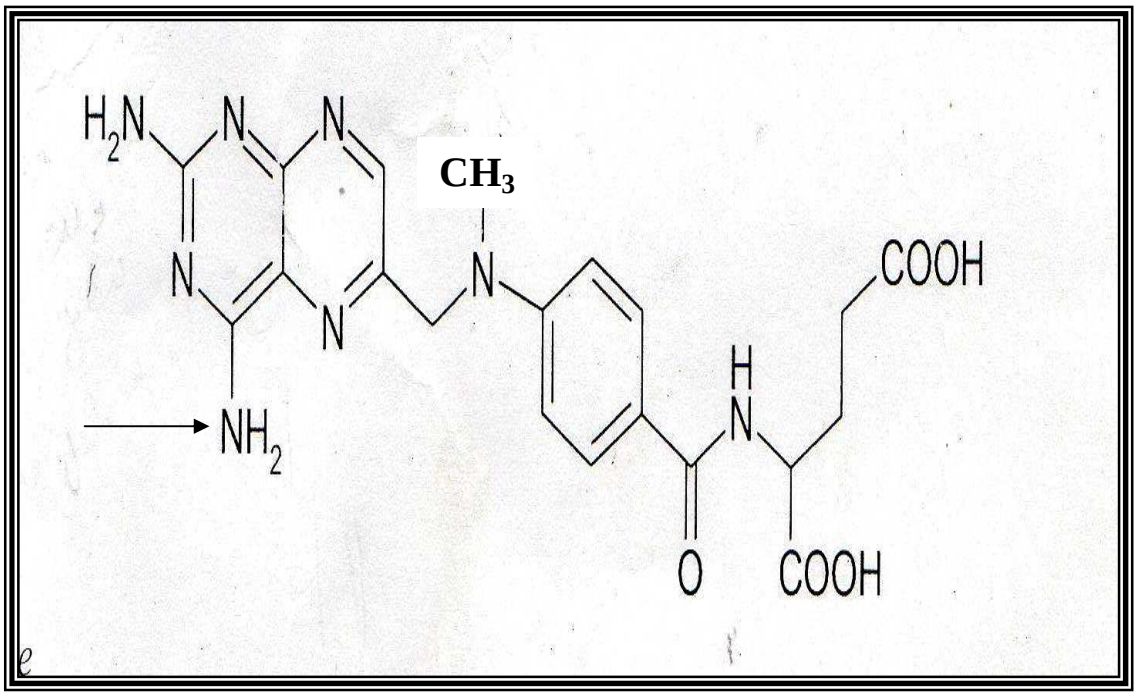
Chronic lymphatic leukaemia is the most common of lymphoproliferative disorders,therefore, the majority of information about (CA) relates to these cases, the most frequent occurring (CAs) in CLL are trisomy 12 occurring in about one third of cytogenetically abnormal cases (Harrison, 1992), t(11;14) has also been identified in 30% to 50% of lymphoma (Williams *et al*., 1991), and the second translocation is the t(14;19) (q32;q13) (Ohno *et al*., 1990). Another CA involves in CLL is deletion (13) (q14) that occur in 10% of CLL cases (Juliusson *et al*., 1990).

2.3: Methotrexate(MTX)

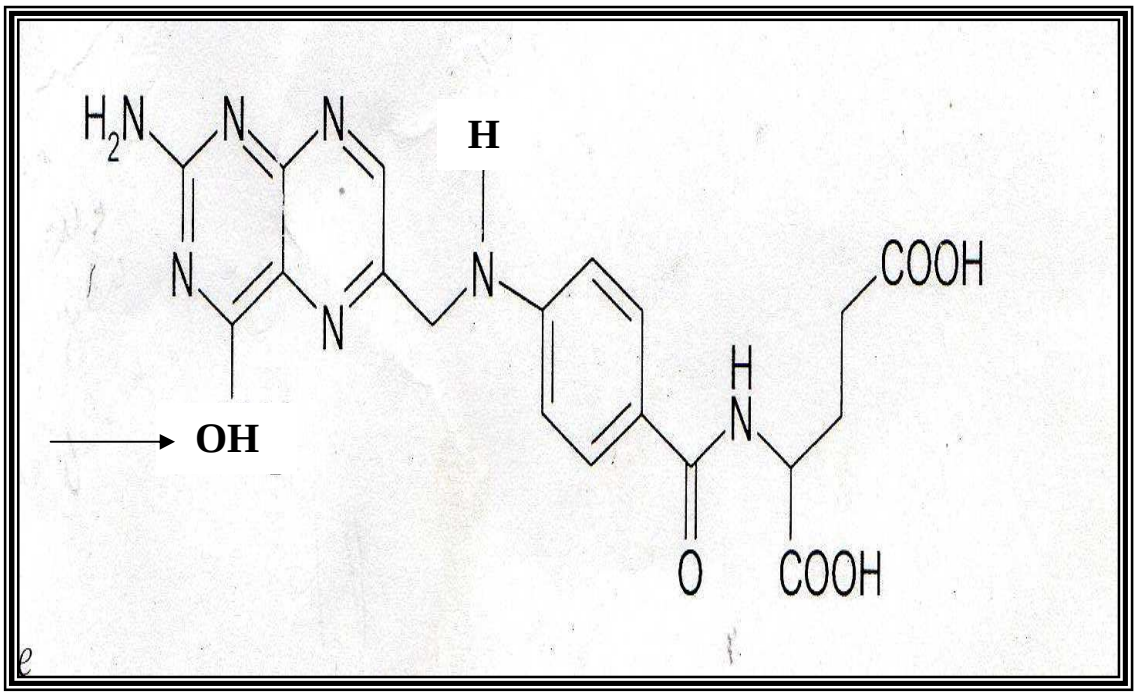
Methotrexate is an antimetabolite drug that acts by inhibiting the metabolism of folic acid. MTX empirical formula is $C_{20}H_{22}N_8O_5$ and it is structural name N-[4-(2,4-diamino-6-pteridinyl) methyl] methyl glutamic acid with a molecular weight 454.4 (Klareskog *et al*., 2004).

Methotrexate is a yellow fluid may be given as an injection intravenously, intramuscularly or intrathecally, also it is available as yellow tablets of 2.5mg and 10mg (Martindale, 2002).

The most widely used and best understood antifolate in cancer therapy is MTX which differs from the essential vitamin, folic acid, by having an amino substituted with a hydroxyl at the 4-position on the pteridine ring (Figure2-2), and this change transforms the enzyme substrate into a tight-binding inhibitor of dihydrofolate reductase (DHFR), a key enzyme required to maintain adequate intracellular levels of reduced folates (Allegra and Collins, 1990).



Methotrexate



Folic acid

(Figure 2-2): The structure of methotrexate and folic acid
(Gescher,2004)

2.3.1: Clinical uses of methotrexate

Almost 50 years ago, MTX was developed as a chemotherapeutic agent for the treatment of cancer especially leukaemia (Cronstein, 1997).Methotrexate has since been used in the treatment of various malignancies including osteosarcoma, non- Hodgkin's lymphoma, Hodgkin's disease, cutanous T cell lymphoma, head and neck cancer, lung cancer, and breast cancer (Jolivet *et al*.,1983). Subsequently MTX was found to play a major therapeutic role in non- neoplastic diseases, acting as an anti-inflammatory and immunosuppressive drug (Seitz, 1999). Currently, MTX is commonly used to treat rheumatoid arthritis (Nash *et al*., 1996; Weinblatt *et al*., 1998), graft-vs-host diseases (Feagen *et al*., 2000), psoriasis, primary biliary cirrhosis, Crohn disease, and intrinsic asthma (Genestier *et al*., 2000).

2.3.2: Mechanism of action

Methoterxate inhibits dihydrofolate reductase(DHFR), an enzyme that is a part of the folate synthesis metabolic pathway and this enzyme (DHFR) catalyses the conversion of folic acid to the reduced folates (i.e., tetrahydrofolate) (Klareskog *et al*., 2004). The tetrahydrofolate is essential cofactor that donates one-carbon group in the enzymatic biosynthesis of thymidylate and purine nucleotide that is a precursor for DNA synthesis (Allegra *et al*., 1986; Allegra *et al*., 1987). Therefore, MTX inhibits the synthesis of DNA,

RNA, thymidylates, and proteins so, MTX is cell cycle S-phase selective, and has a greater negative effect on rapidly dividing cells (such as malignant cells), and thus inhibits the growth and proliferation of these cells (Klareskog *et al*., 2004).

2.4: Cytogenetic analysis

Cytogenetic analysis is a widely employed indication system for the evaluation of physically, chemically and biologically induced mutations that allows for the objective evaluation of the genetic material damages and is a method that permits direct image analysis for the chromosome damage, these analyses have been carried out using *in vivo* and *in vitro* systems and have been proved to be good and reliable for mutagen – carcinogen exposure and chromosomal aberration detection (Nakanishi and Schneider, 1979; Gebhart, 1981). Chick embryos were the first to be utilized (*in vivo*) analysis (Bloom and Hsu, 1975), but later mice became the animals most frequently used due to their fast reproduction, small size, and easy handling (Tice *et al*., 1989; Haung *et al*., 1990). Rabbits also have been used, but less frequently (Stetka and Wolff, 1976). A variety of somatic and germ tissues have been analyzed *in vivo* studies including spermatogonial cells (Allen and Latt, 1976), bone marrow cells (Karm *et al*., 1975) and spleen cells (Nath *et al*., 1988).

2.4.1: Mitotic index (MI)

Mitosis is the type of division in which a cell with 46 chromosomes in human or 40 chromosome in mice produces two daughter cells, each of which also has the same number of chromosomes as in parent cells and it is the shortest portion of cell cycle which can be divided into four phases prophase, metaphase, anaphase, and telophase (Friedman *et al*., 1996).

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

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

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

Mitotic index in lymphocyte cultures may be a useful and sensitive indicator of the cellular toxicity for chemotherapeutic agents (Lialiaris *et al*., 1989; Lialiaris *et al*., 1992). Morris and Heflich, (1984) have shown that MTX at various concentrations (3.1, 6.25, 12.5, 25 and 50nM) was tested *in vitro* and it was found that MTX in human lymphocytes, produced: (a) reduction of MI at concentration of 25 and 50nM,(b) reduction of cell proliferation rates.

2.4.2: 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(CA) assay (Lambert *et al*., 1978; Ardito *et al*., 1980).Paul and Buul, (1977) confirmed that physical and chemical agents have shown the ability to induce CA. The other purpose of the CA test is to identify agents that cause structural CAs in cultured mammalian cells. Structural aberrations may be of two types, chromosome or chromatid and the majority of chemical mutagens induced aberrations of chromatid type but chromosome type aberration occur ,while an increase in polyploidy may indicate that the chemical has the potential to induce numerical aberration (Evans, 1976; Ishidate and Sofuni, 1985; Galloway *et al*., 1987). In addition CA serves to detect the mutageneic ability for some medical drugs especially that has been used in cancer treatment (Miura *et al*., 1983). Mondello and colleagues (1984) have shown the effect of MTX on chromosome morphology in cultured lymphocytes, chromosome anomalies such as gaps and breaks are observed on all the chromosomes and when cells were continuously exposed to the drug, the chromosome damage appeared to be particularly sever and a high proportion of

cells blocked by MTX showing anomalies mainly chromatid and chromosome breaks-when allowed to duplicate DNA and reach mitosis.

Chromosomal aberrations occur spontaneously (Goodenough, 1978), but chromosomes can be broken by ionizing radiation, physical stress, or chemical compounds, if broken ends are not brought together they can remain broken but, if broken chromatid ends are brought into apposition there are several alternative ways in which they can be rejoined, first, the two broken ends of a single chromatid can be reunited, second, the broken end of one chromatid can be fused with the broken end of another chromatid, resulting in an exchange of chromosomal material (Robert, 1996).

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 (Winter *et al*., 1998). Jensen and Nyfros ,(1979) confirmed that the cytogenetic 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. Shubber and colleages (1985) confirmed that C57 black mice showed spontaneous frequencies of CAs and SCE_s in bone

marrow cells which increased due to the effect of carcinogenesis that causes cytogenetic damage. Other studies also done by Shubber and colleagues showed that CAs and SCEs 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).

The malignant cells in virtually all patients who have leukaemia, or lymphoma have acquired CA (Heim and Mitellman, 1995). Cytogenetic changes may lead to cancer development such changes are deletion, translocation, and inversion so, the former often results in loss of tumour suppressor gene (P^{53} and P^{21}) , translocation and inversion can be divided into those found in certain tumours (specific) and those observed only in tumour from one patient (idiopathic). (Rabbits, 1994).

2.4.3: Sister chromatid exchange (SCE)

Sister chromatid exchange (SCE) represents reciprocal interchange of DNA between homologous chromatids observable in metaphase chromosomes (Shubber and Jafar, 2000), and this symmetrical exchanges between newly replicated chromatids and their sisters can be visualized cytologically in vertebrate cells if the DNA of one chromatid is labelled with 5-bromodeoxyuridine (5-BudR) during synthesis (Hagmar *et al*., 1998).

Sister chromatid exchanges have been proposed as a very

sensitive method for detecting mutagens and / or carcinogens, and lately as a valid method for guiding and improving chemotherapy *in vitro* (Tofilon *et al*., 1985; Deen *et al*., 1986) and *in vivo* (Tofilon *et al*., 1985; Mourelatos and Dozi-Vassiliades, 1988).

These SCE have been demonstrated to be an accurate reflection of DNA damage both *in vitro* in cultured cells and *in vivo* in mouse and rat bone marrow and spleen cells (Schneider *et al*., 1979).

More importantly SCEs occur spontaneously in normally cycling human cells, suggesting a link between SCE and DNA replication (Galloway and Evans, 1975; Crossen and Morgan, 1979), but sister chromatid exchanges can be induced by various genotoxic treatment, suggesting that SCE_s reflect a DNA repair process, and the cytological assessment of SCE levels in peripheral blood lymphocytes is used as an index of the mutagenic potential of environmental factors (Hagmar *et al*., 1998). So eukaryotic cells exposed to DNA – damaging agents in G2 show elevated SCE levels only after completing a subsequent replication cycle (Wolff *et al*., 1974). After years of intensive researches, the biological significance and the mechanism of (SCE_s) induction is known (Okey, 1983).

In recent years, more attention has been devoted to the study of SCE levels in relation to health and disease, SCE frequency was

reported to be significantly higher in lymphocytes of patients with ALL (Otter *et al*., 1979), CML (Shirishi and Sandberg, 1980) and in patients with schistosomasis (Shubber, 1987; Shubber *et al*., 1991; Juma *et al*., 1999).

Many halogenated pyrimidine analogues have been assessed for the labeling of DNA including bromodeoxyuridine, iododeoxyuridine, chlorodeoxyuridine, bromodeoxycytidine, chlorodeoxycytidine, and iododeoxycytidine (Dufrain, 1974). When incorporated in to DNA or chromatin, 5-BudR can quench the fluorescence of DNA binding dyes such as 33258 Hoechst (Craig – Holmes and Shaw, 1976).

2.4.4: Blastogenesis index

The index is useful for measuring cellular response to mitogens in term of the induction of proliferation (Soren, 1973).

The theory that inherited this characteristics are classified into three items, one: transmitted from parent to offspring by germ plasma, two: reproduction of an organism by budding, and three: the transformation of small lymphocytes into larger cells that is capable of undergoing mitosis (Evans and Mclean, 1967).

Phytohemagglutinin (PHA) is a mutagen used along with a minimal essential medium or RPMI-1640 medium for initiating the lymphocytes undergo mitosis, and after stimulation of blood lymphocytes with PHA, the cultures soon will contain cells that

have divided different numbers of times (Morimoto and Wolff, 1980). This heterogeneity has been explained variously as a difference in cell-cycle times (Crossen and Morgan , 1979), or in the times when the cells start blastogenesis by responding to PHA (Younkin , 1975). Savage, (1979) indicated that PHA stimulated human lymphocytes are widely used to detect chromosome-damaging agents, possible human exposure to carcinogens and the immune response of blood, the results are detected by the number of cell divisions.

2.4.5: Cell cycle progression (CCP)

The 5-BduR-Giemsa technique is a useful method for identifying, with accuracy, the percentage of cells which have gone through one, two or three division *in vitro* and *in vivo* (Bianchi and Lezana, 1976). *In vitro* CCP was found to be affected by the medium, 5-BduR levels, and colchicine (Shubber Al-Allak, 1986). The nomenclature utilized for the evaluation of CCP pattern, according to the number of cell cycles was (Becher *et al*., 1983):

1. First cell division (M_1):

These groups of cells have incorporated 5-BduR during a single phase. The chromosomes of this phase all appear bright under the light microscope.

2- Second cell division (M_2):

This group contains cells, which incorporated 5-BduR during two "S" phases and display atypical differential staining of sister chromatids (One dull and one bright).

3- Third cell division (M_3):

These metaphases incorporated 5-BduR during three "S" phases and contain 5-BduR – substituted DNA in both sister chromatids and half of number of chromosomes were differential and other half become dark.

2.4.6: Replicative index (RI)

Cell cycle progression (CCP) may be defined using a parameter that considers, at the same time, the number of M_1, M_2 and M_3 metaphase cells. Many indicators were considered, but the most suitable was the RI. This index is formulated using the following equation:

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

(Lamberti *et al.*, 1983).

2.5: The spermatozoon structure and spermatogenesis

The spermatozoon consists of a head and a tail and like other cells the spermatozoon is enclosed within the plasma membrane (Austin and Short, 1982).The shape of the sperm head is characteristics of the species, in mouse it is hook – shaped, and it is composed of two parts, the nucleus and the acrosome. The nucleus contains a highly condensed chromatin while the acrosome is surrounded by the acrosomal membranes and covers the anterior part of the sperm nucleus; the acrosome contains enzymes which are important in penetration of the egg in the fertilization process (Austin and Short, 1972; Sieger, 1997; Saladin and Porth, 1998). The tail is divided in to a neck, middle piece, principle piece and the end piece. The neck connects the tail to the head of spermatozoon (Hafez and Hafez, 2000).

Chemineau and colleagues (1991) showed that abnormal spermatozoon can be classified in to five different classes:

1. Tailless spermatozoon.
2. Spermatozoon with an abnormality in the head (abnormal acrosome, small or narrow head, enlarged head, etc).
3. Spermatozoon with a tail abnormality.
4. Spermatozoon with a proximal cytoplasmic droplet.
5. Spermatozoon with a distal droplet.

The entire process of sperm formation, beginning with

spermatogonia and resulting in mature spermatozoa is referred to as spermatogenesis, the stem cells called spermatogonia, divided through mitosis in which some daughter cells produced from these mitotic divisions remain as spermatogonia and continue to divided, other daughter cells form primary spermatocytes undergo meiosis I giving rise to haploid secondary spermatocytes and each of these undergoes meiosis II to produce spermatids and the spermatids are then transformed into spermatozoa (Arab *et al* ., 1989).

3.1: Materials

3.1.1: Equipments and apparatus

The following equipments and apparatus were employed throughout this study:

Company / Country	Apparatus	No.
Memmert – Germany	Cold incubator	1.
NAPCO – France	Laminar air flow	2.
Beckman – England	Centrifuge	3.
Dixone – UK	Autoclave	4.
Motic – Japan	Microscope	5.
Gelson – France	Micropipette	6.
Sartorius – Germany	Electric balance	7.
Gallenkamp – England	Water bath	8.
Radiometer – Denmark	pH- Meter	9.
Griffin – England	Vortex mixer	10.
BUCHI - Switzerland	Rotary evaporator	11.

3.1.2: Chemical materials

The following chemical materials were used in the study:

Company / Country	Materials	No.
Sigma – USA	Fetal calf serum (FCS)	1.
Radio Biology Center of Ministry of Science and Technology – Iraq	Phytohaemagglutinin (PHA)	2.
Fluka – Switzerland	Methanol	3.
Fluka – Switzerland	Glacial acetic acid	4.
Hexal – German	Methotrexate (MTX)	5.
Sigma – USA	RPMI	6.
Ibn Hayan – Syria Houde – France	Colchicine	7.
Fluka – Switzerland	Giemsa stain	8.
BDH – England	Bromodeoxyuridine	9.
Fluka – Switzerland	Glycerin	10
Sigma – USA	Penicillin	11
Sigma – USA	Streptomycin	12
BDH – England	Hoechst stain	13
Sigma – USA	Heparin	14
Ferak – German	Ethanol	15
Reidal – DE Haen AG seelze – Hanno - ven	Eosin	16.

Fluka – Switzerland	KCl KH_2PO_4	17.
Fluka – Switzerland	HCl NaCl	18.
Fluka – Switzerland	KOH Na_2HPO_4	19.
BDH – England	Sodium bicarbonate	20.
Fluka – Switzerland	NaOH $FeCl_2$	21.
Fluka – Switzerland	Roshail salt	22.
Ferak – German	Sulfuric acid	23.
BDH – England	Chloroform	24.
LA – Chema - Russia	Nigrosin	25.

3.2: Plant Collection

Glycyrrhiza glabra was collected from Al-Nahrain University between July and August(2004), and identified by Proff. Dr.Ali Al-Mousawy, department of Biology, College of Science , University of Baghdad.

Roots of this plant were air dried in shade at room temperature and grinded into powder form by using electric grinder.

3.3: Chemical Preparations

1. Colchicine (Ibn Hayan / Syrian)

Colchicine was prepared by dissolving one tablet (0.5 mg) of colchicine in (0.5 ml) of phosphate buffer saline (PBS) to be used for mouse injection. Each animal was injected with 0.25 ml of this solution in the intraperitoneal membrane (I.P) (Allen *et al*., 1977).

2. Colchicine (Houde / France)

This colchicine was prepared by dissolving one tablet (0.5 mg) of colchicine in (10 ml) of distilled water to make a stock solution. This solution was stored at (-20°C) until used for human blood culture (Allen *et al*., 1977).

3. Phosphate buffer saline (PBS)

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

Sodium chloride (NaCl) 8 gm

Potassium chloride (KCl) 0.2 gm

Sodium phosphate hydrate (Na_2HPO_4) 1.15 gm

Potassium phosphate dihydrate (KH_2PO_4) 0.2 gm

The pH was a adjusted to 7.2 and the solution was sterilized by autoclaving then stored at 4°C(Hudson and Hay, 1989).

4. Potassium chloride (KCl) (hypotonic Solution)

A concentration (0.075 M) of this solution was prepared by dissolving 5.75 gm of KCl salt in 1000 ml of D.W. This solution was sterilized by autoclaving and stored at (4°C) (*Allen et al.,1977).*

5. Fixative Solution

This solution was freshly prepared by mixing 3 parts of absolute methanol with 1 part of glacial acetic acid (Patton, 1967).

6. Sodium bicarbonate ($NaHCO_3$)

Sodium bicarbonate (4.4 gm) was dissolved in 100 ml sterile D.W. and stored at 4°C until used (Allen *et al*., 1977).

7. Bromodeoxyuridine (5-BudR)

5-bromo-2- deoxyuridine powder (50 mg) was suspended in 37.5 ml of D.W, the solution was sterilized by filtration through a 0.2µm sterile filter, distributed in sterile tubes with a final concentration 1.33 mg / ml and stored at (-20°C) until used.

8. Ferric chloride solution

The solution was prepared by dissolving 1g of ferric chloride in 100 ml distilled water. It was used for saponins detection.

9. Leukocyte diluents solution

The solution was preaperd by adding 2 ml of glacial acetic acid and to 98ml of distilled water, with few drops of methylen blue as indicator and stored in refrigerator at 4°C (Sood ,1986) .

10. Leishman kit

Leishman kit was supplied from Institute of Sera and Vaccine, Ministry of Health /Baghdad.

11. Sodium hydroxide solution

This solution was prepared by dissolving 40 mg of NaOH in 1000 ml of D.W.

12. Antibiotic solutions

Streptomycin was prepared by dissolving 1g of streptomycin in 10 ml D.W., penicillin was prepared by dissolving 1000000 unite / 10 ml D.W., both antibiotics were sterilized by filtration under aseptic conditions (Ian-Freshney, 2000).

13. Potassium hydroxide solution

It was prepared by dissolving 50g of potassium hydroxide in 100 ml of D.W. This solution was used to detect the flavonoids.

14. RPMI – 1640 medium

This media contained the following:

RPMI – 1640 medium base	10 gm
Fetal bovine serum	10%
Penicillin	5 ml
Streptomycin	5 ml
Hepes	4 gm
Sodium bicarbonate	1.5%
5-BudR	1 %(1.33 mg/ml)

The volume was completed with sterile D.W. to 1000 ml, and the pH was adjusted to (7.2), sterilized by filtration using (0.22 μm) size filter. After that 2ml of the medium was transferred into sterile test tubes and kept freezed till use (Nara and McCulloch,1985).

15. Giemsa stain (Allen *et al.*, 1977)

Giemsa stock solution was prepared by dissolving 1 gm of Giemsa powder in 33 ml glycerin in a water bath 60°C for two hours with continuous shaking, and then left for 30 min at room

temperature, then 66 ml of absolute methanol was added 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	1 ml
Absolute methanol	1.25 ml
Sodium bicarbonate solution	0.5 ml
D.W.	40 ml

16. Eosin stain

This stain was prepared by dissolving 1 g of yellowish eosin in 100 ml distilled water (Wyrobek and Bruce, 1975).

17. Hoechst (33258) stain

This fluorescent stain was prepared by dissolving 0.025 gm in 100 ml PBS (stock),2 ml from the stock was taken and added to 100 ml of D.W. (Ian-Freshney, 2000).

18. Phytohaemaglutinin (PHA)

Phytohaemaglutinin was obtained as frozen solution from Radio Biology Center-Ministry of Science and Technology then stored at (-20ºC).

19. Fehling reagent(Sarkas *et al*., 1980)

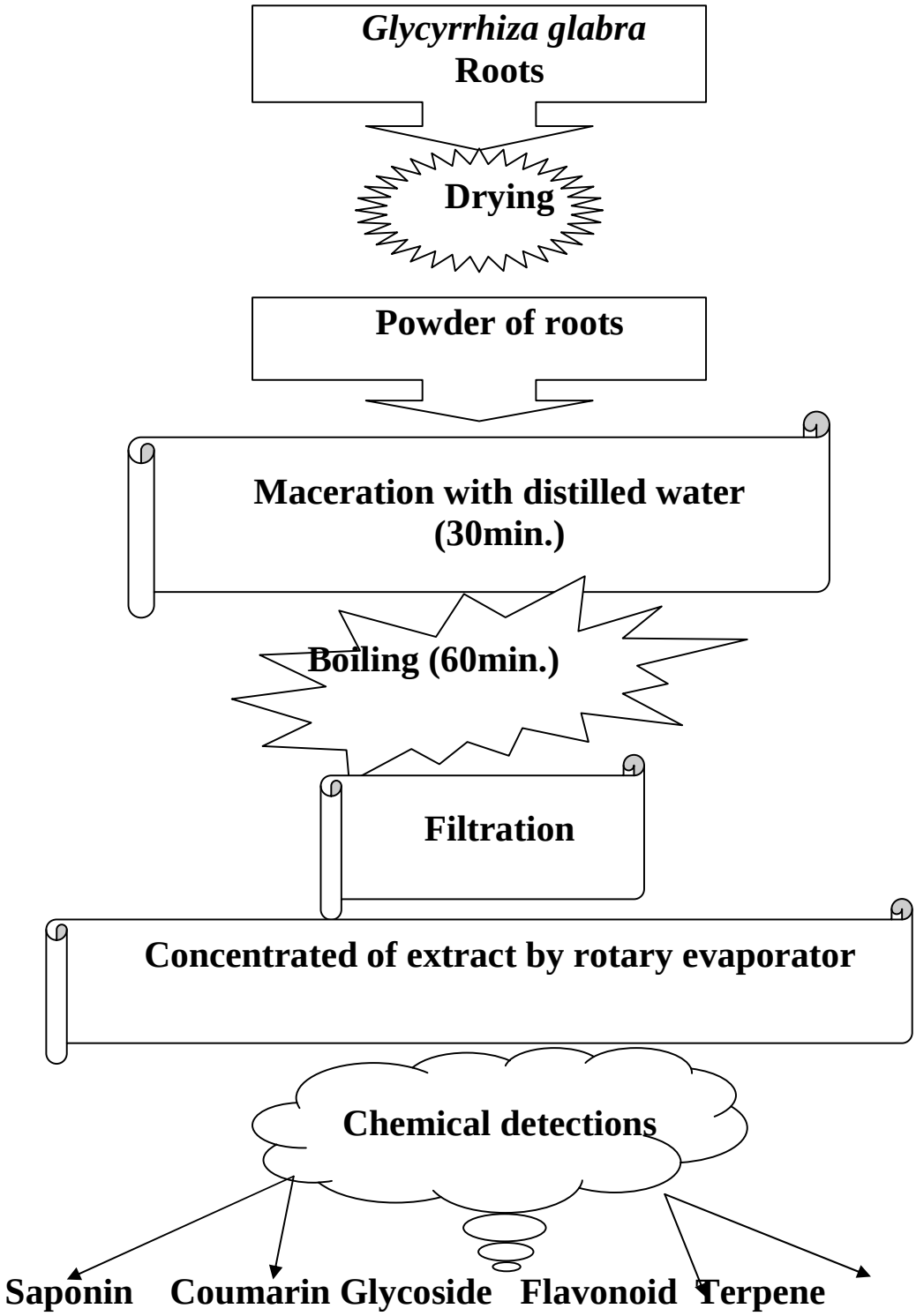
Solution A: 35 g of copper sulfate were dissolved in 100 ml of D.W., then diluted by D.W. until volume 500 ml.

Solution B: 7 g of sodium hydroxide and 175 g of roshail salt were dissolved in 100 ml of D.W., the volume was completed until 500 ml by adding distilled water.

A and B solutions were mixed in equal volume. This reagent used for glycosides detection.

3.4: Preparation of plant extract

The roots powder of *G. glabra* was macerated with sterilized distilled water in ratio of 1:10 (50 g of the powder in 500 ml of sterilized distilled water), the mixture was left for 30 minutes at room temperature, and then boiled for 60 minutes, and finally the suspension was filtered with filter paper (Whatman. No. 2). The filtrate was concentrated using rotary evaporator at 40°C as shown in (Figure 3-1) (Yoshimichi *et al*., 1986).



(Figure 3-1): Diagram of water extract of *Glycyrrhiza glabra*

3.5: Detection of some active compounds in *Glycyrriza glabra* roots extract

3.5.1: Detection of saponins

A: Plant extract was shacked vigorously in a test tube, the formation of foam remaining for few minutes indicated the presence of saponins (Al-Ani, 1998).

B: Five milliliters of the plant extract were added to 3 ml of ferric chloride solution, and the appearance of white precipitate indicated the presence of saponins (Al- Khazraji, 1991).

3.5.2: Detection of glycosides

- Non-hydrolyzed extract:

Equal volumes of the plant extract and Fehling reagent were mixed, and then left in a boiling water bath for 10 minutes, the appearance of red precipitate indicated the presence of free sugars.

- Hydrolyzed extract:

Few drops of 10% hydrochloric acid were added to 5 ml of the plant extract, then left in a boiling water bath for 20 minutes. The acidity was neutralized by adding few drops of sodium hydroxide solution, finally equal volume of Fehling reagent was added and the development of red precipitate was an indication for the aglycon part of the glycoside (Harborne, 1973).

3.5.3: Detection of flavonoids

Ten milliliters of 50% ethyl alcohol were mixed with 10 ml of 50% potassium hydroxide solution, then added to equal volume of the plant extract. The appearance of yellow color indicate the presence of flavonoids (Jafar *et al.*, 1983).

3.5.4: Detection of terpene

One gram of plant extract was precipitated in a few drops of chloroform, and then a drop of acetic anhydride and concentrated sulfuric acid were added. The appearance of dark brown color indicated the presence of terpenes (Al-Abid, 1985).

3.5.5: Detection of coumarins

Coumarins were detected when 3 ml of ethanol (95%) was mixed with 1 ml of plant extract in a test tube, then covered with a wet filter paper with sodium hydroxide solution. The test tube was placed in a boiling water bath for 10 minutes. The filter paper was exposed to UV-light source, the appearance of greenish – yellow color indicated the presence of coumarins (Al-Khazraji, 1991).

3.6: Laboratory animals

Swiss male mice (132) of BALB/c, which were obtained from Biotechnology Research Center, were used. Their age ranged between (8-12) weeks and weighting 23-27 gm. They were divided into 12 groups, each group was put in a separate plastic cage. The cages were put in a room with temperature (23°C-25°C). The animals were fed with a suitable quantity of water and standard pellets.

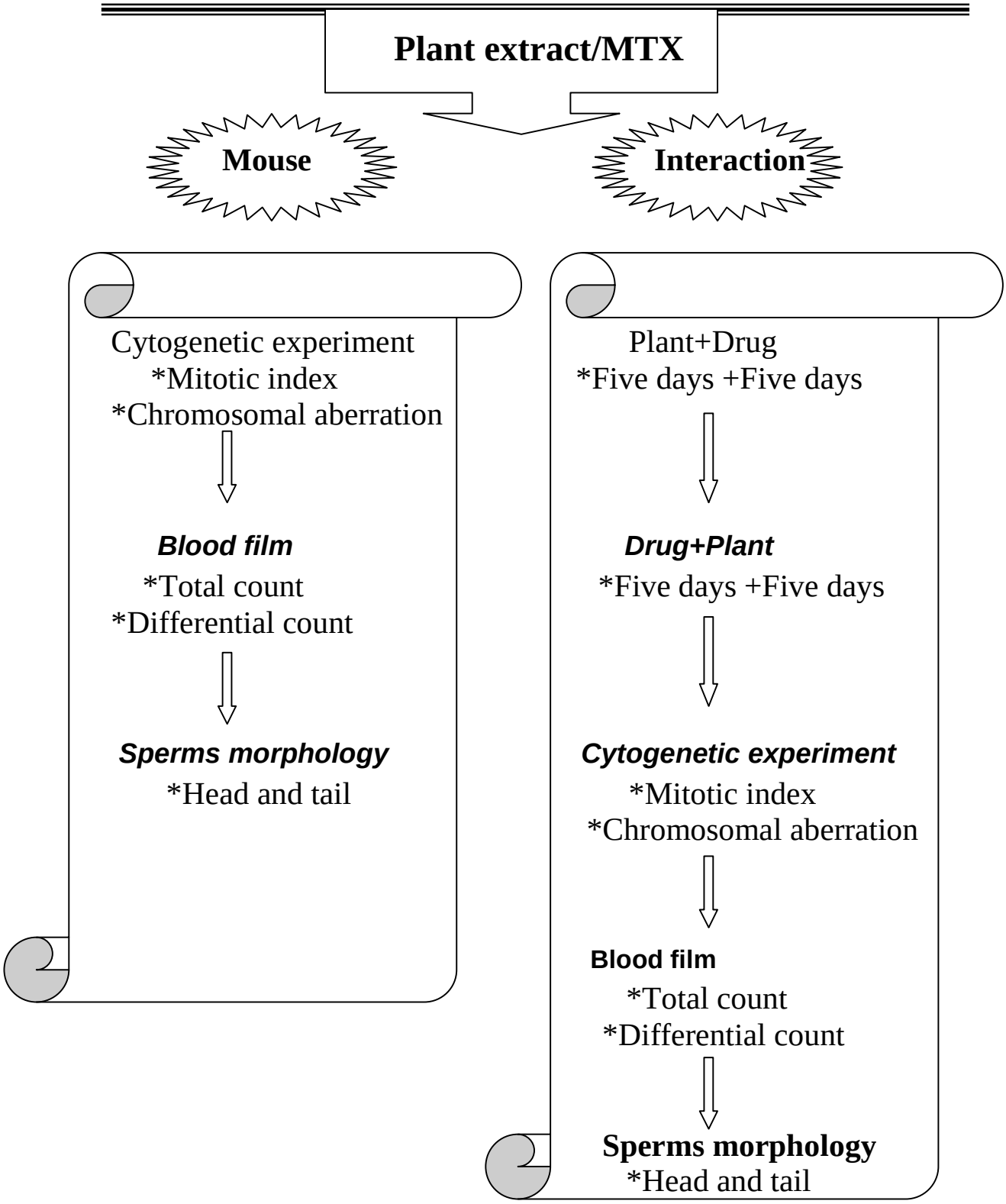
3.7: Preparation of methotrexate

Methotrexate was obtained from Hexal Company at a concentration of 25 mg/ml, and from this stock solution the concentrations 0.6,0.8,and 1 μg/ml were prepared to be used in mice studies. While in human blood culture studies, the stock solution (25 mg/ml) was used to prepare the following concentrations 0.4, 2,and 4 μg/ml, which then sterilized by filtration and kept at (4°C) until used.

3.8:preparation of *G. glabra* extract

One gram of dry extract was dissolved in (10 ml) of D.W. to make a stock solution, and from this stock the concentrations 100, 150, 200 mg/ml were prepared to be used in mice studies. While in human blood culture studies 0.5 gm of dry extract was dissolved in 100 ml of D.W., and this a stock solution, from this stock the

concentrations (50, 100, and 200)µg/ml was prepared and then sterilized by filtration and kept at (4°C) until being used.The genotoxicity assay for plant extract and MTX *in vivo* is outlined in (Figure 3-2).



(Figure3-2):Genotoxicity assay for *G.glabra* aqueous extract and methotrexate *in vivo*

3.9: Administration of drug in laboratory animals

3.9.1: Methotrexate

3.9.1.1: Cytogenetic experiment

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

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

Group I: Negative control (3 mice)

Treated with (0.05 ml) PBS.

Group II: MTX treatment (3 mice)

Treated with (0.05 ml) of MTX (1.2 mg/kg)

Group III: MTX treatment (3 mice)

Treated with (0.05 ml) of MTX (1.6 mg/kg)

Group IIII: MTX treatment (3 mice)

Treated with (0.05 ml) of MTX (2 mg/kg).

Methotrexate was given orally for 5 successive days, and then the mice were scarified after five days along with the first

group of control mice. Bone marrow samples were taken and cytogenetic analyses were carried out as described later (See 3.11.1).

3.9.1.2: Blood film

The aim of this experiment is to evaluate the effect of treatment of MTX on the total count of white blood cells and number of each type of white blood cells by applying total and differential count of leukocytes.

Two groups were taken for this experiment and treated as follow:

Group I: Negative control (3 mice)

Treated with (0.05 ml) PBS.

Group II: MTX treatment (3 mice)

Treated with (0.05 ml) of MTX (2 mg/kg).

Methotrexate was given orally for 5 days, and then mice were sacrificed after 5 days along with first group of control mice. Blood samples were taken from mouse heart, and the analyses of total and differential count were carried out as described later (See 3.12).

3.9.1.3: Sperm head and tail abnormality

Animals in this experiment were treated with MTX to

evaluate the abnormalities of head and tail in mice sperm.

Two groups of mice were taken and treated as follows:

Group I: Negative control (9 mice)

Treated with (0.05 ml) PBS.

Group II: MTX treatment (9 mice)

Treated with (0.05 ml) of MTX (2 mg/kg).

Methotrexate was given orally for 7, 21, 35 days, then mice were scarified along with the first group of control mice. Sperms samples were taken from vas deferens and the experiments were carried out as described later (See 3.13).

3.9.2: *Glycyrrhiza glabra*

3.9.2.1: Cytogenetic experiment

This experiment was carried out to detect the genotoxic effects of *G.glabra* aqueous extract in mice by applying cytogenetic analyses (MI, CA) in normal bone marrow cells. It also aimed to select the most suitable concentration of *G.glabra* aqueous extract to be used in next steps.

Four groups of mice were used and treated as follows:

Group I: Negative control (3 mice)

Treated with (0.1 ml) PBS.

Group II: *G.glabra* aqueous extract treatment (3 mice)

Treated with (0.1 ml) of extract (400 mg/kg).

Group III: *G.glabra* aqueous extract treatment (3 mice)
Treated with (0.1 ml) of extract (600 mg/kg).
Group IIII: *G.glabra* aqueous extract treatment (3 mice)
Treated with (0.1 ml) of extract (800 mg/kg).

Doses were given orally for 5 successive days, and then the mice were scarified along with the first group of control mice. Bone marrow samples were taken and cytogenetic analyses were carried out as described later (See 3.11.1).

3.9.2.2: Blood film

The ability of water extract of *G.glabra* to induce the immune system by increasing the production of white blood cells was the aim of this test.

Four groups of mice were used and treated as in (3.9.2.1). The doses were given orally for 5 successive days, then the mice were scarified along with first group of control mice. Blood samples were taken from heart of mice and the analyses of total and differential count were carried out as described later (See 3.12).

3.9.2.3: Sperm head and tail abnormality

The purpose of this experiment was to evaluate the ability of the water extract for *G.glabra* to reduce the abnormalities in sperm morphology.

Two groups of mice were used and treated as follows:

Group I: Negative control (9 mice)

Treated with (0.1 ml) PBS.

Group II: *Glycyrrhiza* aqueous extract treatment (9 mice)

Treated with (0.1 ml) of extract (800 mg/kg).

The doses were given orally for 7, 21, 35 days, then mice were scarified along with the first group of control mice. Sperms samples were taken from vas deferens and the experiment were carried out as described later (See 3.13).

3.10: The interaction studies

3.10.1: Treatment with *Glycyrrhiza* extract before the treatment with methotrexate

3.10.1.1: Cytogenetic analysis

Three groups of mice were used and divided as follow:

Group I: Negative control (3 mice)

Treated with (0.1 ml) PBS for five days then treated with (0.05 ml) of MTX (2 mg/kg) for five successive days.

Group II: Pre – treatment group (3 mice)

Treated with (0.1 ml) of *G.glabra* aqueous extract (800 mg/kg) for five successive days, then treated with (0.05 ml) of MTX (2 mg/kg) for five successive days.

Cytogenetic analyses (MI, CA) detected the inhibitory effect of extract on abnormalities in B.M cells by MTX treatment.

3.10.1.2: Blood film

Three groups of mice were used and divided as in (3.10.1.1).

The mice of these groups were scarified, and the blood samples were taken from mice heart for total and differential count of leukocyte.

3.10.1.3: Sperm head and tail abnormality

Three groups of mice were used and divided as in (3.10.1.1), and the mice were scarified and the sperm samples were taken from vas deferens to evaluate the rate of reduction in sperm abnormalities by extract treatment.

3.10.2: Treatment with *Glycyrrhiza* extract after the treatment with methotrexate

3.10.2.1: Cytogenetic analysis

Three groups of mice were used and divided as follow:

Group I: Negative control (3 mice)

Treated with (0.05 ml) of MTX (2 mg/kg) for five successive

days then treated with (0.1 ml) PBS for five days.

Group II: Post – treatment group (3 mice)

Treated with (0.05 ml) of MTX (2 mg/kg) for five successive days, then treated with (0.1 ml) of *G.glabra* extract (800 mg/kg) for five successive days.

The mice of these groups were scarified, and bone marrow samples were taken for cytogenetic analysis (MI, CA).

3.10.2.2: Blood film

Three groups of mice were used and divided as in (3.10.2.1).

The mice of these groups were scarified, and the blood samples were taken from mice heart for total and differential count of leukocyte.

3.10.2.3: Sperm head and tail abnormality

Three groups of mice were used and divided as in (3.10.2.1), and the mice were scarified and the sperm samples were taken from vas deferens to evaluate the rate of sperm abnormalities.

3.11: Cytogenetic experiments

3.11.1: Chromosomes preparation from somatic cell of the mouse bone marrow

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

1. Animals were injected with 0.25 ml of colchicine 1 mg/ml

intraperitoneally (I.P.) two hours before scarifying the animals.

2. The animals were scarified by cervical dislocation.

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

4. The femus 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 a centrifuge at a speed of 2000 rpm for (10 min.).

6. The supernatant was removed and (5ml) of 0.075 M potassium chloride (KCl) was added as a hypotonic solution, then the tubes were put in incubator at (37°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 test tube with continuous shaking, the volume was fixed to (5 ml) and the contents were shake well.

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

10. The tubes were centrifuged at 2000 rpm for 10 min. The process was repeated for times and the cells were suspended in (2

ml) of fixative.

11. By a Pasture pipette,a few drops from tube were dropped vertically on 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. Three slides for each animal were prepared for cytogenetic assays.

3.12: Blood film (Catalano, 2002)

3.12.1: Total count of leukocytes

1. The blood was taken by heart puncher and put into heparinized tube.

2. A diluting solution (190 µl) was pipetted into test tube.

3. The heparinized blood (10µl) was pipetted and mixed well with diluting fluid for at least 2 minutes.

4. The hemocytometer was sited up with its cover glass in position, and by Pasture pipette, both sides of the hemocytometer were filled with the diluted blood.

5. The cells were allowed for two minutes to be settled.

6. The cells were count in the four large squares on both sides of chamber using the 40x objective and subdued light.

7. The WBCs were calculated on the basis of cells counted, counted area, and the dilution factor.

No. of cells (cells/mm^3 blood) = no. of cells in four square ×correct volume × correct dilution /4

3.12.2: Differential count of leukocytes

1. A small drop of heprinazed blood was put on the end of clean, dry slides. A pusher slide was place at an angle of 30° to 45° to the slide and then moved back to make contact with the drop. The forward movement of the pusher spreads the blood on the slide.
2. The blood film was allowed to dry in the air.
3. The slides were completely covered with Leishman stain and after 3 minutes the slides were washed gently with Leishman buffer and then examined under light microscope.

No. of cells (cells/mm^3 blood) = (Total no. of leukocyte × Cells %) /100

3.13: Sperm head and tail abnormality

The experiment was done according to (Chemineau *et al*., 1991).

1. The animals were scarified by cervical dislocation, and fixed on it tergal side on the anatomy plate and the abdominal side of the animal was swabbed with 70% ethanol.

2. The caudal epididymis was cut and placed in a petri dish containing (2 ml) of PBS and minced by using microsurgical scissor and forceps.

3. Three drops of eosin stain were placed on the cleaned and dried slide.

4. One drops of diluted semen were added on the slide and mixed with the stain for (10 sec).

5. The mixture was left to stand for about (50 sec.).

6. The mixture, semen + colorant was spread under a second slide or cover slip by drawing a film of the mixture as thinly and regularly as possible.

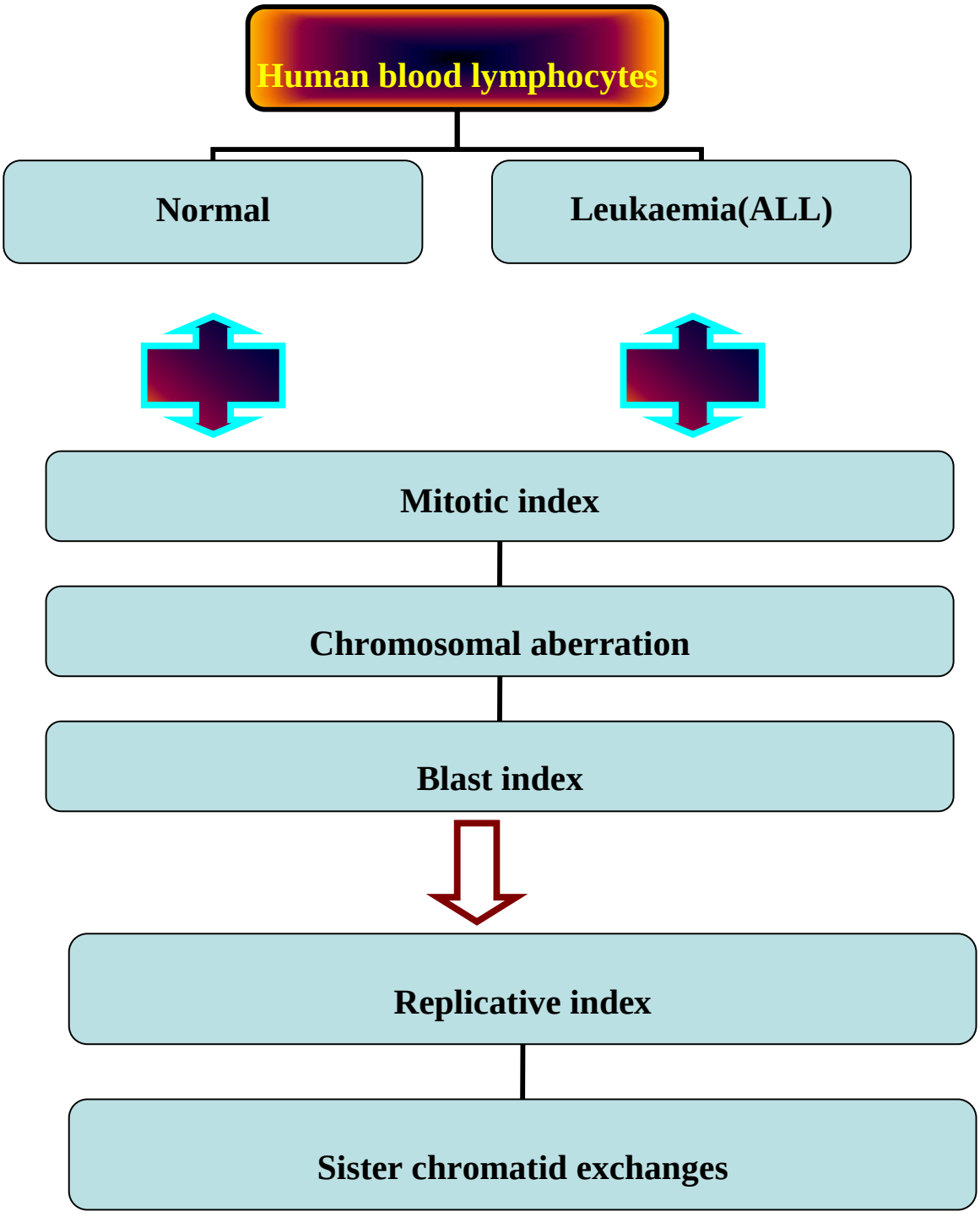
7. The slides were left in the warm place to dry then examined by the microscope.

3.14: Human blood lymphocyte culture

The blood from healthy persons were collected randomly from the students at Al-Nahrain University , their age ranged between (18-20) years , (5 ml) of blood were collected in hyperinizied syringe. Five samples of human blood were collected to examine the three different concentrations of MTX (0.4 , 2 ,and4 μg/ml) then selection the most harmful concentration through it is effects on (BI,MI,RI,SCE, and CA),also these samples were collected to examine the three different concentrations of *G.glabra* extract

(50,100,and 200)µg/ml on (BI,MI,RI,SCE, and CA)in order to select the lowest side effects to be used in next step. A schematic presentation such procedure is outlined in (Figure 3-3).

While the samples for leukaemia of (ALL) type were collected from Baghdad Teaching Hospital, five samples of blood were collected from patients their age range between (15-20) years, and cytogenetic analyses (BI,MI,RI,SCE, and CA) were carried out on the most harmful concentration of MTX(4µg/ml) and on the lowest side effects concentration of *G.glabra* extract(50µg/ml) after 72 hours of incubation.



(Figure3-3):Antileukaemia potency for *G.glabra* aqueous extract and methotrexate *in vitro*

3.15: Experimental design

3.15.1: Normal blood

Seven groups from healthy persons were used for cytogenetic analyses of human blood lymphocytes:

Culture No. 1: Negative control.

Culture No. 2: Positive control (0.4 μg/ml of MTX).

Culture No. 3: Positive control (2 μg/ml of MTX).

Culture No. 4: Positive control (4 μg/ml of MTX).

Culture No. 5: *G.glabra* extract (50 μg/ml of extract).

Culture No. 6: *G.glabra* extract (100 μg/ml of extract).

Culture No. 7: *G.glabra* extract (200 μg/ml of extract).

3.15.2: Leukaemia

After choice the best concentration of water extract and MTX, three groups were used in this experiment and treated as follow:

Culture No. 1: Negative control.

Culture No. 2: Positive control (4 μg/ml of MTX).

Culture No. 3: *G.glabra* extract (50 μg/ml).

3.16: Cytogenetic analysis of human blood lymphocytes (Shubber, 1987)

1. Human blood was collected in a heparinized syringe.
2. (0.25ml) of peripheral blood was added into test tube containing

(2 ml) of culture medium (RPMI – 1640).

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

4. After (24) hours of incubation, different concentrations of MTX (0.4, 2, 4 μg/ml) were added to each test tube (0.1 ml).

5. The test tubes were taken and put in the incubator at (37°C), and shacked gently each (24 hours) one try at least. The incubation period was completed to (72 hours).

6. (0.1mg/ml) of colchicine was added to each test tube (1/2 hours) before harvesting the cells, and gently shacked 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 (0.075 M) KCl was added as a hypotonic solution, then the test tubes were left for (30 min.) in the incubator at (37°C) and the tubes were shaken 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, then, the volume was fixed to (5 ml), and the contents were shaken well.

11. The tubes were transferred into the centrifuge at 2000 rpm for (10 min.). The process was repeated for 3 times, then the cells

were suspended in (5 ml) of the fixative solution.

12. By a Pasture pipette, few drops from the tube were dropped vertically on the chilled 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.

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

14. Three slides for each concentration were prepared for cytogenetic assays.

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

3.16.1: Hoechst (33258) staining

(Ian-Freshney, 2000)

1. The slides were immersed in a coplin jar Hoechst 33258 at a concentration of (20 µl/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 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 slides 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 sourensens 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.

3.17: Cytogenetic analyses test

3.17.1: Mitotic index (MI) assay

The slides were examined under (40X) 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:

$$MI = \frac{\text{No. of the divided cells}}{\text{Total no. of the cells}} \times 100$$ (Shubber *et al*.,1987)

3.17.2: 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 aberration are clear and the percentage of these aberrations could be estimated.

3.17.3: Replicative index (RI) assay

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

$$RI = \frac{(1\times M_1\%) + (2\times M_2\%) + (3\times M_3\%)}{100}$$

3.17.4: Sister chromatid exchang (SCE) assay

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

3.17.5: Blastogenic index (BI) assay

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

$$\frac{\text{No. of the blast cells}}{\text{Total no. of the cells (1000)}}$$ (Al-Shawk.1999)

3.18: The protective value of aqueous extract of *Glycyrrhiza glabra*

The protective value of *G.glabra* extract was calculated according to the following equation:

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

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

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

C = interaction group (treatment with MTX and *G.glabra* aqueous extract).

(Rawat *et al*., 1997).

3.19: Statistical analyses

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

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4.1: Detection of some active compounds in *Glycyrrhiza glabra extract*

By using different chemical reagents and solutions a detection of various active compounds in *Glycyrrhiza* glabra was achieved, those were represented in (Table 4-1), and the results indicated that G. *glabra* contains saponins, glycosides, flavonids, terpenes, and coumarins .

Table (4-1): Detection of some active compounds

Chemical Compounds	Reagents Used	Indication	Results of Detection
Saponins	1.Shaking for water extract 2.Ferric chloride	1.Foaming form 2.White precipitate	+ve +ve
Glycosides (Before)	Fehling	Red Precipitate	+ve
Glycosides (After)	Fehling	Red Precipitate	+ve
Flavonoids	Ethyl alcohol + potassium Hydroxide +plant extract	Yellow Color	+ve
Terpenes	Plant extract +chloroform +acetic anhydride +sulfuric acid	Dark brown Color	+ve
Coumarins	Ethanol+ plant extract (wet filter paper with NaOH) +water bath +U.V.	Greenish- yellow Color	+ve

+ve indicates the presence of the active compound.

4.2: Cytogenetic effect of aqueous extract of *Glycyrrhiza glabra*

4.2.1: Cytogenetic effect of *G.glabra* extract on mouse bone marrow cells

4.2.1.1: Effect on mitotic index (MI)

Three doses of aqueous extract from this plant have been used for mice, those were (400,600,800) mg/kg, and the best concentration has been chosen depending on the induction of MI and reduction in CAs, and the results showed that all these three doses led to an increase in the mitotic index (5.9%, 6.6%, and 7.2%), respectively as compared with negative control (5.2%), but non significantly increase in first concentration while the increase was significant ($P \leq 0.05$) in second and third concentration ,as shown in (Table 4-2) .

Aburada and his partner (1983) have shown that the polysacchride (PLS) of *G. glabra* root has an important role as inducers for immunity through the increase of the activity for T-lymphocytes by the induction of stem cells in mice bone marrow, in addition the extract contained mitogenic agents in its constituents that stimulated cell division after treatment with this extract (Tachipana *et al*., 1993).

These results were in agreement with Al-Obaidi (2002), who indicated that *G. glabra* water extract had a mitogenic activity so could induce cell division.

4.2.1.2: Effect on chromosomal aberrations

Aqueous extract shows the ability to reduce the spontaneous CAs from (0.77%) in the negative control to (0.59%,0.43%,and 0.32%)for the three doses (400,600,800)mg/kg ,respectively (Table 4-2).These results were significantly different ($P \leq 0.05$)in comparison with control. All these reductions in CAs may be related to isoflavans group (glabridin, hispaglabridin A, hispaglabridin B, 4-0- methylglabridin, isoliquiritigenin, and chalcones), which act as anti –oxidant (Vaya *et al*., 1997), additionly the glabridin has the ability of inhibition of spontaneous and induced mutations (Mitscher *et al*., 1985).

Further evidence supporting such results may be observed in mouse bone marrow (Al-khayat), (1999) in which the rate of CAs reduced after extract treatment.

Table (4- 2): Cytogenetic effects of aqueous extract from *G.glabra* in mouse bone marrow *in vivo*.

Groups	Mitotic Index % m±SE	Chromosomal aberration % m±SE						
		Chromatid Break	Chromosome Break	Acentric	Dicentric	Ring	Deletion	Total of CA
Negative control	A 5.2±0.11	A 0.1±0.01	A 0.02±0.005	A 0.12±0.01	AB 0.17±0.02	A 0.06±0.01	AB 0.3±0.01	A 0.77±0.01
Positive control MTX	B 1.8±0.11	B 0.17±0.01	B 0.17±0.02	B 0.27±0.01	B 0.22±0.02	B 0.12±0.005	B 0.36±0.01	B 1.31±0.02
400 mg/kg *G.glabra* extract	AC 5.9±0.12	C 0.05±0.005	C 0.02±0.005	C 0.12±0.01	A 0.13±0.01	AC 0.04±0.005	AC 0.23±0.01	C 0.59±0.01
600mg/kg *G.glabra* extract	CD 6.6±0.54	C 0.05±0.01	C 0.01±0.00	C 0.1±0.01	B 0.05±0.005	CD 0.02±0.01	C 0.2±0.05	D 0.43±0.02
800 mg/kg *G.glabra* extract	D 7.2±0.34	C 0.03±0.005	C 0.00±0.00	C 0.09±0.01	B 0.05±0.005	D 0.00±0.00	C 0.15±0.01	E 0.32±0.005

Numbers with different letters are significantly different (≤ 0.05) to compression columns

4.2.2: The effects of aqueous extract from *Glycyrrhiza glabra* on sperm head and tail morphology

The results of this experiment are displayed in Table 4-3, which indicated that the aqueous extract from this plant had the ability to increase the normal number of sperms from (64.5%) in negative control to (65.67%, 73%, and 77.4%) after 7, 21,and 35 days of treatment, respectively, and this was significant ($P \leq 0.05$) at 21, and 35 days of treatment but non-significant at 7 days of treatment as comparison with negative control.

As a result of extract treatment, the abnormalities of head and tail decreased, in head the abnormalities decreased from (19.83%) in the negative control to (18.83%, 12%, and 10.2%) after 7,21, and 35 days of treatment, respectively. This reduction, however, was not significant after 7 days of treatment but it was significant after 21, and 35 days of treatment, while in tail the abnormalities were decreased from (15.67%) in negative control to (15.5%, 15%, and 12.4%) after 7, 21, and 35 days of treatment, respectively. This reduction was not- significant in all periods of treatment as compared with negative control, as shown in (Table 4-3).

Abnormalities of head and tail decreased especially coiled tail, headless and broken tail, and this decrease was observed especially after 35 days of treatment in which the sperms complete the spermatogenesis and became mature, and this reduction effect of extract on abnormalities may be related to

some active compounds of *G. glabra* like isoflavans groups which act as anti-oxidants or scavengers for free radicals so could cause reduction in abnormalities of sperms. It was found that glabridin in isoflavans group was better than hispaglabridin A, hispaglabridin B, formononetion, o-methyl glabridin, isopropyl chalcone, isoprenylchalcone, isoliquiritigenin, and chalcones as anti-oxidant compounds (Vaya *et al*.,1997).

Table (4-3): Effects of aqueous extract of *G.glabra* in sperm head and tail morphology *in vivo*

Treatment Period	Normal sperms% m±SE	Head abnormality% m±SE	Tail abnormality% m±SE
Negative Control	A 64.5±1.27	A 19.83±2.07	A 15.67±1.67
7 days *G.glabra* extract	A 65.67±2.36	A 18.83±1.44	A 15.5±3.75
21 days *G.glabra* extract	B 73.0±0.57	B 12.0±0.57	A 15.0±0.57
35 days *G.glabra* extract	B 77.4±0.28	B 10.2±0.11	A 12.4±0.17

Numbers with different letters are significantly different (≤ 0.05) to compression columns

4.2.3: Effects of aquous extract of *Glycyrrhiza glabra* on total and differential count of white blood cells

The present study may highlight on the effects of plant extract in the variation of production in leukocytes.

(Table 4-4) shows that the different concentrations of extract caused an increase in the total number of leukocytes, which reached (8550, 9900, 12700) cells/cu.mm.blood for the (400,600,and800) mg/kg respectively after five days of treatment. The first concentration gave non-significantly different, while the second and third concentration were significantly different ($P \leq 0.05$) as compared with the negative control (8050 cells/cu.mm.blood).

The effect of extract treatment on the cells number of immune system were represented in (Table 4-4), which indicated that the number of lymphocytes increase with the increasing of concentrations of extract to reach (4788, 6039, 8255) cells/cu.mm.blood respectively. These were significantly different ($P \leq 0.05$) as compared with negative control (4105.5cells/cu.mm.blood).

Another type of leukocytes that effected by extract treatment was neutrophil, which was in normal mice (2254 cells/cu.mm.blood), and inhibition in number of neutrophil noticed with the increasing of the concentration of extract (2137, 2079, 2032) cells/ cu.mm.blood but the difference was non-significant as compared with negative control (Table 4-4).

Water extract of *G. glabra* as in lymphocytes caused an increased in number of monocyte *in vivo*, which reached to (1624.5, 1782, 2413) cells/cu.mm.blood with the increasing of concentration, this increase was not significant at the doses of (400,600) mg/kg, while the significant increase was clear at the dose of (800mg/kg) when compared with negative control (1529.5 cells/cu.mm.blood)(Table 4-4).

Another effect of water extract was on the number of eosinophil and basophil that reached to zero after treatment with the three concentrations of extract, so significant different ($P \leq 0.05$) was observed as compared with negative control (80.5 cells/cu.mm.blood) of basophil and eosinophil (Table 4-4).

The blood test showed an increase in total number of leukocytes especially lymphocyte and monocyte, while the decreasing observed in neutrophil ,basophil and eosinophil ,all these effects because of the active compounds of the extract which have medicinal activity (Evans,1999),like terpenoids saponins (glycyrrhizin ,glycyrrhetinic acid),glycosids ,flavonoids ,coumarin ,polysaccharide ,volatile oils ,and some minerals and vitamins which have important roles as immunostimulatores (Beresford,1999),and this increase was cleared in leukoctes especially lymphocyte which be as a regulator of immune response through it is secretion to cytokines which act as activator of immune system like phagocytes and the induction of B-cell to produce antibody

(Roitt and Rabson ,2000), additionly ,the increase in number of monocytes may have important role in phagocytosis (Rosenbery and Gallin ,1999) .

The effect of water extract on total number of leukocytes was in agreement with Al-Obaidi (2002), who showed the ability of *G.glabra* extract to increase the total number of leukocytes.

Table (4-4) Effects of aqueous extract of *Glycyrrhiza glabra* in differential and total count of blood *in vivo*

Groups	Total count cells /cu. mm.blood m±SE	No. of lymphocyte cells /cu. mm. blood	No.of neutrophil cells /cu. mm. blood	No. of monocyte cells/ cu. mm.blood	No. of eosinophil cells /cu. mm. blood	No. of Basophil cells / cu. mm. blood
Negative control	A 8050±5.7	A 4105.5±2.75	AB 2254±52	A 1529.5±78.7	A 80.5±3.5	A 80.5±1.9
Positive control MTX	B 5100±2.3	B 1020.0±45	B 2448±114	B 510±5	B 561±65.5	B 561±15.5
400 mg/kg *G.glabra* extract	AC 8550±3.5	C 4788±106	AB 2137±62.5	A 1624.5±96	C 0.00±0.00	C 0.00±0.00
600 mg/kg *G.glabra* extract	C 9900±4.0	D 6039±36	AB 2079±65.5	AC 1782±125	C 0.00±0.00	C 0.00±0.00
800 mg/ kg *G.glabra* extract	D 12700±2.0	E 8255±202.5	A 2032±68.5	C 2413±348.5	C 0.00±0.00	C 0.00±0.00

Numbers with different letters are significantly different (≤ 0.05) to compression columns

4.2.4: Cytogenetic effect of *G. glabra* aqueous extract on human blood lymphocytes from normal individuals (No.=5)

Five cytogenetic parameters were introduced to evaluate the cytogenetic effects, they were BI, MI, RI, SCE and CA.

The first parameter was calculated to detect this cytogenetic effect of *G. glabra* extract on normal individuals was BI, and this calculation indicated the present of increase in BI at the concentrations (50,100)µg/ml, and reduction in BI at the concentration (200µg/ml), this variation was significant ($P \leq 0.05$) at the concentration of (50,200)µg/ml, but gave non-significant variation at the concentration of (100µg/ml) as compared with negative control, as shown in (Table 4-5).

The mitotic response of human blood cells from healthy individuals was (2.38%). This response increased at the concentrations (50,and 100)µg/ml reaching (5.06%, and 2.74%), while a reduction was observed in third concentration(200µg/ml)reached (1.56%), and those effects were dose dependent. These variations were significant ($P \leq 0.05$) at the concentrations of (50,200) µg/ml, but non-significant at the concentration of (100µg/ml) when compared with negative control, as shown in (Table 4-5).

The effect of extract on (RI) in human blood culture was observed, which caused significant variation ($P \leq 0.05$) in RI in

the (50µg/ml), but non significant in (100,200) µg/ml as compared with negative control, as shown in (Table 4-6).

Sister chromatid exchanges evaluation revealed that SCE decreased after treatment with (50,and 100)µg/ml of extract while SCE increased after treatment with (200µg/ml) of extract but it was non significant when compared with negative control, as shown in (Table 4-6).

Other cytogenetic effects of extract *in vitro* were the decrease in the spontaneous frequencies of (CAs) at concentrations of (50,and100)µg/ml, but the increase was observed at concentration of (200µg/ml), these variations were significant ($P \leq 0.05$) as compared with negative control, as shown in (Table4-7).

In human the increase in concentrations of extract led to decrease in MI this was because of some active compounds in extract which have cytotoxic activity like B-sitosterol (Trease and Evans, 1983), that occupied the cholesterol receptor of animal cell membrane and this could inhibit the cell division (Hayatsu *et al*., 1988), also Davidson and his partner (1986) suggested that glycyrrhetinic acid (GA) and carbenoxolene may cause communication intracellular gap-Functional of human fibroblasts, so would delay proteins passing between cells which have important role in cell division.

These results were in agreement with Al-Khayat (1999).

Table (4-5): Cytogenetic effects of methotrexate and *G.glabra* aqueous extract on human blood lymphocytes of healthy individual *in vitro*.

Groups	Blast Index % m±SE	Mitotic Index % m±SE
Negative control	A 42.52±1.5	A 2.38±0.16
Positive control 0.4µg/ml MTX	B 32.18±0.4	B 0.76±0.15
Positive control 2µg/ml MTX	B 29.7±0.9	B 0.26±0.03
Positive control 4 µg/ml MTX	C 27.08±0.7	B 0.0±0.0
50 µg/ml *G.glabra* extract	B 50.54±1.3	C 5.06±0.5
100 µg/ml *G.glabra* extract	A 42.94±0.6	A 2.74±0.1
200 µg/ml *G.glabra* extract	D 33.84±0.4	D 1.56±0.1

Numbers with different letters are significantly different (≤ 0.05) to compression columns

Table (4-6): Effects of methotrexate and *G.glabra* aqueous extract on cell cycle progression on human blood lymphocytes from healthy individual *in vitro*

Groups	Replicative Index % m±SE	Cell Cycle Progression % m			Sister chromatid exchange % m±SE
		M1	M2	M3	
Negative control	AB 1.93±1.1	37	33	30	A 8.2±0.2
Positive control 0.4 µg/ml MTX	AB 1.92±0.8	39	30	31	B 10.7±0.1
Positive control 2 µg/ml MTX	A 1.91±0.6	41	27	32	B 12±0.8
50 µg/ml *G.glabra* extract	B 2.03±0.9	34	29	37	A 7.5±0.2
100 µg/ml *G.glabra* extract	AB 1.97±0.7	36	31	33	A 8±0.04
200 µg/ml *G.glagra* extract	A 1.87±0.8	39	35	26	A 9±0.08

Numbers with different letters are significantly different (≤ 0.05) to compression columns

Table (4-7): Chromosomal aberration variation by methotrexate and *G. glabra* aqueous extract treatment on human blood lymphocyte from healthy individual *in vitro*

Groups	Chromosomal aberration % m±SE						
	Chromatid Break	**Chromosome Break**	Acentric	Dicentric	**Ring**	**Deletion**	**Total of CA**
Negative control	A 0.04±0.003	**A** 0.0±0.0	A 0.05±0.004	A 0.01±0.004	A 0.0±0.0	A 0.1±0.008	**A** 0.2±0.008
***positive control 0.4µg/ml MTX**	**B** 0.08±0.004	B 0.04±0.004	**AB** 0.08±0.004	**A** 0.16±0.003	**A** 0.0±0.0	**B** 0.16±0.003	**B** 0.52±0.008
***positive control 2 µg/ml MTX**	**C** 0.12±0.004	C 0.08±0.004	**B** 0.12±0.04	**A** 0.2±0.01	**B** 0.08±0.004	**C** 0.24±0.004	**C** 0.84±0.01
50 µg/ml *G.glabra* extract	**D** 0.01±0.004	A 0.0±0.0	**A** 0.03±0.004	**A** 0.0±0.0	**A** 0.0±0.0	**A** 0.08±0.004	**D** 0.12±0.01
100 µg/ml *G.glabra* extract	**D** 0.02±0.004	A 0.0±0.0	**A** 0.03±0.004	**A** 0.01±0.004	**A** 0.0±0.0	**A** 0.1±0.004	**D** 0.16±0.004
200 µg/ml *G.glabra* extract	**B** 0.08±0.003	D 0.01±0.004	**A** 0.06±0.004	**A** 0.12±0.004	**A** 0.0±0.0	**D** 0.12±0.004	**E** 0.39±0.008

Numbers with different letters are significantly different (≤ 0.05) to compression columns

*The chromosomal aberration of MTX calculated in 25 mitosis because of the inhibitory effect of drug on MI

4.3: Cytogenetic effect of methotrexate

4.3.1: Cytogenetic effect of methotrexate on mouse bone marrow cells

4.3.1.1: Effect on mitotic index (MI)

Under normal experimental conditions, white mice have a mitotic index of (5.2%) in their bone marrow cells (Table4-2). This considered as a negative control.

Three concentrations of MTX have been used as positive control (0.6,0.8,1)µg/ml, and all these concentrations led to the significant reduction ($p \leq 0.05$) in MI of mouse bone marrow cells as compared with negative control reaching (2.2%, 2%, and 1.8%), respectively, so the concentrations (0.6,0.8)µg/ml hasn't been taken and according to this the selected concentration of MTX was (1µg/ml) because of its high cytotoxic effects on bone marrow cells.

These results indicated that MTX in those range of concentrations resulted in the reduction of MI in mouse bone marrow cells, and this reduction was dose depended. This may be related to MTX that limit the intracellular supply of reduced folates (Tetrahydrofolate) through inhibition of dihydrofolate reductase (DHFR) and, therefore, is considered inducing cytotoxic effect with resultant inhibition of new DNA, thymidylate and purine synthesis so, the MTX is a cell cycle S-phase selective (Li and kaminskas, 1984).

These results were agreed with the results of Al-Amiry (1999), who found that the MTX caused reduction in MI of mouse bone marrow cells.

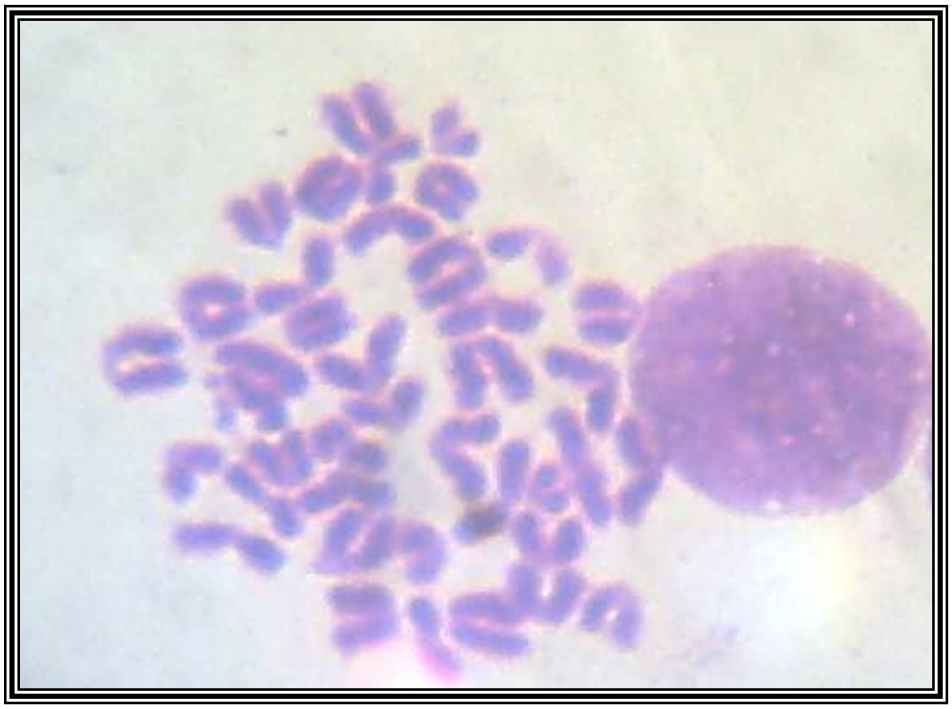
4.3.1.2: Effect on chromosomal aberration

The spontaneous frequency of chromosomal aberration in mouse bone marrow cell was (0.77%) which represented as negative control (Table 4-2).

The results of this experiment indicated that the MTX concentration (1µg/ml) treatment could increase CAs frequencies (chromatid break, chromosome break, acentric, dicentic, ring, and deletion) (Figure 4-1), which reached 1.31% after five days of MTX treatment, and these results were significantly different ($p \leq 0.05$) from the negative control.

In cells treated with MTX, a progressive accumulation of strand break in mature DNA (post-replicate DNA) was detected (Kasahara *et al*., 1992). Hence, DNA strand breaks arise from spontaneous and normally repaired DNA lesions that are not repaired and there is also evidence that MTX influences the cellular topoisomerase II content and causes, therefore, an increase in DNA breaks (Holden *et al*., 1995).

These results are in agreement with Al-Shimary (2004), who indicated that the MTX had the ability to induce the chromosomal abnormalities especially, ring chromosome, chromosome break, chromatid break, and dicentric chromosome.



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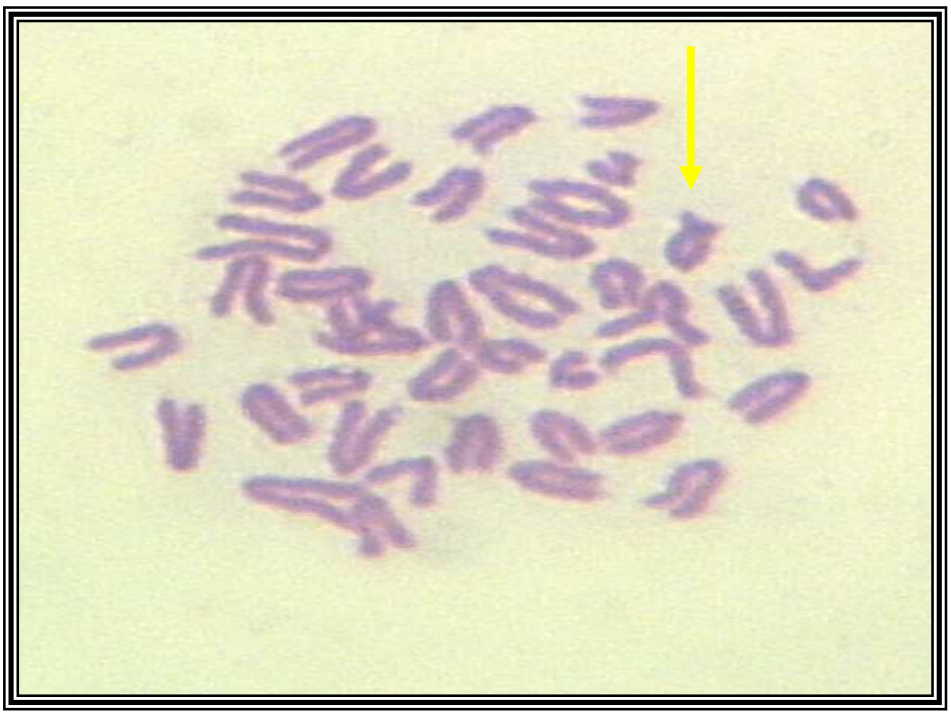

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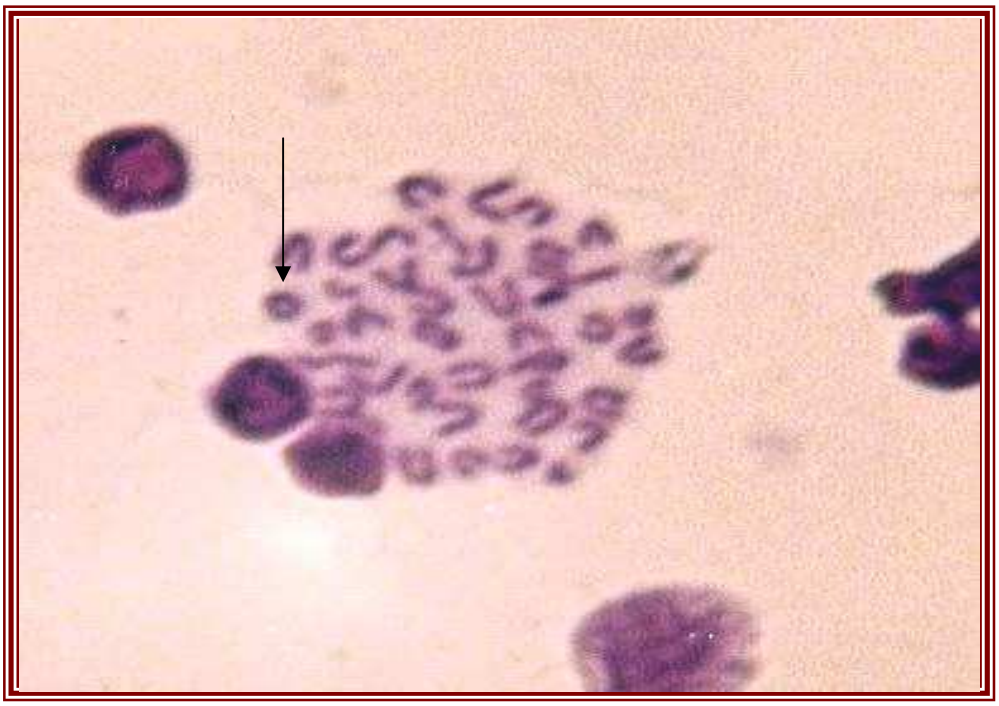

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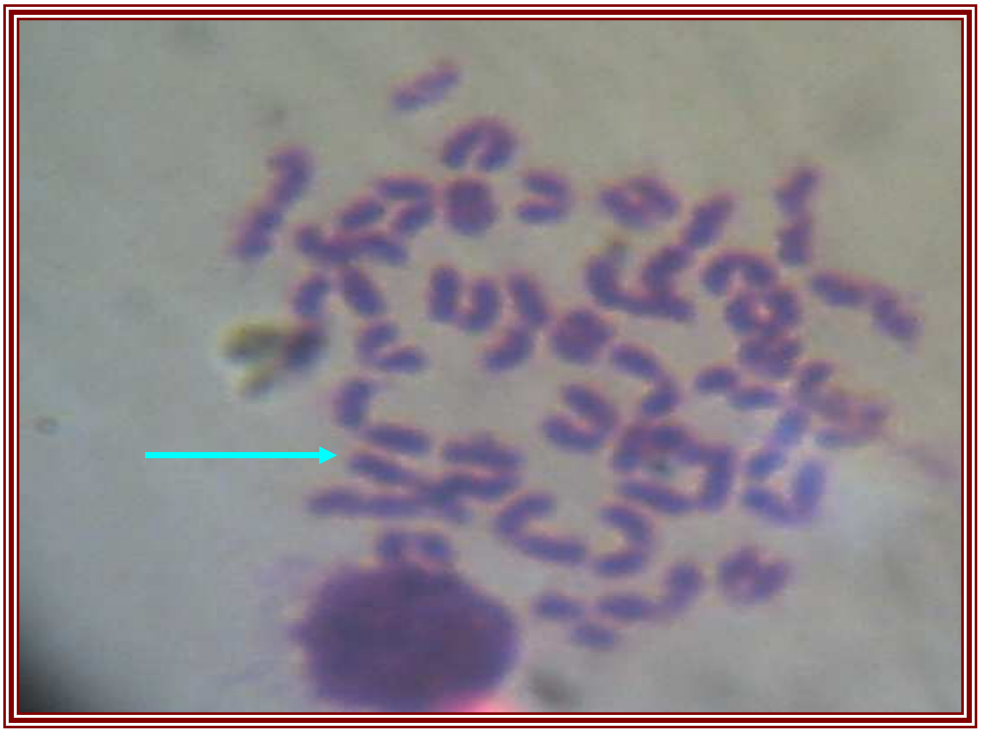

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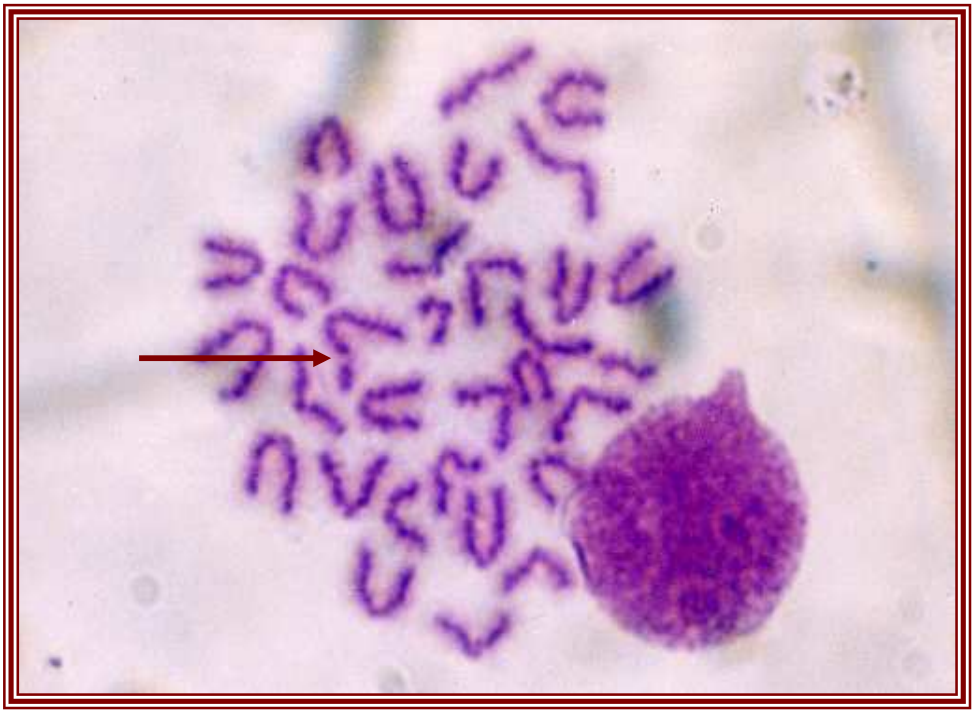

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(Figure 4-1):Metaphase of bone marrow cells from mouse treated with MTX, showing : normal chromosomes (A) ,dicentric Chromosome (B), ring chromosome (C),deletion (D) and chromatid break (E).(100 X)

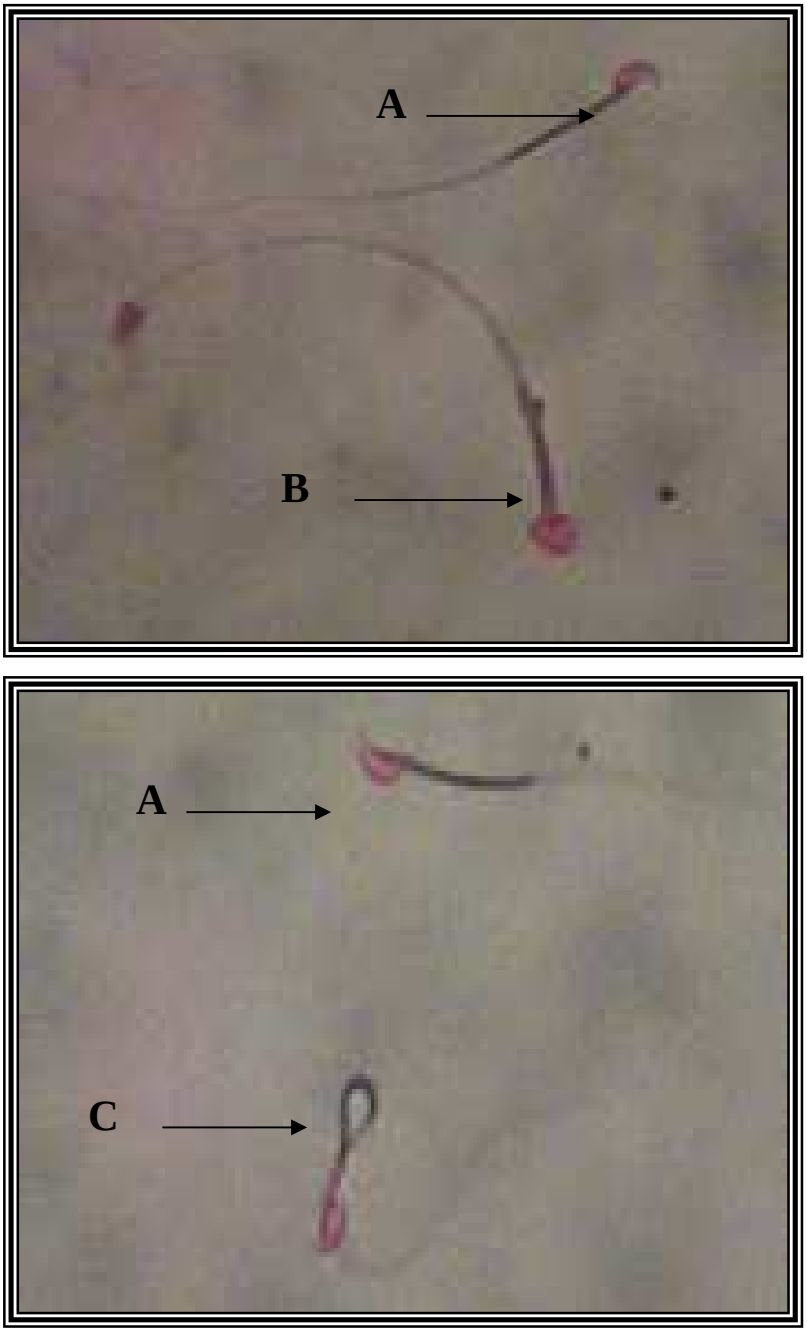
4.3.2: The effect of methotrexate on sperm morphology of tail and head *in vivo*

The investigation of this experiment indicated that the MTX had the ability to reduce the normal number of sperms from (64.5%) in the negative control to (47.7%, 38.3%, 25%) after 7,21, and 35 days of treatment, respectively, and this reduction was significant ($P \leq 0.05$) comprised with negative control (Table 4-8).

As a result of MTX treatment, the abnormalities of head and tail increased as in Figure4-2, in head the abnormalities increase from (19.83%) in the negative control to (27.5%, 31.4%, and 40%) after 7, 21, and 35 days of treatment, respectively, and this was significant ($P \leq 0.05$) in comparison with negative control, also the abnormalities of tail increased from (15.67%) in the negative control to (24.8%, 30.3%, and 35%) after 7, 21 and 35 days of treatment, respectively, and this was significant ($P \leq 0.05$) in comparison with negative control (15.67%), as shown in (Table 4-8).

Male reproductive function is under hormonal control, and spermatogenesis process is under control of FSH and testosterone (Seeley *et al*., 1996), while the formation of type A spermatogonia and conversion of primary spermatocyte into secondary spermatocyte (meiosis I) are dependant on testosterone and the final step of maturation of spermatids are dependant on FSH (Ganong, 1991), so the abnormal sperm

morphology may reflect an abnormal intratesticular maturation as a result of drug treatment (Acosta *et al*.,1988). Also the drug induce an alteration in androgen secretion that usually produce changes in the reproductive system, such changes might include the production of abnormal sperms (Tesarik *et al*., 1992), also any effect on spermatogenesis leads to production of abnormal sperms (Arab *et al*.,1989).



(Figure 4-2): - Effect of methotrexate on sperm morphology, showing: normal sperm (A), sperm of hammer head (B), sperm of curve tail(C) (40 x)

Table (4-8): Effect of methotrexate on sperm head and tail morphology *in vivo*

Treatment period	Normal sperms% m±SE	Head abnormality% m±SE	Tail abnormality% m±SE
Negative control	A 64.5±1.27	A 19.83±2.07	A 15.67±1.67
7 days MTX	B 47.7±4.04	***B*** 27.5±2.88	B 24.8±1.32
21 days MTX	C 38.3±1.78	BC 31.4±1.78	C 30.3±1.03
35 days MTX	D 25±2.07	C 40.0±2	C 35±1.73

Numbers with different letters are significantly different (≤ 0.05) to compression columns

4.3.3: Effects of methotrexate on total and differential count of white blood cells *in vivo*

Depending on our results of untreated mice, the total count of leukocytes was (8050 cells /cu.mm.blood) in their blood (Table 4-4). This considered as a negative control.

(Table 4-4) have shown that the MTX (1µg/ml) caused a significant reduction (P≤0.05) in the total number of leukocytes (5100 cells /cu.mm.blood) as compared with negative control.

The effect of MTX treatment on immune response can be detected by variation in normal count of lymphocytes ,our results indicated that the number of lymphocytes decreased with MTX treatment (1µg/ml) to reach (1020 cells/cu.mm.blood), this result was significantly different (P≤0.05) as compared with negative control (4105.5 cells /cu.mm.blood) (Table 4-4).

Methotrexate also caused a decrease in number of monocyte that reached to (510 cells/cu.mm.blood), and this result significantly different (P≤0.05) as compared with negative control (1529.5cells/cu.mm.blood) (Table 4-4).

While the number of neutrophil in treated mice increase after five days of treatment to reach (2448 cells/cu.mm.blood), this different was non-significant as compared with negative control (2254 cells/cu.mm.blood) (Table 4-4).

The same inducing effect of MTX in neutrophil was observed on the number of eosinophil and basophil which reached 561 cells/cu.mm.blood, so it significant (P≤0.05) in

basophil and eosinophil as compared with negative control (80.5 cells/cu.mm.blood) (Table 4-4).

This test indicated the occurring of an increase in the number of leukocytes especially basophil, eosinophil, and neutrophil, while a decrease observed in lymphocyte, and monocyte. One possible explanation for the action of MTX is the reduction in size and reactivity of the lymphocyte by the induction of apoptosis in activated T-cells (Genestier *et al*., 1998), or by MTX suppressing the production of both TNF and IFN by T-cell, also the MTX treatment had the ability of the reduction in monocytic 1L-1 production (Chang *et al*., 1992).

These results are in agreement with Genestier and his partner (1998) who indicated the presence of immune suppressive properties of MTX.

4.3.4: Cytogenetic effect of methotrexate on human blood lymphocytes from healthy individuals (No.=5)

The result of blast index (BI) of controls in their response to three different concentrations (0.4, 2, and 4) µg/ml of drug MTX are presented in (Table4-5), the three-drug concentration reduced the BI of lymphocytes. The degree of reduction was dependant on the drug concentration, and all these differences were significant ($P \leq 0.05$) as compared with negative control.

The mitotic index of human blood cells from healthy individuals was (2.38%), as shown in (Table 4-5). A gradual

inhibition in MI was noticed after using different concentrations of MTX in human blood culture, this inhibition was does dependent, and the differents were significant (P≤0.05) from the negative control for all concentrations of MTX, so this reduction in MI indicated that MTX had a cytotoxic activity on human blood lymphocytes *in vitro.*

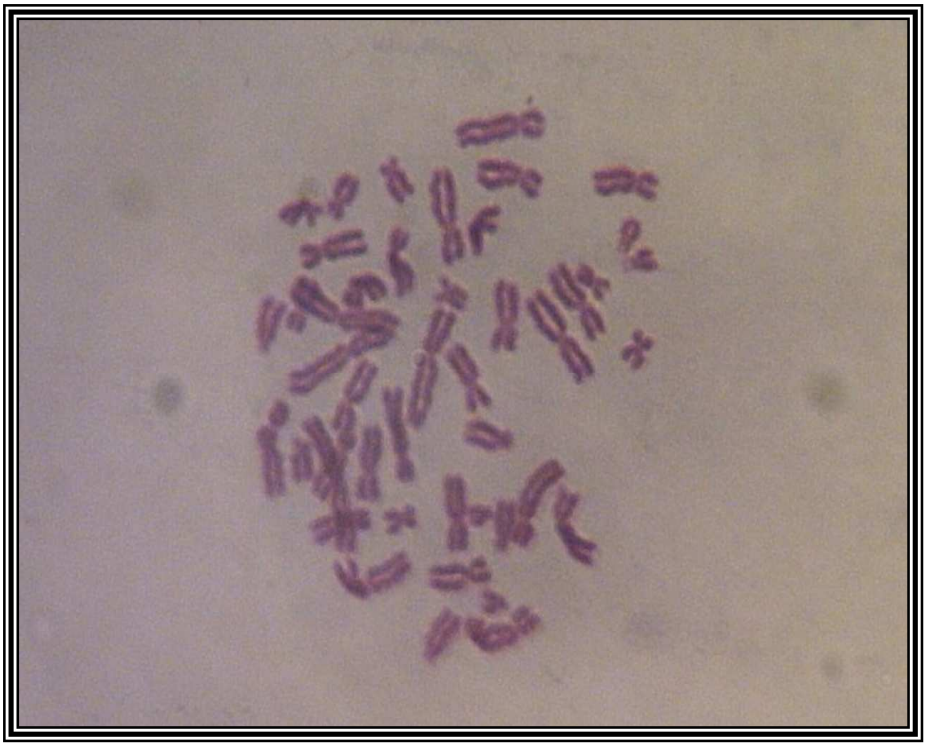
The effect of MTX on the replicative index (RI) in human blood culture was not significant in all concentrations of MTX as compared with the negative control, as shown in (Table 4-6).

Another effect of MTX was an increase in the frequencies of sister chromatid exchange (SCE) (Figure 4-4), it caused significant increase (P≤0.05) in SCE percentage in comparison with negative control, as shown in (Table4-6).

Chromosomal analysis of cultured lymphocytes with MTX revealed the increase in the frequencies of chromosomal aberrations (CAs), as shown in (Table4-7), and these increases in (CAs) were significant (P≤0.05) in all concentrations of MTX as compared with negative control, the frequencies of chromatid break, deletion, dicentric and acentric chromosomes were increased depending on the concentration of MTX in human blood culture, while ring chromosome and chromosome break were not observed in negative control culture , but these types of CAs were induced after the addition of MTX to the culture (Figure 4-3).

It clear that the MTX leads to cell growth inhibition and cell killing (Li and kaminskas, 1984), and this was due to reduction of new DNA synthesis, causes DNA fragmentations through inhibition of different enzyme mechanisms, and this damage in DNA would lead to an increase in the number of incompletely repaired lesion at the time the cells reach S phase, and this lesions may subsequently give rise to SCEs, and causes cell – cycle delays and reduction of MI (Fingert *et al*., 1986).

All these results are in agreement with Maskaleris and Lialiaris, (1998).



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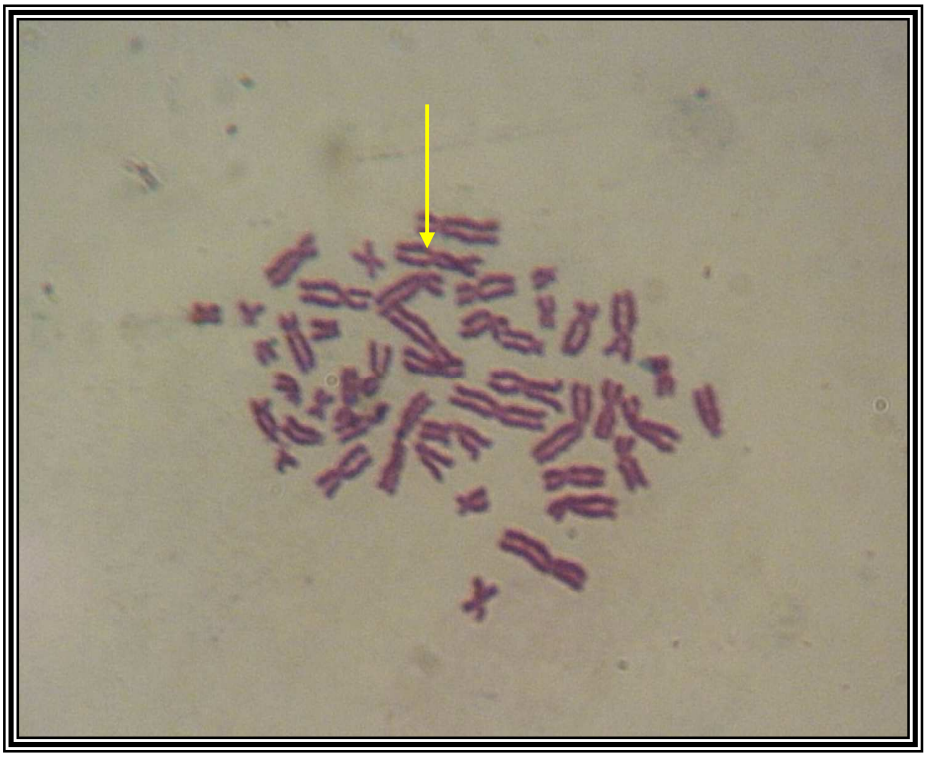

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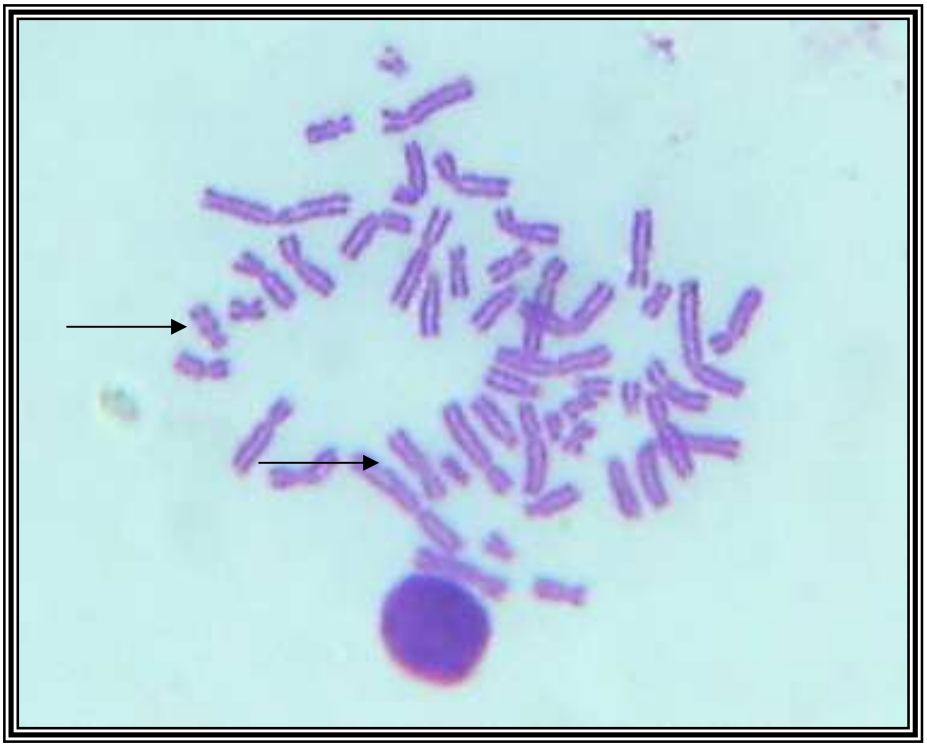
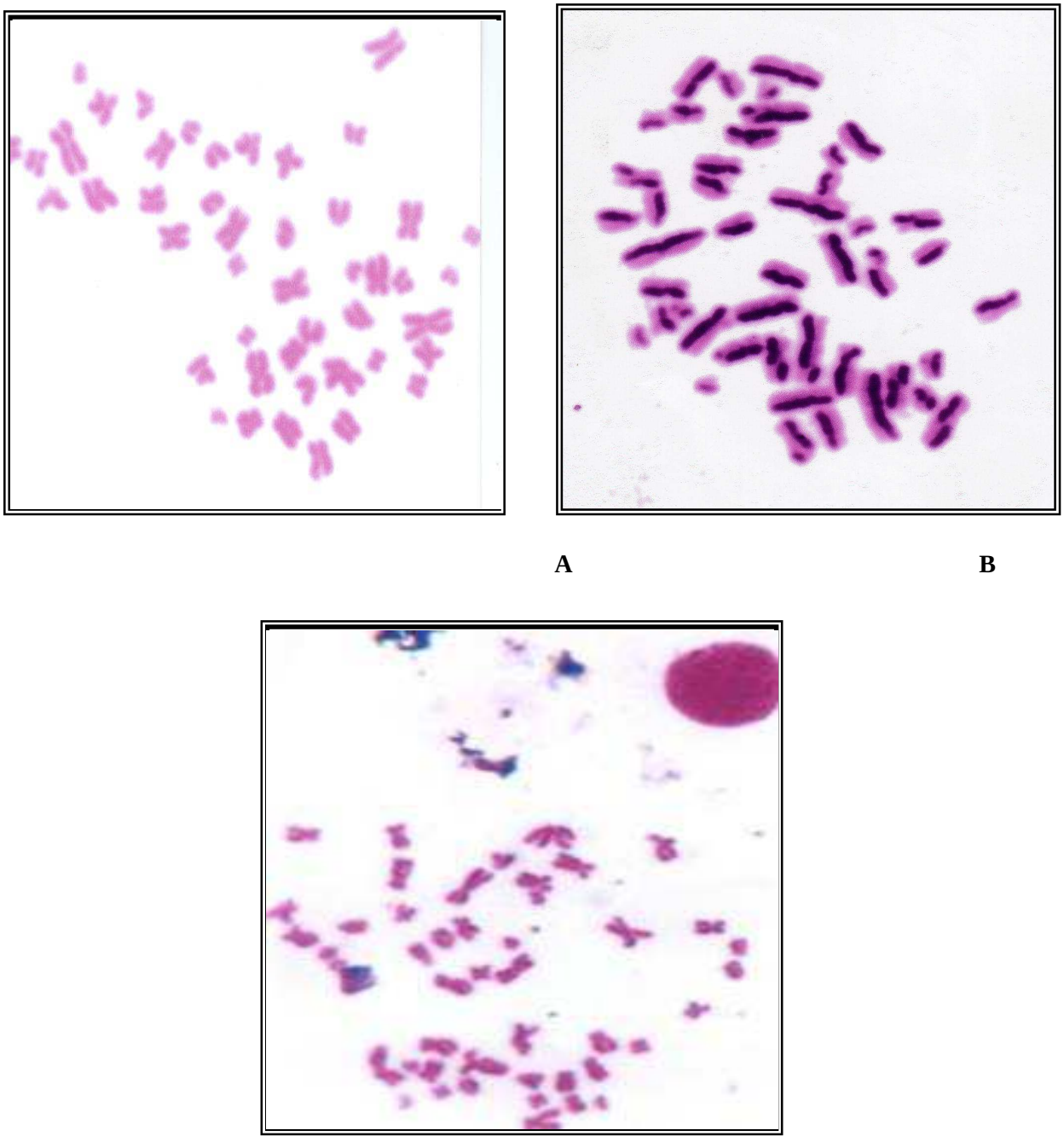


C

Figure(4-3):Metaphase of human blood lymphocytes treated with methotrexate *in vitro*,showing:normal human chromosomes(A),dicentric chromosome(B) , chromatid and chromosome break(C).(100 X)



A **B**

C

(Figure 4-4):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(C) (100 x).

4.4: Interaction between aqueous extract and methotrexate in mouse bone marrow cells

4.4.1: Treatment with aqueous extract before methotrexate

The obtained results of this experiment are shown in (Table 4-9). The MI of mice treated with extract before the drug increased to (4.1%), this result was significantly different ($P \leq 0.05$) from the negative control (1.8%) that treated with the drug only. So, the *G. glabra* extract provided (67.6%) protection against the inhibitory effect of MTX on MI (Figure 4-5).

The percentages of CAs were reduced significantly ($P \leq 0.05$) when water extract used before MTX treatment, which reached (1.02%) in comparison with the negative control (1.31%). So,pre- treatment with *G. glabra* extract provided (54%) protection against the genotoxic effect of MTX (Figure 4-5).

It could be seen that *G. glabra* gave striking results as a protective agent against the genotoxic effect of MTX if it was given before MTX treatment. This might relate to the flavonoids group of *G. glabra*, which may be linked with the inhibition of microsome enzyme (activator enzyme for mutagen) (Francis *et al*., 1989). Webb and his partner (1992) showned that when the extract of *G. glabra* added to rat feed this could induce the damage tissues to increase the level of glutathion-s-transferase and catalase secretion to 50% that play important role in cells protection from mutagens through action as antioxidants or

scavengers for the free radicals in the cells. In addition to other active compounds of extract (GL) may act as anti- mutagenic compounds through the communication intracellular gap-functional of cells (Davidson *et al*., 1986), so this compounds would delay the transfer of MTX between cells and limited its genotoxic and mutagenic activity (blocking agents) (Trosko *et al*., 1990).

These results are in agreement with Al-Khayat (1999), who indicated that pre-treatment with *G. glabra* water extract gave a protective effect against mitomycin C.

Table (4-9): Interaction of aqueous extract from *G.glabra* and methotrexate *in vivo*.

Groups		Mitotic Index % m±SE	Chromosomal aberration % m±SE						
			Chromatid Break	Chromosome Break	Acentric	Dicentric	Ring	Deletion	Total Of CA
Negative Control		A 5.2 ±0.11	A 0.1±0.01	A 0.02±0.005	A 0.12±0.01	A 0.17±0.02	A 0.06±0.01	A 0.3±0.01	A 0.77±0.01
Positive Control MTX		B 1.8±0.11	B 0.17±0.01	B 0.17±0.02	B 0.27±0.01	A 0.22±0.02	B 0.12±0.005	B 0.36±0.01	B 1.31±0.02
Interaction	**Before**	C 4.1±0.05	AC 0.12±0.01	A 0.05±0.005	B 0.24±0.01	A 0.2±0.02	AB 0.09±0.005	AB 0.32±0.01	C 1.02±0.06
	After	D 3.1±0.17	C 0.16±0.01	A 0.09±0.01	B 0.26±0.01	A 0.22±0.03	B 0.1±0.01	AB 0.33±0.01	D 1.16±0.02

Numbers with different letters are significantly different (≤ 0.05) to compression columns

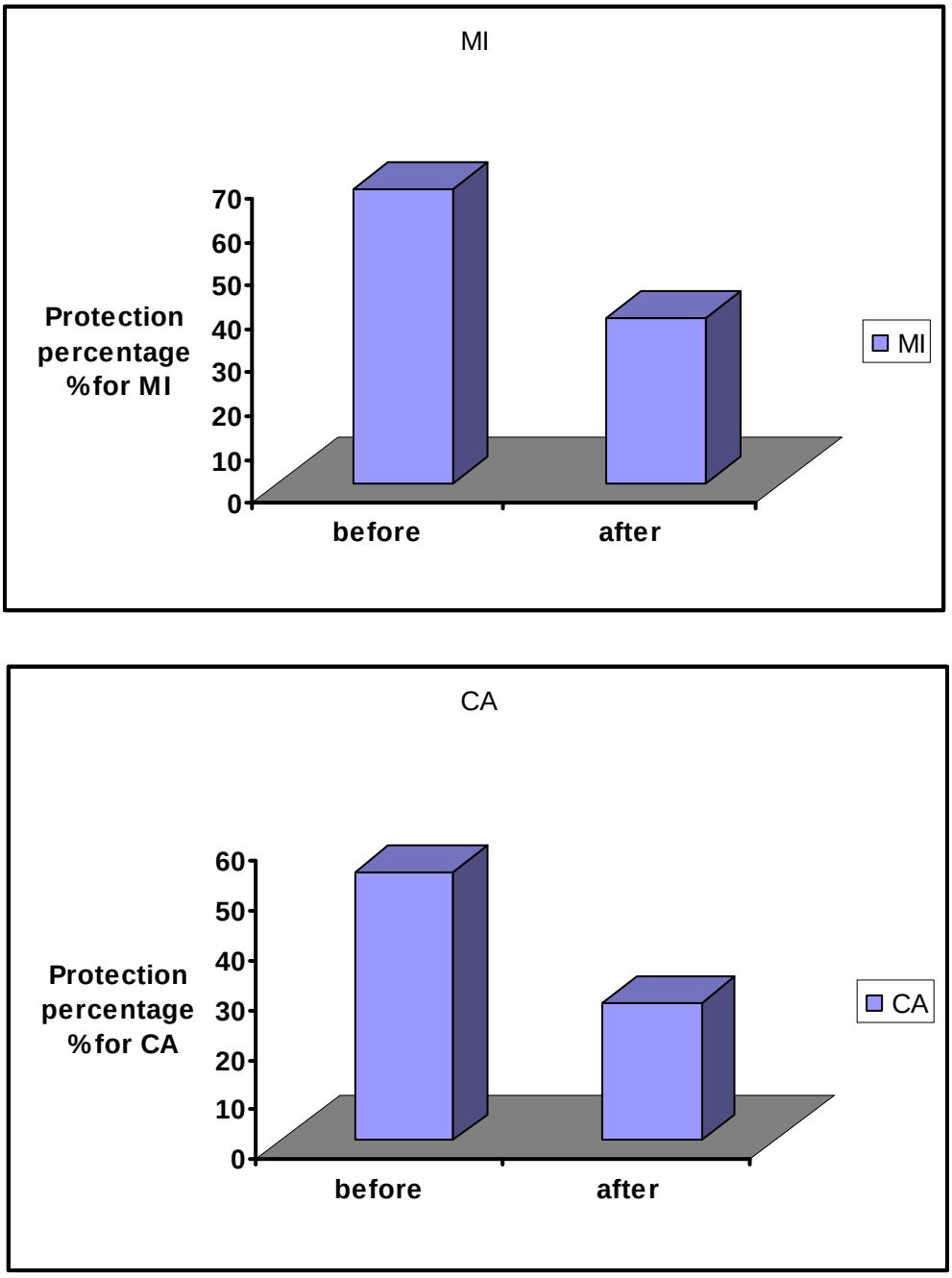


Figure (4-5):- The protections percentage provided by *Glycyrrhiza glabra* when given before and after MTX treatment.

4.4.2: Treatment with aqueous extract of *Glycyrrhiza glabra* after methotrexate.

The results of this experiment were displayed in (Table 4-9). Post-treatment of MTX caused a significant increase ($P \leq 0.05$) in MI (3.1%) when compared with the negative control (1.8%). So, the water extract of *G. glabra* provided (38.1%) protection from the effect of drug on MI (Figure 4-5).

The percentage of CAs after treatment with water extract was (1.16%), this value significantly reduce ($P \leq 0.05$) as compared with negative control (1.31%). so, the post drug treatment provided (27.71%) protection from the effect of drug on CAs (Figure 4-5).

From these results, it was clear that post drug treatment with extract may activate the promoters of DNA repair (Kuroda and Hara, 1999), or may increase the error-free repair fidelity in the cell (Bronzetti, 1994).

Our results were in agreement with Al-Khayat (1999), who indicated that, the use of water extract of *G. glabra* after MMC treatment provides treatment activity against the effect of MMC.

4.5: Interaction between aqueous extract of *Glycyrrhiza glabra* and methotrexate in mouse sperms

4.5.1: Treatment with aqueous extract before methotrexate

The result of this experiment displayed in (Table 4-10). The number of normal sperms that treated with extract before the drug increased to reach (59.5%), this result was significantly different ($P \leq 0.05$) from the negative control (46.2%) that treated with drug only. So, the extract provided (72.9%) protection against the inhibitory effect of MTX (Figure 4-6), but the percentage of head and tail abnormalities decreased, in head abnormalities the reduction reached (20%), so the different was significant ($P \leq 0.05$) as compared to negative control (25.8%), that means that the extract provided (77.2%) protection against MTX, in tail abnormalities the reduction was significant ($P \leq 0.05$) which reached (20.5%) as compared to negative control (28%), hence the extract provided (61.2%) protection against MTX (Table 4-10).

The percentage of head and tail abnormalities reduced when the extract was given before MTX, and this might be related to antimutagenic activity of some active compounds of extract like isoflavans and coumarins that worked as inhibitor for drug activity (Mitscher *et al.*, 1985).

Table (4-10): Interaction between aqueous extract and methotrexate *in vivo*

Groups		Normal sperm% m±SE	Head abnormality% m±SE	Tail abnormality% m±SE
Negative control		A 64.5±1.27	A 19.83±2.07	A 15.67± 1.67
Positive control MTX		B 46.2±0.23	B 25.8±0.92	B 28±1.15
Interaction	**Before**	C 59.5±0.57	A 20±0.57	A 20.5±1.15
	After	B 48.0±1.44	B 25±0.28	B 27±1.73

Numbers with different letters are significantly different (≤ 0.05) to compression columns

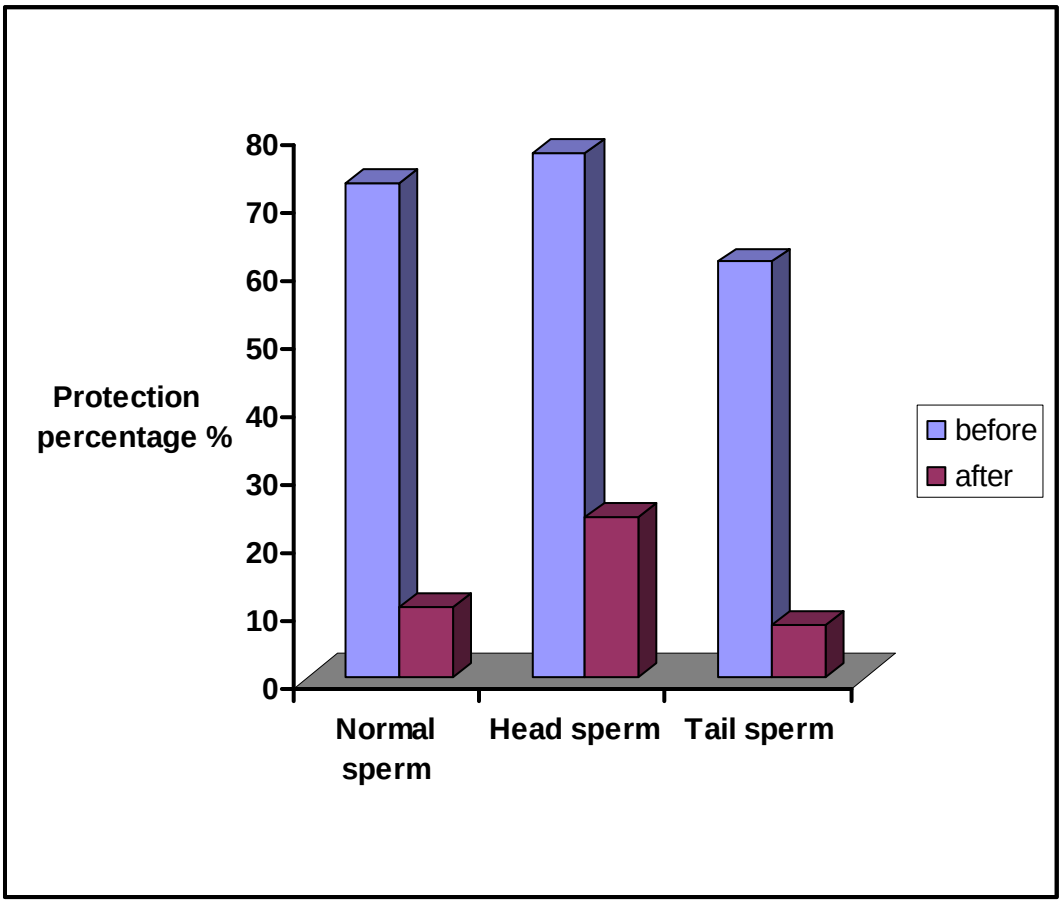


Figure (4-6):- The protections percentage provided by *Glycyrrhiza glabra* when given before and after MTX treatment.

4.5.2: Treatment with water extract after methotrexate

The results of this experiment displayed in (Table 4-10). The number of normal sperms that treated with water extract after the drug treatment was increased to reach (48%), this result was non-significantly different from the negative control (46.2%) that treated with drug only. So, the extract provided (10.3%) protection against the inhibitory effect of MTX (Figure4-6), but the percentage of head and tail abnormalities decreased, in head abnormalities the reduction reached (25%), so the different non-significant as compared to negative control (25.8%). Thus, the extract provided (23.6%) protection against MTX, also in tail abnormalities the reduction were non-significant which reached to (27%) as compared to negative control (28%), that means that the extract provided (7.8%) protection against MTX, as shown (Table 4-10).

These results indicate that the pre-drug treatment with extract gave protection value more than in post-drug treatment and showed that *G. glabra* could be used as a proliferative rather than treating agent against MTX genotoxicity.

4.6: Interaction between aqueous extract and methotrexat in total and differential count *in vivo*

4.6.1: Treatment with aqueous extract before methotrexate

The results of this treatment were shown in (Table 4-11). The total number of leukocytes of mice treated with water extract before MTX was (7600cells/cu.mm.blood), this result was significantly different ($P \leq 0.05$) from the negative control (5100 cells/cu.mm.blood) that treated with the drug only. So, the extract provided (80.16%) protection against the inhibitory effect of MTX on total number of leukocytes (Figure 4-7)

In this experiment the number of lymphocytes were increased significantly ($P \leq 0.05$) when extract used before MTX, which reached (3648 cells/cu.mm.blood) in comparison with the negative control (1020 cells/cu.mm.blood) (Table 4-11).

While the number of neutrophil decreased non-significantly to reach (2280 cells/cu.mm.blood) as compared with positive control (2448 cells/ cu.mm.blood) (Table 4-11)

In this treatment the number of monocyte was affected significantly ($P \leq 0.05$) to reach (1140 cells/ cu.mm.blood) as compared with negative control (510 cells/cu.mm.blood)(Table 4-11). The significant reduction ($P \leq 0.05$) observed also in basophil and eosinophil number, that reached (152,380)(cells/cu.mm.blood) as compared with negative control (561 cells/cu.mm.blood), as shown in (Table 4-11).

It could be seen that *G. glabra* gave striking results as a protective agent against the genotoxic effect of MTX if it was given before treatment, this might related to anti-mutageneic activity of *G. glabra*, which has the ability to inhibit the activity of mutagens and carcinogens like 7, 12-dimethybenz (a) anthracene, and teleocidin (Nishino *et al*., 1984).

These results are in agreement with Al-Khayat (1999), who showed that the water extract of *G. glabra* has the ability to inhibit the mutagenic activity of MMC.

Table (4-11) : Interaction between aqueous extract and methotrexate *in vivo*

Groups		Total count cells /cu. mm. blood m±SE	No. of lymphocyte cells /cu. mm. blood	No.of neutrophil cells /cu. mm. blood	No. of monocyte cells/ cu. mm.blood	No. of eosinophil cells /cu. mm. blood	No. of basophil cells / cu. mm. blood
Negative control		A 8050±5.7	A 4105.5±2.75	A 2245±52	A 1529.5±78.7	A 80.5±3.5	A 80.5±1.9
Positive control MTX		B 5100±2.3	B 1020±45	A 2448±114	B 510±5	B 561±65.5	B 561±15.5
Interaction	**Before**	A 7600±1.5	C 3648±96.5	A 2280.±19	C 1140±64	C 380±10.5	C 152±3
	After	A 6800±3.6	D 2671.5±176.7	A 2397.5±1.8	C 1096±2.5	CB 479.5±29.1	D 205.5±6.3

Numbers with different letters are significantly different (≤ 0.05) to compression columns

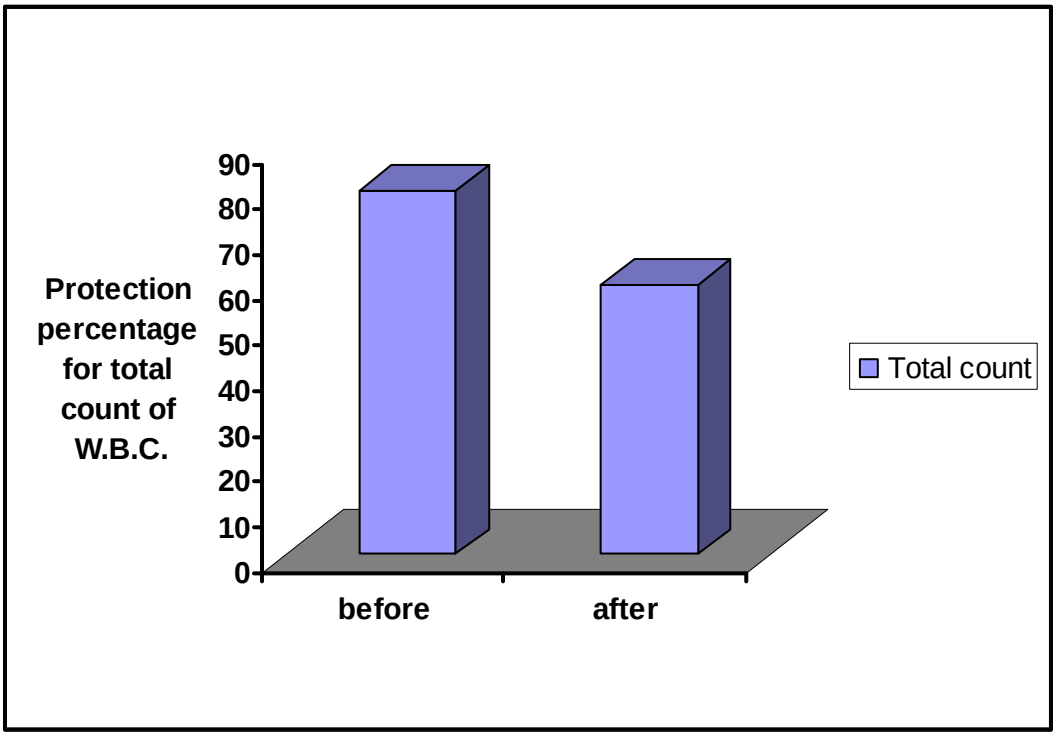


Figure (4-7):- The protections percentage provided by *Glycyrrhiza glabra* when given before and after MTX treatment.

4.6.2. Treatment with aqueous extract after methotrexate

The results of this experiment were displayed in (Table 4-11). Post drug treatment with extract caused a significant increase ($P \leq 0.05$) in total number of leukocytes (6800 cells/cu.mm.blood) when compared with negative control (51 cells/cu.mm.blood). So, the extract provided (59.46%) protection from the effect of drug (Figure4-7).

The numbers of lymphocytes and monocytes increased significantly ($P \leq 0.05$)to reach (2671.5, 1596) cells.cu.mm.blood subsequently when compared with negative control (Table 4-11).

While the number of neutrophil and eosinophil decreased non-significantly, to (2397.5,and 479.5) cells/cu.mm.blood as compared with negative control, Table (4-11).

The numbers of basophil decreased significantly ($P \leq 0.05$) to reach (205.5 cells.cu.mm.blood) as compared to negative control, as shown in (Table 4-11).

Treatment with extract before the drug provided protection ratios more than these ratios when it given after drug treatment. So, the water extract of *G. glabra* could be classified as "desmutagens" in the first order, and "bioantimutagen" in the second order.

4.7: Cytogenetic effects of human blood lymphocyte from leukaemia patients (No.=5)

4.7.1: Cytogenetic effects of *G.glabra* aqueous extract on human blood lymphocytes from leukaemia patients

Five cytogenetic parameters were introduced to evaluate the cytogenetic background of ALL patients and controls, they were BI, MI, RI, SCE and CA.

The control value of BI from healthy individual was (42.52 %), while in the ALL patients, this index decreased to (25%), and this difference reached a significant level ($p \leq 0.05$) as outlined in (Table 4-15).

The treatment of ALL patients with aqueous extract caused a reduction in BI that reached (23.2%), despite it was non significant as compared with negative control of patient (25%), as shown in (Table 4-12).

The mitotic response of lymphocytes obtained from ALL patients was higher than the corresponding value in controls of healthy (2.8 %, and 2.38%) respectively but not significant as compared between them (Table 4-15).

But the MI decreased after treatment of ALL patients with extract, this decrease reaches to (1.84%), so this represent significant reduction ($P \leq 0.05$) as compared to negative control of patient (2.8%), as shown in (Table 4-12).

Considering the RI, although it was decreased in ALL patients compared with the control (healthy) (1.86%, and 1.93

%), the differences failed to reach a significant level, as outlined in (Table 4-15).

In ALL patients, the RI increased after the treatment with extract, this increase was significant ($p \leq 0.05$) as compared with negative control (patient), (Table 4-13).

Sister chromatid exchange evaluation revealed that ALL patients showed a rate of (12.2%), which was higher than control value (healthy) (8.2%). These differences were significant ($p \leq 0.05$), (Table 4-15).

As comparison in ALL patients, SCE were decreased significantly ($p \leq 0.05$) after extract treatment as compared with negative control (patient), (Table 4-13).

The present study also highlight on the chromosomal aberration, the control value of CA (healthy) was (0.2%), while in the ALL patients, this aberration increased significantly ($p \leq 0.05$) reach to (2.56%) (Table 4-15).

In ALL patients the decrease in CA observed after treatment with extract to reach (2.14%), and this represented a significant decreased ($p \leq 0.05$) compared with negative control of patients, (Table4-14).

In patients with ALL leukaemia, treated blood cells with *G.glabra* extract *in vitro* for 72hr showed cytostatic and cytotoxic activity on cancer cells. Decreased rate of cell proliferation, increased proportion of cells in G1 phase of cell cycle, induction of apoptosis and down regulation of bc12

expression, to all these reasons the extract showed this cytostatic and cytotoxic activity on cancer cells (Yano *et al*., 1994).

Similar observation was observed by Juan and colleages (1996).

Table (4-12): Cytogenetic effects of aqueous extract on human blood lymphocytes of leukaemia *in vitro*

Groups	Blast Index % m±SE	Mitotic Index % m± SE
Negative control	**A** 25±0.7	A 2.8±0.08
Positive control MTX	**B** 18.2±0.3	**B** 1.48±0.08
50 µg/ml *G.glabra* extract	**A** 23.2±0.4	B 1.84±0.08

Numbers with different letters are significantly different (≤ 0.05) to compression columns

Table (4-13): Effects of methotrexate and aqueous extract on cell cycle progression on human blood lymphocytes of leukaemia *in vitro*

Groups	Replicative Index % m±SE	Cell Cycle Progression % m			Sister chromatid exchange % m±SE
		M1	M2	M3	
Negative control	A 1.86±0.01	40	34	26	A 12.2±0.1
Positive control MTX	A 1.84±0.7	44	28	28	B 18.8±0.2
50 µg/ml *G.glabra* extract	B 2.01±0.8	33	31	36	C 10.1±0.1

Numbers with different letters are significantly different (≤ 0.05) to compression columns

Table (4-14): Chromosomal aberration variation by *G.glabra* aqueous extract on human blood lymphocyte of leukaemia *in vitro*

Groups	Chromosomal aberration % m±SE						
	Chromatid Break	Chromsome Break	Acentric	Dicentric	Ring	Deletion	Total of CA
Negative control	A 0.46±0.1	A 0.4±0.02	A 0.48±0.01	A 0.3±0.1	A 0.62±0.01	A 0.3±0.02	A 2.56±0.01
Positive control MTX	B 0.632±0.1	B 0.554±0.03	B 0.582±0.02	B 0.536±0.1	B 0.808±0.01	B 0.352±0.02	B 3.464±0.01
50 µg/ml *G.glabra* extract	A 0.4±0.1	C 0.3±0.01	C 0.32±0.03	A 0.3±0.01	A 0.6±0.01	C 0.22±0.01	C 2.14±0.01

Numbers with different letters are significantly different (≤ 0.05) to compression columns

Table (4-15): Cytogenetic effects in human blood lymphocytes in acute lymphoid leukaemia and healthy controls

Parameters m±SE	Healthy No.=5	Patients No.=5
BI	A 42.52±1.52	B 25±0.84
MI	A 2.38±1.34	A 2.8±0.1
RI	A 1.93±0.03	A 1.86±0.42
SCE	A 8.2±0.23	B 12.2±0.21
CA	A 0.2±0.008	B 2.56±0.19

Numbers with different letters are significantly different (≤ 0.05) to compression rows

4.7.2: Cytogenetic effect of methotrexate on human blood lymphocytes from leukaemia patients

In order to evaluate the genotoxic effect of MTX (anti-leukaemia drug), the BI, MI, RI, SCE, and CA were tested. The first parameter was BI, the control value of BI from healthy individual was (42.52%), this value decreased significantly ($P \leq 0.05$) as compared with negative control for patient (Table4-15).

This decrease continued after MTX treatment in ALL patients to reach (18.2%), and this represent significant level as compared with negative control (patient) (Table4-12).

The examination of MI of healthy individuals indicated it reached to (2.38%) so, it is lower than MI of ALL patients (2.8%), but failed to reach a significant level ($P \leq 0.05$), (Table4-15).

In ALL patients, MI decreased after MTX treatment to reach (1.48%) and this represents a significant difference ($P \leq 0.05$) as compared with negative control of patient (2.8%), (Table4-12).

Evaluation of RI from ALL patients showed that the RI rate lower than RI of normal individual (1.93%). This difference was not significant, (Table4-15).

The comparison of RI between treated and untreated ALL patients, showed that the RI decreased after MTX treatment to reach (1.84%), but not significant as compared with negative control of ALL patients, as shown in (Table4-13).

Sister chromatid exchange detection indicated that ALL showed a rate of (12.2%), which was higher than the control value of healthy (8.2%). These differences were significant ($P \leq 0.05$), (Table4-15).

This rate of SCE in ALL patients increased to reach (18.8%) after MTX treatment, which represented significant increase ($P \leq 0.05$) as compared with ALL control (12.2%), as shown in (Table 4-13).

The chromosomal aberration also was one of these parameters, the control value of CA (healthy) was (0.2%), while in the ALL patients this aberration increased significantly ($P \leq 0.05$) that reach to (2.56%), (Table4-15).

In ALL patients the increase in CA observed after MTX treatment reached (3.464%). This difference was significant ($P \leq 0.05$) compared with negative control in patients, (Table4-14).

Methotrexate is taken by cells and tissues and converted to MTX – polyglutamates, which can inhibit (DHFR)enzyme that ultimately affects both purine and pyrimidine nucleotide biosynthesis, thus during the treatment of cancer cells with MTX the malignant cells become starved for purines and pyrimidines which are a precursors of DNA and RNA that required to proliferation (Budzik *et al*., 2000), and this decreased in MI reflects inhibition of cell – cycle progression and /or loss of proliferation capacity ,all this may be an early

marker of cytotoxicity and genotoxicity in human that is generally attributed to it is binding with tubulin causing the impairment of spindle function and chromosome segregation error during cell division (Onfelt,1983).

Summary

The present study was carried out to shed light on the chemical analysis of *Glycyrrhiza glabra* for different active compounds, and on the cytogenetic effects of methotrexate(MTX) and aqueous extract of *G. glabra* in laboratory mice *(in vivo)* and human blood lymphocytes *(in vitro).*

The study also aimed to investigate the role of *G. glabra* aqueous extract in reducing the cytogenetic effects of MTX in mouse through the parameters mitotic index, and chromosomal aberration in bone marrow, the total and differential counts of leukocytes, and sperm head and tail morphology. The cytogenetic analyses were extended to include blast index, mitotic index, replicative index, sister chromatid exchanges and chromosomal aberrations in human blood lymphocytes *in vitro* as a result of *G. glabra* aqueous extract and MTX treatment.

The cytogenetic effects of the drug and plant extract were investigated after five days of treatment in mice with three doses for each. For MTX, the doses were 1.2, 1.6, and 2 mg/kg, while the doses of *G. glabra* were 400, 600, and 800 mg/kg.

Using human blood lymphocyte (72hour) culture, the tested concentrations of MTX were 0.4, 2, and 4 µg/ml, and for *G. glabra* aqueous extract were 50, 100, and 200 µg/ml.

Summary

An interaction between plant extract and MTX was carried out through two types of treatments (before, and after MTX treatment) to determine the activity of *G. glabra* aqueous extract in reducing the drug side effects *in vivo*.

The following results were obtained:

1. Chemical analyses showed that *G. glabra* roots contain different active compounds such as saponins and flavonoids.

2.Methotrexate revealed clear effects in reducing mitotic index and increased chromosomal aberrations in mouse bone marrow cells. In human blood lymphocytes, the drug caused a reduction in blast index, mitotic index, replicative index, while the sister chromatid exchanges and chromosomal aberrations were increased.

3.Aqueous extract of *G. glabra* had a genotoxic effects at high doses in human blood lymphocytes culture (*in vitro*).

*4.*Methotrexate in human blood lymphocytes from patients with leukaemia(ALL) caused reduction in blast index, mitotic index, replicative index, but the increasing observed in chromosomal aberration, and sister chromatid exchanges *in vitro.*

5.In human blood lymphocytes of leukaemia patients *(in vitro*), extract caused reduction in blast index, mitotic index, sister chromatid exchanges, and chromosomal aberration, but the

increasing observed in replicative index, except the concentration (200µg/ml) because of its genotoxic effect.

6.Aqueous extract of *G. glabra* at high dose, however, showed a protective value against the genotoxic effect of MTX in mouse bone marrow, this was more pronounced in pre-treatment than post-treatment.

7.Treatment with *Glycyrrhiza glabra* extract led to increase in the total and differential count of leukocytes, through increasing the number of lymphocytes and monocytes.

8.Methotrexate revealed clear reduction in the total and differential count of leukocytes by the reduction in lymphocytes and monocytes number, while neutrophils, basophils, and eosinophils were increased.

9.The induction in the abnormalities of sperm head and tail morphology were observed in MTX treatment especially after 35 days of treatment.

10.Treatment with *G. glabra* aqueous extract resulted in reduction of sperm abnormal morphology especially after 35 days of treatment.

الاهـداء

على طريقِ منقذِ البشريةِمن الجهلِ وباني مدينةِ العلمِ الرسول الاعظم صلى الله عليه وسلم

الى وطنٍ تجذرت فيه الحضاراتُ منذُ آلاف السنين فأنبتتْ علوماً ومعارفَ

اثمرت ازدهاراً للبشريةِ جمعاء

الى والدي الذي ظلّ يجد نفسَهُ مع كلِ قطرةِ عرقٍ تصببتْ من فوق جبيني

الى امي التي طالما تنفستْ الاملَ لتعيشَ الى يومٍ أنجزُ فيه ما انجزتْ

الى اخوتي ثروتي الاخرى في الحياةِ علياء ، حيدر ، محمد، لينة، ميس

الى رفقةِ دربٍ لن انسى لهم ما قدموه لي ما حييتْ

الى كل هؤلاء اقدمُ جهدي الذي يشكلُ مستقبلَ حياتي

بسم الله الرحمن الرحيم

وَعَلَّمَكَ مَالَمْ تَكُنْ تَعْلَمُ وَكَانَ
فَضْلُ اللهِ عَلَيْكَ عَظِيماً

صدق الله العظيم

سورة النساء {١١٣}

Republic of Iraq
Ministry of Higher Education
and Scientific Research
Al-Nahrain University
College of Science
Department of Biotechnology

The Role of *Glycyrrhiza glabra* Root Aqueous Extract in Modulating the Genotoxic Effects of Methotrexate in Albino Male Mice and Acute Lymphoblastic Leukaemia Patients

A thesis
Submitted to the College of Science / AL-Nahrain University
In partial fulfillment of the requirements for the degree of
Master of Science in Biotechnology

By

Worood Kamil Al-Malikey

B. Sc. Biotechnology. Al-Nahrain University. 2003

Shaban ***1427***

September **2006**

الخلاصة

اجريت هذه الدراسة لتسليط الضوء على الفحوصات الكيميائية للكشف عن مختلف المركبات الفعالة لعرق السوس،وعلى تاثير عقار الميثوتركسيت و المستخلص المائي لعرق السوس في الصفات الوراثية الخلوية لخلايا نقي العظم في ذكور الفئران المختبرية(داخل الجسم) و خلايا الدم المحيطي للانسان(خارج الجسم).

كما هدفت الدراسة ايضا الى اختبار عرق السوس في الحد من هذه التاثيرات الوراثية الخلوية للعقار مثل معامل الانقسام الخيطي والتغيرات الكروموسومية في خلايا نقي العظم ودراسة العدد الكلي و التمايزي في خلايا دم الفئران بالاضافة الى دراسة شكل الراس والذيل لنطف الفئران،اما التحليلات الوراثية الخلوية لخلايا الدم المحيطي للانسان امتدت لتشمل معامل الارومي، الانقسام الخيطي، معامل التضاعف، التبادل الكروماتيدي الشقيق، والتغيرات الكروموسومية خارج الجسم نتيجة المعاملة بمستخلص عرق السوس والميثوتركسيت.

درست التأثيرات الوراثية الخلوية للعقار والمستخلص المائي بعد خمسة ايام من المعاملة في الفئران لثلاث جرع مختلفة لكل عامل للميثوتركسيت كانت(٢،١,٦،١,٢)ملغم/كغم بينما كانت لنبات السوس(٨٠٠،٦٠٠،٤٠٠)ملغم/كغم لاختبار السمية الخلوية للنبات او الميثوتركسيت.

باستخدام خلايا الدم المحيطي (٧٢ ساعة) للانسان التراكيز المختبرة للميثوتركسيت كانت(٤،٢،٠,٤) ميكروغرام/مل ولمستخلص نبات السوس كانت(٢٠٠،١٠٠،٥٠) ميكروغرام/مل.

اجري التداخل بين المستخلص و الميثوتركسيت من خلال نوعين من المعاملات (قبل،بعد المعاملة بالميثوتركسيت) لتحديد قدرة المستخلص في تقليل تاثير العقار داخل الجسم.

وقد تم الحصول على النتائج الاتية:

١.الفحوصات الكيميائية بينت احتواء النبات على مختلف المركبات الفعالة مثل الصابونيات والفلافينويدات.

٢.لقد اظهر الميثوتركسيت تاثيرات سلبية تمثلت بانخفاض معامل الانقسام وزيادة في التغيرات الكروموسومية في خلايا نقي العظم(داخل الجسم) و في خلايا الدم المحيطي(خارج الجسم) ايضا سبب انخفاض المعامل الارومي، معامل الانقسام،معامل التضاعف،لكن سبب تحفيز لتبادل الكروماتيدات الشقيقة والتغيرات الكروموسومية.

٣. المستخلص المائي لعرق السوس يمتلك تاثيرا سميا في التراكيز العالية في خلايا الدم المحيطي(خارج الجسم).

٤.الميثوتركسيت في خلايا الدم المحيطي لمرضى سرطان الدم سبب انخفاض في المعامل الارومي، معامل الانقسام، ومعامل التضاعف ،لكن الزيادة لوحظت في التغيرات الكروموسومية،و تبادل الكروماتيدات الشقيقة(خارج الجسم).

٥. في خلايا الدم المحيطي لمرضى سرطان الدم (خارج الجسم) المستخلص سبب انخفاضا في المعامل الارومي، معامل الانقسام، تبادل الكروماتيدات الشقيقة و التغيرات الكروموسومية لكن الزيادة لوحظت في معامل التضاعف باستثناء تركيز(٢٠٠ميكروغرام/مل) بسبب تاثيراته السمية.

٦.المستخلص المائي لعرق السوس في التراكيز العالية اظهر قابلية حماية ضد التاثير السمي للعقار في خلايا نخاع العظم،وهذا كان واضحاً عند المعاملة قبل اكثر من البعد.

٧.مستخلص عرق السوس ادى الى زيادة في العدد الكلي و التفريقي لكريات الدم البيض من خلال الزيادة باعداد الخلايا اللمفية والوحيدة.

٨.لقد اظهر الميثوتركسيت تاثيرات سلبية تمثلت بانخفاض العدد الكلي و التفريقي لكريات الدم البيض من خلال الانخفاض باعداد الخلايا اللمفية والوحيدة بينما الزيادة لوحظت في خلايا العدلة،الحمضة،والقعدة.

٩.تحفيز التشوهات لراس وذيل النطف لوحظ عند المعاملة مع الميثوتركسيت خصوصا بعد ٣٥يوماً من المعاملة.

١٠.المعاملة بالمستخلص المائي لعرق السوس سبب انخفاضاً في تشوهات الراس والذيل للنطف خصوصاً بعد ٣٥يوماً من المعاملة.

وزارة التعليم العالي والبحث العلمي
جامعة النهرين/ كلية العلوم
قسم التقانة الاحيائية

دراسة دور المستخلص المائي لجذورعرق السوس في تعديل التأثيرات السمية الوراثية لعقار الميثوتركسيت في ذكور الفأران البيض ومرضى ابيضاض الدم اللمفي الحاد

رسالة

مقدمة الى كلية العلوم جامعة النهرين

وهي جزء من متطلبات نيل درجة ماجستير علوم في التقانة الاحيائية

من قبل

ورود كامل المالكي

بكلوريوس تقانة احيائية جامعة النهرين ٢٠٠٣

شعبان ١٤٢٧هـ

ايلول ٢٠٠٦م