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Studying some Immunological and Cytogenetic Effects of *Micromeria myrtifolia* Extracts in Albino Male Mice

A Thesis
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Supervisors 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.

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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.

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Dedication

To my **Mother**, the symbol of tenderness, for her love, compassion, concerning tears; the reason to become what I am.

To my **Father**, who guided me through life, showed me who I want to be, and taught me how to face the problems of life with confidence.

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For having faith in me, and for their endless support.

To my fiance, my soulmate, the source of happiness in my life.

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Summary

The present study was designed to evaluate some immunological and cytogenetic effects of *Micromeria myrtifolia* extracts (methanol and hexane) and methotrexate in albino male mice (*in vivo*). The immunological parameters were total and absolute counts of leucocytes, total serum IgG level and metaphase index of bone marrow and spleen cells, while cytogenetic evaluations included micronucleus formation in polychromatic erythrocytes of bone marrow and sperm-head abnormalities. Additionally, a chemical detection of flavonoids, polysaccharides and alkaloids in methanol extract, and steroids in hexane extract was also carried out.

The evaluations included two main parts. In part I, the immunological and cytogenetic effects of both extracts and methotrexate were carried out. Two doses (200 and 600 mg/kg) of the extracts and one dose of methotrexate (0.33 mg/kg) were investigated, in which the materials was given for seven days (single dose/day) and the evaluation was carried out on day eight. In part II, interactions (pre- and post-treatments) between the two doses of the extracts and methotrexate were evaluated. In both cases, the extracts and methotrexate were administrated orally.

The following results were obtained:

1. The chemical detection revealed that the methanol extract was positive for flavonoids and polysaccharides and it was negative for alkaloids, while the hexane extract was positive for steroids.
2. Methotrexate declared clear immune suppressive and mutagenic effects as judged by the investigated parameters. Reduced indices of metaphase in bone marrow and spleen cells, and increased frequency of micronucleus formation were observed. Additionally, the total count of leucocytes and the absolute count of lymphocytes and monocytes, as well as, the total IgG serum level were significantly decreased as compared with the negative control.
3. The results of part I indicated that *M. myrtifolia* extracts modulated the values of the investigated immunological parameters positively as compared to negative (distilled water and olive oil) and positive (methotrexate) controls. Similarly, the spontaneous formation of micronuclei and sperm-head abnormalities was significantly decreased. In both cases the effect was dependent on dose, as well as, the type of extract. In this regard the low dose (200 mg/kg) was better than the dose 600 mg/kg, and the hexane extract was more effective than the methanol extract, although, the effect was subjected to the parameter of investigation.
4. The results of part II confirmed the observations made in part I, and the two doses of methanol and hexane extracts showed a significant efficiency in protecting the immune system and the genetic make-up from the immune

suppressive and mutagenic effects of methotrexate. In this regard, the pre-treatment with the extract was more effective than post-treatment.

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List of abbreviation

ANOVA	Analysis of Variance
DHFR	Dihydrofolate Reductase
DNA	Deoxyribonucleic Acid
ELISA	Enzyme-Linked Immuno Sorbent Assay
HIV-1	Human Immunodeficiency Virus Type 1
HRP	Horse Radish peroxidase
IgG	Immunoglobulin G

IgM	Immunoglobulin M
IgD	Immunoglobulin D
IgE	Immunoglobulin E
IgA	Immunoglobulin A
IL-1	Interleukin-1
IFN-γ	Interferon- γ
LD50	*Lethal Dose 50%*
MI	Metaphase Index
M. myrtifolia	*Micromeria myrtifolia*
MTX	Methotrexate
OD	Optical Density
PBS	Phosphate Buffer Saline
PHA	Phytohaemoagglutinin
PCE	Polychromatic Erythrocytes
ROS	Reactive Oxygen Species
RNA	Ribonucleic Acid
Spp.	Species
SHA	Sperm-Head Abnormalities

SPSS	Statistical Package for the Social Sciences
SCE	Sister Chromatid Exchange
TMB substrate	Tetraethyl Benzidine Substrate
TLC	Total Leucocyte Count
TLC	Total Leucocyte Count
TNF	Tumour Necrosis Factor
T helper-1	T_H1
T helper-2	T_H2
Trp-p-2	3-Amino-1-methyl-5H-pyrido[4,3-b]indole

Chapter One
Introduction and Review of Literature

1.1 Introduction

Herbal remedies and alternative medicines are used throughout the world, and in the past, herbs often represented the original sources of most drugs. The plant kingdom has provided an endless source of medicinal plants first used in their crude forms as herbal teas, syrups, infusions, ointments, liniments and powders (Rousseaux and Schachter, 2003).

Evidence of use of herbal remedies goes back some 6000 years to a burial site in a cave in northern Iraq, which was uncovered in 1960. An analysis of the soil around the human bones revealed extraordinary quantities of plant pollen of eight species. Seven of these are medicinal plants and still used throughout the herbal world (Saad *et al*., 2005). With the development of chemistry and Western medicine, the active substances of many species have been isolated and in some cases duplicated in the form of synthetic drugs. Nevertheless, the synthetic preparation of some drugs is either unknown or economically impractical. For this reason, scientists continue to search for and test little-known plants and conserve those whose medicinal properties have become crucial in the fight against diseases (Cooper, 2004).

Herbal-derived substances remain the basis for a large proportion of the commercial medications used today for the treatment of heart disease, high blood pressure, pain, asthma and other illnesses. For example, ephedra (*Ephedra sinica*) is an herb used in traditional chinese medicine for more than 2000 years to treat asthma and other respiratory problems, and ephedrine (the

active ingredient in ephedra), is still used in the commercial pharmaceutical preparations for the relief of asthma symptoms and other respiratory problems. Today a great number of modern drugs are still derived from natural sources, and around 25% of all prescriptions contain one or more active ingredients from plants (Saad *et al*., 2005). This fact resulted in a huge development in pharmaceutical science, and pharmacologists and ethnopharmacologists started to search for different ingredients and extracts to be used as remedies, and they even started to study the chemical properties of the materials used in the treatment of various diseases and ailments (Bin Murad, 1991; AlTurkimany, 1993). In this regard, two active fields of research have showed fruitful results; they were the role of medicinal plants in modulating the mutagenesis and/or carcinogenesis, together with their potentials in enhancing the functions of the immune system (Okezie *et al.*, 2005).

With respect to the former field, and over the last two decades, an expanding body of evidence from epidemiological and laboratory studies has demonstrated that some edible plants as a whole, or their identified ingredients, have substantial protective effects on human mutagenesis and/or carcinogenesis (Masahiro, 2000). In this regard, a progress was made to understand the biochemical mechanisms of dietary and medicinal anti-mutagens and anti-carcinogens, and the investigators have broaden the horizons to cover various aspects of chemoprevention by edible phytochemicals or their mixtures (Reviewed by Surh and Ferguson, 2003).

The immune system is a further related target of the medicinal plant research. In this context, it is interesting to note that it has been recognized for several decades that nutrition and health are closely interrelated, and much research has focused on the nutrition effect on the immune system and its proper functioning (Langseth, 2006). More recently, the effect of nutrition on chronic degenerative diseases has become an area of intense study, bringing

about a shift in the concept of optimal nutrition away from merely preventing diseases stemming from nutrient deficiencies to reducing the risk of chronic diseases (Kolida *et al.*, 2000). One group of nutrients thought to play a vital role in such disease prevention is antioxidants. Evidence has been accumulating over the past few years that many plant constituents not previously thought of as separate nutrients, for example the phenolic compounds, can act as powerful antioxidants and immune modulators (Serafini, 2006).

In Iraq, and as in other parts of the world, the research on the anti-mutagenic and immune modulator effects of medicinal plants has gained special interest, and accordingly, the list of investigations in this regard shows a good progress. Examples of these plants are *Hibiscus subdariffa*, *Glycyrrhiza glabra* and *Artemisia herba-alba* (Al-khayat, 1999; Al-Obaidi, 2002), *Allium sativum* (Ad'hiah *et al*., 2004), *Salvia officinalis* (Al-Ezzy, 2006), *Origanum vulgare* (Al-Berikdar, 2007), *Alhagi alhagi* (Ad'hiah *et al*., 2007) and *Rosmarinus officinalis* (AL-Sudany, 2008).

Micromeria spp. (Labiatae) are perennial herbs or chamaephytes. The extract of these plants has been reported to have some medicinal value, for example, the leaves have been reported to possess anti-inflammatory and anti-microbial effects and are also used against some other human ailments (inflamed eyes, wounds, skin infections, stomachache, chest pains, colds, fevers, and others (Palevitch and Yaniv, 1991). These plants are known to be a rich source of essential oil contents (mono and sesquiterpenes especially thymol, carvacrol), and flavanoids, to which, the medicinal effects of *Micromeria* have been ascribed (Shtayeh *et al*., 1997).

1.2 Aims of the Study

Based on the forthcoming presentation, *Micromeria myrtifolia* aerial parts were extracted with two solvents (methanol and hexane), and the two

extracts were tested for their immunological and anti-mutagenic potentials through the following parameters:

- Total and absolute counts of leucocytes.
- Total IgG serum level.
- Mitotic index of bone marrow and spleen cells.
- Micronucleus formation and sperm-head abnormalities.

1.3 Literature Review

1.3.1 *Micromeria myrtifolia* Boiss. and Hohen

The name *Micromeria* is derived from the Greek word *micro* (meaning small), and *meris* (meaning a part), and referred to the tiny flowers and leaves, while *myrtifolia* means that the plant has leaves like the *Myrtle* genus (Stearn, 1996).

1.3.1.1 Common Names and Taxonomy

Internationally, many common names are used to describe the plant *M. myrtifolia*; for instance, bean herb, savory, summer savory, kayakekii and altinbacayi (Gruenwald *et al.*, 2000), while taxonomically (Johnston, 2006), the plant is classified as the following:

Kingdom: Plantae
Phylum: Tracheophyta
Class: Magnoliopsida
Order: Lamiales
Family: Lamiaceae
Genus: *Micromeria*
Species: *myrtifolia* Boiss. and Hohen

1.3.1.2 Distribution

The plant is widely distributed in Europe, North Africa, Australia and North America and in some Mediterranean and sub-Mediterranean regions. The plant requires loamy soils and grows in subtropical climates so it is indigenous to Croatia ,Turkey, Iran, Iraq, Syria, Lebanon and Palestine (Everest and Ozturk, 2005; Shtayeh *et al*., 2007).

1.3.1.3 Plant Description

The plant is a herbaceous annual. It grows up 30 to 45 cm in height with erect, heavily branched, and shortly pubescent stems. The leaves are crossed opposite, up to 3 cm long, short-petioled, lanceolate to linear-lanceolate and entiremargined. They are rather thick with a ciliate margin and are glandular punctate on both surfaces. The lilac or whitish labiate flowers are inaxillary, 5 blossomed and false whorls. The calyx is tubular campanulate and regular, and has 5 tips. The corolla does not have a ring of hair and the upper lip is straight and margined, while the lower lip has 3 divisions and is red-spotted at the mouth of the tube. (Gruenwald *et al.*, 2000). A field picture of the plant is given in figure 1-1.

Figure 1-1: A field picture of *Micromeria myrtifolia* (Johnston, 2006)

1.3.1.4 Active Compounds

The main active compounds, which have been detected as constituents of *M. myrtifolia*, are:

- Polysaccharide: These include galactose, glucose, xylose, arabinose, and rhamnose, in addition to galacturonic acid and pectin. Starch-like α-glucans and xylans have also been detectd (Ebringerova *et al.*, 2002)
- Volatile oils (0.2-3.0%): The chief components are carvacrol (30%), p-cymene (20-30%), alpha-thujene, alpha-pinene, beta-myrcene, alpha- and beta-terpinene, beta-caryophyllene and thymol. (Gruenwald *et al.*, 2000).
- Acids: Caffeic acid derivatives, which are rosmarinic acid (0.2 to 1.3%) and chlorogenic acid, ascorbic acids, carnosic acid, kaempferol, oleanolic acid, labiatic acid and ursolic acid have been detected (Kamatou *et al.*, 2008).

- Essential oil components: They include pinene, 1,8-cineole, linalool, limonene, myrcene, caryophyllene, spathulenol, caryophyllene oxide, viridiflorol, 3-carene and bisabolol (Kaya *et al.*, 2003).
- Flavonoids like apigenin and apigenin-4-methyl ether have been detected (Ghannadi *et al.*, 2000).

1.3.1.5 Folkloric Uses

Micromeria plants have many uses; for example, in Turkey, *Micromeria* species are used as herbal teas due to their pleasant aroma and medicinal properties and as a substitute for mint in folk medicine. ***M. congesta*** is used against kidney stones and stomach ache, ***M. juliana*** is used as an appetizer, carminative and stimulant, while ***M. myrtifolia*** is commonly used as an aromatic herbal tea (Kirimer and Baer, 1997). Several *Micromeria* species have been reported as antiseptic, abortifacient, anti-rheumatic, central nervous system stimulant, and tonic. they are also used against heart disorders, indigestion and headache, and as a topical anesthetic in tooth ache and wounds, inflamed eyes, skin infections, chest pains and colds (Shtayeh *et al.*, 1997). Additionally, the leaves are used as a food flavouring (Facciola, 1990).

1.3.1.6 Biological Potentials and Pharmaceutical Applications

Recent investigations have suggested that the aerial parts are important pharmaceutical part of *Micromeria* with some medicinal applications, especially as a source of anti-viral, anti-mutagen and anti-tumour products (Gulluce *et al.*, 2003). The proposed mechanism of action is related to the high content of phenolic compounds in the essential oil, particularly carvacrol and thymol. It is also suggested that such activities are associated with these chemicals in terms of synergistical effects (Gulluce *et al.*, 2003).

- **Antimicrobial activities:** One study has examined the antimicrobial activities of hexane and methanol extracts of *M. myrtifolia* (Sahin *et al*., 2003). The hexane extract inhibited 4 strains belonging to three *Bacillus* species (*B. amyloliquefaciens*, *B. megaterium* and *B. sphaericus*). The methanol extract inhibited six *Candida albicans* isolates and 23 strains of 11 species belonging to five different bacterial genera including *B. amyloliquefaciens*, *B. atrophaeus*, *B. macerans*, *B. megaterium*, *B. pumilus*, *B. sphaericus*, *B. substilis*, *Escherichia coli*, *Kocuria varians*, *Micrococcus luteus* and *Pantoea agglomerans*. A further study has examined the antifungal activity of the essential oil, and inhibition of growth was found against *Alternaria alternate*, *Aspergillus flavus*, *Aspergillus variecolor*, *Fusarium culmorum*, *Fusarium oxysporum*, *Penicillium* spp., *Rhizopus* spp., *Rhizoctonia solani*, *Moniliania fructicola*, *Trichophyton rubrum*, *Trichophyton mentagrophytes*, *Microsporum canis*, *Sclerotinia sclerotiorum* and *Sclerotinia minor* (Gulluce *et al*., 2003).

- **Anti-HIV-1 activity**: *M. myrtifolia* aqueous extracts showed significant inhibitory effects against HIV-1 induced cytopathogenicity in Molt-4 cells. Additionally, these aqueous extracts inhibited giant cell formation in co-culture of Molt-4 cells with and without HIV-1 infection, and showed inhibitory activity against HIV-1 reverse transcriptase. The active components in the extract samples were found to be water-soluble polar substances (Yamasaki *et al*., 1998).

- **Antispasmodic and antidiarrheal activity**: The essential oil of *M. myrtifolia* inhibited acetylcholine concentration by activating muscarinic receptors, which reduces ileum contraction and mediates the response of acetylcholine. Furthermore, it has been reported that the plant has a spasmolytic effect on isolated smooth muscle and may have an

antidiarrheal effect due to the phenolic compounds in the oil and the tannins contained in the plant (Hajhashemi *et al*., 2000).

- **Anti-inflammatory and anti-oxidant activity**: The anti-inflammatory activity of *M. myrtifolia* was examined on the basis of nitric oxide metabolite measurements and histological changes in rabbits that have been treated for rhinosinusitis. The concentration of nitric oxide metabolites and activity of nitric oxide synthesis in mucosal specimens were reduced by topical administration of *M. myrtifolia* extract (Hajhashemi *et al*., 2002). *M. myrtifolia* also inhibited carrageenan-induced paw edema in rats (Uslu *et al*., 2003). Additionally, antioxidant potentials have also been suggested, and such properties may be related to its secondary metabolites (Dorman and Hiltunen, 2004; Souri *et al*., 2004).

1.3.2 Methotrexate

Methotrexate (MTX) is an anti-metabolite drug that acts by inhibiting the metabolism of folic acid. The empirical formula of MTX is $C_{20}H_{22}N_8O_5$, and its structural name is N-[4-(2,4-diamino-6-pteridinyl) methyl] methyl glutamic acid with a molecular weight 454.4 (Allegra and Collins,1990). Methotrexate was found to have a more favourable therapeutic index than aminopterin **(**Jackson, 1999).

Methotrexate has since been used in the treatment of various malignancies including osteosarcoma, non-Hodgkin's lymphoma, Hodgkin's disease, T cell lymphoma, head and neck cancer, lung cancer, and breast cancer (Jolivet *et al*., 1983). Almost 50 years ago, MTX was developed as a chemotherapeutic agent for the treatment of cancer, especially leukaemia (Cronstein, 1997). 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 (Weinblatt *et al*., 1998), graft-versus-host diseases

(Feagen *et al.*, 2000), psoriasis, primary biliary cirrhosis, Crohn disease, and intrinsic asthma (Genestier *et al.*, 2000).

Methoterxate inhibits dihydrofolate reductase (DHFR), which is 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 an essential co-factor that donates one-carbon group in the enzymatic biosynthesis of thymidylate and purine nucleotide that is a precursor for DNA synthesis (Allegra *et al.*, 1987). Therefore, MTX inhibits the synthesis of DNA, RNA, thymidylates and proteins. Methotrexate 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). Accordingly, MTX was employed as mutagenic and immune suppressive drug in the present study.

1.3.3 Investigated Parameters

The study evaluated some facets of genetics and immunity in mice (*in vivo*) treated with two extracts (methanol and hexane) of *M. myrtifolia*. The parameters of evaluation included total and absolute counts of leucocytes, mitotic index, chromosomal aberrations, micronucleus formation, sperm-head abnormalities and total IgG serum level.

1.3.3.1 Total and Absolute Counts of Leucocytes

The total and absolute counts of leucocytes can give a general picture of the immunity in the peripheral blood, because such counts are sensitive to infections, environmental pollutions (Ad'hiah *et al.*, 2001b) and chemical agents (Ad'hiah *et al.*, 2004). These cells are originated in the bone marrow, which supplies the blood stream with different types of leucocytes through two

types of lineages, the myeloid and lymphoid lineages. The first lineage gives rise to neutrophils, eosinophils and basophils, while the second is the originator of lymphocytes. These cells are involved in the humoral, as well as, the cellular immune response, non-specifically and specifically, although each cell type has its own function in such responses (Male and Roitt, 1998).

1.3.3.2 Mitotic Index

Mitotic index is defined as the ratio of the number of cells in a population undergoing mitosis (different stages) to total number of cells. It is a useful and a sensitive test for the detection of cytotoxic effects of chemical and physical agents, as well as, mutagenic and carcinogenic agents (Ghosh *et al.*, 1991).

Mitosis is only a part, and often only a very brief part, of the cell cycle. Interphase begins with the G1 phase, immediately after mitosis, when the chromosomes from the contracted state become extended (Cooper and Hausman, 2007). The G1 phase of the cell cycle is a far more variable in length than other phases and is responsible for the variability in generation times within a cell population, and cells which stop dividing usually arrest in this phase. During the S phase, which follows, DNA replication occurs and the chromatin material is duplicated. In the final G2 phase, the chromosomes condense in preparation for mitosis (Raven and Johnson, 1989). Therefore, before the cell undergoes mitosis, several molecular events have occurred, and such events are the target for exogenous factors (i.e. mutagens), which have the potentials to induce abnormalities in the dividing cells at the stages of mitosis. These abnormalities can be measured in several assays; for instances, chromosomal aberrations, sister chromatid exchanges, micronucleus formation and mitotic index (Komae *et al.*, 1997). The latter assay is important in this regard, especially if it is considered for two important sites in the body; the bone marrow and spleen. The bone marrow supplies the blood stream with all types of blood cells, while spleen is a secondary lymphoid organ, in which the

confrontation between non-self antigens and the defending cells of the immune system is occurred (Lydyard and Grossi, 1998). Therefore, evaluating the dividing status (mitotic index) in these two sites after a treatment of an animal with different agents (i.e. medicinal plants) may reveal the potential of these agents in enhancing or reducing such activity. In the present study, only cells at metaphase was scored (metaphase index), because this stage of mitosis is the clearest and then it will be easily evaluated to determine the effect of tested materials (Gersen and Keagle, 2005).

1.3.3.3 Micronucleus Formation (Fenech *et al.*,2007)

Micronuclei are small chromatin particles consisting of acentric fragments of chromosome or entire chromosomes, which lag behind at anaphase of cell division. After telophase, these fragments may not be included in the nuclei of daughter cells and form single or multiple micronuclei in the cytoplasm (United States Environmental Protection Agency, 1996). They are the result of a structural chromosomal aberration, or a disturbed function of the mitotic spindle (Miloevi-Dordevi *et al.,* 2003). Therefore, the measurement of micronuclei can reflect chromosome damage and may thus provide a marker of early-stage mutagenesis and carcinogenesis, as well as, they have been considered to represent markers of exposure to mutagens and potential indicators of biological response to genotoxic agents. Cells with micronuclei can be scored *in vivo* in the bone marrow of mammals (polychromatic erythrocytes), as well as, *in vitro* in cultured lymphocytes pre-stimulated with a mitogen i.e. phytohaemoagglutinin (PHA) (Bonassi *et al*., 2003).

1.3.3.4 Sperm-head Abnormality Assay

The entire process of sperm formation, from spermatogonia to spermatozoa is referred to as spermatogenesis, which occurs in the somniferous tubules of the testis (Seely *et al*., 1996). The stem cell (spermatogonia) is divided mitotically to give primary spermatocytes that

undergo meiosis I to give rise to haploid secondary spermatocytes that undergo meiosis II to produce spermatids. the spermatids are then transformed to spermatozoa by a series of morphological changes (Hafez and Hafez, 2000). These events are under a genetic control, especially the morphological changes involved in sperm head formation. Therefore, an assay was developed to detect genotoxic effects induced by physical and chemical agents. The assay scores the percentage of sperms with abnormal head morphology, and such morphological changes are dependent on the stage of spermatogenesis. If the abnormality appeared at the end of first week post-treatment, it means that the genetic abnormality occurred in the stage spermatid formation, while if the abnormality appeared after 3-5 weeks post-treatment, it means that the abnormality occurred in the stages of spermatocytes or spermatogonia. Therefore, this test has been qualified to assess the mutagenic effects of chemical and physical mutagens *in vivo* (Topham, 1980; Wang *et al.*, 2000).

1.3.3.5 Total Serum Level of Immunoglobulin G (IgG)

Immunoglobulins are glycoprotein molecules, which are produced by plasma cells in response to an immunogen and function as antibodies. The immunoglobulins derive their name from the finding that when antibody-containing serum is placed in an electrical field, the antibodies migrate with the globular proteins (Roitt *et al.*, 2001). They bind specifically to one or a few closely related antigens. Each immunoglobulin actually binds to a specific antigenic determinant. Antigen binding by antibodies is the primary function of antibodies and can result in protection of the host. However, often the binding of an antibody to an antigen has no direct biological effect; rather, the significant biological effects are a consequence of secondary "effector functions" of antibodies (Mayer, 2008).

The immunoglobulins can be divided into five different classes based on differences in the amino acid sequences in the constant region of the heavy chains. They are IgG, IgM, IgA, IgE and IgD (Roitt *et al.*, 2001). Immunoglobulin G is the most versatile immunoglobulin due to the followings:

i. It is the major immunoglobulin in serum and extra vascular spaces, as well as in the secondary humoral immune response

ii. It is the only class of immunoglobulin that crosses the placenta.

iii. It is effective in fixation of the complement.

iv. It is able to bind various types of immune cells; especially, monocytes, macrophages and neutrophils. Such binding enhance their phagocytic activity through the process of opsonization.

Chapter Two
Materials and Methods

2.1 Materials

The general laboratory equipments and chemical materials that were employed in the study are given in appendices I and II, respectively.

2.2 The Plant *Micromeria myrtifoila* Boiss. and Hohen

The Professor Dr. Ali Al-Mosawy (Department of Biology, College of Science, University of Baghdad) identified the plant of *Micromeria myrtifoila*, which was collected on December 2006 from a region north the City of Mosul, which is located 440 km north of Baghdad. The plant was left

at room temperature to dry, and after dryness, it was powdered with a coffee grinder.

2.3 Plant Extraction

The plant powder was extracted with two types of solvents (Methanol and Hexane). In both cases, 50 grams of the powder were extracted in the solvent at 45°C for three hours using the Soxhlet apparatus. The resulted extract was concentrated by rotary evaporator at 45°C, and the dry deposit was obtained, which was 2.6 grams (5.2% of the original weight). The collected crude deposit extract was frozen at -20°C until use to prepare the required doses (Sabahi *et al*., 1987).

The methanol deposit extract was dissolved in sterile distilled water to prepare two doses (200 and 600 mg/kg), which were investigated in the laboratory mice. These doses were based respectively on 10 and 30% of the lethal dose (LD_{50}) 2000 mg/kg in mice (Badisa *et al.*, 2002). The extract was sterilized by filtration using millipore filters (0.22 µm). The hexane deposit extract was dissolved in olive oil, and as in the methanol extract, similar doses were prepared and investigated.

2.4 Solutions

i. **Phosphate Buffered Saline (PBS)**: The following chemicals were dissolved in 500 ml of distilled water, and then the volume was made up to 1000 ml. The pH was adjusted to 7.2, and the solution was autoclaved (121°C, 1.5 pound / in^2, 20 minutes) and stored at 4°C (Hunger ford,1965)Hudson and Hay, 1989).

   · Sodium chloride (NaCl): 8.00 gram

- Potassium chloride (KCl): 0.20 gram
- Di-sodium hydrogen phosphate (Na_2HPO_4): 1.15 gram
- Potassium di-hydrogen phosphate (KH_2PO_4): 0.20 gram

ii. Colchicine: One tablet (1 mg) of colchicine was dissolved in 1 ml of PBS, and the solution was freshly used (Allen *et al*., 1977).

iii. Hypotonic Potassium Chloride (KCl) (0.075 M): Potassium chloride (0.575 gram) was dissolved in 50 ml of distilled water, and the volume was made up to 100 ml. The solution was autoclaved and stored at 4°C (Allen *et al*., 1977).

iv. Fixative: This solution was freshly prepared by mixing three parts of absolute methanol with one part of glacial acetic acid. The solution was cooled at 4°C for 30 minutes before use (Allen *et al*., 1977).

v. Giemsa Stain: Giemsa stain stock solution was prepared by dissolving one gram of Giemsa powder in 33 ml of glycerin in a water bath (60°C) for two hours with continuous shaking. Then, the solution was cooled at room temperature for 30 minutes. After that, 66 ml of absolute methanol were added with continuous mixing. The solution was filtered and kept in a dark bottle in the incubator (37°C) (Allen *et al*., 1977). To prepare the Giemsa stain working solution, the following solutions were mixed:

- Giemsa stock solution: 1 ml
- Absolute methanol: 1.25 ml
- Sodium bicarbonate solution: 0.5 ml

- Distilled water: 40 ml

vi. **Leishman Stain**: The stain kit was supplied by the Institute of Sera and Vaccines (Baghdad, Iraq).

vii. **Eosin Stain**: One gram of eosin yellowish stain powder was dissolved in 100 ml of distilled water (Wyrobek and Bruce, 1975).

viii. **Sodium Bicarbonate ($NaHCO_3$)**: Sodium bicarbonate (7.5 gram) was dissolved in 100 ml of distilled water, and the solution was stored at 4°C (Allen *et al*., 1977).

ix. **Normal Saline**: A ready solution (0.9% NaCl) was used. It was the product of ADWIC Company (Egypt).

x. **Leucocyte Diluent**: Two ml of glacial acetic acid were added to 98 ml of distilled water, in addition to three drops of Methylene blue stain as a color indicator (Sood, 1986).

xi. **Human Plasma**: The National Blood Transfusion Centre in Baghdad supplied the human AB plasma. The plasma was transferred to the laboratory in an ice box. In the laboratory, the plasma was divided into aliquots (5 m), and the tubes were placed in a water bath (56°C) for 30 minutes to inactivate the complement, and then stored at -20°C until use in the micronucleus assay (Schmid, 1976). The plasma was filter-sterilized.

xii. **Mayer's Reagent:** Two solutions were firstly prepared; the first one was prepared by dissolving 1.58 gram of Mercuric chloride ($HgCl_2$) in 60 ml

of distilled water, while the second solution was prepared by dissolving 5 grams of potassium iodide (KI) in 10 ml of distilled water. Then both solutions were mixed and the volume was made-up to 100 ml with distilled water (Smolensk *et al*., 1972).

xiii. **Molisch's reagent:** The reagent was prepared by dissolving 0.5 gram of α-naphthol in10 ml of 95% ethanol. The reagent was stored in a dark place at room temperature until use (Mathews *et al*., 1999).

2.5 Laboratory Animals

Albino male mice (*Mus musculus*) were the laboratory animals, which were used to carry out the investigations of the present study. They were obtained from Biotechnology Research Centre (Al-Nahrain University). Their age range was 8-9 weeks, and their weight was 25 ± 2 grams at the beginning of the experiments. They were housed in standard cages with free access to food (standard laboratory rodent pellets) and water. The animal house temperature was maintained at 23 ± 3ºC.

2.6 Experimental Design

Three main types of experiments were carried out to assess the cytogenetic and immunological effects of *M. myrtifoila* extracts (methanol and hexane) in male albino mice.

2.6.1 Experiment Number One

The experiment was designed to assess the cytogenetic effects of two doses (200 and 600 mg/kg) of the plant extracts, as well as, the drug methotrexate (0.33 mg/kg). In all cases, a single dose/day (0.1 ml) of the tested material was orally given for seven days, and on day eight, the animals were scarified to carry out laboratory assessments. The total number of the

animals in this experiment was 5684 mice, which were divided into seven groups:

- **Group 1** (Negative control: 4 mice): The tested material was distilled water (the control of methanol extract).
- **Group 2** (Negative control: 4 mice): The tested material was olive oil (the control of hexane extract).
- **Group 3** (Positive control: 4 mice): The tested material was methotrexate.
- **Group 4** (Micromeria methanol extract: 824 mice): The tested material was two doses (200 and 600 mg/kg) of Micromeria methanol extract.
- **Group 5** (Micromeria hexane extract: 8 mice): The tested material was two doses (200 and 600 mg/kg) of Micromeria hexane extract.

2.6.2 Experiment Number Two

This experiment was designed to assess the cytogenetic and immunological effects of a pre-treatment interaction between the two doses (200 mg/kg and 600 mg/kg) of both extracts and methotrexate. In such treatment, the animals were orally dosed with a single dose/day of the plant extract for seven days, while in day 8, they were given methotrexate. In day 9, they were sacrificed for laboratory assessments. Both extract-interactions were paralleled with control groups, in which the plant extract was replaced with either distilled water (methanol extract) or olive oil (hexane extract).

2.6.3 Experiment Number Three

This experiment was designed to assess the cytogenetic and immunological effects of a post-treatment interaction between the two doses (200 mg/kg and 600 mg/kg) of both extracts and methotrexate. In such

treatment, the animals were orally dosed with a single dose/day of methotrexate in day1, while in days 2-8, they were given the plant extract (single dose/day). In day 9, they were sacrificed for laboratory assessments. Both extract-interactions were paralleled with control groups, in which the plant extract was replaced with either distilled water (methanol extract) or olive oil (hexane extract).

2.7 Laboratory Methods

2.7.1 Chemical Detection of Plant Extract

The chemical analysis of an aqueous extracts of *P. lanceolata* were carried out in The Biotechnology Research Center (Al-Nahrain University) to detect the following compounds:

i. **Flavonoids:** The detecting solution was prepared by mixing 10 ml of ethanol (50%) with 10 ml of potassium hydroxide (50%), and then 5 ml of this solution was added to 5 ml of the methanol extract. The appearance of yellow color was an indicator of the presence of flavonoids (Jaffer *et al.*, 1983).

ii. **Polysaccharides:** Molisch's reagent **(**0.02 ml) was added to 1 ml of the plant extract in a test tube, and after a careful mixing, 0.5 ml of concentrated sulfuric acid (H_2SO_4) was added by pouring it down the side of the tube. A red-violet layer at the interface between the acid (bottom) and aqueous (upper) layers was considered as a positive indication for polysaccharides (Mathews *et al.*, 1999).

iii. **Alkaloids:** One ml of the methanol extract was added to a tube containing 2 ml of Mayer′s reagent. The appearance of gray color after shaking the tube was an indicator of the presence of alkaloids (Harborne, 1973).

iv. **Steroids and Terpens:** The procedure of Al-Maisary (1999) was used, in which 1 ml of the hexane extract was mixed with 2 ml of chloroform and one drop of glacial acetic acid, and then one drop of H_2SO_4 was added. The appearance of brown color was an indicator of the presence of terpens, and if then (5-10 minutes) a blue color was appeared, this time it was an indicator of the presence of steroids.

2.7.2 Total and absolute Counts of Leucocytes

Both counts were carried out on blood obtained by heart puncture using insulin disposable syringe (1 ml) pre-coated with heparin.

2.7.2.1 Total Count of Leucocytes

The conventional method of blood cell counting was employed, following the procedure of Sood (1986). A volume of 0.02 ml blood was dispensed in a test tube containing 0.38 ml of leucocyte diluent solution, and then the contents were mixed and the tube was left for three minutes. One drop of the diluted blood was applied to the surface of a counting chamber (Neubauer hemocytometer) under the cover slip. After that, the chamber was left for two minutes to settle the cells, and by then, the leucocytes were counted using the following equation:

$$\text{Total Count (cell/cu.mm.blood)} = \left(\frac{\text{Number of Cells Counted}}{4} \right) \text{x } 20 \text{ x } 10$$

2.7.2.2 Absolute Count of Leucocytes

A blood smear was made on a clean slide and left for air drying. Then the slide was stained with Leishman's stain for two minutes and buffered for 10 minutes with Leishman's buffer. After that, the slide was rinsed with tap water and left for air drying (Sood, 1986). The stained smear was examined under oil immersion power (100X), and at least 200 leucocytes were

randomly counted. Then, the percentage of each cell type was obtained. The count of each type of leucocytes was calculated according to the following equation:

$$\text{Total Count (cell/cu.mm.blood)} = \left(\frac{\text{Percentage of Cells x Total Count}}{100} \right)$$

2.7.3 Metaphase Index

The metaphase index (MI) was assessed on somatic cells obtained from the bone marrow and spleen of investigated mice, according to a pre-established method (Allen *et al.*, 1977), which was based on the following steps:

i. The animal was injected intraperitoneally with 0.25 ml of colchicine solution, and after two hours, the animal was sacrificed by cervical-dislocation.

ii. The animal was dissected, and femur and spleen were removed and transferred to two Petri dishes containing 5 ml of PBS.

iii. The femur was cleaned from muscles and other tissues, and both ends were cut. Then, the bone marrow was obtained with PBS (5 ml) using disposable insulin syringe, and collected in a test tube.

iv. The spleen was punctured with the needle of insulin syringe, and its cellular content was obtained by repeated injections of PBS. The cells were collected in a test tube.

v. The cell suspension of both tubes was gently pipetted, and centrifuged (2000 rpm) for 10 minutes.

vi. After discarding the supernatant, the cell deposit was suspended in 5 ml of a warm (37°C) hypotonic KCl (0.075M), and incubated for 20 minutes in a water bath (37°C), with shaking every 5 minutes.

vii. The tubes were centrifuged (2000 rpm) for 5 minutes, and the supernatant was discarded.

viii. The cell deposit was slowly suspended in 5 ml of cooled fixative, and incubated for 30 minutes at 4°C.

ix. Step **vii** was repeated, and the cell deposit was gently suspended in 1-2 ml of cooled fixative, to prepare a single cell suspension.

x. Few drops (4-5 drops) of the fixed cell suspension were dropped vertically from a height of about 3 feet on a cleaned slide to give chance for nuclei and chromosomes to spread well.

xi. The slides were air-dried, stained with Giemsa stain for 15 minutes, rinsed with distilled water, and left to dry at room temperature.

xii. The slides were examined under oil immersion lens (100X), and at least 1000 cells (divided and non-divided cells) were scored. Then, the percentage of metaphase cells (metaphase index; MI) was calculated according to the following equation:

$$\text{Metaphase Index (\%)} = \left(\frac{\text{Number of Metaphase Cells}}{\text{Total Count}} \right) \text{x } 100$$

2.7.4 Micronucleus Test

The procedure of Schmid (1976) was followed with some modification to assess the micronucleus formation in the bone marrow of mice. The animal was sacrificed by cervical dislocation, and femur was removed and cleaned from muscles and other tissues, and both ends were cut. Then, the bone was griped from the middle with forceps in a vertical position over the edge of a test tube, and 2 ml of AB human plasma (heat inactivated) were injected in the bone cavity to wash out the bone marrow cells, using insulin syringe. The test tube was centrifuged at (1000 rpm) for 10 min, and after discarding the supernatant, the cell deposit was smeared on a clean slide, which was air-

dried. The smear was fixed with absolute methanol, stained with Giemsa stain for 15 minutes, then washed with distilled water, and air-dried.

The slide was examined under oil immersion lens (100X), and polychromatic erythrocytes (PCE) were inspected for the formation of micronucleus formation. A total of 1000 cells was randomly examined, and the PCE with micronucleus or micronuclei was scored, while micronucleus index was given as micronucleus per 1000 PCE.

2.7.5 Sperm-Head Abnormality Assay

The procedure Wyrobek and Bruce (1978) was followed with some modification, to assess the sperm-head abnormalities (SHA) in mice. The animal was sacrificed by cervical dislocation and dissected to remove the epididymis, which was transferred to a Petri dish containing 5 ml of normal saline. The epididymis was cut into small pieces and dispersed with Pasteur pipette. The spermatozoa containing saline was transferred to a test tube, which was subjected to a centrifugation (1000 rpm) for 5 minutes, and after discarding the supernatant, the spermatozoa deposit was suspended in 1 ml of normal saline and mixed gently. The spermatozoa suspension (2-3 drops) was smeared on a clean slide and air-dried. The smear was stained with eosin for 15 minutes, and then rinsed with distilled water and left to air-drying.

At least 1000 spermatozoae were examined randomly under oil immersion lens (1000X), and the percentage of spermatozoae with abnormal head was scored using the following equation:

$$\text{SHA index (\%)} = \left(\frac{\text{Number of Spermatozoae with Abnormal Head}}{\text{Total Count}} \right) \text{x} \, 100$$

2.7.6 Total IgG Serum Level

2.7.6 Quantification of Serum IgG by Mouse ELISA Kit

The mouse IgG ELISA kit (Murine BioAssay ^{TM}, USBiological, U.S.A.) is an *in vitro* immunoassay for the quantification of IgG circulating in serum or in other qualified samples from tissue fluids (i.e. saliva and mucosa), or in cultures of mouse cells.

2.7.6.1 Principle of the Test

The test is based on the binding of mouse IgG in samples to two antibodies; one is immobilized on the microtiter wells, while the other is conjugated to horseradish peroxidase (HRP) enzyme. After a washing step, chromogenic substrate is added and colour is developed by the enzymatic reaction of the tetraethyl benzidine substrate (TMB substrate), which is directly proportional to the amount of IgG present in the sample. The stop solution is added to terminate the reaction, and absorbance at 450 nm is then measured using an ELISA microtiter well reader. The level of IgG in samples and controls is calculated from a curve of standards containing known concentrations of IgG.

2.7.6.2 Kit Components

- Microtiter plate pre-coated with anti-mouse IgG antibody (1 x 96 wells).
- Sample Diluent.
- Wash solution.
- Anti-mouse IgG antibody conjugated with HRP enzyme.
- Standards of IgG (10, 20, 60, 120 and 200 ng/ml).
- Positive control.

- TMB substrate.
- Stop solution.

2.7.6.3 Collection of Serum Samples

The mouse was anesthetized with chloroform, and the blood then was obtained by heart-puncture using a disposable insulin syringe (1 ml). The blood (0.5 – 0.7 ml) was left at room temperature (20-25ºC) for 15 minutes to clot. The clotted blood was centrifuged (2000 rpm) for 15 minutes, and the serum was collected. The collected serum was frozen (-20 ºC) until the assay was carried out.

2.7.6.4 Assay Method

All reagents were brought to room temperature (30 minutes) equilibration before carrying out the assay, and then the instructions of the manufacturer were followed. They are outlined in the following steps:

i. Working wash solution (200 µl) was added to each well, and the plate was let to stand for 15 minutes. After that, the solution was aspirated, and the wells were dried on a paper towel.

ii. Standards, samples or controls (100 µl each) were added to each predetermined wells, and the plate was tapped gently to mix the reagents and incubated for 60 minutes at room temperature. Then the plate wells were washed four times with washing solution.

iii. Anti-mouse IgG antibody conjugated with HRP enzyme (100 µl) was added to each well, and the plate was incubated for 30 minutes at room temperature. Then the plate wells were washed five times with washing solution.

iv. The substrate TMB (100 µl) was added to each well, and the plate was incubated for 15 minutes in a dark place at room temperature. After that stop solution (100 µl) was added to each well.

v. The absorbency of each well was read at a wave length of 450 nm in less than 30 minutes, and the optical density (OD) was recorded.

vi. The level of IgG was calculated according to the standard curve (Figure 2-1).

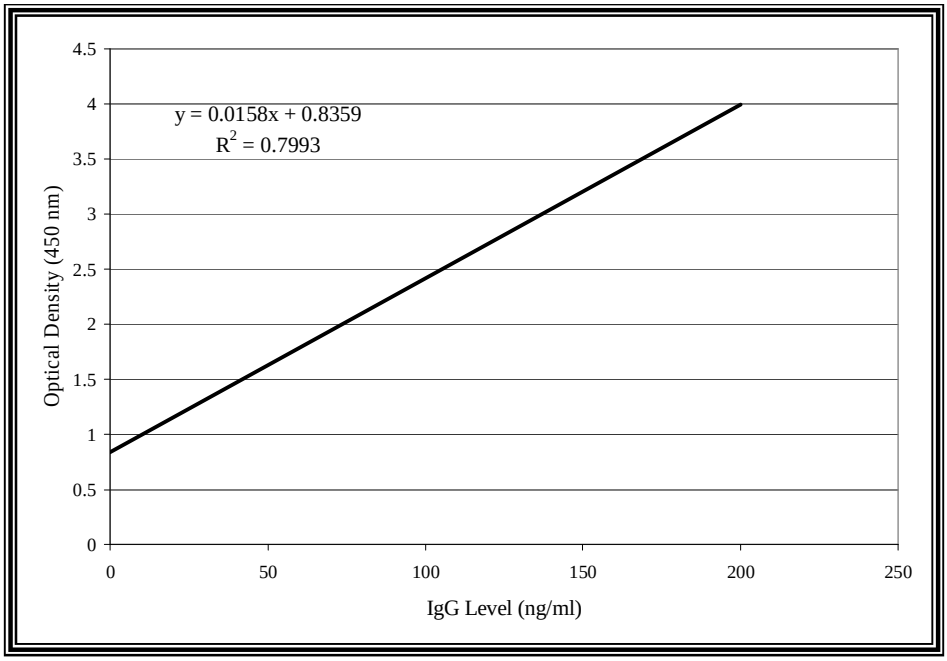

Figure 2-1: Standard curve of mouse IgG serum level.

2.8 Statistical Analyses

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

A further estimation was also given; it was treatment efficiency (Serrano *et al.*, 1997), which was calculated according to the following equation:

$$\text{Treatment efficiency (\%)} = \left(\frac{A - B}{B}\right) \times 100$$

A = Treated groups (plant extracts or methotrexate).

B = Negative control groups (distilled water or olive oil).

Chapter Three
Results

3.1 Chemical Detection of *Micromeria myrtifolia* Extracts

The detection of flavonoids, alkaloids and steroids was carried out in the methanol and hexane extracts of *M. myrtifolia*. The results revealed the presence of flavonoids and polysaccharides in methanol extract, while alkaloids were not detected. On the other hand, steroids showed a positive reaction in the hexane extract of the plant (Table 3-1).

Table 3-1: Chemical detection of *M. myrtifolia* extracts.

Plant extracts	Chemicals Compounds	Reagents	Indication	Reaction
Methanol	Flavonoids	Ethanol with KOH	Dark color	Positive
	Alkaloids	Mayer's reagent	No white precipitate	Negative
	Polysaccharides	Molisch's reagent	red-violet layer	Positive
Hexane	Steroids	Concentrated Sulfuric acid	Blue color to green	Positive

3.2 Immunological and Cytogenetic Effects of Extracts

In this section, two doses (200 and 600 mg/kg) of *M. myrtifolia* extracts (methanol and hexane) were evaluated for their immunological and cytogenetic effects in albino male mice, together with methotrexate (positive control), distilled water and olive oil (negative controls). The parameters of evaluations were total and absolute counts of leucocytes, metaphase index of bone marrow and spleen cells, total IgG serum level, micronucleus formation and sperm head abnormalities.

3.2.1 Total Count of Leucocytes (TLC)

The methotrexate drug was effective in a significant ($P \leq 0.05$) reduction of TLC (5.1 x 10^3 cell/cu.mm.blood) as compared to distilled water (8.1 x 10^3 cell/cu.mm.blood) or olive oil (10.1 x 10^3 cell/cu.mm.blood) negative controls. In contrast, the first dose of methanol extract (200 mg/kg) was significantly effective in increasing the count of leucocytes (12.7 x 10^3 cell/cu.mm.blood) with a treatment efficiency of +57.7%, while the next dose (600 mg/kg) reduced significantly such count (7.3 x 10^3 cell/cu.mm.blood) as compared to the corresponding negative control (8.1 x 10^3 cell/cu.mm.blood). The two doses of hexane extract behaved in a similar manner in their effect on TLC (10.2 and 8.3, respectively *vs*.10.1 x 10^3 cell/cu.mm.blood) as compared to the corresponding negative control, but a significant difference was reached in the second dose (Table 3-2).

Table 3-2: Total leucocyte count in albino male mice treated with methanol and hexane extracts of *Micromeria myrtifolia*, distilled water and olive oil (negative control) and methotrexate (positive control).

Groups	Dose (mg/kg)	Mean ± S.E. x 10^3* (cells/cu.mm. blood)	Treatment Efficiency (%)
Positive Control (Methotrexate)	0.33	5.1± 0.3[b]	- 36.5
Negative Control (H_2O)CONTROL		8.1 ± 0.4[a]	

Negative Control (Olive Oil)			10.1 ± 0.4[a]	
M. myrtifolia Extract	Methanol	200	12.7 ± 0.3[c]	+ 57.7
		600	7.3 ± 0.1[d]	- 9.3
	Hexane	200	10.2 ± 0.1[a]	+ 0.9
		600	8.3 ± 0.2[c]	- 17.1

*Different letters: Significant difference ($P \leq 0.05$) between means of the column.

3.2.2 Absolute Count of Leucocytes

3.2.2.1 Lymphocytes

The two doses of methanol extract (200 and 600 mg/kg) were effective in increasing the count of lymphocytes (8.2 and 4.8 x 10^3 cell/cu.mm.blood, respectively), as compared to the corresponding negative control (4.2 x 10^3 cell/cu.mm.blood), but a significant difference was observed in the first dose, which recorded a treatment efficiency of +97.3%. For hexane extract, a similar picture was drawn, and again the first dose increased the lymphocyte count significantly as compared to the corresponding negative control (7.5 *vs.* 5.7 x 10^3 cell/cu.mm.blood), but with a less treatment efficiency (+31.4%) (Table 3-3).

Table 3-3: Total lymphocyte count in albino male mice treated with methanol and hexane extracts of *Micromeria myrtifolia*, distilled water and olive oil (negative control) and methotrexate (positive control).

Groups	Dose (mg/kg)	Mean ± S.E. x 10^3* (cells/cu.mm. blood)	Treatment Efficiency (%)
Positive Control (Methotrexate)	0.33	1.0 ± 0.04[b]	- 75.5
Negative Control (H_2O)		4.2 ± 0.13[a]	
Negative Control (Olive Oil)		5.7 ± 0.12[a]	

M. myrtifolia Extract	Methanol	200	8.2 ± 0.09[c]	+ 97.3
		600	4.8 ± 0.06[a]	+ 13.8
	Hexane	200	7.5 ± 0.06[c]	+ 31.4
		600	6.2 ± 0.48[a]	+ 8.8

*Different letters: Significant difference ($P \leq 0.05$) between means of the column..

3.2.2.2 Neutrophils

The two doses of methanol and hexane extracts reduced the count of neutrophils as compared to the corresponding negative controls, but none of these differences attended a significant level ($P > 0.05$) (Table 3-4).

Table 3-4: Total neutrophil count in albino male mice treated with methanol and hexane extracts of *Micromeria myrtifolia*, distilled water and olive oil (negative control) and methotrexate (positive control).

Groups		Dose (mg/kg)	Mean ± S.E. x 10^3* (cells/cu.mm. blood)	Treatment Efficiency (%)
Positive Control (Methotrexate)		0.33	2.5 ± 0.19[a]	+ 11.2
Negative Control (H_2O)			2.2 ± 0.15[a]	
Negative Control (Olive Oil)			2.3± 0.18[a]	
M. myrtifolia Extract	Methanol	200	2.0 ± 0.16[a]	- 7.2
		600	1.8 ± 0.07[a]	- 19.6
	Hexane	200	1.9 ± 0.07[a]	- 16.0
		600	1.8 ± 0.13[a]	- 17.2

*Similar letters: No significant difference ($P > 0.05$) between means of the column.

3.2.2.3 Monocytes

The first dose of methanol extract was associated with a significant increased count (2426 cell/cu.mm.blood) with a treatment efficiency of +60.5%, while a significant decrease in monocyte count was reached in the second dose (628 cell/cu.mm.blood) as compared to the distilled water negative control (1510 cell/cu.mm.blood). A further significant reduction in monocyte count was observed in the two doses of hexane extract (610 and 666 respectively *vs*.1763.9 cell/cu.mm.blood) as compared to the olive oil negative control (Table 3-5).

Table 3-5: Total monocyte count in albino male mice treated with methanol and hexane extracts of *Micromeria myrtifolia*, distilled water and olive oil (negative control) and methotrexate (positive control).

Groups		Dose (mg/kg)	Mean ± S.E.* (cells/cu.mm. blood)	Treatment Efficiency (%)
Positive Control (Methotrexate)		0.33	505.4 ± 19.2^{b}	- 66.5
Negative Control (H_2O)			1510.9 ± 84.8^{a}	
Negative Control (Olive Oil)			1763.9 ± 69.1^{a}	
M. myrtifolia Extract	Methanol	200	2426.5 ± 93.3^{c}	+ 60.5
		600	628.9 ± 49.1^{b}	- 58.3
	Hexane	200	610.5 ± 71.7^{b}	- 65.3
		600	666.5 ± 54.6^{b}	- 62.2

*Different letters: Significant difference ($P \leq 0.05$) between means of the column.

3.2.2.4 Eosinophils and Basophils

Neither doses of methanol or hexane extracts were effective to show a significant difference in eosinophil or basophil count when comparisons were

made with the corresponding negative controls. In contrast, the methotrexate was effective in this regard. It increased the count of eosinophils (571.0 cell/cu.mm.blood), as well as, basophils (553.8 cell/cu.mm.blood), with significant differences, when comparisons were made with distilled water negative control (80.5 and 80.5 cell/cu.mm.blood, respectively) or olive oil negative control (0.0 and 100.8 cell/cu.mm.blood, respectively). The treatment efficiencies of the two differences were +609.3 and +587.9%, respectively (Tables 3-6 and 3-7)

Table 3-6: Total eosinophil count in albino male mice treated with methanol and hexane extracts of *Micromeria myrtifolia*, distilled water and olive oil (negative control) and methotrexate (positive control).

Groups		Dose (mg/kg)	Mean ± S.E.* (cells/cu.mm. blood)	Treatment Efficiency (%)
Positive Control (Methotrexate)		0.33	571.0 ± 59.2[b]	+ 609.3
Negative Control (H_2O)			80.5 ± 3.6[a]	
Negative Control (Olive Oil)			0.0± 0.0[a]	
M. myrtifolia Extract	Methanol	200	0.0± 0.0[a]	- 100.0
		600	73.0 ± 1.3[a]	- 9.3
	Hexane	200	101.8 ± 1.2[a]	0.0
		600	41.0 ± 23.7[a]	0.0

*Different letters: Significant difference ($P \leq 0.05$) between means of the column.

Table 3-7: Total basophil count in albino male mice treated with methanol and hexane extracts of *Micromeria myrtifolia*, distilled water and olive oil (negative control) and methotrexate (positive control).

			Treatment

Groups		Dose (mg/kg)	Mean ± S.E.* (cells/cu.mm. blood)	Efficiency (%)
Positive Control (Methotrexate)		0.33	553.8 ± 77.2[b]	+ 587.9
Negative Control (H_2O)			80.5 ± 3.6[a]	
Negative Control (Olive Oil)			100.8 ± 3.5[a]	
M. myrtifolia Extract	Methanol	200	0.0 ±0.0[a]	- 100
		600	73.0 ± 1.3[a]	- 9.3
	Hexane	200	51.3 ± 29.6[a]	- 49.1
		600	40.5 ± 23.4[a]	- 59.8

*Different letters: Significant difference ($P \leq 0.05$) between means of the column.

3.2.3 Metaphase Index

In the present study, only cells at metaphase were scored in samples of bone marrow and spleen, and therefore the metaphase index was based on the percentage of these cells.

3.2.3.1 Bone Marrow Cells

A treatment with Methotrexate caused a significant reduction in the metaphase index (1.87%) as compared to the distilled water and olive oil negative controls (2.57 and 4.51%, respectively. In contrast, the two doses of methanol extract were associated with a significant increased index (4.78 and 3.28%, respectively), as compared to the distilled water negative control (2.57%). For hexane extract, the two doses behaved in a similar manner, in which a significant increased index was also observed (6.93 and 5.68%, respectively), as compared to the olive oil negative control (4.51%). The best treatment efficiency was recorded in the first dose of methanol extract (+86.1%) (Table 3-8).

Table 3-8: Metaphase index of bone narrow cells in albino male mice treated with methanol and hexane extracts of *Micromeria myrtifolia*, distilled water and olive oil (negative control) and methotrexate (positive control).

Groups		Dose (mg/kg)	Mean ± S.E.* (%)	Treatment Efficiency (%)
Positive Control (Methotrexate)		0.33	1.87 ± 0.05[a]	- 27.2
Negative Control (H_2O)			2.57 ± 0.10[b]	
Negative Control (Olive Oil)			4.51 ± 0.23[c]	
M. myrtifolia Extract	Methanol	200	4.78 ± 0.29[c]	+86.1
		600	3.28 ± 0.12[d]	+27.7
	Hexane	200	6.93 ± 0.22[e]	+53.5
		600	5.68 ± 0.19[f]	+25.7

*Different letters: Significant difference ($P \leq 0.05$) between means of the column.

3.2.3.2 Spleen cells

The two doses (200 and 600 mg/kg) of methanol extract were significantly effective in increasing the metaphase index of spleen cells (2.65 and 1.68 %, respectively) as compared to the corresponding negative control (1.38%), especially the first dose, which recorded a treatment efficiency of +92.0%, while the two doses of hexane extract showed a non-significant increase (Table 3-9).

Table 3-9: Metaphase index of spleen cells in albino male mice treated with methanol and hexane extracts of *Micromeria myrtifolia*, distilled water and olive oil (negative control) and methotrexate (positive control).

Groups		Dose (mg/kg)	Mean ± S.E.* (%)	Treatment Efficiency (%)
Positive Control (Methotrexate)		0.33	0.83 ± 0.06^{b}	- 40.0
Negative Control (H_2O)			1.38 ± 0.07^{a}	
Negative Control (Olive Oil)			2.40 ± 0.13^{ac}	
M. myrtifolia Extract	Methanol	200	2.65 ± 0.12^{c}	+ 92.0
		600	1.68 ± 0.06^{d}	+ 21.7
	Hexane	200	3.13 ± 0.14^{c}	+ 30.2
		600	2.80 ± 0.04^{c}	+ 16.7

*Different letters: Significant difference ($P \leq 0.05$) between means of the column.

3.2.4 Micronucleus Index

The micronucleus formation was assessed in polychromatic erythrocytes of bone marrow, in which the methotrexate caused a significant increase of micronucleus frequency (177 micronucleus/1000 cell) as compared to distilled water and olive oil negative controls (73 and 50 micronucleus/1000 cell, respectively). This result was shared by the second dose (600 mg/kg) of methanol and hexane extracts, in which a significant increased frequency was recorded (126 and 100 micronucleus/1000 cell, respectively) as compared to the corresponding negative controls (73 and 50 micronucleus/1000 cell, respectively). In contrast, the first dose of both

extract approximated the corresponding negative control frequency (Table 3-10).

Table 3-10: Micronucleus formation in bone marrow cells of albino male mice treated with methanol and hexane extracts of *Micromeria myrtifolia*, distilled water and olive oil (negative control) and methotrexate (positive control).

Groups		Dose (mg/kg)	Mean ± S.E.* (Micronucleus/1000 cells)	Treatment Efficiency (%)
Positive Control (Methotrexate)		0.33	177 ± 8[b]	+ 142.4
Negative Control (H_2O)			73 ± 10[a]	
Negative Control (Olive Oil)			50 ± 10[a]	
M. myrtifolia Extract	Methanol	200	84 ± 10[a]	+ 15.0
		600	126 ± 20[c]	+ 72.6
	Hexane	200	50 ± 20[a]	0.0
		600	100 ± 19[c]	+ 100

*Different letters: Significant difference ($P \leq 0.05$) between means of the column.

3.2.5 Sperm Head Abnormalities

Both doses (200 and 600 mg/kg) of methanol extract were significantly effective in reducing the frequency of sperm head abnormalities as compared to the corresponding negative control (6.34 and 6.77%, respectively *vs.* 7.02%), but the first dose was the best in this regard (Treatment efficiency = -9.6%). The hexane extract exerted a similar effect (5.79 and 5.83%,

respectively *vs*. 6.63%), but in contrast, the second dose was the best in this context (Treatment efficiency = -12.1%) (Table 3-11).

Table 3-11: Sperm head Abnormalities in albino male mice treated with methanol and hexane extracts of *Micromeria myrtifolia*, distilled water and olive oil (negative control) and methotrexate (positive control).

Groups		Dose (mg/kg)	Mean ± S.E.* (%)	Treatment Efficiency (%)
Positive Control (Methotrexate)		0.33	7.41± 0.035[a]	+11.7
Negative Control (H_2O)			7.02 ± 0.05[a]	
Negative Control (Olive Oil)			6.63 ± 0.17[a]	
M. myrtifolia Extract	Methanol	200	6.34 ± 0.03[b]	-9.6
		600	6.77 ± 0.03[b]	-3.5
	Hexane	200	5.79 ± 0.09[c]	-8.4
		600	5.83 ± 0.04[c]	-12.1

*Different letters: Significant difference ($P \leq 0.05$) between means of the column.

3.2.6 Total serum IgG level

The Methotrexate positive control showed a significant reduced total serum IgG level as compared to both negative controls (8.16 *vs*. 167 and 175 ng/ml). In contrast, the two doses of methanol extract (200 and 600 mg/kg) showed a significant increase in total serum IgG level as compared to the corresponding negative controls (171 and 177, respectively *vs*. 167 ng/ml), with a treatment efficiency of +2 and +5%, respectively. With respect to hexane extract, only the second dose was significantly effective in increasing the total serum IgG level as compared to the corresponding negative control

(183.95 *vs*. 175.00 ng/ml). The treatment efficiency of such effect was +4.5% (Table 4-12).

Table 3-12: Total serum IgG level in albino male mice treated with methanol and hexane extracts of *Micromeria myrtifolia*, distilled water and olive oil (negative control) and methotrexate (positive control).

Groups		Dose (mg/kg)	Mean ± S.E.* (ng/ml)	Treatment Efficiency (%)
Positive Control (Methotrexate)		0.33	8.16± 0.69[a]	-95.0
Negative Control (H_2O)			167.00± 2.50[b]	
Negative Control (Olive Oil)			175.00± 4.00[c]	
M. myrtifolia Extract	Methanol	200	171.00±0.80[c]	+2.0
		600	177.00±0.70[ce]	+5.0
	Hexane	200	172.20±0.69[c]	-1.7
		600	183.95±2.94[f]	+4.5

*Different letters: significant difference ($P \leq 0.05$) between means of the column.

3.3 Extracts-Methotrexate Interactions

Two types of interactions (pre- and post-treatments) were carried out between the two doses of *M. myrtifolia* extracts and methotrexate to evaluate the role of the extracts in modulating the immunological and cytogenetic effects of the drug in albino male mice.

3.3.1 Total Count of Leucocytes

For methanol extract, both doses (200 and 600 mg/kg) in the two types of interaction (pre- and post-treatments) were significantly effective in modulating the suppressive effect of methotrexate as compared to the corresponding control. In pre-treatment group, the first dose was better than the second dose (Treatment efficiency: +37.1 *vs*. +22.5%), and the same picture was drawn in post-treatment group (Treatment efficiency: +43.0 *vs*. +23.0%). Also in this regard, the post-treatment was better than pre-treatment. The hexane extract behaved in a similar manner, but the pre-treatment was the best, which recorded the highest treatment efficiencies in both doses (+75.0 and 47.5%, respectively) (Table 3-13).

Table 3-13: Total leucocyte count in albino male mice after interactions (pre- and post- treatments) between the two doses (200 and 600 mg/kg) of methanol and hexane extract of *Micromeria myrtifolia* and methotrexate.

Groups	Dose mg/kg	Mean ± S.E. x 10^3* (cells/cu.mm. blood)		Probability ≤	Treatment Efficiency (%)	
		Pre	Post		Pre	Post
Control I (H_2O-Methotrexate)	0.33	5.6 ± 0.2[a]	6.9 ± 0.2[a]	0.01		
Control II (Olive Oil-Methotrexate)	0.33	4.0 ± 0.1[d]	7.0 ± 0.2[a]	0.001		
Methanol Extract -Methotrexate	200	7.6 ± 0.3[b]	9.9 ± 0.3[b]	0.001	+ 37.1	+ 43.0
	600	6.8 ± 0.1[b]	8.6 ± 0.2[c]	0.001	+ 22.5	+ 23.0
Hexane Extract - Methotrexate	200	7.0 ± 0.4[b]	8.4 ± 0.2[b]	0.001	+ 75.0	+ 20.5

	600	5.9 ± 0.3[c]	8.4 ± 0.1[b]	0.001	+ 47.5	+ 20.0

*Different letters: significant difference ($P \leq 0.05$) between means of the same column.

3.3.2 Absolute Count of Leucocytes

3.3.2.1 Lymphocytes

The lymphocyte count shared the picture of total leucocyte count, in which both extracts were significantly effective in modulating the suppressive effect of methotrexate, and again the first dose was better than the second dose, as well as, the post-treatment was better than pre-treatment in methanol extract and *vice versa* in hexane extract (Table 3-14).

Table 3-14: Total lymphocyte count in albino male mice after interactions (pre- and post- treatments) between the two doses (200 and 600 mg/kg) of methanol and hexane extract of *Micromeria myrtifolia* and methotrexate.

Groups	Dose (mg/kg)	Mean ± S.E. x 10^3* (cells/cu.mm. blood)		Probability ≤	Treatment Efficiency (%)	
		Pre	Post		Pre	Post
Control I (H_2O-Methotrexate)	0.33	3.7 ± 0.5[a]	3.9 ± 0.7[a]	Not significant		
Control II (Olive Oil- Methotrexate)	0.33	2.5 ± 0.5[a]	4.8 ± 0.7[a]	0.001		
Methanol Extract - Methotrexate	200	5.9 ± 0.2[b]	6.5 ± 0.1[b]	0.01	+ 58.0	+ 64.6
	600	4.9 ± 0.7[c]	5.3 ± 0.6[c]	Not significant	+ 34.3	+ 33.6

Hexane Extract - Methotrexate	200	5.8 ± 0.3^{b}	6.2 ± 0.3^{b}	Not significant	+ 137.7	+ 30.8
	600	4.3 ± 0.9^{c}	5.5 ± 0.8^{c}	Not significant	+ 73.3	+ 14.7

*Different letters: significant difference ($P \leq 0.05$) between means of the same column.

3.3.2.2 Neutrophils

The two doses of methanol extract were significantly effective in increasing the neutrophil count and modulating the suppressive effect of methotrexate. The best treatment efficiency was recorded for the dose 200 mg/kg in post-treatment interaction (+64.8%), while the second dose was more effective in pre-treatment interaction (Treatment efficiency = +28.9%). For hexane extract, only the second dose was significantly effective in this regard, and the pre-treatment was better than the post-treatment (Treatment efficiency: +32.5 *vs.* +23.3%) (Table 3-15).

Table 3-15: Total neutrophil count in albino male mice after interactions (pre- and post- treatments) between the two doses (200 and 600 mg/kg) of methanol and hexane extract of *Micromeria myrtifolia* and methotrexate.

Groups	Dose (mg/kg)	Mean ± S.E. x 10^{3}* (cells/cu.mm. blood)		Probability ≤	Treatment Efficiency (%)	
		Pre	Post		Pre	Post
Control I (H_2O- Methotrexate)	0.33	1.1 ± 0.05^{a}	1.5 ± 0.1^{a}	0.001		
Control II (Olive Oil- Methotrexate)	0.33	0.8 ± 0.04^{b}	1.3 ± 0.05^{a}	0.001		

Methanol Extract - Methotrexate	200	1.4 ± 0.04[c]	2.5 ± 0.18[b]	0.001	+ 29.5	+ 64.8
	600	1.3 ± 0.06[c]	1.8 ± 0.09[c]	0.001	+ 28.9	+ 16.6
Hexane Extract - Methotrexate	200	0.7 ± 0.1[b]	1.4 ± 0.06[a]	0.001	- 11.4	+ 9.3
	600	1.1 ± 0.05[a]	1.6 ± 0.03[a]	0.001	+ 32.5	+ 23.3

*Different letters: significant difference ($P \leq 0.05$) between means of the same column.

3.3.2.3 Monocytes

Only the second dose (600 mg/kg) of both extracts (methanol and hexane) in the post-treatment groups was significantly effective in increasing the count of monocytes in animals treated with methotrexate, as judged by the value of treatment efficiency (+18.3 and +47.2%, respectively) (Table 3-16).

Table 3-16: Total monocyte count in albino male mice after interactions (pre- and post- treatments) between the two doses (200 and 600 mg/kg) of methanol and hexane extract of *Micromeria myrtifolia* and methotrexate.

Groups	Dose mg/kg	Mean ± S.E.* (cells/cu.mm. blood)		Probability =<	Treatment Efficiency (%)	
		Pre	Post		Pre	Post
Control I (H_2O-Methotrexate)	0.33	557.0 ± 57.8[a]	1294.4 ± 37.6[a]	0.001		

Control II (Olive Oil-Methotrexate)	0.33	630.1 ± 10.3[a]	772.2 ± 50.5[b]	0.01		
Methanol Extract -Methotrexate	200	358.4 ± 43.9[b]	1192.6 ± 89.3[a]	0.001	- 35.6	- 7.8
	600	443.0 ± 37.4[c]	1531.8 ± 61.2[c]	0.001	- 20.4	+ 18.3
Hexane Extract - Methotrexate	200	388.0 ± 56.6[b]	770.1 ± 45.6[b]	0.001	- 38.4	- 0.2
	600	469.0 ± 8.7[c]	1137 ± 111.2[a]	0.001	- 25.5	+ 47.2

*Different letters: significant difference ($P \leq 0.05$) between means of the same column.

3.3.2.4 Eosinophils

There was no significant difference between the means of eosinophil count in animals treated with the two doses of both extracts as compared to the corresponding controls, but there was a significant difference between the pre- and post-treatment interactions in animal treated with the two doses of hexane extract. The best treatment efficiency (+80.0%) in this regard was recorded in post-treatment interaction for the dose 600 mg/kg (Table 3-17).

Table 3-17: Total eosinophil count in albino male mice after interactions (pre- and post- treatments) between the two doses (200 and 600 mg/kg) of methanol and hexane extract of *Micromeria myrtifolia* and methotrexate.

Groups	Dose mg/kg	Mean ± S.E.* (cells/cu.mm. blood)	Probability ≤	Treatment Efficiency (%)

		Pre	Post		Pre	Post
Control I (H_2O-Methotrexate)	0.33	55.5 ± 1.7[a]	139.5 ± 28.3[a]	0.01		
Control II (Olive Oil-Methotrexate)	0.33	60.0 ± 11.6[a]	70.0 ± 1.8[a]	Not significant		
Methanol Extract - Methotrexate	200	Not recorded	Not recorded			
	600	Not recorded	Not recorded			
Hexane Extract - Methotrexate	200	32.5 ± 18.8[a]	85.4 ± 1.3[a]	0.001	-45.8	+22.0
	600	59.0 ± 2.7[a]	126.0 ± 24.3[a]	0.001	-1.6	+80.0

*Different letters: significant difference ($P \leq 0.05$) between means of the same column.

3.3.2.5 Basophils

The results of basophils were in favor of no significant difference between the treated groups, but the pre-treatment was significant different from the post-treatment, in which the count of basophils was reduced as compared to the corresponding controls (Table 3-18).

Table 3-18: Total basophile count in albino male mice after interactions (pre- and post- treatments) between the two doses (200 and 600 mg/kg) of methanol and hexane extract of *Micromeria myrtifolia* and methotrexate.

Groups	Dose mg/kg	Mean ± S.E.* (cells/cu.mm. blood)		Probability =<	Treatment Efficiency (%)	
		Pre	Post		Pre	Post
Control I (H_2O-Methotrexate)	0.33	110.0 ±55.0[a]	122.0± 33.2[a]	Not significant		
Control II (Olive Oil-Methotrexate)	0.33	40.0±0.815[a]	142.0 ±31.9[a]	0.001		
Methanol Extract -Methotrexate	200	Not recorded	Not recorded			
	600	Not recorded	Not recorded			
Hexane Extract - Methotrexate	200	0.0 ± 0.0[a]	85.4± 1.3[a]	0.001	- 100.0	- 39.8
	600	30.0±17.5[a]	127.0±43.7[a]	0.001	- 25.0	- 10.5

*Different letters: significant difference ($P \leq 0.05$) between means of the same column.

3.3.3 Metaphase Index

3.3.3.1 Bone Marrow cells

Both extracts were significantly effective in increasing the metaphase index of bone marrow cells, but the extent of the effect was dependent on type of extract, dose and type of treatment. In the pre-treatment interaction the best treatment efficiency was recorded for the first dose of methanol extract, followed by the same dose in the hexane extract (+105.5 and +60.0, respectively). The corresponding figures in post-treatment interaction were

observed in the first and second doses of hexane extract (+74.0 and +32.5%, respectively) (Table 3-19).

Table 3-19: Metaphase index of bone marrow cells in albino male mice after interactions (pre- and post- treatments) between the two doses (200 and 600 mg/kg) of methanol and hexane extract of *Micromeria myrtifolia* and methotrexate.

Groups	Dose (mg/kg)	Mean ± S.E.* (%)		Probability I^	Treatment Efficiency (%)	
		Pre	Post		Pre	Post
Control I (H_2O-Methotrexate)	0.33	2.15±0.07[a]	3.62±0.04[a]	0.001		
Control II (Olive Oil-Methotrexate)	0.33	2.98±0.08[b]	2.00±0.18[b]	0.001		
Methanol Extract -Methotrexate	200	4.42±0.14[c]	4.02±0.17[c]	0.05	+ 105.5	+ 11.0
	600	2.95±0.06[b]	3.75±0.10[c]	0.001	+ 37.2	+ 3.5
Hexane Extract - Methotrexate	200	4.77±0.13[d]	3.48±0.20[c]	0.001	+ 60.0	+ 74.0
	600	3.69±0.18[e]	2.65±0.06[b]	0.001	+ 23.8	+ 32.5

*Different letters: significant difference ($P \leq 0.05$) between means of the same column.

3.3.3.2 Spleen cells

Both extracts were significantly effective in modulating the suppressive effect of methotrexate on spleen cells. For methanol extract, the best treatment efficiency was recorded for the first dose of pre-treatment interaction (+56.8%), followed by the same, but in post-treatment interaction dose (+37.4%). For hexane extract, the pre-treatment interaction was better than post-treatment interaction in both doses (Treatment efficiency: +123.5 and +76.4% *vs*. +36.9 and +19.1%, respectively) (Table 3-20).

Table 3-20: Metaphase index of spleen cells in albino male mice after interactions (pre- and post- treatments) between the two doses (200 and 600 mg/kg) of methanol and hexane extract of *Micromeria myrtifolia* and methotrexate.

Groups	Dose mg/kg	Mean ± S.E.* (%)		Probability ≤	Treatment Efficiency (%)	
		Pre	Post		Pre	Post
Control I (H_2O-Methotrexate)	0.33	1.25±0.06[a]	1.87±0.04[a]	0.001		
Control II (Olive Oil- Methotrexate)	0.33	1.23±0.12[a]	1.57±0.03[b]	0.01		
Methanol Extract - Methotrexate	200	1.96±0.06[b]	2.57±0.08[c]	0.001	+ 56.8	+ 37.4
	600	1.46±0.06[c]	2.10±0.09[d]	0.001	+ 16.8	+ 12.2
Hexane Extract - Methotrexate	200	2.75±0.10[d]	2.15±0.06[d]	0.001	+ 123.5	+ 36.9
	600	2.17±0.13[e]	1.87±0.04[a]	0.05	+ 76.4	+ 19.1

*Different letters: significant difference ($P \leq 0.05$) between means of the same column.

3.3.4 Micronucleus Index

Only the first dose (200 mg/kg) in pre-treatment interaction of methanol extract showed a significant decrease in micronucleus index with treatment efficiency (-13.5%), while the two doses of both pre- and post-treatments of hexane extract showed a significant decrease in micronucleus formation. The post-treatment interaction was more effective in reducing such count than that of pre-treatment (pre: - 28.9 and - 5.2 *vs.* - 37.7 and - 19.7, respectively) (Table 3-21).

Table 3-21: Micronucleus formation of bone marrow cells in albino male mice after interactions (pre-post treatments) between two doses (200 mg/kg, 600 mg/kg) of methanol and hexane extract of *Micromeria myrtifolia* and Methotrexate drug.

Groups	Dose mg/kg	Mean ± S.E.* (%)		Probability ≤	Treatment Efficiency (%)	
		Pre	Post		Pre	Post
Control I (H_2O-Methotrexate)	0.33	10.30±0.60[a]	6.00±0.40[a]	0.001		
Control II (Olive Oil-Methotrexate)	0.33	7.60±0.20[b]	17.20±0.80[b]	0.001		
Methanol Extract -Methotrexate	200	8.90±0.40[c]	8.70±0.40[c]	Not significant	- 13.5	+ 45.0
	600	10.70±0.11[a]	9.20±0.60[c]	Not significant	+ 3.8	+ 53.3
Hexane Extract - Methotrexate	200	5.40±0.05[d]	10.70±0.60[d]	0.001	- 28.9	- 37.7

	600	7.20±0.20[b]	13.80±0.30[e]	0.001	- 5.2	- 19.7

*Different letters: significant difference ($P \leq 0.05$) between means of the same column.

3.3.5 Sperm-Head Abnormalities

The best treatment efficiency was recorded for the first dose of hexane extract (-15.3%), followed by the second dose of the same extract (-9.6%), and such effects were reached in the pre-treatment interaction (Table 3-22).

Table 3-22: Sperm Head Abnormality in albino male mice after interactions (pre- and post- treatments) between the two doses (200 and 600 mg/kg) of methanol and hexane extract of *Micromeria myrtifolia* and methotrexate.

Groups	Dose mg/kg	Mean ± S.E.* (%)		Probability ≤	Treatment Efficiency (%)	
		Pre	Post		Pre	Post
Control I (H_2O-Methotrexate)	0.33	6.76±0.06[a]	6.76±0.03[a]	Not significant		
Control II (Olive Oil-Methotrexate)	0.33	6.99±0.04[a]	6.11±0.04[a]	Not significant		
Methanol Extract -Methotrexate	200	6.47±0.06[a]	6.41±0.04[a]	Not significant	-4.3	-5.2
	600	6.92±0.07[a]	7.03±0.08[b]	Not significant	+2.4	+4.0
Hexane Extract - Methotrexate	200	5.92±0.07[b]	5.89±0.07[c]	Not significant	-15.3	-3.6

	600	6.32±0.05[a]	6.13±0.05[a]	Not significant	-9.6	+0.3

*Different letters: significant difference ($P \leq 0.05$) between means of the same column.

3.3.6 IgG Level in Serum

Both extracts were significantly effective in increasing the IgG level in sera of the treated animals, but the extent of the effect was dependent on type of extract, dose and type of treatment. In the pre-treatment interaction the best treatment efficiency was recorded for the second dose of methanol extract, followed by the same dose in the hexane extract (+27.7 and +43.0%, respectively). The corresponding figures in post-treatment interaction were observed in the first and second doses of hexane extract (+15.3 and +29.4%, respectively) (Table 3-23).

Table 3-23: IgG in albino male mice after interactions (pre- and post- treatments) between the two doses (200 and 600 mg/kg) of methanol and hexane extract of *Micromeria myrtifolia* and methotrexate.

Groups	Dose mg/kg	Mean ± S.E.* (%)		Probability I^	Treatment Efficiency (%)	
		Pre	Post		Pre	Post
Control I (H_2O-Methotrexate)	0.33	113.50±1.49[a]	104.00±0.35[a]	0.01		
Control II (Olive Oil- Methotrexate)	0.33	120.25±0.25[b]	115.20±0.20[b]	0.01		

Methanol Extract - Methotrexate	200	121.00±0.99[b]	110.05±2.05[c]	0.001	+21.0	+5.8
	600	145.00±0.99[c]	120.10±0.90[d]	0.001	+27.7	+15.3
Hexane Extract - Methotrexate	200	142.00±2.00[c]	135.50±0.49[e]	0.01	+18.1	+17.6
	600	172.00±1.49[d]	149.10±0.90[f]	0.001	+43.0	+29.4

*Different letters: significant difference ($P \leq 0.05$) between means of the same column.

Chapter Four

Discussion

Plant extract preparations are effectively and extensively used for their medicinal properties, and they have become increasingly popular worldwide (Astin, 1991). In this setting, herbal medicines generally have fewer side effects than synthetic compounds and their effectiveness can be improved by modern pharmacological methods (Wilasrusmee *et al.*, 2002). Accordingly, the present study came to shed light on some of the anti-mutagenic and immune stimulant potentials of *Micromeria myrtifolia*, because this plant has not been extensively investigated, especially from the point view of immunity and anti-mutagenesis, and the folkloric medicine suggests important biological and pharmaceutical potentials (Craig, 1999). Therefore, and due the paucity of information regarding the interest of the present study, some part of the discussion will be based on the chemical constituents of the plant.

Chemical detection of *M. myrtifolia* extracts (methanol and hexane) revealed that the methanol extract was positive for flavonoids and polysaccharides, while the hexane extract was positive for steroids. Such findings are in agreement with the results of Gruenwald and co-workers (2000), who reported that the methanol and hexane extracts of *M. myrtifolia* aerial parts contains these compounds, in addition to vitamins C and E. Therefore, the positive findings of the present study, which are discussed below, can be ascribed to these constituents, especially if we consider the synergistic effects of these constituents against the drug methotrexate (MTX).

In general, all parameters investigated were negatively affected as a consequence of treatment with the drug MTX, and the reduction reached more than 36% of total and absolute counts of leucocytes in distilled water negative control. Furthermore, the metaphase index of bone marrow and spleen was also decreased in a similar manner, because MTX inhibits dihydrofolate reductase (DHFR), an enzyme that is a part of the folate synthesis metabolic pathway and this enzyme catalyses the conversion of folic acid to the reduced folates (i.e., tetrahydrofolate). 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. Therefore, MTX inhibits the synthesis of DNA, RNA, thymidylates and proteins so, and MTX in this regard 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). The micronucleus formation assay, which is an important parameter for the evaluation of mutagenesis (Petzold *et al.*, 2003), confirmed the forthcoming effects and it was significantly increased in animals treated with MTX; an observation that highlight the mutagenic and genotoxic effects of the drug, and such findings came to confirm previous demonstrations of other investigators (Catalano, 2002; Gescher, 2004). A further inspection of the drug revealed its effect the on immune response, which can be detected by a variation in the normal count of lymphocytes. The possible explanation for such action is the reduction in size and reactivity of the lymphocyte by the induction of apoptosis in activated T-cells (Genestier *et al.*, 2000), or the drug is able to suppress the production of both tumor necrosis factor (TNF) and interferon (IFN)-γ by T-cell, also it has been demonstrated that MTX can reduce interlukin (IL)-1 production by monocytes (Chang *et al.*, 1992). These results have further confirmed by Genestier and colleagues (2000) who suggested the immune suppressive properties of MTX.

As presented earlier in this discussion, the treatment with MTX increased the frequency of micronucleus formation, in addition to sperm-head abnormalities, while the metaphase index of bone marrow and spleen cells was significantly decreased. This may be related to the action of the drug that limit the intracellular supply of reduced folates (tetrahydrofolate) through inhibition of DHFR and, therefore, it is considered a mutagenic agent. Therefore, the MTX were employed in the present study as immune suppressive and mutagenic agent in experiment number one (positive control) and experiment number two (pre- and post-treatment interactions with the plant extract.

The results demonstrated that a treatment with the lower dose (200 mg/kg) of *M. myrtifolia* extracts showed a positive effect on the total count of leucocytes and lymphocytes, metaphase index of bone marrow cell and spleen cell. These parameters manifested a significant increase especially in animal treated with the methanol extract. The detected active constituents can justify these findings, especially if we consider that polysaccharides, flavonoids, steroids have important role as immune stimulators (Williams *et al*., 2002). However, the higher dose (600 mg/kg) was in favour of a cytotoxic effect, and again, the flavonoids may have had contributed to such toxicity. This is reasoned by the fact that the higher doses of flavonoids can inhibit the DNA-maintenance enzymes topoisomerase I and topoisomerase II. These enzymes regulate the supercoiling of chromosomal DNA, and play pivotal roles in chromosome replication, transcription, recombination, segregation, condensation and repair (Wang, 2002).

The increase of leucocyte count was much clear in the lymphocytes, which are considered as the important humoral and cellular arms of the adaptive immune response, and the plant extracts may have exerted their effects on these cells. The lymphocytes are of two main types; T and B

lymphocytes. The T cells are further divided into two functional types; T helper-1 (T_H1) and T_H2, which are based on the profile of cytokines that they produce, and consequently each type is enhancing on arm of the immune response; cell mediated and humoral immune responses, respectively (Roitt *et al.*, 2001). With respect to the humoral immune response, the present study demonstrated a significant increased serum level of IgG, which is produced by B cells after receiving a cytokine signal from T_H2 cells. This observation may suggest that the plant extracts of *M. myrtifolia* may have chemical compounds that have enhancing effects on these cells. In agreement with this scope, Madhavan *et al.* (2002) have recently demonstrated that quercetin (flavonoid compound) can be served as anti-tumor agent, because it showed a modulating effect on the production of T_H1 and T_H2 derived cytokines.

The lymphocyte count and function were investigated in the present study, although the augmentation was indirect. It was firstly determined by a significant increase of peripheral lymphocyte count, and then by a significant increase of spleen cell metaphase index and total serum IgG level. In the spleen, the main immune cells are T and B lymphocytes, as well as, antigen presenting cells, and normally the first two types of cells are divided in response to antigenic challenge presented by antigen presenting cells (Roitt *et al.*, 2001).Therefore, if there is an increase in the metaphase index of spleen cells, it must involve the T and B lymphocytes. T lymphocytes are involved in cellular immune response, while B lymphocytes are the main arm of humoral immune response (William .2008). Such positive effects can also be ascribed to the plant polysaccharides; because it has been recently demonstrated by Zhu *et al.*, (2007) that polysaccharide isolated from *Ganoderma lucidum* induced activation of both T and B lymphocytes.

The lymphocytes are originated in the bone marrow through the lymphoid lineage progenitor, which is the outcome hemopoitic stem cell proliferation. The latter outcome was investigated in term of bone marrow

metaphase index, which was significantly increased in mice treated with the first dose of both plant extracts, and therefore, their absolute peripheral count was expected to be increased. The question is how the plant extract exerted such effect. The answer can be augmented if we consider that *M. myrtifolia* extract contain polysaccharides, and in this regard Biringanine and co-workers (2004) suggested that several plant extracts of the family extracts have some therapeutical activities that could be dependent on the extracts content of polysaccharides, and also it has been recently demonstrated that polysaccharides isolated from fungal spp. (*Ganoderma lucidum*) accelerated the recovery of bone marrow cells and total leucocyte count in immunosuppressed mice (Zhu *et al.*, 2007).

The function of immune system is also genetically determined. To explore the effects of *M. myrtifolia* extracts (methanol and hexane) on the genetic make-up of mice directly or through interactions with MTX, the mitotic index, micronucleus formation in polychromatic erythrocytes of bone marrow and sperm-head abnormalities served as good parameters of mutagenic evaluations (Hajhashemi *et al.*, 2002). The results of genetic evaluations showed that a treatment with *M. myrtifolia* extracts was associated with a significant reduction in micronucleus formation and sperm head abnormalities and caused a significant increase in mitotic index of the bone marrow cells and spleen cells. According to these parameters, the methanol extract was more effective than hexane extract in a dose dependant manner (the lower dose was better than the higher dose). Such findings can be considered important, especially if we consider that methanol and hexane extracts of members of Lamiaceae family showed anti-carcinogenic effects, *in vivo* and *in vitro*, and the dose was effective in this regard (Al-Ezzy, 2006; Mosaffa *et al.*, 2006; Kamatou *et al.*, 2008). Equally important, carcinogenesis is normally preceded by mutations induced by different agents,

especially those that have oxidant effects (Yaseen, 1990; Ad'hiah *et al.*, 2002).

Accordingly, the enhancing effects on the immune response of the plant extracts, together with the effects as anti-mutagen, as demonstrated by the results of micronucleus formation and sperm-head abnormality assay, can be explained in the ground of anti-oxidant activity. In this regard, Burda and Oleszek (2001) demonstrated that the methanol extract of *M. myrtifolia* exhibit an inhibition effect on lipid peroxidation, and such outcome could have lead to the antioxidant properties of the plant extract. Free radicals are the possible result of membrane lipid peroxidation. Membrane lipids (linoleic acid and arachidonic acid) are rich in unsaturated fatty acids that are most susceptible to oxidative processes. Flavonoids, which are rich in the methanol extract as suggested by the chemical detection in the present study, have free radical-scavenging activities which may directly react and quench peroxide radicals to terminate the peroxidation chain reaction and improve the quality and stability of food products (Burda and Oleszek, 2001). Additionally, several flavonoids fix the antioxidant potentials of ascorbic acid by the retarding the conversion of ascorbate to dehydro-ascorbate, and this is based on the ability of flavonoids to act as free radical acceptors since free radical formation is considered to be an important phase of ascorbate oxidation (Miller *et al.*, 1996). Furthermore, vitamin C can protect plasma lipids and membrane lipids from the effect of oxidant compounds through either increase the production of cytokines or interact with the formation of prostaglandins, and both consequences are in favor of immune system enhancement, especially the antibody production (Hughes, 2001). The present methanol extract may mimic these actions of vitamin C, and these findings confirm the increased IgG serum level and the significant increase of metaphase index in spleen cells. However, if we consider the presence of other compounds, as in the hexane extract, a similar augmentation can be

raised, and recently it has been demonstrated that the presence of other compounds in the hexane extract such as olive oil and the essential oils of Lamiaceae family have anti inflammatory effect by inhibition of the cyclo-oxygenase-2 enzyme (one of the key enzymes in the inflammation process) (Gono-Bwalya, 2003).

The terpens are a class of essential oil terpens that have many biological and pharmaceutical activities, which can be useful to treat human diseases; for example, volatile terpens as monoterpens and sesquiterpens are known to have several pharmacological activities including antibacterial, antifungal, antispasmodic, sedative and analgesic (Buchbauer, 1994), also they can be used as potentiators of antitumor agents which can increase bioavailability of an orally administered hydrophobic pharmaceutical compound by inhibition of cytochrome P450 and/or decreasing of P-glycoprotein drug transport (Kim *et al.*, 1995).

The flavonoids compounds, in addition to their immune modulator effects, serve other bio-functions, with special reference to anti-mutagenic activity. It has been demonstrated that the flavonoids compounds, 3-kameferolcoumate and luteolin, which were extracted from some plant of the family Lamiaceae (*S. officinalis, Thymus vulgaris and Mentha piperita)* inhibited the mutagenic activity of Trp-p-2, and the suggestion was that these compounds starts the metabolic inactivation by modifying the action of the enzyme cytochrome p450 (Samejima *et al.*, 1995; Samejima *et al.*, 1998). Such consequence can lead to a decrease in DNA strand breaks, lipid peroxidation and cellular oxidation (Kanazawa *et al.*, 1998). In addition to that, Mimica-Dukic (2001) demonstrated that phenolic diterpenoids extracted from the *M. myrtifolia* showed a strong anti-oxidant activity, and they were potent antioxidants that prevent the formation of free radicals. Free radicals have the ability to cause damage to the DNA and RNA and inhibit some enzymes from reacting with amino acids (Salganik, 2001).

The protecting anti-mutagenic effect of flavonoids can also be occurred through their ability to interact with free radicals when they are associated with DNA via intercalating or external modes, and this makes them a strong antioxidant to protect DNA from harmful damage and prevent disease (Kanakis *et al.*, 2006). Covalent bindings of quercetin (type of flavonoids) to DNA and protein have also been addressed by Lodovici *et al.* (2001) and Walle *et al.* (2003), and protection by quercetin and quercetin-rich fruit juice against induction of oxidative DNA damage has also been demonstrated (Lonneke *et al.*, 2005). Therefore, flavonoids are powerful antioxidants, and prevent DNA damage, and these findings may explain the anti-mutagenic effects against the mutagen MTX in animals treated with the methanol extract of *M. myrtifolia* aerial parts.

The hexane extract was also effective in this regard, and the protective effects of the extract against MTX can be explained in the ground of active constituent compounds including essential oils, terpens and steroids. The plant steroids may provide a protection to the plasma lipids as a result of antioxidant activity, and participate in the integrity of cell membrane (Bramley *et al.*, 2000). Such consequences are able to modify the immune functions in the sense of enhancement (Moller and Loft, 2002). With regard to anti-mutagenicity, dietary steroids are also antioxidants, which are expected to reduce cancer risk by minimizing DNA damage or reducing mutational changes (Elmadfa and Park, 2004). In agreement with these findings, the lower dose of hexane extract was able to reduce the effect of MTX, and a significant reduction of micronucleus formation in mice pretreated with the extract was obtained.

In pre- and post-treatment, both extracts of *M. myrtifolia* could significantly inhibit MTX-induced micronuclei in bone marrow cells, as well as, sperm head abnormalities. Moreover there was a significant enhancement in blood cell counts, mitotic index of bone marrow cells and spleen cells in *M.*

myrtifolia treated animals. By these pathways, the present extracts of *M. myrtifolia* could exert their protection effect against the drug MTX, especially in the pre-treatment interaction, which all may depend on the chemical constituents of the extracts. It could be seen that *M. myrtifolia* gave striking results as a protective agent against the genotoxic effect of MTX if it was given before MTX treatment. This might also be related to the flavonoids group of *M. myrtifolia*, which may be linked with the inhibition of the microsome enzyme (an activator enzyme for mutagens) (Francis *et al.*, 1989). Furthermore, Webb and Eberler (2004) showed that when the extract of *M. myrtifolia* was added to the rat feeding, the level of glutathion-S-transferase and catalase secretion were increased to 50%, and such outcomes play an important role in the cell protection from mutagens through their action as an antioxidant or a scavenger for the free radicals in the cells. Additionally, other active compounds of the extract may also act as anti-mutagenic constituents, but their action was demonstrated to be through the communication intracellular gaps between functional cells (Davidson *et al.*, 1986). These compounds may delay the transfer of MTX between cells and limited its genotoxic and mutagenic activity (blocking agents) (Trosko *et al.*, 1990).These results are also in agreement with Al-Malikey (2006), who indicated that the pre-treatment with *Glycyrrhiza glabra* aqueous extract, rich in flavonoids, gave a protective effect against MTX.

The percentage of sperm-head abnormalities and micronuclei formation were reduced when the extracts were given before MTX, and this might be related to anti-mutagenic activity of some active compounds of the extracts like isoflavans and coumarins that worked as inhibitors for drug activity (Mitscher *et al.*, 1985).

These results indicated that the pre-drug treatment with the extracts gave better protection values for the investigated parameters, as suggested by the treatment efficiency evaluation, than that of post-drug treatment and showed

that *M. myrtifolia* can be used as a prophylactic agent against MTX genotoxicity. These findings have been demonstrated by Hajhashemi *et al*. (2002), who observed that *M. myrtifolia* aqueous extract was able to inhibit the mutagenic and carcinogenic effects of 7, 12-dimethybenz (a) anthracene and teleocidin in mice. Also, Zhu *et al*. (2007) suggested that the pre-treatment with polysaccharides purified from anther medicinal plant (*Plantago lanceolata*) in immunosuppressed mice resulted in a significant increase of total leucocyte count, the absolute counts of lymphocytes, neutrophils and monocyte, as well as, the metaphase index of bone marrow cells. Furthermore, the metaphase index of spleen cells was also increased, and such observation suggests that T and B cell proliferation responses were also enhanced, and the results of total serum IgG level confirm such suggestion.

In post-treatment interaction, also both extracts showed a significant efficiency in reducing the cytogenetic effects of MTX, and again these actions can be attributed to the chemical constituents of the plant with regard to the forthcoming repair mechanisms. It has been found that flavonoids of *M. myrtifolia* can enhance the post-replication repair mechanism (Kuroda and Hara, 1999), while others have demonstrated that flavonoids can stimulate the mechanism of error-free repair mechanism (Sasaki *et al*., 1998). Furthermore, flavonoids can activate recombinational repair mechanism, beside their action in activating the detoxification enzymes (Elson and Yu, 1994; Burke *et al*., 1997). From these findings, it is possible to suggest that the post-drug treatment with the 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).

In conclusion, the methanol and hexane extracts of *M. myrtifolia* may be considered as important materials that are rich in immune stimulating and

anti-mutagenic agents; however, the effect of the extracts depends on the type of interaction, as well as, the dose.

Conclusions

1- The methanol and hexane extracts of *Micromeria myrtifolia* were effective in modulating the immune suppressive and mutagenic effects of methotrexate, *in vivo* (albino male mice).

2- With respect to the investigated immunological and genetic effects, *M. myrtifolia* extracts were effective in enhancing the values of immunological parameters, and reducing the spontaneous formation of micronuclei and sperm-head abnormalities.

Recommendations

1. The immunological effects of methanol and hexane extracts of Micromeria require further investigations to determine the function of lymphocytes in terms of their CD markers and cytokine production.
2. Further investigations are required to determine the genetic effects of the extract, and interactions with other anti-cancer drugs should be investigated with other genetic assessments, especially at the molecular level.
3. Isolation characterization of the active compounds of both extracts to determine the immunological and genetic effects, as a strategy for a drug technology.

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Appendix I: General laboratory equipments.

Equipment	Company / Country
Autoclave	SES little Sister / England
Centrifuge	Beckman / England
Cooled incubator	Memmert / Germany
Digital camera	**Sony / Japan**
Digital balance	Sartorius / Germany
Hemocytometer	Neubauer / Germany
Laminar air flow	Napco / France
Micropipette	Gilson / France
Microscope	Motic / Japan
Oven	Osaw / India
pH meter	Radiometer / Denmark
Rotary evaporator	Buchi / Switzerland
Soxhlet	Electrothermol / England
Vortex	Griffin / England
Water bath	Gallenkamp / England

Appendix II: Chemical materials.

Chemical Material	Company / Country
Ammonium chloride (NH_4Cl)	BDH / England
α-naphthol	BDH / England
Chloroform	BDH / England
Colchicine tablets (0.5 mg)	Ibn Hayan / Syria
Eosin	BDH / England
Ethanol	Ferak / Germany
Ferric chloride ($FeCl_2$)	Fluka / Switzerland
Giemsa stain	Fluka / Switzerland
Glacial acetic acid	Fluka / Switzerland
Glycerin	Fluka / Switzerland
Glycerol	Sigma / U.S.A.
Heparin	Leo Pharmaceutical / Denmark
Hydrochloric acid (HCl)	Sigma / U.S.A.
Lead acetate (CH_3COOPb)	Fluka/ Switzerland
Potassium hydroxide (KOH)	Fluka/ Switzerland
Potassium Iodide (KI)	Fluka/ Switzerland
Mercuric oxide (red)	BDH / England
Mercuric chloride ($HgCl_2$)	Fluka/ Switzerland
Methanol	Fluka / Switzerland
Potassium Chloride (KCl)	Fluka / Switzerland
Sodium bicarbonates ($NaHCO_3$)	BDH / England
Sodium hydroxide (NaOH)	Sigma / U.S.A.
Trypan blue	Sigma/USA
Xylene	BDH / England

خلاصة

صممت الدراسة الحالية لدراسة بعض التأثيرات المناعية والوراثية-الخلوية للمستخلصين الكحولي والهكساني لنبات الزوفة (*Micromeria myrtifolia*) وعقار ميثوتركسيت في ذكور الفئران البيض (في الحي). شمل الجانب المناعي المعاييرالاتية: العد الكلي والتفريقي لخلايا الدم البيض والمستوى الكلي للغلوبيولين المناعي IgG في مصل الدم ومعامل انقسام خلايا نقي العظم وخلايا الطحال في الطور الاستوائي, بينما شمل الجانب الوراثي-الخلوي معامل تكون النوى الصغيرة في خلايا نقي العظم وتشوهات رؤوس النطف. فضلا عن ذالك فقد اجري الكشف الكيميائي للفلافونات والسكريات المتعددة والقلويدات في المستخلص الكحولي والستيرويدات في المستخلص الهكساني.

تضمنت الدراسة مرحلتان أساسيتان: شملت المرحلة الأولى دراسة التأثير المناعي والوراثي-الخلوي للمستخلص الكحولي والهكساني للنبات وبجرعتين (200، 600 ملغم/كغم) وجرعة واحدة من عقار ميثوتركسيت (0.33 ملغم/كغم), وكان التجريع لمدة سبعة أيام (جرعة واحدة لكل يوم) في حين شرحت الحيوانات في اليوم الثامن لغرض إجراء التقييمات الأنفة الذكر. في حين أجري في المرحلة الثانية تداخل ما بين جرعتي مستخلصي النبات والعقار ومن خلال نوعين من التداخل (قبل وبعد المعاملة بالعقار) لأجل اختبار فعالية المستخلصين في منع أو تقليل تأثيرات العقار، وفي كلا المرحلتين كان التجريع عن طريق الفم.

توصلت الدراسة إلى النتائج الآتية:

1. اظهر التحليل الكيميائي بأن المستخلص الكحولي كان موجبا للفلافونات والسكريات المتعددة وسالبا للقلويدات بينما كان مستخلص الهكسان موجبا للستيرويدات.
2. أظهر عقار الميثوتركسيت تأثيرات سلبية واضحة تمثلت بانخفاض الاستجابة المناعية وكما اظهر قابليته التطفيريه من خلال خفض معامل الانقسام و زيادة تكون النوى الصغيرة, فضلا عن ذالك فقد انخفض العد الكلي لخلايا الدم البيض والعد المطلق لخلايا اللمفية ووحيدة النواة والمستوى الكلي للغلوبيولين المناعي IgG في مصل الدم بالمقارنة مع حيوانات السيطرة السالبة.
3. أظهرت نتائج المرحلة الأولى قابلية نبات الزوفة على تعديل قيم المعاملات المناعية المدروسة بالمقارنة مع نتائج السيطرة السالبة (ماء مقطر وزيت الزيتون) والسيطرة الموجبة (عقار ميثوتريكسيت)، كما انخفض معدل تكون النوى الصغيرة وتشوهات رؤوس النطف، و في كلا الحالتين فان التأثير اعتمد على جرعة النبات ونوعية المستخلص وكانت الجرع الوطئة (200ملغم/كغم) أفضل من الجرعة العالية (600ملغم/كغم) وان مستخلص الهكسان كان أكثر فعالية من المستلخص الكحولي
4. ماثلت نتائج المرحلة الثانية المرحلة الأولى، حيث أظهرت الجرع لكلا المستخلصين كفاءة عاليه في حماية الجهاز المناعي و المادة الوراثية من التأثير السلبي للعقار, وفي هذا الصدد كانت المعاملة بالمستخلص قبل العقار اكثر كفاءة من بعد المعاملة بالعقار.

بسم الله الرحمن الرحيم

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صدق الله العظيم

سورة البقرة ألاية "٣٢"

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة النهرين

كليه العلوم
قسم التقانه الاحيائية

دراسة بعض التأثيرات المناعية والوراثية- الخلوية لمستخلصات نبات الزوفة (*Micromeria myrtifolia*) في ذكور الفأر الأبيض

رسالة

مقدمة إلى كلية العلوم جامعة النهرين

وهي جزء من متطلبات نيل درجة ماجستير علوم في التقانة الإحيائية

من قبل

أنس عبد العزيز فرج الربيعي

بكالوريوس تقانة إحيائية جامعة النهرين (2005)

بإشراف

أ.د.خلود وهيب السامرائي

أ.م.د.علي حسين أدحيه

تشرين الأول 2008

شوال 1429