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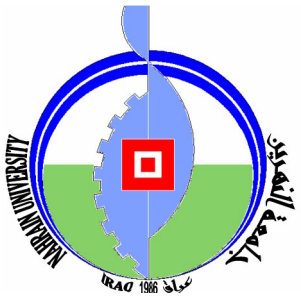

Anti-mutagenic, Immune Modulator and Fertility Effects of Rosemary (*Rosmarinus officinalis*) Extracts in Albino Male Mice

A Thesis
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By

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إهداء

الى

الله جل جلاله وروح المصطفى (صلى الله عليه وأله وصحبه أجمعين)

نور فؤادي كما كان يلقبني دائما .... ابي

الشمعة التي تنور حياتي ... أمي

رفاق الدرب ... إصدقائي

كل من علمني و لو حرفا

سندي في الحياة ... اخواني احمد وعلي وعباس و اختي اسراء

إلى كل هؤلاء اقدمُ بحثي هذا

نور

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Noor

Supervisor Certification

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

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

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Committee Certification

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

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Scientific Degree:

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(Chairman)

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Scientific Degree:

Date:

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I hereby certify upon the decision of the examining committee

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Summary

The present study was designed to evaluate the immunological, cytogenetic and fertility effects of two extracts (methanol and hexane) of rosemary (*Rosmarinus officinalis*) leaves and stems in male albino mice. The immunological parameters included total and absolute counts of leucocytes and phagocytosis, while the cytogenetic evaluation was carried out through the detection of micronucleus formation in polychromatic erythrocytes and sperm-head abnormalities. The fertility assessment was considered in terms of sperm count and motility. Additionally, the extracts were subjected to a chemical detection of flavonoids, terpens and steroids.

The study was carried out through three stages. In the first, the genetic and immunological effects of three doses (0.1, 0.2 and 0.4 mg/kg) of rosemary extracts and one dose (16 mg/kg) of cyclophosphamide (mutagenic and immune suppressive drug) were investigated, while in the second stage, the fertility effects of these doses were evaluated. In the third stage, interactions (pre- and post-treatments) between the ideal dose (0.1 mg/ kg) of both extracts and cyclophosphamide (16 mg/kg) were carried out. In all cases, the tested materials were injected subcutaneous.

Results revealed that the methanol extract was rich in flavonoids, while hexane extract was positive for terpens and negative for steroids. The investigated doses of methanol and hexane extracts showed no mutagenic, immunosuppressive or anti-fertility effects in albino male mice. In contrast, they reduced the spontaneous formation of micronuclei, enhanced the values of immunological parameters (total and absolute counts of leucocytes and phagocytosis), and improved the sperm count and motility. These effects were dose-dependent, and the dose 0.4 mg/kg was effective in these regards. However, in the interaction treatments (pre and post) with the mutagenic and immune suppressive drug (cyclophosphamide), the employed dose (0.1 mg/kg) of both extract failed to reduce the drug effects.

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List of abbreviation

NCCAM	National Center for Complementry and Alternative Medicines
DNA	Deoxyribonucleic acid
PCE	Polychromatic erthrocytes
C3b	Complement component C3b
IgG	Immunoglobulin G
cAMP	Cyclic Adenosine Monophosphate
SHA	Sperm head-Abnormality
ANOVA	Analysis of Variance
SPPS	Statical Programme for Social Sciences
S.D	Standered Deviation
ROE	*Rosemarinus officinalis* extract
LDL	Low density Lipoprotein

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Chapter One

Introduction and Review of the Literatures

Chapter One

Introduction and Review of Literatures

1-1: Introduction

In the past, medicinal plants were considered as the only form of health care readily available to the majority of human populations, and plants or plant derived products were used for medicinal purposes for centuries. Moreover, it is estimated that about 80% of the world population relies on botanical preparations as medicines to meet their health needs (Langner *et al*., 1998).This is reasoned by the fact that they are generally considered as safe and proved to be effective against certain ailments (Ganguly *et al*., 2003).

In recent years, and in the view of their beneficial effects, the use of medicinal plants has shown a gradual increase, especially when the scientific investigations have overwhelmed us with their medicinal potentials (Kalpagam and Nirmala, 2003).Three potentials are considered important in this regard; anti-mutagenic, immune modulators and fertility effectiveness.

With respect to the first potential, medicinal plants and / or their derivatives have shown that they are effective as anti-mutagen against many physical and chemical mutagens (Samejma *et al*., 1995).Their anti-mutagenic effects are exerted either on the mutagen to prevent or reduce the metabolic activation of the mutant, or the genetic material to prevent the fixation of mutation (i.e. stimulating repair mechanisms), therefore, they have been divided into desmutagens and bioantimutagens (Samejma *et al*., 1998).

As immune modulators, the list of medicinal plants that have immunological effects is in an increase, and in Iraq, several medicinal plants have shown modulating effects of the immune response in laboratory animals; for instance, *Nigella Sativa* (Al-Sudany,2005), *Saliva officinalis* (AL-Ezzy, 2006), *Calendula officinalis* (Ebraheem, 2006), *Glycyrrhiza glabra* (Al-Malikey, 2006) and *Origanum vulgare* (Al-Berikdar, 2007).

Equally important, the scope of fertility is a further target of medicinal plant researches, and in this regard, several plants, plant derivatives or their products have shown enhancing effects; for instance, *Tribules terrestris* (Adaikan *et al*., 2005; Zhongga, 2006; Tawfiq, 2007), *Phoenix dactylifera* (Matter, 2005) and *Achillea millefollium* (Butty, 2006).

Rosemary (*Rosmarinus officinalis*), and due to its richness in essential oils and phenolic compounds together with other constituents, has attracted the investigators in the field of medicinal plants. In this regard, the crude extract of the plant and/or isolated compounds have shown immune modulator (Al-Sereiti *et al*., 1999) anti-mutagenic (Alexandrov *et* al., 2006; Peng *et* al., 2007) and anti-oxidant (Yesil-Celiktas *et al*., 2007; Chang *et al*., 2008) effects. In contrast, it has recently been demonstrated that the plant exerted adverse effects on the reproductive function of adult male rats, and its role in fertility has been questionable (Nusier *et al*., 2007).

1-2: Aims of Study

Based on the forthcoming presentation, it was planned to investigate the effects of two extracts (methanol and hexane) of rosemary leaves on some parameters related to the genetics, immunity and fertility of male albino mice.

The parameters of investigation were:

- **i.** Detection of Active Compounds (flavonoids, terpene and steroids).
- **ii.** Immunological parameters (total and absolute counts of leucocytes and phagocytosis).
- **iii.** Genetic parameters (micronucleus formation).
- **iv.** Fertility parameters (sperm count and sperm motility and sperm head abnormalities).

1-3: Medicinal Plants

Medicinal plants are plants, plant parts, plant products, plant extracts and/or plant derived products that are employed in the treatment of diseases or used for their therapeutic properties. They are also used in the sense of improving the health status of human beings (NCCAM, 2005). Most of their effects were discovered through the folkloric medicine, in which the populations around the globe have developed their own strategies to remedy their illness (Gali-Muhtasib *et al*., 2000; Lima *et al*., 2005).

The use of herbs as medicines has played an important role in nearly every culture on earth, including Asia, Africa, Europe and America (Wargovich *et al*., 2001). Herbal medicine is based on the premise that plants contain natural substances that can promote health and alleviate illness. Several herbs provide some protection against cancer and stimulate the immune system. Furthermore, a diet in which culinary herbs are used generously to flavour food provides a variety of active phytochemicals that promote health and protect against chronic diseases (Cheung and Tai, 2007). Additionally, several commonly used herbs have been identified by the National Cancer Institute as possessing cancer-preventive properties (Al-Attar, 2006). Accordingly, many plants have shown medicinal potentials, and furthermore scientific research strategies have refreshed the pharmacy with medicines that are all of plant origins; for instance, aspirin, atropine, artimesinin, colchicine, digoxin, ephedrine, morphine, physostigmine, pilocorpine, quinine, quinidine, reserpine, taxol, tubocurarine, vincristine and vinblastine (Gubri-Fakim, 2005). Most of these plant-derived medicines were originally discovered through the study of traditional cures and folkloric knowledge and some of these could not be substituted despite the enormous advancement in synthetic chemistry (Gilani and Rahman, 2005).

In this regard, rosemary (*Rosmarinus officinalis*) is a further subject of medicinal plants and its medicinal uses in folkloric medicine revealed its medicinal potentials, moreover, the scientific investigations have shown that the

plant is rich in constituents that have immune modulator (Al-Sereiti *et al*., 1999) anti-mutagenic (Alexandrov *et* al., 2006; Peng *et* al., 2007) and anti-oxidant (Yesil-Celiktas *et al*., 2007; Chang *et al*., 2008) properties.

1-4: Rosemary (*Rosmarinus officinalis* L.)

The plant is indigenous to the Mediterranean region and Portugal, and is also cultivated on Central Asia, India, Southeast Asia, South Africa, Australia and the America. The main characteristic of the plant is its very pungent aroma (Haraguchi, 1995).

1-4-1: Common Names and classification

There are several common names used to describe *Rosemary officinalis*; for instance, polar plant, compass-weed, compass plant, in addition to rosemary, which is the most common name (Preus and Mary, 2005). However, from the point view of taxonomists, the plant follows the following classification:

Kingdom: Plantae
Division: Magnoliophyta
Class: Magnoliopda
Order: Lamiales
Family: Lamiaceae
Genus: *Rosmarinus*
Species: *Rosmarinus officinalis* Linn.

1-4-2: Plant Description

Rosemary is a branched bushy shrub, attaining a height of about one meter with up right stem, with whitish-blue flowers and dark green leaves, which are small with edges turned over backward. Underneath these rolled edges are little glands containing aromatic oils (Figure 1-1) (Calabrese *et al*., 2000). Further

descriptions of the plants are presented in the following picture as given by (Haraguchi, 1995):

- ***Medicinal Parts***: The medicinal parts are leaves and leafy stems, flowers, dried twig tips, fresh aerial parts and the flowering branches.
- ***Flower and Fruit***: The flowers grow on tometose inflorescences in the leaf axils of the upper part of the branches. The calyx is 3 to 4 mm., green or reddish, initially tomentose, and later 5 to 7 mm and glabrous. The venation is conspicuous. The corolla is 10 to 12 mm long, bluish, and occasionally pink or white. The nutlet is brown(Haraguchi, 1995).
- ***Leaves, Stem and Root***: The plant is an evergreen, branched subshrub, 50 to 150 cm high with erect, climbing or occasionally decumbent brown branches. The leaves are linear, coriaceous, entire-margined, light green and somewhat rugose above.

Figure 1-1: A field picture of the plant rosemary (Calabrese *et al*., 2000).

1-4-3: Chemical Constituents

Different chemical constituents of rosemary have been described, while the main constituents, which have been presented by Peng *et al*. (2005), Santoyo *et al*. (2005) and Almela *et al.* (2006), are:

i. *Caffeic acid derivatives*: the chief component is rosmarinic acid.

ii. *Diterpenes*: They include carnosolic acid (picrosalvin), isorosmanol, rosmadial, rosmaridiphenol and rosmariquinone.

iii. *Flavonoids*: They include cirsimarin, diosmin, hesperidin, homoplantiginin and phegopolin.

iv. *Triterpenes*: The chief components are oleanolic acid, ursolic acid and their 3-acetyl esters.

v. *Volatile oils* (1.0 to 2.5%): The chief components are 1,8-cineole (20-50%), alpha-pinene (15- 25%) and camphor (10- 25%).

1-4-4: Medicinal Uses

The herb rosemary is used as a food spice and as a medicine since ancient times. Traditional medicinal uses of rosemary leaf preparations include digestive distress, headache and anxiety. The fragrance of rosemary leaf is also said to enhance memory, but the results of one controlled study were inconclusive (Moss *et al*., 2003). Rosemary oil is also applied to the skin to treat muscle and joint pains and taken internally to promote abortions, and like many essential oils, it has antimicrobial properties when it comes in direct contact with bacteria and other microorganisms, therefore its antiseptic potentials are expected (Santoyo *et al*., 2005). Other medicinal potentials of rosemary or its constituents have been suggested; for instance, diuretic (Haloui *et al*., 2000), ulcer-protective (Dias *et al*., 2000) and hepato-protective (Vitaglione *et al*., 2005). Rosmarinic acid (a rosemary rich constituent) has shown potential anti-inflammatory and anti-allergic actions (Osakabe *et al*., 2005).

1-4-5: Biological and Pharmacological Properties

1-4-5-1: Effects on Smooth Muscles

The volatile oils of rosemary have been suggested to have an inhibitory effect on the contraction of smooth muscles, aqel (1991) was able to demonstrate that these oils inhibited the contraction of tracheal smooth muscle

induced by acetylcholine and histamine in rabbits and guinea pigs in Ca^{2+} containing, as well as, Ca^{2+} free solutions, and also inhibited the contraction induced by high K^{+} containing solution. The aqueous extract of rosemary leaves also inhibited the continuous contractions of the rabbit jejunum and inhibited contractions induced by acetylcholine, histamine and barium chloride (Al-Sereiti and Said, 1992).

1-4-5-2: Effects on Central Nervous System

An administration of rosemary oil, both by inhalation and by oral route, has been found to stimulate the central nervous system, respiratory and locomotor activity in mice, suggesting a direct action of one or more of its constituents, moreover, alcoholic extract of rosemary showed antidepressant activity on forced swimming induced immobility test model in mice (Matsunaga *et al*., 1997).

1-4-5-3:-Choleretic and Hepatoprotective Effects

Rosemary has been used empirically as a choleretic and hepatoprotective agent in traditional medicine. These actions have been ascribed to its role in increasing the bile flow and in reduction of plasma liver enzymes, especially when the extracts were given as pre-treatment before carbon tetrachloride (Hoefler *et al*., 1987).

Using an organic hydroperoxide (tetra-butyl-hydroperoxide) to induce injury in freshly isolated rat hepatocytes, it has been shown that the aqueous extract of young sprouts demonstrated an anti-lipoperoxidant activity, as it reduced significantly the formation of malonaldehyde in a dose-dependent manner and significantly decreased the release of lactico-dehydrogenase and aspartate aminotransferase (Joyeux and Rolland, 1990). These results have been further confirmed using different hepatotoxic compounds; for instance carbon tetrachloride (Stelo-Félix *et al*., 2002) and azathioprine (Amin and Hamza, 2005).

1-4-5-4: Antioxidant, Anti-mutagenic and Anti-carcinogenic Effects

The scope of antioxidant, anti-mutagenic and anti-carcinogenic properties of rosemary and/or its constituents have been the subject of intensive investigations, and some the recent ones are presented here.

Carnosol, one of the active constituents of rosemary, showed inhibiting effects on the invasion of B16/F10 mouse melanoma cells, an observation which suggests its anti-carcinogenic properties (Huang *et al*., 2004). Such observation has been further confirmed in a mouse model of myeloid leukemia, in which vitamin D3 derivatives and rosemary preparations showed cooperative anti-tumor effects (Sharabani *et al*., 2006). Additionally, different rosemary extracts showed a modulator effect on induced tumors in laboratory animals (Sancheti and Goyal, 2006; Peng *et al*., 2007).

The anti-carcinogenic and ant-tumor effects suggested that rosemary is also anti-mutagenic, as a mutagenesis is a prerequisite for a carcinogenic transformation. Actually, the literatures are rich in this regard, and anti-mutagenic properties of rosemary have been demonstrated by several investigators. (Del Baño *et al.* ,2006) have been able to demonstrate the anti-mutagenic effects of rosemary phenolics against chromosomal damage induced in human lymphocytes by gamma rays, and a similar radioprotective potential of the plant against the lethal effects of gamma radiation has been confirmed by Jindal *et al*. (2006). Furthermore, DNA damage by benzo(a)pyrene in human cells has been found to be increased by cigarette smoke, while it was decreased by a filter containing rosemary extract (Alexandrov *et al*., 2006). This finding was interpreted that the plant is able to lower free radicals, and its antioxidant effects have been suggested and confirmed by different investigators (Cheung and Tai, 2007; Bozin *et al*., 2007; Yesil-Celiktas *et al*., 2007; Chang *et al*., 2008).

1-4-5-5: Immune Modulator Effects

There has been no direct evidence about the immune modulator effects of rosemary, although Al-Sereiti *et al*. (1999) have suggested such potentials. However, the scope of immune modulation has been conducted in terms inflammation and anti-microbial pathogens. In this regard, rosemary or/and its constituents were effective as anti-inflammatory (Altinier *et al*., 2007; Peng *et al*., 2007) and anti-bacterial, *in vivo* and *in vitro* (Bozin *et al*., 2007; Fu *et al*., 2007), moreover, rosemary oil has been considered a potential in drug-resistance infections (Tawfiq, 2007).

1-4-5-6: Fertility Effects

There has been one investigation (Nusier *et al*., 2007) that tackled the fertility effects of rosemary, which is outlined here. In this investigation, ingestion of rosemary by two groups of adult Sprague-Dawley rats at levels of 250 and 500 mg/kg for 63 days was investigated for its effects on fertility. They found that body weight and absolute and relative testis weights were not affected, but the average weights of epididymides, ventral prostates, seminal vesicles, and preputial glands were decreased significantly. A significant decline in spermatogenesis in testes due to a decrease in the number of primary and secondary spermatocytes and spermatids in treatment group 2 (500 mg/kg) was attributed to a significant decrease in testosterone. Sperm motility and density were also significantly decreased in the epididymis and in the testes of rosemary-treated male rats. Unfortunately, there is no further evidence to confirm or contrast such findings.

1-5: Cyclophosphamide

Cyclophosphamide is a drug that is used primarily for treating several types of cancer due to its alkylating effects (Giralt, et al., 1994). In order to be

effective, cyclophosphamide is first converted by the liver (metabolic activation) into two chemicals, which are acrolein and phosphoramide (Brock, 1996). Acrolein and phosphoramide are the active compounds, and they slow the growth of cancer cells by interfering with the actions of DNA (decreasing its synthesis) within the cancerous cells, although it is a non-specific agent when the cell cycle phases are considered (Brock, 1989). It is, therefore, referred to as a cytotoxic and a mutagenic drug. Normal cells also are affected, and this results in serious side effects. The drug also suppresses the immune system and is also referred to as immunosuppressive (Pang *et al*., 2007). Accordingly, this agent was used in the present study as a mutagenic and an immunosuppressive drug.

1-6: Investigated Parameters

1-6-1: Genetic Parameters

1-6-1-1: Micronucleus Formation

Micronuclei originate from a chromosomal material that has lagged in anaphase. In the course of mitosis, this material is distributed to only one of the daughter cells. It may be included in the main nucleus or form one or more separate small nuclei, i.e., micronuclei (von Ledebur and Schmid, 1973). The micronuclei consist mainly of acentric fragments as demonstrated by DNA content measurements. They may also consist of entire chromosomes and may result from non-disjunction due to malfunction of the spindle apparatus. Micronuclei can be observed in any cell type of a proliferating tissue. They are, however, most easily recognized in cells without the main nucleus; namely erythrocytes (Schmid, 1976).

The scoring of micronuclei in bone-marrow cells was proposed as a screening test by Boller and Schmid (1970) and Heddle (1973).They demonstrated that the frequency of micronuclei can be evaluated most readily in young erythrocytes, shortly after the main nucleus is expelled. The young ones are termed polychromatic erythrocytes (PCEs). With conventional staining

techniques, PCEs stain bluish to purple because of the high content of ribonucleic acid in the cytoplasm. In mouse-bone marrow, the maturing erythroblasts go through six or seven cell divisions with a cell-cycle length of about 10 hours (Cole *et al*., 1979). About 10 hours after the last mitotic division, the expulsion of the main nucleus is completed and the resulting PCE remains in the bone marrow for another 10 hours. Treatment-induced micronuclei derived from chromosomal fragments produced during the preceding cell cycle will thus appear in PCEs not earlier than 10 hours after treatment of the animal with the test material. Therefore, the micronucleus formation assay has been employed by several investigators as a short-time assay of mutagenesis as reviewed by Majer *et al*. (2001).

1-6-1-2: Sperm-head Abnormality Assay

The entire process of sperm formation, from spermatogonia to spermatozoa is referred to as spermatogenesis, which occurs in the seminiferous tubules of the testis (Seely *et al*., 1996). The stem cell (spermatogonia) is divided mitotically to give primary spermatocytes that undergo mieosis I to give rise to haploid secondary spermatocytes that undergo mieosis 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. 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 of 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* (Chatterjee *et al*., 2000).

1-6-2: Immunological Parameters

1-6-2-1: Total and Differential Counts of Leucocytes

Leucocytes are cells of the immune system defending the body against both infections diseases and foreign materials (Alberts, 2005).These cells are originated in the bone marrow through two cell lineages; myeloid progenitor and lymphoid progenitor, which give rise to the five types of leucocytes (neutrophils, lymphocytes, monocytes, eosinophils and basophils). Each type of these cells is commenced to carry out an immune function. The neutrophils are mainly involved in the innate immune system to carry out phagocytosis, while lymphocytes represent the humoral and cellular arms of specific immunity. Monocytes are involved in carrying out phagocytosis, but they are also professional antigen presenting cells. Eosinophils are involved in allergic and inflammatory reactions, as well as, parasitic infections. Basophils release histamine, heparin and some pharmacological mediators of immunological reactions (Kramer, 2003). Due to these diverse immunological functions, the normal counts of leucocytes (total and differential) can be deviated by infections, radiations, environmental pollutants, drugs and products of medicinal plants (Ad'hiah *et al*., 2001; Ad'hiah *et al*., 2002; Ad'hiah *et al*., 2004; Ad'hiah *et al*., 2007).

1-6-2-2: Phagocytosis

Phagocytosis represents the first cellular defense mechanism in the innate immunity. It is carried out mainly by two types of leucocytes; neutrophils and monocytes. Both types of cells can migrate out blood vessels to tissues in response to suitable stimulus, but they differ in that the neurophil is a short-lived cell, while monocyte is developed into a tissue macrophage, which in turn can act as an antigen presenting cell in the secondary lymphoid organs. Their function is to engulf particles (i.e. immune complexes and infectious agents), internalize

them and then achieve their destruction through two pathways; oxygen-dependent and oxygen-independent pathways. The phagocytosis is enhanced by C3b, a complement component, and IgG, which both facilitate the attachment of phagocytes to the engulfed object through the mechanism of opsonization (Lydyard and Grossi, 1998).

It has been reported that phagocytosis can be activated or reduced by several agents; including infectious agents, medicinal plant products, vitamins and environmental pollutants (Ad'hiah *et al*., 2002; AL-Keenani, 2005; Ad'hiah *et al*., 2007)

1-6-3: Fertility Parameters

Male fertility can be defined as the capacity of the male to induce conception in females of the same species. Functionally, it depends on sperm count and motility, and an abnormality in one or both cases may render the male as infertile, although other factors are also operative (Matter, 2005).

Ejaculated mammalian spermatozoa are unable to immediately fertilize ova. Instead, they must first undergo a series of maturational changes within the female tract that confers upon these cells the ability to interact with the oocyte and achieve fertilization. These changes, collectively termed capacitation, were identified independently by Austin and Chang in the 1950s, and collectively reviewed by Ecroyd *et al*. (2003), which are presented in the following. A number of correlates of the process have been identified, including plasma membrane remodeling, an increase in sperm metabolic rate, the expression of hyperactivated motility, and the ability to undergo the acrosome reaction on the surface of the zona pellucida. Recent studies suggest that some, if not all, of these processes are driven by a unique cAMP-dependent signaling cascade that leads to a dramatic increase in the tyrosine phosphorylation status of these cells (Harrison et al., 1996). This signaling pathway is probably ubiquitous among mammals; it has now been identified in spermatozoa from all species that have been studied, including the human, rat, mouse, bull, pig, hamster and monkey.

The process appears to be a necessary prerequisite for plasma membrane remodeling, hyperactivation and sperm-egg binding (Monteiro and Stern, 1996). These processes also related, positively or negatively, to the diet or other agents, including plant and/or plant products that can modify the behavior of spermatozoa (Leclerc et al., 1998). Also, Sakaue *et al*. (2001) showed that two concerns are worth questioning in the quality of spermatozoa; they are sperm count and motility, which were investigated in the present study

Chapter Two

Materials and Methods

Chapter Two
Materials and Methods

2-1: Materials

The general laboratory equipments and chemicals are given in tables 2-1 and 2-2, respectively.

Table 2-1: General laboratory equipments.

Equipment	Company / Country
Autoclave	SES little Sister / England
Centrifuge	Beckman / England
Cooled incubator	Memmert / Germany
Digital camera	Mercury / China
Electrical balance	Sartorius / Germany
Hemocytometer	Neubauer / Germany
Micropipette	Gilson / France
Microscope	Motic / Japan
Rotary evaporator	Buchi / Switzerland
Reflex	Electrothermol \ England
Vortex	Griffin / England
Water bath	Gallenkamp / England

Table 2-2: Chemical materials.

Chemical Material	Company / Country
Chloroform	BDH / England
Cyclophosphamide	Hexal/ France
Ethyl alcohol	BDH / England
Eosin	BDH / England
Giemsa stain	Fluka / Switzerland
Heparin	Leo Pharmaceutical /Denmark
Hexane	Fluka / Switzerland
Methanol	Fluka / Switzerland
Potassium hydroxide	Sigma/ USA
Sodium bicarbonates	BDH / England

2-2: The Plant Rosemary (*Rosemary officinalis* L.)

2-2-1: Plant Collection and Identification

The plant was collected from the gardens of the University of Baghdad, in November 2006 and it was identified as *Rosemary officinalis* by Professor Dr. Ali Al-Mousawi (Plant Taxonomist; Department of Biology, College of Science, University of Baghdad). A field picture of the plant is given in figure 2-1.

Figure 2-1: Field picture of the plant *Rosemary officinalis*.

2-2-2: Preparation of Plant Extracts

The plant was extracted with two types of solvents, which were methanol and hexane. In both cases, the extracted parts of the plant were the aerial parts (leaves and stems), which were air-dried at room temperature (20-25°C), and

then powdered using a coffee grinder. Fifty grams of the powder were extracted for six hours in 250 ml of the solvent (methanol or hexane) using the reflex apparatus and the source of heating was a warm water bath (45°C). The obtained extract solution was then evaporated at 45°C using a rotary evaporator, and the resultant crude extract was kept at -20°C until use to prepare the required doses (Nadir *et al*., 1986).

2-2-3: Rosemary Doses

The plant extracts (methanol and hexane) were assessed in one biological model, which was a mammalian model (albino male mouse; *in vivo*). In such model, three doses (0.1, 0.2 and 0.4 mg/kg) of the two extracts were tested. Dose selection was based on the finding of Fahim (2004), who suggested that these doses are effective as anti-mutagen and immune modulator. To prepare these doses, the methanol extract was dissolved in distilled water, while hexane extract was dissolved in olive oil.

2-3: Solutions

i. **Leucocyte diluent:** The solution was prepared by adding 2 ml of glacial acetic acid to 98 ml of distilled water, in addition to a few drops of methylene blue as a colour indicator (Sood, 1986).
ii. **Normal saline (0.9% NaCl):** A ready prepared solution (Jadda Company, Kingdom of Saudia Arabia) was used. The solution was supplied by the Baghdad Teaching Hospital.
iii. **Sodium bicarbonate:** Sodium bicarbonate (7.5 gm) was dissolved in 100 ml of distilled water, and the solution was stored at 4°C (Allen *et al*., 1977).
iv. **Leishman Stain:** The Institute of Sera and Vaccine (Baghdad) supplied a ready prepared stain kit.
v. **Giemsa stain:** Giemsa stock solution was prepared by dissolving one gram of Giemsa powder in 33 ml glycerin using a water bath (60°C) for

two hours with a continuous shaking. After cooling the solution for 30 minutes at room temperature, 66 ml of absolute methanol were added with a continuous mixing. The solution was then kept in a dark bottle at room temperature (Allen *et al*., 1977). To prepare Giesma 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. **Eosin stain:** The stain was prepared by dissolving one gram of eosin yellowish powder in 100 ml of distilled water. The stain was centrifuged (2000 rpm for 10 minutes) before use (Wyrobek and Bruce, 1975).

vii. **Trypan blue stain:** The stain was prepared by dissolving one gram of trypan blue powder in 100 ml of normal saline (Ad'hiah, 1990).

viii. **Heat-killed Yeast Suspension:** The yeast *Sacchoromyces cerevisiae* (Pakmaya Company, Turkey) was used to prepare the yeast suspension (Metcalf *et al*., 1986). The method is outlined as follows:

- Ten grams of the yeast were suspended in 150 ml of sterile normal saline.
- The suspension was heated in a boiling water bath for 60 minutes.
- After cooling the suspension at room temperature, it was filtered using double layers of gauze.
- The filtered cell suspension was assessed for yeast cell viability by dye exclusion test (trypan blue) to assure that all cells were dead.
- The cell suspension was divided into aliquots (5 ml) after adjusting the cell count to 10^7 cell / ml, and stored at -20ºC until use.

ix. **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 ml) in sterile test tubes. Some of these tubes were stored in the freezer (-20°C) until use in the phagocytosis assay (Metcalf *et al*., 1986). The other 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). In both cases, the plasma was filter-sterilized by Milipore filter (0.2Mm).

x. **Heparin:** The Baghdad Teaching Hospital supplied the solution of heparin (5000 IU/ml), which was the product of Leo Pharmaceutical (Denmark).

xi. **Cyclophosphamide:** The Baghdad Teaching Hospital supplied the drug cyclophosphamide, which was the product of Pharmacia Company (Belgium). A human dose (16 mg/kg) (company leaflet) was considered as an effective dose, which was employed in the present study (Pang *et al*., 2007). The drug solution was diluted with distilled water to prepare the required dose.

2-4: Laboratory Animals

Albino male mice (*Mus musculs*) were the laboratory animals. They were supplied by the Biotechnology Research Centre (Al-Nahrin University). Their age at the start of experiments was 8-10 weeks, and their weight was 25±2 grams. They were divided into groups, and each group was kept in a separate plastic cage (details of these groups are given in the section of experimental design). The animals were maintained at a temperature of 23-25°C, and they had free excess to food (standard pellets) and water (*ad libitum*).

2-5: Experimental Design

The experiments were designed to evaluate (*in vivo*) the genetic, immunological and fertility effects of rosemary extracts (methanol and hexane) in albino male mice. Therefore, such evaluations were carried out through three stages.

2-5-1: First Stage

In this stage, the genetic (micronucleus formation) and Immunological (total and absolute counts of leucocytes and phagocytosis) effects of three doses of rosemary extracts (methanol, hexane) and one dose of cyclophosphamide were investigated. Therefore the animals were divided into five groups:

- **Group I**: The animals were treated with distilled water (negative controls of methanol extract = 4 animals).
- **Group II**: The animals were treated with olive oil (negative controls of hexane extract = 4 animals).
- **Group III**: The animals were treated with cyclophosphamide at a dose of 16 mg/kg (positive controls = 4 animals).
- **Group IV**: The animals were treated with three doses of the methanol extract (0.1, 0.2, 0.4 mg/kg) (12 animals).
- **Group V**: The animals were treated with three doses of the hexane extract (0.1, 0.2, 0.4 mg/kg) (12 animals).

The tested materials were injected subcutaneously as a single dose (0.1 ml) per a day and for 7 days. Then, the mice were sacrificed in day 8 for laboratory assessments. The total number of mice in this stage was 36 animals.

2-5-2: Second Stage

In this stage, the fertility effects (sperm count, actively motile sperms and sperm-head abnormalities) of three doses of rosemary extract (methanol,

hexane) and one dose of cyclophosphamide were investigated. Therefore the animals were divided into five groups:

- **Group I**: The animals were treated with distilled water (negative controls of methanol extract = 12 animals).
- **Group II**: The animals were treated with olive oil (negative controls of hexane extract = 12 animals).
- **Group III**: The animals were treated with cyclophosphamide at a dose of 16 mg/kg (positive controls = 12 animals).
- **Group IV**: The animals were treated with three doses of the methanol extract (0.1, 0.2, 0.4 mg/kg) (36 animals).
- **Group V**: The animals were treated with three doses of the hexane extract (0.1, 0.2, 0.4 mg/kg) (36 animals).

The animals in these groups were injected subcutaneously with a single dose of the tested material on day 1, and then they were sacrificed on days 7, 21 and 35 for laboratory assessments. The total number of mice in this stage was 108 animals.

2-5-3: Third Stage

In this stage, interactions (pre- and post-treatments) between the ideal dose of both extracts (0.4 mg/ kg) and cyclophosphamide (16 mg/kg) were carried out. The criterion of selection for the ideal dose was based on the approximation between the values of total leucocyte count in the extract-treated animals and negative controls of the first stage.

- **In pre-treatment** interaction, the plant extract (methanol or hexane) was given for 6 days (single dose/day), while cyclophosphamide was given on day 7, and then animals were sacrificed on day 8 for laboratory assessments. In both cases, the material was given subcutaneously (0.1 ml). The total number of mice in this interaction was 16 animals.

- **In post-treatment** interaction, the animals was given cyclophosphamide on day one, while the plant extract (methanol or hexane) was given on day 2 till day 7 (single dose/day), and then animals were sacrificed in day 8 for laboratory assessments.

In both cases, the material was given subcutaneously (0.1 ml). The total number of mice in this interaction was 16 animals. For both treatments, control groups paralleled the two types of interactions, in which the plant extract was replaced by either distilled water (methanol extract) or olive oil (hexane extract).

2-6: Laboratory Methods

2-6-1: Chemical Identification of Plant Extracts

The chemical identification of plant extracts (methanol and hexane) was carried out by the staff of Biotechnology Research Centre at Al-Nahrin University using pre-established methods in their laboratories. The constituents of interest in the present study were flavonoids, terpenes and steroids (Al-Malikey, 2006).

2-6-2: Total Leucocyte Count

Blood samples were collected by heart puncture using a disposable insulin syringe (1 ml) pre-coated with heparin. The method of Haen (1995) was followed, in which, an aliquot of 0.02 ml blood was mixed with 0.38 ml of leucocyte diluent in a test tube, and left at room temperature for 5 minutes. A drop of the mixture was applied to the surface of Neubauer chamber under the cover slip, and the chamber was left for 3 minutes to settle the cells. The leucocytes were counted in 4 large squares (each with 16 small squares), and the total count of leucocytes was obtained using the following equation:

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

2-6-3: Absolute Count of Leucocytes

One drop of the blood was smeared on a clean slide using another slide and left to dry at room temperature. The smear was stained with Leishman stain for 5 minutes and buffered for 10 minutes, and then washed with tap water. The slide was air-dried, and then examined under oil immersion lens (100X) (Haen, 1995). At least 100 leucocytes were examined, and the percentage of each type was recorded, while the absolute count of each type was obtained using the following equation:

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

2-6-4: Phagocytic Index

The evaluation of phagocytosis was carried out on phagocytes obtained from the peritoneum of mice. The procedure of Metcalf *et al*. (1986) was followed with some modifications.

i- The animal was anaesthetized with chloroform, and then injected intraperitoneially with 3 ml of normal warm saline (37°). After that, the abdominal region was massaged for 3 minutes.

ii- The animal was dissected, and the peritoneal cells were collected with a pasture pipette and transferred to a clean test tube.

iii- The tube was centrifuged (2000 rpm) for 5 minutes.

iv- The cells were suspended in 1 ml of normal saline, counted and their number was adjusted to 1 x 10^6 cell /ml. Also, the cell viability was assessed using a dye-exclusion test (trypan blue stain).

v- To carry out phagocytosis, 0.2 ml of the cell suspension, 0.1 ml of heat-killed yeast suspension and 0.1 ml of human AB plasma were mixed in a test tube and incubated in a shaking water bath (37°C).

vi- After 30 and 60 minute incubation periods, smears were made and the slides were air-dried, and then stained with Giemsa stain for 15 minutes.

vii- The slides were examined under oil immersion lens (100X), and at least 100 yeast-phagocytic and non-phagocytic cells were randomly counted. The phagocytic activity was expressed as a phagocytic index, which was calculated using the following equation:

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

2-6-5: Micronucleus Formation Assay

To carry out the assessment of micronucleus formation, the procedure of Schmid (1976) was followed, which is outlined in the following steps:

i- The mouse was dissected to obtain the femur, and after removal of muscles, it was cut from both ends and gripped from the middle with a forceps in a vertical position over the edge of a test tube. The cellular content was collected with a heat inactivated (56°C for 30 minutes) human AB plasma (2 ml) using a disposable insulin syringe.

ii- The test tube was centrifuged (1000 rpm) for 10 minutes, and the supernatant was discarded.

iii- The cellular deposit was gently mixed, and a thin smear was made on a clean slide and air-dried at room temperature.

iv- The smear was fixed with absolute methanol for 5 minutes, and then air-dried at room temperature.

v- The smear was stained with Giemsa stain for 15 minutes, and then rinsed with distilled water.

vi- The slides were examined under oil immersion lens (100X), and at least 1000 polychromatic erythrocytes (PCE) were examined for the presence of micronucleus formation. The micronucleus index was considered as number of micronuclei per 1000 cells.

2-6-6: Sperm-head Abnormality Assay

The mouse was dissected to obtain the epididymis, which was collected in as Petri-dish containing 5 ml of normal saline. The epididymis was dispersed with a forceps and a scalpel to free the spermatozoa. The spermatozoa-containing saline was transferred to a test tube, which was centrifuged (1000 rpm) for 10 minutes. The supernatant was discarded, and the spermatozoa deposit was gently suspended in 1 ml of normal saline. A thin smear of the suspension was made on a clean slide, which was then air-dried at room temperature. The slide was stained with eosin for 5 minutes, rinsed with distilled water and air-dried. The slide was examined under oil immersion lens (100X), and at least 1000 spermatozoa were inspected for the morphology of their heads (Wyrobek and Bruce, 1975). The sperm-head abnormality (SHA) index was scored using the following equation:

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

2-6-7: Spermatozoa Count

The counting of spermatozoa was carried out according to a method that was presented by AL-Dujaily (1996). The spermatozoa were collected as described in the previous section (2-6-6), but they were not centrifuged. An aliquot of spermatozoa suspension (10µl) was placed on Neubauer chamber and covered with a cover slip and left for one minute to stabilize. The spermatozoa were counted in four large squares, and then their count was calculated according to the following equation:

$$\text{Spermatozoa Count (million sperm / ml)} = \left(\frac{\text{Number of Spermatozoa}}{4} \right) \text{ x } 10^6$$

2-6-8: Sperm Motility

Sperm motility was recorded according to a method used by AL-Janabi (1992) and AL-Dujaily (1996). Accordingly, a drop (10µl) of sperm suspension was mounted between a warm slide and a cover slip (24 x 24mm). Each sample was recorded at magnification of 400X, using 5 random microscopic fields. The motility of sperms was scored according to the Macleod scale, which is outlined below (Silverberg and Turner, 2001). One hundred spermatozoa were scored and assigned according to that scale, and the percentage of each score was given.

The Macleod scale is as the following:

0 = Immotile sperm, no motion.

1 = Local circular slow motility.

2 = Poor motion, motile sperm with a slow forward progression.

3 = Moderate speed with a relatively straight forward motion (good forward progression, straight-line movement).

4 = Excellent motion, rapid forward progression (very good forward progression movement).

In the present study, only the results of actively motile sperms (Score 4: excellent motion) were given.

2-7: Statistical Analyses

The values of the investigated parameters were given in terms of mean ± standard deviation, and differences between means were assessed by analysis of variance (ANOVA) and Duncan test, using the computer programme 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 (Perez-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 cyclophosphamide).

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

Chapter Three

Results

Chapter Three
Results

3-1: Detection of Active Compounds

Three active compounds were evaluated in the two extracts of rosemary leaves, which were flavonoids in the methanol extract, and terpenes in the hexane extract. The methanol extract gave a positive reaction in flavonoids assays, while the hexane extract was positive for terpenes and negative for steroids (Table 3-1).

Table 3-1: Detection of some active compounds in rosemary extracts.

Rosemary Extracts	Chemical Compounds	Reagents	Indication	Results
Methanol	Flavonoids	Ammonia	Dark yellow color.	Positive
Hexane	Terpenes	Concentrated H_2SO_4	Dark brown color	Positive
	Steroids	Concentrated H_2SO_4	Blue color	Negative

3-2: Immunological and Cytogenetic Parameters

3-2-1: Total Count of Leucocytes (TLC)

Animals treated with distilled water showed a count of 9470 cell/cu.mm.blood, while in animals treated with olive oil, the count was increased to 9730 cell/cu.mm.blood. Both counts were significantly ($P \leq 0.05$) higher than the count of animals treated with cyclophosphamide (7130 cell/cu.mm.blood). Animals treated with three doses of rosemary extracts (methanol or hexane) showed approximated counts (range: 8250 - 8550 cell/cu.mm.blood), but such range was lower than the count of both negative controls and higher than the count of positive control, however the difference was significant with respect to the latter difference (Table 3-2).

Table 3-2: Total leucocyte count in albino male mice treated with methanol and hexane extracts of rosemary.

Groups		Dose (mg/kg)	Mean ± S.D.* (cells/cu.mm. blood)	Treatment Efficiency (%)
Positive Control (Cyclophosphamide)		16	7130 ± 420^{B}	-24.7
Negative Control (Distilled Water)			9470 ± 120^{A}	
Negative Control (Olive Oil)			9730 ± 310^{A}	
Rosemary Extracts	Methanol	0.1	8500 ± 120^{A}	-10.2
		0.2	8300 ± 120^{A}	-12.3
		0.4	8250 ± 300^{A}	-12.8
	Hexane	0.1	8550 ± 300^{A}	-12.1
		0.2	8450 ± 190^{A}	-13.1
		0.4	8350 ± 30^{A}	-14.1

*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

As in TLC, the lymphocyte count behaved in a similar manner of a reduction as a result of treatments with the two extracts of rosemary, but this time, the difference was significant when the comparison was made with the two negative controls. The highest treatment efficiency was recorded in the third dose (0.4 mg/kg) of methanol and hexane extracts (-13.3 and -14.1%, respectively). Also these animals showed a significant increased count as compared to the animals of positive control. The cyclophosphamide was also significantly effective in reducing the lymphocyte count as compared to both negative controls (Table 3-3).

Table 3-3: Total lymphocyte count in albino male mice treated with methanol and hexane extracts of rosemary.

Groups		Dose (mg/kg)	Mean ± S.D.* (cells/cu.mm. blood)	Treatment Efficiency (%)
Positive Control (Cyclophosphamide)		16	4310 ± 252^{C}	-26.4
Negative Control (Distilled Water)			5860 ± 69^{B}	
Negative Control (Olive Oil)			5990 ± 187^{B}	
Rosemary Extracts	Methanol	0.1	5080 ± 73^{A}	-10.4
		0.2	5120 ± 78^{A}	-12.6
		0.4	5080 ± 192^{A}	-13.3
	Hexane	0.1	5250 ± 185^{A}	-12.3
		0.2	5110 ± 188^{A}	-14.6
		0.4	5090 ± 178^{A}	-14.6

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

3-2-2-2: Neutrophils

The cyclophosphamide was effective in reducing the count of neutrophils as compared to animals treated with distilled water or olive oil (2120 *vs*. 2930 and 2850 cells/cu.mm.blood, respectively), and the difference reached a significant level ($P \leq 0.05$). The three doses of methanol and hexane extract behaved in a similar manner, and a reduction of neutrophils was observed in the treated animals (range: 2460 – 2570 cells/cu.mm.blood) as compared to both negative controls, but the differences did not reach a significant level ($P > 0.05$). However, such range was still higher than the count of positive control, and the difference was significant (Table 3-4).

Table 3-4: Total Neutrophil count in albino male mice treated with methanol and hexane extracts of rosemary

Groups		Dose (mg/kg)	Mean ± S.D.* (cells/cu.mm. blood)	Treatment Efficiency (%)
Positive Control (Cyclophosphamide)		16	2120 ± 116^{B}	-2.73
Negative Control (Distilled Water)		0.00	2930 ± 30^{A}	
Negative Control (Olive Oil)		0.00	2850 ± 105^{A}	
Rosemary Extracts	Methanol	0.1	2540 ± 37^{A}	-13.3
		0.2	2480 ± 37^{A}	-15.3
		0.4	2460 ± 99^{A}	-16.0
	Hexane	0.1	2570 ± 99^{A}	-9.8
		0.2	2570 ± 61^{A}	-9.8
		0.4	2940 ± 105^{A}	-12.6

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

3-2-2-3: Monocytes

Both negative controls (distilled water and olive oil) showed approximated counts of monocytes (630 and 660 cells/cu.mm.blood, respectively), but they were significantly higher than the count of positive control (460 cells/cu.mm.blood). The both extracts of rosemary caused a significant decreased count of monocytes as compared to the corresponding negative controls, especially at the third dose of methanol and hexane extracts (540 and 570 cels/cu.mm.blood, respectively). The treatment efficiency of such reductions was -14.2 and -13.6%, respectively, however, such efficiency was significantly still lower than the treatment efficiency of cyclophosphamide, which was -26.9% (Table 3-5).

Table 3-5: Total Monocyte count in albino male mice treated with methanol and hexane extracts of rosemary.

Groups		Dose (mg/kg)	Mean ± S.D.* (cells/cu. mm. blood)	Treatment Efficiency (%)
Positive Control (Cyclophosphamide)		16	460 ± 37^{C}	-26.9
Negative Control (Distilled Water)			630 ± 44^{B}	
Negative Control (Olive Oil)			660 ± 20^{B}	
Rosemary Extracts	Methanol	0.1	560 ± 5^{A}	-11.1
		0.2	560 ± 9^{A}	-11.1
		0.4	540 ± 17^{A}	-14.2
	Hexane	0.1	582 ± 17^{A}	-11.8
		0.2	585 ± 23^{A}	-11.3
		0.4	570 ± 34^{A}	-13.6

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

4-2-2-4: Eosinophils

Animals treated with distilled water showed an eosinophil count of 70 cells/cu.mm.blood, which was significantly lower than the counts of positive control (120 cells/cu.mm.blood) and olive oil negative control (90 cell/cu.mm.blood). In turn, the latter both controls were also significantly different. The three doses of plant methanol extract enhanced the count of eosinophils (95, 90 and 100 cell/cu.mm.blood, respectively), but the differences were not significant as compared to the corresponding negative control (70 cell/cu.mm.blood), although the treatment efficiency was +35.7, +28.5 and +42.8%, respectively. In contrast, the three doses of hexane extract approximated the count of olive oil negative controls. The cyclophosphamide (positive control) contradicted the previous findings and caused a significant increase in the count of eosinophils (120 cells/ci.mm.blood), with a treatment efficiency of +71.4% (Table 4-6).

Table 3-6: Total Eosinophil count in albino male mice treated with methanol and hexane extracts of rosemary.

Groups		Dose (mg/kg)	Mean ± S.D* (cells/cu. mm. blood)	Treatment Efficiency (%)
Positive Control (Cyclophosphamide)		16	120 ± 15^{B}	+71.4
Negative Control (Distilled Water)			70 ± 26^{A}	
Negative Control (Olive Oil)			90 ± 10^{AB}	
Rosemary Extracts	Methanol	0.1	95 ± 5^{A}	+35.7
		0.2	90 ± 11^{A}	+28.5
		0.4	100 ± 12^{A}	+42.8
	Hexane	0.1	90 ± 5^{AB}	0.00
		0.2	100 ± 8^{AB}	-11.1
		0.4	80 ± 49^{A}	-11.1

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

3-2-2-5: Basophils

Animals treated with cyclophosphamide (positive control) showed a significant increased count of basophils (82 cell/cu.mm.blood) as compared to both negative controls (15 and 25 cell/cu.mm.blood, respectively) with a treatment efficiency of +93.9%. A similar picture was shared by both extracts of rosemary, especially the dose 0.4 mg/kg of methanol extract (39 cell/cu.mm.blood) and the dose 0.2 of the hexane extract (42 cell/cu.mm.blood), in which the treatment efficiency was +160 and +68%, respectively. These differences were highly significant (Table 3-7).

Table 3-7: Total Basophil count in albino male mice treated with methanol and hexane extracts of rosemary.

Groups		Dose (mg/kg)	Mean ± S.D.* (cells/cu. mm. blood)	Treatment Efficiency (%)
Positive Control (Cyclophosphamide)		16	82 ± 5^{C}	+93.9
Negative Control (Distilled Water)			15 ± 5^{AB}	
Negative Control (Olive Oil)			25 ± 4^{AB}	
Rosemary Extracts	Methanol	0.1	20 ± 4^{B}	+33.3
		0.2	22 ± 7^{AB}	+46.6
		0.4	39 ± 3^{A}	+160.0
	Hexane	0.1	27 ± 3^{AB}	+8.00
		0.2	42 ± 6^{A}	+68.00
		0.4	40 ± 24^{A}	+60.00

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

3-2-3: Phagocytic Index

3-2-3-1: After 30 Minute Incubation

Both extract were significantly effective in increasing the phagocytic index, especially the second dose of methanol extract (68.35%) and the third dose of hexane extract (85.05%) as compared to the corresponding negative controls (51.86 and 62.33%, respectively), with a treatment efficiency of +31.7 and +36.4%, respectively (Table 3-8).

3-2-3-2: After 60 Minute Incubation

The phagocytic index after 60 minute incubation behaved in a similar manner as after 30 minute incubation, in which the second dose of methanol extract and the third dose of hexane extract were significantly effective in increasing the phagocytic index as compared to the corresponding negative controls (Table 3-9).

Table 3-8: Phagocytic index after 30 minutes of incubation in albino male mice treated with methanol and hexane extracts of rosemary,

Groups		Dose (mg/kg)	Mean ± S.D.* (%)	Treatment Efficiency (%)
Positive Control (Cyclophosphamide)		16	32.36 ± 5.80^{G}	-37.6
Negative Control (Distilled Water)			51.86 ± 0.49^{E}	
Negative Control (Olive Oil)			62.33 ± 0.35^{F}	
Rosemary Extracts	Methanol	0.1	69.57 ± 0.42^{A}	+34.1
		0.2	68.35 ± 0.60^{A}	+31.7
		0.4	67.27 ± 0.55^{A}	+29.7
	Hexane	0.1	77.67 ± 0.68^{d}	+24.6
		0.2	74.90 ± 0.81^{C}	+20.1
		0.4	85.05 ± 0.59^{D}	+36.4

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

Table 3-9: Phagocytic index after 60 minutes of incubation in albino male mice treated with methanol and hexane extracts of rosemary

Groups		Dose (mg/kg)	Mean ± S.D.* (%)	Treatment Efficiency (%)
Positive Control (Cyclophosphamide)		16	40.70 ± 0.78^{f}	-31
Negative Control (Distilled Water)			59.00 ± 0.80^{E}	
Negative Control (Olive Oil)			74.70 ± 0.75^{A}	
Rosemary Extracts	Methanol	0.1	72.90 ± 0.63^{A}	+23.5
		0.2	77.35 ± 2.43^{B}	+31.7
		0.4	73.32 ± 0.61^{A}	+24.2
	Hexane	0.1	80.27 ± 0.66^{B}	+7.4
		0.2	64.12 ± 1.10^{d}	-14.1
		0.4	91.72 ± 0.33^{C}	+22.7

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

3-2-4: Micronucleus Index

A highly significant increase of micronucleus formation was observed in animals treated with cyclophosphamide (28.6 micronucleus/1000 cells) as compared to either negative controls or animals treated with the two types of rosemary extracts, in which the micro nucleus index was ≤ 10 micronucleus/1000 cells. The latter animals showed no significant difference between them with respect to micronucleus formation (Table 3-10).

Table 3-10: Micronucleus formation in bone marrow cells of albino male mice treated with methanol and hexane extracts of rosemary.

Groups		Dose (mg/kg)	Mean ± S.D.* (Micronucleus /1000)	Treatment Efficiency (%)
Positive Control (Cyclophosphamide)		16	28.6 ± 3.5^{B}	+200.0
Negative Control (Distilled Water)			10.0 ± 5.0^{A}	
Negative Control (Olive Oil)			9.3 ± 2.9^{A}	
Rosemary Extracts	Methanol	0.1	8.0 ± 3.6^{A}	-40.00
		0.2	8.2 ± 4.5^{A}	-18.00
		0.4	6.0 ± 2.9^{A}	-20.00
	Hexane	0.1	7.5 ± 5.1^{A}	-24.00
		0.2	9.7 ± 3.5^{A}	+4.00
		0.4	7.0 ± 4.3^{A}	-19.00

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

3-3: Fertility Effects

The fertility effects of rosemary were evaluated in terms of three parameters, which were sperm count, actively motile sperms and sperm head abnormalities. These evaluations were carried out 7, 21 and 35 days post-treatment with a single dose of each of the three doses (0.1, 0.2 and 0.4 mg/kg) of methanol and hexane extracts.

3-3-1: Sperm Count

Seven days post-treatment revealed that both extracts were effective in elevating the sperm count, especially the second and third doses of methanol extract (11.2 and 11.5, respectively *vs*. 8.6 x 10^6 sperm/ml) and the three doses of hexane extract (11.2, 11.5 and 11.7, respectively *vs*. 9.3 x 10^6 sperm/ml) as compared to the corresponding negative controls. Such five differences were significant, and accounted for a treatment efficiency of +32.2, +32.6, +20.4, +23.6 and +25.8%, respectively (Table 3-11).

Twenty one days post-treatment, as well as, 35 days post-treatment behaved in a similar manner as that of 7 days post-treatment with the exception of the third dose of hexane extract, in which the sperm count approximated its value in the corresponding negative control (10.4 *vs*. 10 x 10^6 sperm/ml and 10.7 *vs*. 10.6 x 10^6 sperm/ml, respectively (Table 3-11).

3-3-2: Actively Motile Sperms

In general, the percentage of actively motile sperms was increased 7, 21 and 35 days post-treatment with a single dose of the three doses of the methanol and hexane extracts as compared to the corresponding negative and positive controls. The exception was the third dose of both extracts, in which the activity was reduced as compared to the corresponding negative controls, especially the dose 0.4 mg/kg of hexane extract, in which the activity was also behind the activity of animals treated with cyclophosphamide (60 *vs*. 64%). However, most of these differences failed to attend a significant level ($P > 0.05$) (Table 3-12).

3-3-3: Sperm-head Abnormalities

The percentage of sperms with abnormal heads was subjected to the type of treatment (cyclophosphamide and plant extracts), as well as, days post-treatments (7, 21 and 35 days).

Table 3-11: Sperm count in albino male mice 7, 21 and 35 days post-treatment with a single dose of methanol and hexane extracts of rosemary.

Groups		Dose (mg/kg)	Sperm Count Mean ± S.D. x 10^6 *			Treatment Efficiency (%)		
			7 days	21 days	35 days	7 days	21 days	35 days
Positive Control (Cyclophosphamide)		16	7.6 ± 1.5^{B}	7.0 ± 1.0^{B}	6.6 ± 1.5^{B}	-11.0	-27.0	-35.0
Negative Control (Distilled Water)			8.6 ± 0.5^{B}	9.6 ± 0.5^{AB}	10.3 ± 1.5^{A}			
Negative Control (Olive Oil)			9.3 ± 0.5^{B}	10.0 ± 2.0^{A}	10.6 ± 1.1^{A}			
Rosemary Extracts	Methanol	0.1	9.2 ± 1.5^{B}	10.5 ± 1.7^{A}	10.7 ± 1.5^{A}	+6.7	+8.6	+4.0
		0.2	11.2 ± 1.2^{AB}	12.0 ± 0.9^{A}	12.5 ± 0.8^{AB}	+32.2	+21.5	+16.1
		0.4	11.5 ± 1.2^{A}	12.0 ± 1.4^{A}	13.0 ± 0.8^{B}	+32.6	+24.1	+25.8
	Hexane	0.1	11.2 ± 0.6^{A}	12.1 ± 1.9^{A}	13.2 ± 0.9^{B}	+20.4	+21.0	+24.2
		0.2	11.5 ± 0.4^{A}	12.2 ± 0.9^{A}	12.5 ± 0.5^{AB}	+23.6	+22.0	+17.1
		0.4	11.7 ± 0.9^{A}	10.4 ± 1.7^{A}	10.7 ± 1.7^{A}	+25.8	+4.0	+0.7

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

Table 3-12: Percentage of actively motile sperms in albino male mice 7, 21 and 35 days post-treatment with a single dose of methanol and hexane extracts of rosemary.

Groups		Dose (mg/kg)	Mean ± S.D. (%) *			Treatment Efficiency (%)		
			7 days	21 days	35 days	7 days	21 days	35 days
Positive Control (Cyclophosphamide)		16	64.0 ± 1.0^{AB}	62.0 ± 1.0^{B}	59.0 ± 3.0^{B}	-18.0	-13.0	-13.0
Negative Control (Distilled Water)			70.0 ± 2.6^{A}	71.6 ± 0.5^{A}	72.0 ± 1.7^{A}			
Negative Control (Olive Oil)			71.0 ± 1.7^{A}	72.3 ± 1.5^{A}	72.6 ± 2.1^{A}			
Rosemary Extracts	Methanol	0.1	56.7 ± 4.5^{B}	67.2 ± 4.2^{AB}	70.5 ± 2.6^{A}	-18.9	-6.1	-2.0
		0.2	71.2 ± 4.6^{A}	70.5 ± 5.8^{A}	72.0 ± 4.9^{A}	+1.7	-1.6	+0.0
		0.4	68.7 ± 3.2^{A}	68.7 ± 4.03^{A}	70.2 ± 2.6^{A}	-1.7	-4.0	-2.4
	Hexane	0.1	71.2 ± 3.8^{A}	70.00 ± 3.6^{A}	73.5 ± 2.1^{A}	+0.3	-3.2	+1.1
		0.2	71.5 ± 3.8^{A}	71.0 ± 4.8^{A}	72.0 ± 0.8^{A}	+0.7	-1.8	-0.9
		0.4	60.0 ± 6.6^{B}	73.7 ± 2.6^{A}	72.0 ± 2.8^{A}	-15.7	+1.9	-0.9

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

3-3-3-1: Seven Days Post-treatment

The cyclophosphamide was significantly effective in increasing the sperm-head abnormalities as compared to animals treated with distilled water or olive oil (55.6 *vs*. 49.1 and 47.9%, respectively). In contrast, methanol and hexane extracts were effective in reducing the spontaneous frequency of sperm-head abnormalities, especially the second dose methanol extract (25.8 *vs*.49.1%) and the first dose of hexane extract (28.7 *vs*. 47.9%) as compared to the corresponding negative controls. The treatment efficiency of such effects was -47.5 and -40.1%, respectively (Table 3-13).

3-3-3-2: Twenty One Days Post-treatment

The first dose, as well as, the second and the third doses of hexane extract continued in their reducing effects of sperm-head abnormalities 21 days post-treatment as compared to animals treated with olive oil (20.5, 22.8 and 26.9, respectively *vs*. 41.1%). These differences were significant and scored treatment efficiencies of -50.1, -44.4 and -34.6%, respectively). The methanol extract shared a similar effect, but the third dose (0.4 mg/kg) was the most effective (23.5 *vs*. 41.5%), and scored a treatment efficiency of -43.2% (Table 3-13).

3-3-3-3: Thirty Five Days Post-treatment

The three doses of both extracts were effective in reducing the frequency of spontaneous sperm-head abnormalities in the treated animals as compared to the corresponding negative controls (methanol extract: 24.5, 25.6 and 19.9, respectively *vs*. 35.8%; hexane extract: 16.3, 19.1 and 22.6 *vs*. 35.8%, respectively). These six differences were significant and accounted for a treatment efficiency of -31.4, -28.4, -44.4, -54.4, -46.7 and -36.9%, respectively (Table 3-13).

Table 3-13: Sperm-head abnormalities in albino male mice 7, 21 and 35 days post-treatment with a single dose of methanol and hexane extracts of rosemary.

Groups		Dose (mg/kg)	Mean ± S.D. (%) *			Treatment Efficiency (%)		
			7 days	21 days	35 days	7 days	21 days	35 days
Positive Control (Cyclophosphamide)		16	55.6 ± 0.6^{D}	56.3 ± 0.4^{F}	56.8 ± 0.6^{F}	+13.2	+35.7	+58.7
Negative Control (Distilled Water)			49.1 ± 0.3^{A}	41.5 ± 0.9^{E}	35.8 ± 0.4^{E}			
Negative Control (Olive Oil)			47.9 ± 0.2^{A}	41.1 ± 1.0^{E}	35.8 ± 0.2^{E}			
Rosemary Extracts	Methanol	0.1	48.1 ± 0.9^{A}	37.1 ± 0.9^{C}	24.5 ± 0.9^{B}	-2.00	-15.6	-31.4
		0.2	25.8 ± 1.1^{B}	27.9 ± 0.5^{B}	25.6 ± 0.3^{B}	-47.5	-32.8	-28.4
		0.4	48.4 ± 0.8^{A}	23.5 ± 0.9^{A}	19.9 ± 0.6^{A}	-1.30	-43.2	-44.4
	Hexane	0.1	28.7 ± 0.5^{B}	20.5 ± 0.7^{D}	16.3 ± 0.9^{d}	-40.1	-50.1	-54.4
		0.2	31.5 ± 0.5^{BC}	22.8 ± 0.5^{A}	19.1 ± 0.7^{A}	-34.2	-44.4	-46.7
		0.4	37.0 ± 8.1^{C}	26.9 ± 0.3^{B}	22.6 ± 0.8^{C}	-22.8	-34.6	-36.9

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

3-4: Extract-Cyclophosphamide Interactions

Two types of interactions (pre- and post-treatments) were carried out between the ideal dose of rosemary extracts and cyclophosphamide to evaluate the role of the extracts in modulating the immunological, cytogenetic and fertility effects of the drug in albino male mice. The selection of ideal dose was based on the results of total leucocyte count (Tables 3-2), in which no significant difference was observed between the extract-treated mice and negative controls and such dose was 0.1 mg/kg.

3-4-1: Total and Absolute Counts of Leucocytes

Pre-, as well as, post-treatment animals with the ideal dose of both extracts showed no significant differences in total leucocyte count (Table 3-14), or absolute count of lymphocytes (Table 3-15), neutrophils (Table 3-16), monocytes (Table 3-17), eosinophils (Table 3-18) and basophils (Table 3-19), when comparisons were made with the corresponding controls.

Table 3-14: Total leucocytes count in albino male mice after interactions (pre- and post-treatments) between the ideal dose (0.1 mg/kg) of methanol and hexane extracts of rosemary and cyclophosphamide.

Groups	Mean ± S.D.* (cells/cu. mm. blood)		Treatment Efficiency (%)		$P \leq 0.05$
	Pre	Post	Pre	Post	
Control I (H_2O-Cyclophosphamide)	8333 ± 115^A	8266 ± 115^A			Not Significant
Control II (Olive Oil-Cyclophosphamide)	8400 ± 0.00^A	8266 ± 115^A			Not Significant
Methanol Extract - Cyclophosphamide	8300 ± 115^A	8450 ± 191^A	-0.4	-2.2	Not Significant
Hexane Extract - Cyclophosphamide	8250 ± 100^A	8250 ± 100^A	-1.8	-0.2	Not Significant

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

Table 3-15: Total lymphocyte count in albino male mice after interactions (pre- and post-treatments) between the ideal dose (0.1 mg/kg) of methanol and hexane extracts of rosemary and cyclophosphamide.

Groups	Mean ± S.D.* (cells/cu. mm. blood)		Treatment Efficiency (%)		P ≤0.05
	Pre	Post	Pre	Post	
Control I (H_2O-Cyclophosphamide)	5090 ± 69^A	5092 ± 58^A			Not Significant
Control II (Olive Oil-Cyclophosphamide)	5213 ± 66^A	5094 ± 68^A			Not Significant
Methanol Extract-Cyclophosphamide	5137 ± 75^A	5180 ± 121^A	+0.9	+17.2	Not Significant
Hexane Extract-Cyclophosphamide	5096 ± 70^A	5096 ± 70^A	-2.4	+0.03	Not Significant

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

Table 3-16: Total neutrophil count in albino male mice after interactions (pre- and post-treatments) between the ideal dose (0.1 mg/kg) of methanol and hexane extracts of rosemary and cyclophosphamide.

Groups	Mean ± S.D.* (cells/cu. mm. blood)		Treatment Efficiency (%)		P ≤0.05
	Pre	Post	Pre	Post	
Control I (H_2O-Cyclophosphamide)	2480 ± 71^A	2520 ± 73^A			Not Significant
Control II (Olive Oil-Cyclophosphamide)	2548 ± 32^A	2462 ± 27^A			Not Significant
Methanol Extract-Cyclophosphamide	2494 ± 54^A	2521 ± 58^A	+0.56	+0.03	Not Significant
Hexane Extract-Cyclophosphamide	2479 ± 56^A	2475 ± 58^A	-2.70	+0.52	Not Significant

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

Table 3-17: Total monocyte count in albino male mice after interactions (pre- and post-treatments) between the ideal dose (0.1 mg/kg) of methanol and hexane extracts of rosemary and cyclophosphamide.

Groups	Mean ± S.D.* (cells/cu. mm. blood)		Treatment Efficiency (%)		P ≤0.05
	Pre	Post	Pre	Post	
Control I (H_2O-Cyclophosphamide)	560 ± 14^A	551 ± 17^A			Not Significant
Control II (Olive Oil-Cyclophosphamide)	564 ± 13^A	382 ± 28			Not Significant
Methanol Extract-Cyclophosphamide	545 ± 11^A	559 ± 18^A	-2.60	+1.40	Not Significant
Hexane Extract-Cyclophosphamide	550 ± 8^A	542 ± 13^A	-2.40	+4.2	Not Significant

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

Table 3-18: Total eosinophil count in albino male mice after interactions (pre- and post-treatments) between the ideal dose (0.1 mg/kg) of methanol and hexane extracts of rosemary and cyclophosphamide.

Groups	Mean ± S.D.* (cells/cu. mm. blood)		Treatment Efficiency (%)		P ≤0.05
	Pre	Post	Pre	Post	
Control I (H_2O-Cyclophosphamide)	112 ± 14^A	107 ± 9^A			Not Significant
Control II (Olive Oil-Cyclophosphamide)	112 ± 18^A	101 ± 10^A			Not Significant
Methanol Extract-Cyclophosphamide	109 ± 17^A	111 ± 20^A	-2.60	+3.70	Not Significant
Hexane Extract-Cyclophosphamide	104 ± 2^A	112 ± 9^A	-7.10	+10.8	Not Significant

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

Table 3-19: Total basophil count in albino male mice after interactions (pre- and post-treatments) between the ideal dose (0.1 mg/kg) of methanol and hexane extracts of rosemary and cyclophosphamide.

Groups	Mean ± S.D.* (cells/cu. mm. blood)		Treatment Efficiency (%)		P ≤0.05
	Pre	Post	Pre	Post	
Control I (H_2O-Cyclophosphamide)	46 ± 4^A	52 ± 11^A			Not Significant
Control II (Olive Oil-Cyclophosphamide)	42 ± 9^A	63 ± 12^A			Not Significant
Methanol Extract-Cyclophosphamide	47 ± 8^A	44 ± 12^A	+2.10	-15.3	Not Significant
Hexane Extract-Cyclophosphamide	38 ± 8^A	47 ± 3^A	-9.50	-25.3	Not Significant

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

3-4-2: Phagocytic Index

Both rosemary extracts had no significant effects on the values of phagocytic index (pre- and post-treatments), wherever the incubation period was 30 (Table 3-20) or 60 minutes (Table 3-21).

Table 3-20: Phagocytic index (30 minute incubation) in albino male mice after interactions (pre- and post-treatments) between the ideal dose (0.1 mg/kg) of methanol and hexane extracts of rosemary and cyclophosphamide.

Groups	Mean ± S.D.* (%)		Treatment Efficiency (%)		P ≤0.05
	Pre	Post	Pre	Post	
Control I (H_2O-Cyclophosphamide)	70.6 ± 5.1^A	69.3 ± 6.2^A			Not Significant
Control II (Olive Oil-Cyclophosphamide)	84.1 ± 11.2^A	84.2 ± 2.6^B			Not Significant
Methanol Extract-Cyclophosphamide	72.4 ± 5.2^A	70.5 ± 5.5^B	+2.4	+1.8	Not Significant
Hexane Extract-Cyclophosphamide	86.1 ± 5.3^A	88.5 ± 2.7^B	+2.3	+5.1	Not Significant

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

Table 3-21: Phagocytic index (60 minute incubation) in albino male mice after interactions (pre- and post-treatments) between the ideal dose (0.1 mg/kg) of methanol and hexane extracts of rosemary and cyclophosphamide.

Groups	Mean ± S.D.* (cells/cu. mm. blood)		Treatment Efficiency (%)		P ≤0.05
	Pre	Post	Pre	Post	
Control I (H_2O-Cyclophosphamide)	76.6 ± 2.5^A	73.6± 4.9^B			Not Significant
Control II (Olive Oil-Cyclophosphamide)	86.7 ± 6.3^A	87.3 ±2.4^A			Not Significant
Methanol Extract-Cyclophosphamide	78.8 ± 2.9^A	76.9 ± 3.1^B	+2.9	+4.3	Not Significant
Hexane Extract-Cyclophosphamide	88.7 ± 4.6^A	89.2 ± 6.2^A	+2.3	+2.1	Not Significant

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

3-4-3: Micronucleus Formation

As a general theme, both extracts of rosemary were able to reduce the cyclophosphamide-induced formation of micronuclei in the bone marrow of treated animals, especially the methanol extract in the pre-treatment interaction and hexane extract in the post-treatment-interaction, in which the treatment efficiency was -20.0 and -33.3%, respectively (Table 3-22). However, neither of the two differences was able to reach a significant level.

Table 3-22: Micronucleus formation in bone marrow cells of albino male mice after interactions (pre- and post-treatments) between the ideal dose (0.1 mg/kg) of methanol and hexane extracts of rosemary and cyclophosphamide.

Groups	Mean ± S.D.* (Micronucleus/1000 cell)		Treatment Efficiency (%)		P ≤0.05
	Pre	Post	Pre	Post	
Control I (H_2O-Cyclophosphamide)	5.1 ± 1.5^A	6.2 ± 2.1^A			Not Significant
Control II (Olive Oil-Cyclophosphamide)	7.3 ± 1.1^A	6.2 ± 1.1^A			Not Significant
Methanol Extract-Cyclophosphamide	4.5 ± 1.2^A	5.2 ± 2.1^A	-20.0	-16.6	Not Significant
Hexane Extract-Cyclophosphamide	6.4 ± 3.2^A	4.4 ± 2.1^A	-14.2	-33.3	Not Significant

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

Chapter Four

Discussion

Chapter Four

Discussion

As outlined in the chapter of results, the actions of methanol and hexane extracts of rosemary in relation to the function of the immune system in the treated mice were controversial, and the parameter investigated may determine the effect, as well as, the dose. Similar augmentations can be raised when the antimutagenic properties of the extracts are considered. However, a universal outcome was concluded that was the immune suppressive and mutagenic effects of the anti-cancer drug cyclophosphamide. In general all parameters that evaluated the immune system were devastated as a consequence of treatment with the drug. Furthermore, the micronucleus formation, which is an important parameter for the evaluation of mutagenesis (Hendry and West, 1997; Petzold *et al.*, 2003), confirmed the mutagenic effect; an observation that highlights the mutagenic, genotoxic and fertility effects of cyclophosphamide. The latter regard was demonstrated in patients under treatment with cyclophosphamide, in which oligospermia and azoospermia were pictured (Dollery, 1999), and also confirmed by the present study, which showed that animals treated with cyclophosphamide manifested an increased frequency of sperm-head abnormalities, together with a reduction in sperm count and motility.

Cyclophosphamide is also known to cause several adverse effects; including reproductive toxicity in humans and experimental animals (Anderson *et al*., 1995). Adult male patients treated with cyclophosphamide for more than four months have demonstrated diminished sperm counts and absence of spermatogenic cycles in their testicular tissue (Howell and Shalet, 1998). Previous studies have also shown that male rats exposed to this drug have oligospermia and azoospermia, which were associated with biochemical and histological alteration in the testis and epididymis (Meistrich *et al*., 1995 and Kaur *et al*., 1997).It has also been reported that postmeiotic germ cells are also

specifically sensitive to cyclophosphamide exposure (Qiu et al., 1995). Furthermore, numerous studies showed that cyclophosphamide exposure enhances intracellular reactive oxygen species (ROS) production, suggesting that biochemical and physiological disturbances may result from oxidative stress (Das *et al*., 2002; Ghost *et al*., 2002; Manda and Bhatia, 2003).When produced in excessive amounts, the ROS stimulate DNA fragmentation and a loss of sperm function associated with peroxidative damage to the mitochondria and plasma membrane. Further, spermatozoa are more susceptible to peroxidative damage because of high concentration of polyunsaturated fatty acids and low antioxidant capacity (Vernet et al., 2004). Recently, it has been reported that some of the undesirable effects of cyclophosphamide treatment in adult male rats could be ameliorated by lipoic acid treatment (Selvakumar *et al*., 2005a; Selvakumar *et al*.,2005b), therefore, the rosemary extracts might have a similar effects but with a different chemical constituents.

Chemical analysis of methanol extract revealed the presence of flavonoids, while hexane extract was rich in terpenes. Such findings are in a good agreement with other reported constituents of rosemary. The most important constituents of the plant are carnosol, carnosic acid, caffeic acid and its derivatives such as rosmarinic acid, phenolic diterpenes and flavones (Peng *et al*., 2005; Santoyo *et al*., 2005; Almela *et al.,* 2006). These compounds have a powerful antioxidant activity; especially rosmarinic acid, which is well absorbed from gastrointestinal tract and from the skin. It increases the production of prostaglandin E2 and reduces the production of leukotriene B4 in human leucocytes, and inhibits the complement system. This makes rosmarinic acid a strong anti-inflammatory agent (AL-Sereiti et al., 1999). Also many herbs contain a variety of triterpenes and flavonoids, which have been shown from studies of legumes, fruit and vegetables to be cancer chemoprotective (Steinmetz and Potter, 1991). A plant-based diet that is rich in legumes, fruit and vegetables, is effective in reducing the risk of cardiovascular diseases, which are associated with oxidative stress (Al-Attar, 2006).

Over 4000 flavonoids have been identified in plants (Hollman, 1997). These universal plant pigments are responsible for the colours of flowers, fruits and some times leaves (Bruneton, 1995). The commonly used herbs that provide substantial amounts of flavonoids include rosemary. Flavonoids have extensive biological properties that promote human health and help reduce the risk of disease. Flavonoids extend the activity of vitamin C, act as antioxidant, protect LDL cholesterol from oxidation, inhibit platelet aggregation and act as anti-inflammatory and antitumour agents (Smith and Yang, 1994; Cook and Samman, 1996). However, rosemary and its constituents have a therapeutic potential in treatment or prevention of inflammatory diseases, hepatotoxicity, ischaemic heart diseases, cancer and poor sperm motility (AL-Sereiti et al., 1999; Masuda *et al*., 2002; Sotelo-Fleix *et al*., 2002; Osakabe *et al*., 2005).

Studies by Zhong and Zhu (1993) demonstrated that the flavonoids could improve the phagocytosis of the giant phagocytes in mouse, increase the lysozyme content, increase the periphery T lymphocytes, and enhance the production of interferon in leucocytes, and accordingly, the immune functions can be enhanced; such as phagocytosis, which was significantly increased in animals treated with the plant extracts.

It is evident that the flavonoids display, to a variable extent, a remarkable array of biochemical and pharmacological actions, which suggest that certain members of this group of compounds significantly affect the function of the immune system and inflammatory cells (Middelton and Kandaswami, 1992). Accordingly, the enhancements of phagocytosis that were observed after a treatment with methanol and hexane extracts can be justified. Several flavonoids specifically affect the function of enzymes systems critically involved in the generation of inflammatory processes, especially tyrosine (Hunter, 1995) and serine-threonine protein kinases. Recently it has become evident that these enzymes are involved in signal transduction and cell activation processes that involve cells of the immune system, as well as, growth factors (Middelton *et al*., 2000). These effects suggest that flavonoids can act as chemopreventive agent in

cancer through a variety of mechanisms. It has been reported that flavonoids can inhibit the mitochondrial enzyme succinoxidase, which acts as a bio-transformation enzyme modulator (Eaton, 1996;). Furthermore, they can act as antioxidants and electrophile scavengers, therefore several biological functions can be achieved; a stimulation of the immune system, inhibition of DNA adducts with carcinogens, inhibition of hormonal actions and metabolic pathway associated with the development of cancer, and inducing phase I or II detoxification enzymes (Robbers *et al.,* 1994).

Reduced frequency of micronucleus formation can be consequenced in the light of these functions, but 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). They facilitate the relaxation of supercoiled DNA, essentially through a mechanism involving the breakage of a phosphodiester bond of either one strand (topoisomerase I) or both strands (topoisomerase II) of the DNA. The inhibition of topoisomerases may involve 'conventional' inhibition where the activity of the enzyme is slowed or arrested by, for example, binding of the inhibitor to the active site or alteration of the binding behavior of the enzyme with its substrate. This type of inhibition is generally referred to as inhibition of catalytic activity (Webb and Eberler, 2004). The ability of some flavonoids to act as topoisomerase poisons has been used to explain the results of mutagenicity in higher doses testing, in which it was shown that many flavonoids were positive for clastogenicity in mammalian cell systems (Ferguson, 2001). It has also been suggested that the high maternal consumption of flavonoids may play a role in the development of some childhood leukaemias (Strick *et al*., 2000). In contrast, numerous epidemiological studies suggest that flavonoids consumption is linked to a decrease incidence of certain cancers and, therefore, flavonoids in this regard may act as chemopreventive agents (Peterson *et al.,* 2003).

The chemical analysis of hexane extract revealed that this extract was in terpenes, and as in methanol extract, the dose 0.4 mg/kg was effective in modulating the immune response, and such dose of hexane extract was able to enhance the immune response. The ideal dose of hexane extract was able to reduce the effect of cyclophosphamide, and a significant reduction of micronucleus formation in mice pre- and post-treated with the extract was obtained. Isolation of phenolic diterpene constituents from the freeze-dried leaves of *Rosmarinus officinalis* has been recently achieved by supercritical extraction with carbon dioxide, and the antioxidant activity of each obtained extract was evaluated, including phenolic diterpenes, carnosic acid and carnosol (Chang *et al*., 2008). Their results revealed the potent antioxidant properties of rosemary extracts, which were attributed to its major diterpene; carnosic acid.

Carnosic acid has also been reported to have antioxidant, antibacterial, anticancer and photoprotective activities. In this regard, carnosic acid significantly inhibited collagen-, arachidonic acid and thrombin-induced rabbit platelet aggregation in a concentration-dependent manner, while it failed to inhibit ADP-induced platelet aggregation. In agreement with its antiplatelet activity, carnosic acid blocked collagen-, arachidonic acid-, U46619- and thrombin-mediated cytosolic calcium mobilization. Accordingly, serotonin secretion and arachidonic acid liberation were also inhibited in a similar concentration-dependent manner. However, in contrast to the inhibition of arachidonic acid-induced platelet aggregation, carnosic acid had no effect on the formation of arachidonic acid-mediated thromboxane A2 and prostaglandin D2, thus indicating that carnosic acid has no effect on the cyclooxygenase and thromboxane A2 synthase activity (Lee et al., 2007). Overall, these results suggest that the antiplatelet activity of carnosic acid is mediated by the inhibition of cytosolic calcium mobilization and that carnosic acid has the potential of being developed as a novel antiplatelet agent.

Carnosol, a further constituent of the herb rosemary, has shown beneficial medicinal and antitumor effects (Moran *et al*., 2005). They found that dietary

administration of 0.1% carnosol to the mouse decreased the intestinal tumor multiplicity by 46%, and their study showed that tumor formation in the mouse was associated with alterations in the adherence junctions, including an increased expression of tyrosine-phosphorylated beta-catenin, dissociation of beta-catenin from E-cadherin, and strongly reduced amounts of E-cadherin located at laternal plasma membranes of histological normal enterocytes. These results revealed that the treatment of intestinal tissue with carnosol restored both E-cadherin and beta-catenin to these enterocyte membranes. In this context, carnosol has been suggested to have potent anti-inflammatory activities, through its effect in increasing the tyrosine hydroxylase; Nurr1, and extracellular signal-regulated kinase 1/2 (Kim et al., 2006). Wijeratne and Cuppett (2007) have also suggested that the antioxidant activities of carnosic acid and carnosol could be partly due to their ability to increase or maintain glutathione peroxidase and superoxide dismutase activities.

In order to a further explore of the chemopreventive properties of the crude extracts of rosemary, its anti-proliferative property on several human cancer cell lines and its antioxidant and anti-inflammatory properties *in vitro* in a mouse macrophage/monocyte cell line have been studied. Jindal *et al*. (2006) studied the radioprotective effect of rosemary alcoholic extract in mice exposed to gamma radiation. Treatment of mice with the extract delayed the onset of mortality and reduced the symptoms of radiation sickness when compared with the non-treated irradiated controls. Body weight loss was significantly less in comparison with animals that were given a radiation treatment alone. Furthermore, the irradiation of animals resulted in an elevation in lipid peroxidation, while such effects were reduced after the treatment with rosemary extract. A further study showed that the crude alcoholic rosemary extract has differential anti-proliferative effects on human leukemia and breast carcinoma cells (Cheung and Tai, 2007).

Taking the forthcoming results, together with the findings of the present study, it is possible to suggest that the alcoholic and hexane extracts of rosemary

can be considered as materials contain in agents that have potent anti-mutagenic and immune modulator properties, as well as, fertility enhancements. However, the interaction treatments (pre and post) revealed a non-significant difference in the values of most of the investigated parameters. This is may be reasoned by the fact of dose. The dose used in the interactions may have been not effective, and the higher doses may bridge the gap. Therefore, a further study is certainly required to shed light on these doses in interaction systems; *in vivo* and *in vitro*.

Conclusions

and

Recommendations

Conclusions

Based on the findings of the present study, it possible to conclude that the investigated doses (0.1, 0.2 and 0.4 mg/kg) of methanol and hexane extracts of rosemary (*Rosmarinus officinalis*) showed no mutagenic, immunosuppressive or anti-fertility effects in albino male mice. In contrast, they reduced the spontaneous formation of micronuclei and sperm-head abnormalities, enhanced the values of immunological parameters (total and absolute counts of leucocytes and phagocytosis), and improved the sperm count and motility. These effects were dose-dependent, and the dose 0.4 mg/kg was effective in these regards. However, in the interaction treatments (pre and post) with the mutagenic and immune suppressive drug (cyclophosphamide), the employed dose (0.1 mg/kg) of both extract failed to reduce the drug effects.

Recommendations

The forthcoming conclusions suggest the following recommendations:

1. To understand the anti-mutagenic, immune modulator and fertility effects of rosemary extracts (methanol and hexane), further interactions (pre and post) with the drug cyclophosphamide are required, but this time with a higher doses, especially the dose 0.4 mg/kg.
2. These evaluations can be much fruitful if they are based on purified constituents of the plant extracts; for instance, flavonoids and terpenes.
3. Assessing the anti-mutagenic effects with other cytogenetic parameters; for instance, sister chromatid exchanges.
4. The immune modulating effects require further investigations, but with a more advanced methods, which are based in evaluating the immunological cells in terms of CD markers and cytokine profiles.

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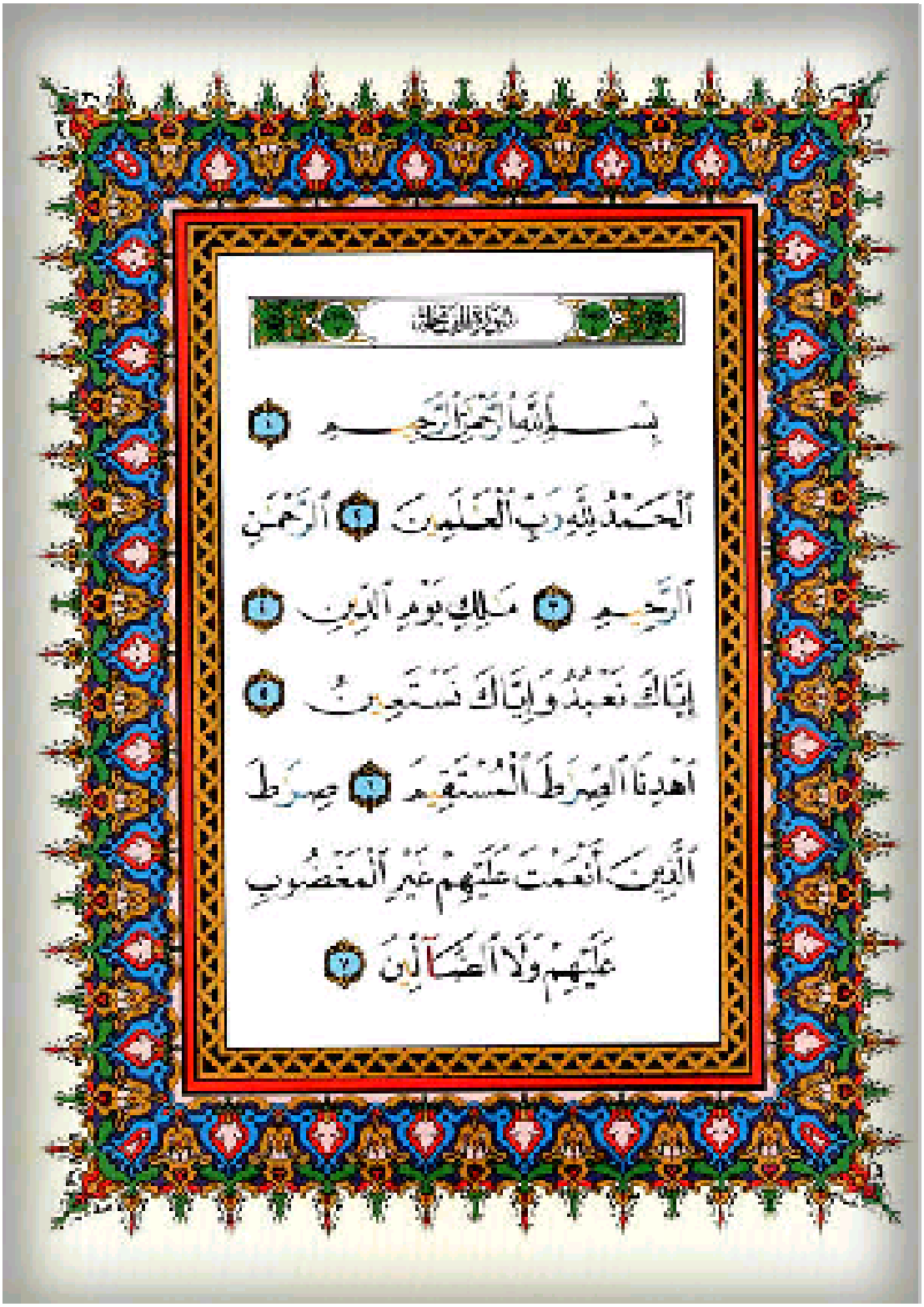
خلاصة

صممت الدراسة الحالية لتقييم التأثيرات المناعية والوراثية-الخلوية والخصوبة لمستخلصين (الكحول المثيلي والهكسان) من أوراق وسيقان نبات اكليل الجبل (*Rosmarinus officinalis*). شمل الجانب المناعي المعايير الأتية: العد الكلي والتفريقي لخلايا الدم البيض والبلعمه لخلايا الخلب، بينما شمل الجانب الوراثي-الخلوي معامل تكون النوى الصغرى في خلايا نقي العظم وتشوهات رؤوس النطف. أما الخصوبة فقد قيمت من خلال عدد النطف وحركة النطف. فضلآ عن ذلك فقد اجري التحليل الكيميائي لكلا المستخلصين للتحري عن الفلافونات والتربينات والستيرويدات.

تضمنت الدراسة ثلاثة مراحل؛ شملت المرحلة الأولى دراسة التأثير الوراثي-الخلوي والمناعي لثلاث جرع (0.1، 0.2، 0.4 ملغم/كغم) من مستخلصي اكليل الجبل و جرعة واحدة (16ملغم-كغم) للسايكلوفوسفامايد(عقار مطفر وراثيآ وكابت مناعيآ)، بينما قيمت في المرحلة الثانية تاثيرات الاخصاب لهذه الجرع. أجري في المرحلة الثالثة تداخل (قبل و بعد المعاملة) ما بين الجرعة المثالية (0.1 ملغم/كفم) لكلا المستخلصين وعقار سايكلوفوسفاما يد (16 ملغم/كغم). وفي جميع الحالات حقنت المواد المختبرة تحت الجلد.

أظهرت نتائج التحليل الكيميائي للمستخلص الكحولي بأنه يحتوي على الفلافونات، في حين أظهر مستخلص الهكسان احتواءه على التربينات وأفتقاره للستيرويدات. كما لم تظهر الجرع المدروسة للمستخلصين أي تاثيرات مطفرة أو كابتة للمناعة أو مضادة للخصوبة في ذكور الفأر الأبيض. وعلى العكس من ذلك، فقد أختزلت التكرار التلقائي للنوى الصغرى وعززت قيم العوامل المناعية (العدد الكلي والتفريقي لخلايا الدم البيض وعملية البلعمة لخلايا الخلب) وحسنت عدد وحركة النطف. كانت هذه التاثيرات معتمدةعلى الجرعة، وكانت الجرعة0.4 ملغم/كلغم هي الأفضل في هذا الصدد. ألا أن الجرعة 0.1 ملغم/كغم لم تكن مؤثرة في تعديل التأثيرات المطفرة وراثيآ والكابتة مناعيآ لعقار سايكلوفوسفامايد عند أجراء التداخل (قبل و بعد) بينها والعقار.


سورة الفاتحة

بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ ﴿١﴾

الْحَمْدُ لِلَّهِ رَبِّ الْعَالَمِينَ ﴿٢﴾ الرَّحْمَٰنِ

الرَّحِيمِ ﴿٣﴾ مَالِكِ يَوْمِ الدِّينِ ﴿٤﴾

إِيَّاكَ نَعْبُدُ وَإِيَّاكَ نَسْتَعِينُ ﴿٥﴾

اهْدِنَا الصِّرَاطَ الْمُسْتَقِيمَ ﴿٦﴾ صِرَاطَ

الَّذِينَ أَنْعَمْتَ عَلَيْهِمْ غَيْرِ الْمَغْضُوبِ

عَلَيْهِمْ وَلَا الضَّالِّينَ ﴿٧﴾

جمهورية العراق
وزارة التعليم العالي و البحث العلمي
جامعة النهرين
كلية العلوم
قسم التقانة الاحيائية

التأثيرات المضادة للتطفر والمعدلة مناعياً والخصبية لمستخلصات نبات اكليل الجبل (Rosmarinus officinalis) في ذكور الفأر الأبيض

رسالة

مقدمة إلى كلية العلوم / جامعة النهرين

وهي جزء من متطلبات نيل درجة ماجستير علوم في التقانة الإحيائية

من قبل

نور عبد الزهرة علي السوداني

بكلوريوس تقانة إحيائية جامعة النهرين (2005)

رجب 1429 هـ

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