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Studying some Immunological and Cytogenetic Effects of *Plantago lanceolata* Aqueous Extract in Albino Male Mice

A Thesis
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By

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

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Dedication

To my late professor **Dr. Mohammed Rafeeq**

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

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

To my brother and sisters

Khalid, Wassan, Hadeel, Shahbaa', Farah and **Wafaa'**

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

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

Anas

To my best friends, who stood for me in every step of my way

Rawaa'

To my friends for their support

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Summary

The present study was designed to evaluate some immunological and cytogenetic effects of *Plantago lanceolata* aqueous extract and cyclophosphamide in albino male mice (*in vivo*). The immunological parameters were total and absolute counts of leucocytes, phagocytic index of peritoneal cells, total serum IgG level, hypersensitivity reactions (Arthus and delayed type), while cytogenetic evaluations included micronucleus formation in polychromatic erythrocytes of bone marrow ,metaphase index of bone marrow and spleen cells and sperm-head abnormalities. Additionally, a chemical detection of tannins, polysaccharides, glycosides, flavonoids, saponins, alkaloids and terpens in the aqueous extract was carried out.The evaluations included four main parts. In part one, the immunological and cytogenetic effects of the plant aqueous extract and cyclophosphamide were carried out. Three doses (1.5, 3 and 4.5 mg/kg) of the extract and one dose (15 mg/kg) of cyclophosphamide were investigated, in which the meterials were given for seven days (single dose/day) and the evaluation was carried out on day eight. In part two, interactions (pre- and post-treatments) between the ideal dose (1.5 mg/kg) of the extract and cyclophosphamide were evaluated. In part three, the animals were given a single dose of the investigated material in day one, and then they were sacrificed in days 7,

21 and 35 to assess the sperm head abnormalities. In all cases, the extract and cyclophosphamide were administrated orally. In part four, two types of immunological reactions (Arthus and delayed type hypersensitivity reactions) were assessed in mice that were immunized intraperitoneally with 0.05 ml of 10% sheep red blood cell suspension. These reactions were assessed in the animals after an oral administration of the plant ideal dose (1.5 mg/kg), cyclophosphamide (15 mg/kg) or distilled water in an immunization-interaction regime. The following results were obtained:

1. The chemical detection revealed that aqueous extract was positive for tannins, polysaccharides, glycosides, flavonoids, saponins, alkaloids and terpens.
2. Cyclophosphamide declared clear immune suppressive and mutagenic effects as judged by the investigated parameters. Reduced indices of metaphase, phagocytosis, Arthus, as well as, delayed type hypersensitivity reactions, and increased frequencies of micronucleus formation and sperm-head abnormalities were observed. The total and absolute counts of leucocytes were also decreased in comparison with negative control animals.
3. The results of parts one, three and four indicated that *P. lanceolata* aqueous extract modulated the innate and adaptive immune responses positively as compared to distilled water negative and cyclophosphamide positive controls. Similarly, the spontaneous formation of micronuclei and sperm-head abnormalities were significantly decreased. In both cases the effect was a dose-dependent, and the lower dose (1.5 mg/kg) was better

than the higher doses (3 and 4.5 mg/kg), and therefore the former dose was considered as an ideal dose in the next experiments.

4. The results of part two confirmed the forthcoming observations, and the ideal dose (1.5 mg/kg) of aqueous extract showed a significant efficiency in protecting the immune system and the genetic make-up from the immune suppressive and mutagenic effects of cyclophosphamide. In this regard, the pre-treatment with the extract was more effective than post-treatment.

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List of abbreviation

WHO	World health organization
AIDS	Acquired Immune Deficiency Syndrome

AST	Aspartate transaminase
ALT	Alanine transaminase
P. lanceolata	*Plantago lanceolata*
DPPH	1,1-diphenyl-2-picrylhydrazyl
NO	Nitric oxide
NOS	Nitric oxide synthase
COX-1	Cyclooxygenase-1
iNOS	Inducible NOS
COX-2	Cyclooxygenase-2
mRNA	Masenger Ribonucleic acid
MTT assay.	Dimethyl thiazolyl diphenyl tetrazolium
PAPA-NONOate	Propylamine Propylamine NONOate
PGE	Prostaglandin E
DSS	Dextran sulphate sodium
MLN	Mesenteric lymph nodes
IL-12	Interleukin-12
IL-2	Interleukin-2
IL-1β	Interleukin-1 β
IL-6	Interleukin-6
TNF	Tumour necrosis factor
INF-γ	Interferon- γ
PBS	Phosphate-buffered saline

A. cepa	*Allium cepa*
ALDH	Aldehyde dehydrogenase
DNA	Deoxyribonucleic acid
C3a	Complement component C3a
C5a	Complement component C5b
IgG	Immunoglobulin G
DTHR	Delayed type hypersensetivity reaction
T_{DTH}	T-helper lymphocyte
IgM	Immunoglobulin M
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IgD	Immunoglobulin D
PCE	Polychromatic erythrocytes
LD	Lethal Dose
HEPES-BSS	HEPES-Buffered balanced salt solution
HEPES buffer	4-(2-hydroxyethyl)-1-piperazine ethanesulfonic buffer
SRBc	Sheep red blood cells
HRP	*Horse radish peroxidase*
TMB substrate	Tetramethylbenzidine substrate
ELISA	Enzyme Linked Immuno Sorbent Assay
ANOVA	Analysis of Variance
OD	Optical Density

TLC	Total Leucocyte Count
T helper-1	T_H1
T helper-2	T_H2
GST	Glutathione Transferase
SOD	Superoxide Dismutase

Chapter one
Introduction and Review of Literature

1.1 Introduction

Plants have played a significant role in maintaining human health and improving the quality of human life for thousands of years, and have served humans as valuable components of seasonings, beverages, cosmetics, dyes and medicines. The World Health Organization (WHO) estimated that around 80% of the earth's inhabitants rely on traditional medicine for their primary health care needs, and most of this therapy involves the use of plant extracts or their active components (Bruneton, 2001). Furthermore, many Western drugs had their origin in a plant extract. Reserpine, which is widely used for the treatment of high blood pressure, was originally extracted from the plant *Rauwolfia serpentina*, whereas digitalis, used as a heart stimulant, was derived from the foxglove plant (*Digitalis purpurea*). The Chinese herb ephedra (*Ephedra sinica*), which contains the active substance ephedrine, was used early for the treatment of asthma, whereas salicylic acid (a precursor of aspirin) was obtained from willow tree bark (*Salix alba*) to help relieve fevers (Gurib-fakim, 2005).

Several commonly used herbs have been identified as possessing anti-mutagenic and cancer-preventive properties (Rizzi *et al.*, 1993). These herbs include members of the *Allium* sp. (garlic, onions and chives); members of the Labiatae family (basil, mints, oregano, rosemary, sage and thyme); members of the Zingiberaceae family (turmeric and ginger); *Glycyrrhiza glabra* root; green tea; flax (*Linum usitatissimum*); members of the Umbelliferae family (anise, caraway, celery, chervil, cilantro, coriander, cumin, dill, fennel and parsley); and tarragon (*Artemisia dracunculus)* (Craig, 1999).

In addition, many herbs contain a variety of phytosterols, triterpenes, flavonoids, saponins, and carotenoids, which have been shown from studies

of legumes, fruits, and vegetables to be anti-mutagenic and cancer chemoprotective (Steinmetz and Potter, 1991). These beneficial substances act as antioxidants and electrophile scavengers, stimulate the immune system, inhibit nitrosation and the formation of DNA adducts with mutagens or carcinogens, inhibit hormonal actions and metabolic pathways associated with mutagenesis and carcinogenesis, and induce phase I or II detoxification enzymes (Smith and Yang, 1994; Zheng *et al*., 1994). Several phytochemicals inhibit mutation and/or tumor formation by stimulating the protective phase II enzyme; glutathione transferase, which is a detoxifying enzyme that catalyzes the reaction of glutathione with electrophiles to form compounds that are less toxic, more water-soluble, and can be excreted easily (Huang *et al.*, 1994). Examples of phytochemicals that stimulate glutathione transferase activity include phthalides, found in umbelliferous herbs; sulfides, found in garlic and onions, curcumin in turmeric and ginger; and terpenoids (limonene, geraniol, menthol, and carvone) found in commonly used herbs (Lam *et al.*, 1994).

Several herbal products that may enhance the function of the immune system are available. These include members of the family Echinacea, *Hibiscus subdariffa*, *Glycyrrhiza glabra*, *Uncaria tomentosa*, and *Allium sativum*, especially herbs that are rich in flavonoids, vitamin C, or the carotenoids. The flavonoid-rich herbs may also possess anti-inflammatory action (Tyler, 1994; Bruneton, 2001). They can promote the activity of lymphocytes, increase phagocytosis, induce interferon production and augment natural killer cell activity (Tyler, 1994). Today, these plants are attracting much attention in the West because of their immunostimulant properties and potentials to help fighting acquired immune deficiency syndrome (AIDS) and leukemia. European researchers have suggested that these plants enhance immune function because they contain various flavonoids, triterpenes, or alkaloids (Rizzi et al., 1993).

In Iraq, the list of medicinal plants that have been investigated for their anti-mutagenic, anti-carcinogenic or immune stimulant potentials is in a progress. Examples include *Hibiscus subdariffa*, *Glycyrrhiza glabra* and *Artemisia herba-alba* (Al-khayat, 1999; Al-Obaidi, 2002), *Allium sativum* (Ad'hiah *et al*., 2004), *Salvia officinalis* (Al-Ezzy, 2006), *Origanum vulgare* (Al-Berikdar, 2007), *Alhagi alhagi* (Ad'hiah *et al*., 2007) and *Rosmarinus officinalis* (Al-Sudany, 2008).

Plantago lanceolata (Plantaginaceae), which is known in English literature as plantain, is a further interest of medicinal plants. It is used in traditional medicine by different populations, and folk medicine recommends the leaf juice for treating blisters, ulcers, insect stings and bites, conjunctival congestion and to reduce the heat and pain of inflammation (Hausmann *et al*., 2007). Moreover, recent investigations have demonstrated several biological and pharmaceutical potentials; for instances, hepatoprotective (Aktay *et al*., 2000), immunostimulant (Ebringerová *et al*., 2003), anti-oxidant (Kardosová and Machová, 2006), anti-parasitic (Kozan *et al*., 2006), anti-viral (Abdin *et al*., 2003), anti-inflammatory (Hausmann *et al*., 2007) and anti-fungal (Yigit *et al*., 2008).

1.2 Aims of Study

The present project was designed to evaluate some immunological and cytogenetic potentials of the plant *P. lanceolata* (aqueous extract) in albino male mice. Interactions between the plant extract and the mutagenic and immune suppressive drug cyclophosphamide were also made to evaluate its action in modulating the drug effects. The parameters of evaluation were:

- **Immunological parameters**
  1. Total and absolute counts of Leucocytes.
  2. Phagocytosis.

3. Arthus and delayed type hypersensitivity reactions
4. Total serum level of IgG.

- **Cytogenetic parameters**

1. Mitotic Index.
2. Micronucleus formation and sperm-head abnormalities.

1.3 Review of Literatures

1.3.1 *Plantago lanceolata* L.

Plantago lanceolata is the most important species of the family Plantaginaceae and has long been used in traditional medicine for the treatment of different illnesses; for instance, wounds, bronchitis, haemorrhagia, cystitis with haematuria and diarrhea (Nakamarua *et al.*, 2005). Moreover, recent investigations have suggested that the plant has some effects on humoral immune response in addition to its potentials as anti-mutagen and anti-carcinogen (Rezaeipoor *et al*., 2000).

1.3.1.1 Common Names and Taxonomy

Plantago is a genus of about 250 species of small inconspicuous plants that are commonly called plantains. Many common names are used to describe *Plantago lanceolata*, which are buckhorn, chimney-sweeps, headsman, narrow-leaved plantain, ribgrass, ribwort, ripplegrass and soldier's herb, although English plantain is the most common used name. Taxonomically, the plant is classified as the following as described by Hjelle *et al*. (2006):

Kingdom: Plantae
Sub-kingdom: Tracheobionta
Superdivision: Spermatophyta
Division: Magnoliophyta
Class: Magnoliopsida

Sub-class: Asterdia

Order: Lamiales

Family: Plantaginaceae

Genus: *Plantago*

Species: *lanceolata* L.

1.3.1.2 Plant Distribution

Plantago is a perennial weed with almost worldwide distribution. There are about 250 species, of which 20 have wide geographical ranges, 9 have discontinuous ranges, 200 are limited to one region, and 9 have very narrow ranges. *P. lanceolata* is among the most widely distributed (Hammond, 1982). The plant is native to Eurasia; but it has become naturalized throughout North America, South America, Australia, New Zealand, Hawaii, Southeast Asia, and eastern Africa (Duke, 1989; Chen *et al.*, 1996). In Iraq, the plant is wildly cultivated in moderately fertile soil in a sunny position and near riversides (Al-Rawi, 1988).

1.3.1.3 Plant Description

Plantago lanceolata is a herbaceous perennial plant that grows from 5 centimetres up to 50 centimetres with a very fibrous root. The leaves are in basal rosettes and are lanceolate or linear lanceolate, deeply 3 to 5 ribbed, entire-margined or shortdentate. They are sessile, but have a narrow part near the stem and have three or five parallel veins that are branching in the wider part of the leaf (Fosberg *et al.*, 1989). Flowers are small narrow-acuminate bracts. The calyx is deeply divided into four parts and has a cylindrical tube and a margin with four ovate tips. There are four long stamens with yellowish-white filaments and anthers and one superior ovary. The fruit is a bivalvular with 3-4 millimetres long capsule. The seeds, are small (1.5-3.5 millimetres), oval, boat-shaped, dark reddish brown, odorless and nearly

tasteless. They are coated with mucilage, which aids in their transportation by allowing adhesion to various surfaces (Brooker *et al.,* 1987). A field picture of the plant is given in figure 1-1.

Figure 2-1: Picture of *Plantago lanceolata* (Glenbrittle, 2002).

1.3.1.4 Chemical Constituents and Active Compounds

An analysis of 8 of 21 Egyptian species of *plantago*, including *P. lanceolata,* identified a variety of sugar and polysaccharide components of the seed mucilage. These include galactose, glucose, xylose, arabinose, and

rhamnose, in addition to galacturonic acid, planteose, plantiobiose, sucrose and fructose (Ahmed *et al.,* 1965). Other plant carbohydrates such as saccharose, stachyose, sorbitol and tyrosol have also been reported. The seeds also contain fixed oil, protein, iridoids and tannins. Further different active compounds have been detected as constituents of *P. lanceolata* including acids (benzoic, caffeic, chlorogenic, cinnamic, p-coumaric, fumaric, salicylic, ursolic, vanillic and ascorbic acids), alkaloids (boschniakine) and amino acids (alanine, asparagine, histidine and lysine) (Bisset, 1994; Newall *et al.*, 1996). Other major and active constituents have been given by Anne (2000), and they are as the following:

- Flavonoids: They include apigenine 7-glucoside, baicaleine, hispiduline, luteoline 7-glucoside, luteoline, plantaginine and scutellareine.
- Iridoids: They include aucubin, plantarenaloside and aucuboside. Iridoid glycosides and phenolic acids have been found in leaf extracts; for instance, asperuloside, aucubine, catapol, majorside, gardoside and geniposidic.
- The plant contains terpenes such as loliolide, oleanolic acid, ursolic acid, 18-beta-glycyrrhetinic concretes, 18-beta-glycyrrhetinic acid and sitosterol (triterpenen).
- Other components of the plant include choline, fats, resins, steroids and vitamins such as carotene, vitamin C and vitamin K.

1.3.1.5 Folkloric Medicinal Uses

The medicinal parts of *P. lanceolata* are dried leaves, dried herbs and fresh plant, which have been suggested to be safe and effective treatment for different illnesses. The leaves contain mucilage and tannin, which have antibacterial properties (Coffey, 1993). They have a bitter flavor and are astringent, demulcent, mildly expectorant, haemostatic and ophthalmic. Internally, leaves are also used in the treatment of a wide range of complaints including diarrhea, gastritis, peptic ulcers, irritable bowel syndrome,

hemorrhage, hemorrhoids, cystitis, bronchitis, catarrh, sinusitis, asthma and hay fever. The leaves also quickly stop blood flow and encourage the repair of damaged tissues, while the heated leaves are used as a wet dressing for wounds and swellings (Chevallier. 1996). The root is suggested as a remedy for the bite of rattle snakes. The seeds are used in the treatment of parasitic worms. These seeds contain up to 30% mucilage, which swells up in the gut, acting as a bulk laxative and soothing irritated membranes (Bown, 1995).

1.3.1.6 Biological Potentials and Pharmaceutical Applications

Recent investigations have demonstrated that extracts of *P. lanceolata*, or some isolated constituents are effective materials that have several biological potentials and pharmaceutical applications. A summary of some these investigations is outlined in the following:

- **Hepatoprotective effects**: Ethanolic extract of *P. lanceolata* was studied for possible hepatoprotective effects using the carbon tetrachloride-induced hepatotoxicity model in rats. The extract significantly prevented the elevation of plasma and hepatic malondialdehyde formation (evidence of lipid peroxidation), as well as, liver function enzyme levels of aspartate transaminase (AST) and alamine transaminase (ALT). Such findings were ascribed to the plant extract constituents that may have a potent hepatoprotective activity (Aktay *et al.*, 2000).

- **Immunostimulant potentials**: From the leaves of *P. lanceolata*, crude polysaccharides have been isolated by extraction with water and further purified and fractionated. The water-soluble polysaccharides obtained were examined for their immunomodulatory activities using the *in vitro* mitogenic and comitogenic rat thymocyte tests. The results indicated that the tested polysaccharides exhibited significant immunomodulatory

properties with a particularly high adjuvant activity (Ebringerová *et al.*, 2003). Furthermore, aqueous and methanol extracts of the plant that were added to the cultures of mouse bone marrow cells and spleen cells, increased the bone marrow cell proliferation and the spleen cell proliferation in a dose-dependent manner (Velasco-Lezama *et al.*, 2006).

- **Anti-oxidant activity**: The hydroalcoholic extract of *P. lanceolata* was evaluated for its antioxidant potentials through a model represented by the respiratory burst of human activated neutrophils (Herold *et al.*, 2003). The investigators demonstrated its potentials as antioxidant. Their antioxidant properties were measured using a minor antioxidant activity, but they suggested that the extract could be a useful tool for obtaining new antioxidant agents. This suggestion has been further inspected by Gálvez *et al*. (2005). They characterized the antioxidative activities of methanol extracts from five *Plantago* species (*P. afra*, *P. coronopus*, *P. lagopus*, *P. lanceolata*, and *P. serraria*) by 1,1-diphenyl-2-picrylhydrazyl (DPPH)scavenging test and the inhibition of Fe^{2+}/ascorbate-induced lipid peroxidation on bovine brain liposomes. All extracts showed antioxidant activity in both methods. Whereas *P. serraria* exhibited the strongest activity as a DPPH scavenger. *P. lanceolata* and *P. serraria* were found to be the most active in the lipid peroxidation inhibition assay. In a further more recent investigation, polysaccharides were isolated from the leaves of *P. lanceolata*, and investigated for their ability to inhibit peroxidation of soyabean lecithin liposomes by OH radicals. A good inhibition was found, and the antioxidant activity accounted for approximately 69% of the activity of the reference compound alpha-tocopherol (Kardosová and Machová, 2006).

- **Anti-inflammatory effects**: Extracts of *P. lanceolata* have been investigated to evaluate their effect on the production of nitric oxide (NO) and prostaglandin E(2), NO synthase (NOS) type II, cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) mRNA expression in the murine macrophage cell line J774A.1 (Vigo *et al.*, 2005). They found that the extracts inhibited NO production in a concentration-dependent manner in this cell line and without obvious cytotoxic effects as tested by dimethyl thiazolyl diphenyl tetrazolium (MTT) assay. The extracts at all doses used showed significant scavenging of NO radicals released by the NO donor propylamine propylamine NONOate (PAPA-NONOate). The data also showed that pre-treatment with these extracts significantly inhibits inducible NOS (iNOS) mRNA production in this cell line, without affecting COX-1 mRNA expression. COX-2 mRNA levels and prostaglandin E2 (PGE 2) levels induced by lipopolysaccharide/interferon-gamma were not modified upon pre-treatment with the extracts. In a further investigation, the herbal phenylethanoid acteoside isolated from *P. lanceolata* was shown to exhibit anti-oxidative potential by using the dextran sulphate sodium (DSS)-induced colitis model (Hausmann *et al.*, 2007). In this study, it was assessed whether systemic application of acteoside affects colitis. Colitis was induced by DSS in Balb/c mice. Treatment with acteoside (120 and 600 µg/mouse/day) was performed intraperitoneally. The colon lengths were determined and colonic tissue was scored histologically. T cells isolated from mesenteric lymph nodes (MLN) were stimulated with anti-CD3 antibody in the presence of interleukin (IL)-2. After incubation for 24 h, IL-1β, IL-6, IL-12 tumour necrosis factor (TNF)-α and interferon (IFN)-γ levels in supernatants were analysed. Histological scoring of colonic tissue revealed that application of acteoside was followed by a significantly improved histological score. In acute colitis the histological score was 3·2 with acteoside *versus* 5·2 with phosphate-buffered saline (PBS). In chronic

colitis, both 120 µg or 600 µg acteoside significantly ameliorated colitis. Stimulated MLN from mice with chronic DSS-induced colitis treated with acteoside showed a significant down-regulation of IFN-γ secretion. Inhibition of oxidative burst activity with acteoside reduced mucosal tissue damage in DSS colitis, and according to the point view of the investigators, this material could be a therapeutic alternative for inflammatory bowel disease treatment.

- **Anti-mutagenic potentials**: For the best knowledge of the investigator, this subject has not been investigated in animal models, but in a plant model, Çelik and Aslantürk (2006) investigated the anti-mitotic and anti-genotoxic effects of *P. lanceolata* aqueous extract on *Allium cepa* root tip meristem cells. For this purpose, two different experiments were performed under the same conditions. In the first experiment, *A. cepa* onion bulbs were treated with 0.7% H_2O_2 for 1 hour. After the H_2O_2 treatment, the onion bulbs were treated with two different concentrations (15 g/L and 30 g/L) of the plant extract for 24 hours. In the second experiment, *A. cepa* onion bulbs were treated with two different extract concentrations (15 g/L and 30 g/L) for 24 hours and then with 0.7% H_2O_2 for 1 hour. As a result, it was determined that the aqueous extract reduced mitotic index and chromosome aberrations in the treatment groups in comparison with controls. These results showed that *P. lanceolata* aqueous extract have anti-mitotic and anti-genotoxic effects.

1.3.2 The Drug Cyclophosphamide

Cyclophosphamide is a drug with a chemotherapeutic activity. It is used to treat various types of cancer; for instance, malignant lymphoma, leukemia, breast and ovarian cancers, and neuroblastoma (Brock, 1996). The drug is also used as immunosuppressive agent following organ transplants or to treat

autoimmune disorder such as rheumatoid arthritis, Wagener's granulomatosis and nephrotic syndrome (Chabner *et al.*, 2001).

It is an alkylating agent and is a derivative of nitrogen mustard. The main effect of cyclophosphamide is due to its metabolite phosphoramide mustard. This metabolite is only formed in cells which have low levels of ALDH (aldehyde dehydrogenase). The phosphoramide mustard forms DNA crosslink between (interstrand cross-linkages) and within (intrastrand cross-linkages) DNA strands at guanine N-7 positions. This action can lead to a cell death (Hazardous Substances Data Bank, 2003). According to this action, cyclophosphamide was used in the present study as mutagenic and immunosuppressive drug.

1.3.3 Evaluations of Immunological Status and Mutagenesis

In the present study, different parameters were employed to evaluate the immunological status and mutagenesis in the investigated animals. The former one included total and absolute counts of leucocytes, phagocytosis, hypersensitivity reactions (Arthus reaction and delayed type hypersensitivity reaction) and total serum level of IgG, while the latter evaluation included mitotic activity of bone marrow cells, micronucleus formation and sperm-head abnormalities.

1.3.3.1 Total and Absolute Counts of Leucocytes

Leucocytes were considered as the active cells in carrying out the functions of the immune system, both non-specifically and specifically, and their count may give a general picture about the function of the immune system (Lydyard and Grossi, 1998).These cells were 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

was commenced to carry out an immune function. The neutrophils were mainly involved in the innate immune system to carry out phagocytosis, while lymphocytes represent the humoral and cellular arms of specific immunity. Monocytes were involved in carrying out phagocytosis, but they were also professional antigen presenting cells. Eosinophils were 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).

1.3.3.2 Phagocytosis

Phagocytosis represents the non-specific cellular immune response, which is carried out mainly by neutrophils and monocytes. These cells function to engulf and attack particles in the host that have been signaled for removal by various mechanisms (Asmis and Jelk, 2000). Both cells can migrate from the blood stream to the site of infections in response to some chemo-attractants (i.e. C3a and C5a), but the monocytes (also known as macrophages in tissues) can migrate back to the secondary lymphoid organs where they act as professional antigen presenting cells. Phagocytes are then motile and roam throughout the bloodstream, the lymphatic system and non-vascular tissue in search for particles to engulf. When a non-self particle is encountered, it is taken into the phagocyte, combined with destructive compounds and destroyed (Hughes, 2001). This process, termed phagocytosis**,** involves several discrete steps**:**

i. Detection of the foreign particle and movement of phagocyte to the area as a response to chemo-attractants.

ii. Attachment of the foreign particle to the phagocyte. Such mechanism is enhanced by IgG and C3b (opsonins), because, the phagocyte expresses surface receptors for both of them.

iii. Engulfment or ingestion of the foreign particle.

iv. Fusion with lysosomes and formation of the phagolysosome.

v. Intracellular killing and digestion, which are carried out through two chemical pathways; oxygen-dependent and oxygen-independent killing.

vi. In the case of macrophages, epitopes of the engulfed object are presented to the lymphocytes in the secondary lymphoid organs.

Several investigations have suggested that phagocytosis can be modulated positively or negatively as a consequence of infections or treatments with drugs, chemicals, natural products and vitamins (Ad'hiah *et al.*, 2002; Al-Keenani, 2005; Abdullah, 2005).

1.3.3.3 Arthus Reaction

Arthus reaction is a type III hypersensitivity reaction, which is mediated by immune complex formation in a second challenge with the same antigen. The immune complexes are formed between antibodies and the challenged antigen in the area of injection. Such formation leads to the activation of the classical pathway of complement system, which in turn leads to the generation of chemo-attractants factors (complement fractions; C3a and C5a) that enhance the migration of neutrophils to the area of injection. As a consequence, a local inflammatory response is generated with the manifestation of a local erythema and oedema after 3-4 hours of the injection. Therefore, this assay can be successively employed to assess the humoral immune response, as well as, the role of some agents in modulating such response *in vivo* (Szalai *et al.*, 2000; Cruse and Lewis, 2001).

1.3.3.4 Delayed type hypersensitivity reaction (DTHR)

Delayed type hypersensitivity reaction represents the fourth type of hypersensitivity reaction (Type IV), which differs from Arthus reaction in the immunological constituents that participate in its generation. It is a cell-mediated reaction, in which a specific T-helper lymphocyte, called T_{DTH}, plays a major role in its initiation together with macrophages (Moore *et al.*, 1999).

It occurs locally after 24-48 hours of a second challenge with the same antigen. Such time is required to activate the T_{DTH} by the antigen that is presented by macrophages, a process that requires the production of cytokines (IL-2, IFN-γ and tumour necrosis factor-β). These cytokines stimulate the migration of more macrophages to the area of injection, which in turn produce extracellular lysozymes that are responsible for the inflammatory reaction in the area of injection. Therefore, the DTHR is employed to assess the cellular immune response *in vivo* (Jacayan *et al.*, 2001).

1.3.3.5 Total Serum Level of IgG

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

The immunoglobulins can be divided into five different classes based on differences in the amino acid sequences in the constant region of the heavy chains. They are IgG, IgM, IgA, IgE and IgD (Roitt *et al.*, 2001).

Immunoglobulin G is the most versatile immunoglobulin due to the followings:

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

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

iii. It is effective in fixation of the complement.

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

1.3.3.6 Mitotic Index

Many studies that determine the activity of immune system and the effect of different agents on it depend on the ability of lymphocytes to proliferate in lymphoid organs and/or depend on dividing cells of bone marrow because it is the source of all blood cells (Hughes, 2001). Mitotic index is defined as the ratio of the number of cells in a population undergoing mitosis (different stages) to total number of cells (Gosh *et al.*, 1991). Mitotic abnormalities often arise directly from defects of centromer and/or mitotic spindles, which then induce prolonged mitotic arrest or delayed mitotic exit and trigger induction of apoptosis (Mollinedo and Gajate, 2003).Therefore, by the employment of this assay the effect of different physical and chemical agents on the mitotic response can be detected. Studies have revealed that the mitotic index can be affected negatively or positively by chemicals, radiations, drugs and medicinal plants (Ad'hiah *et al.*, 2001; Ad'hiah *et al.*, 2002; Ad'hiah *et al.*, 2004; Ad'hiah *et al.*, 2007).. In the present study, only cells at metaphase were scored, therefore the metaphase index was given.

1.3.3.7 Micronucleus Formation

Micronuclei (MN) are cytoplasmic chromation masses with the appearance of small nuclei that arise from chromosome lagging at anaphase or from acentric chromosomal fragments (Tian *et al*, 2003).

The micronucleus test is a mammalian *in vivo* assay, which detects damage of the chromosomes or mitotic apparatus by chemicals. The assay is based on an increase in the frequency of micronucleated polychromatic erythrocytes in bone marrow of treated animals (Cole, 1981). It was found that all chromatid and chromosome breaks will give rise to acentric fragments at mitosis, and these fragments are excluded from the daughter nuclei and appear in following interphase as micronuclei.

Schroder (1970) recommended the use of bone marrow smears to detect *in vivo* genetic damage induced by chemical mutagens and demonstrated the occurrence of micronuclei in bone marrow cells in connection with cytogenetic damages. Furthermore, Von Ledebur and Schmid in 1973 reached the conclusion that the incidence of micronucleated polychromatic erythrocytes (PCE) is a particularly useful index of an *in vivo* bone marrow cytogenetic damage, and such finding formed the basis to develop a simple *in vivo* assay based on an identification of micronuclei in PCE of mouse bone marrow cells. Since then, many researchers have employed this assay for the assessment of mutagenic effects induced by different mutagens (Martino-Roth *et al.*, 2003; Ad'hiah *et al.*, 2007).

1.3.3.8 Sperm-head Abnormalities Assay

Sperm topography is unique among the known cells, and three major parts can be immediately distinguished; head, midpiece and tail (Martin *et al.*, 1994).The shape of sperm head is characteristics of the species. In mouse, it is hook-shaped, and composed of two parts, the nucleus and the acrosome. The nucleus contains a highly condensed chromatin, while the acrosome is surrounded by the acrosomal membranes and covers the anterior part of the

sperm nucleus, and it contains enzymes that are important in penetration of the egg in the fertilization process (Saladin and Porth, 1998).

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

Chapter Two
Materials and Methods

2.1 Materials

The general laboratory equipments and chemicals, which were employed in the present study, were illustrated in appendices I and II, respectively.

2.2 The Plant *Plantago lanceolata*

2.2.1 Plant Collection and Identification

The plant taxonomist Professor Dr. Ali Al-Mosawy (Department of Biology, College of Science, University of Baghdad) identified the plant *P. lanceolata*, which was collected in January 2006 from the gardens of the University of Baghdad. A field picture of the plant is shown in figure 2-1.

Figure 2-1: Field picture of *Plantago lanceolata*.

2.2.2 Preparation of Plant Extract(Sabahi *et. al.*, 1987)

The plant leaves were chopped into small pieces, and 150 grams of them were extracted for four hours in 250 ml of distilled water using the Soxhlet apparatus and the source of heating was a water bath (45°C). The obtained leaf extract solution was then evaporated at 45°C using a rotary evaporator, and the dry extract was obtained and it was seven grams (4.7% of the original weight). The resultant crude extract was frozen at -20°C until use to prepare the required doses (Nadir *et al.*, 1986). The water extracted deposit was dissolved in sterile distilled water to prepare three doses (1.5, 3 and 4.5 mg/kg), which were investigated in the laboratory mice. These doses were based respectively on 10, 20 and 30% of the lethal dose (LD_{50}) (15 mg/kg) in mice (Rezaeipoor *et al.*, 2000). The extract was sterilized by filtration using Millipore filters (0.22 µm).

2.3 Solutions

i. **Colchicine:** One tablet (0.5 mg) of colchicine was dissolved in 1 ml of sterilized normal saline (Allen *et al.*, 1977).The solution was freshly used.

ii. **Potassium chloride (KCl) hypotonic solution (0.075M):** Potassium chloride (5.75 grams) was dissolved in 500 ml of distilled water and the volume was made up to 1000 ml, and then the solution was autoclaved (121°C, 1.5 pound / in^2, 20 minutes) and stored at 4°C (Allen *et al.*, 1977).

iii. **Leucocytes 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).

iv. **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.

v. **Fixative solution:** The solution was freshly prepared by mixing 3 parts of absolute methanol with 1 part of glacial acetic acid (Allen *et al.*, 1977).

vi. **Alsever's solution:** Dextrose (20 grams), sodium citrate (8 grams), citric acid (0.55 gram) and sodium chloride (4.2 grams) were dissolved in 500 ml of distilled water, and then the volume was made up to 1000 ml. After adjusting the pH to 6.1, the solution was filter-sterilized, and stored at 4°C (Hudson and Hay, 1989).

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

viii. **Giemsa stain:** Giemsa stock solution was prepared by dissolving one gram of Giemsa powder in 33 ml glycerin using a shaking water bath (60°C) for 2 hours. After cooling the solution for 30 min 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 Giemsa stain working solution, the following solutions were mixed:

- Giemsa stock solution: 1 ml
- Absolute methanol 1.25 ml
- Sodium bicarbonate solution 0.5 ml
- Distilled water 40 ml

ix. **Eosin stain:** The stain was prepared by dissolving one gram of eosin yellowish powder in 100 ml of distilled water. The stain was centrifuged at 2000 rpm for 10 minutes before use (Wyrobek and Bruce, 1975).

x. **Leishman Stain:** The Institute of Sera and Vaccine (Baghdad) supplied a ready prepared stain kit.

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

xii. **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 at -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.

xiii. HEPES-Buffered Balanced Salt Solution (HEPES-BSS): Six stock solutions were prepared as follows:

- **First solution**: Phosphate buffer was made by dissolving 22.9 grams of KH_2PO_4 and 19.5 grams of K_2HPO_4 in 950 ml of distilled water. The pH was adjusted to 7.2 with KOH, and the volume was made up to 1000 ml.
- **Second solution**: 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic buffer (HEPES buffer) was prepared by dissolving 80 grams of HEPES and 13.4 grams of NaOH in 950 ml of distilled water. The pH was adjusted to 7.2 with NaOH, and the volume was made up to 1000 ml.
- **Third solution**: Sodium chloride (9.83 grams) was dissolved in 950 ml of distilled water and the volume was made up to 1000 ml.
- **Fourth solution**: Potassium chloride (12.5 grams) was dissolved in 950 ml of distilled water and the volume was made up to 1000 ml.
- **Fifth solution**: Calcium chloride (12.45 grams) was dissolved in 950 ml of distilled water and the volume was made up to 1000 ml.
- **Sixth solution**: Magnesium sulfate (41.3 grams) was dissolved in 950 ml of distilled water and the volume was made up to 1000 ml.

The HEPES-BSS working solution was prepared by adding the above stock solutions into the following proportions:

10 ml of first solution

30 ml of second solution

605 ml of third solution

20 ml of fourth solution

15 ml of fifth solution

5 ml of sixth solution

The solution was filter-sterilized and stored at 4°C (Myers, 1995).

xiv. Sheep Red Blood cell (SRBC) Suspension: The procedure of Myers (1995) was followed to prepare the SRBC suspension. It is outlined in the following steps:

- The blood (5 ml) was collected in a sterile test tube from the jugular vein and diluted with an equal volume of Alsever's solution.
- The diluted blood was left in the refrigerator at 4°C overnight.
- The blood was centrifuged at1000 rpm for 15 minutes, and plasma and buffy coat were discarded.
- The SRBC pellet was collected and washed with HEPES-BSS at least three times or until the supernatant was clear, and in each wash the centrifugation speed was 2000 rpm and for 5 minutes. After that, the deposited cells were suspended in HEPES-BSS as required and stored in the refrigerator at 4°C until used.

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

xv. Trypan blue: One gram of trypan blue powder was dissolved in 100 ml of normal saline. The stain solution was filtered (Whattman filter paper No.3) before use (Ad'hiah, 1990, Lillie *et.al.*, 1977).

xvi. **Mayer's Reagent:** Two solutions were firstly prepared; the first one was prepared by dissolving 1.58 gram of Mercuric chloride ($HgCl_2$) in 60 ml of distilled water, while the second solution was prepared by dissolving 5 grams of potassium iodide (KI) in 10 ml of distilled water. Then both solutions were mixed and the volume was made-up to 100 ml with distilled water (Smolensk *et al.*, 1972).

xvii. **Molisch's reagent:** The reagent was prepared by dissolving 0.5 gram of α-naphthol in10 ml of 95% ethanol. The reagent was stored in a dark place at room temperature until use (Mathews *et al.*, 1999 Eaton, 1989)

xviii. **Benedict Reagent:** The reagent was prepared by dissolving 137 grams of sodium citrate and 100 grams of sodium bicarbonate in 800 ml of distilled water and the mixture was filtered (Whatman filter paper No. 3), then cupper sulphate solution (17.3 grams in 100 ml distilled water) was added and the volume was made-up to 1000 ml with distilled water (Al-Janabi, 2004 Simoni *et.al.*, 2002).

xix. **Ferric chloride solution:** The solution was prepared by dissolving one gram of ferric chloride in 100 ml distilled water (Al-Khazraji, 1991 Tarr, 1950).

xx. **Potassium hydroxide solution:** It was prepared by dissolving 50 grams of potassium hydroxide in 100 ml of distilled water (Jaffer *et al.*, 1983 Holleman and Wiberg, 2000).

2.4 Laboratory Animals

Albino male mice (*Mus musculus*) were the laboratory animals. They were supplied by the Biotechnology Research Center (Al-Nahrain 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 were given in the experimental design section 2-5). The animals were maintained at a temperature of 20 – 25°C, and they had free excess to food (standard pellets) and water.

2.5 Experimental Design

The experiments were designed to evaluate (*in vivo*) the immunological and cytogenetic potentials of *P. lanceolata* leaf aqueous extract in albino male mice in vivo. Therefore, such evaluations were carried out through four parts.

2.5.1 Part One

In this part, the immunological and cytogenetic effects of three doses of the plant leaf aqueous extract and cyclophosphamide were investigated. Therefore, the animals were divided into three groups:

- **Group I**: The animals were treated with distilled water (negative controls of aqueous extract = 8 animals).
- **Group II**: The animals were treated with cyclophosphamide at a dose of 15 mg/kg (positive controls = 8 animals).
- **Group III**: The animals were treated with three doses of the aqueous extract (1.5, 3, 4.5 mg/kg) (24 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 on day 8 for laboratory assessments. The total number of mice in this experiment was 40 animals.

2.5.2 Part Two

In this part, interactions (pre- and post-treatments) between the ideal dose of extract (1.5 mg/ kg) and Cyclophoshomide (15 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 control of part one.

- **In pre-treatment interaction**, the plant extract was given for 7 days (single dose/day), while cyclophosphamide was given on day 8, and then the animals were sacrificed on day 9 for laboratory assessments. In both cases, the material was injected 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 was given on day 2 till day 8 (single dose/day), and then the animals were sacrificed on day 9 for laboratory assessments. In both cases, the material was injected 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 with distilled water.

2.5.3 Part Three

In this part, the animals were given a single dose of the investigated material (distilled water, plant extract or cyclophosphamide) on day one, and then they were sacrificed on days 7, 21 and 35 to assess the sperm head abnormalities. The total numbers of animal in this experiment was 60 mice.

2.5.4 Part Four

In this part, the experiment was carried out to assess two types of immunological reactions (Arthus reaction and delayed type hypersensitivity reaction) in mice that were immunized intraperitoneally with 0.1 ml of 10% SRBC suspension. These reactions were assessed in the animals after a subcutaneous injection (0.1 ml) of the plant ideal dose (1.5 mg/kg), cyclophosphamide (15 mg/kg) or distilled water in an immunization-interaction regime, which was given in table 2-1.

Table 2-1: The immunization-interaction regime between SRBC and the ideal dose of aqueous extracts, cyclophosphamide or distilled water in albino male mice.

Days	Material of Injection (M)
1-4	**M (either aqueous extract, cyclophosphamide or distilled water)**
5	M + SRBC
6-8	M
9	M + SRBC
10-11	M
12	Arthus Reaction
13	Delayed type hypersensitivity reaction
Total number of animals = 12 mice (4 animals for each material of injection)	

2.6 Laboratory Methods

2.6.1 Chemical Detection of Plant Extract

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

i. **Tannins:** The procedure of Al-Shami (1982) was used for the detection of tannins. In this procedure, 50 ml of the extract was equally divided into two conical flasks. For the first one, lead acetate solution (CH_3COOPb) (1%; w/v) was added and the appearance of jelly pellet was considered a positive reaction, while for the second flask, ferric chloride solution ($FeCl_2$) (1%; w/v) was added and the appearance of blue color was an indicator for the presence of tannins.

ii. **Saponins**: Two methods were employed in the detection of saponins. In the first one 3 ml of mercuric chloride ($HgCl_2$) was added to 5 ml of the extract, then the formation of white pellet was an indicator of the presence of saponins (Al-Shami, 1982). In the second method, the tube containing extract was shaken, and formation of thick foam that remained for few minutes was an indicator of a saponin's presence (Shihata, 1951).

iii. **Flavonoids:** The detecting solution was prepared by mixing 10 ml of ethanol (50%) with 10 ml of potassium hydroxide (50%), and then 5 ml of this solution was added to 5 ml of the plant extract. The appearance of yellow color was an indicator of the presence of flavonoids (Jaffer *et al.*, 1983 Harborn, 1973).

iv. **Glycosides:** Few drops of HCl were added to a test tube containing 1 ml of plant extract, and then the tube was transferred to a boiling water bath for 2 minutes, and after that 2 ml of Benedict reagent was added.

The appearance of red color was an indicator of glycosides (Evans, 1999).

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

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

vii. **Terpens:** The procedure of Al-Maisary (1999) was followed, in which 1 ml of the plant extract was mixed with 2 ml of chloroform and one drop of glacial acetic acid, and then one drop of concentrated H_2SO_4 was added. The appearance of brown color was an indicator of the presence of terpens.

2.6.2 Total Leucocyte Count

Blood samples were collected by heart puncture using a disposable insulin syringe (1 ml) precoated 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 3 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 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 total count of each type was obtained using the following equation:

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

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 which was at 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 at 2000 rpm for 5 minutes.

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

v. To carry out phagocytosis, 0.2 ml of 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 at 37°C.

vi. After 30 and 60 minute incubations, 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 Metaphase Index Assay

Metaphase index was determined for cells obtained from bone marrow and spleen, following the procedure of Allen *et al*. (1977). Each animal was injected intraperitoneally with 0.25 ml of colchicine solution, and after 1.5 – 2 hours, the animal was sacrificed by cervical dislocation and then dissected to obtain femur and spleen. The femur was cut from both ends, and its cellular contents collected in a test tube using a disposable insulin syringe (1 ml) and normal physiological saline (5 ml). The spleen was placed in a Petri-dish containing 5 ml normal physiological saline, and then punctured several times with the needle of insulin syringe. The cellular content was obtained by repeated infusions of saline in the spleen, and by then the cell suspension (5 ml) was transferred to a test tube.

The cells of both organs were manipulated in a similar way as the following:

i. The cells were gently suspended using Pasteur pipette, and the tubes were centrifuged at 2000 rpm for 10 minutes.

ii. The supernatant was discarded, and the cell deposit was suspended in 5 ml of a warm at 37°C hypotonic solution (KCl; 0.075 M). Then the

tubes were incubated in a water bath (37°C) for 30 min with a gentle shaking every 5 minutes.

iii. The tubes were centrifuged at 2000 rpm for 10 minute, and the supernatant was discarded.

iv. Five ml of the fixative solution was added a drop-wise to the cell deposit with a gentle and a continuous mixing to make a homogeneous cell suspension. Then, the tubes were incubated in the refrigerator at 0°C for 30 minutes,

v. The tubes were centrifuged at 2000 rpm for 10 minutes, and step **iv** was repeated two times.

vi. The cell deposit was well-suspended in 2 ml of the fixative, and 4-5 drops of the cell suspension were dropped on a clean slide from a height of about two feet.

vii. The slide was air-dried at room temperature, and then it was stained with Giemsa stain for 15 minutes and rinsed with distilled water.

viii. The slide was examined under oil emersion lens (100X) at least 1000 cells were examined. The percentage of metaphase cells (metaphase index) was recorded using the following equation:

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

2.6.6 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 sacrificed by cervical dislocation, and then dissected to obtain the femur. After cutting both ends of the bone, it was grapped

from the middle with a forceps in a vertical position over the edge of a test tube, and then 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 at 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 rinsed with distilled water.

The slide was 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 expressed as a number of the observed micronuclei per 1000 PCE cells.

2.6.7 Sperm-head Abnormality Assay

The mouse was sacrificed by a cervical dislocation and then dissected to obtain the epididymis, which was collected in a 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 at 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), 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:

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

2.6.8 Arthus Reaction

After the immunization regime presented in table 2-1, the left foot pad was injected intradermally with 0.05 ml of 10% SRBC, while the right foot pad was injected with 0.05 ml of normal saline. After four hours, the thickness of both pads was measured using a vernier, and the difference represented Arthus reaction index, which was given in units of millimeter (mm) (Triolo *et al.*, 1989).

2.6.9 Delayed Type Hypersensitivity

The index of delayed type hypersensitivity (DTH) was carried out as outlined for Arthus reaction index, but the difference between the thicknesses of both pads was measured after 24 hours.

2.6.10 Quantification of Serum IgG by Mouse ELISA Kit

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

2.6.10.1 Principle of the Test

The test is based on the binding of mouse IgG in samples to two antibodies; the first one was immobilized on the microtiter wells, while the other was conjugated to horseradish peroxidase (HRP) enzyme. After a

washing step, chromogenic substrate was added and colour is developed by the enzymatic reaction of the tetramethylbenzidine substrate (TMB substrate), which was directly proportional to the amount of IgG present in the sample. The stop solution was added to terminate the reaction, and absorbance at 450 nm is then measured using an ELISA microtiter well reader. The level of IgG in samples and controls was calculated from a standard curve (Fig.2-2) containing known concentrations of IgG.

2.6.10.2 Kit Components

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

2.6.10.3 Collection of Serum Samples

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

2.6.10.4 Assay Method

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

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

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

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

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

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

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

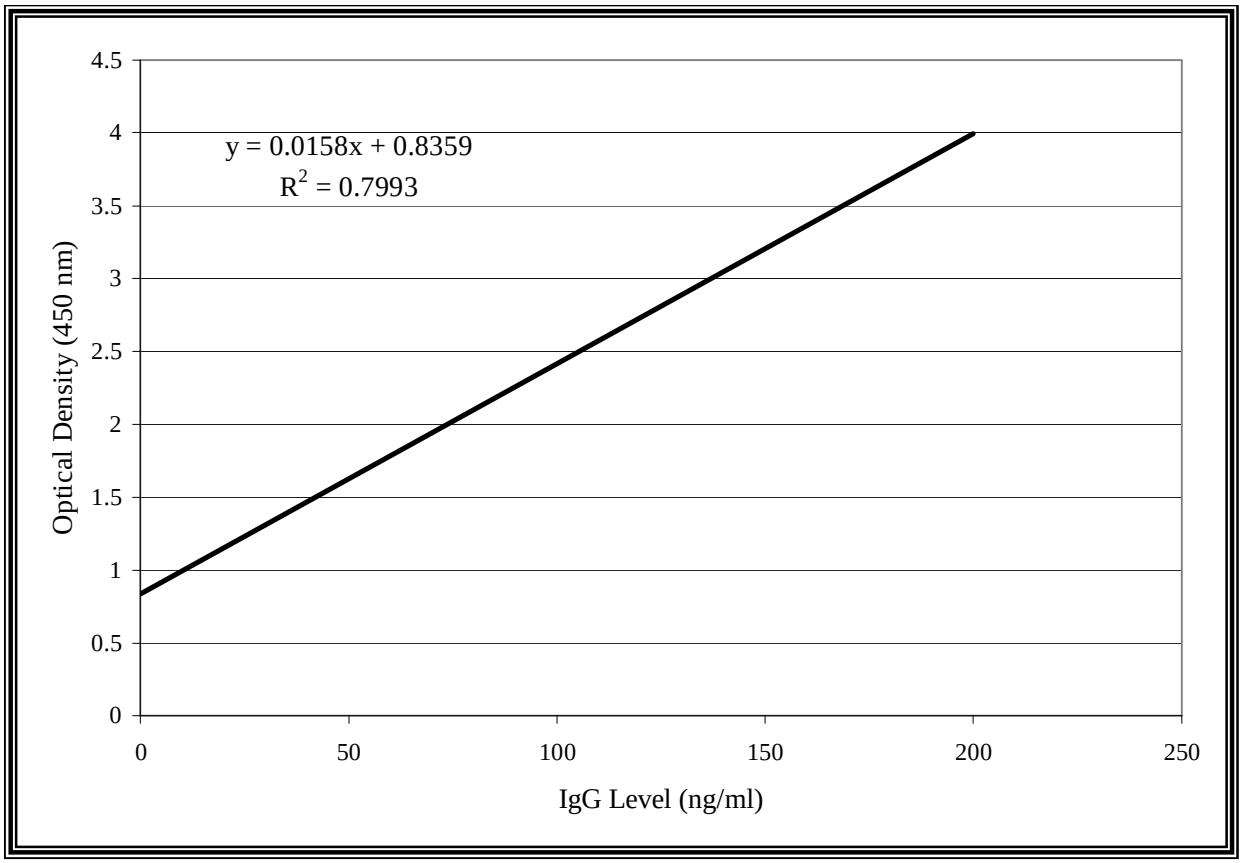

Figure 2-2: Standard curve of mouse IgG serum level.

2.7 Statistical Analyses

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

A further estimation was also given; it was treatment efficiency (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 extract or cyclophosphamide).

B = Negative control groups (distilled water).

Chapter Three
Results

3.1 Chemical Detection of *Plantago lanceolata* Extract

The chemical detection of *P. lanceolata* leaf aqueous extract revealed that the extract was positive for tannins, polysaccharides, glycosides, flavonoids, saponins and terpens (Table 3-1).

Table 3-1: Chemical detection of *Plantago lanceolata* leaf aqueous extract.

	Chemical compounds	Reagents	Indication	Results
Plantago lanceolata Leaf aqueous extract	Tannins	Ferric chloride	Green-blue precipitate	Positive
	Polysaccharides	Molisch's reagent	red-violet layer	Positive
	Glycosides	Benedict reagent	Red precipitate	Positive
	Flavonoids	Ethanol with KOH	Yellow precipitate	Positive
	Saponins	1-Shaking of extract	Foam	Positive
		2-Mercuric chloride	White precipitate	Positive
	Alkaloids	Myers reagent	White precipitate	Positive
	Terpens	Concentrated Sulfuric acid	Brown colour	Positive

3.2 Immunological and Cytogentic Effects of *P. lanceolata*

3.2.1 Total Count of Leucocytes (TLC)

Animals treated with cyclophosphamide (positive control) showed a significant ($P \leq 0.05$) reduction in the TLC as compared to the negative control (5.2 *vs.* 8.1 x 10^3 cell/cu.mm.blood). In contrast, the treatment with these three doses (1.5, 3.0 and 4.5 mg/kg) of *P. lanceolata* aqueous extract showed an increasing in the TLC in the treated mice (13.4, 9.4 and 8.3 x 10^3 cell/cu.mm.blood, respectively) as compared to the negative control (8.1 x 10^3 cell/cu.mm.blood), but the difference was significant only in the first dose, which recorded a treatment efficiency of +65.4% (Table 3-2).

Table 3-2: Total leucocyte count in albino male mice treated with the aqueous extract of *P. lanceolata* leaves, distilled water (negative control) and cyclophosphamide (positive control).

Groups		Dose (mg/kg)	Mean ± S.E. x 10^3 * (cells/cu.mm .blood)	Treatment Efficiency (%)
Negative Control			8.1 ± 0.6[a]	
Positive Control		15	5.2 ± 0.5[b]	-35.8
Plantago lanceolata Aqueous Extract	Dose I	1.5	13.4 ± 0.8[c]	+65.4
	Dose II	3.0	9.4 ± 0.2[a]	+16.0
	Dose III	4.5	8.3 ± 0.2[ab]	+2.4

*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 an increase as a result of treatment with the three doses of *P. lanceolata* aqueous extract (10.3, 6.9 and 6.1 x 10^3 cell/cu.mm.blood, respectively), as compared to the corresponding negative and positive controls (5.2 and 3.6 x 10^3

cell/cu.mm.blood, respectively), but a significant increases was observed only in the first two doses (1.5 and 3.0 mg/kg) as compared to the negative control. The treatment efficiency of the two doses was +65.4 and +16.0%, respectively (Table 3-3).

Table 3-3: Lymphocyte count in albino male mice treated with aqueous extract of *P. lanceolata* leaves, distilled water (negative control) and cyclophosphamide (positive control).

Groups		Dose (mg/kg)	Mean ± S.E. x 10^3 * (cells/cu.mm .blood)	Treatment Efficiency (%)
Negative Control			5.2 ± 0.5[a]	
Positive Control		15	3.6 ± 0.4[b]	-30.7
Plantago lanceolata Aqueous Extract	Dose I	1.5	10.3 ± 0.5[c]	+98.0
	Dose II	3.0	6.9 ± 0.3[d]	+32.6
	Dose III	4.5	6.1 ± 0.5[ad]	+17.3

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

3.2.2.2 Neutrophils

Only the first dose of the plant extract (1.5 mg/kg) was significantly effective in increasing the count of neutrophils as compared to the negative control (2.4 *vs.* 1.6 x 10^3 cell/cu.mm.blood) and this illustrated treatment efficiency of +50.0% (Table 3-4).

Table 3-4: Neutrophil count in albino male mice treated with aqueous extract of *P. lanceolata* leaves, distilled water (negative control) and cyclophosphamide (positive control).

Groups		Dose (mg/kg)	Mean ±S.E. x 10^3 * (cells/cu.mm .blood)	Treatment Efficiency (%)
Negative Control			1.6 ± 0.1[a]	
Positive Control		15	0.9 ± 0.1[b]	-43.7
Plantago lanceolata Aqueous Extract	Dose I	1.5	2.4 ± 0.3[c]	+50.0
	Dose II	3.0	1.3 ± 0.1[a]	-18.7
	Dose III	4.5	1.2 ± 0.1[ab]	-25.0

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

3.2.2.3 Monocytes

The three doses of the plant extract caused a reduction in the count of monocytes, but only the first dose (1.5 mg/kg) contributed to a significant difference (632 cell/cu.mm.blood) as compared to the negative control (1035 cell/cu.mm.blood) with a treatment efficiency of -38.9% (Table 3-5).

Table 3-5: Monocyte count in albino male mice treated with aqueous extract of *P. lanceolata* leaves, distilled water (negative control) and cyclophosphamide (positive control).

Groups		Dose (mg/kg)	Mean ±S.E. * (cells/cu.mm .blood)	Treatment Efficiency (%)
Negative Control			1035.5 ± 83.7[a]	
Positive Control		15	531.5 ± 33.8[b]	-48.6
Plantago lanceolata Aqueous Extract	Dose I	1.5	632.5 ± 59.6[c]	-38.9
	Dose II	3.0	933.0 ± 70.7[ac]	-9.8
	Dose III	4.5	979.0 ± 100.4[a]	-5.4

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

3.2.2.4 Eosinophils and Basophils

The count of eosinophils and basophils counts did not show any a significant difference in animals treated with the three doses of plant extract when the comparison was made with negative or positive controls. However, the first dose of the plant extract (1.5 mg/kg) contributed to a decreased count of either cells (33 and 39 cell/cu.mm.blood, respectively) as compared to the negative control (101.5 and 98.5 cell/cu.mm.blood, respectively) or positive control (103.5 and 79.5 cell/cu.mm.blood, respectively). The treatment efficiency of both were -67.4 and -60.4%, respectively (Tables 3-6 and 3-7).

Table 3-6: Eosinophil count in albino male mice treated with aqueous extract of *P. lanceolata* leaves, distilled water (negative control) and cyclophosphamide (positive control).

Groups		Dose (mg/kg)	Mean ± S.E. * (cells/cu.mm .blood)	Treatment Efficiency (%)
Negative Control			101.5±22.9[a]	
Positive Control		15	103.5±16.0[a]	+1.9
Plantago lanceolata Aqueous Extract	Dose I	1.5	33.0±33.0[a]	-67.4
	Dose II	3.0	94.5±38.4[a]	-6.8
	Dose III	4.5	84.5±35.0[a]	-16.7

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

Table 3-7: Basophile count in albino male mice treated with aqueous extract of *P. lanceolata* leaves, distilled water (negative control) and cyclophosphamide (positive control).

Groups		Dose (mg/kg)	Mean ± S.E. * (cells/cu.mm .blood)	Treatment Efficiency (%)
Negative Control			98.5±38.9[a]	
Positive Control		15	79.5±24.9[a]	-19.2
Plantago lanceolata Aqueous Extract	Dose I	1.5	39.0±39.0[a]	-60.4
	Dose II	3.0	95.0±40.5[a]	-3.5
	Dose III	4.5	73.3±26.5[a]	-25.5

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

3.2.3 Phagocytic Index

After 30 minute incubation, animals treated with cyclophosphamide (positive control) showed a significant reduction in the phagocytic activity as compared to the negative control (19.25 *vs*. 38.5%), while the first and the second doses of the plant extract (1.5 and 3.0 mg/kg) were effective in increasing the phagocytic activity (47.75 and 43.75%, respectively) as compared to the negative control (38.50%), with a treatment efficiency of +24.0 and +13.6%, respectively, but the difference was significant in the first dose only. In contrast, the third dose (4.5 mg/kg) showed a significant decreased phagocytic index (32.25%), and the treatment efficiency of such effect was -16.2% (Table 3-8).

After 60 minute incubation, the phagocytic index behaved in a similar manner as after 30 minutes incubation in the treated animals (Table 3-9).

Table 3-8: Phagocytic index (after 30 minute incubation) in albino male mice treated with the aqueous extract of *P. lanceolata* leaves, distilled water (negative control) and cyclophosphamide (positive control).

Groups		Dose (mg/kg)	Mean ± S.E. * (%)	Treatment Efficiency (%)
Negative Control			38.50 ± 1.85[a]	
Positive Control		15	19.25 ± 0.48[b]	-50.0
Plantago lanceolata Aqueous Extract	Dose I	1.5	47.75 ± 1.38[c]	+24.0
	Dose II	3.0	43.75 ± 3.20[ac]	+13.6
	Dose III	4.5	32.25 ± 0.85[d]	-16.2

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

Table 3-9: Phagocytic index (after 60 minute incubation) in albino male mice treated with the aqueous extract of *P. lanceolata* leaves, distilled water (negative control) and cyclophosphamide (positive control).

Groups		Dose (mg/kg)	Mean ± S.E. * (%)	Treatment Efficiency (%)
Negative Control			45.75 ± 1.65[a]	
Positive Control		15	23.50 ± 0.65[b]	-48.6
Plantago lanceolata Aqueous Extract	Dose I	1.5	53.50 ± 1.85[c]	+16.9
	Dose II	3.0	49.75 ± 2.82[a]	+8.1
	Dose III	4.5	40.25 ± 1.44[d]	-12.0

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

3.2.4 Metaphase Index

In the present study the metaphase index was considered for both bone marrow and spleen, in which only cells at metaphase was scored in a sample of 1000 cells, while their percentage was given

3.2.4.1Metaphase index for bone marrow Cells

The metaphase index of bone marrow cells in the negative control was 3.25%, and such index was significantly decreased (2.35%) when mice were treated with cyclophosphamide (positive control). In contrast, animals treated with the three doses of the plant extract was in favour of enhancing the percentage of metaphase index, especially the dose 1.5 mg/kg (5.27%), in which the treatment efficiency was 62.1%, while a non significant increase was illustrated into the other two doses (4.35 and 3.80%, respectively) as compared to the negative control (3.25%) with a treatment efficiency of 33.8 and 16.9%, respectively (Table 3-10).

Table 3-10: Metaphase index of bone marrow cells in albino male mice treated with the aqueous extract of *P. lanceolata* leaves, distilled water (negative control) and cyclophosphamide (positive control).

Groups		Dose (mg/kg)	Mean ± S.E. * (%)	Treatment Efficiency (%)
Negative Control			3.25 ± 0.17[a]	
Positive Control		15	2.35 ± 0. 20[b]	-27.6
Plantago lanceolata Aqueous Extract	Dose I	1.5	5.27 ± 0.10[c]	+62.1
	Dose II	3.0	4.35 ± 0.10[a]	+33.8
	Dose III	4.5	3.80 ± 0.12[a]	+16.9

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

3.2.4.2 Metaphase index for spleen Cells

The metaphase index of spleen cells shared the picture of the index in bone marrow cells, in which a significant enhancement of dividing cells was observed in animals treated with the first two doses of the plant extract (2.18

and 1.65%, respectively) as compared to the negative control (1.33%) (Table 3-11).

Table 3-11: Metaphase index of spleen cells in albino male mice treated with the aqueous extract of *P. lanceolata* leaves, distilled water (negative control) and cyclophosphamide (positive control).

Groups		Dose (mg/kg)	Mean ± S.E. * (%)	Treatment Efficiency (%)
Negative Control			1.33 ± 0.95[ac]	
Positive Control		15	1.05 ± 0.65[a]	-21.0
Plantago lanceolata Aqueous Extract	Dose I	1.5	2.18 ± 0.21[b]	+63.9
	Dose II	3.0	1.65 ± 0.87[c]	+24.0
	Dose III	4.5	1.45 ± 0.07[ac]	+9.0

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

3.2.5 Micronucleus Formation

The micronucleus formation was observed with a frequency of 8.0 micronucleus/1000 cells in animals treated with distilled water (negative control) and 17.0 micronucleus/1000 cells in animals treated with cyclophosphamide (positive control). Treating the animals with the three doses (1.5, 3.0 and 4.5 mg/kg) of the plant extract revealed a dose-dependent effect. The first dose did not significantly different from the negative control value (8.5 *vs*. 8.0 micronucleus/1000 cells), while a gradual increases in the frequency of micronucleus formation were observed in the other two doses (9.2 and 11.5 micronucleus/1000 cells, respectively), especially the third dose (4.5 mg/kg), in which the difference was significant and the treatment efficiency was +43.8% (Table 3-12).

Table 3-12: Micronucleus formation in albino male mice treated with the aqueous extract of *P. lanceolata* leaves, distilled water (negative control) and cyclophosphamide (positive control).

Groups		Dose (mg/kg)	Mean ± S.E. * (micr-onucleus/1000 cells)	Treatment Efficiency (%)
Negative Control			8.00 ± 0.41[a]	
Positive Control		15	17.00 ± 1.18[b]	+112.5
Plantago lanceolata Aqueous Extract	Dose I	1.5	8.50 ± 0.65[a]	+6.3
	Dose II	3.0	9.20 ± 0.85[ac]	+15.0
	Dose III	4.5	11.50 ± 0.65[c]	+43.8

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

3.2.6 Sperm-head Abnormality Assay

The effect of plant extract on the sperm-head abnormalities was dose and time dependent. **Seven days post-treatment** revealed a gradual increased percentage of abnormalities as compared to the negative control (7.23, 7.95 and 9.35%, respectively *vs*. 6.0%), and the most effective dose was 4.5 mg/kg, in which the difference was significant and the treatment efficiency was +55.8% (Table 3-13). **Twenty-one days post-treatment** contradicted such picture, and instead the spontaneous formation of sperm-head abnormalities was decreased, especially the first two doses (6.65 and 7.30%, respectively *vs*. 9.0%), in which the difference was significant and the treatment efficiency was -26.1 and -18.8%, respectively) (Table 3-14). The latter picture was confirmed **35 days post-treatment**, in which the three doses of the plant extract were significantly effective in reducing the sperm-head abnormalities (6.65, 5.70 and 6.55%, respectively) as compared to the frequency in the negative control (11.72). The treatment efficiency of such effect was -60.1, -51.3 and -44.1%, respectively (Table 3-15).

Table 3-13: Sperm-head abnormalities (7 days post-treatment) in albino male mice treated with the aqueous extract of *P. lanceolata* leaves, distilled water (negative control) and cyclophosphamide (positive control).

Groups		Dose (mg/kg)	Mean ± S.E. * (%)	Treatment Efficiency (%)
Negative Control			6.00 ± 0.53[a]	
Positive Control		15	13.75 ± 0.63[b]	+129.1
Plantago lanceolata Aqueous Extract	Dose I	1.5	7.32 ± 1.15[ac]	+22.0
	Dose II	3.0	7.95 ± 0.67[ac]	+32.5
	Dose III	4.5	9.35 ± 0.63[c]	+55.8

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

Table 3-14: Sperm-head abnormalities (21 days post-treatment) in albino male mice treated with the aqueous extract of *P. lanceolata* leaves, distilled water (negative control) and cyclophosphamide (positive control).

Groups		Dose (mg/kg)	Mean ± S.E. * (%)	Treatment Efficiency (%)
Negative Control			9.00 ± 0.65[a]	
Positive Control		15	14.42 ± 1.02[b]	+60.2
Plantago lanceolata Aqueous Extract	Dose I	1.5	6.65 ± 0.49[c]	-26.1
	Dose II	3.0	7.30 ± 0.58[c]	-18.8
	Dose III	4.5	8.60 ± 0.83[a]	-4.4

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

Table 3-15: Sperm-head abnormalities (35 days post-treatment) in albino male mice treated with the aqueous extract of *P. lanceolata* leaves, distilled water (negative control) and cyclophosphamide (positive control).

Groups		Dose (mg/kg)	Mean ± S.E. * (%)	Treatment Efficiency (%)
Negative Control			11.72 ± 1.06[a]	
Positive Control		15	19.00 ± 1.16[b]	+62.1
Plantago lanceolata Aqueous Extract	Dose I	1.5	4.67 ± 0.64[c]	-60.1
	Dose II	3.0	5.70 ± 0.60[c]	-51.3
	Dose III	4.5	6.55 ± 0.58[c]	-44.1

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

3.2.7 Total IgG level in Serum

Animals treated with cyclophosphamide (positive control) showed a significant reduced total IgG level as compared to the negative control (8.73 *vs.* 149.0 ng/ml), while the first two doses of the extract caused a significant increase IgG level (181.25 and 169.0 ng/ml, respectively) with a treatment efficiency of +21.6 and +13.4%, respectively (Table 3-16).

Table 3-16: Total serum IgG level in albino male mice treated with the aqueous extract of *P. lanceolata* leaves, distilled water (negative control) and cyclophosphamide (positive control).

Groups		Dose (mg/kg)	Mean ± S.E. * (ng/ml)	Treatment Efficiency (%)
Negative Control			149.0 ± 1.0[a]	
Positive Control		15	8.73 ± 1.3[b]	-94.1
Plantago lanceolata Aqueous Extract	Dose I	1.5	181.25 ± 0.8[c]	+21.6
	Dose II	3.0	169.0 ± 1.7[d]	+13.4
	Dose III	4.5	143.8 ± 0.6[a]	-3.4

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

3.3 Extract-Cyclophosphamide Interactions

Two types of interactions (pre- and post-treatments) were carried out between the ideal dose of *P. lanceolata* extract and cyclophosphamide to evaluate the rule of the extract in modulating the immunological and cytogenetic effects of the drug in albino male mice. The selection of ideal dose was based on the results of total leucocyte count (Tables 4-2), in which a significant increase was observed in the extract-treated mice, and such dose was 1.5 mg/kg.

3.3.1 Total Leucocyte Count

In the pre-treatment group, the aqueous extract showed a significant ($P \leq 0.001$) increases in the count of TLC (19.1 *vs*. 6.2 x 10^3cell/cu.mm.blood) as compared to the negative control (6.2 $\times 10^3$cell/cu.mm.blood). In post-treatment group a similar manner was observed, and the extract caused a significant ($P \leq 0.001$) increased count of TLC (11.45 *vs*. 5.5 x 10^3cell/cu.mm.blood), but the treatment efficiency of pre-treatment was better than post-treatment (+208.0 *vs*. 108.1%) (Table 3-17).

Table 3-17: Total leucocyte count in albino male mice after interactions (pre- and post-treatments) between the ideal dose (1.5mg/kg) of aqueous extract of *P. lanceolata* leaves and cyclophosphamide.

Groups	Mean ± S.E. x 10^3* (cells/cu. mm. blood)		Probability ≤	Treatment Efficiency (%)	
	Pre	Post		Pre	Post
Control	6.2 ± 0.1	5.5 ± 0.2	N.S.		
Plant Extract	19.1 ± 0.8	11.45±0.6	0.001	+208.0	+108.1
Probability ≤	0.001	0.001			

N.S.: Not significant

3.3.2 Absolute Count of Leucocytes

3.3.2.1 Lymphocytes

The lymphocyte count shared the picture of TLC, in which, the extract in both interactions was able to modulate the suppressive effect of cyclophosphamide, and again the pre-treatment was better than post-treatment as judged by the assessment of treatment efficiency (+223.2 *vs*. +65.3%) (Table 3-18).

Table 3-18: Total lymphocyte count in albino male mice after interactions (pre- and post-treatments) between the ideal dose (1.5mg/kg) of aqueous extract of *P. lanceolata* leaves and cyclophosphamide.

Groups	Mean ± S.E. x 10^3* (cells/cu. mm. blood)		Probability ≤	Treatment Efficiency (%)	
	Pre	Post		Pre	Post
Control	4.3 ± 0.2	4.9 ± 0.3	N.S.		
Plant Extract	13.9 ± 0.7	8.1 ± 0.6	0.001	+223.2	+65.3
Probability ≤	0.001	0.01			

N.S.: Not significant

3.3.2.2 Neutrophils

In both interactions (pre and post-treatments), the aqueous extract was able to increase the count of neutrophil significantly as compared to the corresponding controls, but the treatment efficiency of post-treatment was better than pre-treatment (+183.3 vs. 81.2%) (Table 3-19).

Table 3-19: Total neutrophil count in albino male mice after interactions (pre- and post-treatments) between the ideal dose (1.5mg/kg) of aqueous extract of *P. lanceolata* leaves and cyclophosphamide.

Groups	Mean ± S.E. x 10^3* (cells/cu. mm. blood)		Probability ≤	Treatment Efficiency (%)	
	Pre	Post		Pre	Post
Control	1.6 ± 0.3	0.6 ± 0.1	0.01		
Plant Extract	2.9 ± 0.3	1.7 ± 0.2	0.01	+81.2	+183.3
Probability ≤	0.01	0.001			

3.3.2.3 Monocytes

In the pre-treatment group, the aqueous extract revealed a significant ($P \leq 0.001$) increased count of monocytes (1982.5 cell/cu.mm.blood) as compared to the negative control (311.5 cell/cu.mm.blood). The post-treatment group showed a similar manner, in which the aqueous extract increased the count of lymphocytes (847.5 *vs*. 269 cell/cu.mm.blood) as compared to the corresponding control, and the difference was also significant ($P \leq 0.001$). However, the pre-treatment effect was better than the effect of pos-treatment (Treatment efficiency: +536.4 vs. +215.0% (Table 3-20).

Table 3-20: Total monocyte count in albino male mice after interactions (pre- and post-treatments) between the ideal dose (1.5mg/kg) of aqueous extract of *P. lanceolata* leaves and cyclophosphamide.

Groups	Mean ± S.E.* (cells/cu. mm. blood)		Probability ≤	Treatment Efficiency (%)	
	Pre	Post		Pre	Post
Control	311.5 ± 72.2	269 ± 20.2	N.S.		
Plant Extract	1982.5 ± 254.0	847.5 ± 62.6	0.01	+536.4	+215.0
Probability ≤	0.001	0.001			

N.S.: Not significant

3.3.2.4 Eosinophils

Both pre- and post-treatments controls showed zero count of eosinophils, while the pre-treatment with the aqueous extract recorded a count of 145.5 cell/cu.mm.blood, while a post-treatment with the extract recorded a higher count of eosinophil (163 cell/cu.mm.blood) (Table 3-21).

Table 3-21: Total eosinophil count in albino male mice after interactions (pre- and post-treatments) between the ideal dose (1.5mg/kg) of aqueous extract of *P. lanceolata* leaves and cyclophosphamide.

Groups	Mean ± S.E.* (cells/cu. mm. blood)		Probability ≤	Treatment Efficiency (%)	
	Pre	Post		Pre	Post
Control	0.0	0.0			
Plant Extract	145.5 ± 97.5	163.0 ± 32.9	N.S.	0.0	0.0
Probability ≤	N.S.	0.01			

N.S.: Not significant

3.3.2.5 Basophils

In the pre-treatment interaction, the aqueous extract showed a non-significant increase of basophile count as compared to the corresponding control (94 *vs*. 30 cell/cu.mm.blood), while in the post-treatment group the control showed zero count of basophile in comparison with the aqueous extract, which showed a count of 109.5 cell/cu.mm.blood (Table 3-22).

Table 3-22: Total basophile count in albino male mice after interactions (pre- and post-treatments) between the ideal dose (1.5mg/kg) of aqueous extract of *P. lanceolata* leaves and cyclophosphamide.

Groups	Mean ± S.E.* (cells/cu. mm. blood)		Probability ≤	Treatment Efficiency (%)	
	Pre	Post		Pre	Post
Control	30.0 ± 17.3	0.0±0.0[a]	N.S.		
Plant Extract	94.0 ± 54.8	109.5±8.6[b]	N.S.	+213.3	0.0
Probability ≤	N.S.	0.001			

N.S.: Not significant

3.3.3 Phagocytic Index

After 30 minute incubation, a pre-treatment with the aqueous extract enhanced the phagocytic activity of peritoneal phagocytes (45 *vs*. 28.15%),

and a similar picture was observed in post-treatment (43 *vs*. 27.05%), in which both differences were significant as compared to the corresponding controls. Also, an approximated treatment efficiency was recorded in both treatment (+59.8 and +58.9%, respectively) (Table 3-23).

After 60 minute incubation, again both pre- and post-treatments with the aqueous extract were significantly effective in enhancing the phagocytic index (pre-treatment: 58.25 *vs*. 30.57%; post-treatment: 49.37 *vs*. 29.12%), in which both differences were significant as compared to the corresponding controls. However, the pre-treatment was better than post-treatment in this regard (+90.5 *vs.* +69.5) (Table 3-24).

Table 3-23: Phagocytic index (after 30 minute incubation) in albino male mice after interactions (pre- and post-treatments) between the ideal dose (1.5mg/kg) of aqueous extract of *P. lanceolata* leaves and cyclophosphamide.

Groups	Mean ± S.E.* (%)		Probability ≤	Treatment Efficiency (%)	
	Pre	Post		Pre	Post
Control	28.15 ± 1.12	27.05 ±1.09	N.S.		
Plant Extract	45.00 ± 3.00	43.00 ± 3.34	N.S.	+59.8	+58.9
Probability ≤	0.01	0.01			

N.S.: Not significant

Table 3-24: Phagocytic index cell (after 60 minute incubation) in albino male mice after interactions (pre- and post-treatments) between the ideal dose (1.5mg/kg) of aqueous extract of *P. lanceolata* leaves and cyclophosphamide.

Groups	Mean ± S.E.* (%)		Probability ≤	Treatment Efficiency (%)	
	Pre	Post		Pre	Post
Control	30.57 ± 0.88	29.12 ± 1.11	N.S.		
Plant Extract	58.25 ± 4.13	49.37 ± 4.98	N.S.	+90.5	+69.5
Probability ≤	0.001	0.01			

N.S.: Not significant

3.3.4 Metaphase Index of Bone Marrow Cells

The metaphase index of bone marrow cells was significantly increased as a consequence of pre-treatment (4.25 *vs*. 2.45%), as well as, post-treatment (4.67 *vs*. 3.0%) with the aqueous extract as compared to the corresponding controls, but the pre-treatment was better than post-treatment (Treatment efficiency: +73.4 *vs*. +55.6) (Table 3-25).

Table 3-25: Metaphase index of bone marrow cells in albino male mice after interactions (pre- and post-treatments) between the ideal dose (1.5mg/kg) of aqueous extract of *P. lanceolata* leaves and cyclophosphamide.

Groups	Mean ± S.E.* (%)		Probability ≤	Treatment Efficiency (%)	
	Pre	Post		Pre	Post
Control	2.45 ± 0.06	3.00 ± 0.23	N.S.		
Plant Extract	4.25 ± 0.18	4.67 ± 0.33	N.S.	+73.4	+55.6
Probability ≤	0.001	0.01			

N.S.: Not significant

4.3.5 Metaphase Index of Spleen Cells

Both types of treatments (pre and post) were not significantly effective in increasing the metaphase index of spleen cells (pre-treatment: 2.35 *vs*. 1.60%; post-treatment: 2.12 and 1.77%) as compared to the corresponding controls (Table 3-26).

Table 3-26: Metaphase index of spleen cells in albino male mice after interactions (pre- and post-treatments) between the ideal dose (1.5mg/kg) of aqueous extract of *P. lanceolata* leaves and cyclophosphamide.

Groups	Mean ± S.E.* (%)		Probability ≤	Treatment Efficiency (%)	
	Pre	Post		Pre	Post
Control	1.60 ± 0.16	1.77 ± 1.14	N.S.		
Plant Extract	2.35 ± 0.49	2.13 ± 0.13	N.S.	+46.8	+20.0

Probability ≤	N.S.	N.S.	

N.S.: Not significant

3.3.6 Micronucleus Formation in Bone Marrow Cells

The distilled water negative controls showed micronucleus indices of 9.20 and 12.5 micronucleus/1000 cell, as a consequence of pre- and post-treatment with cyclophosphamide, respectively. The aqueous extract was able to modulate such effects in both types of treatments, and a significant ($P \leq 0.01$) reduction was observed (7.00 and 7.25 micronucleus/1000 cell, respectively), but the post-treatment had a more effect than pre-treatment in such reduction as judged by the assessment of treatment efficiency of (-42.0 and -23.9%, respectively) (Table 3-27).

Table 3-27: Micronucleus formation in albino male mice after interactions (pre- and post-treatments) between the ideal dose (1.5mg/kg) of aqueous extract of *P. lanceolata* leaves and cyclophosphamide.

Groups	Mean ± S.E.* (micronucleus/1000 cells)		Probability ≤	Treatment Efficiency (%)	
	Pre	Post		Pre	Post
Control	9.20 ±0.47	12.50 ± 0.65	0.01		
Plant Extract	7.00 ± 0.40	7.25 ± 0.20	N.S.	-23.9	-42.0
Probability ≤	0.01	0.01			

N.S.: Not significant

3.3.7 Total IgG Level in Serum

The Total IgG level in serum was significantly increased as a consequence of pre-treatment (167.5 *vs*. 146.0 ng/ml), as well as, post-treatment (154.5 *vs*. 140.4 ng/ml) with the aqueous extract as compared to the corresponding controls. The treatment efficiency of pre-treatment was better than the corresponding one of post-treatment (+14.7 *vs*. +10.0%) (Table 4-28).

Table 3-28: Total IgG level in serum of albino male mice after interactions (pre- and post-treatments) between the ideal dose (1.5mg/kg) of aqueous extract of *P. lanceolata* leaves and cyclophosphamide.

Groups	Mean ± S.E.* (Ng/ml)		Probability ≤	Treatment Efficiency (%)	
	Pre	Post		Pre	Post
Control	146.0 ± 0.2	140.4 ± 0.1	0.01		
Plant Extract	167.5 ± 0.6	154.5 ± 2.0	0.001	+14.7	+10.0
Probability ≤	0.001	0.001			

3.3.8 Arthus Reaction

In this treatment, the aqueous extract was significantly effective in enhancing the values of Arthus reaction index (1.40 mm), as compared to negative control (1.2 mm) or positive control (1.15 mm) (Table 3-29).

Table 3-29: Arthurs reaction in albino male mice treated with the aqueous extract of *P. lanceolata* leaves, distilled water (negative control) or cyclophosphamide (positive control).

Groups	Dose (mg/kg)	Mean ± S.E* (mm)	Treatment Efficiency (%)
Control		1.20 ± 0.12[a]	
Cyclophosphamide	1.5	1.15 ± 0.06[a]	-4.1
Plant extract	15	1.40 ± 0.07[b]	+16.7

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

3.3.9 Delayed Type Hypersensitivity Reaction (DTHR)

The aqueous extract enhanced the DTHR index significantly as compared to negative control (0.892 *vs.* 0.592 mm) with a treatment efficiency of 50.6%, but a much more enhancement was observed with cyclophosphamide (1.415 vs. 0.592%) with a treatment efficiency of 138.8% (Table 4-30).

Table 3-30: Delayed type hypersensitivity in albino male mice treated with aqueous extract of *P. lanceolata* leaves, distilled water (negative control) and Cyclophosphamide (positive control).

Groups	Dose (mg/kg)	Mean ± S.E (mm)	Treatment Efficiency (%)
Control		0.5925 ± 0.0322[a]	
Cyclophosphamide	1.5	1.415 ± 0.0371[c]	+138.8
Plant extract	15	0.8925 ± 0.0353[b]	+50.6

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

Chapter Four
Discussion

Plants are a source of different chemical compounds, which make them of a medicinal importance. These compounds are divided into two types; inert and active constituents, depending on their activity. The inert constituents are defined as compounds that have no medicinal or physiological effects; for instance cellulose, lignin and subrine, while active constituents have these effects. The active constituents in turn are divided into further types, which are alkaloids, tannins, carbohydrates, volatile oils, saponines, steroids and flavonoids, depending on their chemical and physical characteristics (European medicines agency, 2008). Chemical analysis of *Plantago lanceolata* leaf aqueous extract revealed its richness in some of these constituents (tannins, polysaccharides, glycosides, flavonoids, saponines and terpens). Such findings are in agreement with the results of Samuelsen (2000), who reported that the aqueous extract of *P. lanceolata* leaves contains these compounds, in addition to vitamins C and E. Such findings may justify some of the immunological and anti-mutagenic potentials that were investigated and demonstrated in the present study. In this regard, the results demonstrated that the plant extract was effective in increasing the total leucocyte count, as well as, the absolute counts of lymphocytes, neutrophils and monocytes, and it was able to modulate the suppressive effect of cyclophosphamide, especially in the lowest dose. The detected active constituents can justify these findings, especially if we consider that polysaccharides, flavonoids, terpens, saponins and glycosides have important role as immune stimulators (Williams *et al*., 2002).

These augmentations of immune responses involve mainly four immune cells (neutrophils, macrophages and lymphocytes), and the positive effect of *P. lanceolata* leaf extract on these cells, numerically or functionally, can be

explained in the light of active constituents that act either separately or synergistically in enhancing the responsiveness of these cells directly or indirectly (Gabhe *et al.*, 2006).

The neutrophils are important cellular component of the innate immune system, and they are involved in carrying out phagocytosis, and such function is also shared by monocytes that are also called macrophages in tissues (Herant *et al.*, 2003). Both types of cells were significantly increased due to a treatment with the aqueous plant extract, especially at the lowest dose (1.5 mg/kg). These cells are originated in the bone marrow through the myeloid lineage progenitor, which is the outcome hemopoietic stem cell profilration. The latter outcome was investigated in term of bone marrow metaphase index, which was significantly increased in mice treated with the lowest dose of plant extract, and therefore, their absolute peripheral count was expected to be increased. The question is how the plant extract exerted such effect. The answer can be augmented if we consider that *P. lanceolata* aqueous extract contain polysaccharides, especially if we consider the suggestion of Biringanine *et al.* (2004), in which the investigators depicted that *Plantago species* leaves have some therapeutical activities that could depend on their content of polysaccharides, furthermore, it has been recently demonstrated that polysaccharides isolated from another medicinal plant (*Ganoderma lucidum*) accelerated the recovery of bone marrow cells and total leucocyte count in cyclophosphamide-immunosuppressed mice (Zhu *et al.*, 2007).

The forthcoming cells also showed a functional enhancement, which was manifested as a significant increase in the phagocytic index of peritoneal cells of mice treated with the lowest dose of the plant extract. Again, such effect has been explained in the ground of polysaccharide content (Im *et al.*, 2006). The investigators have demonstrated that polysaccharides purified from the hot aqueous extract of *Salicornia herbacea* enhanced the phagocytic activity of mouse monocytic cell line, and the effect was ascribed to the synergistic

action of the plant polysaccharides and interferon-γ (IFN-γ). Furthermore, they suggested that this action is due to an increased production of tumour necrosis factor-α (TNF-α) and nitric oxide (NO). Such suggestion has been more recently demonstrated, in which polysaccharides isolated from *Glycyrrhiza uralensis Fisch* significantly induced NO production and inducible NO synthase transcription in the culture of peritoneal macrophages collected from male mice treated with these polysaccharides (Cheng *et al*., 2008). These findings came in agreement with a previous report that demonstrated the immunostimulatory activity of *Plantago palmata* polysaccharides through their capacity to promote the production of NO and TNF-α by IFN-γ -activated murine cell line macrophages (Biringanine *et al*., 2004). The action of polysaccharides can also be through their effect in the differentiation-inducing, because it has been recently demonstrated that polysaccharides isolated from *Salicornia herbacea* were able to induce differentiation of monocytes to macrophages by morphological changes from slightly adherent monocytic cells to strongly adherent macrophages (Im *et al*., 2006).

Additionally, medicinal plants rich in flavonoids and glycosides, which were detected in the *P. lanceolata* aqueous extract, are further constituents that have a positive effect on the index of phagocytosis (Al-Zendi, 2006). These constituents may exert their effect on phagocytes through providing the cells with the energy required for such process, or enhance their killing ability; however, the latter function was not investigated in the present study. In agreement with this scope, it has been demonstrated that flavonoids and glycosides can modulate the macrophage function and enhance the cellular immune response, in addition to their contribution to the integrity of the immune system function through their antioxidant activity (Lu and Foo, 2000; Rao *et al*., 2003). The effect may be indirect, and other cells may be involved such as lymphocytes, which act as a regulator of the immune response

through their secretion of cytokines, which modulate the cellular functions of the immune system like phagocytosis and the induction of B-cells to produce antibodies (Roitt and Rabson, 2000).

The latter subject (lymphocyte count and function) was also investigated in the present study, although the augmentation was indirect. It was firstly determined by a significant increase of peripheral lymphocyte count, and then by a significant increase of spleen cell metaphase index and total serum IgG level. In the spleen, the main immune cells are T and B lymphocytes, as well as, antigen presenting cells, and normally the first two types of cells are divided in response to antigenic challenge presented by antigen presenting cells (Abbas and Lichtman, 2003). Therefore, if there is an increase in the metaphase index of spleen cells, it must involve the T and B lymphocytes. T lymphocytes are involved in cellular immune response, while B lymphocytes are the main arm of humoral immune response (Abbas and Lichtman, 2003). The cellular immune response was evaluated with delayed-type hypersensitivity reaction, which showed a significant increase in mice treated with the plant extract and injected with sheep red blood cells. Therefore, the cellular adaptive immune response was enhanced due to such treatment. The humoral immune response was also enhanced as suggested by the results of Arthus reaction and total IgG serum level. Such positive effects can also be ascribed to the plant polysaccharides, because it has been recently demonstrated that polysaccharides isolated from *Ganoderma lucidum* induced activation of both T and B lymphocytes (Zhu *et al*., 2007).

The enhancement of the immune system can also be explained in the ground of Saponins, which are a further constituent of *P. lanceolata* and they were detected in the present extract. It has been demonstrated that plants contain saponins can modulate the function of the immune system, due to their action in stimulating the cell-mediated immunity and activating the production of different cytokines, especially those involved in the cellular

immune response. Therefore the present effect of the plant extract can also be explained in this context (Francis *et al.*, 2002).

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

The function of immune system is also genetically determined, and alternations (mutations) in the genetic make-up of animals do have their effect on such function (Abbas and Lichtman, 2003). To explore the effects of *P. lanceolata* leaf aqueous extract on the genetic make-up of mice directly or through interactions with the drug cyclophosphamide, two assessments were carried out; they were micronucleus formation in bone marrow polychromtic erythrocytes and sperm-head abnormalities, which both are good parameters of mutagenic evaluations (Ghaskadbi and Viayda, 1991). The results of genetic evaluations showed that a treatment with the plant extract was associated with a significant reduction in micronucleus formation and sperm head abnormalities especially 35 days post-treatment when a complete cycle of sperm formation is achieved. Such findings can be considered important, especially if we consider that process of carcinogenesis is preceded by mutations induced by different agents, especially those that have oxidant effects (Yaseen, 1990; Ad'hiah *et al.*, 2002). Numbers of studies have been conducted in regard to the antioxidant activity of *P. lanceolata*, and in general

their findings are in agreement with the present results. Mimica-Dukic (2001) demonstrated that phenolic diterpenoids extracted from the plant showed a strong anti-oxidant activity, and they were potent antioxidants that prevent the formation of free radicals. Free radicals have the ability to cause damage to the DNA and RNA and inhibit some enzymes from reacting with amino acids (Salganik, 2001).

The subject of anti-oxidant activity and ability of scavenging free radicals is further highlighted, if we consider other constituents of the plant that have these effects. These constituents are glycosides and flavonoids, which possess anti-mutagenic activity, in addition to their role in the detoxification of mutagenic compounds (Kojima and Kuroda, 1992; Manach *et al*., 1996). These finding have also been further augmented to be involved in increasing the mitotic index of bone marrow and spleen cells, which was observed in the present study (Manach *et al*., 1996; Cook and Samman, 1996). Such effects can be achieved through several metabolic pathways; for instances, inhibit the formation of free radicals, suppress chain initiation and/or breaking chain propagation reaction, increasing the activity of detoxifying enzymes such as glutathione-S-transferase (GST) and superoxide dismutase (SOD) (Noguchi *et al*., 2000).

Free radicals are the possible result of membrane lipid peroxidation. Membrane lipids (linoleic acid and arachidonic acid) are rich in unsaturated fatty acids that are most susceptible to oxidative processes. Flavonoids, which were detected in the extract of *P. lanceolata*, have free radical-scavenging activities that may directly react and quench peroxide radicals to terminate the peroxidation chain reaction and improve the quality and stability of food products (Burda and Oleszek, 2001). Additionally, several flavonoids fix the antioxidant potentials of ascorbic acid by the retarding the conversion of ascorbate to dehydroascorbate, and this is based on the ability of flavonoids to act as free radical acceptors since free radical formation is considered to be an

important phase of ascorbate oxidation (Miller, 1996). Furthermore, the present plant extract is rich in vitamin C as demonstrated by Newall *et al*. (1996), but it was not examined in the present study. Vitamin C can protect plasma lipid and membrane lipid from the effect of oxidant compounds through either increase the production of cytokines or interact with the formation of prostaglandins, and both consequences are in favour of immune system enhancement, especially the antibody production (Hughes, 2001), and these outcomes confirm the increased IgG serum level and the significant increase of metaphase index in spleen cells.

The forthcoming effects were dose-dependent, and the significant enhancement of the investigated parameters was better in the lower dose of the plant extract, while higher doses were in favour of some cytotoxic activity, especially the highest dose (4.5 mg/kg), in which the total and absolute counts of leucocytes, phagocytosis and mitotic index of bone marrow and spleen cells were decreased as compared with the lowest dose (1.5 mg/kg) or negative control, while a significant increase in micronucleus formation and sperm head abnormality was observed in the lowest dose. Such effects of highest doses can also be explained in the ground of a chemical constituent, especially the flavonoids. 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 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).

Accordingly, the dose 1.5 mg/kg *P. lanceolata* leaf aqueous extract was considered as the ideal dose in the present study, which was further investigated to explore its potentials against the immune suppressive and mutagenic effects of cyclophosphamide through the pre-treatment interaction or post-treatment interaction. The results of both types of interactions showed a significant modulation of the drug effects with respect to the investigated parameters, especially genetic parameters, which are the more important, because the genetic make-up and its integrity can be considered the key in controlling the biological functions (Shapiro, 1999).

The integrity of genetic make-up depends on several repair mechanisms; such as, excision repair, photo-reactivation repair, post-replication repair, error- prone repair and error-free repair, and any of these mechanisms can be considered as a target for medicinal plants or their products in terms of enhancement or reduction (Patel and Prince, 2000). In pre-treatment interaction, the plant extract was able show a significant positive effect with respect to most parameters investigated, and the results determined the efficacy of *P. lanceolata* leaf extract treatment for enhancing the activity of immunological effector cells and haematopoiesis in cyclophosphamide-induced immunosuppressed mice. The treatment with the plant extract resulted in a significant increase of total leucocyte count, the absolute counts of lymphocytes, neutrophils and monocytes, as well as, the metaphase index of bone marrow cells. Furthermore, the metaphase index of spleen cells was also increased, and such observation suggests that T and B cell proliferation responses were also enhanced, and the results of Arthus reaction, delayed-type hypersensitivity reaction and total serum IgG level confirm such suggestion. These findings have been the subject of a recent investigation (Zhu *et al.*, 2007) in which polysaccharides purified fro another medicinal

plant (*Ganoderma lucidum*) were able to modulate the immunosuppressive action of cyclophosphamide, and accordingly, the total leucocyte count and mitotic index of bone marrow cells, as well as, the T and B lymphocyte response against mitogens were restored after the cyclophosphamide treatment. The same argument can be raised, but this time is based on to the action of flavonoids, which may be linked with the inhibition of microsome enzymes (activator enzymes for mutagens) (Francis *et al.*, 2002). Several flavonoids affect specifically the function of enzyme systems critically involved in the generation of inflammatory processes, especially tyrosine (Nishizuka, 1988) and serine-threonine protein kinases (Hunter, 1995). More 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 (Middleton *et al.*, 2000).

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

In post-treatment interaction, the ideal dose of plant extract showed a significant efficiency in reducing the mutagenic effects of cyclophosphamide, and again these actions can be attributed to the chemical constituents of the plant extract with regard to the forthcoming repair mechanisms. It has been

found that flavonoids of plants can enhance the post-replication repair (Kuroda *et al*., 1992), while others have demonstrated that tannins, flavonoids and terpens can stimulate the mechanism of error-free repair (Sasaki *et al*., 1998). Furthermore, terpens can activate recombinational repair mechanism, beside their action in activating the detoxification enzymes (Elson and Yu, 1994; Burke *et al*., 1997).

Treatment with the ideal dose of the extract before the drug (pre-treatment interaction) provided protection ratios more than these ratios when it was given after drug treatment (post-treatment interaction). Therefore, it is possible to conclude that the aqueous extract of *P. lanceolata* could be classified as "desmutagen" in the first order, and "bioantimutagen" in the second order from the point view genetics, as well as, the present aqueous extract of *P. lanceolata* leaves can be considered as an immune modulating agent.

Conclusions

Based on the finding of the present study, it possible to conclude:

1. The aqueous extract of *P. lanceolata* leaves is a good immune modulator and has the potential to be anti-mutagenic.
2. Such effects were a dose-dependent, and the lower doses were better than the higher doses in this regard.
3. The higher doses cannot be ignored with respect to their cytotoxic effects, especially if they are considered in terms of anti-cancer agents.
4. A further related conclusion can be augmented in term of the protective effect of the extract against immune suppressive and mutagenic agents. In this regard, the plant extract is desmutagen in the first order and bioantimutagen in the second order.

Recommendations

Based on the forthcoming conclusions, the following suggestions are recommended:

1. The synergistic effects of the plant extract constituents require a further exploration in terms of immune modulator and anti-mutagenic potentials,
2. The immune modulator potentials can be better understood in terms of cytokine profiles and immune cell phenotyping using the CD markers.
3. The anti-mutagenic potentials can be further characterized by other cytogenetic methods, for instance sister chromatid exchanges, and the effect on cell cycle progression is also important in this regard.
4. The latter recommendation can be expanded to understand the plant potential as anti-carcinogen and/or anti-cancer. Such

potential can be investigated *in vitro* (cancer cell lines) and *in vivo* (animal models).

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Appendix I: General laboratory equipments.

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

Appendix II: Chemical materials.

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

خلاصة

صممت الدراسة الحالية لدراسة بعض التأثيرات المناعية والوراثية-الخلوية للمستخلص المائي لنبات اذن العنزة (*Plantago lanceolata*) (in vivo) وعقار سايكلوفوسفومايد. شمل الجانب المناعي المعايير الآتية: العدد الكلي والتفريقي لخلايا الدم البيض وعملية البلعمة لخلايا الخلب والمستوى الكلي للغلوبيولين المناعي IgG في مصل الدم وتفاعلات فرط الحساسية العاجلة والآجلة ومعامل انقسام خلايا نقي العظم والطحال في الطور الاستوائي, بينما شمل الجانب الوراثي-الخلوي معامل تكون النوى الصغيرة في خلايا نقي العظم وتشوهات رؤوس النطف. كما اجري الكشف الكيميائي للدباغيات والسكريات المتعددة والفلافونات والصابونيات والقلويدات والتربينات في المستخلص المائي.

تضمنت الدراسة أربعة مراحل أساسية, شملت المرحلة الأولى دراسة التأثير الوراثي-الخلوي والمناعي للمستخلص المائي للنبات بثلاثة جرع (1.5 و 3 و 4.5 ملغم/كغم) وعقار سايكلوفوسفومايد بجرعة 15 ملغم/كغم, وكان التجريع لمدة سبعة أيام (جرعة واحدة لكل يوم) في حين شرحت الحيوانات في اليوم الثامن لغرض إجراء التقييمات الأنفة الذكر. أجري في المرحلة الثانية تداخل ما بين المستخلص المائي للنبات والعقار بعد اختيار الجرعة المثلى (1.5ملغم/كغم) من خلال نوعين من التداخل (قبل وبعد المعاملة بالعقار). في المرحلة الثالثة، أعطيت للحيوانات جرعة واحدة من المادة المستخلصة لكل مجموعة في اليوم الأول ثم شرحت الحيوانات في الأيام 7 و21 و35 لتقيم نسبة التشوه في رؤوس النطف. كان التجريع في المراحل السابقة عن طريق الفم .آما المرحلة الرابعة, فقد اختبرت نوعين من التفاعلات المناعية وهي تفاعلات فرط الحساسية العاجلة والآجلة على الفئران الممنعة بكريات الدم الحمر للخروف بطريق الحقن في غشاء الخلب وذلك بحقنه0.1 مل من 10% من معلق الخلايا. أجريت هذه التفاعلات بعد تجريعها عن طريق الفم

بالجرعة المثلى (1.5 ملغم/كغم) من المستخلص النباتي أوعقار سايكلوفوسفومايد (15 ملغم/كغم) أو الماء المقطر.

توصلت الدراسة إلى النتائج الآتية:

1. أظهرت نتائج الكشف الكيميائي للمستخلص المائي بأنها كانت موجبة للدباغيات والسكريات المتعددة والفلافونات والصابونيات والقلويدات والتربينات.
2. أظهر عقار سايكلوفوسفومايد تأثيرات مثبطة واضحة للاستجابة المناعية ومطفرة وراثيا وذالك من خلال خفض معامل الانقسام وعملية البلعمة وتفاعل ارثس وتفاعل فرط الحساسية الآجل وزيادة تكون النوى الصغيرة وتشوهات رؤوس النطف, فضلا عن ذالك فقد انخفض العد الكلي والتفريقي لخلايا الدم البيض بالمقارنة مع حيوانات السيطرة السالبة.
3. أظهرت نتائج المرحلة الأولى والثالثة والرابعة قابلية نبات إذن العنزة على تحفيز الاستجابة المناعية المتاصلة والمكتسبة بالمقارنة مع نتائج السيطرة السالبة (ماء مقطر) والسيطرة الموجبة (عقار السايكلوفوسفوامايد)، كما انخفض معدل تكون النوى الصغيرة وتشوهات رؤوس النطف، واعتمد التأثير على جرعة النبات. وفي كل الحالات كانت الجرعة الواطئة (1.5 ملغم/كغم) أكثر كفاءة من الجرع العالية (3 و 4.5 ملغم/كغم). وفي ضوء ذالك اعتبرت الجرعة الأولى كجرعة مثلى في التجارب اللاحقة.
4. أظهرت نتائج المرحلة الثانية بان الجرعة المثلى (1.5ملغم/كغم) للمستخلص كفاءة عالية في حماية الجهاز المناعي والمادة الوراثية من التأثير السلبي للعقار, وفي هذا الصدد كانت المعاملة بالمستخلص قبل العقار اكثر كفاءة من بعد المعاملة بالعقار.

بسم الله الرحمن الرحيم

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صدق الله العظيم

سورة البقرة ألاية "٣٢"

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة النهرين
كليه العلوم
قسم التقانه الاحيائية

دراسة بعض التأثيرات المناعية والوراثية- الخلوية لمستخلص المائي لنبات اذن العنزه (*Plantago lanceolata*) في ذكور الفأر الأبيض

رسالة

مقدمة إلى كلية العلوم جامعة النهرين

وهي جزء من متطلبات نيل درجة ماجستير علوم في التقانة الإحيائية

من قبل

حنين عبد الباقي محمد العلي

بكالوريوس تقانة إحيائية جامعة النهرين(2005)

بإشراف

المرحوم أ.م.د. محمد رفيق توفيق

أ.م.د.علي حسين أدحيه

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