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Supervisor Certification

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

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Chapter One

Introduction

1-1: Introduction

Interest in medicinal plants has burgeoned due to increased efficiency of new plant-derived drugs and the growing interest in natural products. Because of the concerns about the side effects of conventional medicine, the use of natural products as an alternative to conventional treatment in healing and treatment of various diseases has been advanced in the last few decades (Fong, 2002).

The use of plants as medicines dates from the earliest years of man's evolution. Medicinal plants serve as therapeutic alternatives, safer choices, or in some cases, as the only effective treatment. People in separate cultures and places are known to have used the same plants to treat similar medical problems (Dattner, 2003). A larger number of these plants and their isolated constituents have shown beneficial therapeutic ef[illegible] inflammatory, anti-cancer, anti-microbia[illegible] (Huffman, 2003).

Traditional cultures, without the benefits of modern research, somehow understood that culinary spices and herbs added more to food than flavor. They knew that certain spices and herbs were important for health and longevity. Today, scientists have identified the unique compounds responsible for these benefits. Chemical constituents with antioxidant activities were found in high concentrations in plants, and their considerable role in the prevention of various degenerative diseases has been determined, especially their capabilities in deactivating free radicals (Madsen and Bertelsen, 1995). Accordingly, there has been an upsurge of interest in the therapeutic potentials of medicinal plants as antioxidants with the potential to reduce tissue damages induced by free radical (Hu and Willett, 2002).

One culinary herb with profound health promoting properties is oregano (*Origanum vulgare* L). This botanical treasure, as some scientists describe it, from the Lamiaceae family was used internally and externally by ancient Greeks to restore the balance of body, especially the respiratory system. However, recent investigations have verified that oregano is a potent immune enhancer that supports the immune response against pathogens, and this is reasoned by the fact that the plant is rich in phenolic compounds and volatile oils (Force, 2000). Therefore, oregano (leaves and dried herb) is widely employed in medicinal applications by South European and Mediterranean populations to treat indigestion and stomach upsets, influenza, mild feverish illnesses, respiratory tract disorders, cold, coughs, and bronchial mucous membrane inflammation, furthermore, it has been demonstrated that the plant is rich in cancer fighting quercetin, in addition to its anti-microbial, anti-parasitic and anti-fungal properties (Ling and Wang, 2001).

cells, micronucleus formation in bone marrow cells and sperm-head abnormalities), biochemical (glutamate oxaloacetate transaminase; GOT, glutamate pyruvate transaminase; GPT, alkaline phosphatase; ALP and total bilirubin in serum) and histopathological (liver) effects of two extracts (methanol and hexane) of the plant *Origanum vulgare* on albino male mice. Interactions between the plant extracts and carbon tetrachloride (CCl_4) were also made for a further evaluation of the plant extract effects on the forthcoming parameters.

Chapter Two

Literature Review

2-1: Medicinal Plants

The use of traditional medicine and medicinal plants in most developing countries, as a normative basis for the maintenance of good health, has been widely observed (UNESCO, 1996). Furthermore, an increasing reliance on the use of medicinal plants in industrialized societies has been traced to the extraction and development of several drugs and chemotherapeutics from these plants, as well as, from traditionally used rural herbal remedies. Moreover, in these societies, herbal remedies have become more popular in the treatment of minor ailments (UNESCO, 1996).

Since ancient times, natural products notably those from plant origin, have consistently been an important source of therapeutic agents. Currently, about 25–30% of the available therapeutic agents are natural products or their derivatives (Newman *et al.*, 2003). More recently, the World Health Organization (WHO) has shown that about 80% of the world's populations still rely on traditional medicine, and natural products still play a very important role in the medicine of the remaining 20% of the world's populations (Clardy and Walsh, 2004).

The commercial development of plants as sources of antioxidants to enhance health and food preservation is of a current interest, and epidemiological studies have suggested positive associations between the consumption of phenolic-rich foods or beverages and the prevention of diseases (Scalbert and Williamson 2000). These effects have been attributed to antioxidant components such as plant phenolics, which include flavonoids and phenylpropanoids.

Herbs, spices and aromatic plants are good sources of compounds with antioxidant and antimicrobial properties, and among the aromatic plants, the

trade name “oregano” is used for different species of the genus *O*riganum (*O. vulgare*, *O. calcaratum*, O. *majorana*, *O. microphyllum*, *O. ramonense*, *O. rotondifolium* and *O. onites*) (Figue□re□do *et al*., 2006). These perennial plants, collected from the wild or cultivated in different countries, are widely used as spices. The characteristic compound of their essential oil is carvacrol, while their extracts with organic solvents are rich in rosmarinic acid and caffeic acid (Exarchou *et al*., 2001). It has been demonstrated that these plants have antioxidant, antimicrobial and anticytotoxic properties (Ipek *et al*., 2005).

2-2: *Origanum vulgare* L.

The genus *Origanum* is an annual, perennial and shrubby herb that is native to the Mediterranean, Euro-Siberian and Irano-Siberian regions (Aligiannis *et al*., 2001). A total of 38 Origanum species are recognized in the world, and most of them (around 75%) are concentrated in the East Mediterranean situation (Ganer *et al*., 2000). Due to the variability in chemical and aroma characteristics, Origanum plants belong [illegible] industries as a culinary herb, flavouring substances of food products, alcoholic beverages and perfumery for their spicy fragrance (Aligiannis *et al*., 2001). It has also been used as a traditional remedy to treat various ailments, for instance, a spasmodic, antimicrobial, expectorant carminative and aromatic for whooping and convulsive coughs, digestive disorders and menstrual problems (Sokovic *et al*., 2002; Daferera *et al*., 2003).

Origanum vulgare covers several sub-species, particularly the very widespread and common sub-species that are *O. vulgare* spp. vulgare, *O. vulgare* spp. viride and *O. vulgare* spp. hirtum (Sahin *et al*., 2004).

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2-2-1: Common Names and Taxonomy of *Origanum vulgare*

Many common names are used to describe the plant *O. vulgare*; for instance, Greek oregano, mountain mint, wild oregano, winter oregano and winter oregano (Internet, I), and in the present thesis the common name oregano will be used.

From the point view of taxonomy (Internet, II), the plant is classified as the following:

Kingdom: Plantae

Subkingdom: Tracheobionta

Superdivision: Spermatophyta

Division: Magnoliophyta

Class: Magnoliopsida

Subclass: Asteridae

Order: Lamiales

Family: Lamiaceae

Genus: *Origanum*

Species: *Origanum vulgare*

2-2 -2: Plant Description

Oregano is a bushy, semi-woody sub-shrub with upright or spreading stems and branches. Some varieties grow in mound like mats, spreading by underground stems (called rhizomes), and others with a more upright habit. The aromatic leaves are oval-shaped, about 3.8 centimeters long. Throughout the summer, oregano bears tiny purple tube-shaped flowers that are about 0.3 centimeter long. These peek out from whorls of purplish-green leafy 2.5 centimeters long bracts that resemble little pinecones (Figure 2-1). The plant prefers light (sandy), medium (loamy) and heavy (clay) soils, requires well-drained soil and can grow in nutritionally poor soil. The plant prefers acid, neutral and basic (alkaline) soils (Fetrow and Avila, 1999).

Figure 2-1. The plant *Origanum vulgare* (internet 1).

2-2-3. Medicinal Use

Oregano has been used as a culinary and medicinal herb for many years. It has a beneficial effect upon the digestive and respiratory systems and is also used to promote menstruation. The leaves and flowering stems are strongly antiseptic, antispasmodic, carminative, cholagogue, diaphoretic, expectorant, stimulant, stomachic and mildly tonic (Novak *et al*., 2000). The plant is taken internally in the treatment of colds, influenza, mild feverish illnesses, indigestion, stomach upsets and painful menstruation. It is strongly sedative and should not be taken in large doses, though mild teas have a soothing effect and aid restful sleep. Externally, oregano is used to treat bronchitis, asthma, arthritis and muscular pain (Aligiannis *et al*., 2001). Oregano can be used fresh or dried, and it is often used in the form of an essential oil that is distilled from the flowering plant. This plant is one of the best natural antiseptics because of its high thymol content (Daferera *et al*., 2003).

2-2-4: Active Compounds

Different active compounds have been detected as constituents of oregano including:

- **Volatile oil** (0.15–1.0 %): The chief components are carvacrol (40-70%), gamma-terpinene (8-10%) and p-cymene (5-10%), in addition to alpha-pinene, myrcene, tymol. There are also strains with thymol, linalool, caryophyllene or germacren D (Kulisic *et al.*, 2004; Figue□re□do *et al.*, 2006).
- **Flavoniods**: They include luteolin, hispidulin, apigenin, acacetin, diosmetin, herbacetin, quercetin and naringin (Justesen and Knuthsen, 2001; Choi et al., 2002; Cavero *et al.*, 2006).
- **Caffeic acid derivatives**: They are presented mainly by rosmarinic acid (Koukoulitsa *et al.*, 2006).
- **Sesequiterpenes**: They are presented mainly by alpha-humulene (Koukoulitsa *et al.*, 2006).
- **Triterpenoids**: They include ursolic and oleanolic acid (Liu, 2005).
- **Sterols** (Hammer *et al.*, 1999).
- Oregano is also a rich source of a variety of vitamins and minerals, especially vitamins A and C (Ingram, 1995).

2-2-5: Biological Potentials and Pharmaceutical Applications

Phytopharmacological studies of different oregano extracts have demonstrated several biological potentials and pharmaceutical applications, which are antimicrobial, antioxidant, anti-mutagenic and anti-tumor.

2-2-5-1: Antimicrobial Properties

Several investigators have demonstrated that oregano or its extracts are rich in compounds with antimicrobial properties, and in this regard, the essential oils

of the plant, caryophyllene and germacrene-D have been evaluated against a wide range of microorganisms, and the potential of antibacterial and antifungal activities has been observed. Accordingly, it has been recommended to use the plant or its derivatives as natural preservatives in food against the well known causal agents of food borne diseases and food spoilage such as *Escherichia coli*, *Enterobacter* spp., *Bacillus* spp., *Salmonella* spp., *Staphylococcus aureus*, *Candida* spp., *Fusarium* spp., *Aspergillus* spp., *Rhizopus* spp., and *Penicillium* spp. isolates (Kalodera *et* al., 1997; Isman *et al*., 2001; Kazarinova *et al*., 2002; Simic *et al*., 2002). Furthermore, antiviral activities have also been suggested, and oils extracted from oregano have been able to destroy the RNA and DNA of some viruses, including the types causing shingles, cold sore and genital herpes (Internet, IV).

rich in thymol and carvacrol, has a considerable antioxidant effect on the process of the lard oxidation. However, generally the antioxidant activity of the oregano essential oil is less effective than the ascorbic acid, but comparable with the α-tocopherol and the synthetic antioxidant butylated hydroxytoluene. (Lagouri *et al*., 1993; Tsimidou and Boskou, 1994). Similar antioxidant activity has also been demonstrated by hexane extract of oregano (Yanishlieva and Marinova, 1995).

2-2-5-3: Anti-mutagenic and Anti-tumor Activities

Interestingly, oregano leaf extracts and oils have the capacity to inhibit cancer cell proliferation *in vitro*, and such effect has been correlated with the

antioxidant properties of the plant and its constituents (Olsson and Gustavsson, 2004). Chemical analysis of these constituents revealed that they are monoterpenes, biogenetically related phenolics (thymol and carvacrol), as well as, sesquiterpenes, which are responsible for most of the biological activities (Sivropoulou *et al*., 1996; Puertas-Mejia *et al*., 2002; Daferera *et al*., 2003; Kulisic *et al*., 2004; Nostro *et al*., 2004; Bagamboula *et al*., 2004). In this regard, carvacrol is extensively studied. It has been demonstrated that carvacrol is able to inhibit the growth of melanoma, as well as, the DMBA-induced tumor in rats *in vivo* (He *et al*., 1997; Zeytinoglu *et al*., 1998), and similar antimutagenic activities have been demonstrated in Ames test (Stammatia *et al*., 1999). Such findings have been further confirmed in human lymphocyte and mouse myoblast cultures (Zeytinoglu *et al*., 2003; Ipek *et al*., 2004). Accordingly, oregano and its derivatives have been considered as strong anti-mutagenic and anti-tumor agents (Ipek *et al*., 2005).

2.3. Carbon Tetrachloride

Carbon tetrachloride (CCl_4) is a well-k
particularly toxic to the liver, where it causes hepatocellular degeneration and impairs different enzymatic systems. The generation of free radicals appears to be pivotal in CCl_4 hepatotoxicity. It is metabolized by cytochrome P-450 to produce the trichloromethyl radical, which initiates a cascade of free radical reactions resulting in an increase of lipid peroxidation and a reduction in some enzyme activities (Kim *et a*l., 1990; Valles *et al*., 1994). Many investigators have looked for protective agents against CCl_4 toxicity and a variety of compounds with potential antioxidant activity have been tested (Candelario-Jalil *et al*., 2001).

The toxic effects of CCl_4 on liver have been known for years and studied extensively. Acute and chronic renal damage are also very common pathophysiological disturbances caused by CCl_4. The effects of CCl_4 on hepatocytes, depending on dose and exposure time, are manifested histologically

as hepatic steatosis (e.g., fatty infiltration), centrilobular necrosis, and ultimately, cirrhosis. Hepatic steatosis of the liver is a multifactorial phenomenon thought to be caused by a blockage of lipoprotein secretion, impaired synthesis or peroxidation of phospholipids, or both (Guven *et al*., 2003). The endoplasmic reticulum and mitochondria have been shown to be involved in cell damage. The metabolic effects of CCl_4 inside mitochondria have been described, and it has been found that a damage of the calcium pump in mitochondria is dependent upon haloalkylation. However, the profound accumulation of fat following CCl_4 poisoning is considered to be independent of mitochondrial damage. The fatty infiltration of the liver is thought to develop as a result of the action of free alkyl radicals on biomembranes that in turn cause haloalkylation-dependent blocking at the exit of the lipoprotein micelles from the Golgi apparatus (Sarkar *et al*., 2006).

2-4: Antioxidants

Antioxidants are chemicals that reduce the rate of oxidation reactions, which are chemical reactions that involve the transfer of electrons from one substance to an oxidizing agent. Antioxidants can slow these reactions either by reacting with intermediates and halting the oxidation reaction directly or by reacting with the oxidising agent and preventing the oxidation reaction from occurring (Zheng and Wang, 2001). Antioxidants are particularly important in biology, and all organisms maintain a reducing environment inside their cells and contain complex systems of antioxidants to prevent damage by oxidation. These antioxidants include glutathione and ascorbic acid and these chemicals are substrates for enzymes such as peroxidases and oxidoreductases. Low levels of

antioxidants or inhibition of antioxidant enzymes causes oxidative stress and may damage or kill cells (Halliwell, 1993; Manna *et al.*, 2006).

The investigations suggest that antioxidant-rich foods, as well as, some medicinal plants and their derivatives can reduce damages to cells and biochemicals from free radicals. This may slow down, prevent, or even reverse certain diseases that result from cellular damage (Slater, 1984). In this regard, dietary phenolic antioxidants have been shown to play important roles in delaying the development of chronic diseases such as cardiovascular diseases, cancer, inflammatory bowel syndrome and Alzheimer's disease (Shetty, 1997; Akyon, 2002). Phenolic antioxidants are products of secondary metabolism in plants and are good sources of natural antioxidants in human diets (Botsoglou *et al.*, 2002). Aromatic plants such as herbs and spices are rich in their phenolic content, and have been widely used to extend the shelf life of foods (Adam et al., 2006), and as traditional medicine as healing for many diseases (Shetty, 1997). The antioxidant activity of phenolic compounds in plants is mainly due to their redox properties and chemical structure, which can play an important role in adsorbing and neutralizing free radicals, chelating transition metals and triplet oxygen, by delocalization or decomposing peroxides (Zheng and Wang, 2001; Pizzale *et al.*, 2002). These properties are linked to beneficial health functionality of phenolic antioxidants due to their inhibitory effects against development of many oxidative-stress related diseases such as cancer and diabetes (Huang *et al.*, 1992).

Knowledge on the protective mechanisms against toxin and drug induced organ-toxicities leads scientists to look for biologically active relevant compounds from herbal plants, which can possess intrinsic antioxidant activity and protect those organs from unwanted oxidative stress (de Mejia and Ramirez-Mares, 2002). The widespread use of traditional herbs and medicinal plants has been traced to the occurrence of natural products with medicinal properties. The roles of herbs in disease prevention and cure have been attributed, in part, to antioxidant properties of their constituents; liposoluble and water soluble

vitamins, and a wide range of amphipathic molecules (Morel *et al.*, 1994; Rice-Evans *et al.*, 1997)

The use of spices as antioxidants has a long history, and compounds from different medicinal plants, including oregano, have been demonstrated to be powerful antioxidants (Carrubba and Calabrese, 1998). One reason for the continued interest in examining the antioxidant effects of medicinal plants is the desire to find natural antioxidants that have minimal impact on the sensory characteristics of the food (Brown *et al.*, 2006).

2-4-1: Vitamin A

Vitamin A is a fat-soluble vitamin, which is generally derived from two sources, depending on whether the food source is an animal or a plant. Vitamin A found in foods that come from animals is called preformed vitamin A. It is absorbed in the form of retinol, which is one of the most active forms of the vitamin, and sources includes liver, whole milk, and some fortified food products. Vitamin A can be made into retinol in the body (de Pee and West

Vitamin A that is found in colorful fruits and vegetables is called provitamin A carotenoid, which can be made into retinol in the body. Common provitamin A carotenoids found in foods that come from plants are beta-carotene, alpha-carotene, and beta-cryptoxanthin. Among these, beta-carotene is most efficiently made into retinol. Alpha-carotene and beta-cryptoxanthin are also converted to vitamin A, but only half as efficiently as beta-carotene (Pavia and Russell, 1999).

Both type of vitamin A (preformed or as carotenoids) are required for a vast number of biological processes like vision and cellular growth. A major biologic function of vitamin A (as the metabolite retinal) is in the visual cycle, however, investigations also suggest that the vitamin may reduce the mortality rate from bacterial and viral infections, prevent some types of cancer, aid in

growth and development, and improve immune function (Ross and Gardner,1994; Koo, 1997; Semba, 1998).

Some provitamin A carotenoids have been shown to function as antioxidants in laboratory studies; however, this role has not been consistently demonstrated in humans. Antioxidants protect cells from free radicals, which are potentially damaging by-products of oxygen metabolism that may contribute to the development of some chronic diseases (Futoryan and Gilchrest,1994; Olson,1996; Pavia,1999).

Vitamin A deficiency diminishes the ability to fight infections, and in countries where such deficiency is common and immunization programs are limited, millions of children die each year from complications of infectious diseases such as measles (Ross,1992). In vitamin A-deficient individuals, cells lining the lungs lose their ability to remove disease-causing microorganisms, and this may contribute to the precipitous [illegible] associated with deficiency (Ross, 1999).

Dietary intake studies suggest an association between diets rich in beta carotene and a reduced lower risk [illegible] A higher intake of green and yellow vegetables or other food sources of beta carotene and/or vitamin A may decrease the risk of lung cancer (Koo, 1997). Much more recently, Al-Keenani (2005) has demonstrated that vitamin A is anti-mutagenic agent against the mutagen etoposide in albino male mice, and the vitamin enhanced some immunological functions.

2-4-2: Vitamin C

Vitamin C (ascorbic acid) is an essential micronutrient required for normal metabolic functioning of the body, however, humans and other primates have lost the ability to synthesize vitamin C as a result of a mutation in the gene coding for L-gulonolactone oxidase, an enzyme required for the biosynthesis of vitamin C via the glucuronic acid pathway (Woodall and Ames, 1997). Thus, vitamin C must be obtained through the diet. The vitamin is especially plentiful

in fresh fruit, in particular citrus fruit, and vegetables. A deficiency of the vitamin in the diet causes the disease scurvy (Bendich, 1997).

The molecular mechanisms of the antiscorbutic effect of vitamin C are largely, although not completely, understood. Vitamin C is a cofactor for several enzymes involved in the biosynthesis of collagen and neurotransmitters (Burri and Jacob, 1997). The activities of several other enzymes are known to be dependent on vitamin C, although their connection to scurvy has not yet been clearly established. These enzymes include the mono- and dioxygenases involved in peptide amidation and tyrosine metabolism. Vitamin C has also been implicated in the metabolism of cholesterol to bile acids via the enzyme cholesterol 7-monooxygenase. Hydroxylation of aromatic drugs and carcinogens by hepatic cytochrome P450 is also enhanced by reducing agents such as vitamin C (Tsao, 1997; Amy *et al*., 2006)

nitrogen species, such as superoxide and hydroperoxyl radicals, aqueous peroxyl radicals, singlet oxygen, ozone, peroxynitrite, nitrogen dioxide, nitroxide radicals, and hypochlorous acid, thereby effectively protecting other substrates from oxidative damage (Frei *et al*., 1990; Halliwell, 1996; Niki and Noguchi, 1997). Vitamin C can also act as a co-antioxidant by regenerating α-tocopherol (vitamin E) from the α-tocopheroxyl radical, produced via scavenging of lipid-soluble radicals (Packer, 1997).

2-5: Investigated Parameters

2-5-1: Total and Differential Counts of Leucocytes

Leucocytes are blood cells that are responsible for the immunological defense mechanisms of the organism, and originated in the bone marrow from the hematopoietic stem cell through two cell lineage; myeloid and lymphoid. Leucocytes are divided into polymorphonuclear cells, monocytes, which are both of the myeloid lineages and lymphocytes that are of a lymphoid lineage origin (Lydyard and Grossi, 1998).The polymorphonuclear cells, also referred as granulocytes, are recognized as neutrophils, eosinophils and basophils, which are different in their cytoplasm contents of granules, although they are functionally related. The granules have a different affinity towards neutral, acid or basic stains and therefore the cytoplasm is stained with different colors and in such basis, the granulocytes are recognized as the forthcoming three types. Functionally, neutrophils are very active in the cellular innate immune response through the [illegible] phagocytosis, however, eosinophils and basophils also can carry out phagocytosis but is no their main [illegible] and allergy, while basophils [illegible] reactions via secreting vasodilatory substances such as histamines and serotonin (Kramer, 2003).

Monocytes are the precursors of macrophages. They are larger blood cells, which after attaining maturity in the bone marrow, enter the blood circulation where they stay for 24-36 hours. Then, they migrate into the connective tissue, where they become macrophages and move within the tissues. In the presence of an inflammation site, monocytes quickly migrate from the blood vessel and start an intense phagocytic activity. The role of these cells is not solely in phagocytosis because they also have an intense secretory activity. They produce substances which have defensive functions such as lysozymes, interferons and other substances, which modulate the function of other cells. Macrophages cooperate in the immune defenses, and they expose molecules of digested

pathogens on the membrane and present them to more specialized cells, such as T and B lymphocytes (Lydyard and Grossi, 1998).

Lymphocytes are the main constituents of the specific immune response against the attack of pathogenic micro-organisms such as viruses, bacteria and fungi. Most lymphocytes circulating in the blood is in a resting state. They look like smaller cells with a compact round nucleus which occupies nearly all the cellular volume. As a consequence, the cytoplasm is much reduced. The lymphocytes of the lymphoid tissues and organs can be activated following antigenic stimulation (Hosein, 1999).

The total and differential counts of leucocytes can give a general picture of the immunity in the peripheral blood, because such counts are sensitive to infections (Ad'hiah *et al*., 2001a), environmental pollutions (Ad'hiah *et al*., 2001b), and chemical agents (Ad'hiah *et al*., 2004).

2-5-2: Mitotic Index

[illegible] the ratio [illegible] cells undergoing mitosis (different stages) to total [illegible]. It is a useful and a sensitive test for the detection of cytotoxic effects of chemical and physical agents, as well as, mutagenic and carcinogenic agents (Ad'hiah *et al*., 2004).

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). Recent reports have demonstrated that entry into mitosis in the presence of damaged DNA leads to inactivation of centromer, formation of aberrant spindles and blockage of chromosome segregation, which consequently delays mitosis progression and induces mitotic abnormalities (Hut *et al*., 2003; Takada *et al*., 2003). In addition, chemical or pharmacological inhibition of the DNA damage checkpoint at the G2 stage induces a premature entry into mitosis and a subsequent initiation of apoptosis (Sampath and Plunkett, 2001).

2-5-3: Micronucleus Formation

Micronuclei are small particles consisting of acentric fragraments of chromosomes or entire chromosomes, which lag behind at anaphase of cell division. After telophase, these fragraments may not be included in the nuclei of daughter cells and form single or multiple micronuclei in the cytoplasm (Schmid, 1976).

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). Erythrocytes expel their main nucleus before entering the bloodstream, making them ideal for measuring fragramented DNA. The *in vivo* micronucleus assay is capable of detecting compounds that induce clastogenic (chromosome breaking) and aneugenic (whole chromosome loss) activity. When cell division occurs, the chromosome fragaments or whole chromosomes are included in the [illegible] occur simultaneously, but aneu[illegible] in the number of micronucleus relative to the negative control (Torous *et al*., 2005).

2-5-4: Sperm-head Abnormality 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).

Abnormal sperm morphology is classified as defects in the head, midpiece or tail of the sperm. Head defects include large, small, tapered, pyriform, round, and amorphous heads, heads with a small acrosomal area (<40% of the head area) and double heads, as well as, any combination of these (Martin, 2003).

A sperm is basically a package of streamlined genetic information. Intuitively, one might expect that a change in chromosome content is reflected by a change in the size of sperm, thus, it is expected to see a relationship between sperm morphology and genetic abnormalities. Aberrations in the genetic make-up can be reflected in the head of spermatozoa, which then show different abnormal morphologies (Sun *et al*., 2006).

2-5-5: Liver Function Tests

2-5-5-1: Glutamic Oxaloacetic and Glutamic Pyruvic Transaminases

Enzymes are proteins that act as biological catalysts to speed up the rates at which chemical reaction occur by lowering the activation energy. Without [illegible] (Bruice and Benkovic, 1966).

Transamination means the process of transferring an amino group from an amino acid to a keto-acid. Enzymes which catalyze this type of reaction are named transaminases, and the most important transaminase enzymes in the diagnosis of a hepatic damage are glutamic-oxaloacetic-transaminase (GOT) and glutamic-pyruvic-transaminase (GPT) (Charles, 2003).

Many studies have investigated the effects of different drugs or chemicals in inducing hepatic damages and the role of medicinal plants in reducing these effects. Their evaluations were dependent on measuring the levels of GOT and GPT, for instance Kokdil *et al*. (2005) and Sanmugapriya and Venkataraman (2006).

2-5-5-2: *Serum Alkaline Phosphates*

Alkaline phosphatase (ALP) is an enzyme found in all tissues. Its function is to catalyze the hydrolysis of phosphate esters in an alkaline environment, resulting in the formation of an organic radical and inorganic phosphate (Reichling and Kaplan, 1988). Tissues with particularly high concentrations of ALP include the liver, bile ducts, placenta, and bone. Damaged or diseased tissues release the enzyme into the blood, so serum ALP measurements can be abnormal in many conditions, including bone disease and liver disease. Serum ALP is also increased in some normal circumstances (for example, during normal bone growth) or in response to a variety of drugs (Friedman *et al*., 1996).

Markedly elevated serum ALP, hyperalkalinephosphatasemia, is seen predominantly with more specific disorders, including, malignant biliary obstruction, primary biliary cirrhosis, primary sclerosing cholangitis, hepatic lymphoma and sarcoidosis (Neuschhwander-Tetri, 1995). On the other hand, according to the study of Mirfazaelian *et al*. (2006), [illegible] are considered as common causes of hyperalkalinephosphatasemia, whereas diffuse liver metastases, as well as, a number of benign disorders, are relatively less common causes of hyperalkalinephosphatasemia.

Several studies have investigated the correlation between CCl_4 and the level of serum ALP like the study of Manna *et al*. (2006), which indicated that the aqueous extract of *Terminalia arjuna* can prevent carbon CCl_4-induced hepatic and renal disorders by decreasing the marked rise in serum levels of GOT, GPT and ALP caused by CCl_4.

Another study indicated that the aqueous extract of *Asteracantha longifolia* possesses hepatoprotective and antioxidant properties against CCl_4- and paracetamol-induced hepatotoxicities, which resulted in increased levels of the enzymes GOT, GPT and ALP. An administration of the plant extract caused a significant reduction in the levels of these enzymes to the normal level (Hewawasam *et al*. 2003).

2-5-5-3: Serum Bilirubin

Bilirubin is a breakdown product of hemoglobin. Total and direct bilirubin are usually measured to screen for or to monitor liver or gall bladder dysfunction. Bilirubin metabolism begins with the breakdown of red blood cells. Red blood cells contain hemoglobin, which is broken down to heme and globin. Heme is converted to bilirubin, which is then carried by albumin in the blood to the liver (Stocker *et al*., 1987).

In the liver, most of the bilirubin is chemically attached to a glucuronide before it is excreted in the bile. This "conjugated" bilirubin is called direct bilirubin; unconjugated bilirubin is called indirect bilirubin. Total serum bilirubin equals direct bilirubin plus indirect bilirubin. If the bile ducts are obstructed, direct bilirubin will build up, escape from the liver, and end up in the blood. If the levels are high enough, some of it will appear in the urine. Similarly, if bilirubin appears in the urine. Increased direct bilirubin usually means that the [illegible] obstructed. [illegible] clinic-epidemiological studies [illegible] relationship between serum bilirubin levels and oxidative stress-mediated diseases, including in particular atherosclerotic disease (Schwertner *et al*., 1994; Wei *et al*., 2000).

In a recent study the hepatoprotective activity of *Pterocarpus marsupium* stem bark extracts against CCl_4-induced hepatotoxicity has been evaluated. In methanol extract-treated animals, the toxic effect of CCl_4 was controlled significantly by restoration of the normal serum levels of bilirubin in rats (Mankani *et al*., 2005).

A further study carried out by Sanmugapriya and Venkataraman (2006) indicated the hepatoprotective and antioxidant action of *Strychnos potatorum* seeds on CCl_4-induced acute hepatic injury in experimental rats by reducing the elevated levels of serum bilirubin caused by CCl_4.

Chapter Three

Materials and Methods

3-1: Materials

The general laboratory equipments and chemicals, which were employed in the present study, are presented in tables 3-1 and 3-2, respectively.

Table 3-1-1: General laboratory equipments.

Equipment	Company / Country
Autoclave	SES little Sister / England
Centrifuge	Beckman / England
Digital camera	Memmert / Germany
Electronic balance	Sartorius / Germany
Hemocytometer	Neubauer / Germany
Micropipette	Gilson / France
Microscope	Motic / Japan
Microtome	Gallenkamp / England
Oven	Osaw / India
pH meter	Radiometer / Denmark
Rotary evaporator	Buchi / Switzerland
Shaking water bath	Gallenkamp / England
Soxhlet	Electrothermol / England
Vortex	Griffin / England
Water bath	Gallenkamp / England

Table 3-1-2: Chemical materials and kits.

Chemical Material	Company / Country
Alkaline phosphatase Kit	Bio Merieux / France
Amonium alum	BDH / England
Bilirubin Kit	Giesse Company
Calcium chloride	BDH / England
Canada Balsam	BDH / England
Carbon tetrachloride	BDH / England
Chloroform	BDH / England
Eosin	BDH / England
Ethanol	Ferak / Germany
$FeCl_2$	Fluka / Switzerland
Glycerin	Fluka / Switzerland
Glacial acetic acid	Fluka / Switzerland
Glycerin	Fluka / Switzerland
[illegible]	Sigma
[illegible]	Randox Company/U.K
GPT Kit	Randox Company/U.K
Haematoxylin stain	BDH / England
Heparin	Leo Pharmaceutical / Denmark
Hexane	BDH / England
Hydrochloric acid	Sigma / U.S.A.
Lead acetate (CH3Coopb)	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 (NaHCO3)	BDH / England
Sodium hydroxide (NaOH)	Sigma / U.S.A.
Vitamin A (retinol)	Drugs factory of Samara / Iraq
Vitamin C (ascorbic acid)	Drugs factory of Samara / Iraq
Xylene	BDH / England

3-2: The Plant Oregano (*Origanum vulgare* L.)

3-2-1: Plant Collection and Identification

Dr. Ali Hussain Al-Musawe (Department of Biology, College of Science, University of Baghdad) identified the plant oregano (*Origanum vulgare* L.), which was collected from a botanical garden located in Palestine Street (Baghdad) in November 2005.

3-2-2: Preparation of Plant Extracts

The fresh leaves of oregano were cut into small pieces using a food processor for 5 minutes, and then extracted with two types of solvents, which were methanol and hexane. In both cases, 50g of the chopped leaves were extracted in 250 ml of the solvent (methanol or hexane) using the Soxhlet apparatus and the source of heating was a warm water bath (45°C). The obtained leaf extract solution was then evaporated at 45°C using a rotary evaporator, and the resultant crude extract was frozen at -20°C until use to prepare the required doses and concentrations (Nadir *et al*., 1986).

3-2-3: Oregano Doses and Concentrations

The methanol crude extract was dissolved in distilled water to prepare three intraperitonial doses (32, 64 and 96 mg/kg), which were investigated in the laboratory mice. These doses were correspondent to 10, 20 and 30%, respectively of the LD_{50} (320 mg/kg) (Internet, V).

The hexane crude extract was dissolved in olive oil, and as in the methanol extract, similar doses (32, 64 and 96 mg/kg) were prepared and investigated in laboratory mice.

3-3: Solutions

- **Potassium chloride (KCl) hypotonic solution (0.075M):** Potassium chloride (5.75g) was dissolved in 1000 ml of distilled water, and then the solution was autoclaved (121°C, 15 pound / in^2, 20 minutes) and stored at 4°C (Ullyot et al., 1977).

- **Leucocyte diluent (2%):** 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 color indicator (Sood, 1986).

- **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.

- **Fixative:** The solution was freshly prepared by mixing 3 parts of absolute methanol with 1 part of glacial acetic acid (Patton, 1967).

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

- **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. The tubes were placed in a water bath (56°C) for 30 minutes to inactivate the complement, and then stored at -20°C until use in the micronucleus assay (Schmid, 1976).

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

- **Vitamin A:** The Company of Samara Drug Industries supplied the vitamin powder, which was dissolved [illegible] 120 mg/kg (Giacosa *et al*., 1997).

- **Vitamin C:** The Company of Samara Drug Industries supplied the vitamin powder, which was dissolved in distilled water to prepare a dose of 120 mg/kg (Giacosa *et al*., 1997).

- **Carbon tetrachloride (CCl_4):** Carbon tetrachloride solution was diluted with an equal volume of olive oil (1:1) to prepare a dose of 3 ml / kg (3.2 mg/kg) (Lin *et al*., 1998).

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

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

- **Eosin stain (1%):** The stain was prepared by dissolving 1g of eosin yellowish powder in 100 ml of distilled water (Wyrobek and Bruce, 1975).

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

- **Jenner's Stain:** The stain was prepared according to Lillie and Ashburn (1943) and Lillie and Fullmer (1976). Five grams of Jenner's powder were dissolved in 50 ml of ethanol (95%), and 100g of ammonium alum were dissolved in 100 ml of distilled water and heated. Both solutions were mixed and heated to boiling, then cooled and 2.5g of mercuric oxide (red) was added, and the solution was reheated till the mixture showed deep violet color. The solution was cooled and filtered (Whatman filter paper No. 3), and before use, 3 ml of glacial acetic acid was added to each 100 ml of the prepared stain solution.

- **Mayer's Reagent:** Two solutions were firstly prepared; the first one was prepared by dissolving 1.58g 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).

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

- **Ferric chloride solution (1%):** The solution was prepared by dissolving 1g of ferric chloride in 100 ml distilled water. It was used for saponins detection.

- **Sodium hydroxide solution:** This solution was prepared by dissolving 40 g of NaOH in 100 ml of distilled water.

- **Potassium hydroxide solution:** It was prepared by dissolving 50g of potassium hydroxide in 100 ml of distilled water. This solution was used to detect the flavonoids.

3-4: Laboratory Animals

Albino Swiss male mice (*Mus musculs*) were used in the experiments. They were supplied by the Biotechnology Research Centre (Al-Nahrain University). Their age at the start of experiments was 8-10 weeks, and their weight was 23-27g. They were divided into groups, and each group was kept in a separate plastic cage (details of these groups are given in the section of experimental design). The animals were maintained at a temperature of 20- 25°C, and they had free excess to food (standard pellets) and water (*ad libitum*) through out the experimental work.

3-5: Experimental Design

The present study was designed to investigate the immunological, cytogenetic, biochemical and histopathological effects of oregano extracts (methanol and hexane) and their effects on CCl_4-induced acute hepatic injury in [illegible] such evaluation.

3-5-1: First Stage

In this stage, the immunological, cytogenetic, biochemical and histopathological effects of three doses of oregano extracts (methanol and hexane) and CCl_4 were investigated. Therefore, the animals were divided into seven groups:

- **Group I**: The animals were treated with distilled water (negative controls of methanol extract; 12 animals).
- **Group II**: The animals were treated with olive oil (negative controls of hexane extract; 12 animals).

- **Group III:** The animals were treated with vitamin C (positive control of methanol extract; 12 animals).
- **Group IV**: The animals were treated with vitamin A (positive controls of hexane extract; 12 animals).
- **Group V**: The animals were treated with CCl_4 (12 animals).
- **Group VI**: The animals were treated with three doses (32, 64 or 96 mg/kg) of methanol extract (36 animals).
- **Group VII**: The animals were treated with three doses (32, 64 or 96 mg/kg) of hexane extract (36 animals).

The tested materials were injected intraperitoneally as a single dose (0.1 ml) per a day and for 7 days, and then the mice were sacrificed in day 8 for laboratory assessments. In the case of CCl_4, it was injected subcutaneously as a single dose (0.1 ml) for one a day, and then the mice were sacrificed in the second day. The total number of mice in this study was 132 animals as shown in

Table 3-3: Laboratory tests and number of animals in the investigated groups of first stage.

Groups	Tested Material	Dose (mg/kg)	Laboratory Tests and Number of Animals		
			MI	TC, MN and SHAA	Biochemistry and Histpathology
Group I	Distilled H_2O		4	4	4
Group II	Olive Oil		4	4	4
Group III	CCl_4	3.2	4	4	4
Group IV	Vitamin A	1.4	4	4	4
Group V	Vitamin C	120	4	4	4
Group VI	Oregano Extract	32	4	4	4
		64			
		96	4	4	4
Group VII	Oregano Extract	32	4	4	4
		64			
		96			
Total Number of Animals			132		

MI: Mitotic index; TC: Total and differential counts of leucocytes;
MN: Micronucleus; SPAA: Sperm-head abnormality assay.

3-5-2: Second Stage

In this stage, interactions between the three doses of both extracts (32, 64 or 96 mg/ kg), and CCl_4 (3.2 mg/kg) were carried out. In such interactions, the animals were given CCl_4 on day one, while the plant extract (methanol or hexane) was given in day 2 till day 7 (single dose/day), and then animals were sacrificed in day 8 for laboratory assessments. In both cases, the extract was given intraperitoneally (0.1 ml), while CCl_4 was given subcutaneously (0.1 ml). For both extracts, control groups were paralleled the two types of extracts, in which the plant extract was replaced by distilled water (methanol extract

negative controls), vitamin C (methanol extract positive controls), olive oil (hexane extract negative controls) or vitamin A (hexane extract positive controls). The total number of mice in this stage was 120 animals, which were distributed into 6 groups as shown in table 3-4.

Table 3- 4: Laboratory tests and number of animals in the investigated groups of second stage.

Groups	Tested Material	Dose (mg/kg)	Laboratory Tests and Number of Animals		
			MI	TC, MN and SHAA	Biochemistry and Histpathology
Group I	CCl_4 + Distilled H_2O		4	4	4
Group II	CCl_4 +Olive Oil		4	4	4
Group III	CCl_4 +Vitamin A	14	4	4	4
Group IV	CCl_4 +Vitamin C	120			
Group V	CCl_4 +	Methanol Extract	64	4	4	4
			96	4	4	4
Group VI		Oregano	32	4	4	4
		Hexane	64	4	4	4
		Extract	96	4	4	4
Total Number of Animals				120		

MI: Mitotic index; TC: Total and differential counts of leucocytes;
MN: Micronucleus; SPAA: Sperm-head abnormality assay.

3-6: Laboratory Methods

3-6-1: Chemical Detection of Plant Extracts

3-6-1-1: Detection of Tannins

The procedure of Al- Shami (1982) was used for the detection of tannins. In this procedure, 50 ml of each 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.

3-6-1-3: Detection of Resins

The procedure of Shihata (1951) was used by adding 10 ml of each extract to 20 ml of distilled water with HCl (4%).The appearance of turbidity means the presence of resins.

3-6-1-4: Detection of 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 plant extract. The appearance of yellow color was an indicator of the presence of flavonoids (Jaffer *et al*., 1983).

3-6-1-5: Detection of Coumarines

One ml of plant extract was dispensed into test tube, which was then covered by a filter paper and wetted with sodium hydroxide (NaOH). Then, the tube was transferred to a boiling water bath for 5 minutes. After that, the filter paper was examined under UV light and the appearance of greenish blue color indicated the presence of coumarin (Geisman, 1962).

3-6-1-6: Detection of Volatile Oils

According to the Indian Herbal Pharmacopeias (1998), a filter paper was wetted with 10 ml of the plant extract and examined under UV light. The appearance of bright pink color was an indicator of the presence of volatile oils.

3-6-1-7: Detection of Glycosides

Few drops of HCl were added to a test tube containing 1 ml of plant extract and then was transferred to a boiling water bath and after that 2 ml of Benedict reagent was added. The appearance of red color was an indicator of glycosides (Evans, 1999).

3-6-1-8: Detection of 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 (Harbone, 1973).

3-6-1-9: Detection of Steroids and Terpens

The procedure of Al-Maisary (1999) was used by mixing 1ml of plant extract with 2 ml of chloroform and one drop of glacial acetic acid, and then one drop of H_2SO_4 was added. The appearance of brown color was an indicator of

the presence of terpens, and if then a blue color was appeared, this time was indicator of the presence of steroids.

3-6-1-10: Detection of Phenolic compounds

The procedure of Harbone (1973) was used, in which 3 ml of plant extract was added to 2 ml of ferric chloride (1%) and the appearance of greenish blue color was an indicator of phenolic compounds.

3-6-2: Total Leucocyte Count

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

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

3-6-3: Absolute Count of Leucocytes

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

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

3-6-4: Mitotic Index Assay

The mitotic index was determined for cells obtained from bone marrow, following the procedure of Allen *et al*. (1977). The animal was sacrificed by cervical dislocation and then dissected to obtain femur bones. The femur bone was cut from both ends, and its cellular contents were collected in a test tube using a disposable insulin syringe (1 ml) and normal physiological saline (5 ml).

The cells of the bone were manipulated as follows:

- The cell suspension was gently pipetted using Pasteur pipette, and the tube was centrifuged (2000 rpm) for 10 minutes.
- The supernatant was discarded, and the cell deposit was suspended in 5 ml of 0.075 M hypotonic solution (KCl). The tube was incubated in a water bath (37°C) every 5 minutes.
- The tube was centrifuged at 2000 rpm for 10 minute, and the supernatant was discarded.
- 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 tube was incubated in the refrigerator (4°C) for 30 minutes.
- The tube was centrifuged (2000 rpm) for 10 minutes, and the last step was repeated two times.
- 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.

- The slide was air-dried at room temperature, and by then it was stained with Giemsa stain for 15 minutes and rinsed with distilled water.
- The slide was examined under oil emersion lens (100X), and at least 1000 cells were examined. The percentage of divided cells was recorded using the following equation:

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

3-6-5: Micronucleus Formation Assay

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

- The mouse was sacrificed by cervical dislocation, and then dissected to obtain the femur bone. After cutting both ends of the bone, it was flushed from the middle with a forceps in a vertical position over the edge of a test tube containing cellular [illegible] (incubated at 37°C for 30 minutes) using a disposable insulin syringe.
- The test tube was centrifuged (1000 rpm) for 10 minutes, and the supernatant was discarded.
- The cellular deposit was gently mixed, and a thin smear was made on a clean slide, and air-dried at room temperature.
- The smear was fixed with absolute methanol for 5 minutes, and then air-dried at room temperature.
- The smear was stained with Giemsa stain for 15 minutes, and rinsed with distilled water.
- The slides were examined under oil immersion lens (100X), and at least 1000 polychromatic erythrocytes (PCE) were examined for the presence

of micronucleus formation. The micronucleus index was obtained using the following equation:

$$\text{Micronucleus index (\%)} = \left(\frac{\text{Number of Micronuclei}}{\text{Total Count of PCE}} \right) \text{x } 100$$

3-6-6: Sperm-head Abnormality Assay

The mouse was sacrificed by 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 (1000 rpm) for 10 minutes. The supernatant was discarded, and the spermatozoa deposit was gently suspended in one drop of normal saline. A thin smear of the suspension was made on a clean slide, which was allowed to air-dry at room temperature. The [illegible] fixed with distilled water and air-dried again. The slide was examined under an oil immersion lens (100X), and at least 1000 spermatozoa were inspected for the morphology of their heads (Wyrobek and Bruce, 1975). The sperm-head abnormality (SHA) index was scored using the following equation:

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

3-6-7: Biochemical Tests

Four biochemical tests were carried out in the sera of the investigated animals. They were glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), alkaline phosphatase (ALP), and total bilirubin.

3-6-7-1: Glutamic Oxaloacetic Transaminase (GOT)

The enzyme activity of GOT was evaluated in mouse serum following the enzymatic colorimetric method of Reitman and Frankel (1957). For this purpose a commercial kit (Randox Company) was used.

- **Kit Components**
  - GOT substrate reagent (R1)
  - GOT color reagent (R2)
  - Sodium hydroxide (R3)

- **Procedure:** Two test tubes were used and the above solutions were added as shown in table 3-5.

Table 3-5: Method for measuring the GOT activity.

Tubes	Blank (ml)	Test (ml)
Serum	-	0.1
R1	0.5	0.5
Distilled H_2O	0.1	-
The tubes were mixed well and incubated in a water bath (37°C) for 30 minutes		
R2	0.5	0.5
The tubes were mixed well and incubated at room temperature for 20 minutes		
R3	5	5
The tubes were mixed well and left at room temperature for 5 min, and then the absorbency was measured at 546 nm.		
The activity of the enzyme GOT (Unit/L) was calculated from the standard curve of the kit.		

3-6-7-2: Glutamic Pyruvic Transaminase (GPT)

The enzyme activity GPT was evaluated in mouse serum following the enzymatic colorimetric method of Reitman and Frankel (1957). For this purpose a commercial kit (Randox Company) was used.

- **Kit Components**
  - GPT substrate reagent (R1)
  - GPT color reagent (R2)
  - Sodium hydroxide (R3)

- **Procedure:** Two test tubes were used and the above solutions were added as shown in table 3-5.

3-6-7-3: Alkaline Phosphatase (ALP)

The enzyme ALP was evaluated in r[illegible] produced by Bio Merieux Company and the most commonly used method is that of King and Arnistrong, in which the disodium phenyl phosphate is hydrolysed with liberation of phenol and formation of sodium phosphate. The amount of phenol formed is estimated colorimetrically.

- **Kit Components**
  - Buffer (R1)
  - Disodium phenylphosphate 0.01M (R2)
  - Phenol standard (R3)
  - 0.5N NaOH (R4)
  - 0.5N Sodium bicarbonate (R5)
  - 0.6% 4-Aminoantipyrine (R6)
  - Potassium ferric cyanide (R7)

- **Procedure**: Four test tubes (sample, control, standard and blank) were used, and the forthcoming reagents were added as shown in table 3-6.

Table 3-6: Method for measuring the ALP activity.

Tubes / Reagents	Sample	Control	Standard	Blank
R1	1 ml	1ml	1.1 ml	1.1 ml
R2	1 ml	1 ml	-	-
Serum	0.1 ml	-	-	-
R3	-	-	1 ml	-
Distilled Water	-	-	-	1 ml
Incubation at 37°C for 15 min.				
R4	0.8 ml	0.8 ml	0.8 ml	0.8 ml
Serum				
R5	1.2 ml	1.2 ml		
R6	1 ml	1 ml	1 ml	1 ml
R7	1 ml	1 ml	1 ml	1 ml
The absorbency was read immediately at 510 nm				

- **Calculation:** The following equation was employed to assess the serum activity of ALP:

$$\text{ALP activity (Unit/L)} = \frac{\text{Sample} - \text{Control}}{\text{Standard} - \text{Blank}} \times 10$$

3-6-7-4: Total Bilirubin

The total level of bilirubin was evaluated in mouse serum using a commercial kit produced by Giesse Company (Walters, 1970). The principle is that in the presence of diazonium salt of the sulfanilic acid, the bilirubin produces a red-colored azoic coloring in acid environment (azobilirubine).While direct bilirubin, the one conjugated with glucuronic acid, is hydrosoluble and directly react, and total bilirubin is obtained through the presence of an accelerator (DMSO) which separates the bond with albumin. The intensity of the azo-compound color thus produced is directly proportional to the total bilirubin concentration.

- Kit Components

1- Reagent (A)

- Sulfanilic acid 35 mM
- DMSO
- Sulphuric acid 50 Mm

2- Reagent (B)

- Sodium nitrite liquid 35 mM
- Stabilizer

3- Standard

Procedure: Three test tubes were taken, one for the blank, the second for standard and the third for the sample. The solutions were added as in the following table:

Table 3-7: Method for measuring Bilirubin activity.

Materials	Blank	Standard	Sample
Reagent A	1.5 ml	1.5 ml	1.5 ml
Reagent B	50 µl	50 µl	50µl
D.W	100 µl	-	-
Standard	-	100 µl	-
Serum	-	-	100 µl
The contents were mixed and the tubes were incubated for 5 minutes at 37°C,then mixed and read the absorbency putting at zero with blank reagent at 546 nm			

Calculation: The following equation is used for the quantity of Bilirubin:

$$BIL.T\,(mg\ /\ dl) = \frac{Sample}{STD.} \times STD.$$

3-6-8: Histopathological Study

The liver was fixed in 10% formalin for 48 hours, and the procedure of Bancroft and Stevens (1982) was followed to prepare sections for histopathological examinations. The procedure is outlined as follows:

- **Washing:** The sample was placed in 70% ethanol overnight.
- **Dehydration:** The sample was dehydrated with ascending concentrations (50, 70, 90 and 99%) of ethanol. There was two hours for each concentration.
- **Clearing:** The sample was placed in xylene for two hours.
- **Infiltration**: The sample was first placed in paraffin-xylene (1:1) for 30 minutes at 57-58ºC, and then in paraffin alone for 2 hours at 60-70ºC.
- **Embedding**: The sample was embedded in pure paraffin wax (melting temperature: 60-70ºC) and left to solidified at room temperature.
- **Sectioning**: The paraffin block wa[illegible] a thickness of 5 microns and then th[illegible]e covered with Mayer's albumin. The section of tissue was placed in water bath (35-40ºC) for few seconds.
- **Staining**: The slide was first placed in xylene for 15-20 minutes, descending concentrations (90, 80 and 70%) of ethanol (two minutes for each concentration) and finally distilled water. After that, the slide was stained with haematoxylin for 10-20 minutes and then washed with distilled water for 5 minutes. Then, the slide was placed in acidic alcohol for one minutes and washed with distilled water. After washing, the slide was placed in eosin stain for 10-15 seconds, and then in ascending concentrations (70, 80, 90 and 99%) of ethanol (two minutes for each concentration). Finally, the slide was cleared with xylene for 10 minute.

- **Mounting**: The slide was mounted with a Canada balsam and covered with a cover slip. Then, the slide was examined microscopically to inspect the histopathological changes.

3-6-9: Statistical Analyses

The values of the investigated parameters were given in terms of mean ± standard error, and differences between means were assessed by analysis of variance (ANOVA) and Duncan test, using the computer program SPSS version 11.5.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:

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

A = Treated groups (plant extracts, vitamin A, vitamin C or CCl_4).

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

Chapter Four

Results

4-1: Detections of Secondary Metabolites in *O. vulgare* Extracts

Chemical detections of oregano extracts (methanol and hexane) revealed that the plant is rich in several compounds. In methanol extract, flavonoids, coumarins, tannins and phenolic compounds were detected, while hexane extract was positive for terpens, steroids, tannins and volatile oils (Table 4-1).

Table 4-1: Detections of Secondary metabolites in *O. vulgare* Extracts.

Secondary Metabolites	Reagents	Methanol Extract	Hexane extract
Flavonoids	Ethanol + KOH	+	-
Terpens	Acetic acid H_2SO_4	-	+
Saponins	$HgCl_2$	-	-
Coumarins	UV Light	+	-
Tannins	$CH_3Coopb+FeCl_2$	+	+
Alkaloids	Mayer's reagent	-	-
Glycosides	Benedict reagent	-	-
Resins	HCl (4%)	-	-
Volatile oils	UV Light	-	+
Phenols	$FeCl_2$	+	-

+ Indicates the presence of the compound
- Indicates the absence of the compound

4-2: Immunological Effects of *O. vulgare* Extracts

Total and absolute counts (lymphocytes, neutrophils, monocytes and eosinophils) of leucocytes were the immunological parameters for the effect's evaluation of *O. vulgare* extracts (methanol and hexane) in albino male mice.

4-2-1: Total Count of Leucocytes

- **Methanol Extract**: Animals treated with CCl_4 (control III) showed a significant reduced count of leucocytes (4350 cell/cu.mm.blood) as compared to control I (8050 cell/cu.mm.blood) or control II (14900 cell/cu.mm.blood). With respect to the plant extract, the first dose (32 mg/kg) caused a non-significant decreased count of leucocytes as compared to control I (7050 *vs.* 8050 cell/cu.mm.blood). The other two doses (64 and 96 mg/kg) showed a significant increase (9400 and 11100 respectively *vs.* 8050 cell/cu.mm.blood). The best treatment efficiency was recorded for vitamin C (+85.1) followed by the 2nd dose (+16.8) of plant extract (Table 4-2).

Table 4-2: Total leucocyte count (mean ± S.E.) in albino male mice treated with methanol extract of *O. vulgare*.

Treatment Groups	Dose (mg/kg)	Mean ± S.E. (cell/cu.mm.blood)*	Treatment Efficiency (%)
Control I (distilled water)		8050 ± 591a	
Control II (vitamin C)	180	14900 ± 600b	+85.1
Control III (CCl_4)	3.2	4350 ± 435c	-42.0
Plant Methanol Extract	32	7050 ± 457.5a	-12.42
	64	9400 ± 627d	+16.8
	96	11100 ± 557e	+37.9

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

- **Hexane Extract**: The three doses of plant extract (32, 64 and 96 mg/kg) were significantly effective in increasing the total count of leucocytes in a dose-dependent manner (9150, 10850 and 13650 cell/cu.mm.blood, respectively) as compared to control I (7500 cell/cu.mm.blood). Vitamin A (control II) was also significantly effective in increasing the total count of leucocytes (9900 cell/cu.mm.blood) as compared to either control I (7500 cell/cu.mm.blood) or control III (4350 cell/cu.mm.blood). The best treatment efficiency was recorded for the third dose of plant extract (+82.0%) followed by the second dose (+44.7%) and vitamin A (+32.0%) (Table 4-3).

Table 4-3: Total leucocyte count (mean ± S.E.) in albino male mice treated with hexane extract of *O. vulgare*.

Treatment Groups	Dose (mg/kg)	Mean ± S.E. (cell/cu.mm.blood)	Treatment Efficiency(%)
Control I (olive oil)		7500 ± 645a	
Control II (Vitamin A)	1.4	[illegible]	[illegible]
Control III (CCl4)	3.2	[illegible]	[illegible]
Plant Hexane Extract	32	9150 ± 435b	+22.0
	64	10850 ± 1072b	+44.7
	96	13650 ± 634d	+82.0

∗: Different letters: significant difference ($P \leq 0.05$) between means.

4-2-2: Absolute Counts of Leucocytes

4-2-2-1: Lymphocyte Count

- **Methanol Extract**: Animals treated with CCl_4 (control III) showed a significant reduced count of lymphocytes (2106 cell/cu.mm.blood) as compared to control I (5054 cell/cu.mm.blood) or control II (8800 cell/cu.mm.blood). With respect to the plant extract, the first dose (32 mg/kg) caused a non-significant decreased count of lymphocytes (3888

cell/cu.mm.blood as compared to control I (5054 cell/cu.mm.blood), but the next two doses (64 and 96 mg/kg) showed a significant increase (6278 and 7725 cell/cu.mm.blood, respectively). The best treatment efficiency was recorded for vitamin C (control II), which was (+74.1%), followed by the third (+52.8%) and second (+24.2%) doses of the plant extract (Table 4-4).

Table 4-4: Total lymphocyte count (mean ± S.E.) in albino male mice treated with methanol extract of *O. vulgare*.

Treatment Groups	Dose (mg/kg)	Mean ± S.E. (cell/cu.mm.blood)*	Treatment Efficiency (%)
Control I (distilled water)		5054± 385a	
Control II (vitamin C)	180	8800 ± 600b	+74.1
Control III (CCl_4)	3.2	2106 ± 289c	-54.6
Plant Methanol Extract	32	3888± 301a	-23.1
	64	6278 ± [illegible]00d	+24.2
	96	[illegible]	[illegible]

[illegible] letters: significant difference (P ≤ 0.05) between means.

- **Hexane Extract**: The three doses of plant extract (32, 64 and 96 mg/kg) were significantly effective in increasing the count of lymphocytes in a dose-dependent manner (6315, 7220 and 9716 cell/cu.mm.blood, respectively) as compared to control I (4635 cell/cu.mm.blood). Vitamin A (control II) was also significantly effective in increasing the count of lymphocytes (6300 cell/cu.mm.blood) as compared to either control I (4635 cell/cu.mm.blood) or control III (2106 cell/cu.mm.blood). The best treatment efficiency was recorded for the third dose of plant extract (+109.6%) followed by the second dose (+55.8%), the first dose (+36.2%) and vitamin A (+35.9%) (Table 4-5).

Table 4-5: Total lymphocyte count (mean ± S.E.) in albino male mice treated with hexane extract of *O. vulgare*.

Treatment Groups	Dose (mg/kg)	Mean ± S.E. (cell/cu.mm.blood)*	Treatment Efficiency (%)
Control I (olive oil)		4635 ± 481a	
Control II (vitamin A)	1.4	6300 ± 400b	+35.9
Control III (CCl_4)	3.2	2106 ± 289c	-54.6
Plant Hexane Extract	32	6315 ± 182b	+36.2
	64	7220 ± 1124b	+55.8
	96	9716 ± 408d	+109.6

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

4-2-2-2. *Neutrophil Count*

- **Methanol Extract**: Animals treated with CCl_4 (control III) showed a significant reduced count of neutr... compared to control I (1996 cell/c... cell/cu.mm.blood). With respect to the plant extract, the first and second doses (32 and 64 mg/kg) approximated the count of control I (1840 and 2012, respectively *vs*. 1996 cell/cu.mm.blood), while the third dose (96 mg/kg) showed a significant increase (2339 cell/cu.mm.blood). Much more significant increase of neutrophil count was observed after a treatment with vitamin C (4200 cell/cu.mm.blood).The best treatment efficiency was recorded for vitamin C (+110.5%), followed by the third dose (+17.2%) of plant extract (Table 4-6).

Table 4-6: Total neutrophil count (mean ± S.E.) in albino male mice treated with methanol extract of *O. vulgare*.

Treatment Groups	Dose (mg/kg)	Mean ± S.E. (cell/cu.mm.blood)*	Treatment Efficiency (%)
Control I (distilled water)		1996 ± 88a	
Control II (vitamin C)	180	4200 ± 600b	+110.5
Control III (CCl_4)	3.2	1265 ± 103c	-27.2
Plant Methanol Extract	32	1840 ± 198a	-7.8
	64	2012 ± 165a	+0.8
	96	2339 ± 52d	+17.2

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

Table 4-7: Total neutrophil count (mean ± S.E.) in albino male mice treated with hexane extract of *O. vulgare*.

Treatment Groups	Dose (mg/kg)	Mean ± S.E. (cell/cu.mm.blood)*	Treatment Efficiency (%)
Control I (olive oil)		1738 ± 154a	
Control II (vitamin A)	1.4	3700 ± 400b	+112.9
Control III (CCl_4)	3.2	1265 ± 103c	-27.2
Plant Hexane Extract	32	2061.5 ±74a	+18.6
	64	2212 ± 23d	+27.3
	96	2733± 325d	+57.3

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

4-2-2-3: *Monocyte Count*

- **Methanol Extract**: The distribution of monocyte count in animals treated with distilled water, vitamin C, CCl_4 and the plant extract showed no significant variation, with the exception of first dose of plant extract, which was significantly effective in increasing the count as compared to control I (1166 *vs*. 849 cell/cu.mm.blood). The treatment efficiency of such effect was +37.3% (Table 4-8).

Table 4-8: Total monocyte count (mean ± S.E.) in albino male mice treated with methanol extract of *O. vulgare*.

Treatment Groups	Dose (mg/kg)	Mean ± S.E. (cell/cu.mm.blood)∗	Treatment Efficiency (%)
Control I (distilled water)		849.0 ± 122.0a	
Control II (vitamin C)	180	926.4 ± 60.5a	[illegible]
Control III (CCl_4)	3.2	[illegible] ± 203.6a	+5.8
[illegible] Methanol Extract	32	[illegible]	[illegible]
	64	[illegible]	[illegible]
	96	957.0 ± 136.3a	+12.7

∗: Different letters: significant difference (P ≤ 0.05) between means.

- **Hexane Extract**: The hexane extract shared the picture of methanol extract, in which the count of monocytes showed no significant variations between animals treated with the three doses (32, 64 and 96 mg/kg) of plant extract, olive oil (control I) or CCl_4 (control III). However, animals treated with vitamin A (control II) manifested a significant decreased count of monocytes (733.5 cell/cu.mm.blood) as compared to the counts of the forthcoming groups (1140.5, 1068.0, 1136.0, 982.5 and 1039.5 cell/cu.mm.blood, respectively) (Table 4-9).

Table 4-9: Total monocyte count (mean ± S.E.) in albino male mice treated with hexane extract of *O. vulgare*.

Treatment Groups	Dose (mg/kg)	Mean ± S.E. (cell/cu.mm.blood)*	Treatment Efficiency (%)
Control I (olive oil)		982.5 ± 68.6a	
Control II (vitamin A)	1.4	733.5 ± 26.4b	-25.3
Control III (CCl_4)	3.2	1039.5 ± 203.5a	+5.8
Plant Hexane Extract	32	1140.5 ± 168.4a	+16.1
	64	1068.0 ± 156.9a	+8.7
	96	1136.0 ±203.75a	+15.6

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

4-2-2-4: Eosinophil and Basophil Counts

Both counts of eosinophils and basophils showed no significant variation

[illegible] treated with the three doses of plant extracts (methanol or hexane), [illegible] and vitamin [illegible] and 4-13).

Table 4-10: Total eosinophil count (mean ± S.E.) in albino male mice treated with methanol extract of *O. vulgare*.

Treatment Groups	Dose (mg/kg)	Mean ± S.E. (cell/cu.mm.blood)*	Treatment Efficiency (%)
Control I (distilled water)		66.5 ± 43.7a	
Control II (vitamin C)	180	46.9 ± 1.7a	-29.47
Control III (CCl_4)	3.2	43.5 ± 4.4a	-34.6
Plant Methanol Extract	32	87.5 ± 24.8a	+31.6
	64	50.0 ± 20.0a	-24.8
	96	51.5 ± 29.9a	-22.55

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

Table 4-11: Total eosinophil count (mean ± S.E.) in albino male mice treated with hexane extract of *O. vulgare*.

Treatment Groups	Dose (mg/kg)	Mean ± S.E. (cell/cu.mm.blood)*	Treatment Efficiency (%)
Control I (olive oil)		70.5 ± 22.8a	
Control II (vitamin A)	1.4	44.8 ± 11.9a	-36.5
Control III (CCl_4)	3.2	43.5 ± 4.35a	-38.3
Plant Hexane Extract	32	89.2 ± 33.1a	+26.5
	64	85.3 ± 35.2a	+20.9
	96	86.5 ± 30.5a	+22.7

*: Different letters: significant difference (P ≤ 0.05) between means.

Table 4-12: Total basophil count (mean ± S.E.) in albino male mice treated with methanol extract of *O. vulgare*.

Treatment Groups	Dose (mg/kg)	Mean ± S.E. (cell/cu.mm.blood)*	Treatment Efficiency (%)
Control I (distilled water)		85.5 ± 34.4a	
Control II (vitamin C)	180	66.5 ± 21.3a	
Control III (CCl_4)	3.2	71.0 ± 36.2a	+29.1
Plant Methanol Extract	32	49.0 ± 30.8a	-42.7
	64	44.0 ± 25.6a	-48.5
	96	47.5 ± 27.5a	-67.8

*: Different letters: significant difference (P ≤ 0.05) between means.

Table 4-13: Total basophil count (mean ± S.E.) in albino male mice treated with hexane extract of *O. vulgare*.

Treatment Groups	Dose (mg/kg)	Mean ± S.E. (cell/cu.mm.blood)*	Treatment Efficiency (%)
Control I (olive oil)		55.0 ± 37.7a	
Control II (vitamin A)	1.4	58.8 ± 19.7a	
Control III (CCl_4)	3.2	71.0 ± 36.23a	+29.09
Plant Hexane Extract	32	44.6 ± 24.3a	-56.36
	64	50.1 ± 20.4a	-63.63
	96	55.3 ± 35.1a	-36.36

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

4-3: Cytogenetic Effects of *O. vulgare* Extracts

Mitotic, micronucleus formation and sperm head abnormality indices were ... the effect ... hexane) in albino male mice.

4-3-1: Mitotic Index

- **Methanol Extract**: Animals treated with CCl_4 (control III) showed a significant decreased mitotic index (8.25%) as compared to control I (14.0%) and control II (13.6%). With respect to the plant extract, the first two doses (32 and 64 mg/kg) caused a non-significant increase (16.25 and 16.0%, respectively), while the third dose (96 mg/kg) showed a significant increased mitotic index (19.0%) as compared to control I (14.0%). Vitamin C (control II) showed no effect on mitotic index, and the value of control I was approximated (13.6%). The best treatment efficiency was recorded for the third dose (35.7%), followed by the first (16.1%) and second (14.3%) doses of plant extract (Tables 4-14).

Table 4-14: Mitotic index (mean ± S.E.) of bone marrow cells in albino male mice treated with methanol extract of *O. vulgare*.

Treatment Groups	Dose (mg/kg)	Mean ± S.E. (%)*	Treatment Efficiency (%)
Control I (distilled water)		14.00 ± 1.22a	
Control II (vitamin C)	180	13.60 ± 1.90a	-2.6
Control III (CCl_4)	3.2	8.25 ± 0.75b	-42.6
Plant Methanol Extract	32	16.25 ± 1.03a	+16.1
	64	16.00 ± 2.12a	+14.3
	96	19.00 ± 0.70d	+ 35.7

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

• **Hexane Extract**: The second and third doses of plant extract showed [illegible] increased mitotic index (22.75%, respectively) as compared to controls (14.37%), I[illegible] efficiency of +20.0% and +39.1% respect[illegible]

Table 4-15: Mitotic index (mean ± S.E.) of bone marrow cells in albino male mice treated with hexane extract of *O. vulgare*.

Treatment Groups	Dose (mg/kg)	Mean ± S.E. (%)*	Treatment Efficiency (%)
Control I (olive oil)		14.37 ± 1.79a	
Control II (vitamin A)	1.4	15.10 ± 0.20a	+5.1
Control III (CCl_4)	3.2	8.25 ± 0.75b	-42.6
Plant Hexane Extract	32	17.25 ± 1.37a	+20.0
	64	20.00 ± 1.08c	+39.1
	96	22.75 ± 1.1c	+58.3

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

4-3-2: Micronucleus Index

- **Methanol Extract**: Animals treated with CCl_4 (control III) showed a significant increased micronucleus index (2.65%) as compared to control I (0.8%) and control II (0.51%). With respect to the plant extract, the second (64 mg/kg) and third (96 mg/kg) doses caused a significant decreased in micronucleus formation (0.50 and 0.25%, respectively) as compared to control I (0.8%).Vitamin C also decrease the micronucleus formation (0.51%). The best treatment efficiency was recorded for the third dose (-68.75%), followed by the second dose (-37.5%) of the plant extract and finally vitamin C (-36.3%) (Table 4-16) and (Figure 4-1).

Table 4-16: Micronucleus index (mean ± S.E.) in bone marrow cells of albino

Treatment Groups	Dose (mg/kg)	Mean ± S.E. (%)	Treatment Efficiency (%)
Control II (vitamin C)	180	0.51 ± 0.10b	-36.3
Control III (CCl_4)	3.2	2.65 ± 0.15c	+231.3
Plant Methanol Extract	32	0.80 ± 0.08a	0.0
	64	0.50 ± 0.06b	-37.5
	96	0.25 ± 0.05d	-68.8

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

- **Hexane Extract**: Animals treated with CCl_4 (control III) showed a significant increased in micronucleus formation (2.65%) as compared to control I (0.75%) and control II (0.54%). With respect to the plant extract, the second and third doses (64 and 96 mg/kg) caused a significant decreased in micronucleus formation (0.35 and 0.15%, respectively) as compared to control I and control II, while the first dose (32 mg/kg) caused a significant

decrease (0.55%) compared to control I only. The best treatment efficiency was recorded for the third dose (-80.0%), followed by the second dose (-53.3%) of the plant extract (Table 4-17).

Table 4-17: Micronucleus index (mean ± S.E.) in bone marrow cells of albino male mice treated with hexane extract of *O. vulgare*.

Treatment Groups	Dose (mg/kg)	Mean ± S.E. (%)*	Treatment Efficiency (%)
Control I (olive oil)		0.75 ± 0.09a	
Control II (vitamin A)	1.4	0.54 ± 0.20b	-28.0
Control III (CCl_4)	3.2	2.65 ± 0.15c	+231.3
Plant Hexane Extract	32	0.55 ± 0.09b	-26.7
	64	0.35 ± 0.05d	-53.3
	96	0.15 ± 0.09e	-80.0

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

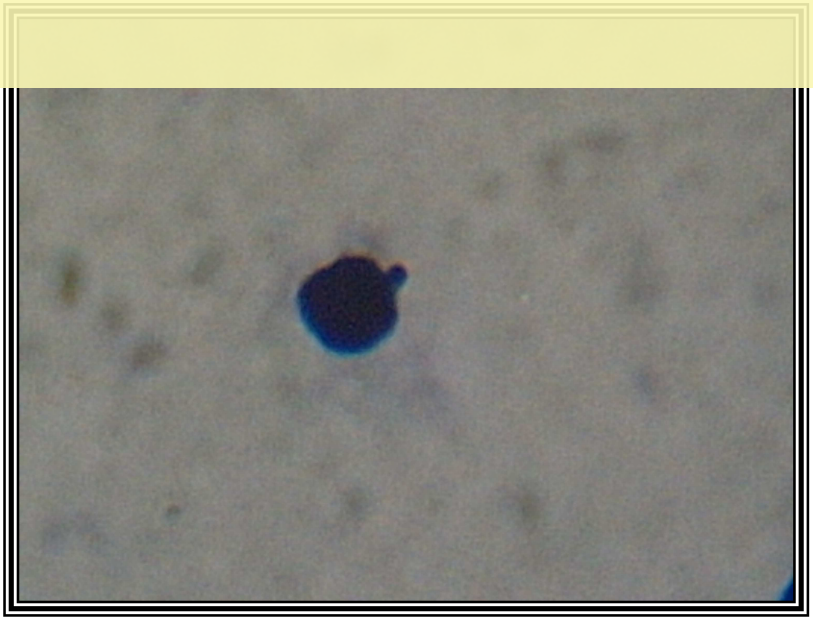


Figure 4-1: Micronucleus formation in a bone marrow cell of mouse treated with CCl_4 (100X).

4-3-3: Sperm Head Abnormality Index

- **Methanol Extract**: Animals treated with CCl_4 (control III) showed a significant increased sperm head abnormality index (10.30%) as compared to control I (5.95%).With respect to the plant extract, the third dose (96 mg/kg) caused a significant decrease in sperm head abnormalities (4.15%) as compared to control I, While the first (32 mg/kg) and second (64 mg/kg) doses showed a non-significant difference (5.5 and 5.05%, respectively). Vitamin C shared the picture of third dose of plant extract in which the index was 4.25%.The best treatment efficiency was recorded for the third dose of plant extract, which was -30.25%, followed by vitamin C -28.6% (Table 4-18) and (Figure 4-2).

Table 4-18: Sperm head abnormality index (mean ± S.E.) in albino mice treated with methanol extract of *P. vulgare*.

Groups	dose (mg/kg)		
Control I (distilled water)		5.95 ± 0.63a	
Control II (vitamin C)	180	4.25 ± 0.12b	-28.6
Control III (CCl_4)	3.2	10.30 ± 0.72c	+136.8
Plant Methanol Extract	32	5.50 ± 0.44a	-7. 6
	64	5.05 ± 0.20a	-15.1
	96	4.15 ± 0.30b	-30.3

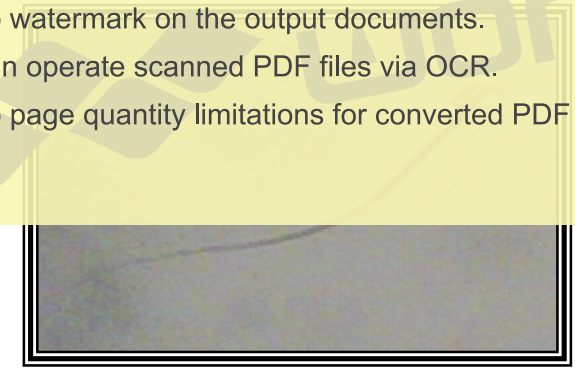
*: Different letters: significant difference ($P \leq 0.05$) between means.

- **Hexane Extract**: Only the third dose of plant extract (3.25%) and vitamin A (3.45%) were effective in decreasing the sperm head abnormalities as compared to controls I (4.35%) or III (10.30%), therefore, the treatment efficiencies were -25.3 and -20.7%, respectively (Table 4-19).

Table 4-19: Sperm head abnormality index (mean ± S.E.) in albino male mice treated with hexane extract of *O. vulgare*.

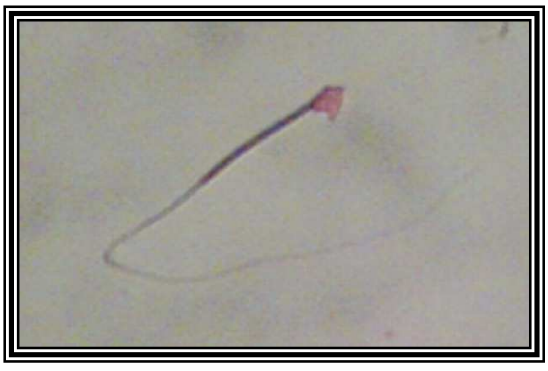
Treatment Groups	Dose (mg/kg)	Mean ± S.E. (%)*	Treatment Efficiency (%)
Control I (olive oil)		4.35± 0.24a	
Control II (vitamin A)	1.4	3.45 ± 0.22b	-20.7
Control III (CCl_4)	3.2	10.30 ± 0.72c	+136.8
Plant Hexane Extract	32	5.30 ± 0.38a	+ 21.8
	64	4.00 ± 0.21a	-8.04
	96	3.25 ± 0.22b	-25.3

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



(A)

(B)



(C)

Figure 4-2: Normal (A) and abnormal (B and C) sperm- heads in mice treated with CCl_4 (100X).

4-4: Biochemical Effects of *O. vulgare* Extracts

Glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), alkaline phosphatase (ALP) and total bilirubin were the biochemical parameters for the effect's evaluation of *O. vulgare* extracts (methanol and hexane) in albino male mice.

4-4-1: Glutamic Oxaloacetic Transaminase (GOT)

- **Methanol Extract**: Animals treated with CCl_4 (control III) showed a significant increased in GOT value (381 IU/L) as compared to control I (192 IU/L). With respect to the plant extract, the first dose (32 mg/kg) and the third dose (96 mg/kg) caused a non-significant decreased in GOT value (164.0 and 159.75 IU/L, respectively) as compared to control I, but the second dose (64 mg/kg) showed a significant decreased activity (146.5 IU/L). Maximum treatment efficiency was recorded for the second dose (-23.7%), followed by the third (-16.8%) and first (-14.6%) doses (Table 4-20).

Table 4-20: Glutamate oxaloacetate transaminase activity (mean ± S.E.) in sera of albino male mice treated with methanol extract of *O. vulgare*.

Treatment Groups	Dose (mg/kg)	Mean ± S.E. (IU / L)*	Treatment Efficiency (%)
Control I (distilled water)		192.00 ± 10.82a	
Control II (vitamin C)		Not Tested	
Control III (CCl_4)	3.2	381.00 ± 5.96b	+103.5
Plant Methanol Extract	32	164.00 ± 22.88a	-14.6
	64	146.50 ± 17.22c	-23.7
	96	159.75 ±20.49a	-16.8

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

● **Hexane Extract**: Animals treated with CCl_4 (control III) showed a significant increased activity of serum GOT (381 IU/L) as compared to control I (187.25 IU/L). With respect to the plant extract, the three doses (32, 64 and 96 mg/kg) caused a non-significant decrease in GOT activity (168.25, 164.5 and 162.25 IU/L, respectively) as compared to control I (Table 4-21).

Table 4-21: Glutamate oxaloacetate transaminase activity (mean ± S.E.) in sera of albino male mice treated with hexane extract of *O. vulgare*.

Treatment Groups	Dose (mg/kg)	Mean ± S.E. (IU / L)*	Treatment Efficiency (%)
Control I (olive oil)		187.25 ± 16.49a	
Control II (vitamin A)		Not Tested	
Control III (CCl_4)	3.2	381.00 ± 5.96b	+103.5
Plant Hexane Extract	32	168.25 ± 13.27a	-10.1
	64	164.50 ± 10.08a	-13.8
	96	[illegible]	[illegible]

[illegible] letters: significant difference (P ≤ 0.05) between means.

4-4-2: Glutamic Pyruvic Transaminase (GPT)

- **Methanol Extract**: Animals treated with CCl_4 (control III) showed a significant increased activity of serum GPT (154.75 IU/L) as compared to control I (67.75 IU/L). With respect to the plant extract, the first dose (32 mg/kg) caused a non-significant decrease in GPT activity (60 IU/L respectively) as compared to control I, while the second and third doses (64 and 96 mg/kg, respectively) showed a significant decrease (46.5 and 56.0 IU/L, respectively). The best treatment efficiency was recorded for the second dose (-31.4%), followed by the third (-17.3%) and first (-11.4%) doses of the plant extract (Tables 4-22).

Table 4-22: Glutamate pyruvic transaminase activity (mean ± S.E.) in sera of albino male mice treated with methanol extract of *O. vulgare*.

Treatment Groups	Dose (mg/kg)	Mean ± S.E. (IU / L)*	Treatment Efficiency (%)
Control I (distilled water)		67.75 ± 1.25a	
Control II (vitamin C)		Not Tested	
Control III (CCl_4)	3.2	154.75 ± 1.93b	+99.0
Plant Methanol Extract	32	60.00 ± 5.43a	-11.4
	64	46.50 ± 3.07c	-31.4
	96	56.00 ±4.10c	-17.3

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

Table 4-23: Glutamate pyruvic transaminase activity (mean ± S.E.) in sera of albino male mice treated with hexane extract of *O. vulgare*.

Treatment Groups	Dose (mg/kg)	Mean ± S.E. (IU / L)*	Treatment Efficiency (%)
Control I (olive oil)		77.75 ± 4.66a	
Control II (vitamin A)		Not Tested	
Control III (CCl_4)	3.2	154.75 ± 1.93b	+99.0
Plant Hexane Extract	32	73.75 ± 7.12a	-5.14
	64	57.00 ± 3.74c	-26.7
	96	63.75 ± 5.81c	-18.0

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

4-4-3: Alkaline Phosphatase (ALP)

- **Methanol Extract**: Animals treated with CCl_4 (control III) showed a significant increased activity of serum ALP (153 IU/L) as compared to control I (85.62 IU/L). With respect to the plant extract, the three doses (32, 64 and 96 mg/kg) showed a significant decreased activity of ALP (68.50, 48.0, 61.0 IU/L, respectively) as compared to control I, and accordingly, the treatment efficiencies were -19.9, -43.9 and -28.8%, respectively (Table 4-24).

Table 4-24: Alkaline phosphatase activity (mean ± S.E.) in sera of albino male mice treated with methanol extract of *O. vulgare*.

Treatment Groups	Dose (mg/kg)	Mean ± S.E. (IU/L)*	Treatment Efficiency (%)
Control I (distilled water)		85.62 ± 4.4a	
Control II (vitamin C)		Not Tested	
Control III (CCl_4)	32	68.50 ± 4.52c	-19.9
Plant Methanol Extract	64	48.00 ± 4.79e	-43.9
	96	61.00 ± 2.27d	-28.8

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

- **Hexane Extract**: The three doses (32, 64 and 96 mg/kg) of plant extract showed a significant decreased activity of ALP (65.50, 55.25, 64.25 IU/L, respectively) as compared to control I (75.11 IU/L), and accordingly, the treatment efficiencies were -12.7, -26.3 and -14.3%, respectively (Table 4-25).

Table 4-25: Alkaline phosphatase activity (mean ± S.E.) in sera of albino male mice treated with hexane extract of *O. vulgare*.

Treatment Groups	Dose (mg/kg)	Mean ± S.E. (IU / L)*	Treatment Efficiency (%)
Control I (olive oil)		75.00 ± 3.34a	
Control II (vitamin A)		Not Tested	
Control III (CCl_4)	3.2	153.25 ± 7.60b	+104.3
Plant Hexane Extract	32	65.50 ± 3.07c	-12.7
	64	55.25 ± 4.77d	-26.3
	96	64.25 ± 8.95c	-14.3

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

4-4-4: Total Bilirubin

Methanol Extract Administration treated [illegible] (Control III) showed significant increased serum level of bilirubin (0.58 mg/dL) compared to control I (0.58 mg/dL). With respect to the plant extract, the second dose (64 mg/kg) showed a significant decreased serum level of bilirubin value (0.29 mg/dL) as compared to controls I, while the first and third doses (32 and 96 mg/kg) showed no significant variation , and therefore, the treatment efficiency of the second dose was -50.0% (Table 4-26).

Table 4-26: Total bilirubin level (mean ± S.E.) in sera of albino male mice treated with methanol extract of *O. vulgare*.

Treatment Groups	Dose (mg/kg)	Mean ± S.E. (mg / dL)*	Treatment Efficiency (%)
Control I (distilled water)		0.58 ± 0.05a	
Control II (vitamin C)		Not Tested	
Control III (CCl_4)	3.2	2.24 ± 0.22b	+273.3
Plant Methanol Extract	32	0.48 ± 0.04a	-17.2
	64	0.29 ± 0.04c	-50.0
	96	0.55 ± 0.11a	-5.2

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

Table 4-27: Total bilirubin level (mean ± S.E.) in sera of albino male mice treated with hexane extract of *O. vulgare*.

Treatment Groups	Dose (mg/kg)	Mean ± S.E. (mg / dL)*	Treatment Efficiency (%)
Control I (olive oil)		0.60 ± 0.08a	
Control II (vitamin A)		Not Tested	
Control III (CCl_4)	3.2	2.24 ± 0.22b	+273.3
Plant Hexane Extract	32	0.81 ± 0.11a	+35.0
	64	0.51 ± 0.13a	-15.0
	96	0.86 ± 0.15a	+43.3

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

4-5: Effect of CCl_4 - *O. vulgare* Extracts Interaction

4-5-1: Total Count of Leucocytes

- **Methanol Extract**: The second (64 mg/kg) and third dose (96 mg/kg) of plant extract and vitamin C were able to modulate the effect of CCl_4 and increase the leucocyte count significantly (6000, 7700 and 6950 cell/cu.mm.blood, respectively) as compared to control I (4200 cell/ cu. mm. blood). Such modulation was associated with treatment efficiencies of +42.9, +83.3 and +65.5%, respectively (Table 4-28).

Table 4-28: Total leucocyte count (mean ± S.E.) in albino male mice after interactions between CCl_4 and methanol extract of *O. vulgare*.

Interaction Groups	Dose (mg/kg)	Mean ± S.E. * (cell/cu.mm.blood)	Treatment Efficiency (%)
Control I (CCl_4 + distilled water)		4200 ± 47[illegible]	
Control II (CCl_4 + vitamin C)	100	6950 ± 63[illegible]	+65.5
+ Plant Methanol Extract	32	[illegible]	[illegible]
	64	6000 ± 648b	+42.9
	96	7700 ± 412b	+83.3

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

- **Hexane Extract**: The three doses of plant extract (32, 64 and 96 mg/kg) were significantly effective in increasing the total count of leucocytes and in a dose-dependent manner (6050, 7200 and 7950 cell/cu.mm.blood, respectively) as compared to control I (4150 cell/cu.mm.blood). Vitamin A had a similar effect (5750 cell/cu.mm.blood). The best treatment efficiency was recorded for the third dose of plant extract (+91.6%), followed by the second (+73.5) and first (+45.8%) doses of the plant extract, and finally vitamin A (+38.6%) (Tables 4-29).

Table 4-29: Total leucocyte count (mean ± S.E.) in albino male mice after interactions between CCl_4 and hexane extract of *O. vulgare*.

Interaction Groups	Dose (mg/kg)	Mean ± S.E. * (cell/cu.mm.blood)	Treatment Efficiency (%)
Control I (CCl_4 + olive oil)		4150 ± 298a	
Control II (CCl_4 + vitamin A)	1.4	5750 ± 298b	+38.6
CCl_4 + Plant Hexane Extract	32	6050 ± 411b	+45.8
	64	7200 ± 469c	+73.5
	96	7950 ± 801c	+91.6

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

increasing the count of lymphocytes (4169 cell/cu.mm.blood) as compared to control I. The best treatment efficiency was recorded for the third dose of plant extract (+138.6%) followed by vitamin C (+97.2%), and finally the second (+74.2%) and first (+38.9%) doses of plant extract (Tables 4-30).

The hexane extract had a similar effect, but the second and third doses of plant extract were more effective in this regard, and a significant increased count of lymphocytes was observed as compared to control I (4670 and 5430, respectively *vs.* 2035 cell/cu.mm.blood). Such two increases were correspondent to treatment efficiencies of +129.5 and +166.8%, respectively. Vitamin A was also effective in modulating the effect of CCl_4 and the treatment efficiency of such increase was +76.3% (Table 4-31).

Table 4-30: Total lymphocyte count (mean ± S.E.) in albino male mice after interactions between CCl_4 and methanol extract of *O. vulgare*.

Interaction Groups	Dose (mg/kg)	Mean ± S.E. * (cell/cu.mm.blood)	Treatment Efficiency (%)
Control I (CCl_4 + distilled water)		2114 ± 417a	
Control II (CCl_4 + vitamin C)	180	4169 ± 458b	+97.2
CCl_4 + Plant Methanol Extract	32	2938 ± 345c	+38.9
	64	3684 ±515bc	+74.2
	96	5044± 314d	+138.6

*: Different letters: significant difference (P ≤ 0.05) between means.

Table 4-31: Total lymphocyte count (mean ± S.E.) in albino male mice after interactions between CCl_4 and hexane extract of *O. vulgare*.

Interaction Groups	Dose (mg/kg)	Mean ± S.E. * (cell/cu.mm.blood)	Treatment Efficiency (%)
Control I (CCl_4 + olive oil)		2035 ± 188a	
Control II (CCl_4 + vitamin A)	1.4	3587 ± 165b	+76.3
CCl_4 + Plant Hexane Extract	32	3598 ± 230b	+76.8
	64	4670 ± 366c	+129.5
	96	5430 ± 501c	+166.8

*: Different letters: significant difference (P ≤ 0.05) between means.

4-5-2-2: Neutrophil Count

The third dose of methanol extract and vitamin C were significantly effective in increasing the count of neutrophil as compared to control I (1793 and 1795, respectively *vs*. 1184 cell/cu.mm.blood), with treatment efficiencies of +51.4 and +51.6%, respectively (Table 4-32). However, for hexane extract, only the second dose (64 mg/kg) of plant extract was significantly effective

(1580.5 *vs*. 1185.0 cell/cu.mm.blood) in this regards with a treatment efficiency of +33.4% (Table 4-33).

Table 4-32: Total neutrophil count (mean ± S.E.) in albino male mice after interactions between CCl_4 and methanol extract of *O. vulgare*.

Interaction Groups	Dose (mg/kg)	Mean ± S.E. ∗ (cell/cu.mm.blood)	Treatment Efficiency (%)
Control I (CCl_4 + distilled water)		1184 ± 102a	
Control II (CCl_4 + vitamin C)	180	1795 ± 185b	+51.6
CCl_4 + Plant Methanol Extract	32	1305 ± 194a	+10.2
	64	1404 ± 89a	+18.6
	96	1793 ± 195b	+51.4

Table 4-33: Total neutrophil count (mean ± S.E.) in albino male mice after interactions between CCl_4 and

Interaction Groups	Dose (mg/kg)	Mean ± S.E. ∗ (cell/cu.mm.blood)	Treatment Efficiency (%)
Control I (CCl_4 + olive oil)		1185 ± 111a	
Control II (CCl_4 + vitamin A)	1.4	1310 ± 89a	+10.5
CCl_4 + Plant Hexane Extract	32	1392 ± 143a	+17.5
	64	1580 ± 109b	+33.4
	96	1508 ± 252a	+27.3

∗: Different letters: significant difference ($P \leq 0.05$) between means.

4-5-2-3: Monocyte, Eosinophils and Basophil Counts

The absolute counts of monocytes, eosinophils and basophils showed no significant variations between animals treated with the three doses (32, 64, and 96 mg/kg) of methanol or hexane extracts and controls. The vitamins A and C shared a similar picture (Tables 4-34, 4-35, 4-36, 4-37, 4-38 and 4-39).

Table 4-34: Total monocytes count (mean ± S.E.) in albino male mice after interactions between CCl_4 and methanol extract of *O. vulgare*.

Interaction Groups	Dose (mg/kg)	Mean ± S.E. * (cell/cu.mm.blood)	Treatment Efficiency (%)
Control I (CCl_4 + distilled water)		831.0 ± 47.9a	
Control II (CCl_4 + vitamin C)	180	854.5 ± 77.1a	+2.8
CCl_4 + Plant Methanol Extract	32	792.0 ± 204.4a	-4.7
	64	831.0 ± 51.4a	0.0
	96	803.0 ± 55.1a	-3.4

*: Similar letters: no significant difference (P > 0.05) between means.

Table 4-35: Total monocytes count (mean ± S.E.) in albino male mice after interactions between CCl_4 and hexane extract of *O. vulgare*.

Interaction Groups	Dose (mg/kg)	Mean ± S.E. * (cell/cu.mm.blood)	Treatment Efficiency (%)
Control I (CCl_4 + olive oil)		856.0 ± 118.8a	
Control II (CCl_4 + vitamin A)	1.4	780.0 ± 107.1a	-8.9
CCl_4 + Plant Hexane Extract	32	950.0 ± 174.4a	+10.9
	64	864.0 ± 78.8a	+0.9
	96	948.0 ± 120.8a	+10.7

*: Similar letters: no significant difference (P > 0.05) between means.

Table 4-36: Total eosinophils count (mean ± S.E.) in albino male mice after interactions between CCl_4 and methanol extract of *O. vulgare*.

Interaction Groups	Dose (mg/kg)	Mean ± S.E. * (cell/cu.mm.blood)	Treatment Efficiency (%)
Control I (CCl_4 + distilled water)		52.0 ± 10.5a	
Control II (CCl_4 + vitamin C)	180	83.5 ± 40.3a	+60.6
CCl_4 + Plant Methanol Extract	32	62.0 ± 8.3a	+19.2
	64	64.5 ± 28.3a	+24.0
	96	61.0 ± 21.0a	-59.6

*: Similar letters: no significant difference (P > 0.05) between means.

Table 4-37: Total eosinophils count (mean ± S.E.) in albino male mice after interactions between CCl_4 and hexane extract of *O. vulgare*.

Interaction	Dose	Mean ± S.E. *	Treatment
Control I (CCl_4 + olive oil)		51.5 ± 9.0a	
Control II (CCl_4 + vitamin A)	14	56.5 ± 23.0a	+9.7
	32		
+	64	68.0 ± 25.0a	+32.0
Plant Hexane Extract	96	42.5 ± 25.3a	-17.5

*: Similar letters: no significant difference (P > 0.05) between means.

Table 4-38: Total basophil count (mean ± S.E.) in albino male mice after interactions between CCl_4 and methanol extract of *O. vulgare*.

Interaction Groups	Dose (mg/kg)	Mean ± S.E. * (cell/cu.mm.blood)	Treatment Efficiency (%)
Control I (CCl_4 + distilled water)		25.0 ± 11.0a	
Control II (CCl_4 + vitamin C)	180	48.0 ± 16.3a	+152.6
CCl_4 + Plant Methanol Extract	32	52.5 ± 39.7a	+176.3
	64	54.0 ± 35.2a	+184.2
	96	39.0 ± 22.6a	+105.3

*: Similar letters: no significant difference (P > 0.05) between means.

Table 4-39: Total basophils count (mean ± S.E.) in albino male mice after interactions between CCl_4 and hexane extract of *O. vulgare*.

Interaction Groups	Dose (mg/kg)	Mean ± S.E. * (cell/cu.mm.blood)	Treatment Efficiency (%)
Control I (CCl_4 + olive oil)		22.5 ± 13.1a	
Control II (CCl_4 + vitamin A)	1.4	26.0 ± 16.0a	-28.9
CCl_4 + Plant Hexane Extract	32	46.5 ± 46.5a	+106.7
	64	17.0 ± 17.5a	-24.4
	96	29.0 ± 21.0a	-6.7

*: Similar letters: no significant difference (P > 0.05) between means.

4-5-3: Mitotic Index

Table 4-40: Mitotic index (mean ± S.E.) in albino male mice after interactions between CCl_4 and methanol extract of *O. vulgare*.

Interaction Groups	Dose (mg/kg)	Mean ± S.E. (%) *	Treatment Efficiency (%)
Control I (CCl_4 + distilled water)		9.00 ± 0.40a	
Control II (CCl_4 + vitamin C)	180	10.50 ± 0.64a	+16.7
CCl_4 + Plant Methanol Extract	32	10.50 ± 1.70a	+16.7
	64	11.25 ± 0.47a	+25.0
	96	14.25 ±1.03b	+58.3

*: Different letters: significant difference (P ≤ 0.05) between means.

Table 4-41: Mitotic index (mean ± S.E.) in albino male mice after interactions between CCl_4 and hexane extract of *O. vulgare*.

Interaction Groups	Dose (mg/kg)	Mean ± S.E. (%) *	Treatment Efficiency (%)
Control I (CCl_4 + olive oil)		10.25 ± 0.40a	
Control II (CCl_4 + vitamin A)	1.4	11.75 ± 0.62a	+14.6
CCl_4 + Plant Hexane Extract	32	12.50 ± 1.44a	+21.9
	64	14.00 ± 1.58ab	+36.6
	96	15.75 ± 0.85b	+53.7

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

4-5-4: Micronucleus Index

The third dose (96 mg/kg) of methanol extract showed a significant reduction in micronucleus formation (1.05%) as compared to control I (2.10%). The first and second doses (32 and 64 mg/kg) showed a similar significant effect (1.75 and 1.40%, respectively). Vitamin A [illegible] with a frequency of vitamin C (-58%), followed by second (-52%) and first (-30%) doses of plant extract (Tables 4-42).

Administration of the three doses (32, 64 or 96 mg/kg) of hexane extract after CCl_4 treatment showed a significant effect in decreasing the frequency of induced micronucleus formation (1.45, 1.10 and 0.75%) in a dose-dependent manner as compared to control I (2.10%). Vitamin A had a similar effect, and the induced micronucleus formation was significantly decreased (1.30%) as compared to control I. The best treatment efficiency was recorded for the third dose (-64.3%), followed by the second dose (-47.6%), vitamin A (-38.1%) and finally the first dose (-30.9%) of the plant extract (Table 4-43).

Table 4-42: Micronucleus index (mean ± S.E.) in albino male mice after interactions between CCl_4 and methanol extract of *O. vulgare*.

Interaction Groups	Dose (mg/kg)	Mean ± S.E. (%) *	Treatment Efficiency (%)
Control I (CCl_4 + distilled water)		2.50 ± 0.17a	
Control II (CCl_4 + vitamin C)	180	1.05 ± 0.09b	-58.0
CCl_4 + Plant Methanol Extract	32	1.75 ± 0.09c	-30.0
	64	1.20 ± 0.08d	-52.0
	96	1.05 ± 0.12b	-58.0

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

Table 4-43: Micronucleus index (mean ± S.E.) in albino male mice after interactions between CCl_4 and hexane extract of *O. vulgare*.

Interaction Groups	Dose (mg/kg)	Mean ± S.E. (%) *	Treatment Efficiency (%)
Control I (CCl_4 + distilled water)		2.50 ± 0.17a	
Control II (CCl_4 + vitamin A)	1.4	1.30 ± 0.05b	-38.1
CCl_4 + Plant Hexane Extract	32	1.45 ± 0.12b	-30.9
	64	1.10 ±0.05c	-47.6
	96	0.75 ± 0.05d	-64.3

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

4-5-5: Sperm Head Abnormality Index

For methanol extract, the third dose (96 mg/kg), as well as, vitamin C were significantly effective in decreasing the sperm head abnormality index as compared to control I (5.90 and 6.05, respectively *vs*. 9.5%), with treatment efficiencies of -37.9 and -36.3%, respectively (Table 4-44). The hexane extract had a similar effect, but the second dose was also effective in addition to the

third dose and vitamin A (5.90, 5.35 and 5.70, respectively *vs*. 9.0%), with treatment efficiencies of -34.4, -40.6 and -36.7%, respectively (Table 4-45).

Table 4-44: Sperm head abnormality index (mean ± S.E.) in albino male mice after interactions between CCl_4 and methanol extract of *O. vulgare*.

Interaction Groups	Dose (mg/kg)	Mean ± S.E. (%) *	Treatment Efficiency (%)
Control I (CCl_4 + distilled water)		9.50 ± 0.28a	
Control II (CCl_4 + vitamin C)	180	6.05 ± 0.40b	-36.3
CCl_4 + Plant Methanol Extract	32	9.90 ± 0.79a	+4.2
	64	8.75 ± 0.15a	-7.9
	96	5.90 ±0.41b	-37.9

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

Table 4-45: Sperm head abnormality index (mean ± S.E.) in albino male mice after interactions between CCl_4 and [illegible]

Interaction Groups	Dose (mg/kg)	Mean ± S.E. (%) *	Treatment Efficiency (%)
Control I (CCl_4 + olive oil)		9.00 ± 0.40a	
Control II (CCl_4 + vitamin A)	1.4	5.70 ± 0.41b	-36.7
CCl_4 + Plant Hexane Extract	32	8.55 ± 0.59a	-5.0
	64	5.90 ± 0.20b	-34.4
	96	5.35 ± 0.50b	-40.6

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

4-5-6: Glutamic Oxaloacetic Transaminase (GOT)

The three doses of methanol extract (32, 64 and 96) and vitamin C were effective in a significant reduction of GOT serum activity (283.5, 231.25, 253.5 and 256.25 IU/L, respectively) as compared to control I (336.0 IU/L). The treatment efficiencies of such reductions were -15.6, -31.2, -24.6 and -23.7%,

respectively (Table 4-46). In hexane extract, the three doses and vitamin A were non-effective in a significant reduction of GOT serum activity (Table 4-47).

Table 4-46: Glutamic oxaloacetic transaminase activity (mean ± S.E.) in sera of albino male mice after interactions between CCl_4 and methanol extract of *O. vulgare*.

Interaction Groups	Dose (mg/kg)	Mean ± S.E. (IU/L) *	Treatment Efficiency (%)
Control I (CCl_4 + distilled water)		336.0 ± 61.59a	
Control II (CCl_4 + vitamin C)	180	256.25 ± 23.36b	-23.7
CCl_4 + Plant Methanol Extract	32	283.50 ± 13.55b	-15.6
	64	231.25 ± 16.98b	-31.2
	96	253.50 ± 9.17b	-24.6

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

Table 4-47: Glutamic oxaloacetic transaminase activity (mean ± S.E.) in sera of albino male mice after interactions between CCl_4 and methanol extract of *O. vulgare*.

Interaction Groups	Dose (mg/kg)	Mean ± S.E. (IU/L) *	Treatment Efficiency (%)
Control I (CCl_4 + olive oil)		302.5 ± 4.55a	
Control II (CCl_4 + vitamin A)	1.4	265.00 ± 27.25a	-12.4
CCl_4 + Plant Hexane Extract	32	282.00 ± 19.16a	-6.7
	64	286.00 ± 8.15a	-5.4
	96	286.75 ± 4.83a	-5.2

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

4-5-7: Glutamic Pyruvic Transaminase (GPT)

The three doses of methanol extract and vitamin C were effective in a significant reduction of GPT serum activity (106.25, 79.50, 89.25 and 102.25 IU/L, respectively) as compared to control I (141.25 IU/L), with treatment efficiencies of -24.8, -43.7, -36.8 and -27.6%, respectively (Table 4-48). The three doses of hexane extract showed a similar effect but the treatment efficiencies were different (-15.4, -33.3 and -21.7%, respectively (Table 4-49).

Table 4-48: Glutamic pyruvic transaminase activity (mean ± S.E.) in sera of albino male mice after interactions between CCl_4 and methanol extract of *O. vulgare*.

Interaction Groups	Dose (mg/kg)	Mean ± S.E. (IU/L) ∗	Treatment Efficiency (%)
Control I (CCl_4 + distilled water)		141.25 ± 3.9a	
[illegible]	[illegible]	[illegible]	[illegible]
CCl_4	32	106.25 ± 6.65b	[illegible]
+	[illegible]	[illegible]	-43.7
[illegible]	96	[illegible]	[illegible]

∗: Different letters: significant difference (P ≤ 0.05) between means.

Table 4-49: Glutamic pyruvic transaminase activity (mean ± S.E.) in sera of albino male mice after interactions between CCl_4 and hexane extract of *O. vulgare*.

Interaction Groups	Dose (mg/kg)	Mean ± S.E. (IU/L) ∗	Treatment Efficiency (%)
Control I (CCl_4 + olive oil)		135.00 ± 5.21a	
Control II (CCl_4 + vitamin A)	1.4	93.75 ± 4.01b	-30.6
CCl_4	32	114.25 ± 6.75c	-15.4
+	64	90.00 ± 4.04b	-33.3
Plant Hexane Extract	96	105.75 ± 6.47c	-21.7

∗: Different letters: significant difference (P ≤ 0.05) between means.

4-5-8: Alkaline phosphatase (ALP)

Only the second and third doses of methanol extract were effective in a significant reduction of serum ALP activity as compared to control I (97.0 and 109.0, respectively *vs*. 152.25 IU/L), with treatment efficiencies of -36.3 and -28.4%, respectively (Table 4-50), while the three doses of hexane extract or vitamin A were significant in this regard with treatment efficiencies of -18.6, -27.2, -14.9 and -20.3%, respectively (Table 4-51).

Table 4-50: Alkaline phosphatase activity (mean ± S.E.) in sera of albino male mice after interactions between CCl_4 and methanol extract of *O. vulgare*.

Interaction Groups	Dose (mg/kg)	Mean ± S.E. (IU/L) ∗	Treatment Efficiency (%)
Control I (CCl_4 + distilled water)		152.25 ± 9.95a	
Control II (CCl_4 + vitamin A)	1.4	120.00 ± 7.88b	-20.3
CCl_4 + Plant Methanol Extract	32	134.50 ± 5.31a	-11.7
	64	97.00 ± 9.03b	-36.3
	96	109.00 ± 8.75b	-28.4

∗: Different letters: significant difference (P ≤ 0.05) between means.

Table 4-51: Alkaline phosphatase activity (mean ± S.E.) in sera of albino male mice after interactions between CCl_4 and hexane extract of *O. vulgare*.

Interaction Groups	Dose (mg/kg)	Mean ± S.E. (IU/L) ∗	Treatment Efficiency (%)
Control I (CCl_4 + olive oil)		150.50 ± 6.18a	
Control II (CCl_4 + vitamin A)	1.4	120.00 ± 7.88b	-20.3
CCl_4 + Plant Hexane Extract	32	122.50 ± 8.0b	-18.6
	64	109.50 ± 11.02b	-27.2
	96	128.00 ± 8.75b	-14.9

∗: Different letters: significant difference (P ≤ 0.05) between means.

4-5-9: Total Bilirubin

The three doses of methanol extract and vitamin C were significantly effective in decreasing the total serum level of bilirubin as compared to control I (1.99, 1.25, 180 and 1.49, respectively *vs*. 2.40 mg/dL) with treatment efficiencies of -17.0, -47.9, -25.0 and -37.9%, respectively (Table 4-52), while in hexane extract, only the second dose and vitamin A were effective in this regard with treatment efficiencies of -23.1 and -35.6%, respectively (Table 4-53).

Table 4-52: Total bilirubin level (mean ± S.E.) in sera of albino male mice after interactions between CCl_4 and methanol extract of *O. vulgare*.

Interaction Groups	Dose (mg/kg)	Mean ± S.E. (mg/dL) *	Treatment Efficiency (%)
Control I (CCl_4 + distilled water)		2.40 ± 0.18a	
Control II (CCl_4 + vitamin C)	180	1.49 ± 0.32b	-37.9
CCl_4 + Methanol Extract	32	1.99 ± 0.26b	-17.0
	64	1.25 ± 0.21c	-47.9
	96	1.80 ± 0.22b	-25.0

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

Table 4-53: Total bilirubin level (mean ± S.E.) in sera of albino male mice after interactions between CCl_4 and hexane extract of *O. vulgare*.

Interaction Groups	Dose (mg/kg)	Mean ± S.E. (mg/dL) *	Treatment Efficiency (%)
Control I (CCl_4 + olive oil)		2.08 ± 0.05a	
Control II (CCl_4 + vitamin A)	1.4	1.34 ± 0.31b	-35.6
CCl_4 + Plant Hexane Extract	32	2.09 ± 0.15a	+0.5
	64	1.60 ± 0.17b	-23.1
	96	2.14 ± 0.12a	+2.9

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

4-6: Histopathological Effects in Liver of Mouse

4-6-1: Methanol Extract of *O. vulgare*

Liver sections of animals treated with the first (32 mg/kg) or third (96 mg/kg) dose of methanol extract showed a normal looking histological structure (Figure 4-3).The second dose (64 mg/kg) showed a mild congestion and normal looking appearance (Figure 4-4) as compared to mice treated with distilled water (Figure 4-5).

Figure 4-3: Liver section of mouse treated with the first dose (32 mg/kg) of methanol extract showing normal looking structure (H. and E 20X).

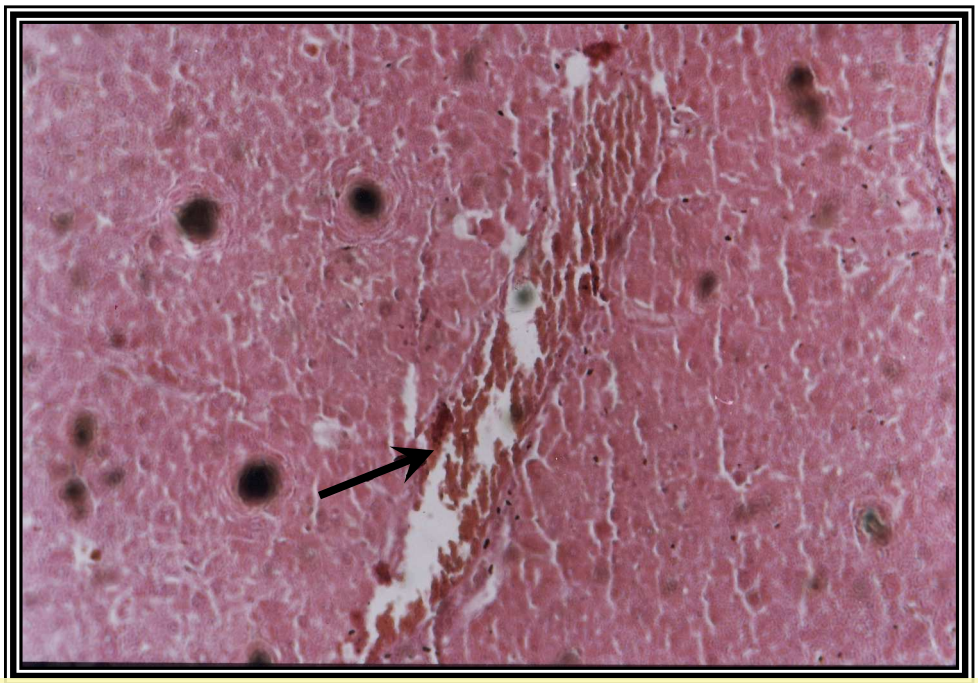


Figure 4-4: Liver section of mouse treated with the second dose (64 mg/kg) of methanol extract showing mild congestion (X) (H. and E.

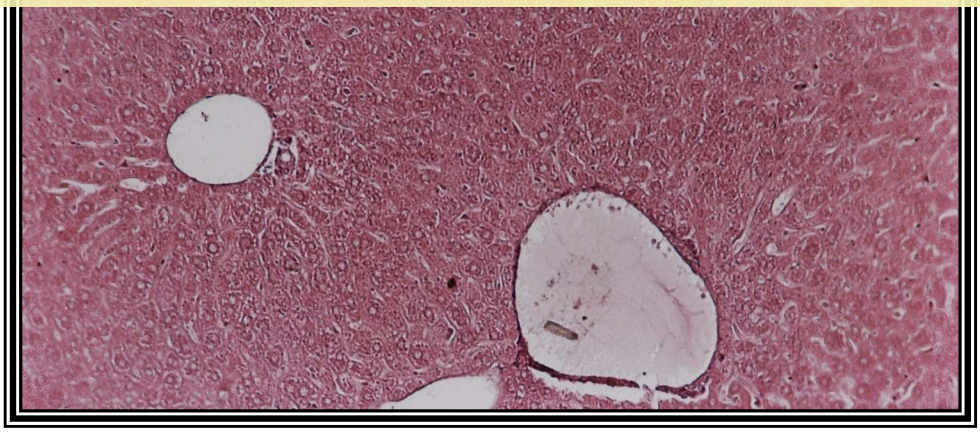


Figure 4-5: Liver section of mouse treated with distilled water showing normal looking structure consisting hepatic lobule with central vein (H. and E 20X).

4-6-2: Hexane Extract of *O. vulgare*

Liver sections of animals treated with the first (32 mg/kg) and second (64 mg/kg) does of hexane extract showed a normal looking histological structure with discrete focal degenerative cells (Figure 4-6). The second dose (96 mg/kg) showed a mild congestion with normal looking appearance (Figure 4-7) as compared to mice treated with olive oil (Figure 4-8).

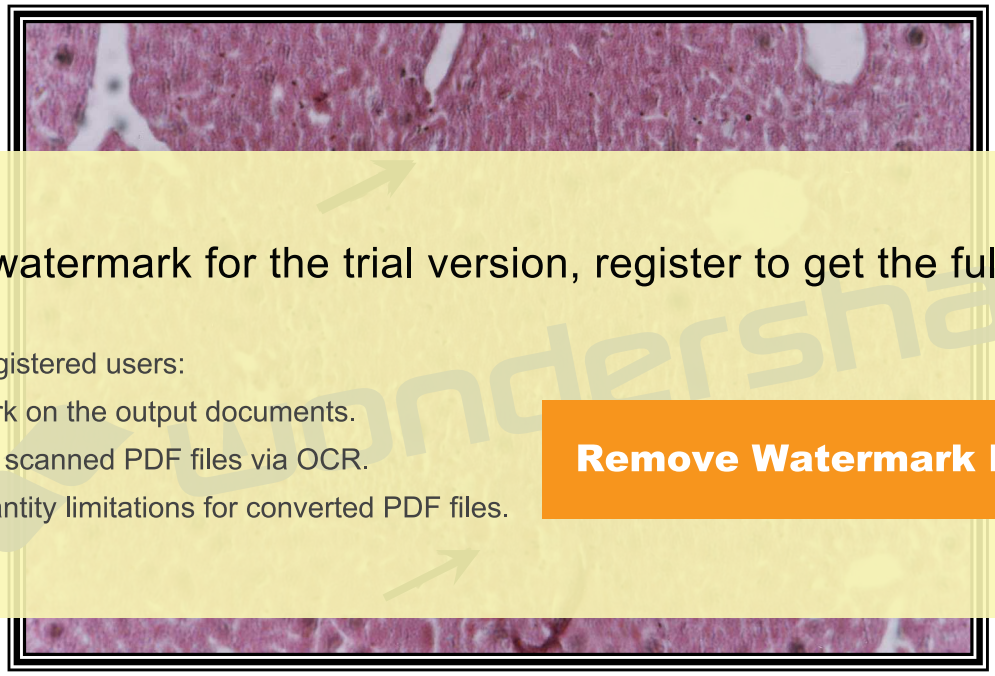
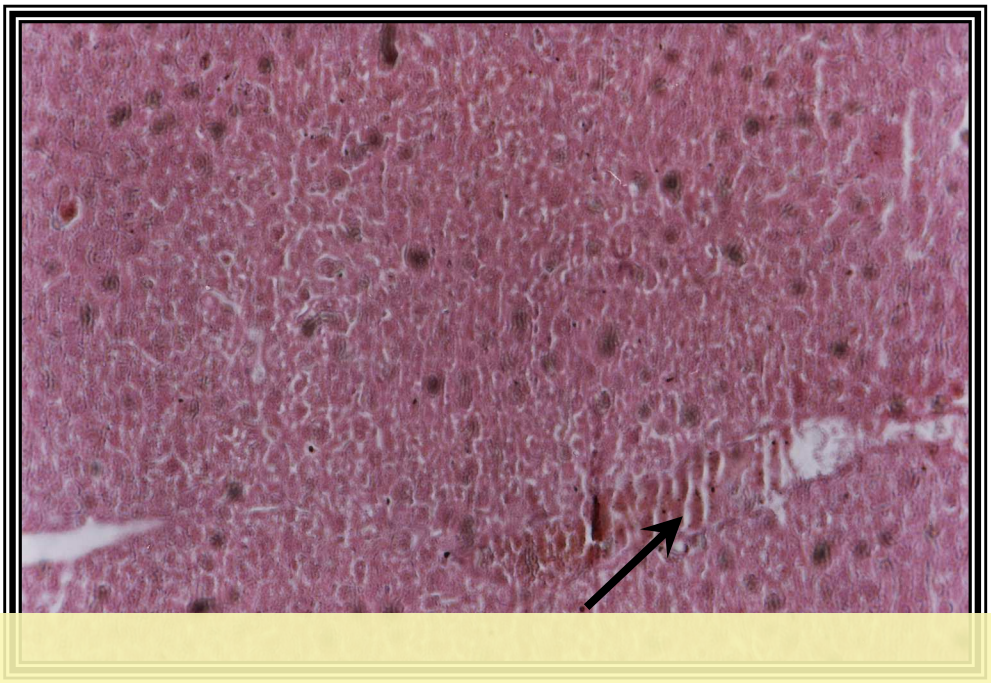


Figure 4-6: Liver section of mouse treated with the first dose (32 mg/kg) of hexane extract showing focal degenerative cells changes (⟶) (H. and E 20X).



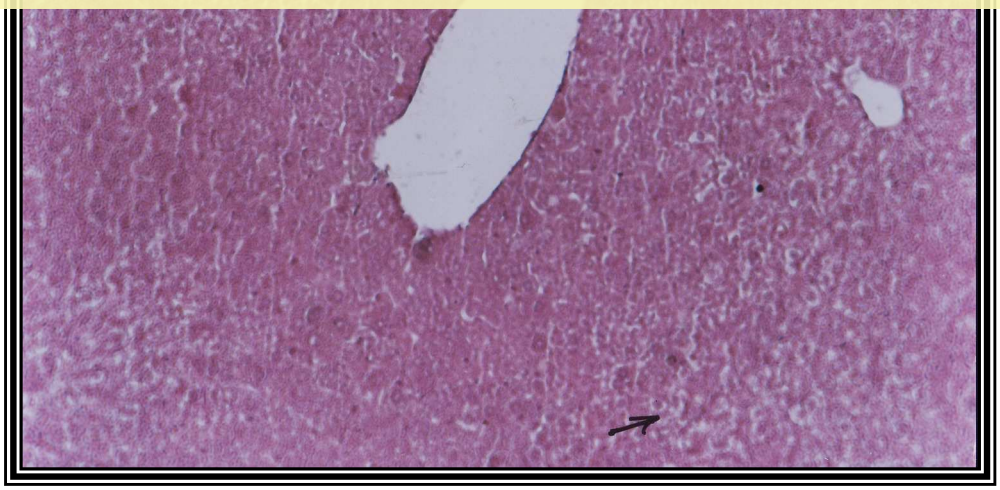
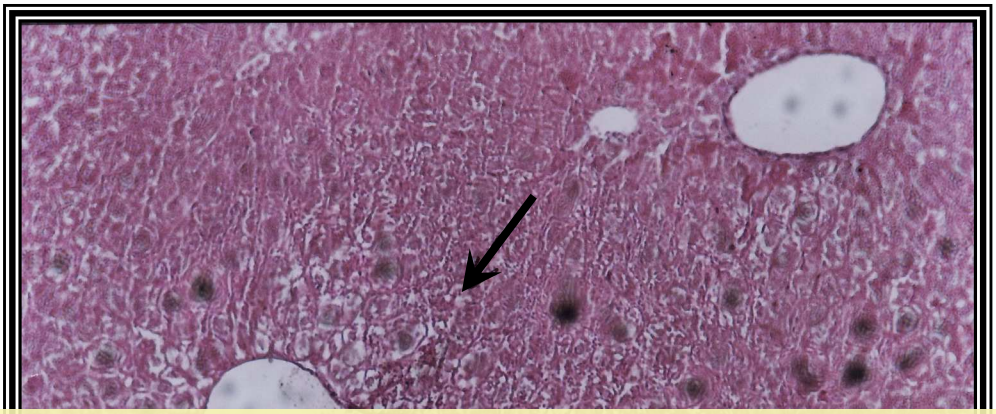


Figure 4-8: Liver section of mouse treated with olive oil showing normal looking structure (H. and E 20X).

4-6-3: Carbon Tetrachloride (CCl_4)

Liver sections of animals treated with CCl_4 showed marked fatty accumulation, degenerative areas and necrosis of hepatocytes around the central vein (Figure 4-9).



4-6-4: Methanol Extract – CCl_4 Interaction

Liver sections of animals treated with the first (32 mg/kg) or third (96 mg/kg) dose of methanol extract after CCl_4 treatment showed the still discrete areas of degeneration and necrosis (Figure 4-10), while in the sections of the first dose the degenerated areas were widely distributed (Figure 4-11).The second dose (64 mg/kg) and vitamin C were much more effective, and a mild congestion with a mild areas of degeneration and necrosis of hepatocytes were observed (Figure 4-12) as compared to the mice treated with distilled water after the treatment with CCl_4, in which the liver sections showed abundant degenerative changes and necrosis of hepatocytes (Figure 4-13).

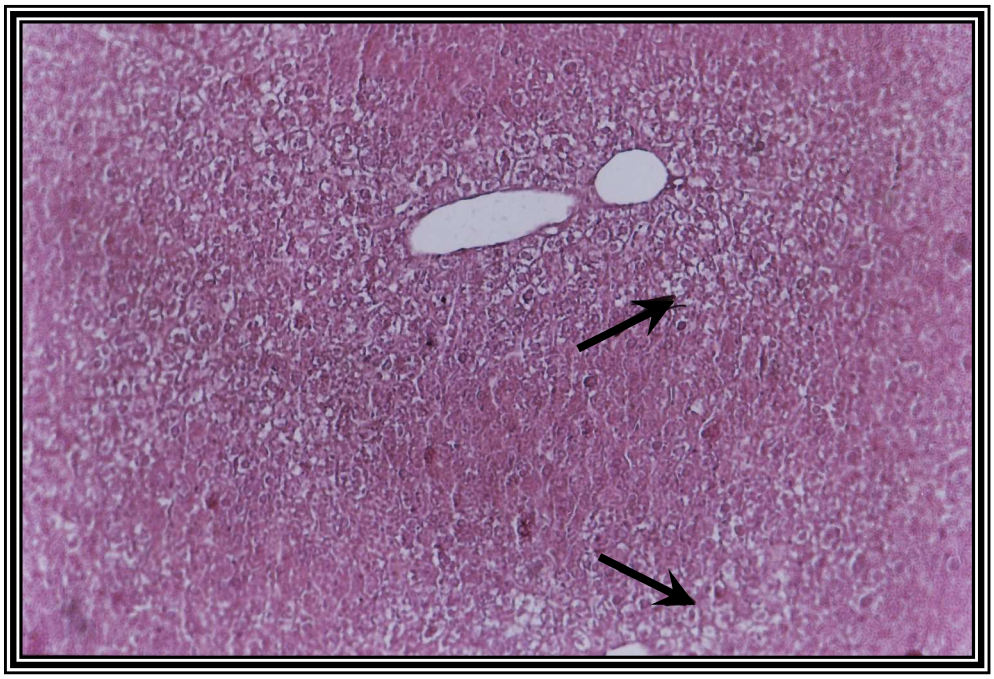


Figure 4-10: Liver section of mouse treated with the third dose (96 mg/kg) of methanol extract after CCl_4 treatment showing degeneration and necrosis (⟶) of hepatocytes (H. and E 10X).

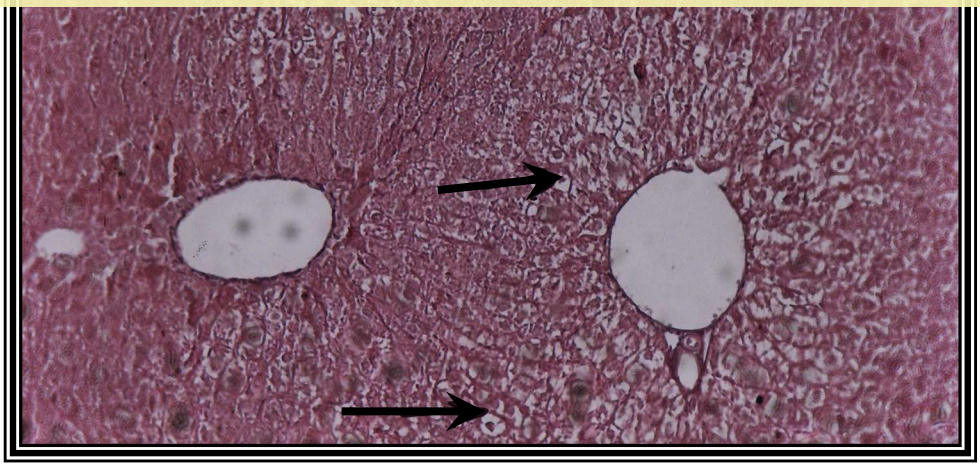


Figure 4-11: Liver section of mouse treated with the first dose (32 mg/kg) of methanol extract after CCl_4 treatment showing wide degeneration and necrosis (⟶) of hepatic cells (H. and E 20X).

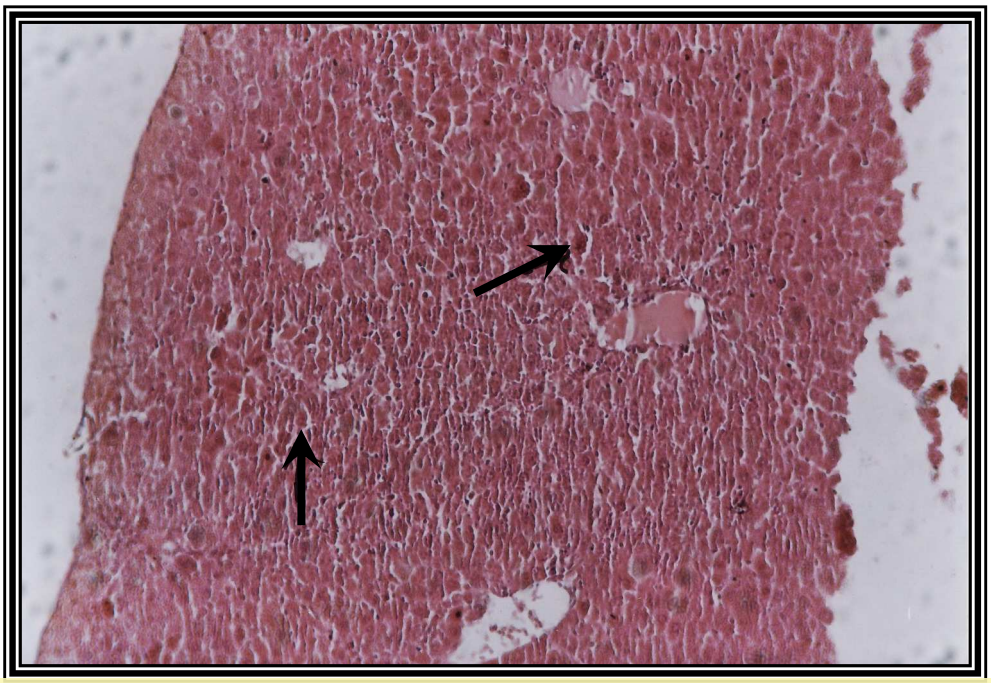


Figure 4-12: Liver section of mouse treated with the second dose (64 mg/kg) of [illegible] necrosis (⟶) of hepatic cells (H. and E 20X).

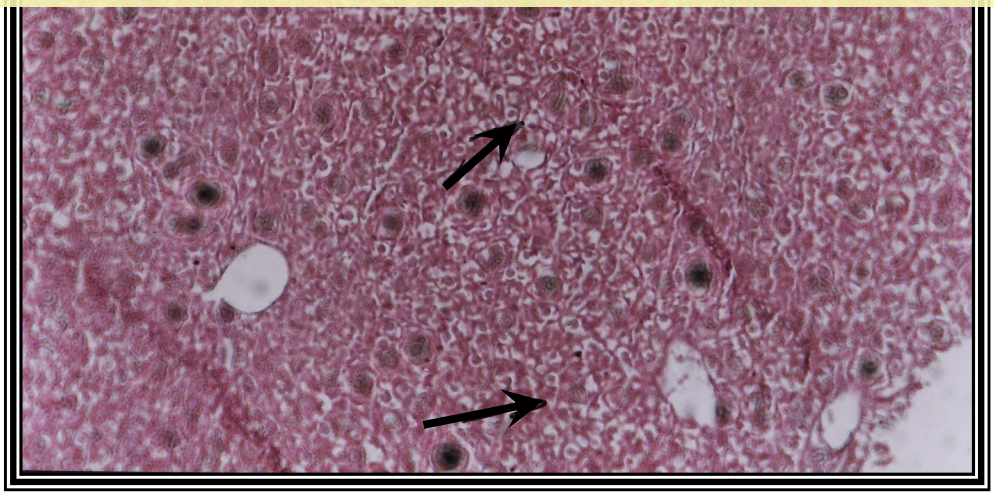


Figure 4-13: Liver section of mouse treated with distilled water after treatment with CCl_4 showing abundant degenerative cells and necrosis (⟶) of hepatic cells (H. and E 20X).

4-6-5: Hexane Extract – CCl_4 Interaction

Liver sections of animals treated with the first (32 mg/kg) dose of hexane extract after CCl_4 treatment showed the still appearance of degenerative changes and necrosis with inflammatory cells (Figure 4-14). The second dose (64 mg/kg) showed focal discrete areas of degeneration and necrosis of hepatocytes (Figure 4-15), but the third dose (96 mg/kg) showed mild degenerative areas and necrosis of hepatocytes with few inflammatory cells (Figure 4-16) as compared to mice treated with olive oil after CCl_4 treatment, in which the liver sections showed wide degenerative changes and necrosis of hepatocytes (Figure 4-17). Vitamin A showed a mild congestion with still abundant degenerative effect and necrosis after treatment with CCl_4 (Figure 4-18).

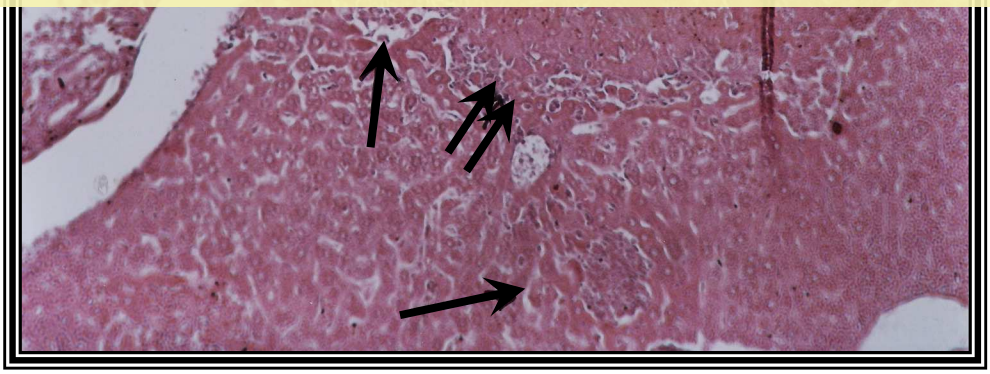


Figure 4-14: Liver section of mouse treated with the first dose (32 mg/kg) of hexane extract after CCl_4 treatment showing degeneration and necrosis (⟶) with inflammatory cells infiltrate (⟹) (H. and E 20X).

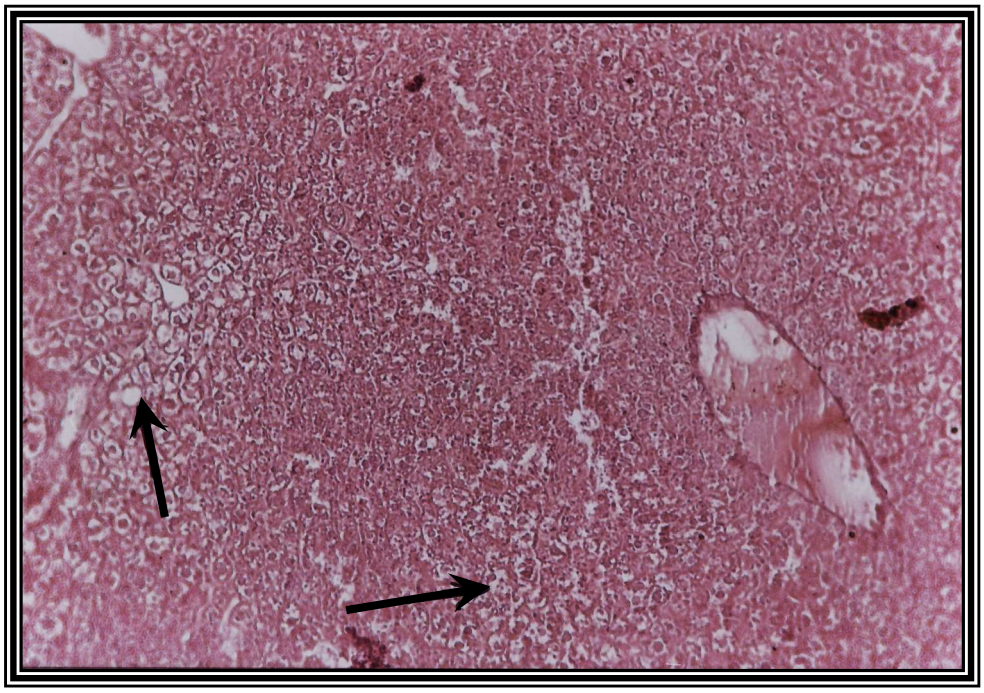


Figure 4-15: Liver section of mouse treated with the second dose (64 mg/kg) of hexane extract after CC_4 treatment showing focal discrete areas of degeneration

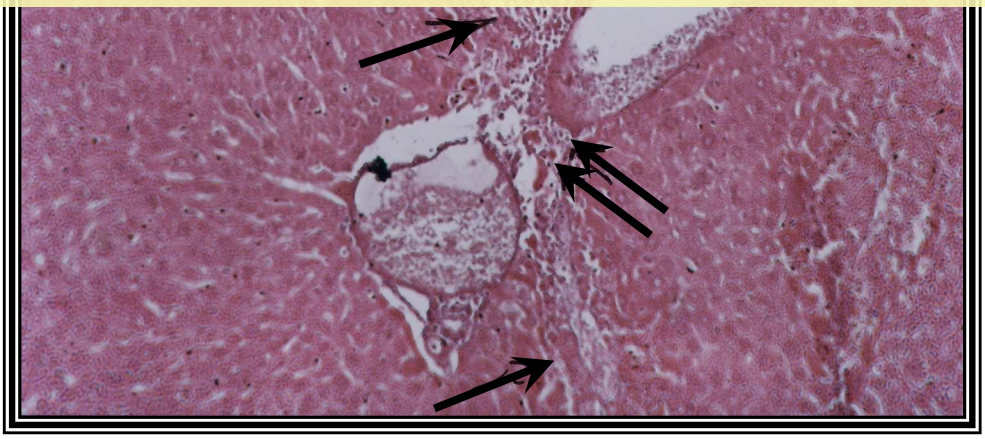


Figure 4-16: Liver section of mouse treated with the third dose (96 mg/kg) of hexane extract after CCl_4 treatment showing mild degeneration and necrosis (⟶) with few inflammatory cells infiltrate (⟹) (H. and E 20X).

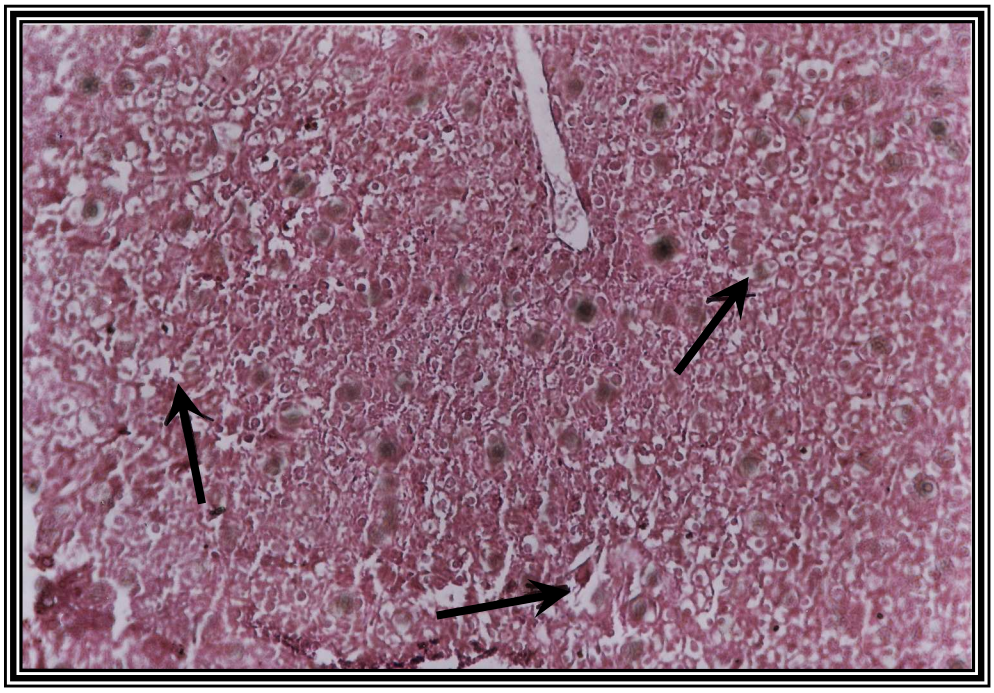


Figure 4-17: Liver section of mouse treated with olive oil after CCl_4 treatment showing abundant degeneration and necrosis (⟶) of hepatocyte (H. and E 20X).

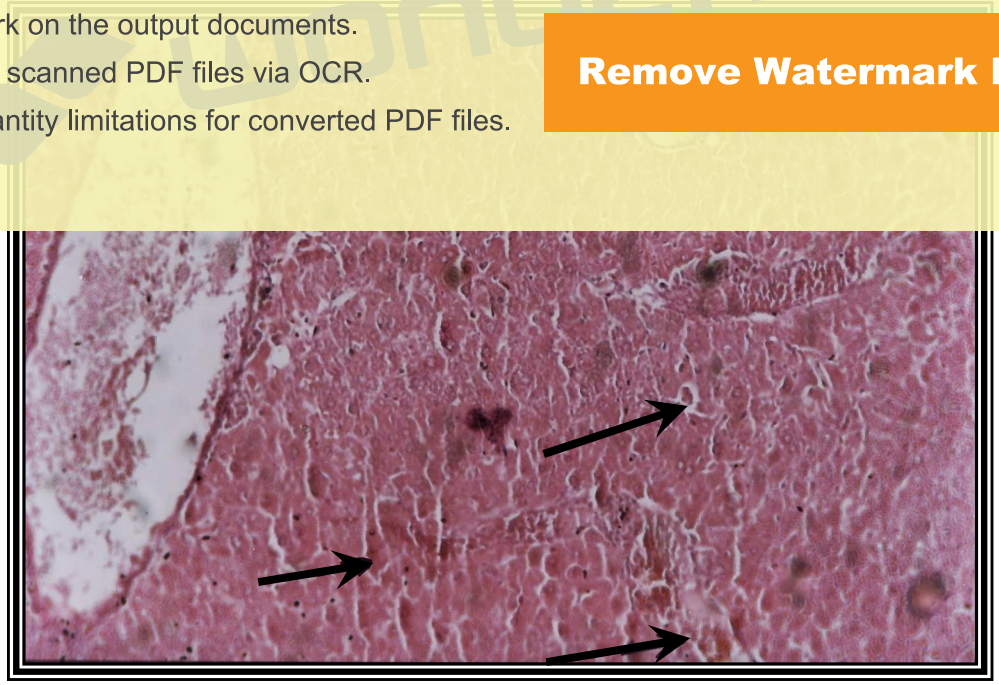


Figure 4-18: Liver section of mouse treated with vitamin A after CCl_4 treatment showing mild congestion with abundant degeneration and necrosis (⟶) of hepatocyte (H. and E 20X)

Chapter Five

Discussion

The role of various plant extracts as immune modulators, anti-mutagens and hepatoprotective is being increasingly recognized (Dhir *et al*., 1990). These properties were evaluated in the present study for two extracts (methanol and hexane) of oregano against the oxidant CCl_4. As expected, such agent demonstrated a wide range of devastating effects to the biological system of the laboratory animal male mouse. Such effects were manifested as a decreased count of total leucocytes, lymphocytes and neutrophils (immune suppressive), decreased mitotic activity of bone marrow cells, increased frequency of micronucleus formation and sperm-head abnormalities (genotoxic) and increased activities of liver functions enzymes (hepato-damage). These findings have been explained mainly in the ground of indirect genotoxic effects, because it has been reported that CCl_4-induced mutations may not be a result of direct damage to DNA but may occur via indirect [illegible] functions important in DNA synthesis and/or repair (Zemlin *et al*., 1988). Moreover, an oxidative damage produced by CCl_4 in mice has been reported (Chaurasia *et al*., 2000), and such demonstration may suggest that the antioxidant enzymes are depressed as a consequence of CCl_4-treatment, and the DNA damage may be due to depletion of these enzymes.

In contrast, the oregano two extracts had no such effects and the general outcome of animal treatments was in favour that the plant is neither immune suppressive, mutagenic nor hepato-toxic; moreover, some positive augmentations of the investigated parameters were observed. Such picture was further cleared when the plant extracts were given to the animals after CCl_4 administration, and most of the CCl_4-induced immunological, genetic and hepatic damages were almost repaired and normalizations of the parameter's

values was reached. Such properties were mostly dose-dependent, as well as, the type of solvent of extraction had some effect.

Some of these properties have been previously demonstrated, although different approaches were employed, and the effects were ascribed to the anti-oxidant potentials of the plant or their products (Lima *et al*., 2004; Capecka *et al*., 2005; Sanmugapriya and Venkataraman, 2006). Moreover, the antioxidant potentials were explained in the ground of chemical constituents that are available in the plant extracts (Mojca- Skerget *et al*., 2005). Chemical analysis of oregano extracts (methanol and hexane) revealed the presence of volatile oils, steroids and terpens in hexane extract and flavonoids, tannins and coumarin in methanol extract. Similar findings have been demonstrated by other investigators (Daferera *et al*., 2000; Isman, 2000; Gracia and Sanz, 2001; Mockute *et al*., 2001; Daferera *et al*., 2003). These investigators demonstrated that antimicrobial properties of oregano are related to the volatile oils due to flavonoids. Moreover, these two constituents are important anti-mutagenic and anti-carcinogenic compounds *in vitro* and *in vivo* (Puertas-Mejia *et al*., 2002; Nostro *et al*., 2004; Nostro *et al*., 2004). Accordingly, the volatile oils of oregano have shown a broad spectrum of *in vitro* and *in vivo* antibacterial (Lambert *et al*., 2001; Nostro *et al*., 2004), antifungal (Manohar *et al*., 2001), insecticidal (Isman *et al*., 2001), antioxidants (Kulisic *et al*., 2004) and anti-carcinogenic effects (Teissedre and Waterhouse, 2000). Chemical compositions of these oils have been well documented and they included monoterpenes, biogenetically related phenolics, as well as, sesquiterpenes (Daferera *et al*., 2003).

Therefore, both extracts may serve as good antioxidants, and in this regard Yanishlieva and Marinova (1995) examined the antioxidant activity of methanol and hexane extracts of oregano grown in Bulgaria. Their results suggested that methanol extract behaved as a strong free radical scavenger, whereas the oil showed a weaker activity. Total phenolic constituents based on gallic acid equivalents revealed the presence of total soluble phenolics in the

extract and, most probably, they were responsible for the radical scavenging activity of methanol extracts (Sahin *et al*., 2004). However, little information is available about the antioxidant activity of other oregano compounds, although some authors have found free radical scavenging activity in aqueous and organic extracts of oregano leaves (Cervato *et al*., 2000; Bendini *et al*., 2002).

The results demonstrated that a treatment with oregano extracts showed a positive effect on total and absolute counts of leucocytes (lymphocytes and neutrophils), and these counts manifested a significant increase especially in animal treated with the hexane extract. Hexane extract (64 and 96 mg/kg) was even better than vitamin A in increasing total and absolute counts of leucocytes with the exception of neutrophils, while vitamin C was better than methanol extract in this regard. Vitamin C can protect plasma lipid and membrane lipid from the effect of oxidant compounds through either increase the production of cytokines or interfere with the formation of prostaglandins, both consequences are in favour of immune system enhancement, especially the antibody production (Hughes, 2001).

Some antimutagens act directly on mutagens or on those agents that inhibit the generation of active mutagenic forms (Kada *et al*., 1987). The present study investigated the antimutagenic activity of oregano in terms of its inhibitory effect against formation of micronuclei induced by CCl_4 in bone marrow cells of mice. Micronuclei are chromatid/chromosome fragments that are left behind after expulsion of the main nucleus during maturation of erythroblasts to erythrocytes in the bone marrow (Khanam and Devi, 2005).

These represent the consequence of DNA damage caused by externally administered substances. In the present study the genotoxicity of CCl_4 was evident in the micronucleus test. Administration of CCl_4 (3.2 mg/kg) caused a significant increase in percentage frequency of micronuclei.

The extracts of oregano could significantly inhibit CCl_4-induced micronuclei in bone marrow cells, as well as, sperm head abnormalities. Moreover there was

a significant enhancement in blood cell counts and mitotic index of bone marrow cells in oregano-treated animals.

The function of immune system is also genetically determined. To explore the effects of oregano extracts (methanol and hexane) on the genetic make-up of mice directly or through interactions with CCl_4, the mitotic index, micronucleus formation in polychromtic cells of bone marrow and sperm-head abnormalities served as good parameters of mutagenic evaluations. The results of genetic evaluations showed that a treatment with oregano extracts was associated with a significant reduction in micronucleus formation and sperm head abnormalities and caused a significant increase in mitotic index of the bone marrow cells. Accordingly to these parameters, the hexane extract was more effective than methanol extract in a dose dependant type. Such findings can be considered important, especially if we consider that most cancers are preceded by mutations induced by different agents, especially those that have oxidative (Ames, 1990; Ad'hiah *et al*., 2002) like CCl_4, which was used in this study and showed genotoxic effects.

The protective effect of oregano hexane extract may be due to the presence of active constituent compounds including essential oils, terpens and steroids. The responsible constituent of action in oregano is the essential oil, which contains carvacrol and thymol as the primary components (Kokkini, *et al*., 1994).The major constituents of essential oils are phenolic monoterpens such as thymol and carvacrol which are responsible for the main biological activities (Aeschbach *et al*., 1994; Bagamboula *et al*., 2004; Nostro *et al*., 2004). It has been suggested that carvacrol exerts its activities by interacting with the cytoplasmic membrane via its own hydroxyl group, thus changing the permeability of membrane for protons and potassium ions (Ultee *et al*., 2002).The plant steroids may provide a protection to the plasma lipids as a result of antioxidant activity, and participate in the integrity of cell membrane. Such consequences are able to modify the immune functions in the sense of enhancement (Bramely *et al*, 2000; Lyons *et al*., 2001; Moller and Loft, 2002).

With regard to antimutagenicity, dietary steroids are also antioxidants, which are expected to reduce cancer risk by minimizing DNA damage or reducing mutational changes (Elmadfa and Park, 2004).

The present study was also conducted to evaluate the protective effect of the methanol and hexane extracts of oregano against CCl_4 induced hepatic disorder in mice. Results suggest that the extracts possess protective action against hepatic dysfunction induced by the potent hepatotoxin, CCl_4.

A number of chemicals including various environmental toxicants and clinically useful drugs can cause severe cellular damages in different organs of the body through the metabolic activation to highly reactive substances such as free radicals, and CCl_4 is one of such extensively studied environmental toxicant. The reactive metabolite trichloromethyl radical ($\cdot CCl_3$) is one of the formed forms that result from a metabolic conversion of CCl_4 by cytochrome P-450 (Recknagel *et al.*, 1989). The CCl_3 radical in the presence of O_2 and consequently many reactive free radicals are formed from CCl_4. These free radicals are able to initiate lipid peroxidation of membrane phospholipids (Recknagel *et al.*, 1989), generate PUFA and covalently bind to microsomal lipids and proteins. This phenomenon results in the generation of reactive oxygen species (ROS), (like the superoxide anion O_2^-, H_2O_2 and the hydroxyl radical, ·OH). Evidence suggests that various enzymatic and non-enzymatic systems have been developed by the cell to cope up with the ROS and other free radicals. However, when a condition of oxidative stress establishes, the defense capacities against ROS becomes insufficient (Halliwell and Gutteridge, 2000).The ROS also affects the antioxidant defense mechanisms, reduces the intracellular concentration of reduced glutathione (GSH) and decreases the activity of superoxide dismutase (SOD) and catalase (CAT). It has also been known to decrease the detoxification system produced by glutathione-S-transferase (GST) (Yamamoto and Yamashita, 1999). Increasing evidence

indicates that oxidative stress causes organ injury and carcinogenesis (Stal and Olson, 2000).

In the liver, CCl_4 is metabolized by the cytochrome P450-dependent monooxygenase systems followed by its conversion to more chemically active form, trichloromethyl radical ($\cdot CCl_3$). The enzymes involved in this process are located in the endoplasmic reticulum of the liver and their activities are dependent on many environmental factors. Some herbal extracts are known to prevent the oxidative damages in different organs by altering the levels of cytochrome P-450 through their antioxidant properties (Rajesh and Latha, 2004).

In biochemical analysis, the levels of liver function enzymes were also significantly decreased. These findings suggest that oregano extracts are effective in preventing DNA damage, and one of the mechanisms of action might involve scavenging of active oxygen species generated by the mutagens initiated by the mutagens. Earlier studies with known antioxidants have also reported that they help in counteracting CCl_4 damage, for instance vitamin C dose of Vitamin C that was used in the present experiments was 120 mg/kg and at this dose the vitamin did not change the mitotic index value notably and did not have mutagenic effects. Carbon tetrachloride showed strong reduction in the mitotic index of bone marrow cells at the test dose and markedly induced micronucleus formation and sperm head abnormalities. However, the inhibitory effect of CCl_4 on the mitotic activity was significantly decreased with treatment by vitamin C. Micronucleus formation and sperm head abnormalities induced by CCl_4 also decreased markedly. Therefore, vitamin C acts as either a radical scavenger or a pro-oxidant producing hydrogen peroxide and free radicals by autoxidation (Shamberger, 1984; Anderson, 1996).

The antimutagenic effects of oregano extracts were further evaluated by the mouse sperm morphology assay that was developed by Wyrobek, and its relevance in evaluating mammalian germ cell mutagens is well accepted

(Wyrobek and Bruce, 1975; Wyrobek *et al*., 1983). Presence of abnormal sperm head suggests an induction of a genetic damage in the male germ cells. Sperm head abnormalities may arise due to small deletions or point mutations (Jha and Bharti, 2002). Abnormality in sperm head may occur by physiological, cytotoxic or genetic mechanisms (Odeigah, 1997) and alteration in testicular DNA, which in turn disrupts the process of differentiation of spermatozoa (Bruce and Heddle, 1979). In the present experiment, CCl_4 induced an increase in the frequency of abnormal sperm and significantly higher percentage of abnormal sperm was recorded after one day of treatment. However, the percentage of abnormal sperm heads decreased significantly after a treatment with methanol or hexane extract and in a dose-dependent manner. Therefore, this assay further qualifies the oregano extracts as antimutagens.

plant extracts possess favorable metabolic effects on liver function in treated mice, but by which mechanism it is not well defined and require further investigations, although the suggested antioxidant profile of plant can not be ignored. In the literature, some experiments have shown antioxidant properties of oregano or its products, and these studies suggested that the plant protects against free radicals and inhibits inflammatory mediator synthesis and release (Kokdil *et al*., 2005).

This protective effect has been attributed in part to flavonoids, through modulation of several enzymes of the P450 family involved in precarcinogen metabolism (Zhai *et al*., 1998). The extracts of oregano contain triterpenes and flavonols; for this reason, it is not surprising that extracts of this polyphenolic

plant have been used for multiple medicinal purposes in folk therapy (Ramos *et al*., 1998). Therefore, the suggestion is that this effect is caused by flavonols, although a possible synergistic or antagonistic effect of flavonols with other compounds in the extracts can not be excluded.

Since the changes associated with CCl_4-induced liver damage are similar to that of acute viral hepatitis (Suja *et al*., 2004), CCl_4-mediated hepatotoxicity was chosen as the experimental model. The ability of a hepatoprotective drug to reduce the injurious effects or to preserve the normal hepatic physiological mechanisms that have been disturbed by a hepatotoxin is the index of its protective effects (Yadav and Dixit, 2003). The hepatotoxicity induced by CCl_4, as mentioned earlier, is due to its metabolite ·CCl3, a free radical that alkylates cellular proteins and other macromolecules with a simultaneous attack on polyunsaturated fatty acids, in the presence of oxygen, to produce lipid peroxides, leading to liver damage (Chatterjee *et al*., 1997). Hepatocellular necrosis leads to elevation of the serum marker enzymes which are released from the liver into blood (Shirwaikar *et al*., 2003). The increased levels of SGOT, SGPT, ALP and bilirubin are considered as indicators of liver damage (Achliya *et al*., 2004). The present study revealed a significant increase in the activities of SGOT, SGPT, ALP and serum bilirubin levels on exposure to CCl_4, indicating considerable hepatocellular injury. Administration of both, methanol or hexane extracts at three different dose levels, attenuated the increased levels of the serum enzymes produced by CCl_4 and caused a subsequent recovery towards normalization almost like that of vitamin A, vitamin C or untreated controls. The hepatoprotective effect of the plant extracts was further concluded by the histopathological examinations. The results showed that both extracts at different dose levels offer hepatoprotection, but the dose 64 mg/kg was more effective than all other doses and this may be due to its rich content of polysaccharides, and hepatoprotective action of certain polysaccharides has been well-documented in the literature (Chiu *et al*., 1992; Ye *et al*., 2001).

It has been hypothesized that one of the principal causes of CCl_4-induced liver injury is formation of lipid peroxides by free radical derivatives of CCl_4 (•CCl3). Thus, the antioxidant activity or the inhibition of the generation of free radicals is important in the protection against CCl_4-induced hepatopathy. The body has an effective defense mechanism to prevent and neutralize the free radical-induced damage. This is accomplished by a set of endogenous antioxidant enzymes such as SOD, catalase and glutathione peroxidase (GPX). These enzymes constitute a mutually supportive team of defense against ROS (Venukumar and Latha, 2002). In CCl_4-induced hepatotoxicity, the balance between ROS production and these antioxidant defenses may be lost, 'oxidative stress' results, which through a series of events deregulates the cellular functions leading to hepatic necrosis. Unfortunately, it was not possible to investigate these enzymes in the present study.

The histopathological examinations of liver sections in the present study revealed a regeneration of hepatic cells after treatments with the plant extracts; therefore it is possible to suggest that these extracts being able to condition [illegible] accelerated [illegible] 1996). The observed protective effect of oregano against the hepatotoxins may be attributed to the presence of flavonoids, terpenoids and sterols, which are among the important plant constituents (Misra *et al*., 2001). Flavonoids are a group of polyphenolic compounds with known properties which include free radical scavenging, inhibition of hydrolytic and oxidative enzymes and anti-inflammatory action leading to hepatoprotection (Hewawasam *et al*., 2003). However, in several plants with hepatoprotective properties the crude drug effect is known to be mediated by the action of a number of different active components, and the potencies of the individual active principles being less than that of the crude extract (Jayatilaka *et al*., 1989).

The mechanism by which oregano exerts its protective action against the hepatotoxin-induced alterations in the liver is not clear. The fact that treatment with the plant extracts is also capable of bringing about a recovery indicates that

the protective action of oregano may not simply be due to an antioxidative property, and other mechanism may be operating and further investigations are certainly required.

To conclude the present discussion, the investigated oregano extracts were able to modulate the immune-supressive, mutagenic and hepatotoxic effects of CCl_4 in mice, and their modulations shared the effect of two well-known anti-oxidant vitamins; A and C. However, the mechanism of action, as suggested by many investigators, is related to the plant constituents that have anti-oxidant activities, although other mechanisms may underline the effects.

Conclusions:

1. The plant of oregano (*Origanum vulgare*) is rich in several active compounds including flavonoids, coumarins, phenolic compounds, terpens, steroids, tannins and volatile oils**.**
2. Carbon tetrachloride (CCl_4) was immune suppressive, mutagenic and hepatotoxic agent as suggested by the results of total and absolute counts of leucocytes, mitotic index, micronucleus formation, sperm-head abnormalities, biochemical and histopathological studies.
3. Methanol and hexane extracts of *Origanum vulgare* were significantly effective in enhancing the values of the investigated parameters, especially the mitotic index, which showed a significant increase, and the [illegible] which showed a significant decrease, in addition to their enhancing effects on the immune system by increasing total and absolute counts of [illegible] Natural oregano extracts were significantly effective in modulating the immune suppressive and mutagenic effects of CCl_4, and the biochemical parameters confirmed the hepatoprotective effect of oregano extracts through decreasing or normalizing the level of serum enzymes and repairing the induced cellular damage of liver.
5. Oregano hexane extract was even better than vitamin A in increasing total and absolute counts of leucocytes with the exception of neutrophils, while vitamin C was better than methanol extract in this regard.
6. Vitamins A and C were significantly effective in modulating the immune suppressive and mutagenic effects of CCl_4 especially in decreasing the micronucleus formation and sperm head abnormalities.

7. The biochemical and histopathological studies showed the hepatoprotective effect of both vitamins through decreasing the level of serum enzymes and repairing the cellular damage of liver induced by CCl_4.

Recommendations:

1. Isolation and characterization of active compounds from the *Origanum vulgare* leaves and investigating their immunological, cytogenetic and histopathological effects *in vitro*, and in this regard, the parameters of evaluations should be more advanced and based in the recent advanced technologies.
2. Further studies are needed to reveal the potentials of the plant in relations to other oxidant agents, and in this regard the anti-oxidant properties should be examined.
3. [illegible] histopathological effects of carbon tetrachloride.

- **Achliya,** G.S.; Wadodkar, S.G. and Dorle, A.K. (2004). Evaluation of hepatoprotective effect of Amalkadi Ghrita against carbon tetrachloride induced hepatic damage in rats. *Journal of Ethnopharmacology*, **90:** 229–232.
- **Ad'hiah,** A. H.; Al-Kashaly, S. S. and Abbas, T. A. A. (2002). Group A Streptococcus (*Streptococcus piygoenes*) and the mitotic activity of lymphoid organs in albino mice. *The Eight Scientific Conferences of the Technical Education Committee*, **pp**. 302-208.
- **Ad'hiah,** A. H.; Ghali, K. H. and El-Hassani, M. (2001a). An epidemiological approach to bladder cancer in Iraq from 1976 to 1998. *AL-Mustansirya Journal of Science*, **11**: 25- 30.
- **Ad'hiah,** A. H.; Hassan, M. K. A. and Kadhim, K. K. (2001 b).The hematological and cytogenetic effect of gamma radiation on white Mouse (*Mus Musculus*). Ibn Al – Haitham *Journal of Pure and Applied Science*, **14**: 1-6.
- **Ad'hiah,** A. H.; Jalood, Y. D. and S[illegible] hematological and cytogenetic effects of tamoxifen by alcoholic extract of garlic (*Allium Sativum*). *Nucleus*, **47**:10 - 16.
- **Adam,** K.; Sivropoulou A.; Lanalas, T. and Arsenakis, M. (1998). Antifungal activity of *Origanum vulgare* subsp. *Hirtum*, *Mentha spicata*, *Lavandula angustifolia* and *Salvia fruticosa* essential oils against human pathogenic fungi. *Journal of Agricultural and Food Chemistry*, **46**:1739–1745.
- **Aeschbach,** R.; Loliger, J.; Scott, B. C.; Murcia, A.; Butler, J. and Halliwell, B. (1994). Antioxidant action of thymol, carvacrol, 6-gingerol, zingerone and hydroxytyrosol. *Food and Chemical Toxicology*, **32**: 31–36.
- **Akyon, Y**. (2002). Effect of antioxidants on the immune response of *Helicobacter pylori*. *Clinical Microbiology*, **8**:438 – 441.

- **Al – Janabi**, A.A. (2004). Effects of *Calendula officinalis* extracts on the Growth of some pathogenic Microorganisms. M.Sc. thesis, College of Science/University of Al- Nahrain.
- **Allen**, J. W.; Shuler, C. V.; Mendes, R. W. and Latt, S. A. (1977). A simplified technique for *in vivo* analysis of sister chromatid exchanges using 5-bromo-deoxy uridine tablets. *Cytogenetics and Cell Genetics*, **18**: 231-237.
- **Aligiannis**, N.; Kalpoutzakis, E.; Mitaku, S. and Chinou, I. B. (2001). Composition and antimicrobial activity of the essential oils of two Origanum species. *Journal of Agricultural and Food Chemistry*, **49**: 4168–4170.
- **Al-Keenani**, I. B. (2005) .The role of Vitamins A, C and E in modulating the genetic and immunological effects of etoposide in albino mice *Mus musculus*. M.Sc. thesis. College of Education/Ibn Al-Haitham/University of Baghdad.
- **Al-Maisary**, M. (1999).Effect of oil and alcoholic extract of *Azdirachta indica* on some pathogenic fungi of pl[illegible] Mustansiria University.
- **Al-Shami**, S.A. (1982). Study of some pharmacological and toxicological properties of Achillea flowers. M.Sc. thesis, College of veterinary medicin/University of Baghdad.
- **Al-Sudany**, A. M. (2005). Inhibitory effects of black seed oil and honey on the genotoxicity of tamoxifen in mice. M.Sc. thesis, College of Science/University of Al- Nahrain.
- **Amy**, L.; Stump, Pharm.D; Terri Mayo, Pharm .D. and *Alan* Blum, M.D. (2006). Management of Grapefruit-Drug Interactions. *American Family Physician*, **74:**605-608.
- **Anderson, D.** (1996). Antioxidant defenses against reactive oxygen species causing genetic and other damage, *Mutatation Research*, **350**: 103–108.

- **Atlas,** R. M. (1995). Principles of Microbiology.1st ed., Robert J. Callanan (ed). Chapter 4, P.122.
- **Bagamboula**, C. F.; Uyttendaele, M. and Debevere, J. (2004). Inhibitory effect of thyme and basil essential oils, carvacrol, thymol, estragol, linalool and p cymene towards Shigella sonnei and S. flexneri. *Food Microbiology*, **21**: 33–42.
- **Bancroft**, J.D. and Stevens, A. (1982). Theory and Practice of Histological Technique.2 ed. Churchill living stone. Edinburgh, London, pp: 662.
- **Bendich** A. (1997). Vitamin C Safety in Humans. In: Packer L, Fuchs J, eds. Vitamin C in health and disease. New York: Marcel Dekker Inc, 367-379.
- **Bendini,** A.; Toschi, T.G. and Lercker, G. (2002). Antioxidant activity of oregano (*Origanum vulgare*) leaves, Ital. *Journal of Food Science*, **14**: 17-24.
- **Bishayee,** A.; Sarkar, A. and Chatterjee, M. (1995). The hepatoprotective activity of Carrot (*Daucus carota* L) against carbon tetrachloride intoxication in mouse liver. *Journal of Ethnopharmac*[illegible]
- **Botsoglou**, N .A.; Christaki , E.; Fletour[illegible] , AB.(2002). The effect of dietary oregano essential oil on lipid oxidation in raw and cooked chicken during refrigerated storage. *Meat Science*, **62**:259–265.
- **Bramley,**P.M.;Elamadfa,I.;Kafatos,A.;Kelly,F.J.;Manios,Y.;OXborough, H.E.; Schuch, W.; Sheehy, P.J.A. and Wgner,K.H. (2000). Vitamin E. *Journal of Science and Food Agriculture***, 80:**913-938.
- **Brown,** M .J.; Henderson, D. E. and Hunt,C. H . (2006). Composition of antioxidant properties of supercritical fluid extracts of herbs and the confirmation of pinocembrin as a principle antioxidant in Mexican Oregano (*Lippa gravolens*).*Electronic Journal of Environmental Agriculture and Food Chemistry*, **5** :1265-1277.

- **Bruce,** W. and Heddle, J. (1979). The mutagenicity of 61 agents as determined by the micronucleus, *Salmonella* and sperm abnormality assays, Can. *Journal of* Cytology *and Genetic*, **21**: 319–334.
- **Burri,** B.J. and Jacob, R.A. (1997). Human metabolism and the requirement for vitamin C. In: Packer L, Fuchs J, eds. Vitamin C in health and disease. New York: Marcel Dekker Inc, 341–366.
- **Candelario –Jalil , E.**; Al-Dalain, M. S.; Le´on Fern´andez, O. S. ; Men´endez, S.; P´erez-Davison, G.; Merino, N.; Sam, S. and Ajamieh, H. H. (2001) .Oxidative Preconditioning Affords Protection Against Carbon Tetrachloride-induced Glycogen Depletion and Oxidative Stress in Rats. *Journal of Applied Toxicology*, **21**: 297–301.
- **Cao,** G.; Alessio, H.M. and Cutler, RG. (1993). Oxygen-radical absorbance capacity assay for antioxidants. *Free Radical Biology and Medicin*, **14**:303-311.
- Capecka, E.; Mareczek, A. and Leja, M. (2005). Antioxidant activity of fresh and dry herb of some Lamiaceae species. *Food Chemistry*, **93**: 223-226.
- **Carrubba**, A. and Calabrese,I. (1998). Antioxidant compounds in some herbaceous aromatic plants. *Acta Hort.*, **457**: 85-93.
- **Cavero** , S. ; M´onica, R.; Garc´ıa-Risco ; Francisco, R. M. ; Jaime, L.; Santoyo, S.; Javier, F.S. ; Reglero , G. and Iba˜nez, E . (2006). Supercritical fluid extraction of antioxidant compounds from oregano Chemical and functional characterization via LC–MS and in vitro assays. *Journal of Supercritical Fluids, **38***:62–69.
- **Cervato,** G.; Carabelli, M.; Gervasio, S.; Cittera, A.; Cazzola, R. and Cestaro, B. (2000). Antioxidant properties of extracts of oregano (*Origanum vulgare*) leaf extracts, *Journal of Food Biochemistry*, **24**: 453.
- **Charles,** E.O. (2003). Diagnostic serum enzymes.Virtual Chemistry Book, Elnhmrst College.

- **Chaurasia**, S.S.; Panda, S. and Kar, A. (2000). *Withania somnifera* root extract in regulation of lead-induced oxidative damage in male mouse. *Pharmacological Research*, **41**:663-666.
- **Chiu,** H.F.; Lin, C.C.; Yen, M.H.; Wu, P.S. and Yang, C.Y. (1992). Pharmacological and pathological studies on hepatic protective crude drugs from Taiwan (V): the effects of *Bombax malabarica* and *Scutellaria rivularis*. *American Journal of Chinese Medicine*, **20**: 257–264.
- **Choi** , C. W.; Kim, S. C.; Hwang, S. S.; Choi, B. K.; Ahn, H. J.; Lee, M. Y.; Park, S. H. and Kim, S. K. (2002). Antioxidant activity and free radical scavenging capacity between Korean medicinal plants and flavonoids by assay-guided comparison. *Plant Science*, **163**: 1161–1168.
- **Clardy,** J. and Walsh, C. (2004). Lessons from natural molecules. *Nature* **432:**729–837.
- **Cole,** R.J. (1981). Short-term tests for transplacentally active carcinogens: Micronucleus formation in fetal and maternal mouse cerebroblasts. *Mutation*
- **Daferera,** D. J., Tsagkarakis, N., Ziogas, B. N. and Polissiou, M. G. (2003). In vitro effectiveness of plant essential oils on *Botrytis cinerea*, *Fusarium* sp. and *Clavibacter michiganensis* subsp. *michiganensis*. *Crop Protection*, **22**:39–44.
- **Daferera,** D.J.; Ziogas, B.N. and Polissiou, M.G. (2000). GC-MS analysis of essential oils from some Greek aromatic plants and their fungitoxicity and *Penicillium digitatum*. *Journal of Agricultural and Food chemistry*, **48**:2576-2581.
- **Dattner,** A.M. (2003). From medical herbalism to phytotherapy indermatology: back to the future. *Dermatologic Therapy*, 16.
- **de Pee,** S. and West, CE. (1996). Dietary carotenoids and their role in combating vitamin A deficiency: A review of the literature. *European Journal of Clinical Nutrition*, **50**: 38-53.
- **de Mejia**, E.G. and Ramirez-Mares, M.V. (2002). Leaf extract from Ardisia compressa protects against 1-nitropyrene-induced cytotoxicity and its

antioxidant defense disruption in cultured rat hepatocytes. *Toxicology*, **179**:151-162.

- **Dhir,** H.; Roy, A.K.; Sharma, A. and Talukdar, G. (1990). Modification of clastogenicity of lead and aluminium in mouse bone marrow cells by dietary ingestion of *Phyllanthus emblica* fruit extract. *Mutation Research*, **241**: 305-312.
- **Elmadfa**, I. and Park, E. (2004). Impact of diets with oil or olive/sunflower oils on DNA damage in healthy Young men. *European Journal of Nutrition*, **38**:1436-6207.
- **Enstrom,** J.E. (1997). Vitamin C in prospective epidemiological studies. In: Packer L, Fuchs J, eds. Vitamin C in health and disease. New York: Marcel Dekker Inc, 381–98.
- **Evans, W.C**. (1999).Trease and Evan's pharmacognosy, 14th (Ed.) W.B.Saunders Company Ltd.U.K.
- **Exarchou**, V.; Troganis, A.; Gerothana[illegible]; Tsim[illegible], M. and Boskou, D. (20[illegible]). Ide[illegible] and quantifica[illegible] complex plant extracts by the use of v[illegible] nuclear magnetic resonance spectros-copy. *Journal of Agricultural and Food Chemistry*, **49**: 2-8.

- **Fetrow,** C.W. and Avila, J.R. (1999). Professional s Hands book of Complementary and Alternarive medicines. *Spring House Corporation*, spring house PA., 473-475.
- **Figuérédo,** G.**;** Cabassu, P.; Jean-Claude, C. and Pasq, B. (2006). Studies of Mediterranean oregano populations. VIII—Chemical composition of essential oils of oreganos of various origins and Sons, Ltd. *Flavour and Fragrance Journal* , **21**: 134–139.
- **Fong,** H.H. (2002). Integration of herbal medicine into modern med-ical practices: issues and prospects. *Integrative Cancer Therapies*, **1**:287-293.
- **Fontham**, E.T.H. (1990). Protective dietary factors and lung cancer. *International Journal of Epidemiology*, **19:32**-42.

- **Force**, M. (2000). Inhibition of enteric parasites by emulsified oil of oregano *in vivo*. *Phytotherapy Research*, **14**:213-214.
- **Frei,** B; Stocker, R.; England L. and Ames, B.N. (1990). Ascorbate: the most effective antioxidant in human blood plasma. *Advanced in Experimental Medicin and Biology*, **264**:155–163.
- **Friedman**, L .S.; Martin, P. and Munoz, S. J. (1996) . Liver Function tests and the objective evaluation of the patient with liver disease**.** *In: Hepatology:a Textbook of Liver Disease. Philadelphia, WB Saunders*, 791-833.
- **Futoryan**, T. and Gilchrest, B.E. (1994). Retinoids and the skin. *Nutrition Review*, **52**:299-310.
- **Geisman,** T.A. (1962). Chemistry of Flavonoid compounds. Macmillan Co. New York. USA.
- **Gey,** K .F. (1998). Vitamins E plus C and interacting conutrients required for optimal health. *Biofactors*, 113–174.
- **Giacco**[illegible] A.; Hilbert, K.;Hill M.J. [illegible] cancer chemoprevention.*European Jour*[illegible]
- **Gosh,** B. B.; Talukder, G. and Shorama, A. (1991). Effect of culture media on spontaneous residence of mitotic index,chromosomal aberration, micronucleus counts, sister chromatid exchange and cell cycle. Kinetics in principle blood lymphocytes of male and female donars. *Cytobios*, **67**: 71-75.
- **Gracia,** M.A. and Sanz, J. (2001). Analysis of Origanum vulgare volatiles by direct thermal desorption coupled to gas chromatography-mass spectrometry .*Journal of Chromatography*, **918**:189-194.
- **Guner**, A.; Ozhatay, N.; Ekim, T. and Baser, K. H. C. (2000). Flora of Turkey and the East Aegean Islands (Vol. 11 (supplement-II)). Edinburgh: Edinburgh University Press.
- **Guven,** A.; Guven A. and Gulmez M. (2003). The effect of kefir on the activities of GSH- Px,GST,CAT,GSH and LPO levels in carbon

tetrachloride-induced mice tissue. *Journal of Veterinary Medicin series B-Infectious Diseases and Veterinary Public Health*, **50**:412-416.

- **Haen**, P. J. (1995). Principles of Hematology. Edited by L. H. Young and W. B. Publisher, London.310-325.
- **Halliwell,** B. and Gutteridge, J.M.C. (2000). Free radicals in Biology and medicine. Oxford University Press, 148-149.
- **Halliwell,** B. (1996). Vitamin C: antioxidant or pro-oxidant in vivo. *Free Radical Research*, **25**:439–54.
- **Halliwell**, B. (1993). Oxygen Species in Pathology with Special Reference to the Skin.In Oxidative Stress in Dermatology. Marcel Dekker, Inc., New York, 3-11.
- **Hammer,** K.A.; Carson, C.F.and Riley, T.V. (1999). Antimicrobial activity of essential oils and other plant extracts. *Journal of Applied Microbiology*, 86-98.
- **Harborne,** J.B. (1973). Phytochemical methods .Science paper backs, Chapman and Hall London, UK
- **He,** L.; Mo, H.; Hadisusilu, S.; Qures[illegible] Isoprenoids suppress the growth of murine B16 melanomas in vitro and in vivo. *Biochemical and Molecular Journal of Nutrition*, **127**: 668–674.
- **Hewawasam,** R. P. , Jayatilaka, K. A. P. W.; Pathiranaand, C. and Mudduwa, L. K. B. (2003). Protective effect of *Asteracantha longifolia* extract in mouse liver injury induced by carbon tetrachloride and paracetamol. *Journal of Pharmacy and Pharmacology*, **55:**1413-1418.
- **Hossein,** S. (1999). Management your health. Health Canada, Under the Canadian Strategy on HIV/AIDS. Internet: //www.jci.org/cgi/content.
- **Hu,** F. B. and Willett, W. C. (2002). Optimal diets for prevention of coronary heart disease. *Journal of American Medical Association*, **2888**: 2569–2578.
- **Huang,** M.T; Ho, C.T. and Lee, C.Y. (1992). Phenolic compounds in food and their effects on Health II. *ACS Symposium Series*, **507**: 8–34.

- **Huffman**, M.A. (2003). Animal self-medication and ethno-medicine: exploration and exploitation of the medicinal properties of plants. *Proceedings of the Nutrition Society*, **62**:371–381.
- **Hughes,** D. A. (2001) .Dietary carotenoids and human immune function. *Nutrition*, **1**:823-827.
- **Hut,** H.M.; Lemstra, W.; Blaauw, E.H; Van Cappellen, G.W.; Kampinga, H.H.; and Sibon, O.C. (2003). Centrosomes split in the presence of impaired DNA integrity during mitosis. *Molecular and Biology of the cell*, **14**:1990-2000.
- **Indian herbal** pharmacopeias. (1998). Ajoint publication of Regional Research Laboratory, *Council of scientific and Industrial research*. Jammutawi .P: **1**:1-10.
- **Ingram**, G. (1995). Oregano: Nature s Healer. In internet file: http://www.amazon.com
- **Internet (I)** Plants Profile for Origanum vulgare L. USDA Plants
- **Internet (II)** http://www.genome.cornell.edu/
- **Internet (III)** http://www.rz.uni-karlsruhe.de/db26/fotos
- **Internet (IV)** http://www.oregano oleoresin.htm
- **Internet (V)** http://www.science lab.com
- **Ipek,** E.; Tu¨ylu¨, B. A. and Zeytinog˘lu, H. (2004). Effects of carvacrol on sister chromatid exchanges in human lymphocyte culture. *Cytotechnology*, **43**: 145–148.
- **Ipek,** E.; Zeytinoglu, H.; Okay, S.; Berrin A.T.; Kurkcuoglu, M. K. and Base, C.H. (2005). Genotoxicity and antigenotoxicity of Origanum oil and carvacrol evaluated by Ames Salmonella/microsomal test. *Food Chemistry*, **93**: 551–556.
- **Isman**, M. B. (2000). Plant essential oils for pest and disease management. *Crop Protection*, **19:** 603–608.

- **Isman**, M. B.; Wan, A. J. and Passreiter, C. M. (2001). Insecticidal activity of essential oils to the tobacco cutworm Spodoptera litura. *Fitoterapialix*, **72**:65–68.
- **Jaffer, H.T**.; Mahmoud, M.; Jawad, A.;Nagi, A. and Alnailb, A. (1983). Phytochemical and biological Screening of some Iraqi plant .*Fitoterapialix*, pp.299.
- **Jayatilaka** , K. A. P. W. ; Thabrew, M. I.; Pathirana, C.; De Silva, D. G. H. and Perera, D. J. B. (1989). An evaluation of the potency of *Osbeckia octandra* and *Melothria maderaspatana* as antihepatotoxic agents. *Planta Medicin*, **55**: 137-139.
- **Jayatilaka** , K. A. P. W. ; Thabrew, M. I. and Perera, D. J. B. (1990) .Effect of *Melothria maderaspatana* on carbon tetrachloride induced changes in rat hepatic microsomal drug metabolizing enzyme activity. *Journal of Ethnopharmacology*, **30**: 97-105.
- **Jha**, A.M. and Bharti, M.K. (2002). Mutagenic profiles of carbazole in the germ cells of male mice. *Mutat*
- Jensen, U. and Knuthsen, P. (2001). herbs and calculation of flavonoids intake by use of herbs in traditional Danish dishes. *Food Chemistry*, **73**: 245.
- **Kada,** T.; Jain, A.K. and Shimoi, Y. (1987). Preliminary study on the desmutagenic and antimutagenic effect of some natural products. *Current Science*, **56**:1266-1269.
- **Kalodera,** Z.; Pepeljnjak, S.; Blazevic, C. N. and Petrac, T. (1997). Chemical composition and antimicrobial activity of Tanacetum parthenium essential oil. *Pharmazie*, **52**: 885–886.
- **Karmer,** R.J. (2003). Complete Blood Count. Internet://www.jci.org/cgi/content.
- **Kazarinova** , N. V. ;Tkachenco, K. G.; Uzychenko, L. M.; Safonova, N. G.; Tkachev, A. V. and Koruljuk, E. A. (2002). Component composition and

analysis of antibiotic activity of essential oil of Origanum vulgare L. grown in regions of West Siberia. *Rastitel'nye-Resursy*, **38**: 99–103.

- **Khanam**, S. and Devi, K. (2005). Effect of *Withania somnifera* root extract on lead-induced DNA dama. *Journal of Food and Agriculture Environment*, **3**:31-33.
- **Kim,** H.J.; Bruckner, J.V.; Dallas, C.E. and Gallo, J.M. (1990). Effect of dosingvehicles on the pharmacokinetics of orally administered carbon tetrachloride in rats. *Toxicology and Applied Pharmacolology*, **102**: 50–60.
- **Kokkini,** S.; Karousou, R. and Vokou, D. (1994). Pattern of geographic variation of Origanum vulgare trichomes and essential oil content in Greece. *Biochemical Systematics and Ecology*, **22**: 517–528.
- **Ko** , K. M.; Ip, S. P.; Poon, M. K. T. ;Wu, S. S.; Che, C. T.; Ng, K. M. and Kong, Y. C. (1995). Effect of lignan enriched *Fructus schisandrae* extract on hepatic glutathione status in rats: Protection against CCl4-induced liver injury. *Planta Medicin*, **61**:134-137.
- [illegible] (2006). Cholinesterase inhibition and docking studies of some secondary metabolites, isolated from Origanum vulgare L. ssp. hirtum. *Bioorganic and Medicinal Chemistry*, **14**: 1653–1659.
- **Koo**, L.C. (1997). Diet and lung cancer 20+ years later: more questions than answers. *International Journal of Cancer*, **l10**:22-9.
- **Kokdil**, G.; Tamer, L.; Ercan, B.; ˙Ilcim, A.; Aras, N. and Atik, U. (2005). Effects of *Nigella unguicularis* fixed oil on blood biochemistry and oxidant/antioxidant balance in rats. *Journal of Ethnopharmacology*, ***99*:** 131–135.
- **Kulisica, T.;** Radonicb, A.; Katalinicc, V. and Milosa, M. (2004). Use of different methods for testing antioxidative activity of oregano essential oil .*Food Chemistry*, **85**: 633–640.

- **Lagouri,** V.; Blekas, G.; Tsimidou, M.; Kokkini, S. and Boskou, D. (1993). Composition and antioxidant activity of essential oil from Oregano plants grown in Greece. Lebensmittel- Unters. Forsch, **197**: 20–23.
- **Lambert**, R. J. W.; Skandamis, P. N.; Coote, P. J. and Nychas, G. J. E. (2001). A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. *Journal of Applied Microbiology*, **91**: 453–462.
- **Lima**, C.F. ; Carvalho , F. ; Fernandes , E.; Bastos, M.L. ; Santos-Gomes , P.C. ; Fernandes-Ferreira , M. and Pereira-Wilson , C. (2004). Evaluation of toxic/protective effects of the essential oil of Salvia officinalis on freshly isolated rat hepato cytes. *Toxicology in Vitro*, **18**: 457–465.
- **Lin,** C.C.; Yen, M.H.; Lo, T.S. and Lin, J.M. (1998). Evaluation of the hepatoprotective and antioxidant activity of *Boehmeria nivea* var. *nivea* and *B.nivea* var. *tenacissima*. *Journal of Ethnopharmacology*, **61**: 211-217.
- **Liu**, J. (2005). Oleanolic acid and Ursolic acid. Journal of Ethnopharmacology, **99**: 175-184.
- **Lyons,**N.M.;Woods,J.A. and Obrien,N.M. (2001). Alfa-tocopherol but not gamma-tocopherol inhibits 7-bete-hydroxyl cholesterol induced apoptosis in human U937 cell. *Free Radical Research***, 5:**320-339**.**
- **Madsen,** H.L. and Bertelsen,G. (1995). Spices as antioxidants.*Trends in Food Science and Technology*, **6**:271-276.
- **Maldonado, O.**; Demasi, R.; Maldonado, Y.; Taylor, M.; Troncale, F. and Vender, R.(1998). Extremely high levels of alkaline phosphatase in hospitalized patients. *Journal of Clinical Gastroenterology*, **27**:342-345.
- **Manna**' P.; Sinha, M. and Sil, C.P. (2006). Aqueous extract of *Terminalia arjuna* prevents carbon tetrachloride induced hepatic and renal disorders. *BMC Complementary and Alternative Medicine*, **6:**33.

- **Mankani** , K.L. ; Krishna, V. ; Manjunatha, B.K. ; Vidya, S.M. ; Jagadeesh Singh, S.D. ; Manohara ,Y.N. ; Raheman, A. and Avinash, K.R. (2005). Evaluation of hepatoprotective activity of stem bark of Pterocarpus marsupium Roxb. *Indian Journal of Pharmacology*, **37**:165-168.
- **Manohar,** V.; Ingram, C.; Gray, J.; Talpur, N. A.; Echart, B. W. and Bagchi, D. (2001). Antifungal activities of origanum oil against Candida albicans. *Molecular and Cellular Biochemistry*, **228**:111–117.
- **Martin,** R.H.; Ko, E. and Barclay, L. (1994). Human sperm karyotypes**.** In Human chromosomes: manual of basic techniques. New York, McGraw-Hill; 56-65.   .
- **Martin,** R.H. (2003). Chromosomal abnormalities in human sperm**.** In Advances in Male-Mediated developmental toxicity. New York, Plenum Press, **518:**181-188.
- **Misra,** T. N.; Singh, H. S.; Pandey, B. K.; Singh, R. P. [illegible] Pandey, R. [illegible] (2001). Constituents of *Asteracantha longifolia* [illegible]*itoter*[illegible]*lix*, **72**: 194-196.
- **Mock**[illegible] D.; Bernotiene, G. and Judze[illegible] *Origanum vulgare* L. ssp.vulgare growi[illegible] *Phytochemistry*, **57**:65-69.
- **Mojca-Skerget**; Kotnik, P.; Hadolin, M.; Hras, R.A.; Simonic, M. and Knez, Z. (2005). Phenols, proanthocyanidins, flavones and flavonols in some plant materials and their antioxidant activities. *Food Chemistry*, **89**:191–198.
- **Moller,** P. and Loft, S. (2002). Oxidative DNA damage in human white blood cells in dietary antioxidant intervention studies. *American Journal of Clinical and Nutrition*, **76**:303-310.
- **Mollinedo,** F. and Gajate, C. (2003). Microtubules, microtubule-interfering agents and apoptosis. *Apoptosis*, **8**:413-450.
- **Morel,** I.; Lescoat, G.; Cillard, P. and Cillard, J. (1994). Role of flavonoids and iron chelation in antioxidant action. *Methods of Enzymoogy*, **234**: 437–443.

- **Nadir,** M.T.; Salih, F. M.; Dhahir, A. J.; Nori, M. and Hussain, A. M. (1986). Antimicrobial activity of *Salvia* species indigenous to Iraqi. *Journal of Biological Science Research*, **17**: 109-117.
- **Nefic**, H. (2001). Anticlastogenic effect of Vitamin C on cisplatin induced chromosome aberrations in human lymphocyte cultures. *Mutation Research*, **498**: 89–98.
- **Neuschhwander-Terti,** B.A. (1995). Common blood tests for liver disease.Which ones are most usefule. *Postgraduate Medical Journal*, ***98:49***-56.
- **Newman,** D.J.; Cragg, G.M. and Snader, K.M. (2003). Natural products as sources of new drugs over the period 1981–2002. *Journal of Natural Products*, **66**: 1022–1037.
- **Niki,** E. and Noguchi, N. (1997). Protection of human low-density lipoprotein from oxidative modification by vitamin C. In: Packer L. and Fuchs J. eds. Vitamin C in health and disease. New York: Marcel Dekker Inc, 183–
- L.; Poyer, J.L. and Mccay, P.B. (1982). Specificity of aphenobarbital-induced cytochrome P450 for metabolism of carbontetrachloride to the trichloromethyl radical**.** *Biochemistry and Pharmacology*, **31:**615-624.
- **Nostro** , A.; Blanco, A. R.; Cannatelli, M. A.; Enea, G. F.; Morelli, I. and Roccaro, A. S. (2004). Susceptibility of methicillin-resistant Staphylococci to oregano essential oil, carvacrol and thymol. *FEMS Microbiology Letters*, **230**: 191–195.
- **Novak**, J.; Christina, B.; Langbehn, B.; Pank, F.; Skoula, M.; Gotsiou, Y. and Franz, C. M. (2000). Ratios of cis- and trans-sabinene hydrate in Origanum majorana L. and Origanum midrophyllum (Bentham) Vogel. *Biochemical Systematics and Ecology*, **28**: 697–704.
- **Olson**, J.A. (1996). Benefits and liabilities of vitamin A and carotenoids. *Journal of Nutrition*, **126**:8-12.

- **Olsson,** M. E. and Gustavsson, K. E. (2004). Inhibition of cancer cell proliferation in vitro by fruit and berry extracts and correlations with antioxidant levels. *Journal of Agricultural and Food Chemistry*, **52**: 7264–7271.
- **Odeigah**, P.G.C**.** (1997). Sperm head abnormalities and dominant lethal effects of formaldehyde in albino rats, *Mutatation Research*, **189**: 141–148.
- **Packer,** L. (1997). Vitamin C and redox cycling antioxidants. In: Packer L, Fuchs J, eds. Vitamin C in health and disease. New York: Marcel Dekker Inc, 95–121.
- **Patton,** J. L. (1967). Chromosome studies of certain pocket genus perogenathus (Radentia: Heteromyidae). *Journal of Mammalogy*, **48**: 27-37.
- **Pavia,** S.A. and Russell, R.M. (1999). Beta-carotene and other carotenoids as antioxidants. *Journal of American College and Nutrition*, **18**:426-433.
- **Perez –Serrano,** J.; Denegri, G.; Casado, N. and [illegible] (1997). *In vivo* effect of oral albendazole and albendazole sulphoxide on development of secondary [illegible] *Parasitology*, 27: 1341-1345.
- **Pizzale**, L.; Bortolomeazzi, R.; Vichi , S. ; Uberegger, E. and Conte L. (2002). Antioxidant activity of sage [*Salvia officinalis and S. fruticosa*] and oregano [*Origanum onites and Origanum indercedens*] extracts related to their phenolic compound content. *Journal of Scientific and Food Agricultural*, **82**:1645–1651.
- **Puertas -Mejia**, M.; Hillebrand, S.; Stashenko, E. and Winterhalter, P. (2002). In vitro radical scavenging activity of essential oils from Colombian plants and fractions from oregano (Origanum vulgare L.) essential oil. *Flavour and Fragrance Journal*, **17,** 380–384.
- **Rajesh**, M.G. and Latha, M .S. (2004). Protective activity of *Glycyrrhiza glabra* Linn. on Carbon tetrachloride-induced peroxidative damage. *Indian Journal of Pharmacology*, **36**:284-287.

- **Ramos**, A.; Edreira, A.; Vizoso, A.; Betancourt, J.; Lo´ pez, M. and Decalo, M. (1998). Genotoxicity of an extract of Calendula officinalis L. *Journal of Ethnopharmacology*, **61**: 49–55.
- **Recknagel,** R .O.; Glendek, E A. ; Dolazk, J.A. and Waller, R .L. (1989). Mechanism of carbon tetrachloride toxicity. *Pharmacological Therapies*, **43:**139-154.
- **Reichling,** J.J. and Kaplan, M . M. (1988). Clinical use of serum enzymes in liver diseases**.** *Digestive Diseases and Sciences*, **33**:1601-1614.
- **Reitman, S**.; and Frankel,A.S. (1957).A colorimetric method for the determination of serum glutamic oxaloacetatic and glutamic pyruvic transaminases .*American Journal of Clinical Pathology* ,**28**:56-58.
- **Rice -Evans**, C.A.; Miller, N.J. and Pagang , G. (1997) . Antioxidant properties of phenolic compounds. *Trends of Plant Science*, **2**:152–159.
- **Ross,** A .C. and Gardner, E .M. (1994). The function of vitamin A in cellular growth and differentiation, and its roles during pregnancy and lactation. *Advances in Experimental Medical Biology*
- **Ross,** A .C. (1999). Vitamin A and retin and Disease. 9th Edition (edited by Shils ME, Olson J, Shike M, and Ross AC).
- **Ross,** A .C. (1992). Vitamin A status: Relationship to immunity and the antibody. *Proceedings of the Society in Experimental Biology and Medicin* , **200**:303-320.
- **Sahin** , F**.** ; Gulluce , M.; Daferera , D.; Sokmen , A.; Sokmen ,M.; Polissiou, M.; Agar ,G. and Ozer, H. . (2004). Biological activities of the essential oils and methanol extract of Origanum vulgare ssp. vulgare in the Eastern Anatolia region of Turkey. *Food Control*, **15**:549–557.
- **Saladin,** K.S.; and Porth, C.M. (1998). Anatomy and physiology, the unity of form and function. McGraw-Hill, USA.

- **Sampath,** D. and Plunkett,W. (2001). Design of new anticancer therapies targeting cell cycle checkpoint pathways. *Current Opinion in Oncology*, **13**:484-490.
- **Sanmugapriya, E.** and Venkataraman, S. (2006). Studies on hepatoprotective and antioxidant actions of *Strychnos potatorum* Linn. Seeds on CCl4-induced acute hepatic injury in experimental rats. *Journal of Ethnopharmacology*, **105**:154-160.
- **Sarkar**, K.; Ghosh, A.; Kinter, M.; Mazumder, B. and Sil, P.C. (2006). Purification and characterization of a 43 kD hepatoprotective protein from the herb Cajanus indicus L.Protein.
- **Scalbert,** A. and Williamson, G. (2000). Dietary intake and bioavailability of polyphenols. *Journal of Nutrition*, **130**:2073–2085.
- **Schmid,** W. (1976). The micronucleus test for cytogenetic analysis. Chemical Mutagens, Principles and Methods for their [illegible] Vo[illegible] 4 Hollaender A. (Ed.) Plenum New York and London.pp.3[illegible]–53.
- **Schwertner**, H.A.; Jackson, W.G. and T[illegible] serum concentration of bilirubin with [illegible] disease. *Clinical Chemistry*, **40**: 18-23.
- **Semba**, R.D. (1998). The role of vitamin A and related retinoids in immune function. *Nutrition Review*, **56**: 38-48.
- **Shamberger**, R.J. (1984). Genetic toxicology of ascorbic acid. *Mutatation Research*, **133**:135–159.
- **Shenoy,A.** K. ; Somayaji, S.N. and Bairy, K.L. (2002) . Evaluation of hepatoprotective activity of *Gingo biloba* in rats. *Indian Journal of Pharmacology*, **46**:167–174.
- **Shetty,** K. (1997). Biotechnology to Harness the benefits of dietry phenolics: focus on Lamiaceae. *Asia Pacific Journal of Clinical Nut*rition, **6**:162–171.
- **Shihata, I.M**. (1951).Apharmacological study of Anagllis arvensis,M.D.Vet.Thesis,Cairo University.

- **Silva,** F. M. O.; Vergara-Parente, J. E.; Gomes, J. K. N. ; Teixeira, M. N. and Lima, R. P. (2007). A Contribution for the Definition of Serum Chemistry Values in Captive Adults Antillean Manatees (*Trichechus manatus manatus* Linnaeus, 1758). *Journal of Veterinary Medicine Series*, **54**:119–122.
- **Simic,** N.; Palic, R.; Vajs, V.; Milosavljevic, S. and Djkovic, D. (2002). Composition and antibacterial activity of Achillea asplenifolia essential oil. *Journal of Essential Oil Research*, **14**:76–78.
- **Sivropoulou,** A.; Papanikolaou, E.; Nikolaou, C.; Kokkini, S.;Lanaras, T. and Arsenakis, M. (1996). Antimicrobial and cytotoxic activities of Origanum essential oil. *Journal of Agriculture and Food Chemistry*, **44**: 1202–1205.
- **Slater**, T.F. (1984). Free radical mechanism in tissue injury. *Biochemistry Journal*, **222**:1-15.
- **Smolensk,** S.J.; Silinis,H. and Fransworth,N.R. (1972).Alkaloid screening. *Annals of Biochemistry*, **35**:31-34.
- **Sokovic,** [illegible] Pitarokili, D. [illegible] activities of selected aromatic plants gro[illegible] **46**: 317–320.
- **Sood,** R. (1986). Hematology for students and practitioners. Jaypee Brothers, New Delhi, India.
- **Stal,** P. and Olson, J . (2000). Oxidative stress, and liver carcinogenesis**.** In *Coenzyme Q:* Molecular Mechanisms in Health and Disease. CRC Press, Boca Raton; 317-329.
- **Stammatia,** A.; Bonsia, P.; Zuccob, F.; Moezelaarc, R.; Alakomid, H. - L. and von Wrightd, A. (1999). Toxicity of selected plant volatiles in microbial and mammalian short-term assays. *Food Chemical and Toxicology*, **37**:813–823.
- **Stocker,** R.; Yamamoto, Y.; McDonagh, A.F.; Glazer, A.N. and Ames, B.N. (1987). Bilirubin is an antioxidant of possible physiological importance. *Science.* , **235:**1043-1046.

- **Suja,** S.R.; Latha, P.G.; Pushpangadan, P. and Rajasekharan, S. (2004). Evaluation of hepatoprotective effects of *Helminthostachys Zeylanica* (L.) Hook against carbon tetrachloride induced liver damage in Wistar rats. *Journal of Ethnopharmacology*, **92**: 61–66.
- **Sun**, F.; Ko, E. and Martin, H.R. (2006). Relationship between sperm chromosome abnormalities and sperm morphology. *Reproductive Biology and Endocrinology*, **4:**1.
- **Takada,** S.; Kelkar, A.; and Theurkauf, W.F. (2003). Drosophila checkpoint kinase 2 couples centrosome functions and spindle assembly to genomic integrity. *Cell*, **113:**87-99.
- **Teissedre,** P. L. and Waterhouse, A. L. (2000). Inhibition of oxidation of human low-density lipoproteins by phenolic substances in different essential oils varieties. *Journal of Agricultural Food Chemistry*, **48**: 3801–3805.
- **Torous** , D.; Hall, N. ; Diehl, M.; Cederbrant, K.; Sandberg, A.; Edvard, E. Bolesfoldi, G. ; Tometsko, A. and Dellinger, S. (2003) . Mouse Micronucleus assay. *Environmental and Molecular Mutagenesis*, **45:** 44-55.
- **Tsao,** C.S. (1997). An overview of ascorbic acid chemistry and biochemistry. In: Packer L, Fuchs J, eds. Vitamin C in health and disease. New York: Marcel Dekker Inc, 25–58.
- **Tsimidou,** M. and Boskou, D. (1994). Antioxidant activity of essential oils from the plants of the Lamiaceae family. In G. Charalambous (Ed.), *Spices, herbs and edible fungi*, pp. 273–284.
- **Ultee**, A.; Bennik, M. H. J. and Moezelaar, R. (2002). The phenolic hydroxyl group of carvacrol is essential for action against the foodborne pathogen Bacillus cereus. *Applied Environmental Microbiology*, **68**: 1561–1568.
- **UNESCO.** (1996). Culture and Health, Orientation Texts –World Decade for Cultural Development 1988 – 1997,Document CLT/DEC/PRO, Paris, France, p. 129.

- **UNESCO.** (1998). FIT/504-RAF-48 Terminal Report:Promotion of Ethnobotany and the Sustainable Use of PlantResources in Africa, Paris, p.60.
- **Valles,** E.G; De Castro, C.R and De Castro, J.A. (1994). N-Acetylcysteine is an early but also a late preventive agent against carbon tetrachloride-induced liver necrosis. *Toxicology Letter*, **71**: 87–95.
- **Vekiari,** S. A., Oreopoulou, V., Tzia, C. and Thomopoulos, C. D. (1993). Oregano flavonoids as lipid antioxidants. *Journal of American Oil Chemistry Society*, **70**: 483–487.
- **Venukumar,** M.R. and Latha, M.S. (2002). Antioxidant activity of *Curculigo orchioides* in carbon tetrachloride induced hepatopathy in rats. Indian *Journal of Clinical Biochemistry*, **17**: 80–87.
- **Walters,** M.I. (1970). Assay of total bilirubin.*Microchemistry*, **15**:231.
- **Weber,** P.; Bendich, A. and Schalch, W. (1996). Vitamin C and human health - a review of recent data relevant to human requirements. *International Journal for Vitamins Nutrition*
- Schwetz, H.A.; Zeng, Q. a
bilirubin concentrations and the risk of subsequent coronary heart disease death in men (abstract). *Hepatology*, **32:**314.
- **Woodall,** A.A. and Ames, B.N. (1997). Diet and oxidative damage to DNA: the importance of ascorbate as an antioxidant. In: Packer L, Fuchs J, eds. Vitamin C in health and disease. New York: Marcel Dekker Inc, 193–203.
- **Wyrobek,** A.J.; Gordon, D.A. ; Bukhari, J.K.; Francies, M.W.; Kapp, R.W.; Letz, G.; Malling, H.V.; Topham, J.C. and Whorton, M.D. (1983). An evaluation of the mouse sperm morphology test and other sperm tests in non-human animals. A Report of the U. S. Environmental Protection Agency Gene-Tox Program. *Mutatation Research*, **115**: 1–72.
- **Wyrobek,** A.J. and Bruce, W.R. (1975). Chemical induction of sperm abnormalities in mice, *Proceeding Natural and Acadimic Science*. (U.S.A.), **72**: 4425–4429.

- **Yadav,** N.P. and Dixit, V.K. (2003). Hepatoprotective activity of leaves of *Kalanchoe pinnata* Pers. *Journal of Ethnopharmacology*, **86**: 197–202.
- **Yamamoto,** Y. and Yamashita, S. (1999). Plasma ubiquinone to ubiquinol ratio in patients with hepatitis, cirrhosis, and hepatoma, and in patients treated with percutaneous transluminal coronary reperfusion. *Biological Factors*, **9:**241-245.
- **Yanishlieva**, N. V. and Marinova, E. M. (1995). Antioxidant activity of selected species of the family Lamiaceae grown in Bulgaria. *Die Nahrung*, **39**: 458–463.
- **Yaseen,** N. Y. (1990).Cytogenetic study of human colorectal cancer. Ph.D. thesis/University Sheffield. U. K.
- **Ye ,** Y.N.; Liu, E.S. ; Li, Y.; So, H.L.,;Cho, C.C.; Sheng, H.P.; Lee, S.S. and Cho, C.H. (2001). Protective effect of polysaccharides-enriched fraction from *Angelica sinensis* on hepatic injury. *Life Sciences*, **69**: 63[illegible]
- **Zelikoff**, J.T.; Li, J.H.; Hartig, A.; Wa[illegible] Costa, M. and Rossman, T.G. (1[illegible] Cellular toxicology of [illegible]
- **Zeytinoglu,** H.; Incesu, Z. and Baser, K. H. C. (2003). The inhibition of DNA synthesis by carvacrol in mouse myoblast cells bearing ahuman N-ras oncogene. *Phytomedicine*, **10**: 292–299.
- **Zeytinoglu,** M.; Aydin, S.; Ozturk, Y. and Baser, K.H.C. (1998). Inhibitory effects of carvacrol on DMBA induced pulmonary tumorigenesis in rats. *Acta Pharmaceutica Turcica*, **40**, 93–98.
- **Zhai ,** S. ; Dai, R.; Friedman, F.K. and Vestal, R.E. (1998). Comparative inhibition of human cytochromes P450 1A1 and 1A2 by flavonoids. *Drug Metabolism and Disposition*, **26**: 989–992.
- **Zheng**, W. and Wang, S.Y. (2001). Antioxidant activity and phenolic compounds in selected herbs. *Journal of Agricultural and Food Chemistry*, **49:**5165–5170.

Summary

The study was conducted to evaluate some immunological (total and absolute counts of leucocytes), cytogenetic (mitotic index of bone marrow cells, micronucleus formation in bone marrow cells and sperm-head abnormalities), biochemical (glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), alkaline phosphatase (ALP) and total bilirubin in serum) and histopathological (liver) effects of oregano (*Origanum vulgare* L.) fresh leaf extracts (methanol and hexane), and their effects on carbon tetrachloride (CCl_4)-induced acute hepatic injury in albino male mice. Therefore, such evaluations were carried out through two stages.

In stage I, three doses (32, 64 or 96 mg/kg) of oregano extracts and a single dose (0.2 mg/kg) of CCl_4 were investigated. Extracts were administered intraperitoneally as a single dose (0.1 ml) per a day and for 7 days and then the mice were sacrificed on day 8 for laboratory assessments. In the case of CCl_4, it was administered as a single dose on day one and mice were sacrificed in the second day.

In stage II, interactions between the three doses of both extracts and CCl_4 were carried out. In such interactions, the animals were given CCl_4 on day one, while the plant extract was given in day 2 till day 7 (single dose/day), and then animals were sacrificed in day 8 for laboratory assessments.

Chemical detections of oregano extracts revealed that the plant is rich in several compounds. In methanol extract, flavonoids, coumarins, tannins and phenolic compounds were detected, while hexane extract was positive for terpens, steroids, tannins and volatile oils.

The results revealed that CCl_4-treated animals showed significant decreased counts of total and absolute counts of leucocytes, and such observations suggest the CCl_4 is an immune suppressive agent. Furthermore, significant increased

percentages of induced micronucleus formation and sperm head abnormalities and a significant decreased mitotic activity of bone marrow cells in the treated mice were also observed, therefore CCl_4 can be considered as a mutagenic agent. In contrast, methanol and hexane extracts of oregano were significantly effective in enhancing the values of the investigated parameters, especially the mitotic index, which showed a significant increase, and the spontaneous micronucleus formation and sperm head abnormalities, which showed a significant decrease. However these effects were dependent on dose and type of extract.

The results of interactions between CCl_4 and both oregano extracts confirmed the forthcoming effect of the plant, and the two extracts were significantly effective in modulating the immune suppressive and mutagenic effects of CCl_4, although the effects were also subjected to the dose, type of extract and the parameter of evaluation.

Liver sections of animals treated with CCl_4 showed marked degenerative areas and necrosis of hepatocytes around the central vein. Moreover, significant elevations in the serum AST, GPT, ALP and [illegible] animals and such findings reflected induced hepatic damages. In contrast, biochemical parameters confirmed the hepatoprotective effect of oregano extracts through decreasing or normalizing the serum level of these enzymes and repairing the induced cellular damage of liver. However, a histopathological examination of liver sections was still showing some of the CCl_4-induced hepatic histological damages.

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List of abbreviations

ALP	Alkaline Phosphatase
CAT	Catalase
CCl_3	Trichl
CCl_4	Carbon Tetrachloride
DMBA	Dimethyl Benz Anthracene
DNA	Deoxyribonucleic Acid
GOT	Glutamic Oxaloacetic Transaminase
GPT	Glutamic Pyruvic Transaminase
GPX	Glutathione Peroxidase

GSH	Reduced Glutathione
GST	Glutathione-S-Transferase
LD_{50}	Lethal Dose 50
MI	Mitotic Index
MN	Micronucleus
O.vulgare	*Origanum vulgare*
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
rpm	rotation per minute
SHA	Sperm-Head Abnormality
SOD	Superoxide Dismutase

TLC	Total Leucocyte Counts
UV	Ultraviolet
WHO	World Health Organization

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

Assessment of Immunological and Cytogenetic Properties of Oregano (*Origanum vulgare* L.) Leaf Extracts on CCl_4-Induced Acute Hepatic Injury in Albino Male Mice

A Thesis
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By

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جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية العلوم

تقييم الخواص المناعية والوراثية الخلوية لمستخلصات أوراق نبات المردقوش (*Origanum vulgare* L.) ضد

وهي جزء من متطلبات نيل درجة ماجستير علوم في التقانة الإحيائية

من قبل

زينب طلال حسين البيرقدار

بكالوريوس تقانة إحيائية / جامعة النهرين ٢٠٠١

ربيع الثاني **ايار**

١٤٢٨ **٢٠٠٧**

الإهداء

الى كل من عشق هذا الوطن الغالي...

الى ارواح الشهداء في سبيل الله ووحدة التراب...

الى كل من سار في درب العلم واوقد شمعة لينير ظلامها

الى ابي و امي...

زينب

الخلاصة

صممت الدراسة بهدف تقييم بعض التاثيرات المناعية (العدد الكلي والتفريقي لخلايا الدم البيض) و الوراثية (معامل انقسام خلايا نقي العظم ومعامل تكون النوى الصغيرة في خلايا نقي العظم وتشوهات رؤوس النطف) و الكيموحيوية (الانزيمات GOT و GPT والفوسفات القاعدي والبليروبين الكلي) والنسيجية (الكبد) لمستخلصين (الكحولي و الهكساني) من اوراق نبات المردقوش (*Origanum vulgare* L.) وتاثيرهما على رباعي كلوريد الكربون (CCl_4) المسبب لاصابة الكبد الحادة في ذكور الفئران البيض. وقد انجزت الدراسة على مرحلتين.

في المرحلة الاولى، اعطيت ثلاث جرع (٣٢،٦٤،٩٦ ملغم/كلغم) من مستخلصي نبات المردقوش وجرعة واحدة من رباعي كلوريد الكربون (٣،٢ ملغم/كلغم)، وقد كان التجريع عن طريق الحقن تحت غشاء البريتون بجرعة واحدة (٠،١ مل) يوميا ولمدة ثمانية ايام. ضحي بالحيوان في اليوم الثامن، اما في حالة رباعي كلوريد الكربون فقد كان الحقن لمدة يومين (٠،١ مل) ليوم واحد ثم بعد ذلك ضحي بالحيوان في اليوم الثالث من الكربون حيث اعطيت جرعة واحدة من رباعي كلوريد الكربون في اليوم الاول ثم اعطي المستخلص في اليوم الثاني ولغاية اليوم السابع وبعد ذلك ضحي بالحيوان في اليوم الثامن.

لقد اظهرت التحليلات الكيميائية لمستخلصات نبات المودقوش بانة يحتوي على العديد من المركبات الفعالة ، ففي مستخلص الميثانول ظهر وجود فلافونويدات و كومارينات وتانينات و مركبات فينولية، في حين أظهر مستخلص الهكسان وجود تربينات وستيرويدات وتانينات و زيوت طيارة.

اظهرت النتائج بان الفئران المعاملة برباعي كلوريد الكربون قد اظهرت انخفاض واضح في العد الكلي والتفريقي لخلايا الدم وهذا يدل على ان رباعي كلوريد الكربون هو عامل مثبط للمناعة. كما تسبب هذا المركب بزيادة نسبة تكون النوى الصغيرة وتشوهات رؤوس النطف وانخفاض واضح في معامل انقسام خلايا نقي العظم ولهذا فهو عامل مطفر.وعلى العكس من ذلك فأن مستخلصي الميثانول والهكسان لنبات المردقوش كانت

فعالة في زيادة معامل انقسام خلايا نقي العظم وانخفاض تكون النوى الصغيرة وتشوهات رؤوس النطف . وكانت فعالية المستخلصات معتمدة على التركيز ونوع المستخلص.

اظهرت نتائج التداخل بين رباعي كلوريد الكربون وكلا المستخلصين لنبات المردقوش بان كلا المستخلصين كانا فعالين في تعديل التثبيط المناعي والوراثي لرباعي كلوريد الكربون وكان ذلك ايضا معتمد على نوع المستخلص والتركيز.

اظهرت الدراسة النسيجية لمقاطع الكبد للحيوانات المعاملة برباعي كلوريد الكربون تنخروتنكس خلايا الكبد في المناطق الواقعة حول الوريد المركزي. بالاضافة الى ذلك فقد ارتفع المستوى المصلي للانزيمات GOT وGPT والفوسفات القاعدي والبليروبين الكلي وهذا يعكس الضرر الكبدي المستحث. وعلى العكس من ذلك فأن العوامل الانفة الذكر قد اكدت اهمية مستخلصي نبات المردقوش في توفير الحماية الكبدية من خلال خفض قيم

هذه العوامل أو جعلها ضمن المستوى الطبيعي واصلاح الضرر الكبدي المستحث، مع

بِسْمِ اللهِ الرَّحْمنِ الرَّحِيم

وَاللهُ أَخْرَجَكُمْ مِّنْ بُطُونِ أُمَّهَاتِكُمْ لا

صَدَقَ اللهُ العَظِيم

سورة النحل الآية (٧٨)