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Biochemical, Cytogenetic and Histopathological studies on the effects of *Agrimonia eupatoria* extracts on albino male mice

A thesis

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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 and- tail abnormalities), biochemical (aspartate amin**o**-transferase; AST, alanine amino-transferase; ALT and alkaline phosphatase; ALP), histopathological (liver) and antitumor (human cervical cancer; HeLa and Rhabdomyosarcoma; RD cell lines and a normal cell line; mouse embryo fibroblast; MEF) effects of agrimony (*Agrimonia eupatoria* L.) extracts (aqueous and methanol), and their effects on carbon tetrachloride (CCl_4)-induced acute hepatic injury, in albino male mice. Additionally, a chemical detection of flavonoids, tannins, alkaloids, glycosides and saponins was carried out and furthermore, flavonoids were detected by a thin layer chromatography (TLC) method. Such evaluations were carried out through three stages.

In stage I, three doses (1.0, 2.0 or 3.0 mg/kg) of agrimony extracts and a single dose (3.2 mg/kg) of CCl_4 were investigated. The tested materials were injected intraperitoneally as a single dose (0.1 ml) per a day and for 6 days, and then the mice were sacrificed on day 7 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. In stage II, interactions between the ideal dose (3.0 mg/kg) of each extract 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 6 (single dose/day), and then, the animals were sacrificed on day 7 for laboratory assessments. In stage III, the percentage growth inhibition (PGI) of five plant concentrations (6, 12, 24, 48 and 96 µg/ml) were assessed *in vitro* using two tumor cell lines (HeLa and RD) and one normal cell line (MEF).

Chemical detections of agrimony extracts revealed that the aqueous extract was positive for flavonoids, tannins and phenolic compounds, while the methanol extract was positive for flavonoids, and tannins. The TLC analysis showed that the aqueous extract manifested four types of flavonoids (rutin, kaempferol, quercetin, isorhamnetin and myricetin), while in the methanol extract five flavonoids were detected (rutin, myricetin, azoleatin, vitexin and iso-orientin).

The results revealed that CCl_4-treated animals showed significant decreased counts of total and absolute counts of leucocytes, Furthermore, significant increased percentages of induced micronucleus formation and sperm-head and-tail abnormalities and a significant decreased mitotic activity of bone marrow cells were also observed. In contrast, the aqueous and methanol extracts of agrimony 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 and-tail 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 agrimony extracts confirmed the forthcoming effects 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.

Histopathological examinations of liver sections of animals treated with CCl_4 showed marked degenerative areas and necrosis of hepatocytes. Moreover, significant increased serum levels of liver function enzymes (AST, ALT and ALP) were observed in the treated animals. In contrast, biochemical parameters demonstrated the hepatoprotective effect of agrimony 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.

The results of stage III revealed that the five concentrations of the plant extracts showed anti-tumor properties in a concentration-dependent manner, and the methanol extract recorded better values of PGI than aqueous extract in HeLa and RD cell lines, while, less PGI values were recorded in the MEF cell line.

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List of Abbreviations

ALP	Alkaline phosphatase
ALT	Alanine transaminase
ANOVA	Analysis of Variance
AST	Aspartate transaminase
CAT	Catalase
DNA	Deoxyribonucleic acid
DPPH	1,1-diphenyl-2-picrylhydrazyl
ELISA	Enzyme Linked Immunosorbent Assay
GSH	Glutathione
GST	glutathione-S-transferase
HBSS	Hank's balanced salt solution
HBV	Hepatitis B virus
HeLa	Henrietta Lacks (human cervical cancer cell line)
HIV-1	Human immunodeficiency virus-1
HOAc	Acetic acid
IL-1	interleukin -1
LD_{50}	Lethal dose
MEF	Mouse embryo fibroblast
n-BuOH	*n*-butanol
OD	Optical Density
PBS	Phosphate buffer saline
PCE	Polychromatic erythrocytes
PUFA	Poly-unsaturated fatty acids
RD	Human rhabdomyosarcoma
Rf	Mobility relative to front
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TLC	Thin layer chromatography
TLC	Total count of leucocytes
WHO	World Health Organization

Chapter One

Introduction and Review of Literatures

Chapter One
Introduction and Review of Literatures

1.1 Introduction

Herbal medicines derived from plant are being increasingly utilized to treat a wide variety of diseases in folkloric medicine and accordingly, interest in the pharmacological evaluation of various plants shows a progress worldwide, although a little knowledge is available about their mode of action (Gupta, 2004). The use of plants as medicines dates from the earliest years of man's evolution, and they not only provided food and shelter but also served the humanity to cure different ailments. In this regard, the world health organization (WHO) reported that about 75% of the world populations relies upon traditional remedies (mainly herbs) for the health care of its people (Calixto, 2005). This is reasoned by the fact that medicinal plants have advantages (low cost and less side effects) over the conventionally used drugs, which are expensive and known to have harmful side effects (Ateyyat, *et al* 2009).

A larger number of these plants, plant extracts, plant derivatives and/or their isolated constituents have shown beneficial biological effects; including immunomodulatory, anti-oxidant, anti-inflammatory, anti-mutagenic, anti-carcinogenic and anti-microbial effects (Huffman, 2003). Modulation of immune response by using medicinal plant products has become a subject of scientific investigations, which revealed that several plants have important secondary metabolites that can interfere with different immunological functions; for instance, activation of humoral- and cell-mediated immune responses, which are crucial for the integrity of immune system against invading pathogens or a carcinogenic transformation (Arokiyaraj *et al*., 2007). Their secondary metabolites have different structures that correlate with different chemical terminologies (flavonoids, alkaloids, tannins,

glycosides and others), and therefore they may have different functions with regard to their biological potentials (XD *et al*., 2008).

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

Agrimonia eupatoria (Rose Family: Rosaceae) is a further interest of medicinal plants. The plant is known as agrimony in English literature, and it is used in folkloric medicine to treat a wide range of ailments; for instance, eye infections, diarrhea and disorders of gall bladder, liver and kidneys (Abdul, 2007). However, experimental investigations have demonstrated several other biological and pharmaceutical potentials; for instance, anti-mutagenic and anti-tumor (Horikawa *et al*., 1994), hepatoprotective (Park *et al*., 2004), anti-viral (Kwon *et al*., 2005), anti-bacterial (Bae and Sohn, 2005) and anti-oxidant and anti-inflammatory (Correia *et al*., 2007) effects.

Aims of Study

The present project was designed 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 and -tail abnormalities), biochemical (aspartate amin**o**-transferase; AST, alanine amino- transferase; ALT and alkaline phosphatase; ALP) and histopathological (liver) effects of two *Agrimonia eupatoria* extracts (aqueous and methanol) on albino male mice. Interactions between the plant extracts and carbon tetrachloride (CCl_4) were also carried out. Furthermore, the cytotoxic effects of both extracts were evaluated *in vitro* against three

cell lines (Human rhabdomyosarcoma; RD, human cervical cancer; HeLa and mice embryo fibroblast; MEF).

1.2 Review of Literatures

1.2.1 *Agrimonia euptatoria* L.

Agrimonia eupatoria is the most important species of the family Rosaceae and has long been used in traditional medicine for the treatment of different illnesses; for instance, eye ailment, diarrhea, wounds and disorders of gall bladder, liver and kidneys (Abdul, 2007). Moreover, recent investigations suggest that the plant is rich in chemical compounds that are able to stimulate immune system, in addition to their potentials as anti-oxidant, anti- inflammatory, anti-mutagenic and anti-carcinogenic (Correia *et al*., 2007) .

1.2.1.1 Common Names and Taxonomy

Agrimonia is a genus of 12-15 species of perennial herbaceous flowering plants that commonly called agrimony. However, *Agrimonia eupatoria* may be considered as the most important species of the family Rosaceae due to its wide applications in folkloric medicine (Sala *et al*., 2003). Many common names are used to describe the plant, and these are cocklebur, stickwort, church steeples, sticklewort, philanthropos and ackerkraut, but the most commonly used name is agrimonia, and in Iraq, the plant is known locally as Ghafath. Taxonomically, the plant is classified (Eriksson *et al*., 2003) as the following:

- Kingdom: Plantae
- Subkingdom: Tracheobionta
- Superdivision: Spermatophyta
- Division: Magnoliophyta
- Class: Magnoliopsida

- Subclass: Rosidae
- Order: Rosales
- Family: Rosaceae
- Genus: *Agrimonia*
- Species: *Agrimonia eupatoria* L**.**

1.2.1.2 Plant Distribution

Agrimony is native to the temperate regions of the northern hemisphere, but it has become naturalized throughout North America, Eastern Asia**,** Southwest Asia and Eastern Europe, as well as, Canada (Eriksson *et al*., 2003). In Iraq, the plant is found in the northern regions (Dr. Ali Al-Mosawi, Department of Biology, College of Science, University of Baghdad: personal communication).

1.2.1.3 Plant Description

Agrimonia eupatoria is herbaceous perennial plant that grows up to between 0.5 - 2 meters. The leaves are alternate, terminal pinnate, and they are divided into pairs of serrate oblong-oval leaflets, with several smaller leaves arranged in intervals between them. They are finely haired on top and rough beneath. Near the ground, they are often 7 or 8 inches long, while the upper leaves are about 3 inches in length. Upper leaves have far fewer leaflets than the lower ones **(**Chevallier, 1996). The flowers are small, slightly aromatic, stalkless, five petaled, bright yellow and numerous, and they are arranged closely on slender with a terminal spike. As flowers mature, they become rather woody and thickly covered with a mass of small bristly hairs. Flowers bloom from June to August. The seeds are very sticky and persist through the winter (Chiej, 1984). A taxonomical picture of the plant is given in figure 1-1.

Figure 1-1: A taxonomical picture of *Agrimonia eupatoria* (Eriksson *et al*., 2003).

1.2.1.4 Chemical Constituents

The aerial parts of agrimony are rich in flavonoids (tiliroside, 3-O-alpha-L-rhampyranoside, quercetin 3-O-alpha-L-rhampyranoside, quercetin 3-O-beta-D-glucopyranoside, kaempferol 3-O-beta-D-glucopyranoside, kaempferol 3-gucside, kaempferol 3-rhamnoside, kaempferol 3-rutinoside, apigenin and luteolin), tannins (epigallocatechin, epigallocatechin gallate, tannic acid and oligomeric hydrolysable tannins), aromatic acids, triterpenes, coumarins, terpenoids and glycosides, as well as, vitamins B and K (Xu *et al*., 2005; Jung and Park, 2007; Pan *et al*., 2008).

1.2.1.5 Folkloric Medicinal Uses

The medicinal parts of agrimony are aerial parts, which are either used as a dried herb or a fresh plant, and in both cases, it has been suggested that

the plant is safe to use and effective treatment for different illnesses, which include gall-bladder problems, diarrhea, throat and mouth inflammation, and skin irritations (Duke, 1985). The plant is also believed to relieve acute breathing disorders, tuberculosis, bronchitis, kidney and urinary problems, jaundice and liver conditions, gout, internal bleeding, menstrual problems, irritation from wounds and bites, skin eruptions, rheumatism, pain and swelling associated with sprains and bruises and urogenital problems in pre- and post-menopausal women (Duke, 2002).

1.2.1.6 Biological Potentials and Pharmaceutical Applications

The biological potentials and pharmaceutical applications of *Agrimonia eupatoria* have not investigated extensively, and the PubMed line revealed that up to June 2009, there have been only 45 papers about the genus *Agrimonia*, and most of them about the species *Agrimonia pilosa*. However, some of these investigations demonstrated that the crude extracts of agrimony or some isolated chemical constituents are effective materials that may have several biological potentials and pharmaceutical applications. A summary of some these potentials and applications is outlined in the following:

- **Hepatoprotective effects:** There has been no direct evidence that agrimony has hepatoprotective effects, but a phytochemical investigation of an aqueous extract of roots of a different species (*Agrimonia pilosa*), as guided by hepatoprotective activity *in vitro*, demonstrated two isocoumarin compounds, which showed hepatoprotective effects on both tacrine-induced cytotoxicity in human liver-derived Hep G2 cells and tert-butyl hydroperoxide-induced cytotoxicity in rat primary hepatocytes (Park *et al*., 2004).

- **Anti-mutagenic and Anti-tumor Activities**: the first investigation that evaluated the anti-tumor activity of *Agrimonia pilosa* was carried out in 1985 (Koshiura *et al*., 1985), in which the effects of the methanol extract from roots of the plant on several transplantable rodent tumors were investigated. Their results indicated that the roots of the plant contain some anti-tumor constituents, and they also suggested that the possible mechanisms of the anti-tumor activity may be related to some host-mediated actions and/or a direct cytotoxicity. Based on such finding, Miyamoto *et al*. (1988) investigated the effect of agrimoniin, a tannin contained in *Agrimonia pilosa*, on ascites type and solid type rodent tumors. Their results indicated that agrimoniin is potent anti-tumor tannin and they suggested that the anti-tumor effect may be due to this tannin, which can enhance the immune response of the host animals through the actions on tumor cells and some immunocytes. In 1992, it was found that agrimoniin is able to induce interleukin (IL)-1 production *in vitro* (human peripheral blood mononuclear cells) and *in vivo* (mouse adherent peritoneal exudate cells), and accordingly the anti-tumor effects can be justified (Murayama *et al*., 1992). The anti-mutagenic and anti-carcinogenic effects of *Agrimonia pilosa* were further confirmed *in vitro* against the environmental mutagens and carcinogens benzo[a]pyrene, 1,6-dinitropyrene and 3,9-dinitrofluoranthene (Horikawa *et al*., 1994).

- **Anti-oxidant and anti-inflammatory activities**: It has been demonstrated that agrimony has antioxidant properties and other important activities, especially anti-inflammatory effects (Santos-Buelga and Scalbert, 2000; Li *et al*., 2001), and accordingly the plant can be considered as a rich source of water-soluble antioxidants and/or phenolic compounds (Ivanova. *et al*., 2005). Phytochemical studies on an agrimony hydro-alcoholic extract and a polyphenol-enriched fraction obtained from it were found to possess a high concentration of flavan-3-ols, flavonols, flavones and phenolic acids.

The extract and fraction antioxidant potential and scavenging activity against the reactive species formed during inflammation were established, and the results showed that both the extract and the fraction promptly reacted by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) denoting a general radical scavenger activity and a potential antioxidant capacity. They also reacted with superoxide anion, peroxyl and hydroxyl radicals, as well as, with the oxidant species; hydrogen peroxide, hypochlorous acid and peroxynitrite, strengthening their radical scavenger and antioxidant activities. In most assays, the polyphenol-enriched fraction was more efficient, pointing to a significant contribution of the polyphenols content to those activities. (Venskutonis *et al*., 2007; Correia *et al*., 2007).

- **Antimicrobial properties**: Several investigators have demonstrated that Agrimony or its extracts are rich in compounds with antimicrobial properties, and in this regard, hexane, dichloromethane and methanol extracts of the seeds of agrimony have been assessed for antibacterial activity. These extracts showed inhibitory effect against *Bacillus cereus* and *Bacillus subtilis* (Copland *et al*., 2003). Accordingly, it has been recommended to use the plant or its derivatives against food-born pathogens. The antimicrobial activity of *Agrimonia pilosa* extracts was determined using a paper disc method against food-born pathogens and food spoilage bacteria. The petroleum ether extracts showed a strong antimicrobial effect against *Pseudomonas aeruginosa*, *Bacillus cereus* and *Salmonella Enteritidis* (Bae and Sohn, 2005). Furthermore, antiviral activities have also been suggested, and the aqueous extract of agrimony (whole plant) was found to have inhibitory effects against HIV-1 activities (Min *et al*., 2001). Furthermore, many researchers have tried to develop compounds from plants that have potent anti-hepatitis B virus (HBV), and an inhibition of HBV antigen release was achieved by an aqueous extract prepared from the aerial part (stem and leaves) of *Agrimonia eupatoria* (Kwon *et al*., 2005).

1.2.2 Carbon Tetrachloride

Carbon tetrachloride (CCl_4) is a well-known environmental biohazard. It is particularly toxic to the liver, where it causes hepatocellular degeneration and impairs different enzymatic systems, and the generation of free radicals appears to be pivotal in CCl_4 hepatotoxicity (Valles *et al*., 1994). It is metabolized by the mitochondrial monooxygenase system, and during metabolism, an unstable trichloromethyl (CCI_3) free radical is formed, and rapidly converted to trichloromethyl peroxide (CI_3COO^-).These free radicals lead to the peroxidation of fatty acids found in the phospholipids that make up the cell membranes (Kuzu *et al.,* 2007).

The toxic effects of CCl_4 on liver have been known for years and studied extensively, and the effects on hepatocytes are dependent on dose and exposure time, and they are manifested histologically as hepatic steatosis (i.e. fatty infiltration), centrilobular necrosis, and ultimately cirrhosis. Hepatic steatosis of the liver is a multifactorial phenomenon that is 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 the cell damage, and the metabolic effects of CCl_4 inside mitochondria have been described, however, the profound accumulation of fat following CCl_4 poisoning is considered to be independent of the 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). Due to the forthcoming toxic effects, a treatment with the CCl_4 has been served as a model to test the anti-toxic potentials of various agents, including medicinal plants and their derivatives, in different biological systems (Ko *et al*., 1995; Achliya *et al*., 2004; Sanmugapriya and Venkataraman, 2006).

1.2.3 Anti-oxidants

Anti-oxidants are molecules capable of slowing or preventing the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent. Oxidation reaction can produce free radicals, which start chain reactions that damage cell. Antioxidants terminate these chain reactions by removing free radical intermediates and inhibit other oxidation reactions (Bjelakovic *et al.*, 2007). 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 (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 (Shetty, 1997). 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 (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., 1998), and in traditional medicine as treatment for many diseases (Shetty, 1997).

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 anti-oxidant 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).One reason for the continued interest in examining the anti-oxidant effects of medicinal plants is the desire to find natural anti-oxidants that have a minimal impact on the characteristics of food (Brown *et al*., 2006).

1.2.4 Vitamin C

Vitamin C (ascorbic acid) is a monosaccharide anti-oxidant that is found in both animals and plants, but 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), therefore they must obtain it from diet, and a deficiency of the vitamin in the diet causes the disease scurvy (Bendich, 1997). The molecular mechanisms of the anti-scorbutic effect of vitamin C are largely, although not completely, understood (Burri and Jacob, 1997).The pharmacophore of vitamin C is the ascorbate ion, and in living organisms, ascorbate is an anti-oxidant, since it protects the body against oxidative stress (Padayatty *et al*., 2003).

Vitamin C is a cofactor in several vital enzymatic reactions (Rojas-Grau *et al*., 2008). These enzymes include the mono- and di-oxygenases 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). Biochemical, clinical, and epidemiologic studies have suggested that vitamin C may be important in reducing the risk to develop chronic diseases such as cardiovascular diseases, cancer, and cataract, and in this regard the antioxidant properties of the vitamin have been augmented (Weber *et al*., 1996; Enstrom, 1997; Gey, 1998).

The vitamin is considered as an important water-soluble anti-oxidant in biological fluids, and can scavenge reactive oxygen and nitrogen species, such as superoxide and hydroperoxyl radicals, aqueous peroxyl radicals, singlet oxygen, ozone, peroxynitrite, nitrogen dioxide, nitroxide radicals, and hypochlorous acid, thereby it effectively protects 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).

1.2.5 Investigated Parameters

1.2.5.1 Total and Absolute Counts of Leucocytes

The total and absolute counts of leucocytes can give a general picture of the immunity in the peripheral blood, because such counts are sensitive to infections, environmental pollution and chemical agent (Ad'hiah *et al*., 2001b; Ad'hiah *et al*., 2004). These cells are originated in the bone marrow from the hematopoietic stem cell through two cell lineage; myeloid and lymphoid, which give rise to the five types of leucocytes (neutrophils, lymphocytes, monocytes, eosinophils and basophils), and each type of these cells is commenced to carry out an immune function (Lydyard and Grossi, 1998).

The neutrophils are mainly involved in the innate immune system to carry out phagocytosis, while lymphocytes represent the humoral and cellular arms of specific immunity. Monocytes are involved in carrying out phagocytosis, but they are also professional antigen presenting cells. Eosinophils are involved in allergic and inflammatory reactions, as well as, parasitic infections. Basophils release histamine, heparin and some pharmacological mediators of immunological reactions (Richard and Pamela, 2008).

1.2.5.2 Mitotic Index

Many studies that determine the activity of immune system and the effect of different agents on it depend on the ability of lymphocytes to proliferate in lymphoid organs and/or depend on dividing cells of bone marrow because it is the source of all blood cells (Hughes, 2001). Mitotic index is defined as the ratio of the number of cells in a population undergoing mitosis (different stages) to total number of cells (Gosh *et al*., 1991). 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 the induction of apoptosis (Mollinedo and Gajate, 2003). Investigations have demonstrated that entry into mitosis in the presence of a damaged DNA may lead 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).

1.2.5.3 Micronucleus Formation

Micronuclei are formed during the metaphase \ anaphase transition of mitosis. It may arise from a whole lagging chromosome (aneugenic event leading to chromosome loss) or an acentric chromosome fragment detaching from a chromosome after breakage (clastogenic event) that do not integrate in the daughter nuclei (Fenech *et al*., 2003). Micronuclei occur spontaneously, but aneugens and clastogens cause an increase in the number of micronuclei relative to the negative control (Torous *et al*., 2005).

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 are the cells that are scored in the bone marrow or the blood for presence of micronuclei, and these cells arise from "stem cell" in the bone marrow and are produced by a series of divisions in a precursor cell population. The constant, rapid turnover of precursor cells makes erythrocytes an ideal cell type for a micronucleus test (Witt *et al*., 2008).

1.2.5.4 Sperm-head and Tail Abnormality Assay

Sperm topography is unique among the known cells, and three major parts can be immediately distinguished; head, mid-piece 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 the penetration of ovum in the fertilization process. The tail is divided into a neck, middle piece, principle piece and the end piece. The neck connects the tail to the head of spermatozoon (Saladin and Porth, 1998).

Abnormal sperm morphology is classified as defects in the head, mid-piece 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).

1.2.5.5 Liver Function Tests

1.2.5.5.1 Aspartate Amino- and Alanine Amino-Transferases

Enzymes are proteins that act as biological catalysts to speed up the rates at which chemical reactions occur by lowering the activation energy, and therefore without assistance of enzymes most of the chemical reactions of metabolism would barely proceed at all (Atlas, 1995). In this regard, 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 transferases in the diagnosis of a hepatic damage are aspartate amino-transferase (AST) and alanine amino-transferase (ALT) (Charles, 2003). With respect to this scope, studies have investigated the effects of different drugs or chemicals in inducing hepatic damages and the role of medicinal plants in reducing these effects. Therefore, the evaluations of AST and ALT levels are important in assessing the degree of hepatic damages (Kokdil *et al*., 2005; Sanmugapriya and Venkataraman, 2006).

1.2.5.5.2 Serum Alkaline Phosphataes

Alkaline phosphatase (ALP) is an enzyme found in all tissues, and 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 liver, bile ducts, placenta, kidneys and bones, and in a lower amount in intestines and leukocytes (Kenton *et al*., 2001). 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 is associated predominantly with more specific disorders; including malignant biliary obstruction, primary biliary cirrhosis, primary sclerosing cholangitis, hepatic lymphoma and sarcoidosis (Neuschhwander-Terti, 1995). On the other hand, according to a study of Maldonado *et al*. (1998), sepsis and malignant obstruction are identified 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 AST, ALT) and ALP caused by CCl_4. Another study demonstrated that the methanol extract of *Bauhinia racemosa* possessed hepatoprotective and anti-oxidant properties against CCl_4- and paracetamol-induced hepatotoxicities, which resulted in increased levels of the enzymes AST, ALT and ALP, while the plant extract caused a significant reduction in the level of these enzymes down to the normal level (Hewawasam *et al.*, 2003).

1.2.5.6 Cancer Cell Lines

Two cancer cell lines (HeLa and rhabdomyosarcoma) and a normal cell line (mouse embryonic fibroblast) were employed in the present study to assess the *in vitro* tumoricidal potency of agrimony extracts.

- **HeLa cell line**: HeLa is immortal cell line used in scientific research. The cell line was derived from cervical cancer cells taken from Henrietta Lacks, who died from her cancer on October 4, 1951. These cells are termed "immortal" in that they can divide an unlimited number of times in a laboratory cell culture plate as long as fundamental cell survival conditions are met (i.e. being maintained and sustained in a suitable environment (Sharrer, 2006). HeLa cells have an active version of the enzyme telomerase during cell division, which prevents the incremental shortening of telomeres that is implicated in aging and eventual cell death. In this way, HeLa cells circumvent the Hayflick Limit, which is the limited number of cell divisions that most normal cells can later undergo before dying out in cell culture (Roland, 2006).

- **Rhabdomyosarcoma cell line**: Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma of childhood and adolescence (Naini *et al*., 2008). There are two major histologic subtype of rhabdomyosarcoma; alveolar rhabdomyosarcoma and embyonal rhabdomyosarcoma (Wysoczynski *et al*., 2007), and several models have been described for investigation of RMS *in vitro* and *in vivo* (Seitz *et al*., 2007).The biological properties of these cells are still a subject of controversy. Degrees of differentiation and proliferation rate were estimated morphologically and by means of immunohistochemistry and a monolayer proliferation assay. The activation of a telomere maintenance mechanism (TMM) is crucial for the immortalization of tumor cells. The TMM and its association with survival in RMS is important implications for understanding the role of TMM in the

development of RMS tumors, and for future designing of adapted treatment strategies (Ohali *et al*., 2008**).**

- **Primary Mouse Embryonic Fibroblasts**: Mouse embryonic fibroblast (MEF) cells are used as a feeder layer for the culture of mouse embryonic stem (ES) cells to maintain them as pluripotent stem cells. The inhibition of ES-cell differentiation provided by the MEF feeders appears to be due to their production of leukemia inhibitory factor (David, 2000). These normal cells are known to have a limited life span *in vitro*, and after a certain number of divisions the cells pass into a "crisis" stage and dies. Some cells survive and acquire the ability for unlimited division; forming so-called stable or immortalized cell lines. These cells were prepared and employed in the present study as a normal cell line (Matise *et al*., 2000).

1.2.5.7 Thin Layer Chromatography

Thin Layer chromatography (TLC) provides a quick, economical and reliable method for rapid screening of pharmaceuticals (Kenyon and Layloff, 2000). The TLC is performed on a sheet of glass, plastic, or aluminum foil, which is coated with a thin layer of adsorbent material, usually silica gel. This layer of adsorbent is known as the stationary phase (Jonathan *et al*., 2007). After a sample application on the activated plate, the plate is placed in a closed system containing a solvent or solvent mixture (known as the mobile phase). Because different analytes ascend the TLC plate at different rate, separation of compounds is achieved (Fair and Kormos, 2008).

Chapter Two

Materials and Methods

Chapter two
Materials and Methods

2.1 Materials

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

2.2 The Plant *Agrimonia eupatoria*

The plant taxonomist Professor Dr. Ali Al-Mosawy (Department of Biology, College of Science, University of Baghdad) identified the plant *A. eupatoria*, which was collected in July 2008 from the mountain regions (Binqlat village) surrounding the city of Irbil (around 400 Km north the capital Baghdad) (Figure 2-1). The plant was left at room temperature (20-25ºC) to dry (around 5 days).

Figure 2-1: Field picture of *Agrimonia eupatoria*.(Taken by researcher)

2.3 Plant Extraction

The dried aerial parts of the plant (leaves, stem and flowers) were powdered using a coffee grinder for 5 minutes, and then extracted with two types of solvents (distilled water or methanol). In both cases, 50 grams of the processed plant were extracted in 250 ml of the solvent using the Soxhlet apparatus and the source of heating was a warm water bath (45°C). The obtained extract solution was then evaporated at 45°C using a rotary evaporator, and the resultant crude extract was frozen at -20°C until use to prepare the required doses and concentrations ((Arokiyaraj *et al*., 2007)**.**

The aqueous or methanol crude extract was dissolved in sterile distilled water to prepare three intraperitonial doses (1.0, 2.0 and 3.0 mg/kg), which were investigated in the laboratory mice. These doses were based respectively on 10, 20 and 30% of the LD_{50} dose (10 mg/kg) in mice (Murayama *et al*., 1992).

2.4 Solutions

i. **Phosphate Buffer Saline (PBS)**: The solution was prepared by dissolving the following chemicals in 950 ml of distilled water and then the volume was made-up to 1000 ml:

Sodium chloride (NaCl):	8.0 g
Potassium chloride (KCl):	0.2 g
Di-sodium hydrogen phosphate (Na_2HPO_4):	1.15 g
Potassium di-hydrogen phosphate (KH_2PO_4):	0.2 g

The pH was adjusted to 7.2, and the solution was compeleted to 1000ml with D.W, autoclved and stored at 4° C (Hudson and Hay, 1989).

ii. **Potassium chloride (KCl) hypotonic solution (0.075M)**: was prepared by dissolving (5.75g) in 1000 ml of distilled water, and then the solution was autoclaved and stored at 4°C (Allen *et al*., 1977).

iii. Colchicine: It was prepared by dissolving one tablet (0.5 mg) of colchicine in 0.5 ml of sterilized PBS, and it was freshly used (Allen *et al*., 1977).

iv. Hanks Balanced Salt Solution (HBSS): Each of the following chemicals were dissolved in 50 ml of distilled water:

$CaCl_2$ (anhydrous)	0.14 g
KCl	0.4 g
K_2HPO_4	0.06 g
$MgCl_2.6H_2O$	0.1 g
$MgSO_4.7H_2O$	0.1 g
NaCl	8.0 g
$NaHCO_3$	0.35 g
Na_2PO_4	0.09 g
D-glucose	1.0 g
Hepes	2.08 g

The volumes were combined together (the solution of $CaCl_2$ was the last added), the pH was adjusted to 7.2 and the volume was made-up to 1000 ml with distilled water. The solution was filter-sterilized (Millipore filter; 0.22 μm) and stored at 4ºC (Freshney, 2000).

v. Antibiotic solutions: Two antibiotics were used; they were penicillin and streptomycin. The penicillin (1000000 IU) was dissolved in 10 ml of sterilized distilled water, while one gram of streptomycin was dissolved in 10 ml of sterilized distilled water. Both solutions were filter-sterilized (Millipore filter; 0.22 μm) and stored at -20ºC after dividing them into aliquots (1 ml) (Freshney, 2000).

vi. **RPMI-1640 Medium**: Ten grams of RPMI-1640 medium, 2 grams of sodium bicarbonate and 4 grams of hepes were dissolved in 500 ml of distilled water, then, 100 ml of heat inactivated fetal calf serum, 5 ml penicillin (100000 IU/ml) and 5 ml streptomycin (100 mg/ml) were added. The volume was made up to 1000 ml with distilled water, and the medium was filter-sterilized (Millipore filter; 0.22 μm) after adjusting the pH to 7.2. The filtered medium was divided into aliquots (5 ml) and stored at -20°C until use (Nara and McCulloh, 1985).

vii. **Neutral red**: Ten milligrams of neutral red dye were dissolved in 100 ml of PBS and filtered (Whatman filter paper No. 1) (Freshney, 2000).

viii. **The eluent**: The solution was prepared by mixing equal volumes of 0.1M NaH_2PO_4 (0.156 grams of NaH_2PO_4 per 10 ml distilled water) and absolute ethanol (Freshney, 2001).

ix. **Trypan blue stain**: Trypan blue powder (1 gram) was dissolved in 100 ml HBSS. The solution was filtered (Whatman filter paper No. 1) and stored at 4ºC (stock solution), and then it was diluted (1:10) in HBSS to prepare working solution (Freshney, 2001).

x. **Leucocyte diluent**: The solution was prepared by adding 2 ml of glacial acetic acid to 98 ml of distilled water, in addition to a few drops of methylene blue as a color indicator (Sood, 1986).

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

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

xiii. **Vitamin C:** The vitamin powder was dissolved in distilled water to prepare a dose of 120 mg/kg (Giacosa *et al*., 1997).

xiv. **Carbon tetrachloride (CCl_4)**: Carbon tetrachloride solution was diluted with an equal volume of distilled water (1:1) to prepare a dose of 3.2 mg/kg (Lin *et al*., 1998).

xv. **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).

xvi. **Giemsa stain**: Giemsa stock solution was prepared by dissolving one gram of Giemsa powder in 33 ml glycerin using a water bath (60°C) for 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

xvii. **Eosins stain (1%)**: The stain solution was prepared by dissolving a gram of eosin yellowish powder in 100 ml of distilled water, and then the solution was filtered (Whatman filter paper No. 1) (Wyrobek and Bruce, 1975).

xviii. **Leishman stain**: The stain solution was prepared by dissolving 0.15 gram of Leishman powder in 100 ml of absolute methanol, and then the solution was filtered (Whatman filter paper No. 1) (Collee *et al*., 1996).

xix. **Leishman stain buffer**: The buffer was prepared by mixing together 5.447 grams of Na_2HPO_4 (anhydrous) and 4.752 grams of KH_2PO_4. Then, a gram of the mixture was dissolved in 2 liters of distilled water, and the pH was adjusted to 7.0 (Collee *et al*., 1996).

xx. **Haematoxylin stain**: The stain solution was ready supplied by the Histopathology Department at Educational Laboratories of Medical Baghdad City.

xxi. **Mayer's reagent:** Two solutions were firstly prepared; the first one was prepared by dissolving 1.58 grams 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. Both solutions were mixed and the volume was made-up to 100 ml with distilled water (Smolensk *et al*., 1972).

xxii. **Benedict reagent**: The reagent was prepared by dissolving 137 grams of sodium citrate (CH_3COONa) and 100 grams of sodium bicarbonate ($NaHCO_3$) in 800 ml of distilled water and the mixture was filtered

(Whatman filter paper No. 3), and then cupper sulphate ($CuSO_4$) solution (17.3 grams in 100 ml distilled water) was added and the volume was made-up to 1000 ml with distilled water (Al-Janabi, 2004).

xxiii. **Ferric chloride solution (1%):** The solution was prepared by dissolving 1gram of ferric chloride in 100 ml of distilled water (Collee *et al*., 1996).

xxiv. **Sodium hydroxide solution**: The solution was prepared by dissolving 40 mg of sodium hydroxide in 1000 ml of distilled water (Collee *et al*., 1996).

xxv. **Potassium hydroxide solution**: It was prepared by dissolving 50 grams of potassium hydroxide in 100 ml of distilled water (Collee *et al*., 1996).

xxvi. **Trypsin stock solution**: Trypsin (2.5 grams) was dissolved in 100 ml of 0.85% NaCl and stirred for 60 minutes at room temperature. The solution was filter-sterilized (Millipore filter; 0.22 μm), divided into aliquots (10 ml), and stored at -20ºC. The working solution was prepared by diluting the stock solution (1:10) with PBS (Freshney, 2000).

xxvii. **Trypsin / versin solution:** It was prepared by dissolving 2 grams of trypsin / versin powder in 100 ml of distilled water and the pH was adjusted to 7.0 using sodium bicarbonate solution. Then, it was filter-sterilized (Millipore filter; 0.22 μm) and kept at 4ºC (Freshney, 2000).

2.5 Laboratory Animals

Albino male mice (*Mus musculs*) were the laboratory animals that were employed in carrying out the experiments of the study. They were supplied by the Biotechnology Research Centre (Al-Nahrain University), and their age at the start of experiments was 8-10 weeks, and their weight was 23-27grams. 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 through out the experimental work.

2.6 Experimental Design

The experiments were designed to evaluate some immunological, cytogenetic, biochemical, and histopathological effects of agrimony extracts (aqueous and methanol), and their effects on CCl_4-induced acute hepatic injury in albino male mice. Therefore, such evaluations were carried out through two stages (experiments number one and two).

2.6.1 Experiment Number One

The experiment was designed to assess the immunological, cytogenetic, biochemical and histopathological effects of three doses (1.0, 2.0 and 3.0 mg/kg) of agrimony extracts (aqueous or methanol), as well as, CCl_4 (positive control I), vitamin C (positive control II) and distilled water (negative controls). Therefore, the animals were divided into nine groups:

- **Group I (negative controls):** The animals were treated with distilled water (4 animals).
- **Group II (positive control I):** The animals were treated with CCl_4 (3.2 mg/kg) (4 animals).

- **Group III (positive control II):** The animals were treated with vitamin C (120 mg/kg) (4 animals).
- **Groups IV, V and VI)**: The animals were treated with three doses (1.0, 2.0 and 3.0 mg/kg, respectively) of aqueous extract (12 animals).
- **Group VII, VIII and IX)**: The animals were treated with three doses (1.0, 2.0 and 3.0 mg/kg, respectively) of methanol extract (12 animals).

The tested material was injected intraperitoneally as a single dose (0.1 ml) per a day and for 6 days, and then the mice were sacrificed in day 7 for laboratory assessments. In the case of CCl_4, it was injected subcutaneously with a single dose (0.1 ml) in day 1, and then the mice were sacrificed day 2.

2.6.2 Experiment Number Two

The experiment was designed to assess the interaction between the ideal dose of each extracts (3.0 mg/ kg) and CCl_4 (3.2 mg/kg). The selection of the plant extract ideal dose was based on the results of experiment number one. In such interaction, the animals were given CCl_4 on day 1, while the plant extract (aqueous or methanol) was given in day 2 till day 6 (single dose/day), and then the animals were sacrificed in day 7 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 paralleled the two types of extracts, in which the plant extract was replaced by distilled water (negative control) or vitamin C (positive control; 120 mg/kg).

2.7 Laboratory Methods

2.7.1 Chemical Analysis of Plant Extracts

2.7.1.1 Chemical Detection of Plant Extracts

The chemical detection of the plant aqueous and methanol extracts was carried out at the Biotechnology Research Center (Al-Nahrain University).

i. **Tannins**: The procedure of Harbone (1984) 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.

ii. **Saponins**: Two methods were employed in the detection of saponins. The first one included adding 3 ml of mercuric chloride ($HgCl_2$) to 5 ml of the extract (aqueous or methanol), and then the formation of white pellet was an indicator of the presence of saponins . In the second method, the tube containing the extract was shaken, and the formation of thick foam that remained for few minutes was an indicator of a saponin's presence (Stahl, 1969).

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

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

v. **Alkaloids**: One ml of the plant extract was added to a tube containing 2 ml of Mayer′s reagent. The appearance of white residue after shaking the tube was an indicator of the presence of alkaloids (Sousek, 1999).

2.7.1.2 Detection of Flavonoid Compounds by TLC

The procedure of Harborne (1984) was followed, in which a silica gel TLC plate (200×200×0.25mm) was activated in the oven at 110^5C for one hour, and after cooling the plate at room temperature (around 30 minutes), a marginal line was drawn on the upper and lower side of the plate with a distance of 2 centimeters from the margins. On the lower line of the plate, 0.25 ml of concentrated sample (aqueous or methanol extract) and a flavonoid standard (rutin) was applied. After around 5-7 minutes, the plate was placed in a jar-closed-system containing a solvent (*n*-BuOH:HOAc:H_2O at a ratio 4:1:5), and the system was left for two hours at room temperature. Then plate was examined under UV light to detect the separated flavonoid compounds in the aqueous and methanol extract samples as compared to the flavonoid standard and the R_F value.

The R_F value is the distance compound moves in the chromatography relative to the solvent front. It is obtained by measuring the distance from the origin to the center of the spot produced by the substance, and this is divided by the distance between the origin and the solvent front (i.e. the distance the solvent travels). This always appears as a fraction and lies between 0.01 and 0.99. It is convenient to multiply this by 100 and R_F is quoted as R_F (×100) (Harborne, 1984). Such value was used to assess the presence of flavonoid compounds in the two plant extracts (Table 2-1).

Table 2-1: Properties (R_F values) of common flavonoid compounds*.

R_F (×100)	
Flavonoid Compounds	Solvent (*n*-BuOH:HOAc:H_2O)
Flavonols	
Kaempferol	83
Quercetin	64
Myricetin	43
Isorhamnetin	74
Azaleatin	48
Cossypetin	31
Flavones	
Apigenin	89
Luteolin	78
Chrysoeriol	82
Tricin	73
Glycosylflavones	
Vitexin	41
Isovitexin	56
Orientin	31
Iso-orientin	41
Biflavonyl	
Kayaflavone	98

* After Harborne (1984).

2.7.2 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 3 minutes. A drop of the mixture was applied to the surface of Neubauer chamber under the cover slip, and the chamber was left for 3 minutes to settle the cells. The leucocytes were counted in 4 large squares (each with 16 small squares), and the total count of leucocytes was obtained using the following equation:

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

2.7.3 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, buffered for 10 minutes with Leishman buffer, and finally 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 cell type was recorded, while the absolute count of each type was obtained using the following equation:

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

2.7.4 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. The femur was cut from both ends, and its cellular contents were collected in a test tube using PBS (5 ml), with aid of a disposable insulin syringe (1 ml). These cells were manipulated as the following:

i. The cell suspension was gently pipetted using Pasteur pipette, and the tube was centrifuged (2000 rpm) for 10 minutes.

ii. The supernatant was discarded, and the cell deposit was suspended in 5 ml of a warm (37°C) hypotonic solution (KCl; 0.075M). Then the tube was incubated in a water bath (37°C) for 30 minutes with a gentle shaking every 5 minutes.

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

iv. Five ml of the fixative solution was added a drop-wise to the cell deposit with a gentle and a continuous mixing to make a homogeneous cell suspension. Then, the tube was incubated in the refrigerator (4°C) for 30 minutes.

v. The tube was centrifuged (2000 rpm) for 10 minutes, and the last step was repeated two times.

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

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

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

$$\text{Mitotic Index (\%)} = \left(\frac{\text{Number of Divided Cells}}{\text{Total Count (1000)}} \right) \text{x } 100$$

2.7.5 Micronucleus Formation Assay

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

i. The mouse was sacrificed by cervical dislocation, and then dissected to obtain the femur. After cutting both ends of femur, it was gripped from the middle with a forceps in a vertical position over the edge of a test tube, and then the cellular content was collected with a heat inactivated (56°C for 30 minutes) human AB plasma (1 ml) using a disposable insulin syringe.

ii. The test tube was centrifuged (1000 rpm) for 5 minutes, and the supernatant was discarded.

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

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

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

vi. The slide was examined under oil immersion lens (100X), and at least 1000 polychromatic erythrocytes (PCE) were examined for the presence of micronucleus formation. The micronucleus index was obtained using the following equation:

$$\text{Micronucleus index (\%)} = \left(\frac{\text{Number of Micronuclei}}{\text{Total Count of PCE (1000)}} \right) \text{x } 100$$

2.7.6 Sperm-head and -tail 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 PBS. The epididymis was dispersed with a forceps and a scalpel to free the spermatozoa. The spermatozoa-containing PBS was transferred to a test tube, which was centrifuged (1000 rpm) for 10 minutes. The supernatant was discarded, and the spermatozoa deposit was gently suspended in 1 ml of PBS. A thin smear of the suspension was made on a clean slide, which was then air-dried at room temperature. The slide was stained with eosin for 5 minutes, rinsed with distilled water and air-dried. The slide was examined under oil immersion lens (100X), and at least 1000 spermatozoa were inspected for the morphology of their head and tail (Wyrobek and Bruce,

1975). The sperm-head (SHA) or tail abnormality (STA) index was scored using the following equation:

$$\text{SHA or STA index (\%)} = \left(\frac{\text{Number of Spermatozoae with Abnormal Head or Tail}}{\text{Total Count (1000)}} \right) \text{x } 100$$

2.7.7 Biochemical Tests

Three biochemical tests were carried out in the sera of investigated animals. They were aspartate amino-transferase (AST), alanine amino-transferase (ALT), and alkaline phosphatase (ALP).

2.7.7.1 Aspartate Amino-Transferase (AST)

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

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

- ***Procedure***: Two test tubes (blank and sample) were used and the above solutions were added as shown in table 2-2.

Table 2-2: Method of measuring AST activity.

Reagents	**Tubes**	
	Blank (ml)	**Sample (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 AST (Unit/L) was calculated from the standard curve of the kit		

2.7.7.2 Alanine Amino-Transferase (ALT)

The enzyme activity ALT 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***
  - ALT substrate reagent (R1)
  - ALT color reagent (R2)
  - Sodium hydroxide (R3)
- ***Procedure***: Two test tubes (blank and sample) were used and the above solutions were added as shown in table 2-2.

2.7.7.3 Alkaline Phosphatase (ALP)

The enzyme ALP was evaluated in mouse serum using a commercial kit produced by Bio Merieux Company and the most commonly used method is that of King and Arnistrong (2003), in which di-sodium phenyl phosphate is

hydrolyzed with liberation of phenol and formation of sodium phosphate. The amount of phenol formed is estimated colorimetrically.

- ***Kit Components***
  - Buffer (R1)
  - Di-sodium phenyl phosphate 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 2-3.

Table 2-3: Method of measuring ALP activity.

Reagents	**Tubes**			
	Serum sample	**Serum blank**	**standard**	**Reagent blank**
Reagent 1	2 ml	2 ml	2 ml	2 ml
The tubes were incubated in a water bath (37°C) for 5 minutes				
Serum Reagent 2	50 µl -	- -	- 50 µl	- -
The tubes were mixed well and incubated in a water bath (37°C) for 15 minutes				
Reagent 3	0.5 ml	0.5 ml	0.5 ml	0.5 ml
The tubes were mixed well and vortexed				
Reagent 4 Serum Distilled water	0.5 ml - -	0.5 ml 50 µl -	0.5 ml - -	0.5 ml - 50 µl
The tubes were well-mixed and left to stand for 10 minutes in dark place. Then the optical density (O.D.) was measured at a wave length of 510 nm.				

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

$$\text{Serum Level of ALP (I.U./ml} = \left(\frac{\text{O.D. Serum Sample - O.D. Serum Blank}}{\text{O.D. Standard}} \right) \times 142$$

2.7.8 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 the following:

i. **Washing:** The sample was placed in 70% ethanol overnight.
ii. **Dehydration:** The sample was dehydrated with ascending concentrations (50, 70, 90 and 99%) of ethanol. There were two hours for each concentration.
iii. **Clearing:** The sample was placed in xylene for two hours.
iv. **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.
v. **Embedding**: The sample was embedded in pure paraffin wax (melting temperature: 60-70ºC) and left to solidified at room temperature.
vi. **Sectioning**: The paraffin block was sectioned (rotary microtome) at a thickness of 5 microns, and then the sections were transferred to a slide covered with Mayer's albumin. The section of tissue was placed in a water bath (35-40ºC) for few seconds.
vii. **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.

viii. **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.

2.7.9 Assessment of Anti-Tumor Activity (*in vitro*)

The *in vitro* anti-tumor activity of agrimony aqueous and methanol extracts was carried out at the Iraqi Center for Cancer and Medical Genetic Research (Al-Mustansiryah University) and the Biotechnology Research Centre (Al-Nahrain University). In this study, the cytotoxic activity of the two plant extracts was evaluated against two tumor cell lines (human rhabdomyosarcoma; RD and human cervical cancer; HeLa) and a normal cell line (mouse embryonic fibroblasts; MEF).The percentage of growth inhibition was calculated according to an equation presented by Phuangsab *et al*. (2001):

$$\text{Growth inhibition}\,(\%) = \left(\frac{\text{Control Absorbance - Treated Absorbance}}{\text{Control Absorbance}}\right) \times 100$$

2.7.9.1 Growth Inhibition Assessment in RD and HeLa Cell Lines

The RD cell line was on passage number 195, while HeLa cell line was on passage number 185. The laboratory assessment of growth inhibition was carried out according to a method that was adopted by Freshney (2000). The method included the following steps:

i. The cells (RD or HeLa) were supplemented as a monolayer attached cells in Falcon culture flasks (25 ml) containing RPMI-1640 medium. The cells were washed with PBS, and then 1 ml of trypsine –versine solution

was added with a gentle shaking until the cells were detached from the flask surface. Such manipulation was carried out with aid of phase contrast inverted microscope. Then, the contents of the flask were transferred to another flask and incubated at 37ºC for 15 minutes (sub-culture).

ii. The cells were counted and their number was adjusted to 1 x 10^6 cell/ml. At the same time, viability was assessed using a dye-exclusion test (trypan blue stain), and it was always greater than 96%.

iii. The cells were seeded in the wells of 96-well tissue culture plate, which was carried out by pipetting 150 µl of the cell suspension into each well, and the plate was incubated overnight at 37ºC.

iv. The day after, the wells were examined to inspect the formation of cell monolayear, and then five concentrations (6.0, 12.0, 24.0, 48.0 and 96.0 µg/ml) of each plant extract were prepared and the solvent was RPMI-1640 medium. In each well, 50 µl of each concentration was pipetted and the plate was wrapped with a cling film and incubated at 37ºC. In this regard three plates were prepared to cover three incubation periods; 24, 48 and 72 hours.

v. After the end of each incubation period, the medium in each well was discarded and the wells were washed once with PBS, and then 0.8 ml of neutral red solution was added to each well. The plate was re-incubated at 37ºC for 2 hours.

vi. After incubation, the neutral red solution was discarded and each well was washed once with PBS, and in each well, 0.1 ml of phosphate buffered-ethanol (0.1M NaH_2PO_4-ethanol; 1:1) was added.

vii. The wells were read ELISA reader at wave length of 492 nm, and the absorbance was recorded. The growth inhibition of the tested materials was calculated using the equation given in section 2.7.9.

2.7.9.2 Mouse Embryonic Fibroblast (MEF) Cell Line

The MEF was the normal cell line, which was prepared by the student following the instruction of Freshney (2000). It was prepared from a pregnant mouse at a gestation period of 11-13 days, in which the mouse was sacrificed by cervical dislocation and dissected to obtain the embryos, which were transferred to sterilized Petri-dishes under aseptic conditions, and then the following steps were followed:

i. The embryos were washed with PBS three times, and then they were transferred to another Petri-dish and cut into small pieces. These pieces were transferred to a 25ml universal tube and washed several times with PBS (15 ml) until the supernatant was clear and free of erythrocytes.

ii. The washed embryo pieces were transferred to a 50ml-tissue culture flask containing a magnetic bead and 15 ml of trypsin solution. The flask was transferred to a magnetic warm plate (37ºC), and stirring continued for 15 minutes.

iii. The contents of flask were filtered using double layers of autoclaved gauze, and the filtrate was centrifuged (1000 rpm) for 5 minutes in a cooled centrifuge. The cell pellet was collected and washed two times with RPMI-1640 medium at a centrifugation speed of 1000 rpm and for 5 minutes.

iv. The cell pellet was gently suspended in a 10ml round bottom tube containing 5 ml of RPMI-1640 medium. The cell suspension was transferred to Falcon tissue culture flask, and the volume was made up to 25 ml with RPMI-1640 medium.

v. The flask was incubated at 37ºC for 72-96 hours, and the formation of monolayer cell was inspected using a phase contrast inverted microscope.

vi. These cells were harvested and employed in the assessment of growth inhibition after treatments with the plant extracts as mentioned earlier in

the section 2.7.9.1**,** but the assessment was carried out for one period, which was 48 hours.

2.8 Statistical Methods

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), least significant difference (LSD) 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:

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

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

B = Negative control groups (distilled water).

Chapter Three

Results

Chapter Three
Results

3.1 Chemical Analysis of Plant Extracts

3.1.1 Chemical Detection of Plant Extracts

Chemical detections of *A. eupatoria* extracts (aqueous and methanol) revealed that the plant was positive for several secondary metabolites. In aqueous extract, flavonoids, alkaloids, tannins and glycosides were detected, while methanol extract was positive for flavonoids, alkaloids, tannins and glycosides. Whereas, both extracts were negative for saponins (Table 3-1).

Table 3-1: Chemical detections of secondary metabolites in the aerial part extracts (aqueous and methanol) of *A. eupatoria*.

Secondary Metabolites	Reagents	Aqueous Extract	Methanol Extract
Flavonoids	Ethanol with KOH	+	+
Tannins	$CH_3COOPb+FeCl_2$	+	+
Alkaloids	Mayer's reagent	+	+
Glycosides	Benedict reagent	+	+
Saponins	$HgCl_2$	-	-

+: Positive reaction.
- : Negative reaction.

3.1.2 Detection of Flavonoids Compounds by TLC

The plate was examined under UV light to detect the separated flavonoid compounds in the aqueous and methanol extract samples as compared to the flavonoid standard and the R_F value (Figure 3-1). As

suggested by RF values of the separated extracts, the aqueous extract contained myricetin, azoleatin, vitexin and iso-orientin, while the methanol extract contained kaempferol, quercetin , isorhamnetin and myricetin .

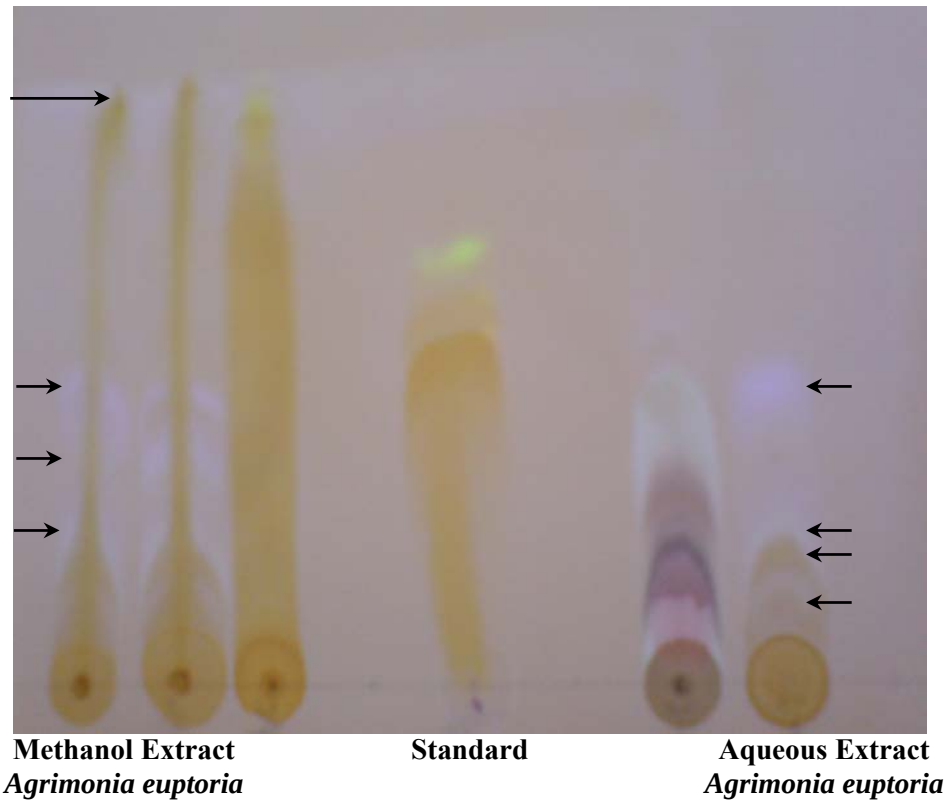


Figure 3-1: Thin layer chromatography of agrimony extracts.

3.2 Immunological and Cytogentic Effects of *A. eupatoriaa*

3.2.1 Total Count of Leucocytes (TCL)

Animals treated with CCl_4 (control III) showed a significant reduced count of leucocytes (3512.5 cell/cu.mm.blood) as compared to control I (7637.5 cell/cu.mm.blood) or control II (12037.5 cell/cu.mm.blood). With respect to the aqueous extract, the three doses (1.0, 2.0, and 3.0 mg/kg) showed a significant increase count of leucocytes as compared to control I (7787.5, 8937.5, 9987.5, respectively *vs*. 7637.5 cell/cu.mm.blood). The methanol extract showed a similar effect and the three doses were significantly effective in increasing the total count of leucocytes in a dose-

dependent manner (8375.0, 9162.5 and 10087.5 cell/cu.mm.blood, respectively) as compared to control I (7637.5 cell/cu.mm.blood). Vitamin C (control II) was also significantly effective in increasing the total count of leucocytes (12037.5 cell/cu.mm.blood) as compared to either control I (7637.5 cell/cu.mm.blood) or control III (3512.5 cell/cu.mm.blood). The best treatment efficiency was recorded for vitamin C (+57.6%), followed by the third dose of methanol (+32.0%) and aqueous (+30.7%) extracts (Table 3-2 and Figure 3-2).

Table 3-2: Total leucocyte count in albino male mice treated with two extracts (aqueous and methanol) of *A. eupatoria.*

Treatment Groups		Dose (mg/kg)	Mean ± SE* (cell/cu.mm.blood)
Control I (distilled water)		0.0	7637.5 ± 274.2^{e}
Control II (vitamin C)		120	12037.5 ± 235.7^{a}
Control III (CCl_4)		3.2	3512.5 ± 233.1^{f}
Agrimonia eupatoria	Aqueous extract	1.0	7787.5 ± 215.4^{e}
		2.0	8937.5 ± 219.3^{c}
		3.0	9987.5 ± 96.6^{b}
	Methanol extract	1.0	8375.0 ± 96.8^{d}
		2.0	9162.5± 108.7^{c}
		3.0	10087.5 ± 87.5^{b}

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

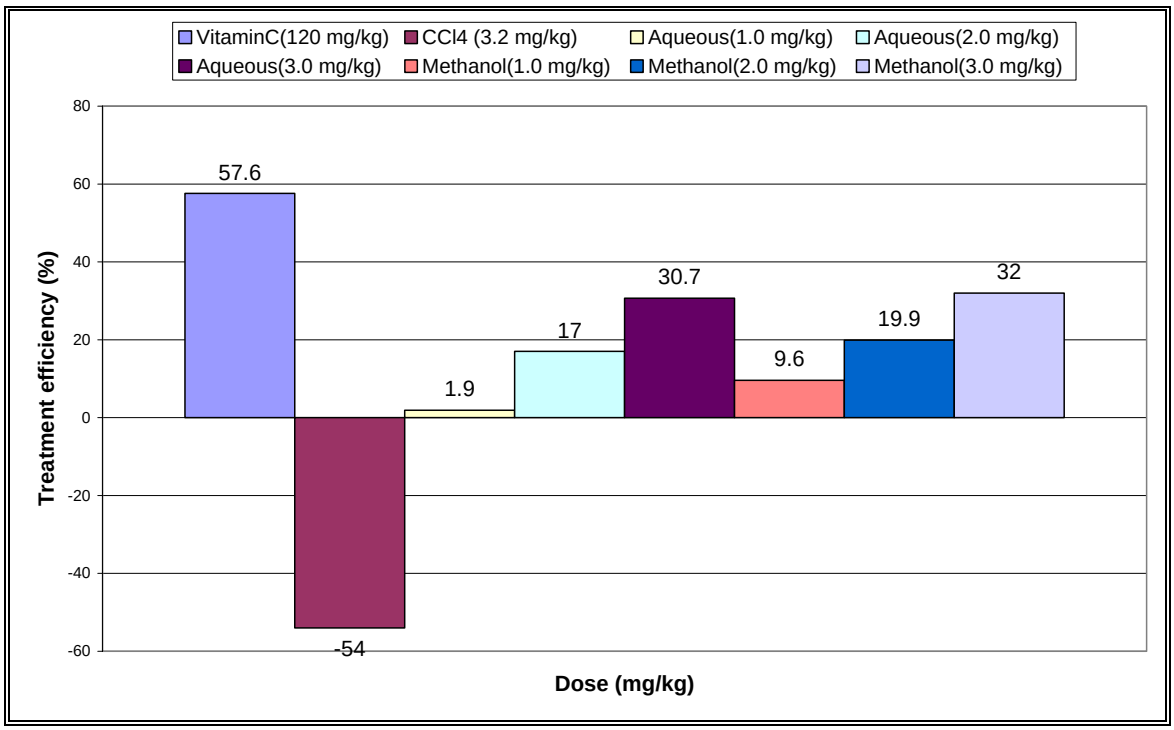


Figure 3-2: Treatment Efficiency of total leucocyte count in albino male mice treated with two extracts (aqueous and methanol) of *A. eupatoria.*

3.2.2 Absolute Counts of Leucocytes

3.2.2.1 Lymphocytes

A treatment with CCl_4 (control III) showed a significant reduced count of lymphocytes (2717.8 cell/cu.mm.blood) as compared to control I (6108.4 cell/cu.mm.blood) or control II (6463.3 cell/cu.mm.blood). With respect to the aqueous extract, the first dose (1.0 mg/kg) caused a significant decreased count of lymphocytes (3364.8 cell/cu.mm.blood) as compared to control I (6108.4 cell/cu.mm.blood), but the third dose (3.0 mg/kg) showed a significant increased count (7895.73 cell/cu.mm.blood, respectively). With respect to the methanol extract, the first dose also caused a significant decreased count of lymphocytes as compared to control I (5469.8 *vs*. 6108.4 cell/cu.mm.blood), but the third dose showed a significant increase (6280.6 *vs*. 6108.4 cell/cu.mm.blood).

The best treatment efficiency was recorded for the third dose of aqueous extract (+29.2%), followed by vitamin C (control II) (+5.8%) (Table 3-3 and Figure 3-3).

Table 3-3: Total lymphocyte count in albino male mice treated with two extracts (aqueous and methanol) of *A. eupatoria.*

Treatment Groups		Dose (mg/kg)	Mean ± SE* (cell/cu.mm.blood)
Control I (distilled water)		0.0	6108.4 ± 165.1^{b}
Control II(vitamin C)		120	6463.2 ± 255.3^{b}
Control III (CCl_4)		3.2	2717.8 ± 175.4^{e}
Agrimonia eupatoria	Aqueous extract	1.0	3364.8 ± 40.3^{d}
		2.0	6351.2 ± 161.2^{b}
		3.0	7895.7 ± 85.8^{a}
	Methanol extract	1.0	5469.8 ± 198.8^{c}
		2.0	5949.8 ± 95.6^{b}
		3.0	6280.6 ± 50.8^{b}

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

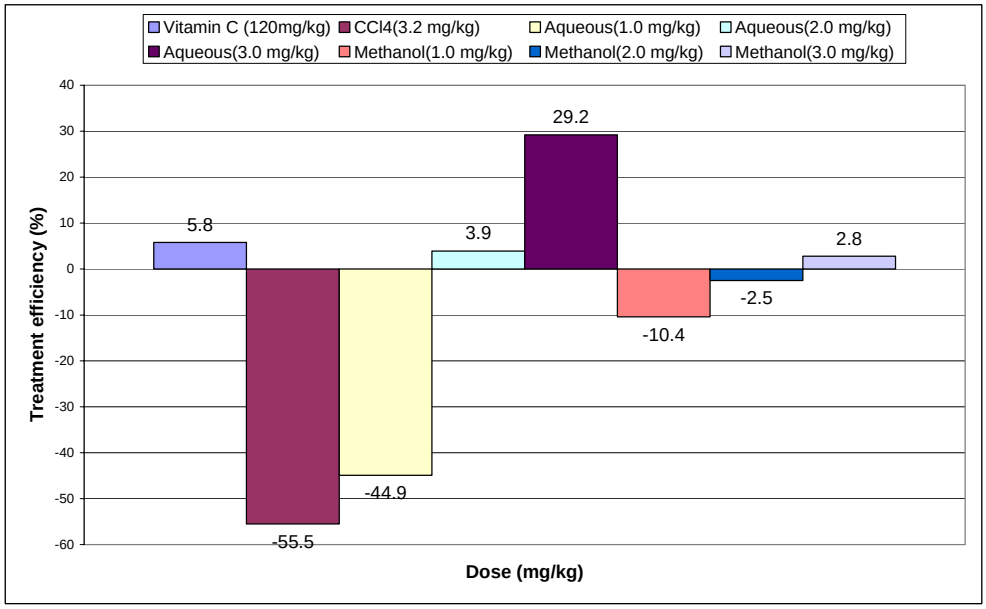


Figure 3-3: Treatment Efficiency of total lymphocyte count in albino male mice treated with two extracts (aqueous and methanol) of *A. eupatoria*.

3.2.2.2 Neutrophils

The animals treated with CCl_4 showed a significant reduced count of neutrophils (681.9 cell/cu.mm.blood) as compared to control I (1176.0 cell/cu.mm.blood) or control II (5195.2 cell/cu.mm.blood). With respect to the aqueous extract, the three doses (1.0, 2.0 and 3.0 mg/kg) showed a significant increase neutrophil count as compared to control I (1851.7, 2269.2 and 4217.7**,** respectively *vs*. 1176.0 cell/cu.mm.blood). Much more significant increase of neutrophil count was observed after a treatment with vitamin C (6463.3 cell/cu.mm.blood). The methanol extract behaved in a similar manner and the three doses showed a significant increase count of lymphocytes as compasred to control I (2651.1, 2810.3 and 3335.2**,** respectively *vs*. 1176.0 cell/cu.mm.blood). The best treatment efficiency was recorded for vitamin C (+341.7%), followed by the third dose of aqueous (+258.6%) and methanol (183.6%) extract (Table 3-4 and Figure 3-4).

Table 3-4 Total neutrophil count in albino male mice treated with two extracts (aqueous and methanol) of *A. eupatoria.*

Treatment Groups		Dose (mg/kg)	Mean ± SE* (cell/cu.mm.blood)
Control I (distilled water)		0.0	1176.0 ± 57.2^{g}
Control II(vitamin C)		120	5195.2 ± 104.4^{a}
Control III (CCl_4)		3.2	681.9 ± 62.4^{k}
Agrimonia eupatoria	Aqueous extract	1.0	1851.7 ± 113.2^{f}
		2.0	2269.1 ± 42.6^{e}
		3.0	4217.7 ± 132.1^{b}
	Methanol extract	1.0	2651.1 ± 100.7^{d}
		2.0	2810.3 ± 31.9^{d}
		3.0	3335.2 ± 135.9^{c}

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

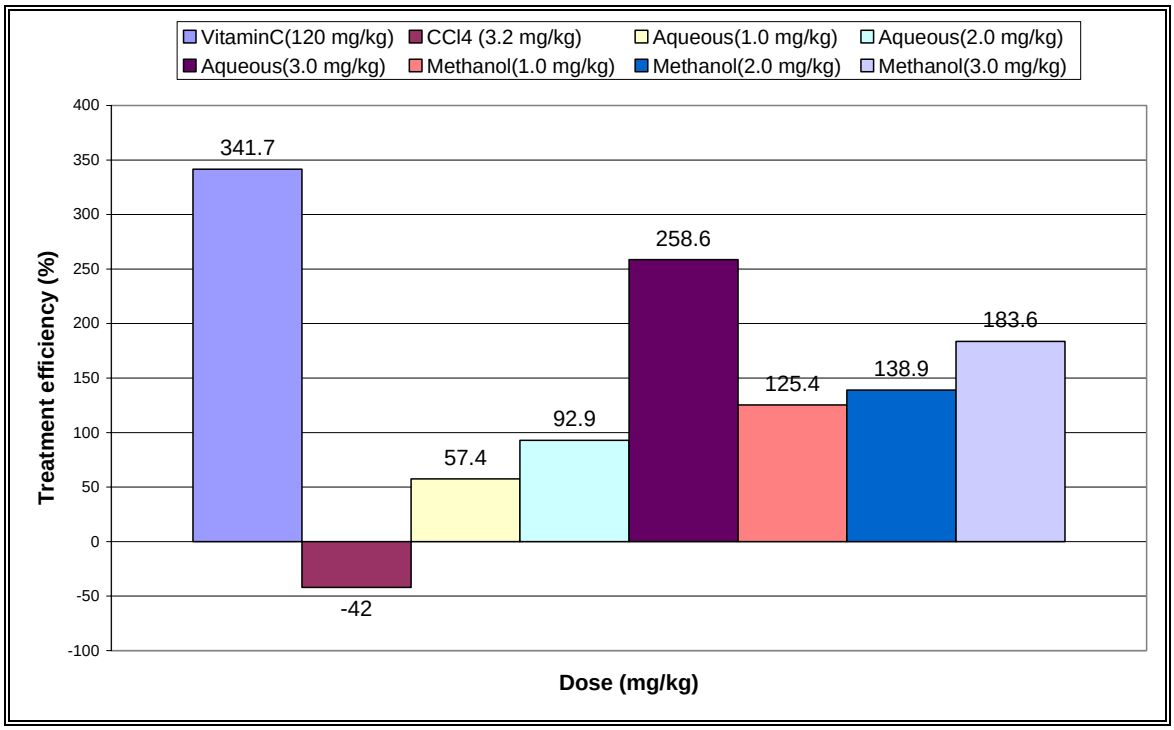


Figure 3-4 Treatment Efficiency of total neutophil count in albino male mice treated with two extracts (aqueous and methanol) of *A. eupatoria*.

3.2.2.3 Monocytes

The animals treated with CCl_4 (control III) showed a significant reduced count of monocytes (91.06 cell/cu.mm.blood) as compared to control I (113.95 cell/cu.mm.blood) or control II (738.10 cell/cu.mm.blood). With respect to the aqueous extract, the three doses (1.0, 2.0 and 3.0 mg/kg) showed a significant increase count of monocytes as compared to control I (150.05, 246.95, 274.85**,** respectively *vs*. 113.95 cell/cu.mm.blood). The three doses of methanol extract were also significantly effective in increasing the count of monocytes as compared to control I (371.00, 352.75 and 288.98**,** respectively *vs*. 1135.95 cell/cu.mm.blood), but the difference was higher than that observed in the aqueous extract. The best treatment efficiency was recorded for vitamin C (+547.7%), followed by the first and second doses of

methanol extract (+225.5 and +209.5%, respectively) (Table 3-5 and Figure 3-5).

Table 3-5: Total monocyte count in albino male mice treated with two extracts (aqueous and methanol) of *A. eupatoria.*

Treatment Groups		Dose (mg/kg)	Mean ± SE* (cell/cu.mm.blood)
Control (distilled water)		0.0	113.95 ± 15.40^{e}
Control I (vitamin C)		120	738.10 ± 47.18^{a}
Control II (CCl_4)		3.2	91.01 ± 12.75^{e}
Agrimonia eupatoria	Aqueous extract	1.0	150.05 ± 14.97^{e}
		2.0	246.95 ± 14.83^{d}
		3.0	274.85 ± 16.70^{d}
	Methanol extract	1.0	288.79 ± 38.17^{cd}
		2.0	352.75 ± 7.10^{bc}
		3.0	371.00 ± 29.71^{b}

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

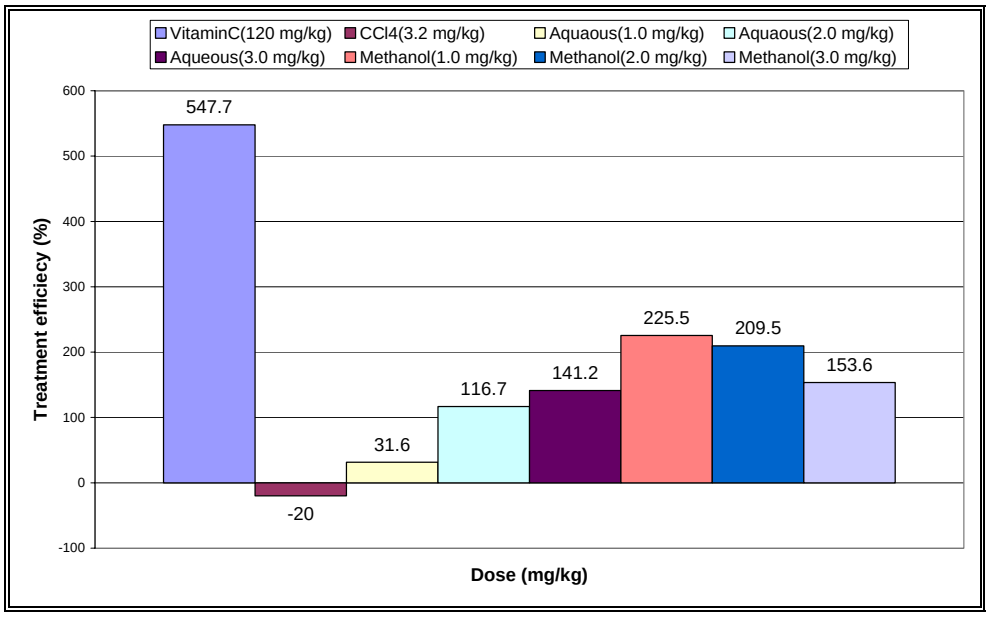


Figure 3-5: Treatment Efficiency of total monocyte count in albino male mice treated with two extracts (aqueous and methanol) of *A. eupatoria*.

3.2.2.4 Eosinophils and Basophils

The CCl_4 was significantly effective in reducing the count of eosinophils (5.26 cell/cu.mm.blood) as compared to control I (31.13 cell/cu.mm.blood) or II (65.20 cell/cu.mm.blood). Whereas, the third dose of aqueous (50.02 cell/cu.mm.blood) and methanol (75.52 cell/cu.mm.blood) extracts were significantly effective in increasing the count of such cells as compared to control I, and such effect was associated with a treatment efficiency of 60.6 and 142.5%, respectively (Table 3-6 and Figure 3-6). With respect to basophils, no such variations were observed, with the exception of the first dose of aqueous extract, in which the count of cells was significantly decreased in comparison with control I (11.69 *vs*.15.60 cell/cu.mm.blood). The treatment efficiency of such effect was -25% (Table 3-7and Figure 3-7).

Table 3-6: Total eosinophil count in albino male mice treated with two extracts (aqueous and methanol) of *A. eupatoria*

Treatment Groups		Dose (mg/kg)	Mean ± SE* (cell/cu.mm.blood)
Control I (distilled water)		0.0	31.13 ± 1.31^{de}
Control II (vitamin C)		120	65.20 ± 5.68^{ab}
Control III (CCl_4)		3.2	5.26 ± 1.09^{f}
Agrimonia eupatoria	Aqueous extract	1.0	27.70 ± 2.87^{e}
		2.0	47.75 ± 5.28^{bc}
		3.0	50.02 ± 4.49^{bc}
	Methanol extract	1.0	19.00 ± 3.90^{ef}
		2.0	34.45 ± 2.06^{cd}
		3.0	75.52 ± 14.83^{a}

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

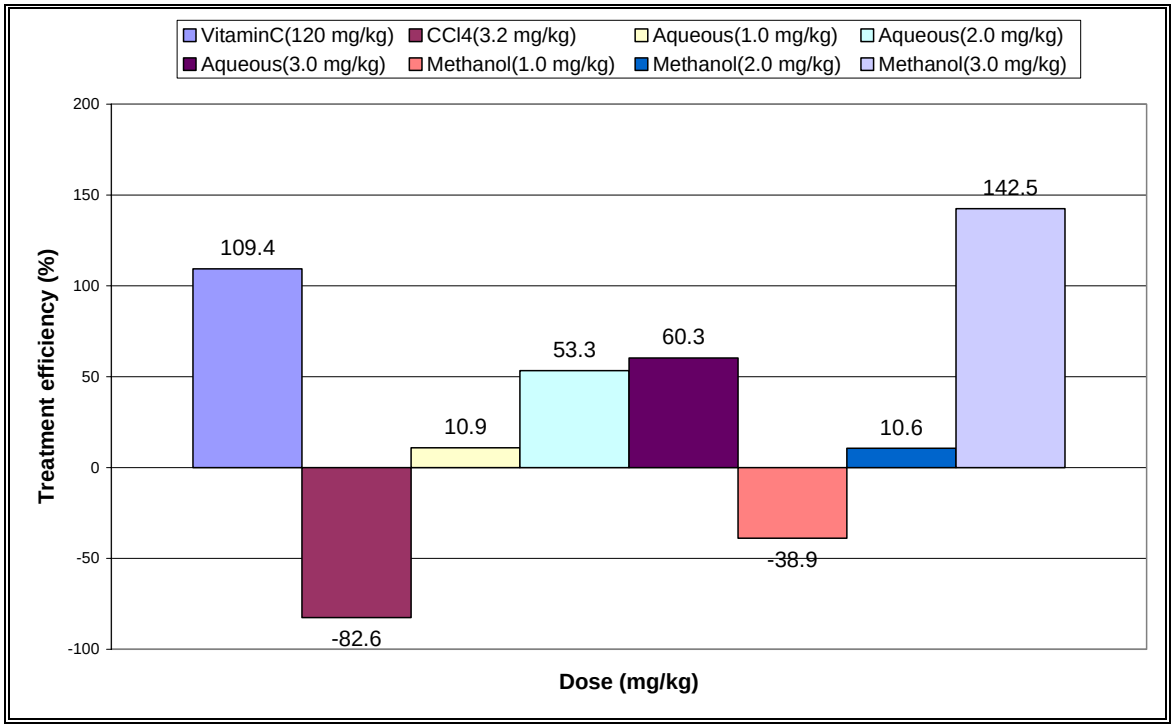


Figure 3-6: Treatment of total eosinophil count in albino male mice Efficiency treated with two extracts (aqueous and methanol) of *A. eupatoria*

Table 3-7: Total basophil count in albino male mice treated with two extracts (aqueous and methanol) of *A. eupatoria*

Treatment Groups		Dose (mg/kg)	Mean ± SE* (cell/cu.mm.blood)
Control I (distilled water)		0.0	15.60 ± 0.62^{ab}
Control II (vitamin C)		120	26.83 ± 3.34^{a}
Control III (CCl_4)		3.2	16.52 ± 3.50^{ab}
Agrimonia eupatoria	Aqueous extract	1.0	11.69 ± 2.29^{b}
		2.0	22.45 ± 5.65^{ab}
		3.0	20.02 ± 4.13^{ab}
	Methanol extract	1.0	21.27 ± 4.58^{ab}
		2.0	20.62 ± 2.34^{ab}
		3.0	25.20 ± 2.84^{a}

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

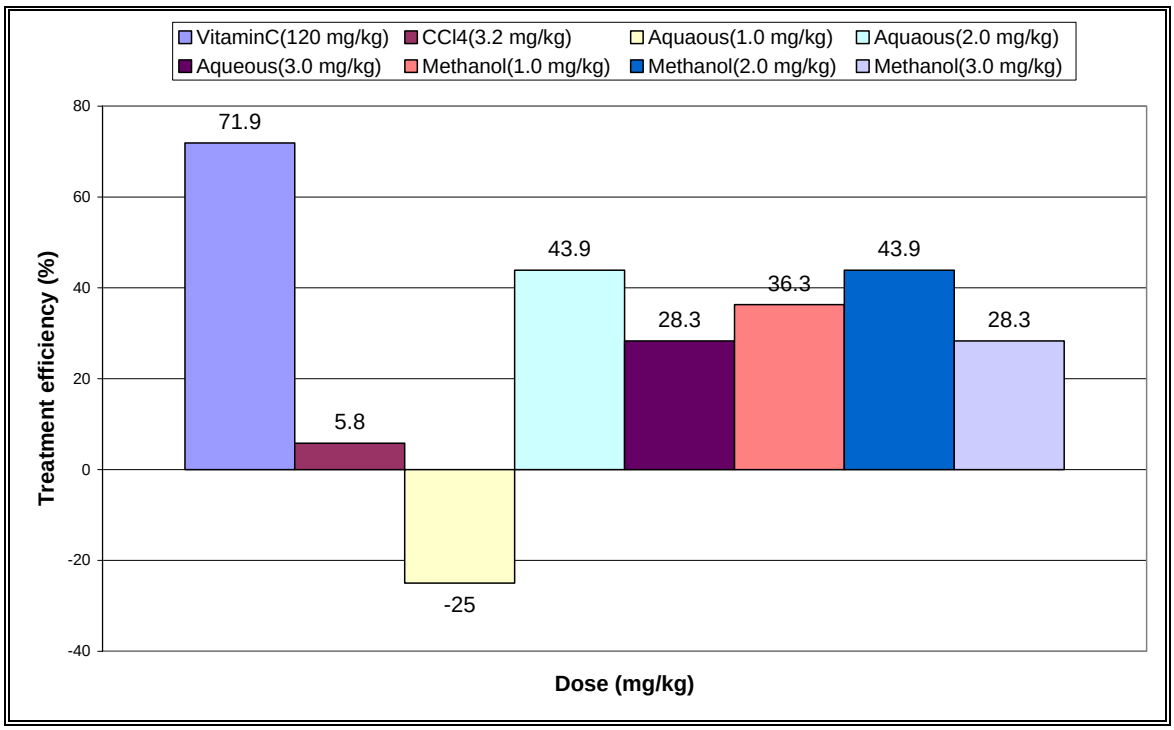


Figure 3-7: Treatment of total basophil count in albino male mice Efficiency treated with two extracts (aqueous and methanol) of *A. eupatoria*

3.2.3 Mitotic Index

Animals treated with CCl_4 (control III) showed a significant decreased mitotic index (1.55%) as compared to control I (4.38%) or control II (7.20%). With respect to the aqueous extract, the first and second doses (1.0 and 2.0 mg/kg) caused a significant decreased mitotic activity (3.0%, 3.0%, respectively), as compared to control I, but the third dose (3 mg/kg) was able to increase the mitotic index to 8.20%, and such value was significantly higher than the corresponding value of negative control (control 1). Moreover, such value approximated the mitotic index percentage in animals treated with vitamin C (7.20). The treated efficiency of the latter groups was 87.2 and 64.3%, respectively. Whereas, the three doses of methanol extract did not show such variations. (Table 3-8 and Figure 3-8).

Table 3-8: Mitotic index of bone marrow cells in albino male mice treated with two extracts (aqueous and methanol) of *A. eupatoria*.

Treatment Groups		Dose (mg/kg)	Mean ± SE (%)*
Control I (distilled water)		0.0	4.38 ± 0.34^{b}
Control II (vitamin C)		120	7.20 ± 0.97^{a}
Control III (CCl_4)		3.2	1.55 ± 0.18^{e}
Agrimonia eupatoria	Aqueous extract	1.0	3.00 ± 0.13^{c}
		2.0	3.00 ± 0.13^{c}
		3.0	8.20 ± 0.36^{a}
	Methanol extract	1.0	3.93 ± 0.20^{bc}
		2.0	4.13 ± 0.42^{bc}
		3.0	4.93 ± 0.33^{b}

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

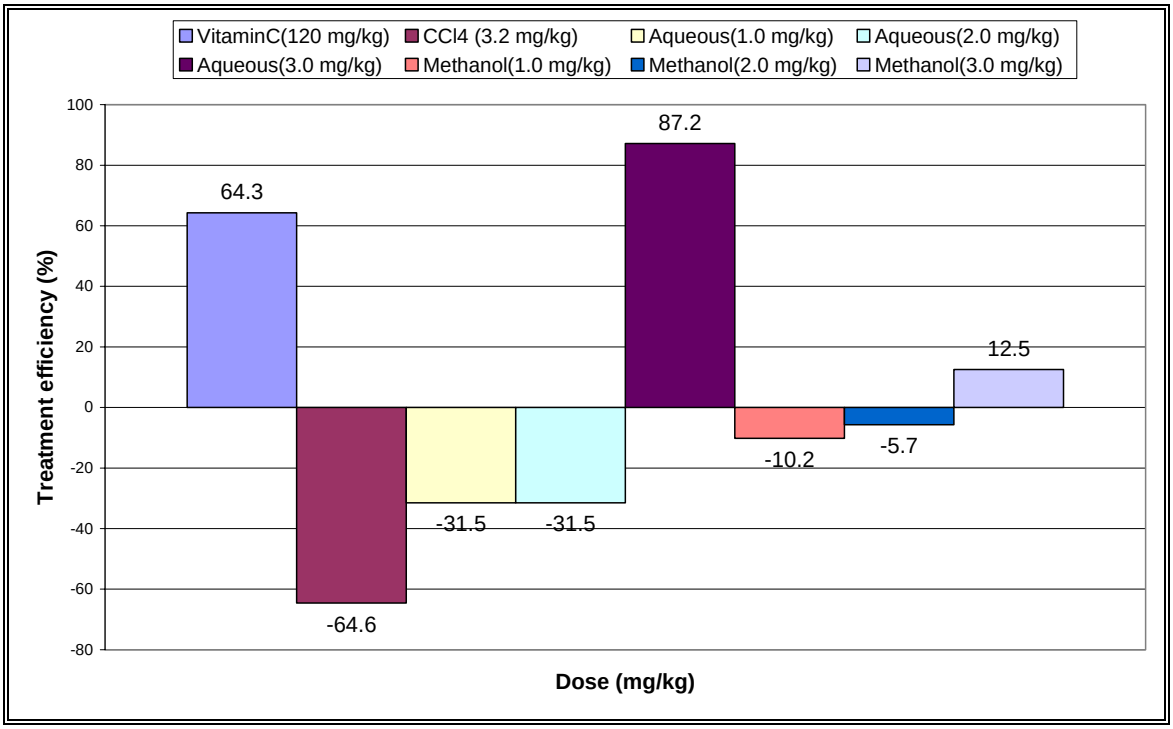


Figure 3-8: Treatment Efficiency of Mitotic index of bone marrow cells in albino male mice treated with two extracts (aqueous and methanol) of *A. eupatoria*

3.2.4 Micronucleus Index

The micronucleus formation was scored in polychromatic erythrocytes obtained from the femur of investigated mice (Figure 3-9).

The frequency of micronucleus spontaneous formation was 2.93% (control I), and such frequency was significantly decreased after a treatment with vitamin C (1.88%), while the CCl_4 contributed in a significant increased frequency (4.80%). When the animals treated with the aqueous or methanol extract, the third dose was the most important in reducing the micronucleus formation in comparison with control I (1.95 and 1.68, respectively *vs*. 2.93%), and such significant reduction was associated with a treatment efficiency of -33.4 and -42.6%, respectively (Table 3-9 and Figure 3-10).

Table 3-9: Micronucleus index of bone marrow cells in albino male mice treated with two extracts (aqueous and methanol) of *A. eupatoria*

Treatment Groups		Dose (mg/kg)	Mean ± SE (%)*
Control I (distilled water)		0.0	2.93 ± 0.34^{b}
Control II (vitamin C)		120	1.88 ± 1.49^{c}
Control III (CCl_4)		3.2	4.80 ± 0.27^{a}
Agrimonia eupatoria	Aqueous extract	1.0	2.38 ± 0.18^{bc}
		2.0	2.12 ± 0.22^{c}
		3.0	1.95 ± 0.16^{c}
	Methanol extract	1.0	1.88 ± 0.13^{c}
		2.0	2.00 ± 0.28^{c}
		3.0	1.68 ± 0.14^{c}

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

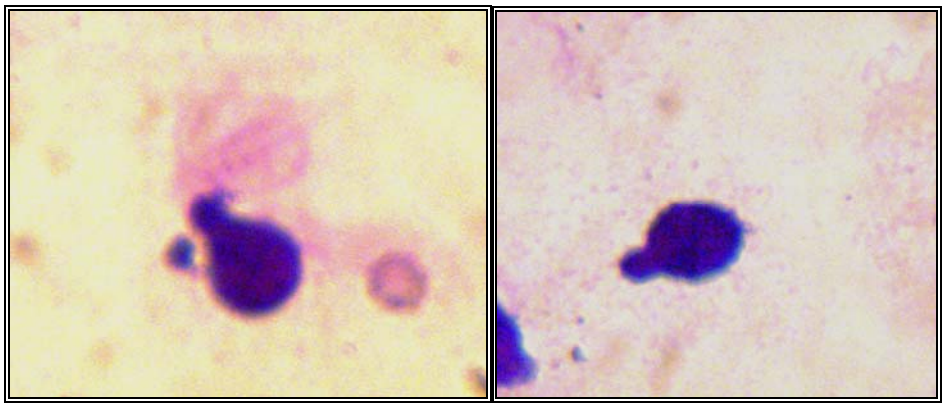


Figure 3-9: Micronucleus formation in two polychromatic erythrocytes.

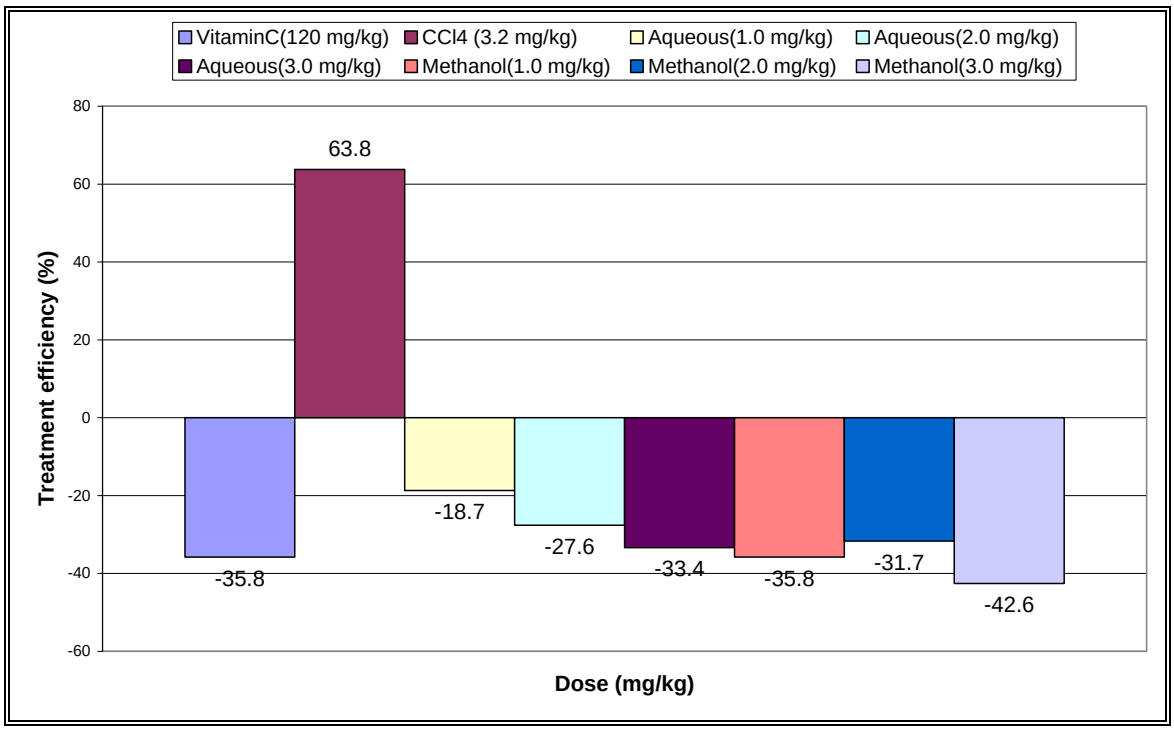


Figure 3-10: Treatment Efficiency of Micronucleus index of bone marrow cells in albino male mice treated with two extracts (aqueous and methanol) of *A. eupatoria*

3.2.5 Sperm-Head Abnormality Index

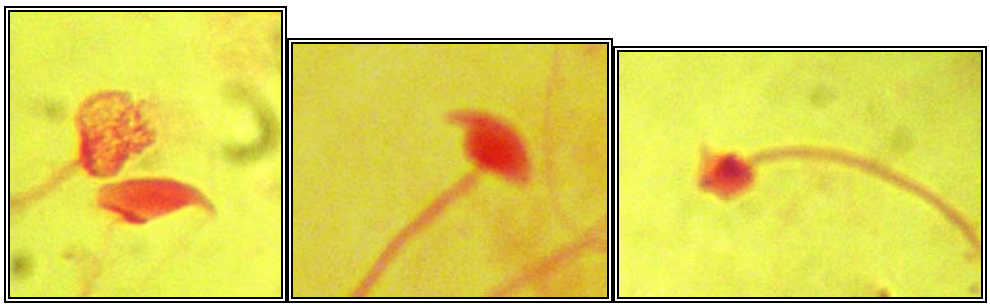
Different sperm-head abnormalities were observed in the investigated animals (Figure 3-11), and they are collectively presented as a percentage value. A treatment with CCl_4 (control III) showed a significant increased sperm-head abnormality index (10.93%) as compared to control I

(5.75%).With respect to the plant extracts, the third dose (3.0 mg/kg) of aqueous and methanol extracts was significantly effective in reducing the spontaneous formation of sperm-head abnormalities as compared to control I (4.07 and 5.13, respectively *vs*. 5.75%) (Table 3-10). The treatment efficiency of such effects was -29.2 and -17.3%, respectively (Figure 3-12).

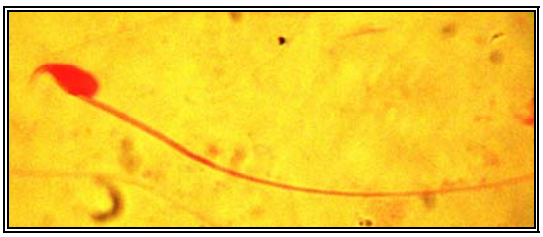
Table 3-10: Sperm-head abnormality index in albino male mice treated with two extracts (aqueous and methanol) of *A. eupatoria*

Treatment Groups		Dose (mg/kg)	Mean ± SE (%)*
Control I(distilled water)		0.0	5.75 ± 0.35^{bc}
Control II (vitamin C)		120	4.73 ± 0.23^{de}
Control III(CCl_4)		3.2	10.93 ± 0.43^{a}
Agrimonia eupatoria	Aqueous extract	1.0	6.13 ± 0.26^{bc}
		2.0	4.93 ± 0.39^{de}
		3.0	4.07 ± 0.14^{e}
	Methanol extract	1.0	6.68 ± 0.27^{b}
		2.0	4.75 ± 0.58^{de}
		3.0	5.13 ± 0.31^{cd}

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



(A)



(B)

Figure 3-11: Sperms with abnormal heads (A) and normal head (B).

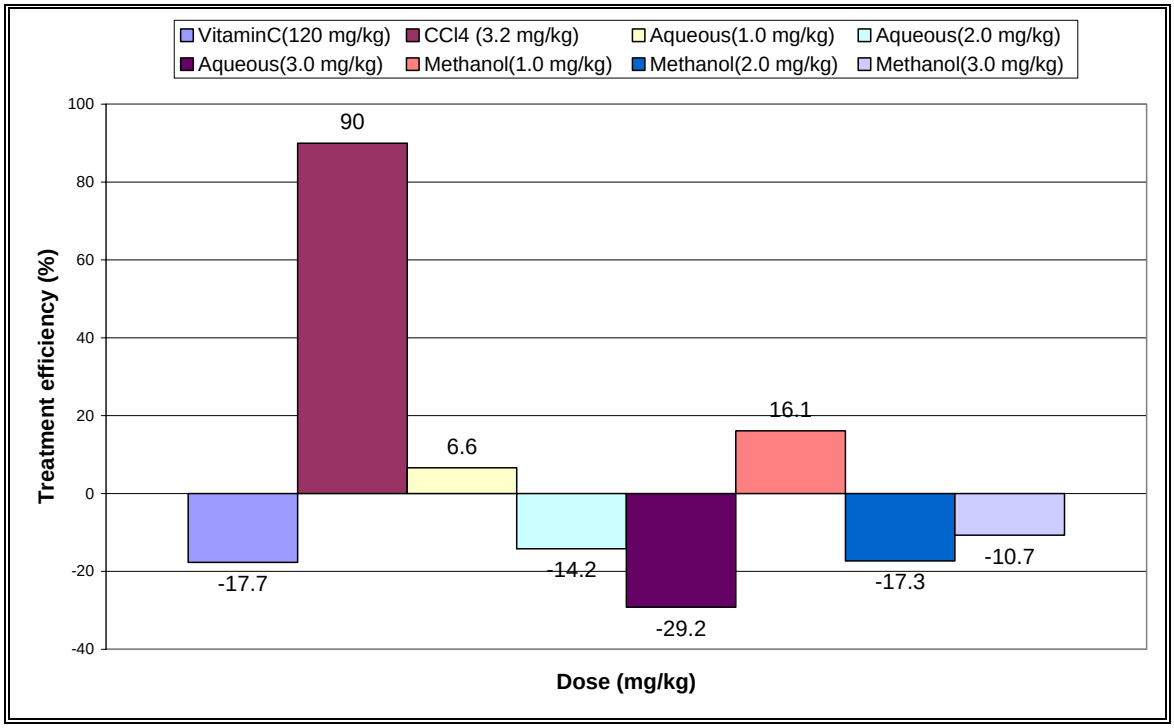


Figure 3-12 Treatment Efficiency of Sperm-head abnormality index in albino male mice treated with two extracts (aqueous and methanol) of *A. eupatoria*

3.2.6 Sperm-Tail Abnormality Index

The most frequently encountered sperm-tail abnormalities were coiled tail (Figure 3-13).

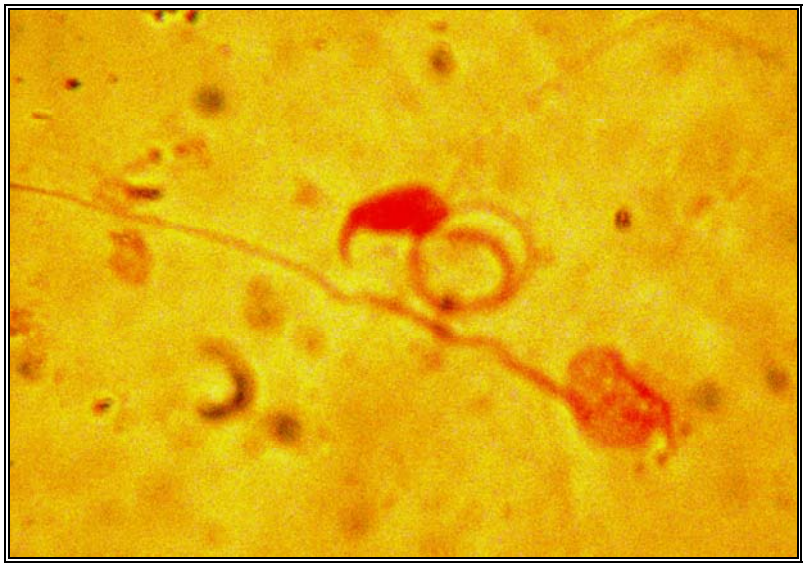


Figure 3-13: A sperm with a coiled tail.

Two doses (2.0 and 3.0 mg/kg) of aqueous extract were significantly effective in reducing the frequency of sperm-tail abnormalities as compared to the corresponding negative control (3.58 and 3.38, respectively *vs*. 4.30%). Methanol extract was also effective, but the third dose recorded the best reduction (1.62%), and vitamin C shared a similar effect (2.50%) (Table 3-11). The best treatment efficiency was recorded for the third dose of methanol extract (-62.3%), followed by of three doses of methanol extract in which the index was (2.50 %, 2.50%, 1.62 % respectively *vs*2.50 %). The best treatment efficiency was recorded for the third dose of methanol extract, which was (-62.3%), followed by the first and second doses of the same extract (-41.8 and -41.8%) (Figure 3-14).

Table 3-11: Sperm-tail abnormality index in albino male mice treated with two extracts (aqueous and methanol) of *A. eupatoria*

Treatment Groups		Dose (mg/kg)	Mean ± SE (%)*
Control I (distilled water)		0.0	4.30 ± 0.27^{bc}
Control II (vitamin C)		120	2.50 ± 0.19^{ef}
Control III (CCl_4)		3.2	7.78 ± 0.53^{a}
Agrimonia eupatoria	Aqueous extract	1.0	4.98 ± 0.33^{b}
		2.0	3.58 ± 0.25^{cd}
		3.0	3.38 ± 0.39^{de}
	Methanol extract	1.0	2.50 ± 0.21^{ef}
		2.0	2.50 ± 0.21^{ef}
		3.0	1.62 ± 0.21^{F}

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

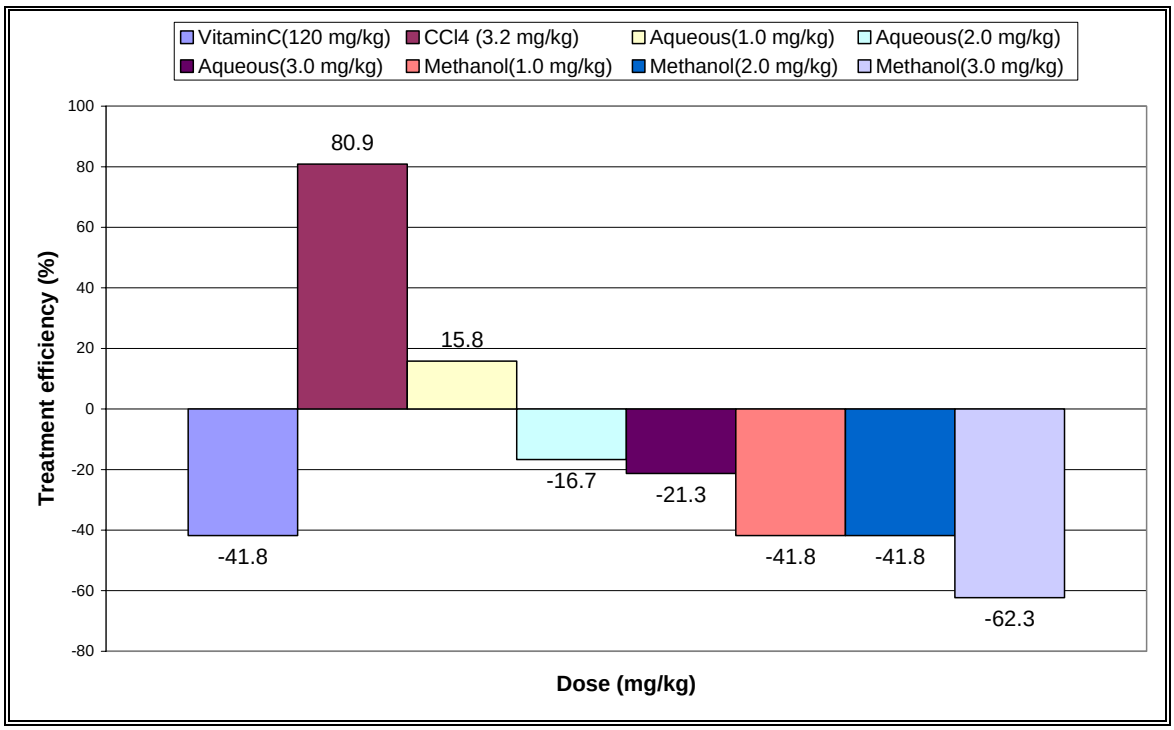


Figure 3-14: Treatment Efficiency of Sperm-tail abnormality index in albino male mice treated with two extracts (aqueous and methanol) of *A. eupatoria*

3.3 Biochemical Effects of *A. eupatoria* Extracts

3.3.1 Aspartate Amino-Transferase (AST)

A treatment with CCl_4 (control III) showed a significant increased activity of AST (263.0 IU/L) as compared to control I (176.3 IU/L). With respect to the aqueous extract, the first (1.0 mg/kg) and the second doses (2.0 mg/kg) caused a significant reduction in the enzyme activity (57.33 and 65.00 IU/L, respectively) as compared to control I, but much more a significant decreased activity (23.33 IU/L) was observed in the third dose (3.0 mg/kg). The methanol extract was less effective in this regard, but the second and third doses also contributed in a significant reduction of AST activity (130.0 and 91.67 IU/L, respectively) as compared to control I (Table 3-12). The best treatment efficiencies were recorded for the three doses of aqueous extract (-67.4, -63.1 and -86.7%, respectively) (Figure 3-15).

Table 3-12: Aspartate amino-transferase activity in sera of albino male mice treated with two extracts (aqueous and methanol) of *A. eupatoria*.

Treatment Groups		Dose (mg/kg)	Mean ± SE (IU/L)*
Control I (distilled water)		0.0	176.3 ± 15.28[b]
Control II (vitamin C)		120	137.3 ± 6.43[c]
Control III (CCl_4)		3.2	263.0 ± 34.04[a]
Agrimonia eupatoria	Aqueous extract	1.0	57.33 ± 7.50[e]
		2.0	65.00 ± 5.00[e]
		3.0	23.33 ± 5.77[f]
	Methanol extract	1.0	175.0 ± 5.00[b]
		2.0	130.0 ±18.03[c]
		3.0	91.67 ± 7.64[d]

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

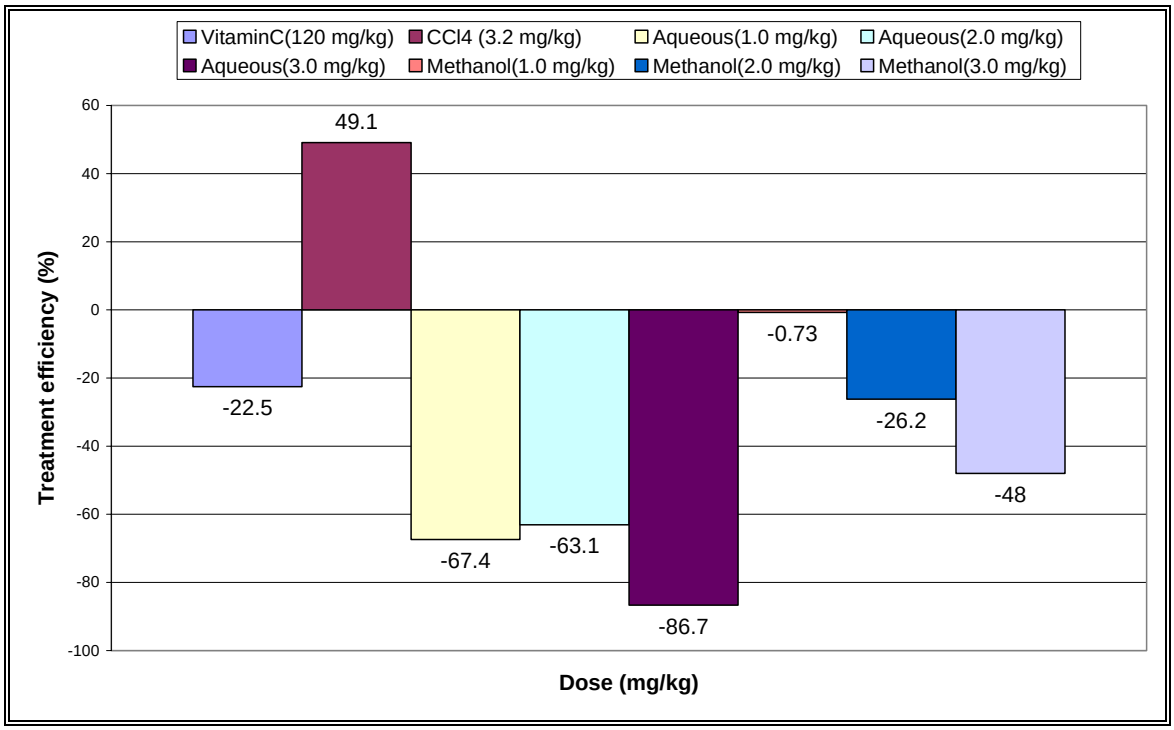


Figure 3-15: Treatment Efficiency of aspartate amino-transferase activity in albino male mice treated with two extracts (aqueous and methanol) of *A. eupatoria.*

3.3.2 Alanine Amino-Transferase (ALT)

A treatment with CCl_4 (control III) showed a significant increased activity of serum ALT (910.0 IU/L) as compared to control I (110.7 IU/L). With respect to the aqueous extract, the three dose (1.0, 2.0 and 3.0 mg/kg) caused a significant decreased activity of ALT (48.33, 47.33 and 50.67 IU/L, respectively) as compared to control I, while the second and third doses of methanol extract were much more effective in reducing the activity of ALT (25.67 and 24.00 IU/L, respectively). Therefore, the best treatment efficiency was recorded in these two doses (-76.8 and -78.3%, respectively) (Table 3-13 and Figure 3-16).

Table 3-13: Alanine amino-transferase activity in sera of albino male mice treated with two extracts (aqueous and methanol) of *A. eupatoria*

Treatment Groups		Dose (mg/kg)	Mean ± SE (IU.L)*
Control I (distilled water)		0.0	110.7 ± 5.81^{b}
Control II (vitamin C)		120	45.00 ± 2.89^{d}
Control III (CCl_4)		3.2	910.0 ± 5.78^{a}
Agrimonia eupatoria	Aqueous extract	1.0	48.33 ± 3.33^{cd}
		2.0	47.33 ± 1.45^{cd}
		3.0	50.67 ± 1.76^{cd}
	Methanol extract	1.0	56.33 ± 0.67^{c}
		2.0	25.67 ± 2.33^{e}
		3.0	24.00 ± 3.05^{e}

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

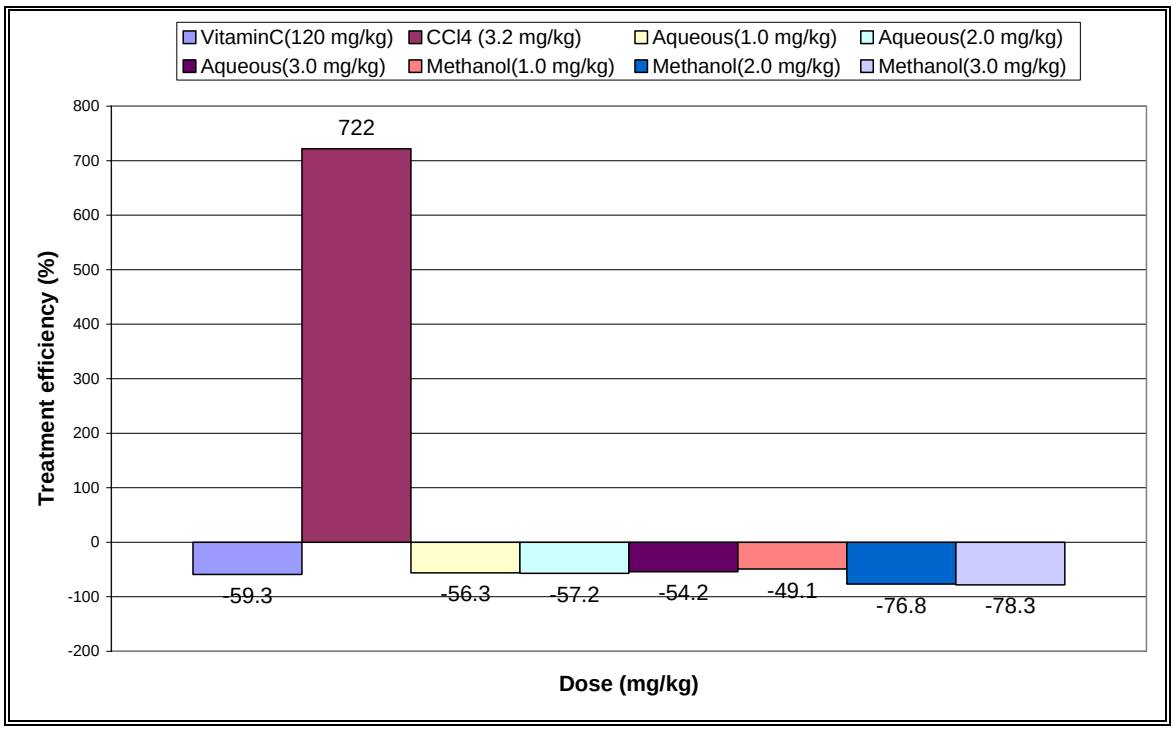


Figure 3-16: Treatment Efficiency of alanine amino-transferase activity in albino male mice treated with two extracts (aqueous and methanol) of *A. eupatoria*.

3.3.3 Alkaline Phosphatase (ALP)

A treatment with CCl_4 (control III) showed a significant increased activity of serum ALP (111.3 IU/L) as compared to control I (81.50 IU/L). With respect to the aqueous and methanol extracts, the three doses (1.0, 2.0 and 3.0 mg/kg) were significantly effective in reducing the activity of the enzyme in a dose-dependent manner (aqueous: 66.73, 29.77 and 19.43 IU/L, respectively; methanol: 64.80, 48.43 and 16.50 IU/L, respectively) as compared control 1 (Table 3-14). The treatment efficiencies for such effects were -18.1, -63.4 and -76.1%, respectively for aqueous extract and -20.4, -40.5 and -79.7%, respectively for methanol extract (Figure 3-17).

Table 3-14: Alkaline phosphatase activity in sera of albino male mice treated with two extracts (aqueous and methanol) of *A. eupatoria*.

Treatment Groups		Dose (mg/kg)	Mean ± SE (IU/L)*
Control I (distilled water)		0.0	81.50 ± 1.76[b]
Control II (vitamin C)		120	17.17 ± 1.88[f]
Control III (CCl_4)		3.2	111.3 ± 5.55[a]
grimonia eupatoria	Aqueous extract	1.0	66.73 ± 1.75[c]
		2.0	29.77 ± 3.25[e]
		3.0	19.43 ± 1.39[f]
	Methanol extract	1.0	64.80 ± 6.18[c]
		2.0	48.43 ± 1.15[d]
		3.0	16.50 ± 2.02[f]

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

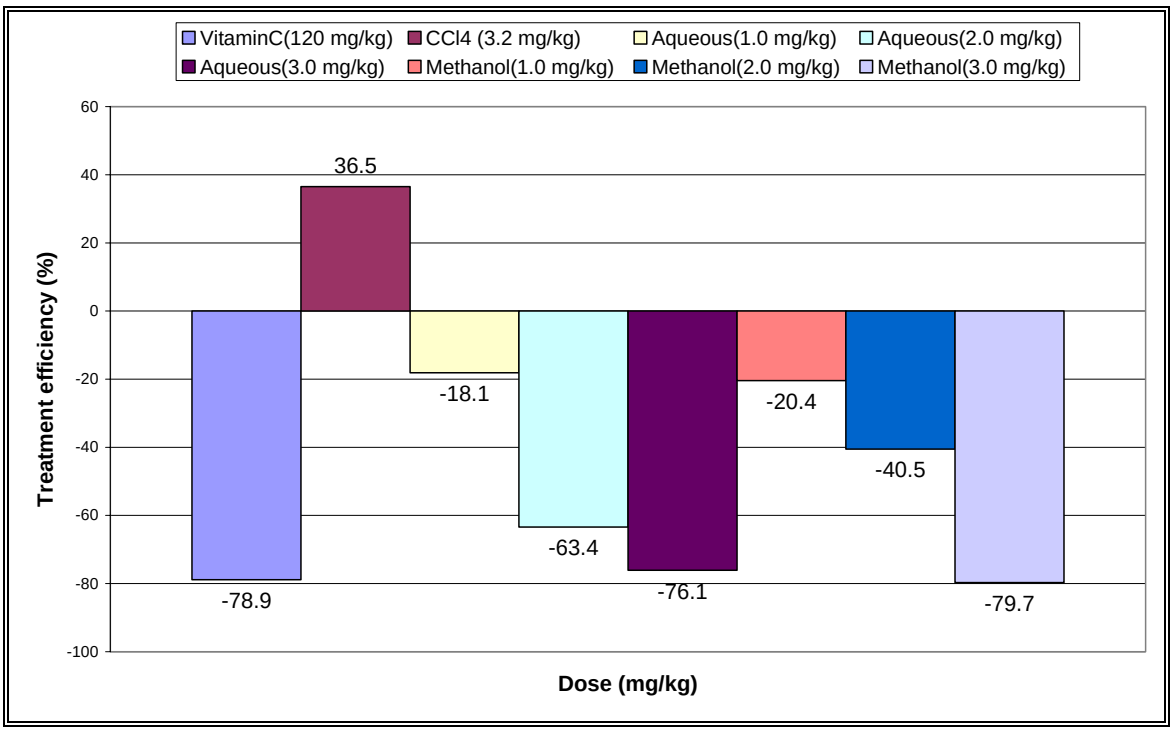


Figure 3-17: Treatment Efficiency of Alkaline phosphatase activity in albino male mice treated with two extracts (aqueous and methanol) of *A. eupatoria.*

3.4 Effect of CCl_4 - *A. eupatoria* Extracts Interaction

The interaction involved giving a single dose of CCL_4 in day 1, and then it was followed (post-treatment) by vitamin C or the ideal dose of the plant aqueous or methanol extract in the next six days (single dose/day). The selection of ideal dose was based collectively on the findings of the forthcoming results, in which the dose 3.0 mg/kg was almost the best in enhancing the values of the investigated parameters.

3.4.1 Total Count of Leucocytes

Both aqueous and methanol extracts were effective in modulating the effects of CCl_4, and the total count of leucocytes was significantly increased to 5025.0 and 5750.0 cell/ cu.mm.blood, respectively, as compared to control I (3412.5 cell/cu.mm.blood), but vitamin C was more effective in this regard (7550.0 cell/cu.mm.blood) (Table 3-15). Therefore, the best treatment efficiency was recorded for vitamin C (+121.2%), followed by methanol (+68.4%) and aqueous (+47.2%) extracts (Figure 3-18).

Table 3-15: Total leucocyte count in albino male mice after interactions between CCl_4 and two extracts (aqueous and methanol) of *A. eupatoria*.

Interaction Groups	Dose (mg/kg)	Mean ± S.E.* (cell/cu.mm.blood)
Control I (CCl_4 + distilled water)	0.0	3412.5 ± 151.0^{c}
Control II (CCl_4 + vitamin C)	120	7550.0 ± 226.4^{a}
CCl_4 + Plant aqueous Extract	3.0	5025.0 ± 253.7^{b}
CCl_4 + Plant methanol Extract	3.0	5750.0 ± 686.5^{b}

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

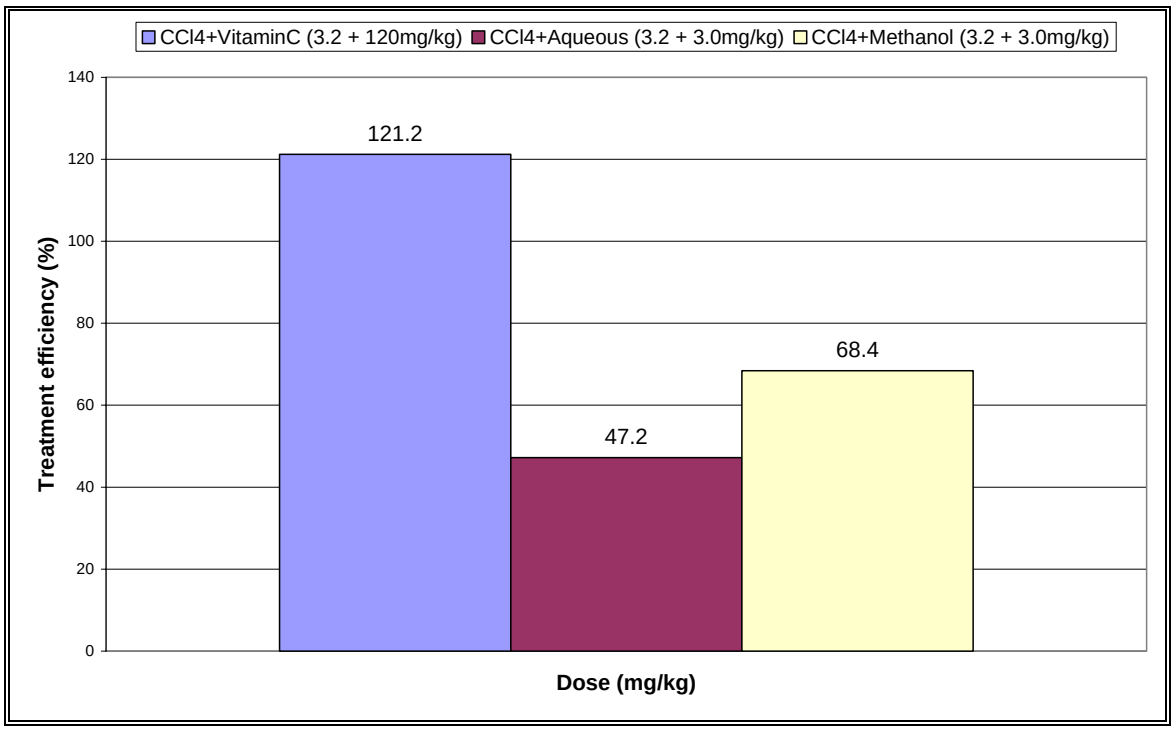


Figure 3-18: Treatment Efficiency of total leucocyte count in albino male mice after interactions between CCl_4 and two extracts (aqueous and methanol) of *A. eupatoria*.

3.4.2 Absolute Count of Leucocytes

3.4.2.1 Lymphocytes

The lymphocyte count shared the picture of total leucocyte count, in which vitamin C and both extracts (aqueous and methanol) were significantly effective in modulating the effects of CCl_4 **(**6148.6**,** 2820.8**,** 3377.4, respectively *vs*. 1879.8 cell/cu.mm.blood) (Table 3-16). The corresponding treatment efficiencies were 227.0, 50.0 and 79.6%, respectively (Figure 3-19).

Table 3-16: Total lymphocyte count in albino male mice after interactions between CCl_4 and two extracts (aqueous and methanol) of *A. eupatoria*.

Interaction Groups	Dose (mg/kg)	Mean ± S.E.* (cell/cu.mm.blood)
Control I (CCl_4 + distilled water)	0.0	1879.8 ± 75.05^{c}
Control II (CCl_4 + vitamin C)	120	6148.6 ± 200.28^{a}
CCl_4 + Plant aqueous Extract	3.0	2820.8 ± 165.92^{b}
CCl_4 + Plant methanol Extract	3.0	3377.4 ± 320.65^{b}

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

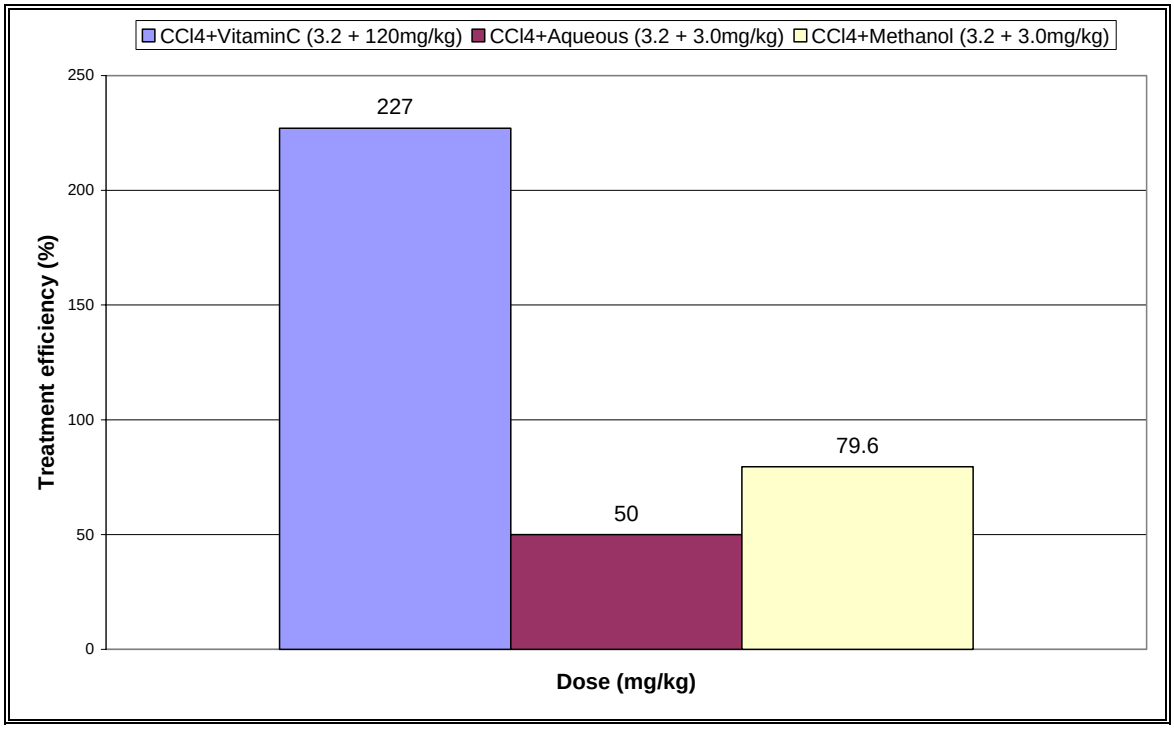


Figure 3-19: Treatment Efficiency of total lymphocyte count in albino male mice after interactions between CCl_4 and two extracts (aqueous and methanol) of *A. eupatoria*.

3.4.2.2 Neutrophils

The aqueous and methanol extracts were able to modulate the effect of CCl_4 and increase the neutrophil count significantly (1960.6 and 2142.6

cell/cu.mm.blood, respectively) as compared to control I (1265.5 cell/cu. mm.blood), while vitamin C failed to show such effect (Table 3-17). The treatment efficiency of methanol extract (+69.3%) was better than that of aqueous extract (+54.9 %) (Figure 3-21).

Table 3-17: Total neutrophil count in albino male mice after interactions between CCl_4 and two extracts (aqueous and methanol) of *A. eupatoria*.

Interaction Groups	Dose (mg/kg)	Mean ± S.E.* (cell/cu.mm.blood)
Control I (CCl_4 + distilled water)	0.0	1265.5 ± 78.24^{b}
Control II (CCl_4 + vitamin C)	120	1100.4 ± 28.20^{b}
CCl_4 + Plant aqueous Extract	3.0	1960.6 ± 79.51^{a}
CCl_4 + Plant methanol Extract	3.0	2142.6 ± 357.46^{a}

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

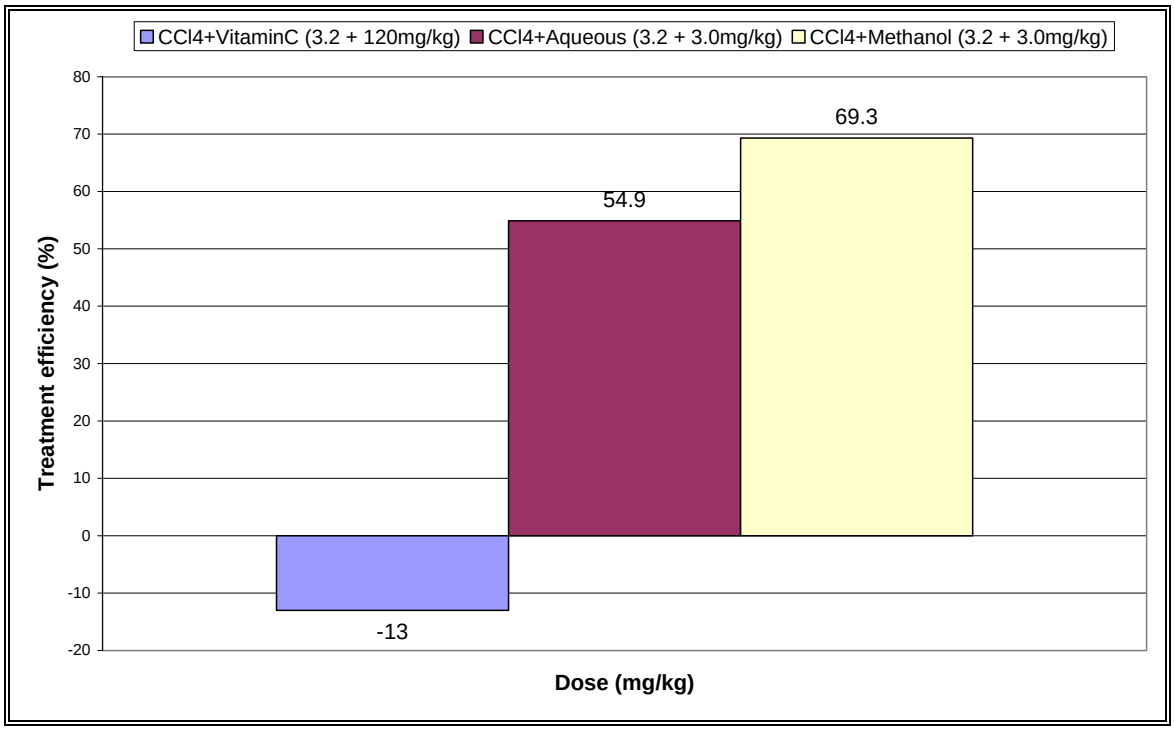


Figure 3-20: treatment efficiency of total neutrophil count in albino male mice after interaction between CCl_4 and two extracts (aqueous and methanol) of A .*eupatoria*

3.4.2.3 Monocytes, Eosinophils and Basophils

The monocytes showed a decreased count after post-treatment with vitamin C, aqueous extract or methanol extract (150.5, 145.5 and 140.3 cell/cu.mm.blood, respectively) as compared to control I (197.0 cell/cu.mm.blood), but none of these differences reached a significant (Table 3-18 and Figure 3-21). The eosinophil count revealed a similar outcome, but vitamin C was significantly effective increasing the count of the cells as compared to control I (75.25 *vs.* 43.50 cell/cu.mm.blood) and the corresponding treatment efficiency was +72.9% (Table 3-19and Figure 3-22). The basophil count was an exception, and the cells were significantly increased in vitamin C (75.25 cell/cu.mm.blood), aqueous extract (50.25 cell/cu.mm.blood) or methanol extract (57.50 cell/cu.mm.blood) treated animals in comparison with control I (34.13 cell/cu.mm.blood) (Table 3-20). The corresponding treatment efficiencies were +120.4, +47.2 and 68.4%, respectively (Figure 3-23).

Table 3-18: Total monocyte count in albino male mice after interactions between CCl_4 and two extracts (aqueous and methanol) of *A. eupatoria*.

Interaction Groups	Dose (mg/kg)	Mean ± S.E.* (cell/cu.mm.blood)
Control I (CCl_4 + distilled water)	0.0	197.0 ± 15.78[a]
Control II (CCl_4 + vitamin C)	120	150.5 ± 4.52[a]
CCl_4 + Plant aqueous Extract	3.0	145.5 ± 9.17[a]
CCl_4 + Plant methanol Extract	3.0	140.3 ± 29.90[a]

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

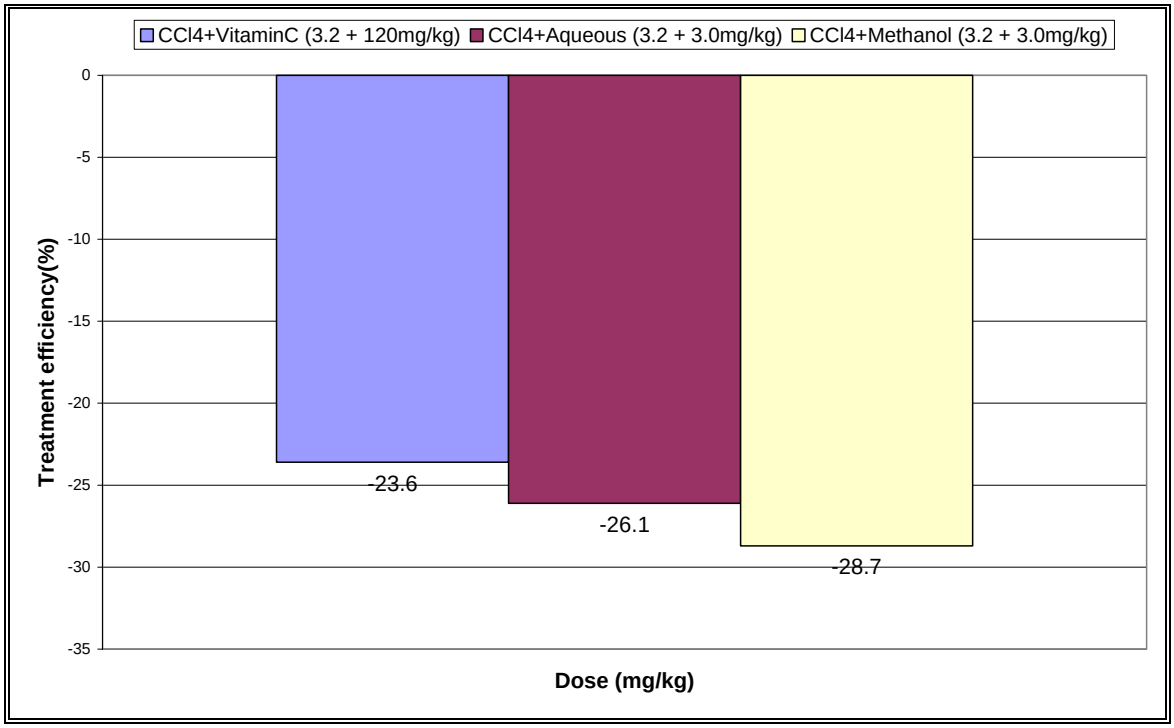


Figure 3-21: Treatment Efficiency of total monocyte count in albino male mice after interactions between CCl_4 and two extracts (aqueous and methanol) of *A. eupatoria*.

Table 3-19: Total eosinophil count in albino male mice after interactions between CCl_4 and two extracts (aqueous and methanol) of *A. eupatoria*.

Interaction Groups	Dose (mg/kg)	Mean ± S.E.* (cell/cu.mm.blood)
Control I (CCl_4 + distilled water)	0.0	43.50 ± 10.56^{b}
Control II (CCl_4 + vitamin C)	120	75.25 ± 2.26^{a}
CCl_4 + Plant aqueous Extract	3.0	50.25 ± 2.54^{b}
CCl_4 + Plant methanol Extract	3.0	57.50 ± 6.86^{ab}

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

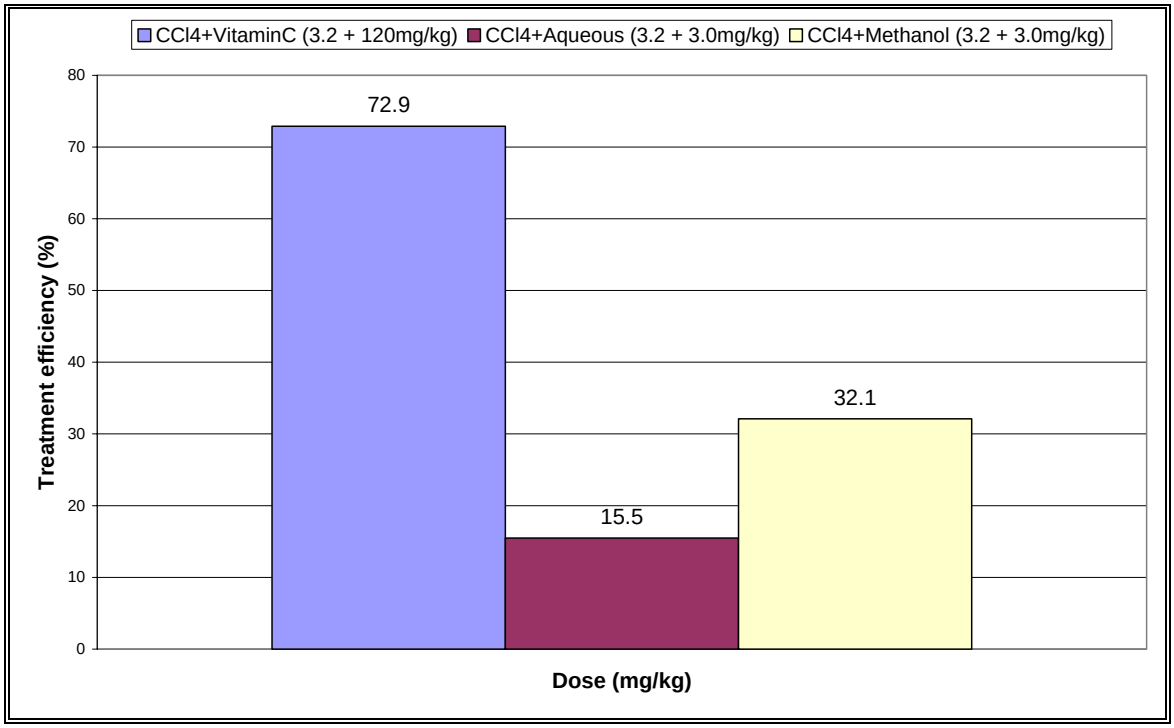


Figure 3-22: Treatment Efficiency of total eosinophil count in albino male mice after interactions between CCl_4 and two extracts (aqueous and methanol) of *A. eupatoria*.

Table 3-20: Total basophil count in albino male mice after interactions between CCl_4 and two extracts (aqueous and methanol) of *A. eupatoria*.

Interaction Groups	Dose (mg/kg)	Mean ± S.E.* (cell/cu.mm.blood)
Control I (CCl_4 + distilled water)	0.0	34.13 ± 1.56^{c}
Control II (CCl_4 + vitamin C)	120	75.25 ± 2.26^{a}
CCl_4 + Plant aqueous Extract	3.0	50.25 ± 2.54^{b}
CCl_4 + Plant methanol Extract	3.0	57.50 ± 6.86^{b}

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

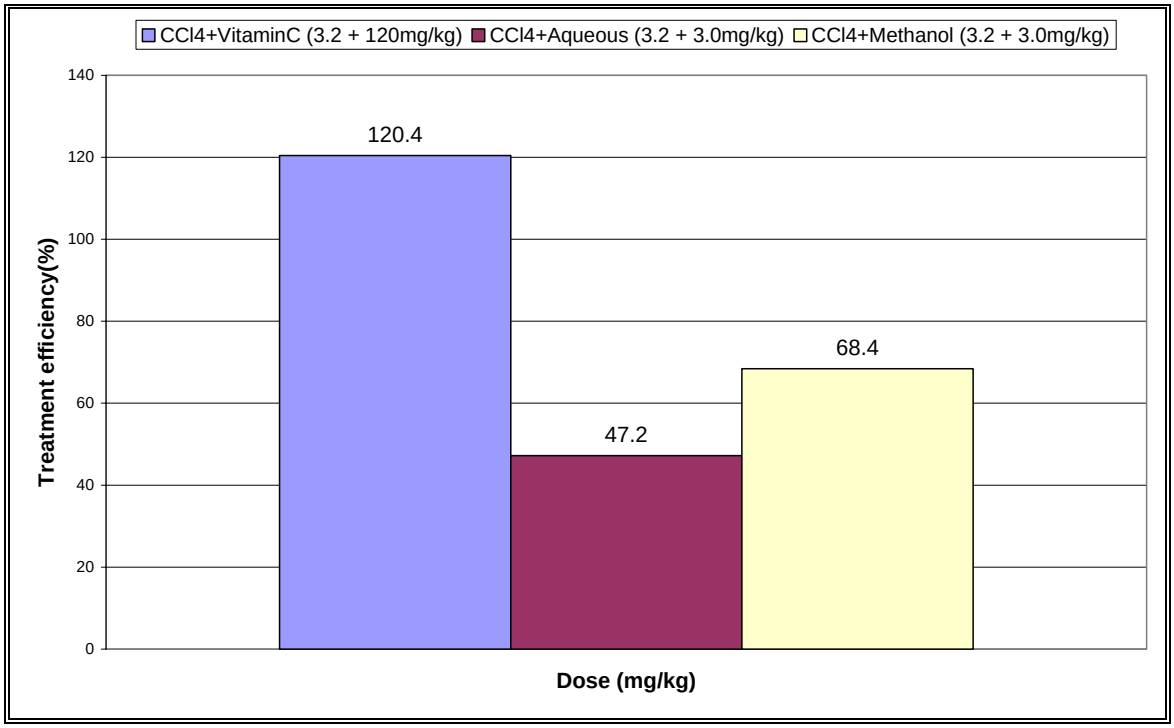


Figure 3-23: Treatment Efficiency of total basophil count in albino male mice after interactions between CCl_4 and two extracts (aqueous and methanol) of *A. eupatoria*.

3.4.3 Mitotic index

Vitamin C, as well as, aqueous and methanol extracts were significantly effective in increasing the mitotic index of bone marrow cells (8.50, 5.47 and 6.13%, respectively) as compared with control 1 (1.53%) (Table 3-21). Such effects were associated with a treatment efficiency of +455.5, +257.5 and +300.6%, respectively (Figure 3-24).

Table 3-21: Mitotic index in albino male mice after interactions between CCl_4 and two extracts (aqueous and methanol) of *A. eupatoria*.

Interaction Groups	Dose (mg/kg)	Mean ± SE (%)*
Control I (CCl_4 + distilled water)	0.0	1.53 ± 0.20^{c}
Control II (CCl_4 + vitamin C)	120	8.50 ± 0.81^{a}
CCl_4 + Plant aqueous Extract	3.0	5.47 ± 0.67^{b}
CCl_4 + Plant methanol Extract	3.0	6.13 ± 0.69^{b}

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

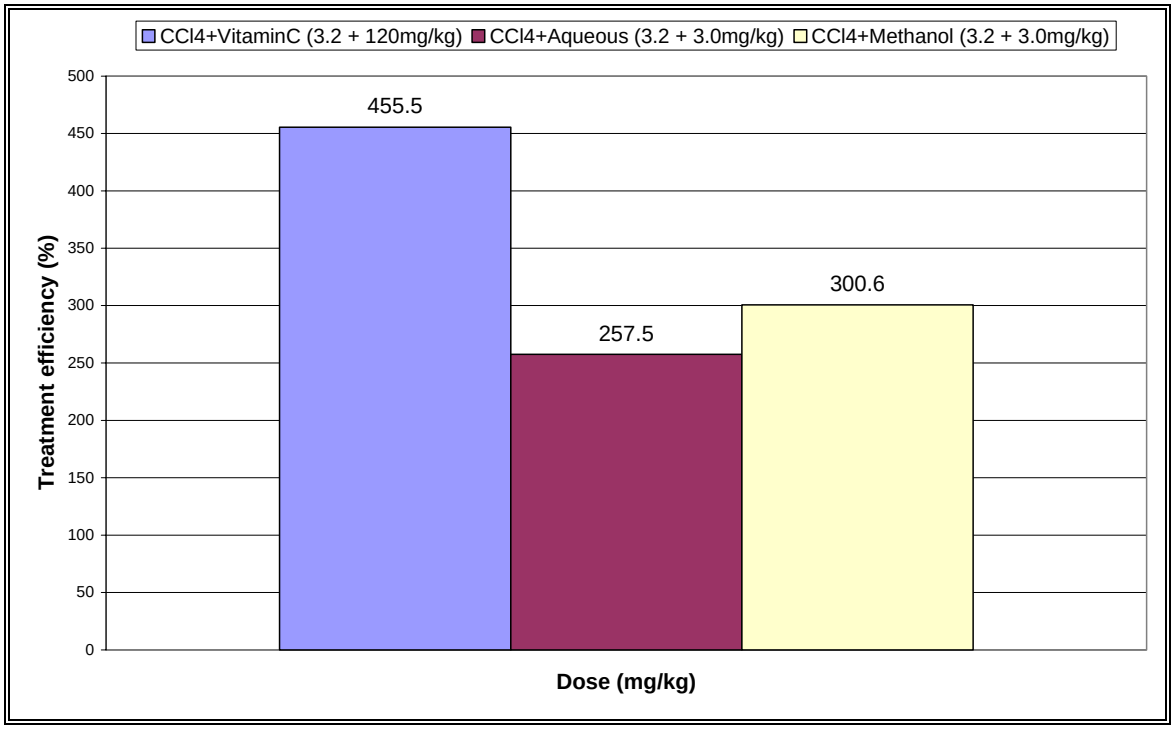


Figure 3-24: Treatment Efficiency of mitotic index in albino male mice after interactions between CCl_4 and two extracts (aqueous and methanol) of *A. eupatoria*.

3.4.4 Micronucleus Formation

The methanol extract showed a significant reduction in micronucleus formation (1.50%) as compared to control I (4.60%). Vitamin C was also effective in this regard (1.27%), as well as, the aqueous extract (1.80 %) (Table 3-22). The corresponding treatment efficiencies were -67.3, -72.3 and -60.9%, respectively (Figure 3-25).

Table 3-22: Micronucleus index in albino male mice after interactions between CCl_4 and two extracts (aqueous and methanol) of *A. eupatoria*.

Interaction Groups	Dose (mg/kg)	Mean ± SE (%)*
Control I (CCl_4 + distilled water)	0.0	4.60 ± 0.35^{a}
Control II (CCl_4 + vitamin C)	120	1.27 ± 0.15^{b}
CCl_4 + Plant aqueous Extract	3.0	1.80 ± 0.150^{b}
CCl_4 + Plant methanol Extract	3.0	1.50 ± 0.23^{b}

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

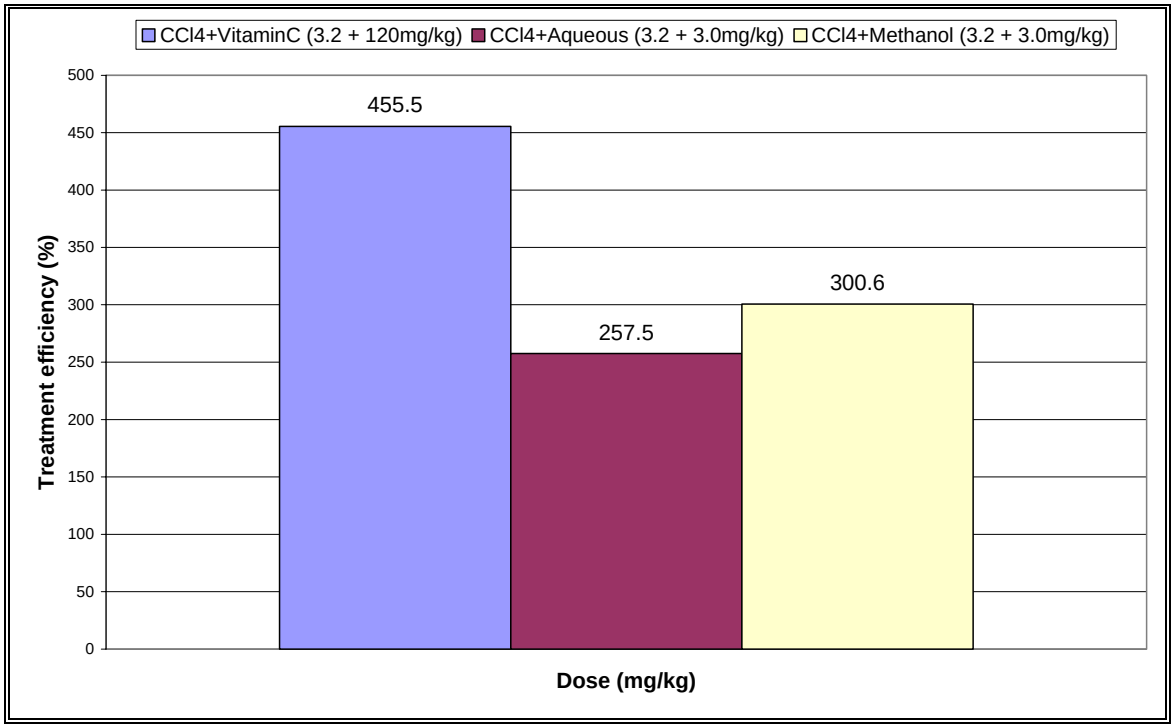


Figure 3-25: Treatment Efficiency of micronucleus index in albino male mice after interactions between CCl_4 and two extracts (aqueous and methanol) of *A. eupatoria*

3.4.5 Sperm-Head and Sperm-Tail Abnormalities

Vitamin C, as well as, aqueous and methanol extracts were significantly effective in reducing the sperm-head abnormalities (7.97, 10.07 and 8.03%, respectively) as compared to control I (16.83%) (Table 3-23). Such reducing effects were associated with a treatment efficiency of -52.6, -40.1 and -52.2, respectively (Figure 3-26). The sperm-tail abnormalities shared the theme of sperm-head abnormalities, and the corresponding treatment efficiencies were -71.3, -61.0 and -58.3%, respectively (Table 3-24 and Figure 3-27).

Table 3-23: Sperm - head abnormality index in albino male mice after interactions between CCl_4 and two extracts (aqueous and methanol) of *A. eupatoria*.

Interaction Groups	Dose (mg/kg)	Mean ± SE (%)*
Control I (CCl_4 + distilled water)	0.0	16.83 ± 1.05[a]
Control II (CCl_4 + vitamin C)	120	7.97 ± 0.26[b]
CCl_4 + Plant aqueous Extract	3.0	10.07 ± 0.23[b]
CCl_4 + Plant methanol Extract	3.0	8.03 ± 0.64[b]

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

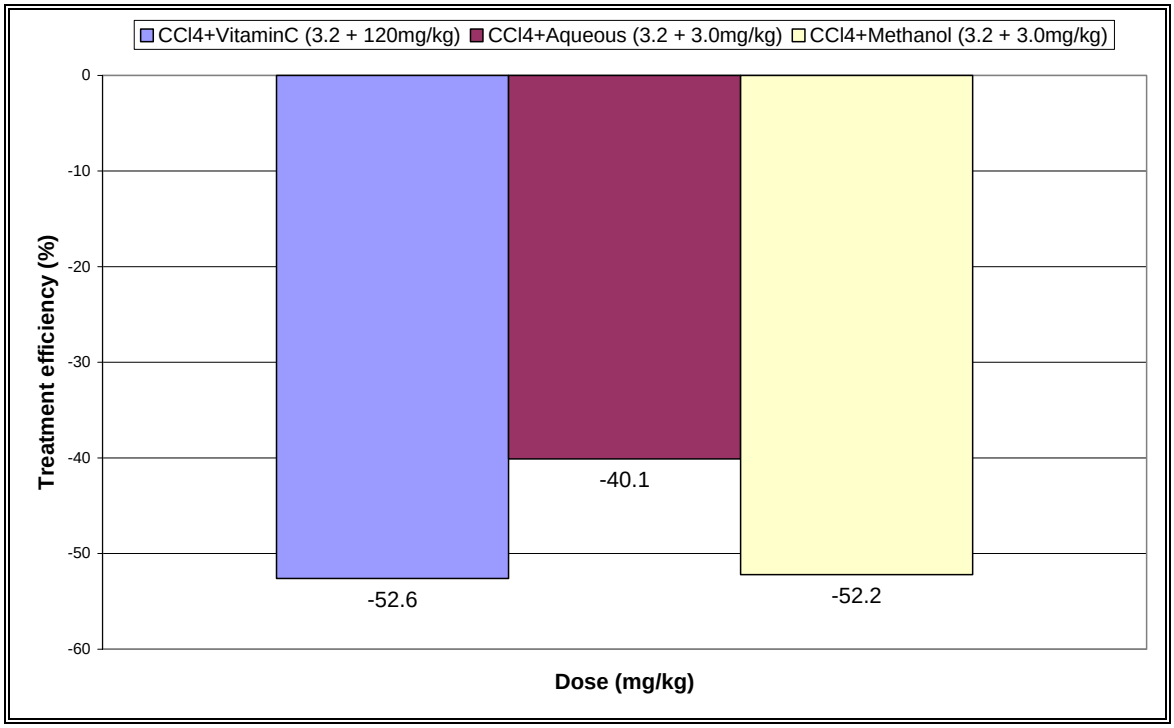


Figure 3-26: Treatment Efficiency of Sperm-head abnormality index in albino male mice after interactions between CCl_4 and two extracts (aqueous and methanol) of *A. eupatoria*.

Table 3-24: Sperm-tail abnormality index in albino male mice after interactions between CCl_4 and two extracts (aqueous and methanol) of *A. eupatoria*.

Interaction Groups	Dose (mg/kg)	Mean ± SE (%)*
Control I (CCl_4 + distilled water)	0.0	8.47 ± 0.48[a]
Control II (CCl_4 + vitamin C)	120	2.43 ± 0.26[b]
CCl_4 + Plant aqueous Extract	3.0	3.30 ± 0.40[b]
CCl_4 + Plant methanol Extract	3.0	3.53 ± 0.55[b]

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

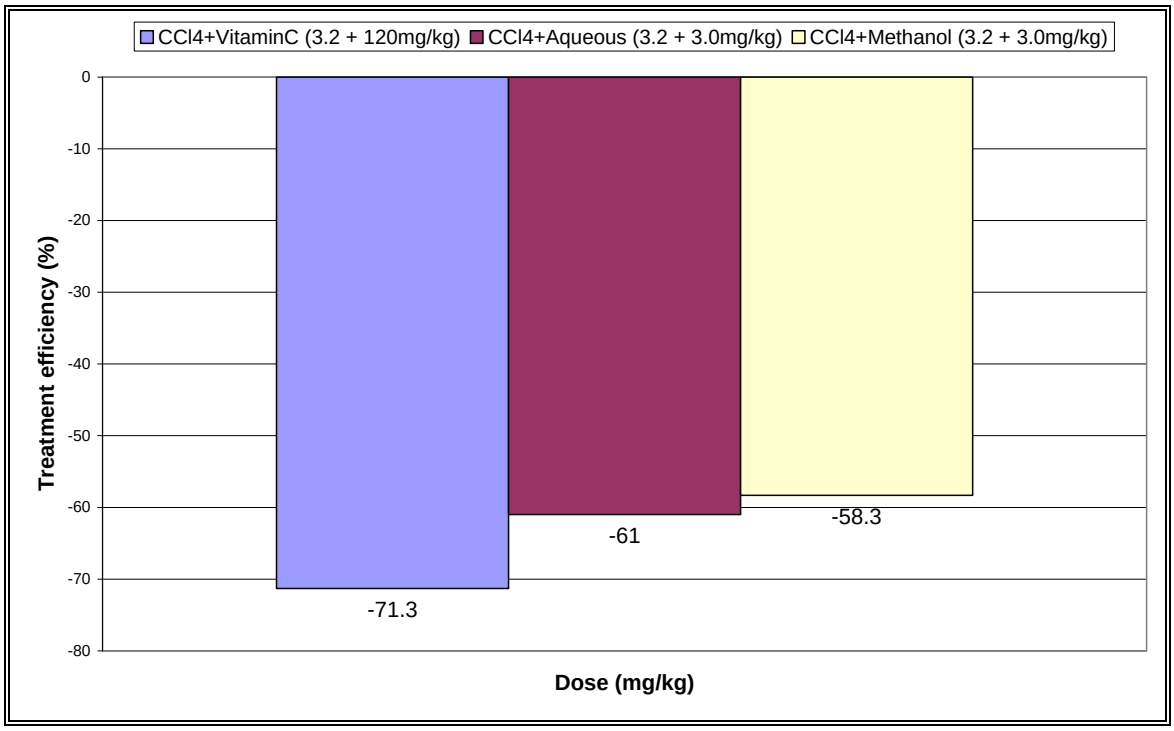


Figure 3-27: Treatment Efficiency of Sperm - tail abnormality index in albino male mice after interactions between CCl_4 and two extracts (aqueous and methanol) of *A. eupatoria*.

3.4.6 Aspartate Amino-Transferase (AST)

The enzymatic activity of AST, which was increased as a consequence of CCl_4 treatment (846.7 IU/L), was significantly decreased after a post-

treatment with vitamin C (517.3 IU/L), aqueous extract (210.0 IU/L) or methanol extract (534.0 IU/L), and such modulating effects were associated with a treatment efficiency of -38.9, -75.1 and -36.9%, respectively (Table 3-25and Figure 3-28).

Table 3-25: Aspartate amino-transferase activity in sera of albino male mice after interactions between CCl_4 and two extracts (aqueous and methanol) of *A. eupatoria*.

Interaction Groups	Dose (mg/kg)	Mean ± SE (IU/L)*
Control I (CCl_4 + distilled water)	0.0	846.7 ± 73.71[a]
Control II (CCl_4 + vitamin C)	120	517.3 ± 2.52[b]
CCl_4 + Plant aqueous Extract	3.0	210.0 ± 10.00[c]
CCl_4 + Plant methanol Extract	3.0	534.0 ± 15.00[b]

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

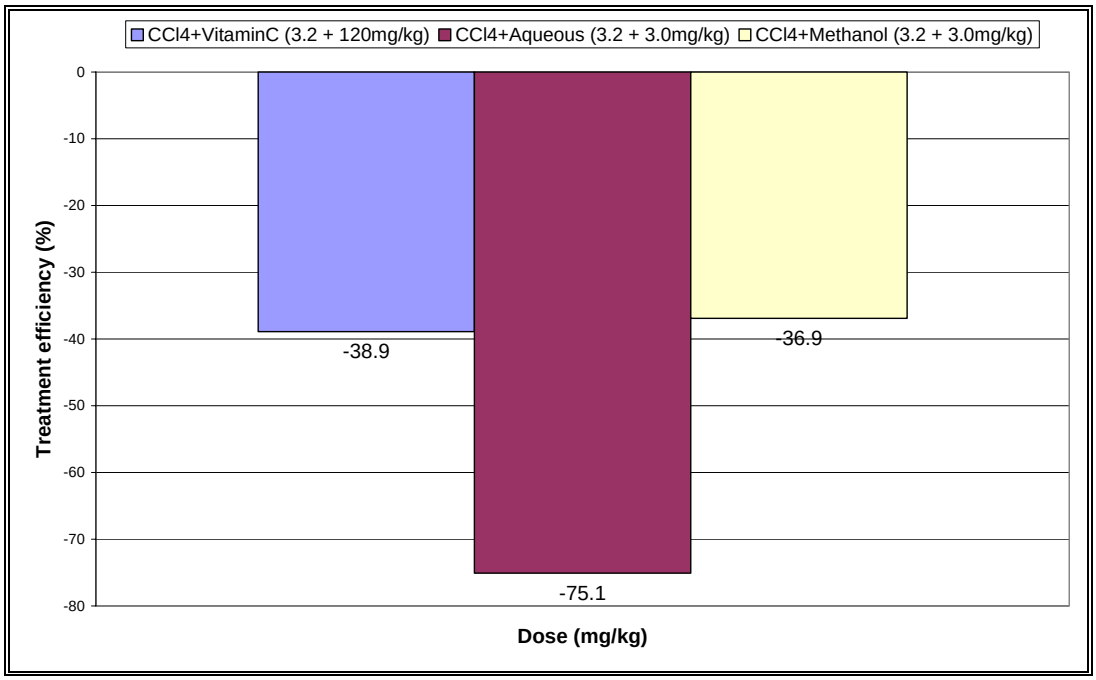


Figure 3-28: Treatment Efficiency of Aspartate amino-transferase activity in albino male mice after interactions between CCl_4 and two extracts (aqueous and methanol) of *A. eupatoria*.

3.4.7 Alanine Amino-Transferase (ALT)

The ALT activity was also modulated by the tested materials (vitamin C, aqueous or methanol extract) as in AST (section 3.4.6), and the associated

treatment efficiencies were -81.4, -88.4 and -81.8%, respectively (Table 3-26and Figure 3-29).

Table 3-26: Alanine amino-transferase activity in sera of albino male mice after interactions between CCl_4 and two extracts (aqueous and methanol) of *A. eupatoria*.

Interaction Groups	Dose (mg/kg)	Mean ± SE (IU/L)*
Control I (CCl_4 + distilled water)	0.0	916.7 ± 3.33[a]
Control II (CCl_4 + vitamin C)	180	170.0 ± 5.78[b]
CCl_4 + Plant aqueous Extract	3.0	105.7 ± 1.20[c]
CCl_4 + Plant methanol Extract	3.0	166.7 ± 4.41[b]

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

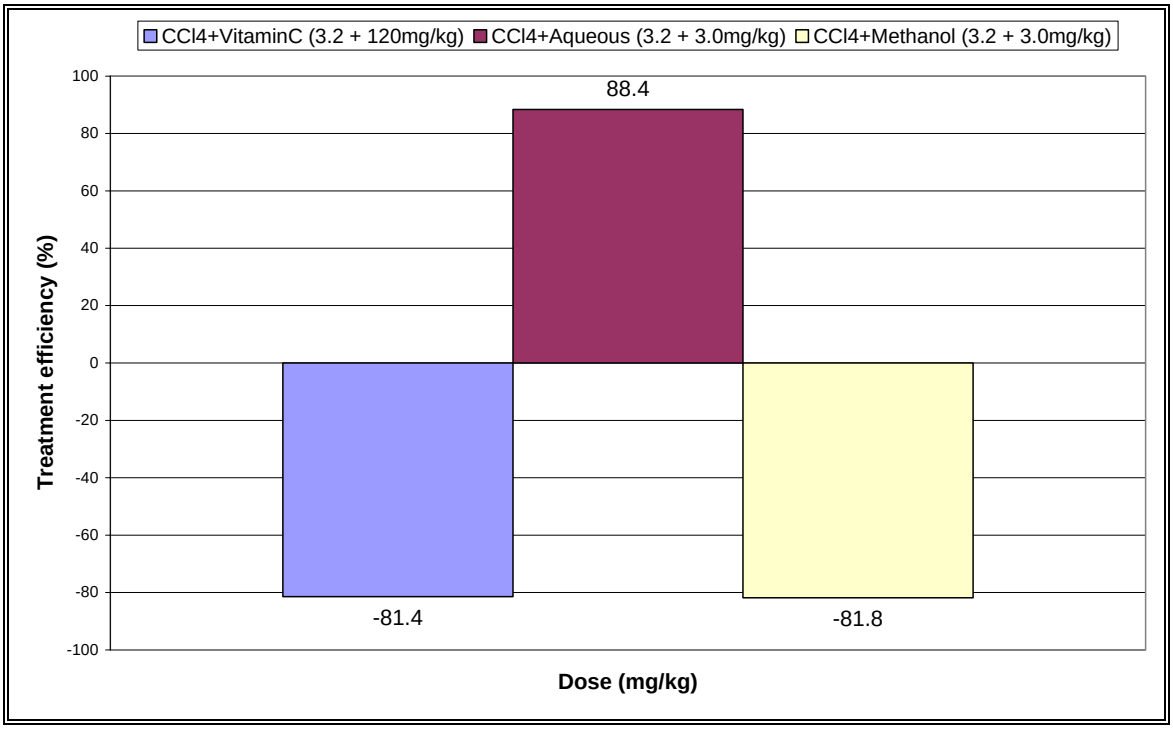


Figure 3-29: Treatment Efficiency of Alanine amino-transferase activity in albino male mice after interactions between CCl_4 and two extracts (aqueous and methanol) of *A. eupatoria*.

3.4.8 Alkaline phosphatase (ALP)

Vitamin C contributed in a significant reduced activity of ALP (95.87 IU/L), but the aqueous extract was better in this regard (59.13 IU/L), followed by the methanol extract (68.23 IU/L) as compared to control I (112.0 IU/L) (Table 3-28). The associated treatment efficiencies were -14.4, -47.2 and -39.0%, respectively (Figure 3-31).

Table 3-27: Alkaline phosphatase activity in sera of albino male mice after interactions between CCl_4 and two extracts (aqueous and methanol) of *A. eupatoria*.

Interaction Groups	Dose (mg/kg)	Mean ± SE (IU/L)*
Control I (CCl_4 + distilled water)	0.0	112.0 ± 6.11[a]
Control II (CCl_4 + vitamin C)	120	95.87 ± 2.26[b]
CCl_4 + Plant aqueous Extract	3.0	59.13 ± 2.31[c]
CCl_4 + Plant methanol Extract	3.0	68.23 ± 4.45[c]

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

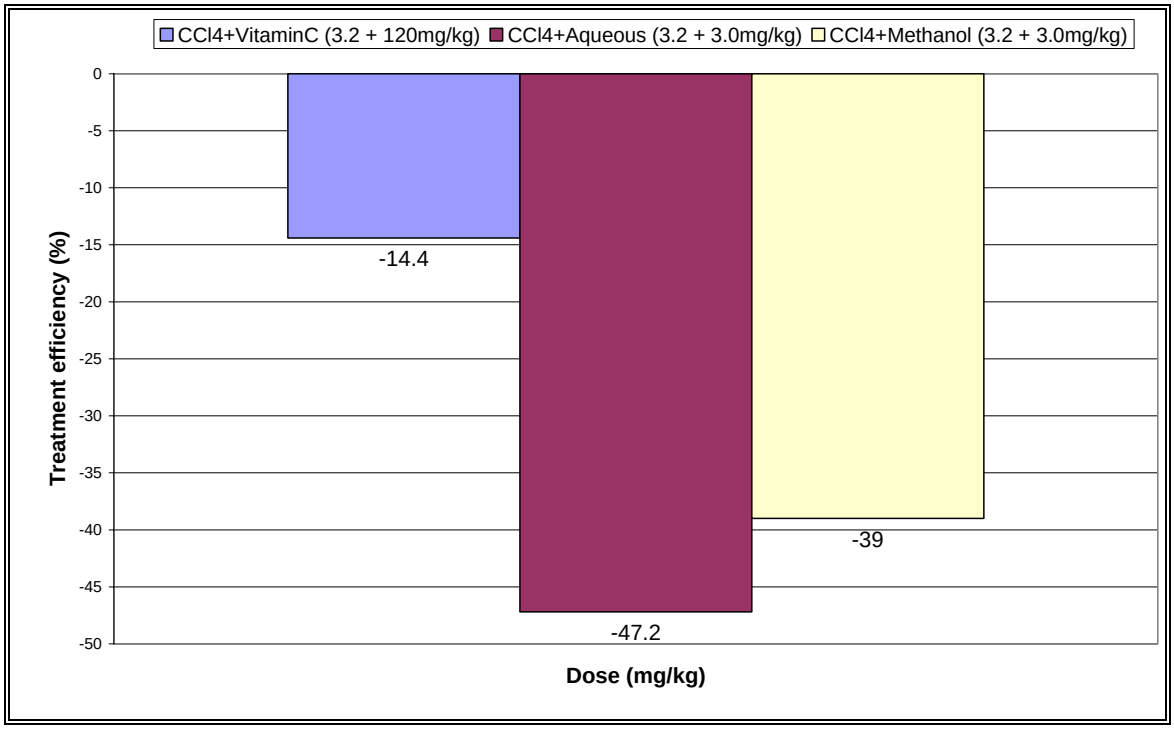


Figure 3-30: Treatment Efficiency of Alkaline phosphatase activity in albino male mice after interactions between CCl_4 and two extracts (aqueous and methanol) of *A. eupatoria*.

3.5 Histopathological Effects in Liver of Mouse

3.5.1: Plant Extracts

The normal appearance of a liver section (mouse treated with distilled water) is given in figure 3-31, and a similar appearance was observed in animals treated with vitamin C.

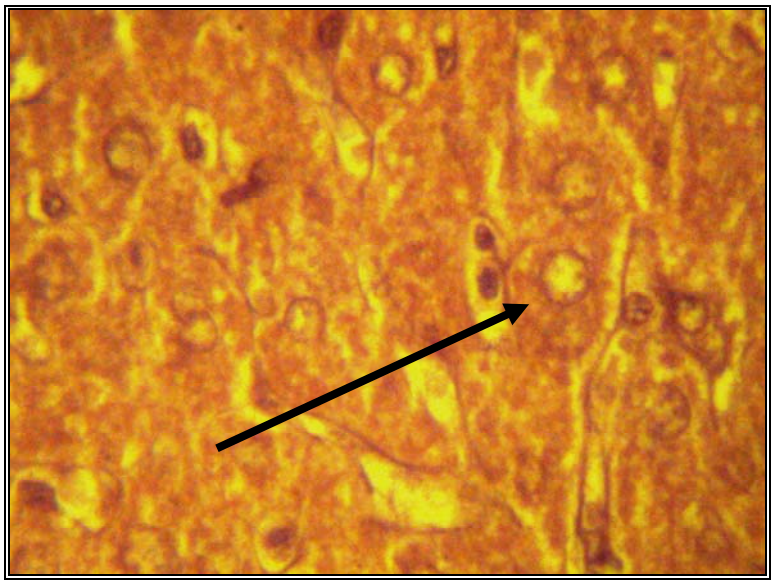


Figure 3-31: A liver section with a normal histological appearance (40x).

In animals treated with CCl_4 for one day, the liver section showed a marked fatty accumulation, degenerative areas and necrosis of hepatocytes (Figure 3-32).

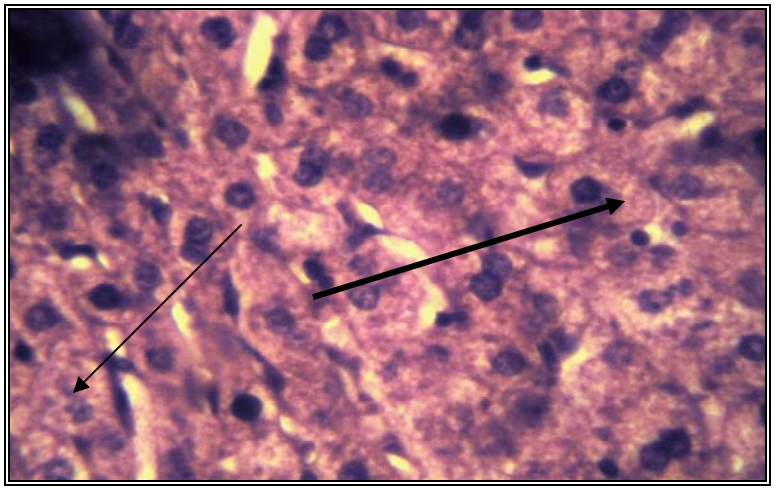


Figure 3-32: A liver section of mouse treated with CCl_4 for one day showing marked fatty accumulation (→), degenerative areas and necrosis of hepatocytes (40x).

With respect to the aqueous extract, the liver sections of mice treated with the first and second doses (1.0 and 2.0 mg/kg, respectively) showed vacuoles in the cytoplasm of hepatocytes, in addition to a degeneration of the cells with inflammatory cell infiltrate (Figures 3-33 and 3-34). Whereas, the third dose (3.0 mg/kg) was associated with a normal-looking appearance of hepatocytes.

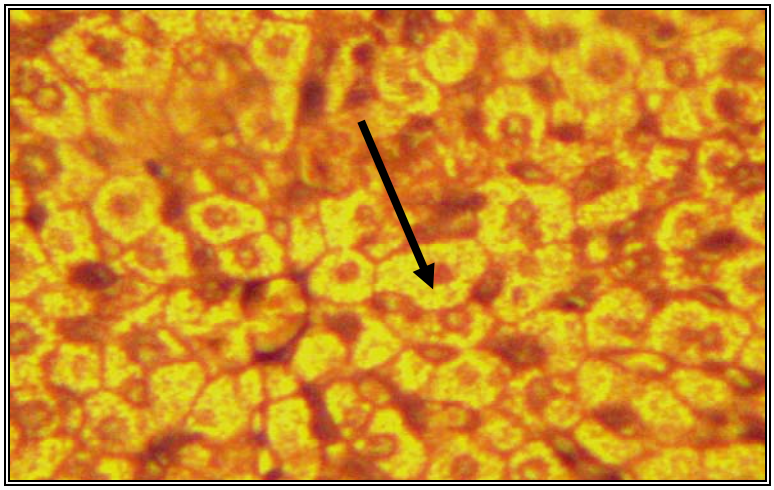


Figure 3-33: A liver section of mouse treated with the first dose (1.0 mg/kg) of agrimony aqueous extract showing vacuoles in the cytoplasm of hepatocytes, in addition to a degeneration of the cells with inflammatory cell infiltrate (40x).

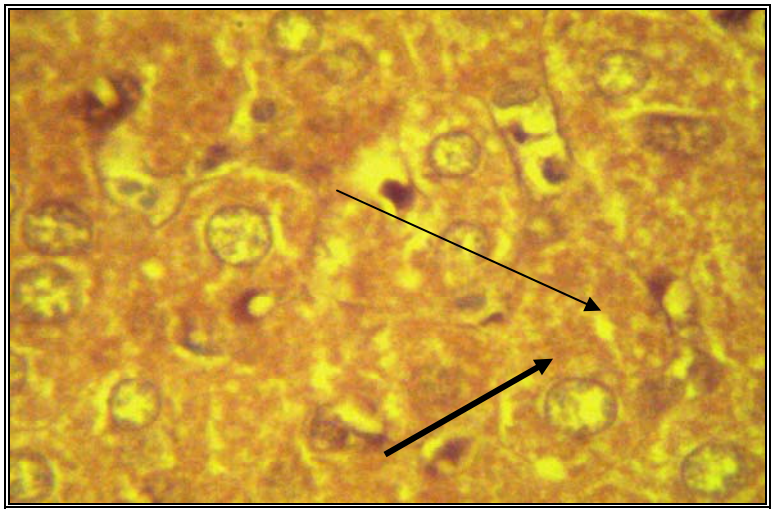


Figure 3-34: A liver section of mouse treated with the second dose (2.0 mg/kg) of agrimony aqueous extract showing vacuoles in the cytoplasm of hepatocytes, in addition to a degeneration of the cells with inflammatory cell infiltrate (40x).

The first dose (1.0 mg/kg) of methanol extract showed accumulation of vacuoles in the cytoplasm (3-35), and the second dose (2.0 mg/kg) showed marked fatty accumulation and the hepatocytes appeared like a plant cell (3-36). Whereas, the third dose (3.0 mg/kg) was associated with a normal-looking appearance of hepatocytes.

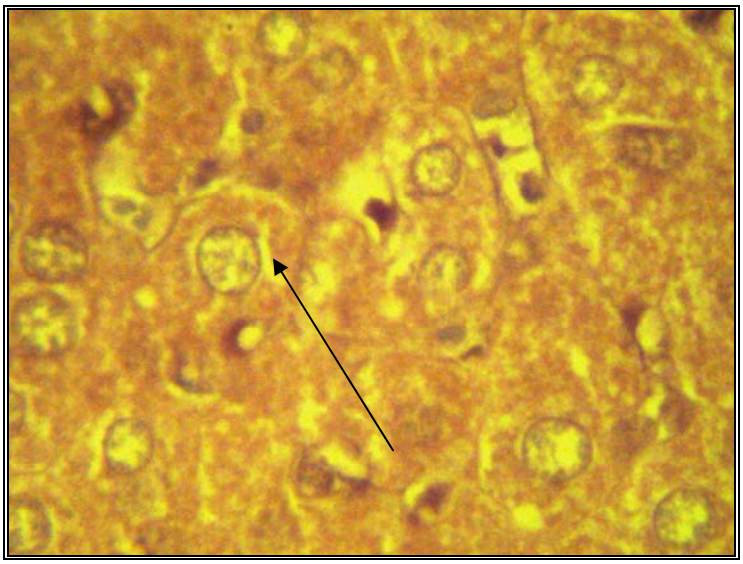


Figure 3-35: A liver section of mouse treated with the first dose (1.0 mg/kg) of agrimony methanol extract showing vacuoles in the cytoplasm of hepatocytes (40x).

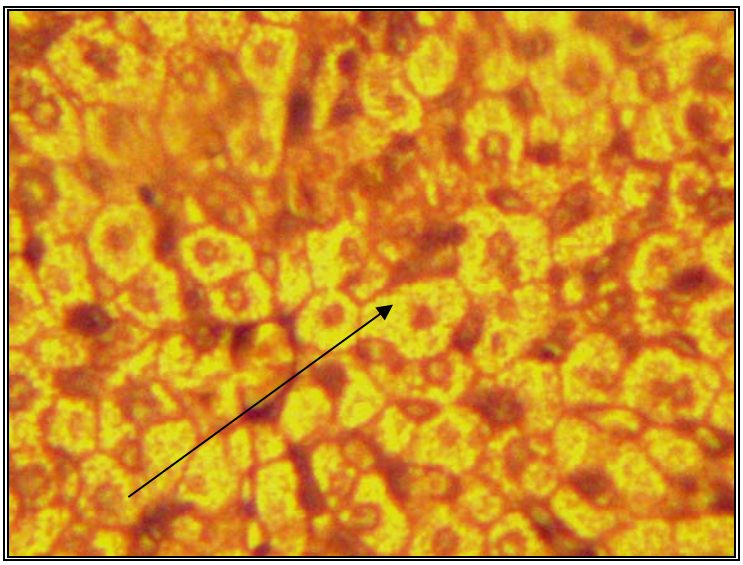


Figure 3-36: A liver section of mouse treated with the second dose (2.0 mg/kg) of agrimony methanol extract showing marked accumulation of fatty acids (40x).

3.5.2 Plant Extract – CCl_4 Interaction

The liver section of mouse treated with distilled water after CCl_4 treatment showed a marked fatty accumulation, degenerative areas and necrosis of hepatocytes, which were also observed in mouse treated with CCl_4 for one day as shown in the previously presented figure (Figure 3-32). The third dose of methanol extract was not effective in modulating these effects and the liver sections showed degeneration of hepatocytes and vacuoles in the cytoplasm with inflammatory cell infiltrate (Figure 3-37). In contrast, the third dose as well as vitamin C were able to modulate the CCl_4-induced effects and the liver sections showed a normal looking histological structure with inflammatory cell infiltrate (Figures 3-38 and 3-39).

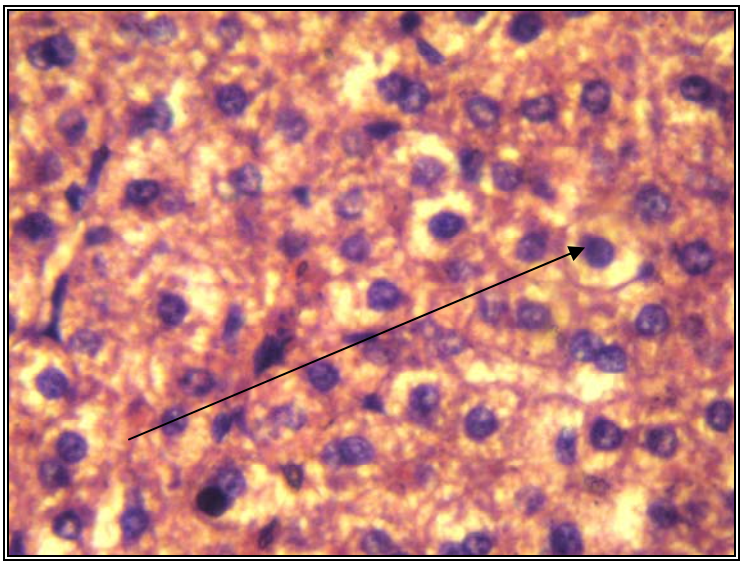


Figure 3-37: A liver section of mouse treated with the third dose of agrimony aqueous extract after CCl_4 treatment showing a normal looking histological structure with inflammatory cell infiltrate (40x).

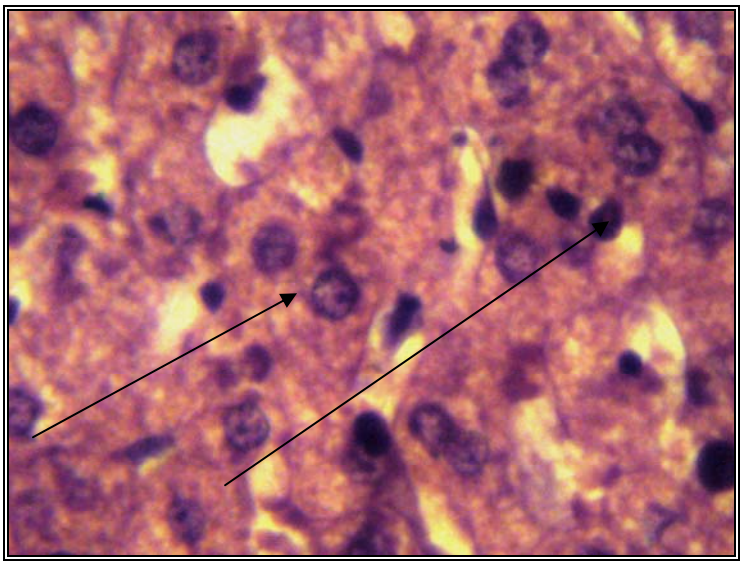


Figure 3-38: A liver section of mouse treated with vitamin C after CCl_4 treatment showing a normal looking histological structure with inflammatory cell infiltrate (40x).

3.6 Cytotoxicity of Agrimony Extracts against Cell Lines

Five concentrations (6.0, 12.0, 24.0, 48.0 and 96.0 µg/ml) of agrimony aqueous and methanol extracts were evaluated for their cytotoxic effect (Percentage of Growth Inhibition; PGI) against two tumor cell lines (HeLa and RD) and one normal cell line (MEF) after three periods of incubation (24, 48 and 72 hours) for HeLa and RD cells and one period (48 hours) for MEF cells.

3.6.1 Aqueous Extract

- **Twenty-Four Hour Incubation**: There was a gradual increase the value of PGI as the concentration of the extract was increased (15.07, 21.37, 23.80, 23.97 and 35.77 % for the concentrations 12.0, 24.0, 48.0 and 96.0 µg/ml, respectively) against RD cells**.** The 1^{st} and 5^{th} concentrations showed a significant difference in comparison with the 2^{nd}, 3^{rd} and 4^{th} concentrations, and the latter concentrations showed no significant

differences between them. A similar observation was made in HeLa cells, but the 4th concentration showed a significant difference in comparison with the 1st, 2nd and 3rd concentrations. This concentration also showed a significant difference (P = 0.007) in the PGI value when a comparison was made between RD (33.03%) and HeLa (23.97%) cells (Table 3-28).

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Table 3-28: Growth inhibition effects of *Agrimonia eupatoria* aqueous extract on RD and HeLa cell lines after 24 hours incubation.

Extract Concentration (µg/ml)	Percentage of Growth Inhibition (Mean ± S.E.)*		LSD Probability
	RD Cell Line	HeLa Cell Line	
6	15.07 ± 0.43^{c}	20.97 ± 0.24^{c}	Not significant
12	21.37 ± 1.72^{b}	26.70 ± 0.40^{b}	Not significant
24	23.80 ± 4.33^{b}	27.25 ± 2.63^{b}	Not significant
48	23.97 ± 4.33^{b}	33.03 ± 0.88^{a}	0.007
96	35.77 ± 6.04^{a}	33.37 ± 1.92^{a}	Not significant

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

- **Forty-eight Hour Incubation**: The five concentrations of the aqueous extract were effective in reducing the growth of both cell lines, and almost the effect was similar (no significant differences between them) on RD and HeLa cells. The effect was a dose-dependent, and accordingly the 5th dose recorded the highest PGI, which was 67% in RD cells and 72% in HeLa cells. These two values were significantly different in comparison with PGI values of the other concentrations of the same cell line (Table 3-29).

Table 3-29: Growth inhibition effects of *Agrimonia eupatoria* aqueous extract on RD and HeLa cell lines after 48 hours incubation.

Extract Concentration (µg/ml)	Percentage of Growth Inhibition (mean ± S.E.)*		Probability ≤
	RD Cell Line	HeLa Cell Line	
6	42.00 ± 4.61^{c}	36.33 ± 2.67^{d}	Not significant
12	42.00 ± 4.61^{c}	53.33 ± 2.67^{c}	Not significant
24	53.00 ± 3.00^{b}	53.33 ± 2.67^{c}	Not significant
48	53.00 ± 3.00^{b}	59.67 ± 3.00^{b}	Not significant
96	67.00 ± 9.81^{a}	72.67 ± 16.33^{a}	Not significant

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

- **Seventy-two Hour Incubation**: Much more growth reducing effects were observed after 72 hour incubation, and the 5th concentration was the most efficient on both cell lines, in which no significant difference was recorded between them in all concentrations, with the exception of 24 µg/ml that showed a significantly ($P = 0.026$) higher PGI in HeLa cells (69%) than the PGI in RD cells (60.33%) (Table 3-30).

Table 3-30: Growth inhibition effects of *Agrimonia eupatoria* aqueous extract on RD and HeLa cell lines after 72 hours incubation.

Extract Concentration (µg/ml)	Percentage of Growth Inhibition (mean ± S.E.)*		Probability ≤
	RD Cell Line	HeLa Cell Line	
6	57.33 ± 1.20^{b}	60.00 ± 0.58^{e}	Not significant
12	58.67 ± 0.33^{b}	65.67 ± 0.67^{c}	Not significant
24	60.33 ± 1.45^{b}	69.00 ± 0.58^{bc}	0.026
48	70.00 ± 1.53^{a}	75.33 ± 0.67^{b}	Not significant
96	73.00 ± 0.58^{a}	84.00 ± 0.58^{a}	Not significant

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

3.6.2 Methanol Extract

- **Twenty-Four Hour Incubation**: The first four concentrations recorded a PGI range of 13-25.33% in RD cell line, while such range in HeLa cell line was 14-30.67%. Such range was exceeded dramatically in the 5th concentration (96 µg/ml), but the PGI in RD cell line was a significantly (P = 0.001) higher than that of HeLa cell line (80 *vs.* 53.67%). A further significant differences (P = 0.013) was observed in the concentration 12 µg/ml, in which the PGI in RD cell line was higher than that of HeLa cell line (25 *vs.* 16%)(Table 3-31).

Table 3-31: Growth inhibition effects of *Agrimonia eupatoria* methanol extract on RD and HeLa cell lines after 24 hours incubation.

Extract Concentration (µg/ml)	Percentage of Growth Inhibition (mean ± S.E.)*		Probability ≤
	RD Cell Line	HeLa Cell Line	
6	13.00 ± 1.00 [c]	14.00 ± 1.15 [c]	Not significant
12	25.00 ± 0.58 [b]	16.67 ± 0.67 [c]	0.013
24	24.00 ± 0.57 [b]	27.67 ± 1.45 [b]	Not significant
48	25.33 ± 0.33 [b]	30.67 ± 30.67 [b]	Not significant
96	80.00 ± 0.58 [a]	53.67 ± 0.67 [a]	0.001

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

- **Forty-eight Hour Incubation**: Approximated values of PGI were recorded in the five tested concentrations for both cell lines, especially the 5th concentration in which the PGI was 82 and 84% for RD and HeLa cell lines, respectively (Table 3-32).

Table 3-32: Growth inhibition effects of *Agrimonia eupatoria* methanol extract on RD and HeLa cell lines after 48 hours incubation.

Extract Concentration (µg/ml)	Percentage of Growth Inhibition (mean ± S.E.)*		Probability ≤
	RD Cell Line	HeLa Cell Line	
6	24.00 ± 2.00^{c}	27.33 ± 1.45^{c}	Not significant
12	27.33 ± 4.70^{c}	33.67 ± 3.38^{b}	Not significant
24	32.67 ± 0.66^{b}	34.67 ± 6.56^{b}	Not significant
48	36.67 ± 1.67^{b}	36.67 ± 5.90^{b}	Not significant
96	82.00 ± 1.00^{a}	84.00 ± 1.00^{a}	Not significant

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

- **Seventy-two Hour Incubation**: Again the 5th concentration recorded the highest PGI in RD and HeLa cell lines (90 and 87%, respectively) with no significant difference between them. However, the first four concentrations contradicted such scope, in which the PGI recorded significantly (P = 0.001) higher values in HeLa cells than in RD cells (Table 3-33).

Table 3-33: Growth inhibition effects of *Agrimonia eupatoria* methanol extract on RD and HeLa cell lines after 72 hours incubation.

Extract Concentration (µg/ml)	Percentage of Growth Inhibition (mean ± S.E.)*		Probability ≤
	RD Cell Line	HeLa Cell Line	
6	13.50 ± 0.29^{c}	58.00 ± 7.94^{c}	0.001
12	27.00 ± 2.08^{b}	62.00 ± 6.56^{b}	0.001
24	30.33 ± 0.67^{b}	63.00 ± 2.00^{b}	0.001
48	33.00 ± 1.00^{b}	65.00 ± 1.00^{b}	0.001
96	90.00 ± 3.05^{a}	87.00 ± 2.65^{a}	Not significant

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

3.6.3 Mouse Embryonic Fibroblast (MEF) Cell Line

The aqueous and methanol extract recorded an approximated range of PGI against the MEF cell line (aqueous extract: 10.90-14.80%; methanol extract: 10.53-16.58%) with the exception of the concentrations 12 µg/ml, in which the methanol extract demonstrated a significantly increased PGI as compared with the PGI of the corresponding aqueous extract concentration (15.63 *vs*. 10.90%) (Table 3-34).

Table 3-34: Growth inhibition effects of *Agrimonia eupatoria* extracts (aqueous and methanol) on MEF cell line after 48 hours incubation.

Extract Concentration (µg/ml)	Percentage of Growth Inhibition (mean ± S.E.)*		Probability ≤
	Aqueous extract	Methanol extract	
6	14.80 ± 0.72^{a}	16.50 ± 1.44^{a}	Not significant
12	10.90 ± 1.30^{b}	15.63 ± 0.73^{a}	0.01
24	12.40 ± 1.56^{ab}	12.70 ± 1.24^{b}	Not significant
48	13.00 ± 1.53^{a}	14.13 ± 2.92^{ab}	Not significant
96	14.00 ± 1.15^{a}	10.53 ± 0.74^{b}	0.01

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

Chapter Four

Discussion

Chapter four

Discussion

Medicinal plant extracts are reported to have a wide range of beneficial properties including hepatoprotective, anti-inflammatory, anti-oxidant, immune modulators and anti-mutagenic and accordingly they are subjected to intensive research by many investigators to explore their potency in the treatment of a wide range of disorders, including cancer (Dhir *et al*., 1990; Perera *et al*., 2008; Saif, 2008). Some of these properties (immune modulation, anti-mutagenic, hepatoprotective and anti-tumor) were evaluated in the present study for two extracts (aqueous and methanol) of agrimony against the immune suppressive, mutagenic and hepatotoxic effects of CCl_4. As expected, such agent demonstrated a wide range of devastating effects to the biological system of the laboratory 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 and -tail abnormalities (genotoxic); and increased activities of liver function enzymes (hepatic damage). These findings can be explained mainly in the ground of indirect genotoxic effects, because it was reported that CCl_4-induced mutations may not be as a result of a direct DNA damage but may occur via indirect mechanisms; including disturbances in enzyme functions, which are important in the DNA synthesis and/or repair (Zelikoff *et al*., 1988). Carbon tetrachloride-induced hepatic injury is also associated with global DNA hypomethylation and homocysteinemia (Moreiras *et al*., 1995). Moreover, CCl_4-induce oxidative stress in mice has been reported (Khurana and Mukhopadhyay, 2007), 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 agrimony 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 hepatotoxic; moreover, some positive augmentations of the investigated parameters were observed. Such potentials were further confirmed 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 a normalization of the parameter values was almost reached. Such properties were mostly a dose-dependent, as well as, the type of solvent used in the 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, its products or derivatives (Buelga and Scalbert, 2000), and the antioxidant potentials were explained in the ground of chemical constituents that are available in the plant extracts (Venskutonis *et al.,* 2007).

Chemical analysis of *Agrimonia eupatoria* extracts (aqueous and methanol) revealed the presence of flavonoids, tannins and alkaloids in aqueous extract and flavonoids, tannins, and alkaloids in methanol extract. Similar findings have been obtained by other investigators (Horikawa *et al*., 1994; Pan *et al*., 2008). These investigators demonstrated that the anti-oxidant properties of agrimony are related to the flavonoids in addition to tannins. These two constituents are important anti-mutagenic and anti-carcinogenic compounds *in vitro* and *in vivo* (Koshiura *et al*., 1985; Horikawa *et al*., 1994), and accordingly, the flavonoids and tannins of agrimony have shown a broad spectrum of *in vitro* and *in vivo* antibacterial (Copland *et al*., 2003; Bae and Sohn, 2005), antiviral (Yaolan *et al*., 2004; Kwon *et al*., 2005) and antioxidant (Murayama *et al*., 1992) properties.

Due to the forthcoming potentials of flavonoids, the two plant extracts were chemically analyzed by means of a TLC method to inspect the types of flavonoids that were exist in the extract. Such analysis revealed that the aqueous extract contained myricetin, azoleatin, vitexin and iso-orientin, while the methanol extract contained kaempferol, quercetin, isorhamnetin and myricetin. These findings came to confirm previous chemical analyses for flavonoids of the

plant (Xu *et al*., 2005; Jung and Park, 2007). The chemical analysis carried out by these investigators revealed that the plant contains the following flvaonoids; tiliroside, kaempferol 3-O-alpha-L-rhampyranoside, quercetin 3-O-alpha-L-rhampyranoside, quercetin 3-O-beta-D-glucopyranoside, kaempferol 3-O-beta-D-glucopyranoside, kaempferol, kaempferol 3-gucside, kaempferol 3-rhamnoside, kaempferol 3-rutinoside, apigenin, luteolin, quercetin, catechin, hyperoside, quercitrin, rutin, tiliroside and 3-methoxy quercetin. Some of these constituents were not detected in the present study analysis, and such difference may be related to either the type of chemical analysis that was employed (they employed HPLC) or the type of extract that was analyzed (they used different plant extracts). The tannins are further chemical constituents that were inspected in the plant extracts, but by a detection method rather than a TLC method, because the tannin standard was not available. Such detection revealed that the two plant extract contained tannins.

To discuss the immune stimulant, anti-mutagenic, heptoprotective and anti-tumor effects of aqueous and methanol extracts that were demonstrated in the present study, the chemical constituents are going to be considered, with special reference to flavonoids and tannins. In this regard Venkutonis *et al*. (2007) examined the antioxidant activity of aqueous extract and methanol extract of agrimony grown in Kaunas Botanical Garden of Vytautas Magnus University. Their results suggested that the aqueous and methanol extracts behaved as a strong free radical scavenger. Such demonstration supports the results of the present study, and the effects can be ascribed to the flavonoid content, because it has been demonstrated that agrimony extracts (aqueous, acetone and hexane) contained phenol fractions that have an antioxidant potential and a scavenging activity against the reactive species formed during inflammation, and accordingly a relationship between such activity and the phenolic composition was established, and the fractions were found to possess a high concentration of flavonols, flavones and phenolic acids (Correia *et al*., 2007). However, little information is available about the antioxidant activity of other agrimony

compounds, but Tomlinson *et al*. (2003) have also demonstrated the free radical scavenging activity of aqueous and organic extracts of agrimony aerial parts.

The results demonstrated that a treatment with agrimony extracts showed a enhancing effect on total and absolute counts of leucocytes (neutrophils and lymphocytes and monocytes), and these counts manifested a significant increase especially in animal treated with both extract (aqueous extract and methanol extract), and such effects were shared by vitamin C. The general outcome of these findings is that these materials might have immunostimulating properties, because the profile of leucocytes in the peripheral blood can give a general picture about the functional status of the immune system (Hughes, 2001). This reasoned by the fact that each type of leucocytes is commenced to carry out a special immunological function in the innate and adaptive immune response, and the numerical count of these cells may correlate with their function (Herant *et al*., 2003). The question is how the aqueous and methanol extract modulate or restore the count of leucocytes especially after CCL_4 treatment. The answer can be augmented if we consider that both extracts contain tannins and flvavonoids. In this regard, it was demonstrated that the adherent peritoneal exudate cells of mouse treated with an agrimony tannin (agrimoniin) were able to produce a relatively high levels of interleukin (IL)-1(Murayama *et al*., 1992), and such interleukin is important cytokine involved in recruiting blood neutrophils and monocytes to sites of infection (Abbas and Lichtman, 2004). Furthermore, it has been recently demonstrated that agrimony flavonoids showed immunostimulating properties, and the effect was ascribed to their modulating potentials of cytokines (Gerhauser, 2008). Accordingly, the enhancement of the total and absolute counts of leucocytes can be ascribed to these chemical constituents (flavonoids and tannins), which may be able to modulate the immune response through the interaction between cytokines that were affected by the plant extracts treatment.

The function of immune system is also genetically determined (Roitt, 2001). Therefore, the forthcoming effects of the plant extracts or their chemical

constituents may have a genetic effect. To test this suggestion, the present study investigated the antimutagenic activity, directly or through interactions with CCl_4, of agrimony extracts in mice. The mitotic index, micronucleus formation in polychromtic erythrocytes of bone marrow and sperm-head and -tail abnormalities were considered as parameters of mutagenic evaluations as suggested by other investigators in this regard (Martino-Roth *et al*., 2003; Jha and Kumar, 2006; Moura *et al*., 2008).

The results of genetic evaluations showed that a treatment with agrimony extracts was associated with a significant reduction in micronucleus formation and sperm-head and -tail abnormalities and caused a significant increase in mitotic index of bone marrow cells. According to these evaluations, both extracts (aqueous and methanol) can be considered as effective anti-mutagens, especially at the third dose of aqueous extract and methanol extract (3.0 mg/kg), which exerted the highest effect. 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 oxidant effects (Yassen, 1990; Ad'hiah *et al*., 2002) like CCl_4, which was used in this study and showed cytotoxic effects. However, it is not possible to judge that these effects were on the mutagen (CCl_4) or its metabolism, or the effects are related to the biological systems that are responsible of protecting the genetic material of the organism, but both mechanisms can be possible especially if we consider the tannin's and flavonoid's constituents of the two extracts.

The anti-oxidant potentials of these compounds may partially explain some of the observed effects, and evidence of a relevant antioxidant activity of *A. eupatoria* aqueous or alcoholic extract has been clarified by Rice-Evans *et al*. (1996) attributed the pharmacological effect to the detected flavonoids (quercetin, kaempferol, flavone glycoside and flavan-3-ols), but other synergistic compounds have also been suggested, and they are grouped under tannins (catechin and proanthocyanidins) (Li *et al*., 2001). However, valuable antioxidant and anti-iflammatory activities have been referred to kaempferol 3-

0-(6”-0-p-coumaroyl)-glucoside, quercetin 3-0-galactoside and quercetin 3-0-gllucoside (Sala *et al*., 2003), and *in vivo* studies further suggested that the tannin catechin and smaller oligomeric procyanidins are also important in this regards (Crespy *et al*., 2001; Cos *et al*., 2004). Accordingly, the present anti-mutagenic properties of the two extracts can be related to the anti-oxidant activities of these compounds, especially if we consider that anti-oxidant and anti-mutagenic effects are interrelated (Matkawski, 2008).

The present study was also conducted to evaluate the hepatoprotective effects of the aqueous and methanol extracts of agrimony against CCl_4-induced hepatic damage in mice. The evaluation included measuring of liver function enzymes and a hisopathological examination of liver sections. The first evaluation demonstrated that the concentrations of AST, ALT and ALP were almost normalized after agrimony extract treatments in mice with CCl_4-induced hepatic damage, and the results were even better than that observed in animals treated with vitamin C, especially the third dose (3.0 mg/kg), but the results of histopathological examinations did not revealed a perfect protection, and only a partially histological repair was occurred.

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 of highly reactive substances such as free radicals, and CCl_4 is widely used for inducing a hepatic injury in mice. The localized liver injury is mainly occurred by the biotransformation of CCl_4, which is mediated by the cytochrome P-450 and leads to the generation of free radicals through the process of lipid peroxidation (Valcheva-Kuzmanova *et al*., 2004). These activated radicals bind covalently to the macromolecules and induce a peroxidative degradation of membrane lipids of endoplasmic reticulum (Palanivel *et al*., 2008). As O_2 tension rises, a greater fraction of $\cdot CCl_3$ present in the system reacts very rapidly with O_2 and consequently many reactive free radicals are generated from $\cdot CCl_3$. These free radicals are able to initiate the peroxidation of membrane poly-unsaturated fatty acids (PUFA) (Manna *et al*.,

2006), 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) (Kebieche *et al*., 2008). Evidences suggest 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; Stal and Olson, 2000).

In the liver, CCl_4 is metabolized by the cytochrome P450-dependent monooxygenase systems followed by its conversion to a more chemically active form, which is trichloromethyl radical ($\cdot CCl_3$). The enzymes involved in this process are located in the endoplasmic reticulum of hepatocytes and their activities are subjected to the influence of internal and external factors. In this regard, some herbal extracts are known to prevent the oxidative damages in hepatoctes by altering the levels of cytochrome P-450 through their antioxidant properties (Rajesh and Latha, 2004), and therefore, the present observed hepatoprotective effects (decreased levels of AST, ALT and ALP) of agrimony extracts can be explained. These findings also suggest that agrimony extracts are effective in preventing DNA damage, and one of the mechanisms of action might involve scavenging of active oxygen radicals generated in reactions initiated by the mutagen and hepatotoxic agent CCl_4, because materials with known antioxidant activities have been proved to be useful in counteracting CCl_4 damage; for instance vitamin C (Nefic, 2001).

The 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 tested dose and markedly induced micronucleus formation and sperm-head and tail-abnormalities. However, the inhibitory effect of CCl_4 on the mitotic activity was significantly decreased after a treatment with vitamin C. Micronucleus formation and sperm-head and -tail abnormalities induced by CCl_4 also decreased markedly. Therefore, vitamin C acts as either a radical scavenger or a pro-oxidant agent (Shamberger, 1984; Anderson, 1996), and such outcome can also be high-lighted after a treatment with agrimony extracts.

The antimutagenic effects of agrimony extracts were further evaluated by the mouse sperm morphology assay that was developed by Wyrobek and Bruce, and its relevance in evaluating mammalian germ cell mutagens is well accepted (Wyrobek and Bruce, 1975; Wyrobek *et al*., 1983). In the present experiments, CCl_4 induced an increase in the frequency of abnormal sperms, but, the percentage of these abnormalities was significantly decreased after a treatment with the aqueous or methanol extracts and in a dose-dependent manner. Therefore, this assay can further qualify the agrimony extracts as antimutagens, and the anti-mutagenic activity may be related to their contents of flavonoids. A presence of abnormal sperm-head and -tail suggests an induction of a genetic damage in the male germ cells, and such abnormalities may arise due to small deletions or point mutations. Furthermore, abnormalities in sperm-head and -tail may occur by physiological, cytotoxic or genetic mechanisms and alteration in testicular DNA, which in turn disrupts the process of differentiation of spermatozoa (Bruce and Heddle, 1979; Odeigah, 1997; Jha and Bharti, 2002).

The protective anti-mutagenic effect of flavonoids can be occurred through their ability to interact with free radicals when they are associated with DNA via intercalating or external modes and prevent disease (Lodovici *et al*., 2001; Walle *et al*., 2003). One of these flavonoids is quercetin, which was detected in agrimony methanol extract. This flavonoid binds covalently to DNA and protein to start it is protective action (Lonneke *et al*. 2005). A protection by quercetin and quercetin-rich fruit juice against induction of oxidative DNA damage has

also been demonstrated (Kanakis *et al*., 2006). Therefore, flavonoids are considered as powerful antioxidants, and prevent DNA damage, and these finding may explain the anti-mutagenic effect against the mutagen CCl_4 in animals treated with the two extracts of agrimony, especially the methanol extract in the present study.

Agrimony extracts also caused a significant decrease in serum transaminases (AST and ALT) and alkaline phosphatase activities and almost down to the normal levels after CCl_4 treatment in mice. These parameters are the commonly employed biological markers for evaluating a hepatic injury and efficacy of hepatoprotective interventions (Achliya *et al*., 2004). The present study revealed that agrimony extracts at the given doses had no deleterious effect on liver function enzymes in normal mice. These findings suggest that the 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 also been conducted and showed the antioxidant properties of agrimony or its products, and these findings suggest that the plant can protect against free radicals and inhibits inflammatory mediator synthesis and release. This protective effect has been attributed in part to flavonoids, through a modulation of several enzymes of the P-450 family that are involved in the pre-carcinogen metabolism (Zhai *et al*., 1998; Kokdil *et al*., 2005).

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 $\cdot CCl_3$; 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 a liver damage (Bishayee *et al*., 1995). Hepatocellular necrosis leads to elevation of the serum marker enzymes, which are released from the liver into blood (Shenoy *et al*., 2002) and the increased levels of AST, ALT and ALP are conventional indicators of liver injury (Achliya *et al*., 2004). The present study revealed a significant increase in the activities of AST, ALT and ALP levels after a treatment with CCl_4; indicating a considerable hepatocellular injury. An administration of aqueous or methanol extract at three different dose levels reduced the increased levels of serum liver function enzymes induced by CCl_4 and caused a subsequent recovery towards normalization almost like that of vitamin C or untreated controls. The hepatoprotective effect of the plant extracts was further conduced by the histopathological evaluation, and the results showed that both extracts at different dose levels offered a good hepatoprotection, but the dose 3.0 mg/kg was more effective than all other doses and this may be due to its content of tannis, and a hepatoprotective action of certain tannins has been well-documented recently (Tomisato *et al*., 2007).

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 ($\bullet CCl_3$). Thus, the antioxidant activity or the inhibition of the generation of free radicals is important for the protection against CCl_4-induced hepatotoxicity. 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 superoxide dismutase(SOD), catalase(CAT) 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, and an oxidative stress can result, in which a series of events deregulates the cellular functions leading to hepatic necrosis (Mochizuki *et al*., 2009). Unfortunately, it was not possible to investigate SOD, CAT and GPX in the experiments of present study.

The histopathological examinations of liver sections in CCl_4-treated mice revealed a regeneration of hepatic cells after treatments with the plant extracts; therefore it is possible to suggest that these plant extracts being able to condition the hepatic cells to a state of accelerated regeneration (Jayatilaka *et al*., 1989, 1990). The observed protective effect of plants belong to the family Rosaceae against the hepatotoxins may be attributed to the presence of agrimoniin (a tannin compound and kaempferol (a flavonoid compound), which are among the important plant constituents (Tomczyk and Latte, 2008). Flavonoids are a group of polyphenolic compounds with known properties that include free radical scavenging, inhibition of hydrolytic and oxidative enzymes and anti-inflammatory action. These consequences can lead to a hepatoprotection (Hewawasam *et al*., 2003). However, the use of herbal drugs for the treatment of liver diseases has a long tradition in many countries, because the easy accessibility without the need for laborious pharmaceutical synthesis has drawn increased attention towards herbal medicines (Girish and Pradhan, 2008).

The *in vivo* anti-mutagenic effects of agrimoniy aqueous and methanol extracts were further inspected, but this time in terms of *in vitro* anti-tumor potentials against two tumor cell liens (human rhabdomyosarcoma; RD and human cervical cancer; HeLa) and a normal cell line (mouse fibroblasts; MEF) after three periods of incubation (24, 48 and 72 hours) for HeLa and RD cells and one period (48 hours) for MEF cells. Five concentrations (6.0, 12.0, 24.0, 48.0 and 96.0 µg/ml) of each extract were tested, and these concentrations were assessed after running the experiments with the concentrations 1.0, 2.0 and 3.0 µg/ml, which were not effective against RD, HeLa cells and MEF cell lines. Therefore, five serial duplications of the third concentration were investigated. These concentrations were found to be cytotoxic against RD and HeLa cell lines, but the cytotoxicity was a concentration-dependent and in both cell lines, the concentration 96.0 µg/ml was the most effective for the three investigated periods. Such anti-tumor effects can be ascribed to the flavonoid and tannin contents of the two extracts, because it has been suggested that the members of

the family Rosaceae, which agrimony is belong, may be served as good anti-cancer agents due to their richness in flavonoids and tannins (Munoz *et al*., 2004). The mechanism of such anti-tumor cytotoxicity is not well understood, but the anti-oxidant potential of the two chemical constituents can not be ignored (Russo *et al*., 2005), although the correlation between their antioxidative and anti-carcinogenic activities remains unclear (Tomisato *et al*., 2007).

Among the polyphenols is the tannin compound epigallocatechin-3-gallate, which has been been demonstrated to possess anti-oxidative activity and to induce apoptosis in tumor cells (Lih-Geeng *et al*., 2009). The apoptosis was found to occur through two main pathways; in the first the death receptors are triggered, while the second pathway involves an intrinsic mitochondrial damage, which consequence in the release of cytochrome *c* and activation of caspase-9 (Gosse *et al*., 2005). Furthermore, polyphenolic can cause a down-regulation of factors that are necessary for cell-cycle progression, and this series of events lead to stoppage of cell cycle progression at the G1-S phase transition and thereby causing G0/G1 arrest and subsequent apoptotic cell death (Cilla *et al*., 2009). Accordingly, natural polyphenols have gained a great interest in pharmaceutical industry due to their demonstrated inhibitory activity against tumorgenesis, and a comparative study of Hakimuddin *et al*. (2008) proposed that total polyphenolic isolated from wine were more effective in reducing tumor growth as compared with a hydrophobic polyphenol fraction isolated from the wine. Analyses of gene expression showed that genes belong to signaling pathways were down-regulated in the induced tumor of grape polyphenol-treated mice.

Flavonoids are a further constituent of agrimony extracts that may exerted anti-tumor effects, and a study carried out by Aheren and O'Brien (2000) showed that flavonoids from different fruits and vegetables were capable to modulate H_2O_2-induced DNA damage in two cell line models (colon carcinoma and hepatic cell carcinoma). In agreement with such finding, it has been demonstrated that flavonoids are acting as a scavenging system, in addition to

their role in arresting the DNA replication in S phase, and inducing apoptosis due to the inactivation of *BCL-2* gene as reported in different types of leukemais (Elangovan *et al*., 1994; Caceres-Cortes *et al*., 2001; Pellecchia and Reed, 2004). Such findings suggest that polyphenolic fraction may affect the topological state of the DNA by increasing or decreasing the level of cleavage of DNA topoisomerases, especially DNA topoisomerase II; causing DNA topoisomerase II poisoning (Gonzalez de Mejia *et al*., 2006). The DNA topoisomerase II is a ubiquitous enzyme that regulates DNA unwinding and removes knots and tangles from the genetic material by creating transient breaks in the sugar phosphate back bone of the double helix (Fortune and Osheroff, 2000). Topoisomerases alo maintain genomic integrity during this process by forming covalent attachments between active site residues and terminal DNA phosphates that are generated during the cleavage reaction (Champoux, 2001).

Several *in vitro* studies have demonstrated these selective tumoricidal action of natural products are occurred without harming the normal cell (Parakash and Gupta, 2004; Griffin *et al*., 2007), a finding which was also observed in the present study against the MEF cell line. In this context, Weber (2009) demonstrated that that the secondary metabolites flavonoids of *Scutellaria* are not only cytostatic but also cytotoxic to various human tumor cell lines *in vitro* and inhibit tumor growth *in vivo*, but most importantly, almost no or minor toxicity of these flavonoids against normal epithelial and normal peripheral blood and myeloid cells was observed.

To conclude the present discussion, the investigated agrimony extracts were able to modulate the immune-suppressive, mutagenic and hepatotoxic effects of CCl_4 in mice, and their modulations shared the effect of vitamin C, which is a well-known anti-oxidant. 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 and Recommendations

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• Conclusions

1. Different active compounds were detected in the aqueous and methanol extracts of agrimony (*A. eupatoria*) including flavonoids, tannins alkaloids and glycosides**.** The flavonoid compound rutin was also detected using the TLC method.
2. Aqueous and methanol extracts of *A. eupatoria* showed different effects on the investigated parameters, especially the mitotic index, which showed a significant increase, and the spontaneous micronucleus formation and sperm-head and -tail abnormalities, which showed a significant decrease, in addition to their enhancing effects on the immune system by increasing total and absolute counts of leucocytes.
3. Both agrimony extracts were significantly effective in modulating the immunosuppressive and mutagenic effects of CCl_4, and the biochemical parameters confirmed the hepatoprotective effect of agrimony extracts through decreasing or normalizing the level of serum enzymes and repairing the induced cellular damage of liver.
4. Vitamin C was significantly effective in modulating the immunosuppressive and mutagenic effects of CCl_4 especially in decreasing the micronucleus formation and sperm-head and -tail abnormalities. A similar outcome was observed when the hepatoprotective effects were considered. Agrimony extracts (aqueous and methanol) were similarly effective as vitamin C or even better in increasing the total and absolute counts of leucocytes, with the exception of lymphocytes, while in

biochemical assessments, the aqueous extract was better than the methanol extract and vitamin C in this regard.

5. Both agrimony extracts were *in vitro* effective as anti-tumor agents, and the affect was dependent on the cell line (tumor or normal cell line) that was investigated.

- **Recommendations**

1. Isolation and characterization *A. eupatoria* active compounds and investigating their immunological, cytogenetic and histopathological effects *in vitro* and *in vivo*, in addition to their anti-tumor effects by a more advanced methodologies and parameters; for instance, sister chromatid exchanges, commet assay, apoptotic consequences, cytokine levels and liver immunohistochemical and *in situ* hybridization for the expression of immunological markers. Further studies are also needed to reveal the anti-oxidant potentials of the plant extract.
2. The forthcoming evaluations can also be explored, but this testing the synergistic potentials of the active compounds and other modulators, for instance vitamins and cytokines.

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Appendix I: General laboratory equipments

Equipment	Company / Country
Autoclave	SES little Sister / England
Centrifuge	Beckman / England
Cooled incubator	Memmert / Germany
Digital camera	Mercury / China
Electrical balance	Sartorius / Germany
ELISA reader	Ovganon Teknika
Hemocytometer	Neubauer / Germany
icroscope	Motic / Japan
Incubator	Memmert /Germany
Laminar flow hood	Heraeus / Germany
Microfuge	Eppendorf / Germany
Micropipette	Gilson / France
Microtome	Gallenkamp / England
Oven	Osaw / India
pH meter	Radiometer / Denmark
Rotary evaporator	Buchi / Switzerland
Shaking water bath	Gallenkamp / England
Soxhlet	Electrothermol / England
Vortex	Giffin / England
Water bath	Gallenkamp / England

Appendix II: Chemical materials.

Chemical Material	Company / Country
Alkaline phosphatase Kit	Bio Merieux / France
ALT Kit	Randox Company/U.K
AST Kit	Randox Company/U.K
Calcium chloride	BDH / England
Canada Balsam	BDH / England
Carbon tetrachloride	BDH / England
Chloroform	BDH / England
Colchicine	Ibn Hayan /Syria
Eosin stain	BDH / England
Ethanol	Ferak / Germany
$FeCl_2$	Fluka / Switzerland
Fetal calf serum	Sigma / U.S.A.
Giemsa stain	Fluka / Switzerland
Glacial acetic acid	Fluka / Switzerland
Glycerin	Fluka / Switzerland
Haematoxylin stain	BDH / England
Heparin	Leo Pharmaceutical / Denmark
Human plasma	Biotest pharma /Germany
Hydrochloric acid	Sigma / U.S.A.

Lead acetate (CH3Coopb)	Fluka/ Switzerland
Lieshman Stain	Merck/ Germany
Mercuric oxide (red)	BDH / England
Methanol	Fluka / Switzerland
Methylene Blue	Fluka/ Switzerland
Neutral Red	Sigma / U.S.A.
Penicillin	Sigma / U.S.A.
Potassium Chloride (KCl)	Fluka / Switzerland
Potassium hydroxide (KOH)	Fluka / Switzerland
Potassium Iodide (KI)	Fluka / Switzerland
RPMI-1640	Sigma / U.S.A.
Sodium bicarbonates ($NaHCO_3$)	BDH / England
Sodium hydroxide (NaOH)	Sigma / U.S.A.
Streptomycin	Sigma / U.S.A.
Trypan Blue stain	Pharmaciafine chemical Uppsala / Sweden
Trypsin	Sigma / U.S.A.
Versene	BDH/ England
Vitamin C (ascorbic acid)	Drugs factory of Samara / Iraq
Xylene	BDH / England

خلاصة

اجريت الدراسة بهدف تقييم بعض التاثيرات المناعية (العد الكلي والمطلق لخلايا الدم البيض) والوراثية-الخلويه (معامل انقسام نقي العظم وتكون النوى الصغرى في خلايـا نقـي العظم وتشوهات رووس واذناب النطف) والكميائيـه الحياتيـة (AST, ALT and ALP) , والنسجية المرضية للكبد والمضادة للأورام وبإسـتخدام خطـين مـن الخلايـا السـرطانية (Human cervical cancer; HeLa and Rhabdomyosarcoma; RD cell lines) وخط من الخلايا الطبيعية (Mouse embryonic fibroblast; MEF) للمستخلصين المائي والكحولي لنبات الغافث (*Agrimonia eupatoria*) وتأثيراتهما في الحـد مـن الاضـرار الكبدية المستحثه لرابع كلوريد الكاربون. وفضلا عن ذلك فقد أجري كشفا كيميائية للتحـري عن وجود الفلافونات والتانينات ومركبات فينولية أخرى في المستخلصين، كمـا حلـل المستخلصين بطريقة كروماتوغرافيا الطبقه الرقيقه للكشف عن انواع الفلافونات الموجـودة في المستخلصين. اجريت هذه التقييمات من خلال ثلاثة مراحل.

شملت المرحلة الاولى دراسة تاثير ثلاثة جرع من مستخلصي النبات (1.0،2.0،3.0 ملغم/كلغم) وجرعة واحدة (3.2 ملغم/كلغم) من رابع كلوريد الكـاربون. زرقـت المـواد المدروسة في غشاء الخلب كجرعة واحده في اليوم الواحد (0.1 مل) ولمد ة ستة ايـام وفـي اليوم السابع ضحي بالحيوانات لغرض اجراء التقييمات المختبرية. وفي حالة رابـع كلوريـد الكاربون فقد زرق الحيوان بجرعه واحدة تحت الجلد في اليوم الاول وضحي بـالحيوان فـي اليوم الثاني. وفي المرحلة الثانية اجري تداخل مابين الجرعة المثلى (3.0 ملغم/كلغم) لكـل مستخلص ورابع كلوريد الكاربون. وفي هذا التداخل زرقت الحيوانات بجرعة واحدة من رابع

كلوريد الكاربون في اليوم الاول اما في اليوم الثاني وحتى اليوم السادس فقد زرقت الحيوانات بجرعة واحدة من كل مستخلص ثم ضحي بالحيوانات في اليوم السابع لغرض التقييم المختبري. اما في المرحلة الثالثة فقد تم تقدير النسبة المئوية لتثبيط النمو لخمسة تراكيز من كل مستخلص (6, 12, 24, 48, 96 مايكروغرام/مل) في الزجاج باستخدام خطين من الخلايا السرطانية (HeLa and RD) وخط من الخلايا الطبيعية (MEF).

اظهرت نتائج الكشف الكميائي بان المستخلص المائي للنبات كان موجبآ للفلافونات والتانيينات والقلويدات، اما المستخلص الكحولي فقد كان موجبآ للفلافونات والتانيينات والقلويدات أيضآ. كما اظهر تحليل كروماتوغافيا الطبقة الرقيقة احتواء المستخلص المائي على اربعة مركبات فلافونية (Rutin, Myricetin, Azoleatin, Vitexin and Iso-orientin)، في حين احتوى المستخلص الكحولي على خمسة مركبات فلافونية (rutin, kaempferol, quercetin , isorhamnetin and myricetin).

اظهرت النتائج بأن معاملة الحيوانات برابع كلوريد الكاربون قد تسبب بانخفاض معنوي في العد الكلي والمطلق لخلايا الدم البيض وكذلك ازدادت النسب المئوية لتكون النوى الصغرى المستحثة وتشوهات رؤوس واذناب النطف وانخفضت الفعالية الانقسامية لخلايا نقي العظم. وعلى العكس من ذلك فقد كان للمستخلصين المائي والكحولي للنبات فعلا مؤثرا في المحافظة على قيم المعاملات المدروسة خصوصا معامل الانقسام والذي ارتفع معنويا وتكون النوى الصغرى المستحثة وتشوهات الرؤوس واذناب النطف واللذين انخفضا معنويا. اعتمدت درجة فعل هذين المستخلصين على جرعة ونوع المستخلص.

جاءت نتائج التداخل مابين رابع كلوريد الكاربون ومستخلصي النبات معززا للتاثيرات ألانفة الذكر وكان للمستخلصين فعلا معنويا مؤثرا في تعديل التأثيرات المثبطة مناعيآ

والمطفرة وراثيا لرابع كلوريد الكاربون, وكانت هذه التأثيرات معتمدا على مقـدار الجرعـة ونوع المستخلص والعامل المدروس.

اظهرت الفحوصات النسجية-المرضية لمقاطع الكبد في الحيوانات المعاملـة برابـع كلوريد الكاربون تنخر وتنكس للخلايا الكبدية. كما ارتفعـت معنويـا المسـتويات المصـلية لانزيمات وظائف الكبد (AST, ALT and ALP). وعلى العكس من ذلـك فقـد اظهـرت المعاملات الكميائية الحياتية دور مستخلصي النبات في حمايـة الكبـد مـن خـلال خفـض المستويات المصلية لهذه الانزيمات فضلا عن الاصلاح الخلوي للانسجة الكبدية و لم يكن ذلك الاصلاح كاملا فقد لوحظت بعض الاضرار الكبدية بعد المعاملة بالمستخلصين.

جاءت نتائج المرحلة الثالثة لتظهر أمتلاك التراكيـز الخمسـة لمستخلصـي النبـات المواصفات المضادة للاورام وكان التأثيرمعتمدا على التركيز. كما سجل المستخلص الكحولي نتائج افضل من المستخلص المائي في هذا الصدد في الخطين الـورمين (HeLa and RD)، في حين لوحظت قيم اقل بكثير من التثبيط في الخط الخلوي MEF.

الإهداء

إلى قبس النور الذي أضاء طريقي و هيأ لي أسباب النجاح ... إلى سندي في الحياة و فخري و اعتزازي... والدي حفظه الله و مد في عمره.

إلى البساطة و الطيبة... إلى بحر الحنان و الحب...نور عيني ...والدتي حفظها الله و أمد في عمرها.

إلى أحبائي و قرة عيني و سندي في مسيرتي...اخوتي وأخواتي ثروتي الأخرى في الحياة: يعرب, نوفل, نبيل, أستبرق, رونق, فرزدق, محمد, غسق.

إلى رفقة دربٍ لن أنسى لهم ما قدموه لي ما حييت...

عروبه

بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

قَالَ علمُهَا عِندَ رَبِّي فِي كِتَابٍ

لَا يَضِلُّ رَبِّي وَلَا يَنسَى (٥٢)

صدق الله العظيم

سورة طـــه

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية العلوم

دراسات كيموحيويه، مناعيه، ونسيجيه عن تأثير مستخلصات نبات الغافث في ذكور الفأر الابيض

رسالة
مقدمة الى كلية العلوم– جامعة النهرين
وهي جزء من متطلبات نيل درجة ماجستير علوم
في التقانة الإحيائية

من قبل
عروبه ناظم حربي البديري
بكلوريوس أحياء مجهرية/ كلية العلوم/الجامعة المستنصرية/1997

بإشراف

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ذي الحجة 1430 كانون الاول 2009