Republic of Iraq

Ministry of Higher Education and Scientific Research

Al- Nahrain University/ College of Science

Department of Biotechnology

Cytotoxic Evaluation of *Alchemilla vulgaris* Flavonoids in Normal Blood Lymphocytes

A thesis

Submitted to College of Science/ Al-Nahrain University as a partial fulfillment of the requirement for the Degree of Master of Science in Biotechnology

By

Saif Luay Al-osaj

B.Sc. Biotechnology/ College of Science /Al-Nahrain University (2013)

Supervised by

Dr. Khulood W. Abbood

(Professor)

July 2016 Shawwal 1437

بسم الله الرحمن الرحيم

أَلَمْ تَرَ أَنَّ اللَّهَ أَنزَلَ مِنَ السَّمَاءِ مَاءً
فَسَلَكَهُ يَنَابِيعَ فِي الأَرْضِ ثُمَّ يُخْرِجُ بِهِ
زَرْعاً مُّخْتَلِفاً أَلْوَانُهُ ثُمَّ يَهِيجُ فَتَرَاهُ
مُصْفَرّاً ثُمَّ يَجْعَلُهُ حُطَاماً إِنَّ فِي ذَلِكَ
لَذِكْرَى لأُوْلِي الأَلْبَابِ

سورة الزمر21

Acknowledgments

First of all Praise to Allah the lord of the universe, peace be upon Mohammed the messenger of Allah and upon his relatives and companions.

Great thank for my supervisor Professor ***Dr. Khulood W. Abbood*** *for her support and important guidance throughout my study.*

I would like to extend my thankful, respect and appreciation to ***Dr. Zainab Yaseen***, ***Dr. Rehab subhi*** *and* ***Dr. shahla`a Mahdi*** *who had given me a hand, support, and important guidance throughout this study.*

My deep appreciation to the Head of Biotechnology Department College of Science of Al-Nahrain University: prof. ***Dr. Hameed M. Jasim*** *and all staff of Biotechnology Department for their support*

I would gratefully like to express my gratitude and feelings of love to my mother, sisters and my dear wife for their encouragement, patience and the many sacrifices they presented during all stage of my life.

To all these and other forgotten names who assist me in accomplishing this study, my great thankful and gratitude.

For all of them, I said "thank you"…

Summary

The discovery and identification of a new drugs, which can potentiate the immune function has become an important goal of researches in immune-pharmacology. This study demonstrates the favorable effects of wild type *Alchemilla vulgaris* active components as immunomodulating agent. *A. vulgaris* is an important medicinal plant, represented a rich sources of biologically active phytocompounds, which are beneficial for humans. *A. vulgaris* which is widely known as lady's mantle, bear's foot or lion's foot is traditionally used due to their tannin content for the treatment of inflammation of the upper digestive tract, diarrhea internally and as wound healing and astringent externally. It is also used as gargle against mouth and throat inflammation. Lady's mantle is reported to be an important remedy in folk medicine in Europe. It is used to heal inflammations in mouth, bleeding of the nose and gynecological diseases. The plant aerial parts dried and extracted in 80% methanol by maceration. Chemical detection of crud plant extracts was performed. The total flavonoids were isolated, subjected to thin layer chromatography (TLC) using different mobile systems. The purified material was read by using high performance liquid chromatography (HPLC).A.*vulgaris* contain total flavonoids (9.8mg/g dried whole plant) calculated as Rutin. *A.vulgaris* also contains quareciten and kaempferol, both of them were investigated qualitatively and quantitatively in the present study but their quantities in this herbal plant were less than Rutin.The immunomodulation occur by treated extracted semi-purified flavonoids with Lymphocytes isolated from healthy people to measure the cytotoxic effect of extracted Flavonoids on normal lymphocytes by using MTT dye. MTT is widely chosen as a cell viability-measurement for optimal end point. MTT is a

yellow water-soluble tetrazolium dye {3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide} reduced by live, but not the dead cells to a purple formazan product that is insoluble in aqueous solution. The cytotoxic evaluation was read by using sandwich ELISA technique at 450nm. The result of treatment the isolated flavonoids with normal lymphocytes show significant effect by suppressing the lymphocytes proliferation with the decrease in the extracted flavonoids concentration as compared with control (untreated lymphocytes in suitable media). The immunomodulation effects of *A.vulgaris* were caused alteration in the IL-2 & IL-10 level estimated by ELISA technique, The extracted flavonoids cause significant increase in both IL-2 and IL-10 level that produced from treated lymphocytes as compared with negative control (normal lymphocytes with media). The reduction in the extracted *A.vulgaris* concentration accompanied with significant increase in both IL-2 and IL-10 in 2hours and 4hours. The present study reveal the effects of semi-purified flavonoids on IL-2 and IL-10 production indicated by alteration in gene expression of normal lymphocytes in vitro toward IL-2 and IL-10 production in response to all concentrations.

1.1 Introduction

Alternative treatment methods have been used for curing the disease and for amelioration health for hundreds of years. Studies on the alternative herbal treatment usage in the community have shown a constantly increasing rate. It is reported that (42.1%) of the United State population use alternative treatment methods. The rate of using an alternative treatment method at least once is (48%) in Australia, (70%)in Canada,(90%) in Germany and (75%)in France [al-Handh et al 2012].

Plants that have therapeutic properties or may do beneficial pharmacological effects on the human body are commonly called medicinal plants. Medicinal plants naturally produced and generally accumulate some secondary metabolites such as alkaloids, flavonoids, steroids, terpenes, saponines, glycosides, tannis and volatile oil.

The medicinal plants have been used for treatment of illness and diseases, since the dawn of history. Ancient Chinese scriptures and Egyptian papyrus hieroglyphics explain the uses of medicinal plants. Indigenous culture mostly used plants for curing rituals, nevertheless other people evolve traditional medical system such as Ayurvedic in which herbal therapies are being used.(motaleb 2011).

Alchemilla vulgaris or lady's mantle, is an uncommon herbaceous member of the rose family (Rosaceae).There are approximately 300 species of *Alchemilla* native to Europe and Asia, although fewer species are commonly cultivated. Lady's mantle has been used for many centuries in Europe and Asia. Some experts consider ladies mantle to be good for treating wounds due to its coagulation (blood clotting), astringent and styptic (stops bleeding) properties, anti-convulsant, anti-inflammatory. It has also been used as a mouth rinse after dental

procedures to help stop bleeding. A.vulgaris is useful in a variety of female conditions such as menstrual disorders including excessive menstruation and menopause, as an aid during conception, in the prevention of miscarriages, and to help the body heal after childbirth. (Bradshaw *et al.*, 2006).

Immune cells which are Monocytes, macrophages, NK cells, dendritic cells, T lymphocytes and B lymphocytes play an important role in the immune system. Immune cells can recognize the antigen, activate immune cells and then generate a series of immune responses, including innate and acquired immunity. Therefore, the study of the effects of polysaccharides on the immune cells is of great significance. (Chen *et al*., 2008).

Lymphocytes are small white blood cell (leukocyte) that play large role in defending the body against disease. There are two main types of lymphocytes: B cells and T cells. The B cells make antibodies that attack bacteria and toxins while the T cells attack body cells themselves when they have been taken over by viruses or have become cancerous. Lymphocytes secrete products (lymphokines) that modulate the functional activities of many other types of cells and are often present at sites of chronic inflammation.

During the functioning of the immune system, such as in phagocytosis, reactive oxygen and nitrogen species are generated. If they are left unchecked they can affect the components of the immune system by inducing oxidative damage. This is more so in the elderly or during inflammation where there is excess generation of these reactive species than can be taken care of by the defenses in the form of antioxidants. Dietary supplementation with antioxidants may greatly help in such conditions. There are some indications of possible benefits of antioxidant

supplementation. Natural compounds (alkaloids, polyphenols,..) from medicinal plants having antioxidant and immunomodulatory activities have potential as therapeutic agents in this regard.(Devasagayam, Sainis *et al.*, 2002)

Another major constituent of *Alchemilla vulgaris* is flavonoids which comprise a large class of low-molecular-weight plant metabolites ubiquitously distributed in food plants. These dietary antioxidants exert significant antitumor, antiallergic, and anti-inflammatory effects. The molecular mechanisms of their biological effects remain to be clearly understood (Nair *et al.*,2006).

1.2. Aims of this project

1) Investigation of the cytotoxic effect of the *Alchemilla vulgaris* estimated flavonoids on lymphocytes by MTT assay.

2) The Effect of *Alchemilla vulgaris* flavonoids on cytokines level in lymphocytes cultured cells represented by (IL-10& IL-2) as a mediators of immune regulation by ELISA technique at cellular level.

1.3 Literatures Review

1.3.1. *Alchemilla vulgaris*

Alchemilla was named in the 16th century by Jerome Bock, also known as Tragus, and it appears under his name in the book History of Plants, published in 1532. Alchemilla is referred to as lady's cloak or mantle because of its association with the Virgin Mary. The lobes of the leaf are said to resemble the scalloped edges of a mantle. It has also been referred to as lion's foot and bear's foot, most likely because of the resemblance of its spreading root leaves to such feet. (*Fraisse and Carnat et al 1999)*

Scientific classification	
Kingdom:	Plantae
Order:	Rosales
Family:	Rosaceae
Subfamily:	Rosoideae
Tribe:	Potentilleae
Genus:	*Alchemilla*

Figure (1-1) Scientific Classification of Alchemilla vulgaris

Lady mantles are invaluable garden plants; perhaps not the flashiest, but easy to grow in diverse landscapes, and the perfect foil for many other perennials. The vibrancy of their chartreuse flowers is singular, while a string of pearly dewdrops accentuates the elegance of the foliage. Who hasn't marveled at a raindrop moving languidly over the leaf surface like a bead of mercury? The discovery of an early morning, dew-covered lady's mantle delights children and seasoned gardeners alike.

Alchemilla, or lady's mantle, is an uncommon herbaceous member of the rose family *Rosaceae*. The insignificant flowers, which lack eye-catching petals, *Alchemilla* do not have much freatures like flamboyant cousins like roses (Rosa) and cinquefoils (Potentilla). There are

approximately 300 species of Alchemilla native to Europe and Asia, although fewer species are commonly cultivated.

Most lady's mantles are mounded, clump-forming perennials with basal leaves arising from woody rhizomes. The palmately lobed to divided leaves are typically fan-shaped with small apical teeth. The long stalked grey-green to green leaves are often covered with soft hairs, which slow water drops on the surface and along the margins. The green to bright chartreuse flowers are small, apetalous and borne in clusters above the foliage in late spring and summer.

Lady's mantles grow best in moist, well drained soils in full sun to partial shade. Extra water may be required in full sun, especially in high temperature areas. Drought and stress can cause leaves to shrivel, but new leaves sprout quickly if cut back. Lady's mantles self-sow readily; dead-heading reduces the number of seedlings produced as well as regenerate the plants. Lady's mantles does not suffer from any significant disease or pest problems.

The small stature of lady's mantles is ideal for the front of perennial borders, as edging along paths and walls, in rock gardens and as ground covers. The grey-green leaves of *Alchemilla mollis* and other species blend well with many colors in the garden, including purple, blue, pink, yellow and white. Lady's mantles combine well with hardy geraniums (*Geranium*), bellflowers (*Campanula*) and sages (*Salvia*), as well as foliage plants like coral bells (*Heuchera*) and ferns.*(Hawke et al 2004)*

1.3.2. Phytochemical Contents of *Alchemilla vulgaris*:

The anti-microbial, anti-oxidation, and anticancer activities of this herbal plant is due to it`s pharmaceutical contents. The pharmaceutical compounds found in the lady mantle are flavonoids, Tannis, phenols and saponine. But the flavonoids considered as the major content. It is very well known that the amount of bioactive compounds in plant extracts may be subjected to change according to several factors such as growth stages, cultivation, insect invasion, season of collection and the method of extraction. *(Ding W et al 2014*).

Duckstein *etal., 2012* tested the phenolic constituent of *Alchemilla vulgaris* and *Alchemila mollis* and they noticed that the sanguiin H-10, castalagin/vescalagin, and galloyl-bis- hexahydroxydiphenoyl (HHDP) hexose constituted the major phenolic fraction of both plant species. Also, gallic and chlorogenic acids were found in both extracts.

Denev , Kratchanova and Ciz (2014) worked on the *Alchemilla glabra* aerial part to investigate the phytochemical compounds in this plant. By comparing the Alchemilla with five other herbal plants, they founded that the Alchemilla is the richest source of flavonoids, containing catechin (250 mg/100 g), epicatechin (524 mg/100 g) and a significant amount of rutin (1057 mg/100 g), as described in the table below:

Component	Quantity (mg\100g)
Gallic acid	63
3,4-dihydroxy-benzoic acid	135
Chlorogenic acid	80
Caffeic acid	151
Totlal phenolic	429

Catechin	250
Epicatechin	524
Rutin	1057
Total flavonoids	1831
Total polyphenols	8377

Table (1-1) Alchemilla vulgaris components

1.3.3. Parts used medically:

The whole herb plant is used medically after it`s collected at the flowering seasons which is in June and July Sometime the roots also can be used medically.

1.3.4. Medical uses of *Alchemilla vulgaris* and their benefits:

1- Vasorelaxant and blood pressure lowering:

Takır *et al.*, 2015 demonstrated that the methanol extract of *Alchemilla vulgaris* has more prominent and favourable vascular effects in normal and experimental hypertensive conditions reinforcing its traditional use in cardiovascular disorders, in particular hypertension, because *A.vulgaris* produced relaxations in $PGF_{2\alpha}$ (3×10^{-5} M) pre-contracted arteries which were insensitive to the inhibitors of endothelium derived vasoactive substances namely, L^G-nitro-L-arginine (10^{-4} M), ODQ (10^{-5} M) and indomethacin (10^{-5} M) or removal of endothelium.

2-Antioxidant and Antimicrobial Activities:

Ergene and Acikara (2010) worked on the extracts prepared using aerial parts and roots of Alchemilla persica Rothm. They evaluated for their antioxidant activity by using 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay and measurement of malondialdehyde (MDA) levels. HPLC analyses of the extracts were also performed using some

phenolic acid and flavonoid standards. The hydro-methanolic extract of the aerial parts was found to possess significant antioxidant activity in both assays.

Denev and Kratchanova **(**2014**)** provides a comprehensive data on the antimicrobial activities of aerial parts of *A.vulgaris* as compared with other five medicinal plants**.** Denev and Kratchanova revealed the antimicrobial activity against *Escherichia coli, Staphylococcus aureus, Proteus vulgaris, Klebsiella pneumoniae* and *Candida albicans.*

3-Anti-influenza activity:

Makau and Watanabe (2013) explained the using of *Alchemilla mollis* as traditional medicine in Europe for different indications for minimizing the symptoms of a sore throat. The exposure of the virus to *A. mollis* extract prior to infection and treatment of cells during virus infection significantly suppressed plaque formation. Influenza virus induced hemagglutination of chicken red blood cells was inhibited by *A. mollis* extract treatment. The inhibitory effect was observed against influenza A virus subtypes H1N1, H3N2, and H5N2.

4-Wound Healing:

Shrivastava and Cucuat (2007) studied the effects of *A. vulgaris* on cell growth. Shrivastava and Cucuat investigated in Chang liver and Madin Darby Bovine Kidney (MDBK) epithelial cell lines and rat aortic myofibroblast cultures. In rats *A. vulgaris* (3%)-treated lesions were significantly decreased in diameter by 10.0 ± 0.7% ($p < 0.005$) after 2 days of treatment. Shrivastava and Cucuat demonstrated that the wound-healing properties of A. vulgaris associated with promitotic activity in epithelial cells and myofibroblasts.

5-Weight Loss:

Said and Saad (2011) prepared a mixture of extract of four plants used in traditional Arabic and Islamic medicine as well as in European herbal medicine, assessed for its safety and efficacy in weight loss. Leaves of *Alchemilla vulgaris*, *Olea europaea* and *Mentha longifolia* L., as well as seeds of *Cuminum cyminum*, were used. Progressive and significant weight loss was observed in chickens given this mixture weekly for 4 weeks compared with controls.

1.3.5. Principles for *In Vitro* Determination of Cell Toxicity (Cytotoxic assay):

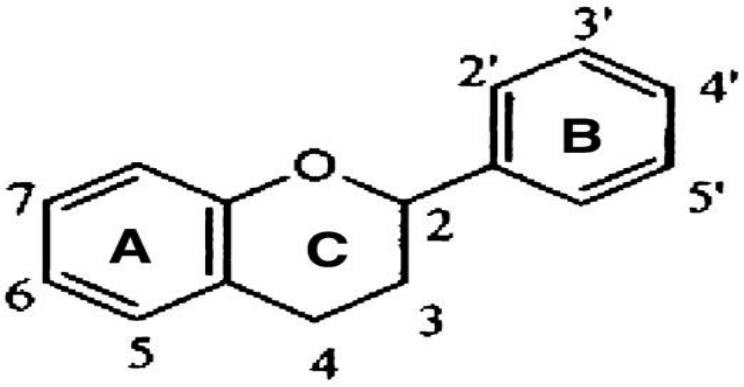
Once a cell is explanted from its normal *in vivo* environment, determination of viability in the course of experimental manipulations, becomes fundamental. These determinations can over simply all events and measurements that employed in the test because they are cheap, easily quantified and reproducible. The traditional approach to cytotoxicity is concentrated on cell growth or survival which can be measured by cloned growth, net change in population size and change in cell mass or metabolic activity as in MTT reduction or other spects. (Freshney, 2012).Definition of cytotoxicity varies (Kroemer *et al.*, 2009),depending on the nature of the study and whether cells are killed or phenotypically altered. In addition cells may die by necrosis, apoptosis, self –digestion or may become terminally differentiated(Galluzi *et al.*,2009).Where as in an anticancer agent; assay may require a cytocidal effect(cell killing) of that agent(Freshney, 2012).

Among the Applications of cytotoxicity assays are: anticancer drug screening(by staining the cells with MTT), predictive drug testing for tumors(which has been considered as measurement of the chemo-sensitivity of cells derived from a patient's tumor) and testing

pharmaceuticals to maintain an *in vitro* program that might enforcing with animal tests for an *in vivo* assay(Freshney, 2012).

1.3.6. Pharmacological Activities of Flavonoids:

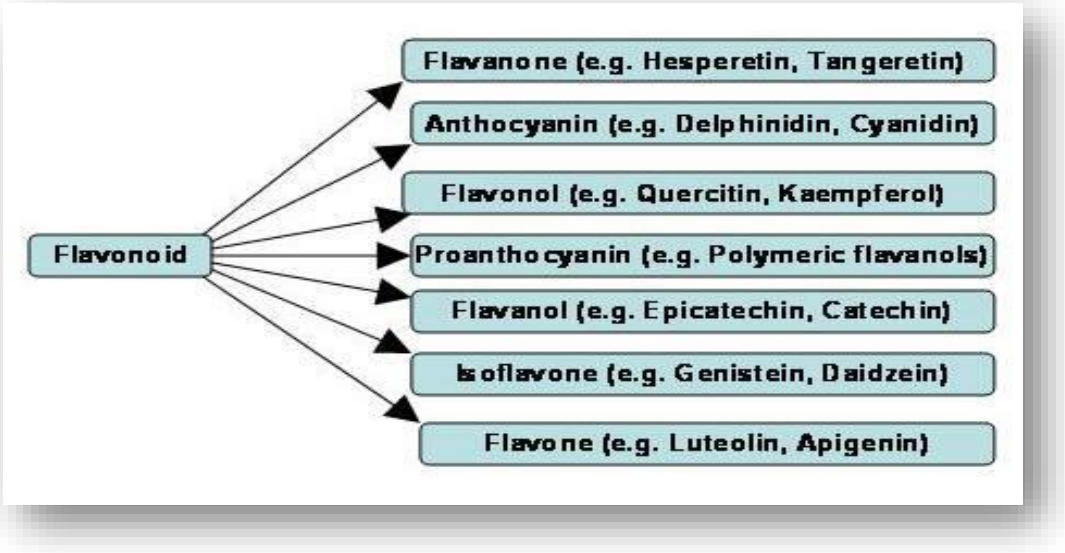
Flavonoids are polyphenol compounds that are ubiquitous in nature. Flavonoids are natural products widely distributed in plant kingdom and currently consumed in large amounts in the daily diet (Hanneken *et al.*,2006).



3): Figure (1-3) Basic structure of flavonoids

Tsuchiya *et al.*, (2010) suggested that flavonoids differ in their arrangement of hydroxyl, methoxy and glycosidic side groups and in the conjunction between A and B rings. A variation in C ring provides division of subclasses figure (1-3). According to their molecular structure.

Figure (1-4) show the subclass of Flavonoids:



flavonoids have been reported to exert wide range of biological activities. These includes: anti-inflammatory, antibacterial, antiviral, antiallergic, cytotoxic antitumour, treatment of neurodegenerative diseases, vasodilatory action.

In addition flavonoids are known to inhibit lipid-peroxidation, platelet aggregation, capillary permeability and fragility, cyclooxygenase and lipoxygenase enzyme activities, flavonoids exert these effects as:-

1. Antioxidants, free radical scavengers, chelators of divalent cation. (Narayana *et al., 2001*).
2. In treatment of hepatotoxicity: Flavonoids bind to subunit of DNA-dependent RNA polymerase I, thus activating the enzyme. As a result, protein synthesis gets increased leading to regeneration and production of hepatocytes (Murray, 1998).
3. In treatment of allergy: Flavonoids inhibit cyclic AMP phosphodiesterase and calcium-dependent ATPase which are responsible for histamine release from mast cells and basophils (Murray, 1998).
4. In treatment of inflammation: Flavonoids have been found to be prominent inhibitors of COX or LOX (Pal *et al.*, 2009).

1.3.7. Immunomodulation

It`s refers to the action undertaken by the medication on autoregulating processes that steer the immunological defense system. A lot of antihomotoxic medication intervene here and are more than useful as beside a proven therapeutic action they are extremely safe. The microdoses or nanodoses used in antihomotoxic medications exclude intoxication by the therapeutic components which are used in the formula, a phenomenon we often see in conventional medication. Macrodoseses have, beside the blocking effect they often induce, often side effects or interactions with other medications or substances (like alcohol) as consequence.

In the subtle regulations induced by a microdoses or nanodoses of cytokines and other mediators, only microdoses or nanodoses therapy is at his place.

As time is not in favour of the cell as it comes under the influence of dysregulation and intoxication, the sooner detoxification and drainage of homotoxines take place, the better it is for the cell. The Detoxification consider as the pillar of homotoxicological treatment.

The second pillar is immunomodulation. As the fastest cleansing of the extra cellular matrix is an active defense system, immunomodulation is very important in a homotoxicological treatment protocol, especially in the case of chronic disease. To activate or regulate immune reactions not only put the defense system on the right purposeful level of action, it also keeps the clinical symptoms of inflammation within for the patient acceptable levels, or stimulate a non-reactive immune system.

1.3.7.1. Basic actors of defense scene(aleckearg *et al.*, 2007)_

1) APC's: antigen presenting cells will use phagocytosis to process and present antigens. Characteristic proteins of the antigen will be presented at the external wall of the APC in a changed MHC class, to chemotactically attracted T-lymphocytes (naïve prolymphocytes/TH0).

The antigen characteristic (antigen peptide) will be taken from the MHC of the APC and bind to the TCR (T-Cell Receptor). From that moment on the T-cell becomes a fully in charge helper cell (T-helper cell) that will focus on the antigen defense and stimulate other actors of the defense system to eliminate it.

As the motif or pattern on the TCR of the TH1 or TH2-cell is specific in function of the antigen, the tasks of the TH1/TH2 are purely to be seen as specific defense.

2) Dendritic cell: dendritic cells play a central role in stimulating and modulating cell mediated responses. Infections have a profound effect on dendritic cells, which in turn interact with T cells and determine whether Th1 or Th2 type responses develop.

3) T-Helper cells: are special subpopulations of CD4+ T cells that provide help to other immune competent cells in mounting immune responses by causing cell activation or the secretion of cytokines. We divide the TH-cells into 3 classes: TH-1, TH-2 and TH-3 cells.

TH-1 cells are responsible for the cellular immunity. They trigger natural killer cells (NK-cells) and macrophages micro organisms or even deviating cells from proper origin (e.g. viral infected organ cells).

TH-2 cells direct humoral immunity. Once triggered by a motif on their TCR their activity results in a stimulation of antibody production (B-

cells, plasma cells) so that antigens outside the cells ('humoral' = body liquids) are eliminated.

An inflammatory process can go into a mainly TH-1 mediated pathway or in a mainly TH-2 mediated one. TH-1 activity will inhibit TH-2 activity and vice versa. It is known that few contacts with micro-organisms in childhood (TH-1 reaction) will increase the risk on TH-2 mediated pathologies like bronchial asthma and other allergic reactions. In healthy persons there is a harmonious balance between TH-1 and TH-2 activity.

TH-3 cells are regulator cells. Their main inflammation regulating mediator is TGF-β, Transforming Growth Factor beta. Treg cells will inhibit both the TH-1 and TH-2 pathway and are therefore down regulating inflammation. In antihomotoxic medicine the stimulation of Treg cells is a common technique to intervene in inflammation processes

1.3.7.2. Enumeration of Lymphocytes Proliferation

The enumeration of cells proliferation is a simple method for measuring lymphocyte activation and proliferative ability, which can be measured either by [3H]thymidine incorporation into DNA synthesis, upon harvesting cell cultures after stimulating lymphocytes artificially with a mitogens, or by measurement of cell functional with the MTT proliferation assay (Freshney,2012). Amplification of the immune response usually involves proliferation of particular subpopulations of lymphoid cells that are normally in the resting state. Thus, lymphoid cell responses to antigens, superantigen, alloantigens, autoantigens, cytokines, and agents such as antibodies, allinduce signals at the cell membrane which are primarily measured by proliferation assays(Passlick*et al.*, 1989).

Proliferative assays have the following applications in clinical studies: (1) Assessment of overall immunologic competence of T cells or B cells as manifested in their ability to respond to polyclonal proliferation signals. Defects in the proliferation may be indicative of fundamental cellular immunologic defect. Low proliferation is often found as a nonspecific secondary effect of chronic disease. (2) Assessment of an individual's response to specific antigens, where low responses are indicative of general or specific immunologic defect. (3) Determination of MHC compatibility by the mixed lymphocyte reaction culture (MLR) (Pileri *et al.*, 1988).

In addition, proliferative assays are useful in basic research for estimating lymphokine production, investigating signal transduction, and assessing growth factor requirements (e.g., lymphokines) for T or B cells. Cell proliferation in response to external stimuli is a very complex process often involving delivery of a signal or set of signals to the cell membrane, activation of intracellular enzymatic pathways that are not well understood, activation and transcription of multiple genes, DNA and protein synthesis, and finally, cell division(Stephen,1994).

1.3.7.3. Interleukin

Interleukin (IL), any of a group of naturally occurring proteins that mediate communication between cells. Interleukins regulate cell growth, differentiation, and motility. They are particularly important in stimulating immune responses, such asinflammation.

Interleukins are a subset of a larger group of cellular messenger molecules called cytokines, which are modulators of cellular behaviour. Like other cytokines, interleukins are not stored within cells but are

instead secreted rapidly, and briefly, in response to a stimulus, such as an infectious agent. Once an interleukin has been produced, it travels to its target cell and binds to it via a receptor molecule on the cell's surface. This interaction triggers a cascade of signals within the target cell that ultimately alter the cell's behaviour.(Abdullah, *et al.*, 2015)

The first interleukins were identified in the 1970s. Initially investigators believed that interleukins were made chiefly by leukocytes (white blood cells) to act primarily on other leukocytes, and for this reason they named them interleukins, meaning "between leukocytes." Because leukocytes are involved in mounting immune responses, interleukins were thought to function only as modulators of immune functions. Although this is an important function of interleukins, it is now known that interleukins also are produced by and interact with a host of cells not involved in immunity and are involved in many other physiological functions. Thus the role that interleukins play in the body is much greater than was initially understood.*(Brocker et al 2010)*

Fifteen different types of interleukins are known, and they are designated numerically, IL-1 through IL-15. The immunological functions of most of the interleukins are known to some degree. IL-1 and IL-2 are primarily responsible for activating T and B lymphocytes(white blood cells integral to bringing about the acquired immune response), with IL-2 being a stimulant of T- and B-cell growth and maturation(*Menachem et al.,2010*). IL-1, along with IL-6, is also a mediator of inflammation. IL-4 often leads to an increase in antibody secretion by B lymphocytes, while IL-12 causes a greater number of the leukocytes cytotoxic T cells and natural killer cells to be made. The set of interleukins stimulated by a specific infectious agent determines which cells will respond to the infection and influences some of the clinical manifestations of the disease. (*Menachem et al.,2011*)

1.3.8. Herbs and Immunity

The using of the plant products as immunostimulants has a traditional history. However, the isolation of the active principles did not gain momentum till the 19th century (Benny *et al.,2004*). A study in 1990 showed that 64% of the world's population use herbal drugs to fighting health problems (Colic *et al.*,2002). Currently, it is estimated that almost 50% of the synthetic medicines are derived from, or patterned as, phytochemicals by which plants synthesize chemicals as part of their defense against pathogens (Wilasrusmee *et al.*, 2002).Many such compounds occur in nature as anti-feedant and anti-infectant chemicals, that are found effective against microbes. Flavonoids and hydroxylated phenols, for example, are naturally synthesized by plants in response to infection (Klein *et al*., 2000). Flavones and flavanones, also have natural anti-feedant effects. Alkaloids are the most common plant metabolites that have important biological effects (Goel *et al.*, 2002). Quinine for example, alkaloid isolated from the bark of the *Cinchona* tree, is the first effective antimalarial drug (Wu *et al.*, 1994). Nicotine, another example of alkaloid, has insecticidal activity (Tao and Lipsky, *et al.*, 2000),

Immunomodulation by using medicinal plants can provide an alternative to conventional chemotherapy for a variety of diseases(Yang *et al.*,2008) especially when host defense mechanism has to be activated under the conditions of impaired immune response or when a selective immunosuppression is desired in situations like autoimmune disorders(Falini *etal.*,2010).Studies have demonstrated that plants possess various biological activities including antitumor and immunomodulatory activity and the capacity of medicinal plants to enhance or reduce the

immune response has been studied. For example: The immunomodulatory effect of garlic on cell mediated immunity has been reported (Colic *et al.,* 2002). Milk thistle has been proven to increase humoral and cellular activity (Wilasrusmee *et al., 2002*). *Ginseng* enhances production of macrophages, B and T cells(Klein *et al., 2000*).Also *Echinacea* act as immune stimulant(Goel *et al., 2002*) while *Tripterygium wilfordi* is used for both anti-inflammatory and immunosuppressive effect(Tao and Lipsky, 2000).

1.3.9. Determination of *Alchemilla vulgaris* Extracts Cytotoxicity using MTT

The colorimetric cytotoxic assay by MTT is widely chosen as a cell viability-measurement for optimal end point. MTT is a yellow water-soluble tetrazolium dye {3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide} reduced by live, but not the dead cells to a purple formazan product that is insoluble in aqueous solution. The amount of MTT-formazan produced can be determined spectro-photometrically once solubilized in suitable solvent after cells have been espoused to different concentration at different time intervals to that material of concern(Abraham *et al.*,2008).

Chapter Two

Material & Method

2. Materials and Methods

Materials used in this work were provided from Different sources; chemicals are supplied from the local markets. Instrument used in this study were present in Biotechnology department laboratory in the college of sciences Al-Nahrain University. Kits Were supplied from different sources by different local companies. The Lymphocytes cytotoxicity by MTT assay for viability of different cell lines and the Cytokine profile were carried out in the Biotechnology Research Centre in Al-Nahrain university. The High-performance liquid chromatography (HPLC) assay was also accomplished in the Biotechnology Research Centre in the Al-Nahrain university.

2.1. Materials

Materials included different types of instruments, apparatus, chemicals and kits.

Table(2-1) Instruments and their Origins

Apparatus	Company / country
Autoclave	Tomy / Japan
Bench centrifuge	VEB / Germany
CO2 incubator	Sanyo-UK
Deep Freezer	Sony/UK
Enzyme Linkede Immuno Sorbent Assay(ELISA) reader	OrganonTeknikamicrowell system reader 230s/Belgium
Eppendorf cool Mirocentrifuge	Eppendorf / Germany
Haemocytometer	Neubaur/Germany
High Performance Liquid Chromatography(HPLC)	Shimatzu/Japan
Hot plate with magnetic stirrer	Gallenkamp / England
Incubator	Sanyo / Japan

Laminar air flow cabinat	Gelaire class 100 gelman instrument/U.K.
Light microscope	Olympus / Japan
Micropipettes	Eppendorf/Germany
pH meter	WTW/ Germany
Phase inverted contrast microscope	Optical/Germany
Rotary evaporator	Quikfit/England
Sensitive balance	Mettlev / Switzerland
Spectrophotometer	Shimadzu / Japan
Tissue culture plate &flask	Nune/Denimark
Thin layer chromatography(TLC) plates(0.5&0.2mm thickness)	Merck/Germany
Vortex	Buchi /Switzerland
Water bath	Memmert / Germany

2.1.2. Chemicals

Most of chemicals were supplied from the local markets as shown in Table (2-2).

Material	Company/origin
Aluminum chloride anhydrous	Laboratory Reagent/India
Amikacin solution	COX \UK
Concentrated hydrochloric acid(HCl) &Glacial acetic acid	Fluka/ Switzerland
Copper sulfate	Sigma/USA
Ceftriaxone , Cefotaxime solution	AGUETTANT \FRANCE
Diethylether& chloroform	GCC/UK
Dimethylsulphoxide(DMSO)	BDH/UK
3-(dimethylthiozol-2-yl)-2,5-dimethyltetrizoliumbromide(MTT)stain	Sigma/USA

Ficoll lymphoprep Flow	laboratory/Scotland
Formic acid BDH/UK	Formic acid BDH/UK
n-hexane & Methanol & Ethanol & Toluene	BDH/UK
Penicillin/streptomycin solution	Hyclone/U.S.A
Phenol crystal Thomas	Baker/India
RPMI1640 with L-Glutamine	Gibo/USA
Sodium citrate & sodium nitrite	Fluka/ Switzerland
Sulfuric acid	Sulfuric acid
Trypan blue dye &Ethidium bromide	US biological/U.S.A.
Quercetin, Kaempferol, Luteolin, Rutin standard powder	Sigma/USA

2.1.3. Kits

All Kits used in this research are listed in Table (2-3).

Kits	Company	Country
IL-2 human (ELISA kit)	US Biological	USA
IL-10 human (ELISA kit)	US Biological	USA

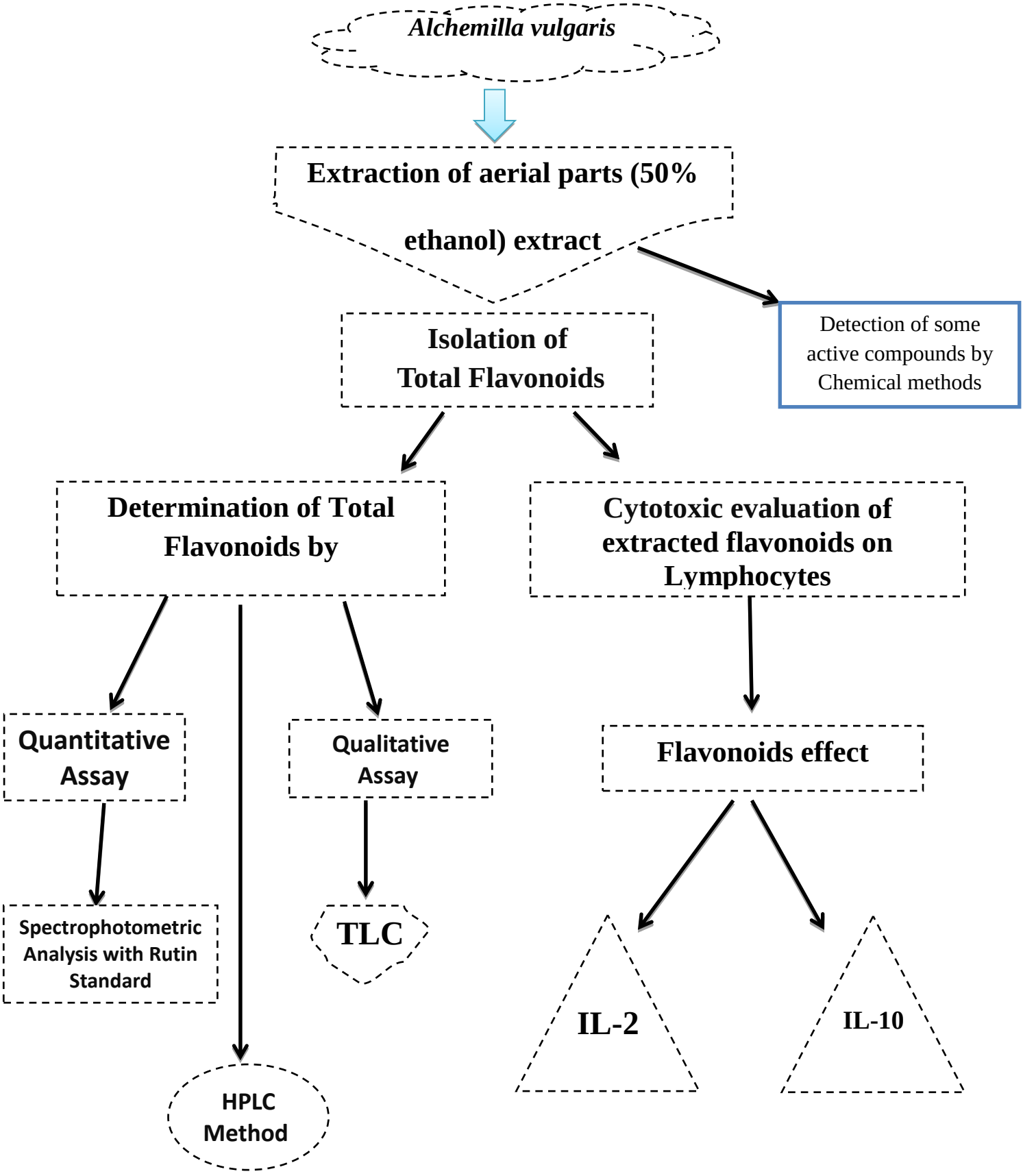


Figure (2-1): Main steps of the research plan

2.2Methodology

2.2.1. Extraction of Flavonoids from *Alchemilla valgaris* (Mingbo *et al*., (2005).)

Extraction method used in this study was a modification of the method exercised by Mingbo *et al*., (2005). Dried and powdered plant material (10gr) was extracted with 50% aqueous ethanol in a flask. The extract was subjected to maceration process in incubator for four hours at 150 rpm, at 50°C. It was left to maceration for 20 hours. Then, extract was filtered using gauze and quantitative filter paper, the extract was evaporated by rotary evaporation at 50c^{o} for 4 hours, then the petroleum ether (40-60c^{o}) were added to the extract and separated by separatory fennels. Then the defatted extract was reflected for 5 hours after filtration using 20 ml of 2M HCl solution. The filtrate was cooled and transferred to a separator funnel. The aglycon moiety was extracted three times each with (50 ml) ethyl acetate. The collected ethyl acetate layers were washed with distilled water to get rid of the excess acid then evaporated to dryness by rotary evaporator at 40ºC .The dried residue was weighted then resolved in 30 ml 50% ethanol. The obtained extract represented the total flavonoids.

2.2.2 Chemical Detection of Active Compounds in the *Alchemilla vulgaris* Extract:

1-Detection of tannins

One gram of plant powder was mixed with 5 ml distilled water in a magnetic stirrer .The mixture was boiled in a boiling water bath for few minutes, then filtered and the filtrate was treated with few drops of 1% lead acetate solution. The development of greenish-blue precipitate is an indicator for the presence of tannins (Evans, 1989).

2- Detection of saponins

A few drops of aqueous extract of the plant was added to 1-3 drops of 3% ferric chloride solution, a white precipitate was developed which indicates a positive result (Alsereita and Abu-Amer, 1996)

3- Detection of terpenes and steroids

One milliliter of ethanol extract was participated in a few drops of chloroform, then a drop of acetate anhydride and drop of concentrated sulfuric acid were added, brown precipitate appeared which representing the presence of trepan, and the appearance of dark blue color after few minutes would represent the present of steroids (Harborne, 1984).

4- Detection of flavonoids

Ethanol extract was partitioned with petroleum ether; the aqueous layer was mixed with the ammonia solution. The appearance of dark color is an evidence for the presence of flavonoids (Harborne, 1984).

5- Detection of phenolic compounds:

This detection is composed of mixing an equal volume of 1%aqueous ferric chloride solution with ethanol plant extract. The appearance of blue-green color indicates to the presence of phenols (Harborne, 1984).

6- Detection of alkaloids

One gram of the extract was boiled with 5 milliliters of distilled water and 4% of hydrochloric acid was added, then the solution was filtered and cooled. 0.5 ml of the supernatant was tested with Mayer solution; appearance of white precipitate indicates the presence of alkaloids (Harborne, 1984).

7- **Detection of glycosides:**

About 3ml from the extract was hydrolyzed with HCl for few hours on water bath. Then the hydrolysate transferred to glass tube and heated with 7ml Benedict's reagent. The reagent contained blue copper(II) ions(Cu^{+2}) which were reduced to copper(I)ions(Cu^{+1}) in the presence of reducing sugar and heating, which precipitated as insoluble red copper(I) oxide (Harborne, 1998).

2.2.3. Determination of Total Flavonoids

A. Quantitative determination (Wang *et al.,* 2009).

Rutin standard stock solution was prepared at concentration 1mg/ml in 50%ethanol, then serial dilutions were made to obtain different Rutin standard solutions at concentrations represented by 0.5, 0.25 and 0.1 mg/ml in 50% ethanol. Aliquot of 1ml from each concentrations of standard Rutin solution and from the redisolved extracted residue(total flavonoids) were transferred into a glass tubes, then 0.75 ml of 5% sodium nitrite solution was added and mixed well and left to stand at room temperature for 5 minutes. To all tubes 1.5 ml of 10% AlCl3 in 50% ethanol was added, shacked well and left to stand at room temperature for another 5 minutes. Finally 5ml of 1N NaOH solution was added to all tubes. The absorbance was read by spectrophotometer at 510nm, and a standard curve was plotted between each concentration and the absorbance, then the amount of total flavonoid was calculated as Rutin from the equation of straight line that obtained from the plotted curve.

B. Qualitative determination (Marcica *et al.*,2009)**:**

Thin layer chromatography was performed on silica gel Gf254 aluminum sheets by dropping one spot from each standard solution for rutin, kaempferol, quercetin and luteolin prepared at concentration 0.1mg/ml and from the extracted total flavonoids, in each time with the mobile phase was used to move until reaching about one centimeter beneath the upper sheet margin. To obtain the most suitable separation for *A.vulgaris*, three solvent system have been used which are n-Hexane: Ethanol: Glacial acetic acid (31:14:5), Conc. HCl: Glacial acetic acid: Distilled water (1.2:12:4) and n-Hexane: Etylacetate: Glacial acetic acid (30:20:1.5).The mobile system able to separate different flavonoids was selected.

C. Quantitative determination (Fang *et al*., 2007)

Qualitative analysis of flavonoids glycosides was achieved by high performance liquid chromatography with direct injection by autosampler. Qualification was carried out with a HPLC Dionex (U.S.A). The system consisted of an ASI-10 autosampler, a P580 pump, STH 585 column heater and UVD 170S UV Visible detector. The data were collected and analyzed with the Chromelon Chromatography Software. Separation and identification was carried out using C18 silica column. This column was 250 x 4.6mm i.d. 5µm particle size (Dionex Corp., USA). Column was placed in the oven set to 27 degrees Celsius. The flow rate was adjusted to 1ml/min isocratic elution. Mobile phase used included acetonitrile -water (85:15). Before loading sample mobile phase was put into the column. The injection volume was adjusted to 10µl. The monitoring peaks were detected at 254, 280 and 360nm by automatically Chromelon Chromatography software (Fang *et al*., 2007). *Alchemilla* L.

species samples, standard solutions and mobile phases were filtered by a 0.45-µm pour size membrane filter. The filtered standard and ethyl acetate extract were injected under these conditions. The identity of HPLC peaks was confirmed by injection of authentic standards.

2.2.4. Determination of Cytotoxicity

The cytotoxic effects of extracted flavonoids from *A.vulgaris* were investigated according to MTT assay as a cell functional assay to determine cell viability of lymphocytes.

2.2.4.1 Isolation of lymphocytes from whole blood (Khakdan, Piri and Talebi 2013)

3ml of blood were taken from normal healthy individuals and collected in heparinized test tube. Five ml of Phosphate Buffered Saline (PBS) were added and mixed well. Two ml of ficoll hypaque solution were taken and carefully layered blood PBS mixture on to the ficoll hypaque solution. It was centrifuged at 2000 rpm for 30 minutes. The opaque interface containing mononuclear cells was collected, mixed with PBS, and centrifuged at 1500 rpm for 10 minutes, and supernatant was discarded. The centrifugation was repeated twice, and normal lymphocytes were resuspended in RPMI medium with 10% fetal bovine serum. Then the cells were plated in 96-well plates at 104 cells/100 µl/well for the normal lymphocytes and used for cytotoxicity analysis.

2.2.4.2 **Lymphocytes Counting:**

The Lymphocytes counting manually can be carried out by the haemocytometer chamber using Trypan blue dye. The lymphocytes that prepared to be count must be mixed by gentle agitation of the flask

containing the cells**.** Some cell suspension containing trypan blue must be draw up by using pipette Carefully fill the haemocytometer by gently resting the end of the pipette tip at the edge of the chambers. Avoid overfill of the chamber. Then the haemocytometer should be put under the microscope and focus on one set of 16 corner squares of the haemocytometer chamber .Always be attention to count the number of live cells (unstained cell) in this area of 16 squares. The cells that are within the square and any positioned on the right hand or bottom boundary line were counted only. Dead cells stained blue with trypan blue can be counted separately for a viability count. Move the haemocytometer to another set of 16 corner squares and carry on counting until all 4 sets of 16 corner squares are counted.

Cell concentration (cell/ml), total cell count and %viable cell count were calculated as follow:

$$\text{Cell concentration}\left({}^{\text{cell}}/_{ml}\right) = \text{number of counted cells} \times \text{dilution factor} \times 10^4$$

$$\text{Total cell count} = \text{cell concentration(cell/ml)} \times \text{original fluid volume}$$

$$\%\ \text{viable cells} = \frac{\text{number of living cells}}{\text{total number of cells}}\ \text{X}\ 100$$

2.2.4.3 Measurement of the Viable Lymphocytes by MTT Assay (Freshney, 2012):

-Aliquot of 100µl of the suspended cells was cultured in each of the 96 well microtiterplate, (104cell/well).The plate was incubated at least for 2 hours in a CO2incubator.

- Serial concentrations from purified flavonoids extract were prepared from each stock solution (1000µg/ml) to get (500,250, 125, 62.5, 31.25, 15.625, and7.8125) µg/ml, then sterilized with 0.22 µm Millipore filter.
- Then 100 µl from each concentration of the extract was added in triplicate to each well of the lymphocytes seeding plate. Positive control was employed as 10 µl of 0.1% PHA solution (phytohemagglutinin), while untreated medium consider as negative control.
-The plate was incubated in a CO2 incubator for 24 hours at 37^{o}C.
-Finally, 50 µl of MTT dye (2mg/ml) was added to all wells, and then incubated for further 4 hours.
-The medium was removed gently by fine gauge needle after centrifugation.
-The MTT-formazan crystals which formed only by live cells were dissolved with100µl DMSO added to all wells.
-Absorbance at 620 nm was recorded immediately by ELISA reader.

-Viable cell Lymphocytes as a percentage was calculated as followed:

$$\frac{\text{Absorbance of the test}}{\text{Absorbance of negative control}} \text{X } 100.$$

2.2.5 Estimation of the Cytokine Level by ELISA Technique (De-Waal and Moree, 1988):

*1000µl of the suspended cells were seeded in each of the 24 well tissue culture plate(1X106cell/well), two plates were needed, one plate incubated for 2 hours intervals and the other incubated for 4 hours.
*Both plates were incubated at least for 2 hours in a CO2incubator before treatments.
*From MTT results done in the previous step, three effective concentrations from extracted flavonoids were chosen, these are: (1500,

150 and 30) µg/ml as they showed the potent proliferative effect for the normal lymphocytes. They were prepared from flavonoids stock solution, Then all solutions were sterilized with 0.22 µm Millipore filter.

*1ml/well were added to each plate from each flavonoids concentration in triplicate. Negative control represented by untreated cells suspended in growth medium was included.

* One plate was incubated for 2 hours, the other incubated four 4hours in CO2 incubator at 37^0C.

*At the end of each period time all wells were aspirated and transferred in separated vacuum tubes and centrifuged for 20 minutes at 2000 rpm**.**

*****Finally read the treated lymphocytes with different concentrations of extracted flavonoids at 450nm by sandwich ELISA. At the end of experiment a standard curve for different standard concentrations verses absorbance at 450nm was plotted, then a calculation of each IL-2 and IL-10 concentration for all test readings were applied according to straight line equation. Results were also evaluated statistically.

2.2.5.1 The Cytokine IL-2 Level

The human interleukin 2 ELISA Kit is ready to be used for the in vitro quantitative determination of human IL-2 concentration in serum, plasma, cell culture supernatants and other biological fluids.

i. Sample preparation according to human IL- 2 ELISA Kit:

1-cell culture supernatant: Centrifuge to remove any visible particular material.

2-serum: blood should be drawn using standard venipuncture techniques and serum separated from the blood cell as soon as possible. Samples

should be allowed to clot for 1 hour at room temperature, centrifuged for 10 min (4^0C), and serum extracted.

3-Plasma: blood should be drawn using standard venipuncture techniques and plasma collected by sodium citrate, EDTA or heparin as an anticoagulant. To ensure optimal recovery and minimal platelet contamination, after collection there must be quick separation of plasma with less than 30 min on ice. Centrifuge for 10 min (4^0C) to remove particular.

4-(A) Serum, Plasma and Cell Culture supernatant Sample must be used within 24-48 hours may be stored at 4^0C; otherwise sample can be stored at -20^0C to avoid loss of bioactivity and contamination. Avoid freeze-thaw cycles.

4-(B) The samples were got slowly to room temperature when preforming the assay.

4-(C) It is recommended that all samples should be assayed in duplicate.

4-(D) heat-treated specimens should be avoid.

ii. Preparation of Reagents according to human IL- 2 ELISA Kit:

all Kit reagents were removed from refrigerator and allowed to reach the room temperature. The following reagents must be prepared :(Mix thoroughly by gently swirling before pipetting, foaming must be avoid)

1-Wash buffer: 60ml of wash buffer were diluted to a final volume of 1200ml with ddH_2O and mix Thoroughly. If smaller volume of wash buffer is desired, 1ml of wash buffer was added to 19 volume of ddH_2O. Wash buffer is stable for 1 month at 4^oC .

2- Substrate Solution: substrate A and substrate B should be mixed togather to equal volume up to 15 min before use.

3- IL-2 Standard:

A) Two vials of standard are provided in this Kit to allow both serum\plasma and cell culture supernatant testing. Reconstitute IL-2 Standard with 2.0ml of Calibrator Diluent 1 or Calibrator Diluent 2. This reconstitution provides a stock solution of 2500pg\ml. Allow solution to set for at least 15 min with gentle agitation prior for making dilution. The IL-2 stock solution can be stored frozen (-20^0C) for up to 30 days.

B) the above stock solution were used to produce a serial 2-fold dilution series. 0.5 ml of appropriate Calibrator Diluent was added to each test tube. The undiluted IL-2 stock solution (2500pg\ml) were served as the high standard and the Calibrator Diluent will serve as the Zero standard (0pg\ml).

iii. Assay procedure according to human IL- 2 ELISA Kit:

1- Wash Buffer and IL-2 standards were Prepared before starting the assay procedure.

2-100ul of the sample were added to the appropriate well of the microliter plate (pre-coated with anti-human IL-2 monoclonal antibody) and incubated for one hour at room temperature.

3-without discarding the standard and samples, 50ul of IL-2 Pab (Biotin) were added to each well .mix well. Cover and incubated for 1 hour at room departure.

4- the microliter plate was washed using one of the specified methods indicates bellow:

A-Manual Washing: incubation mixture was removed by aspirating contents of the plats into a sink or proper waste container. By using a squirt bottle , each well should be filled completely with Wash Buffer then aspirate contents of the plats into the a sink or proper waste container. Repeat this step four more times for a total of five washes. After five wash , the plate was inverted and dried by hitting plate onto absorbent paper or paper of towels until no moisture appears.

Note: the sides of the plate frame have to hold firmly when washing the plate to assure that all strips remain securely in frame.

B- Automated Washing: all wells were aspirated, then plate was washed five times using Washing Buffer. Always adjust your washer to aspirate as much liquid as possible and set fill volume at 350ul\well\wash (range: 350-400 ul). After five wash, the plate was inverted and dried by hitting plate onto absorbent paper or paper of towels until no moisture appears.

5- One hundred microliter of Avidin solution were added to each well. Then covered and incubated for 1 hour at room temperature.

6- Substrate solution should be prepared no more than 15 min before end of incubation.

7- Washing procedure was repeated as described in step 4.

8- One hundred microliter Substrate solution were added into each well. Cover and incubate for 30 min. at room temperature.

9- One hundred microliter of Stop Solution were added to each well .Mix well.

10- The optical density (O.D.) was read at 450 nm using microtiter plate reader within 30 min.

2.2.5.2 The Cytokine IL-10 Level:

Human IL-10 ELISA kit contain the key components required for the quantitative measurement of natural and/or recombinant HIL-10 in a sandwich ELISA format within the range of50-1600pg/ml. The kit exhibits no significant cross activity with human IL-1a, IL-2, IL-3, IL-4, IL-6, IL-7, -IL-8, TNF-α, TNF-β, and IFN-γ.

i. Reagent preparation and storage according to human IL- 10 ELISA Kit:

A. Reconstitution of the IL-10 standard:

IL-10 standard solution should be prepared no more than 2 hour prior to the experiment. Two tubes of IL-10 standard (10ng per tube) are included in each kit. Use one tube for each experiment.

1) 10,000pg\ml of human IL-10 standard solution: 1ml of sample Diluent buffer was added into one tube, the tube been kept at room temperature for 10 min and mix thoroughly.

2) 250pg\ml of human IL-10 standard solution: 50 ul of the above 10ng\ml IL-10 standard solution were added into 950 ul sample diluent buffer and mix thoroughly.

3) 250pg/ml →7.8 pg/ml of human IL-10 standard solutions. Label 6 Eppendorf tubes with 250pg/ml, 125pg/ml, 62.5pg/ml, 31.3pg/ml, 15.6 pg/ml, 7.8pg/ ml respectively.300 ul of sample diluent buffer should be aliquoted into each tube. 300 ul of the above 500pg/ml IL-10 standard solution were added into 1^{St}. tube and mixed . 300 ul were transfered from 1^{st} to 2^{nd} tube and mixed. 300 ul were transfered from 2^{nd} tube to 3^{rd} tube and so on.

B. Preparation of anti-human IL-10 (biotin) antibody working solution: the solution should be prepared no more than 2 hours prior to the experiment.

1) The total volume should be: 100ul/well x (number of wells). (Allowing 100-200 ul more than total volume).

2) Biotinylated anti-human IL-10 antibody should be diluted in 1:100 with antibody diluent buffer and mix thoroughly (I.e. added 1ul biotinylated anti-human IL-10 antibody to 99 ul diluent buffer).

C. Preparation of Avidin-Biotin- proxidase complexs (ABC) working solution: the solution should be prepared no more than 1 hour prior to the experiment.

1) The total volume should be: 100 ul /well x (number of wells). (Allowing 100-200 ul more than total volume).

2) Avidin-Biotin- proxidase complexs (ABC) should be diluted in 1:100 with ABC Diluent buffer and mix thoroughly. (I.e. add 1 ul ABC to 99 ul ABC diluent buffer.

ii. Assay procedure according to human IL- 10 ELISA Kit:

The ABC working solution and TMP color developing agent must be kept worm at 37^0C for 30 min before use. When diluting samples and reagents, the must be mix completely and evenly. Standard IL-10 detection Curve should be prepared for each experiment. The User will decide sample dilution fold by crude estimation of IL-10 amount in sample

1. Aliquot 100 ul per well of the 500 pg/ ml , 250 pg/ml , 125pg/ml, 62.5pg/ml ,31.3pg/ml, 15.6 pg/ml , 7.8pg/ ml human IL-10 standards

solutions in to the precoated 96-well plate . then100 ul of the sample diluent buffer were added into the control well (Zero well). 0.1 ml of each properly diluted sample of human cell culture supernatants, serum or plasma (heparin, EDTA, citrate) was added to each empty well.

2. The plate should be sealed with the cover and incubated at 37c^{o} for 90 min.

3. Remove the cover discard plate content and blot the plate onto paper towels or other absorbent material. DO not let the wells completely dry at any time.

4. One hundred microliters of biotinylated anti-human IL-10 antibody working solution were added into each well and incubated the plate at 37c^{o} for 60 min.

5.The plate have to wash 3 times with 0.01M TBS or 0.01 M PBS, and each time let washing buffer stay in the wells for 1 min. the washing buffer were discarded and blot the plate onto paper towels or other absorbent material.(plate washing method : discard the solution in the plate without touching the side walls. Blot the plate onto paper towels or other absorbent material. Soak each well with at least 300 ul PBS or TPS buffer for 1 -2 min. repeat this process 2 additional times for a total 3 washes.

6. One hundred microliter of prepared ABC working solution were added into each well and incubated the plate at 37^{o}C for 30 min.

7. The plate were washed 5 times with 0.01M TBS or 0.01 M PBS, and each time let washing buffer stay in the wells for 1--2 min. the washing buffer were discarded and blot the plate onto paper towels or other absorbent material.(See step five for plate washing method).

8. Ninty microliters of prepared TMP color developing agent were added into each well and incubated the plate at 37^{o}C in dark for 20-25 min.

9. One hundred microliter of prepared TMP stop solution were added into each well. The color change into yellow immediately.

10. The O.D. absorbance were read at 450nm in a microplate reader within 30 min. after adding stop solution.

Chapter Three

Result & Discussion

3. *Alchemilla vulgaris*

Alchemilla vulgaris distributed most of the countries, in Bulgarian folklore *A. vulgaris* is known as king's herb. The rural people in these countries used it as astringent, antidiarrhetic and inti-inflammatory agent. *A.vulgaris* contains about 10% tannins ,flavonolglycosides ,leucoanthocyanidins, sugars, resins and vitaminC. **.**

3.1.Plant Active Components Extraction

3.1.1. Methanol extract

The dried powdered areal parts (10g) yield a quantity of (120mg) which represents 12% of the *Alchemilla vulgaris* original sample. The appearance of the residue was brown in color.

3.1.2. Chemical Detection of active compounds in the plant extract:

By using different chemical reagents, the result of detection classes of secondary metabolities in *A.vulgaris* methanolic extracts indicated the presence of flavonoids, glycosides, saponins ,Tannins and terpenes and steroids, while no alkaloids were found using mayer`s reagent. All results were illustrated in table (3-1).

Table 3-1: Chemical detections of secondary metabolites in the aerial parts of *A.vulgaris* methanolic extract*[1]

	Secondary Metabolites	Reagents	Indication	Result of detection
1	Alkaloids	Mayer's reagent	No white ppt.	-

2	Flavonoids	Ethanol with KOH	Yellow ppt.	+
3	Glycosides	Benedict reagent	Red ppt.	+
4	Saponins	Shaking Extract ferric chloride	foam white ppt.	+
5	Terpenes and Steroids	chloroform, acetic anhydride, sulphuric acid	brown precipitate	+
6	Tannins	ferric chloride	Greenish-blue color	+

Note:(+ve) indicates the presence of this active compound. (-ve) indicates the absance of this active compound.

Afifi and Kasabri *(2013)* tested the Antidiabetic Activities of selected medical plants from Jordan. Afifi F. and Kasabri V. showed that the *Alchemilla spp* contains Polyphenols, flavonoids, tannins and gallic acid. These compounds are responsible for biological activities of the plant. While Another study carried out by *Olafsdottir et al.*, 2001 who worked on three different species of *Alchemilla* from Iceland, they mentioaned that no flavonoids compounds were detected in three Alchemilla species, which are *A.faeroensis, A.alpina and A.vulgaris***.**

3.2. Determination of Total Flavonoids:

The dried powder yielded about (120mg) residue which was reflected with acidic solvent (HCl) to break down the glyosidic linkage. The non-aqueous aglycon residue was dissolved in 30 ml of 50%v/v ethanol for the following investigations

A. Quantitative Assay.

The absorbance of the spectrophotometric was recorded by *A.vulgaris* extract total flavonoids and rutin standard solutions at 510nm as shown in Figure (3-1).

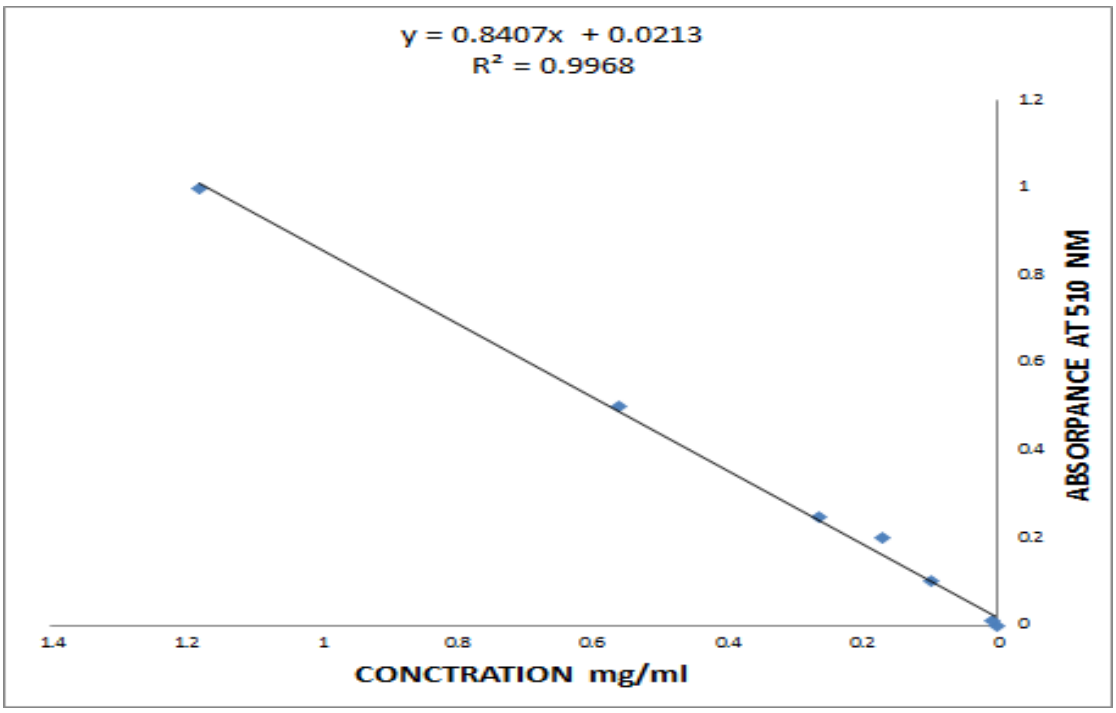


Figure (3-1): Standard curve for rutin as determined spectrophotometrically at 510 nm.

Results indicated that the total determined flavonoids in (1g) *A.vulgaris* extracted was 9.8 mg determined as rutin according to straight line equation.

Denev and Kratchanova (*2014*) stated that (1.831g) total flavonoids were produced from (100g) of *A.vulgaris* extract. This result was achieved as a step for studying the antioxidant, antimicrobial and neutrophil-modulating activities of this plant.

D'Agostino *et al*., 1998 concluded that the *A.vulgaris* contain 145mg of flavonoids in 6g of this dried plant.

B. Qualitative Assay:

Thin Layer Chromatography assay was applied for qualitative study of extracted *A.vulgaris* .Many types of solvents system were used but the solvent system N- hexane, ethanol and Glacial acetic acid (31:14:5) was the best one as long as it gave good separation of the components as shown in figures (3-2).

Figure (3-2): TLC chromatogram for the mobile phase.
A.vulgaris flavonoids extract (S), Querecten (Q), Kaempferol (K), Luteoline (L) Rutin (R).

Pervious study by Kaya *et al*., (2012) proved the appearance of flavonoids compounds (Rutin, Isoquercetin, Kaempferol and Quercitrin) on the TLC chromatogram of the A. vulgaris collected from north-eastern black sea region of Turkey. Another study done by Ondrejovic *et al*., (2009) showed that spots of methanolic extracted *Alchemilla xanthochlora* on TLC chromatogram indicated the presence of Terpenes, tannins, polyphenols and coumarins. A research written by Smolyakova (2012) showed that *A.vulgaris* aerial parts methanolic extract contains luteolin, quercetin, apigenin, rutin and Kaempferol.

C. HPLC Analysis

HPLC analysis of the methanolic extract for *A.vulgaris* flavonoids extract indicated the presence of**:**

A. Rutin, with retantion time (1.023)minutes, figure(3-6) in comparsion with Rutin standard (1.07) figure (3-3).

B. Quareciten, with retantion time (2.587)minutes, figure(3-6) in comparsion with Quareciten, standard (2.535) figure (3-4).

Retantion time of Kaempferol standard (it`s spot appeared in TLC) is 4.6 min (figure 3- 5)

When applied the data for peak area under the curve and retention time of the standard and extracted flavonoids, the concentration for total flavonoids were calculated as follow:

$$\text{Total flavonoids(mg) in 1g dried extract} == \frac{Peak\ area\ of extracts}{Peak\ area\ of standard} \times Standard\ solution\ concentration \times total\ volume\ of\ extract$$

So:

- **Rutin**

$$\frac{3582289}{3810650} \times 1\frac{mg}{ml} \times 100ml = 94\,mg$$

- **Quareciten**

$$\frac{1556}{3044626} \times 1\frac{mg}{ml} \times 100ml = 0.05mg$$

- **One gram of dried extract contains**:-

Rutin 94 % of total flavonoid $\Longrightarrow$ Rutin = 9.212mg

Quareciten 0.05% of total flavonoid $\Longrightarrow$ Quareciten =0.5mg

The rest quantities 0.088mg may be suggested as Lueteolin and Kaempferol.

The present study focuses on estimation of total flavonoid in *A.vulgaris* areal parts. It became clear that *A.vulgaris* plant is rich with flavonoids [9.8 mg\1g dried powder] that might give an emphasis for the plant pharmacological action.

According to our study on *A.vulgaris* (areal parts flavonoids extract) Rutin are found to be the major flavonoids constituents of the extract. These results are in agreement with Denev *et al*. (2014). They revealed that *A.vulgaris* contains 1.057g of rutin in each 100g of dried *A.vulgaris* extract. Also Sytar *et al*. (2015) worked on the leaves *Alchemilla mollis* methanol extract and they founded that the *A.mollis* contains 0.98mg flavonoids in each gram of dried *A.mollis* plant.

Our HPLC result is in accordance with study by Kaya *et al*,. (2012) which also illustrated that Rutin is the major flavonoid constituents of the *A.vulgaris* followed by Quareciten, also in this study some unidentified

peaks were observed on chromatograms which may belong to Lueteolin and Kaempferol (already their spots appeared in TLC paper) perhaps it requires more future work using different solvent systems for excellent identification.

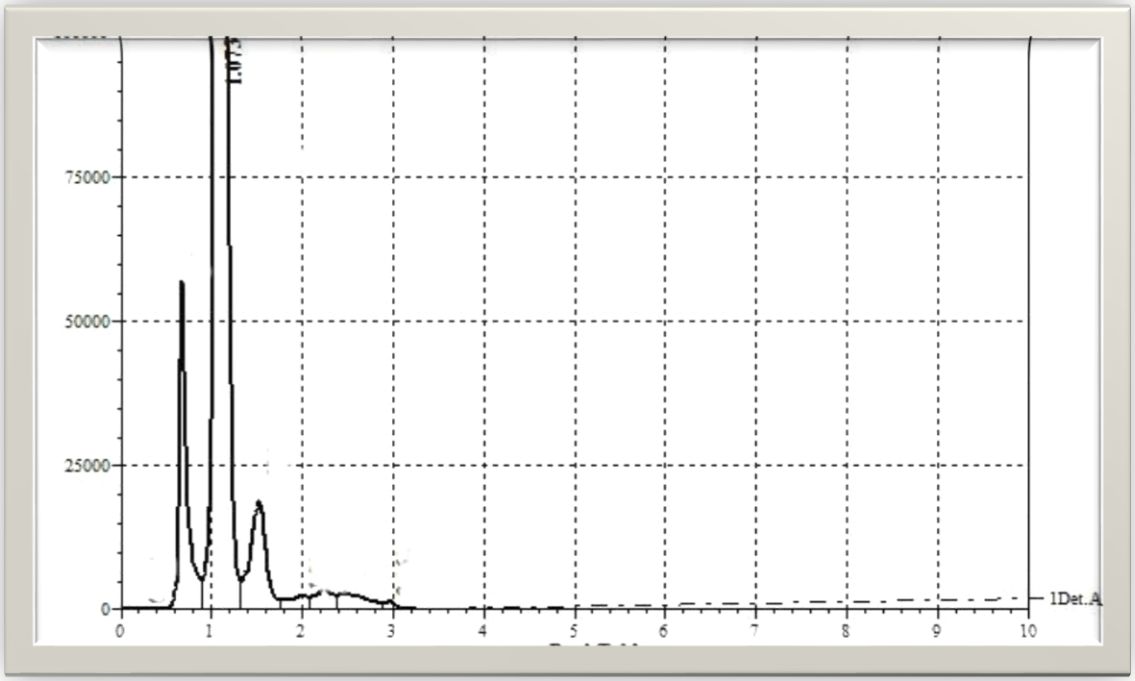


Figure (3-3) HPLC anlysis of Rutin standard

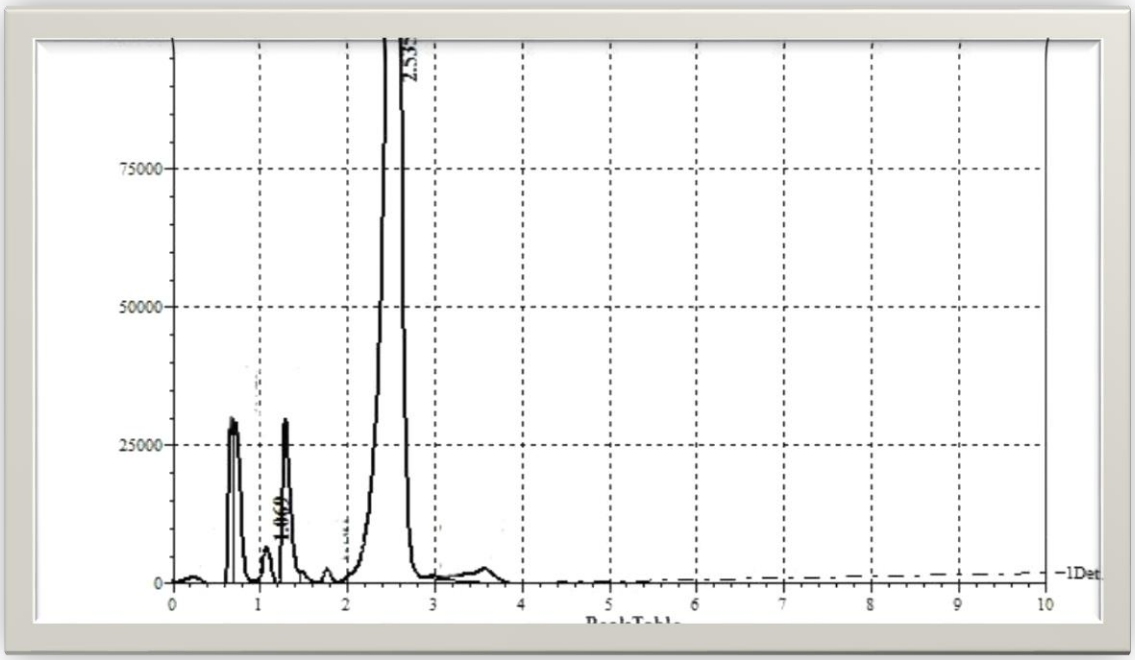


Figure (3-4) HPLC analysis of Querecten standard

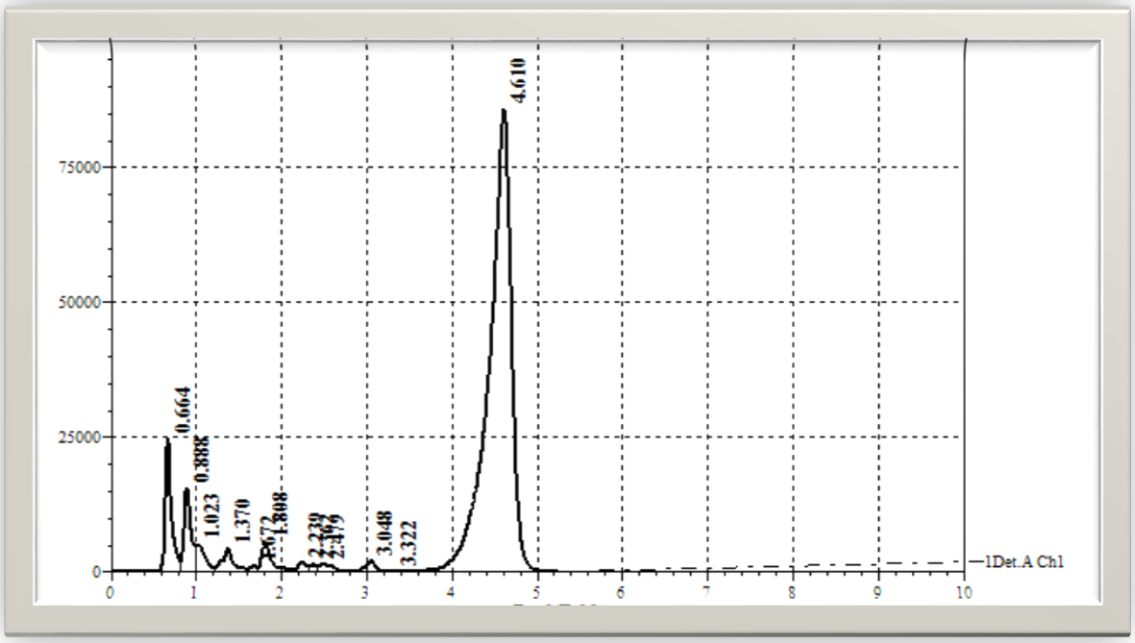


Figure (3-5) HPLC analysis of Kaempferol standard

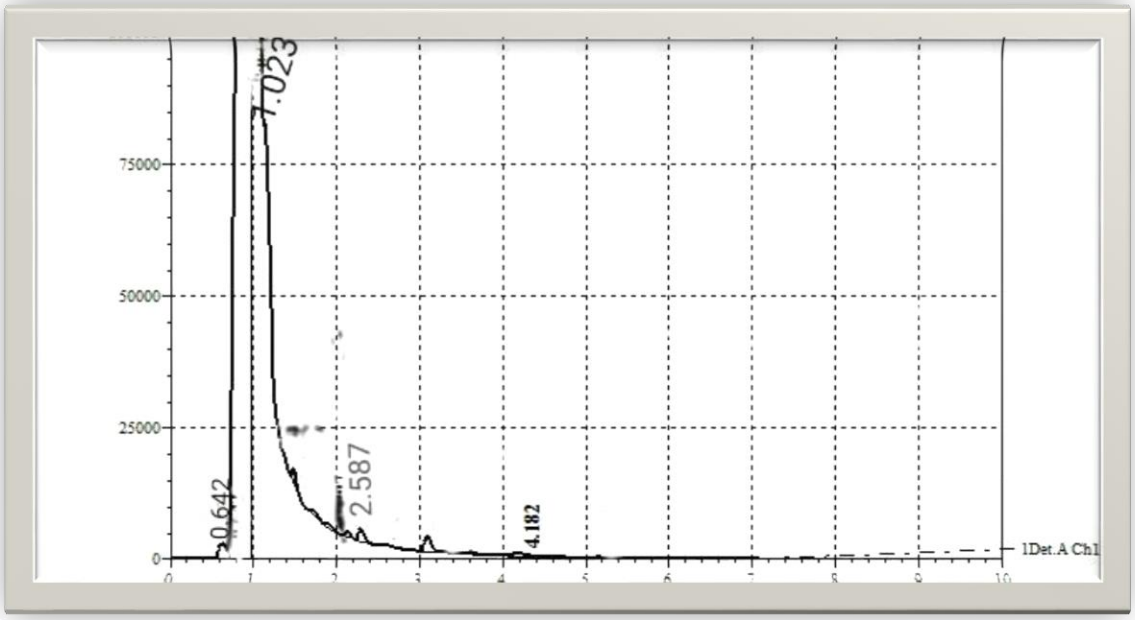


Figure [3-6]: HPLC analysis of the *A.vulgaris* dried aerial parts flavonoid extract

3.3. Extracted Flavonoids from *Alchemilla vulgaris* as Immunomodulator (In vitro):

In order to achieve the study of immune-modulation and the mechanism for regulation of the immune system, two of parameters were employed ; Lymphocyte proliferation and cytokines(IL-10 and IL-2) level. Results of the effect for different *A. vulgaris* active components on these parameters were analyzed statistically

3.3.1 Effect of extracted Flavonoids from *Alchemilla vulgaris* on Lymphocytes Proliferation :

This work was held at Al-Nahrain Biotechnology Research Center Laboratories. Lymphocyte proliferation was determined using MTT assay. Results of the effect of different concentrations of purified extracts of *A.vulgaris* on proliferation of normal human lymphocyte are shown in Table (3-2).

Table (3-2) Effect of purified flavonoid extracted from *A.vulgaris* on Normal human lymphocytes treated for 24 hours

Conc. of extracted flavonoids in µg/µl		% viable Lymphocytes treated by the Extracted flavonoids
1	3000	89.0
2	1500	76.4
3	750	63.5
4	375	45.8
5	187.5	37.8
6	93.75	37.3
7	46.87	37.3
8	23.43	45.7
9	11.71	55.6
10	5.85	59.2
LSD value		9.336 *

*** (P<0.05).**

Results indicated that treatment of lymphocytes using different concentration of extracted flavonoids a significant difference in the viability of lymphocytes were observed as compared with results of the untreated lymphocytes as negative control. As shown in Table (3-2). Flavonoids suppress lymphocytes proliferation with significances between all concentrations in respect to the control result. The decrease in the *Alchemilla vulgaris* concentration led to increase the suppression of Lymphocytes proliferation.

Flavonoids are known to have an effect on immune cells function by Inhibits Pro-inflammatory cytokine gene expression in normal peripheral blood mononuclear cells via modulation of the NF-κβ System **(**Nair *et al*., 2006). Nair showed that the aglycon part of these flavonoids possessed inhibitory effects on human normal lymphocytes**.**

Immunomodulatory therapy represented an important field in the treatment of infection and is more actual, in influencing a specific immune function or modifies one or more complements of the immunomodulatory network to achieve an indirect effect on specific

immune function. If the model for measuring the immunomodulatory activity is evaluation of proliferation of the lymphocytes and cytotoxic effect on macrophage. The flavonoids and alkaloids are considered tobe responsible of these activities. (lyu and park, 2005). Other suggested that the mechanism of this effect may be modulated by the interaction between active components of the extract and cell surface molecules, or growth factors involving nitrogen activation. There active components could be alkaloids or polyphenolics compounds. Another possible mechanism of action may be interference with cell signaling and cytokine production. . (Liu, 2004).It was that the effect of herbal products on host defense against pathogens and tumor were directly correlated with their ability to stimulate lymphocytes proliferation.

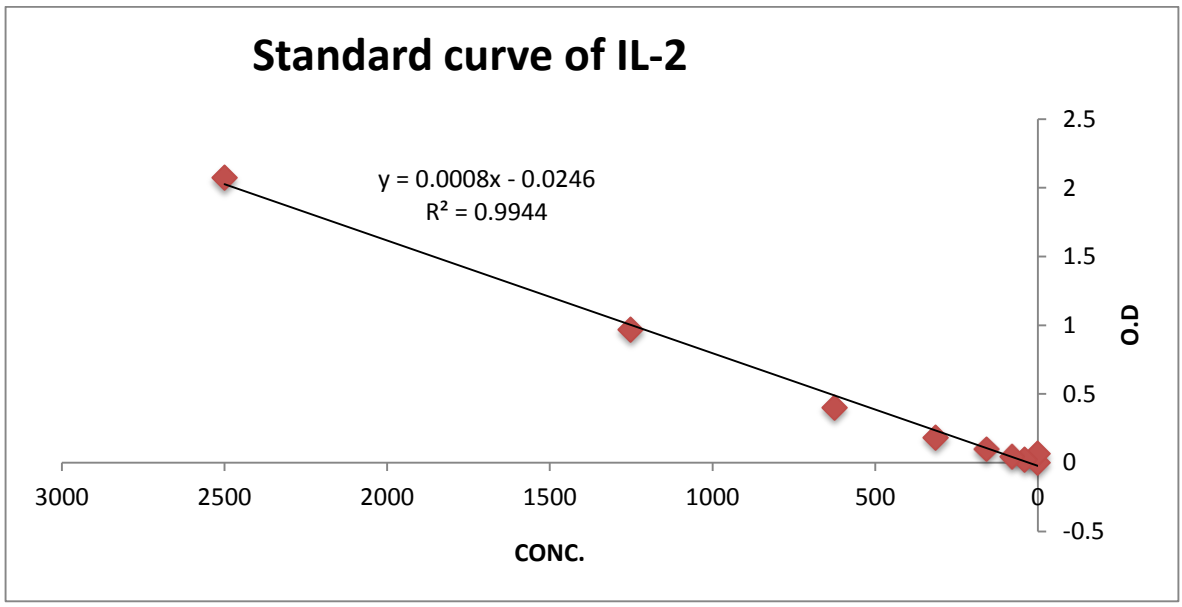
Renewed attention to natural therapies has stimulated a new wave of research interest in traditional practices, herbs have become a target for the search for new anticancer drugs. About half of the drugs used in clinical practice come from natural products(Butler,2005).Various in vitro studies about the mechanism of the plant cytotoxicity were differ from one cell culture to another depending on whether whole plant extract was used or any of the plant component, in fact, many nutritive and nonnutritive phytochemicals with diversified pharmacological properties have shown promising responses for the prevention and treatment of various cancers, including different types of cancer. Most of the animal studies done on *A.vulgaris* explained that the anti-cancer effects of the plant were through immune enhancements and prevent the development of complications or even tendency to carcinogenesis by increasing numbers of CD4+ and CD8+ T-cells to relieve the immunosuppression and enhance the anti-tumor function of the immune system(Wu et al., 2006). T lymphocytes play a central role in adaptive

immunity, and Chen et al (2008), found that *A.vulgaris* components are able to activate T cells. At the same time, the percentage of cells in G0/G1 phase was increased (Chen et al.,2008), thus because T cells spontaneously arrest in G0 and may remain quiescent for long period of time until exposed to specific antigen or mitogens that initiates a cascade of biochemical events leading the resting T cells to enter the cell cycle then proliferating and differentiating(Liu, 2004),for this reason the plant active components had been used as immune stimulant or immune adjuvant.

3.4 Effect of Flavonoids fraction on cytokine levels (IL-2 and IL-10)

In order to measure cytokines (IL-10 and IL-2) level in the supernatant of the treated lymphocytes with different concentrations and exposure time (2 and 4 hours) as well as control culture and standard solutions, ELISA technique was used and standard curve for both interleukins was plotted separately (Figure 3-7).

(A)



(B)

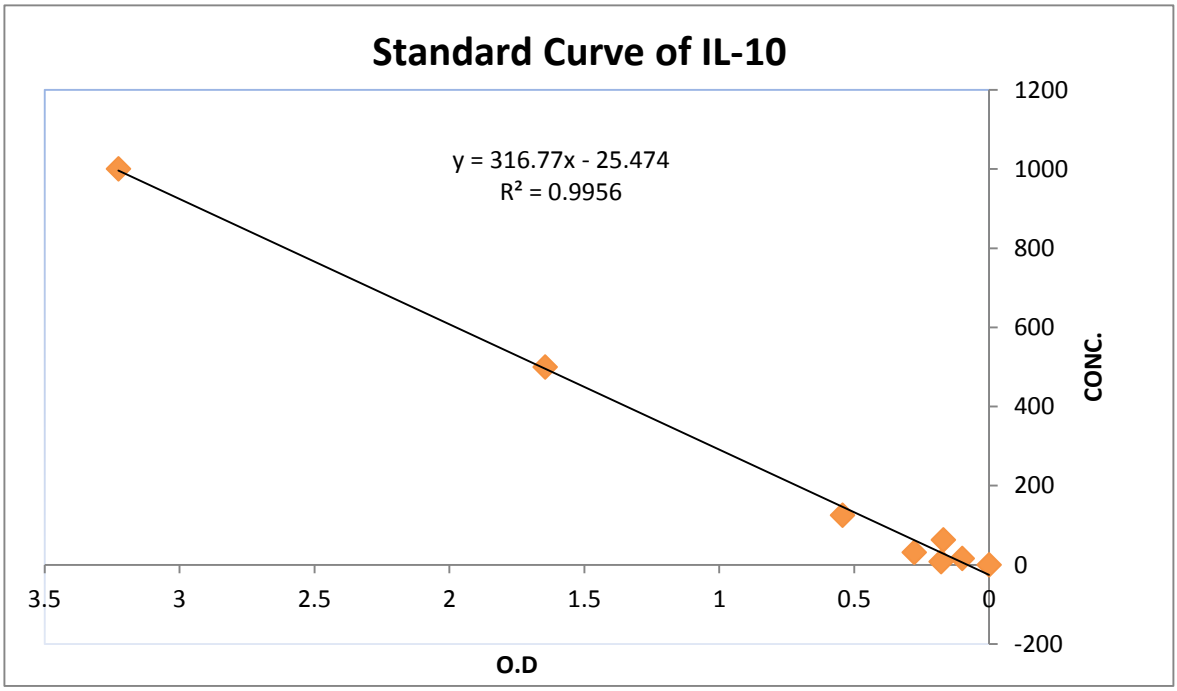


Figure (3-7)Standard curve of IL-2 (A) and IL-10 (B) analyzed by ELISA, R_2=0.994 And 0.995 respectively.

The effect of different concentrations of extracted flavonoids on cytokine level produced by treated lymphocytes after (2-4) hours was shown in Table (3-3). The extracted flavonoids caused significant increase in both IL-2 and IL-10 level that produced from treated lymphocytes as compared with negative control (normal lymphocytes with media).The reduction in the extracted *A.vulgaris* concentrations accompanied with significant increase in both IL-2 and IL-10 in 2hours and 4hours.

In IL-2, the exposure time factor has no significant effect on $1.5_{ug/ul}$ and $0.15_{ug/ul}$ concentrations of *A.vulgaris* while the $0.035_{ug/ul}$ conc. of A.vulgaris had significant effect due to the significant difference between the IL-2 conc. produced at 2hours from the IL-2 conc. produced at 4 hours. As shown in table (3-3)

In IL-10, the exposure time factor has significant effect using 1. $5_{ug/ul}$ and $0.03_{ug/ul}$ concs. of extracted *A.vulgaris* while the exposure time factor has no significant effect in $o.15_{ug/ul}$ conc. because there is no significant difference between the IL-10 conc. produced at 2hours from IL-10 conc. produced at 4 hours. As shown in table (3-3).

Conc. Of flavonoids in (µg/µl)	IL-2 conc.		T-test	IL-10 conc.		T-test
	2 Hours	4 Hours		2 Hours	4 Hours	
1.5	19.3 ± 0.81	15.7 ± 0.61	4.982 NS	176.4 ± 8.92	214.3 ± 10.3	22.573 *
0.15	26.6 ± 1.35	23.6 ± 0.82	4.543 NS	179.6 ± 9.31	178.2 ± 8.63	14.603 NS
0.03	26.5 ± 1.09	41.6 ± 1.76	6.473 *	199.1 ± 9.75	147.3 ± 6.59	19.862 *
Control	16.6 ± 0.73	13.5 ± 0.64	3.973 NS	161.0 ± 7.62	123.4 ± 6.42	18.025 *
LSD value	5.331 *	7.043 *	---	21.974 *	26.512 *	----
* : significant (P<0.05), NS: Non-significant.						

Table (3-3) Effect of different concentrations of Flavonoids and exposure time (2 , 4 hours) on lymphocytes IL-2 &IL-10 concentrations.

Our results disagree with Tenq *et al*,.2008 who study the effect of icariin (which is a type of flavonoids) on the intermediate and advanced activation of murine T lymphocytes stimulated by concanvalin A (ConA) in vitro. Tenq concluded that the icariin decreased the secretion of IL-2, IL-4 and IL-10 but increased that of TNF-alpha and IFN-gamma. Icariin can not only decrease CD25, IL-2, IL-4 and IL-10 but also enhance the cell-mediated immunity reaction. So it may have a function of two-way regulation of immune balance.

In our study, the down-modulating effects of Alchemilla flavonoids on T lymphocyte activation have been established. T cell activation occurs in a specific immune response when an antigen is presented by

specialized cells, while the histocompatibility complex class II molecules attach to specific, naive T helper (Th) lymphocytes. After the specific recognition of antigenic peptides by T cell receptors, an intracellular signals cascade is triggered that involves the production of IL-2 (Powell et al. 1998). IL-2 binds to and signals through a receptor complex consisting of three subunits designated IL-2Ra (CD25), IL-2Rb (CD122) and IL-2Rg (CD132). All three subunits are required for high-affinity binding of IL-2.Interactation between the IL-2 with its specific cell receptor leads to the stimulation of a set of complex signal transduction pathways resulting in cell proliferation (Nelson & Willerford, 1998). In addition to its potent T cell growth-stimulatory activity, IL-2 mediates multiple biological processes, including growth and differentiation of B cells.B cell grow and differentiate in the bone morrow.Activated Th-lymphocytes can be divided into two distinct subsets of effector cells based on the profile of cytokines that they produce. The Th1 subset secretes cytokines usually associated with inflammation, such as IFN-g and TNF-a and induces cell-mediated immune responses. (Constant& Bottomly, 1997)

The Th2 subset produce cytokine such as IL-4 and IL-5 that help B cells to proliferate and differentiate and is associated with humeral immune responses. Th2 cells also produce IL-10 which suppresses Th1 cells (Constant & Bottomly, 1997). IL-4 is mainly produced by activated Th2 cells and plays an important role in regulating Th1/Th2 balance. It has been found that IL-4 down-regulates Th1 function involved in autoimmune diseases such as psoriasis and arthritis(Ghoreschi et al. 2003).

Conclusions and Recommendations

4.1 Conclusions

1. The aerial parts of *A.vulgaris* were rich with flavonoids and the major components were Rutin, chase by Quareciten and Kaempferol, proved by TLC and HPLC technique.
2. Immunomodulation study indicated that *A.vulgaris* flavonoids were able to suppress the normal lymphocytes proliferation using MTT assay.
3. *A.vulgaris* flavonoids induce the secretion of Th1 and Th2 due to the increase in the IL-2 and IL-10 Levels.

4.2. Recommendations

1. In order to realize the entire mechanism of Immunomodulation further studies with purified phytoconstituents of *A.vulgaris* extract are needed.
2. More isolation and identification of major active principles present in this plant.
3. Another analytical technique was needed to determine other flavonoids in the plant, qualitatively and quantitatively.

Reference

Reference

1. Afifi F. and Violet Kasabri. Faculty of Pharmacy, The University of Jordan, Queen Rania Al-Abdullah Street, 11942 Amman, Jordan.

2. Aleckearg(2007) . immunomodulation.. Intenational academy of Homotoxicology.
3. Alsereita, M. and Abu-Amer, K. (1996). Therapeutic potential and pharmacology of medicinak plants. In 40th Annul of the Egy. Soc. Of Pharmacol. Ant Therap., Cairo,44, 19.
4. Ben Menachem-Zidon O, Avital A, Ben-Menahem Y, Goshen I, Kreisel T, Shmueli EM, Segal M, Ben Hur T and Yirmiya R (2010). Astrocytes support hippocampal-dependent memory and long-term potentiation via interleukin-1 signaling. *Brain, Behaviour, and Immunity* 25 (5): 1008–1016.
5. Benny, K.; Tan, H.; and Vanitha, J.(2004). Immunomodulatory and antimicrobial effects of some traditional Chinese medicinal herbal. Journal of Current Medicinal Chemistry, vol.11,pp:1423-1430.
6. Blackmer J and Jefromova L. (2002). The use of alternative therapies in the Saskatchewan stroke rehabilitation population. BMC Complement Altern Med.; 2(1): 7.
7. Bradshaw, M. E. (2006). The survey of three meadow plants in Teesdale revisited, *Teesdale Record Society* Journal 3rd series 14: 25–30.
8. Brocker C, Thompson D, Matsumoto A, Nebert DW and Vasiliou V (2010).Evolutionary divergence and functions of the human interleukin (IL) gene family. *Human Genomics* **5** (1): 30–55. doi:10.1186/1479-7364-5-1- 0.
9. Burçin E., Özlem , Filiz , Gülçin and Serpil.(2010). Antioxidant Activity and phytochemical Analysis of *Alchemilla persica rothm*. Ankara Ecz. Fak. Derg. 39 (2) 145-154.
10. Butler, M.S.(2005). Natural products to drugs: natural product derived compounds in clinical trials. Nat Prod Rep.vol.22, pp:62–195.
11. Colic, M.; Vucevic, D.; Kilbarda, V. and Savic, M.(2002). Modulatory effects of Garlic extracts on proliferation of T-Lymphocytes in vitro stimulated with concavelin A. Phytomedicine, vol. 9, pp: 117-124.
12. D'Agostino, M., Dini, I., Ramundo, E., Senatore, F. (1998). Flavonoid Glycosides of *Alchemilla vulgaris* L., Phytother. Res., 12: 162–163.
13. Devasagayam, T P A and Sainis, K B.(2002). Immune system and antioxidants, especially those derived from Indian medicinal

plants. Indian Journal of Experimental Biology (IJEB).IJEB Vol.40. IJEB Vol.40(06).

14. Evans, W. C. (1989). Pharmacognosy 13th (Eds) Balliere Tindal, London, pp: 419-420.
15. Falini, B.; Canino, S.; Sacchi, S.; Ciani, C.; Martinelli, M. F.; Gerdes, J.; Stein, H.; Fang, M.; Bingxiun, X.; Zhen, J.; and Junming, G.(2010). Medical Oncology, vol. 9, pp: 9415-9420.
16. Fang, Zhang and Wang.(2007). HPLC-DAD-ESIMS analysis of phenolic compounds in bayberries (Myrica rubra Sieb. et Zucc.), *Food Chemistry*, 100: 845-852.
17. Khakdan F.,Piri K, Talebi1 A.(2013). Antiproliferative activity of aqueous extract from Arictum lappa L. root in human erythroleukemia cell line (k562) and lymphocyte cell. Life Sci J 2013;10(6s):656-661] (ISSN:1097-8135).
18. Fraisse D, Carnat A and Carnat A.P., et al. [Standardization of the aerial parts of Alchemilla]. Ann.Pharm Fr. 1999;57(5):401-405.
19. Freshney, R.I. (2012). Culture of Animal Cell. Sixth Edition.Wily-Liss,New York.
20. Freshney, R.I. (2012). Culture of Animal Cell. Sixth Edition.Wily-Liss,New York.
21. Galluzzi, L.; Aaronson, S.; and Abrams,J. (2009).Guidelines for the use and interpretation of assays for monitoring cell death in higher eukaryotes. Cell Death Differ. vol. 16, pp:1093-1107.
22. Goel,V.; Chang, C.; Slama, J. and Basu, T.(2002). Echinacea stimulates
23. Haleem A., Esmaeel K. and Abdul Wahid Al-Shaibani.(2013). The Study of Inhibitory Effects of Some Plant Extracts on Lymphocytes from Healthy Person. Eng. & Tech. Journal .Vol31,Part (B), No. 6 .
24. Harborne, J. B. (1984). Phytochemical Methods. A guide to Modern Technique of Plant Analysis. Chapman Hall, London.
25. Harborne, J. B. (1998). Phytochemical Methods: A Guide to Modern Techniques of Plant Analysis, 3rd edition, Chapman and Hall Int. Ed., New York.

26. Hawke and Richard G.(2008).An Evaluation Study of Alchemilla. *Plant Evaluation Notes*. Chicago Botanic Garden.
27. Hirano, T.; Oka, K.; Kawashima, E.; and Akiba, M. (1989).Effects of synthetic and naturally occurring flavonoids on human peripheral-blood lymphocytes. Life Sci.vol.45,pp:1407-1411
28. Hubeek, I, Peters, GJ, Broekhuizen, R, Zwaan, CM, Kaaijk, P, Wering, ES, Gibson, BE, Creutzig, U, Janka-Schaub, GE, Boer, ML, Pieters, R and Kaspers, GJ (2006) In vitro sensitivity and cross-resistance to deoxynucleoside analogs in childhood acute leukemia. Haematologica. 91: pp. 17-23 immunosuppressive herbal remedy Tripterygium wilfordi Hook. Clin North Am. vol.26,pp:29-50.
29. Ivancheva S. and Stancheva, B. (2000) J. Ethnopharmacol., 69, 165.
30. Jing, Z.; Dong, Y. Lu.; and Wang, Y.(2009).Analysis of flavonoid from leaves of cultivated Lycium sp. Plant foods for Human Nutrition, vol. 5, pp: 1007.
31. Kaya, B., Menemen, Y., and Saltan, F.(2012).Flavonoids in the endemic species of Alchemilla L.,(section Alchemilla L. Subsection Calycanthum Rothm. ser. Elatae Rothm.).from North-East Black sea region in Turkey. Pak. J. Bot., 44(2): 595-597.
32. Klein, C.; Sato, T.; Meguid, M. and Miyata, G.(2000).From blood to nutritional support to specific nutraceuticals: a journey across time in the treatment of disease.J. Gastroenterol.vol. 12,pp:1-6.
33. Kroemer, G.; Galluzzi, L.; Vandenabeel, P and Peter, M.E.(2009). Classification of Cell Death: Recommendation of the Nomenclature Committee on Cell Death. Cell Death Differ, vol.16,pp:3-11.
34. Lin, F.Y.; Lai, Y.K.; Yu, H.C.; Chen, N.Y.; Chang, C.Y.; Lo, H.C.; and Hsu,T.H.(2008). Effects of Lycium Spp. extract on production and immunomodulatory activity of the extracellular polysacchardes peptides from submerged fermentation culture of Coriolusversicolor. Food Chem., vol. 110,pp:446–453.
35. Liu, R. H. (2004). Potential synergy of phytochemicals in cancer prevention mechanism and action. Nutr. Cancer, vol. 35, pp: 108-112.
36. Liu, Y.; Wei, S. H.; Ho, A. S.; de-Waal, M.R.; and Moore, K. W. (1994).Expression cloning and characterization of a human IL-10 receptor. J. Immunol., vol. 152, pp:1821-1829.macrophage

function in lung and spleen of normal rats .J. Nutr. Biochem. vol. 13,pp: 487.

37. Lyu S. and Park W. (2005). Production of Cytokine and NO by RAW 264.7 Macrophages and PBMC In Vitro Incubation with Flavonoids. Arch Pharm Res Vol 28, No 5, 573-581.

38. Makau J. N., Watanabe K. and Kobayashi N.(2013)..Anti-influenza activity of *Alchemilla mollis* extract: Possible virucidal activity against influenza virus particles. *Drug Discoveries & Therapeutics.; 7(5):189-195.*

39. Marcica, M.S.; Vesna, R.; Mirza, B. and Zeljan, M. (2009). From functional food to medicinal product: Systematic approach in analysis of polyphenolics from popolis and wine. Nutrition Journal, vol.8, page:33.

40. Mingbo, Z., I. Yoichiro and T. Pengfei. 2005. Isolation of a novel flavanone 6-glucoside from the flowers of *Carthamus tinctorium* Honghua) by high-speed countercurrent chromatography. *J. Chromatogr. A*., 1090: 193-1.

41. Mohammed A. Motaleb.(2011). Selected Medicinal Plants Of Chittagong Hill Tracts.

42. Nair, Mahajan, Reynolds, Aalinkeel, Schwartz and Kandaswami.(2006).The Flavonoid Quercetin Inhibits Proinflammatory Cytokine (Tumor Necrosis Factor Alpha) Gene Expression in Normal Peripheral Blood Mononuclear Cells via Modulation of the NF-κβ System. Clinical and Vaccine Immunology, vol. 13, pp:319-328.

43. Olafsdottir, Omarsdottir and Jaroszewski.(2001).Constituents of three Icelandic *Alchemilla* species. *Biochemistry Systematic and Ecology*, 29: 959-962.

44. Ondrejovic M., Ondrigova Z. and Kubincova J. (2009). ISOLATION OF ANTIOXIDANTS FROM Alchemilla xanthochlora. *ova Biotechnologica 9-3 .313*.

45. Passlick, B.; Flieger, D.; and Ziegler, H.(1989).Identification and characterization of a novel monocyte subpopulation in human peripheral blood. Journal of the American society of hematology, vol. 74, pp:2527-2534.

46. Petko Denev, Maria Kratchanova, Milan Ciz, Antonin Lojek, Ondrej Vasicek, Denitsa Blazheva, Plamena Nedelcheva, Libor Vojtek and Pavel Hyrsl.(2014). Antioxidant, antimicrobial and neutrophil-modulating activities of herb extracts. Polish Biochemical Society Journal. Vol.61, p.359–367.

47. Pileri, S., Gobbi, M., Fagioli, M., Minelli, O., Flenghi, L.(1988). Immunocyto chemical evaluation of the percentage of proliferation cells in pathological bone marrow and peripheral blood samples with the Ki-67 and antibromodeoxyuridine antibodies. Br. J. Hematol.vol. 69, pp: 311-320.
48. Ramiro, Franch1, Castellote, Cristina and Castell. (2005).Effect of Theobroma cacao flavonoids on immune activation of a lymphoid cell line. British Journal of Nutrition (2005), 93, 859–866.
49. Richard G. Hawke. An Evaluation Study of Alchemilla. Chicago Botanic Garden. issue 24,2004.
50. Said O, Saad B, Fulder S, Khalil K and Kassis E.(2011). Weight loss in animals and humans treated with "weighlevel", a combination of four medicinal plants used in traditional arabic and islamic medicine.Evid Based Complement Alternat Med. 2011;2011:874538. doi: 10.1093/ecam/nen067.
51. Sarina M. Ducksteina,Lottera, Meyera, Lindequistb, and Florian C. (2012).Phenolic Constituents from Alchemilla vulgaris L. and Alchemilla mollis (Buser) Rothm. at Different Dates of Harvest. Z. Naturforsch. 67 c, 529 – 540.
52. Sheikhly and Al-azawy, (1993). natural compounds in plant. Mousal university.
53. Shrivastava R, Cucuat N and John GW.(2007). Effects of Alchemilla vulgaris and glycerine on epithelial and myofibroblast cell growth and cutaneous lesion healing in rats. Phytother Res. ;21(4):369-73.
54. Shrivastava R., Cucuat N. and Gareth W. John.(2007). Effects of Alchemilla vulgaris and glycerine on epithelial and myofibroblast cell growth and cutaneous lesion healing in rats.Phytotherapy Research,.1002/ptr.2060.
55. Siess, M.H.; Le-Bon, A.M.; Canivenc-Lavier, M.C.; and Suschetet, M. (2000). Mechanism involved in the chemoprevention of flavonoids. Biofactors, vol.12, pp:193-199 .
56. Smolyakova IM, Andreeva VY, Kalinkina GI, Avdeenko SN, Shchetinin PP (2012) Development of extraction techniques and

standardization methods for a common lady's mantle (Alchemilla vulgaris) extract. Pharm Chem J-USSR 45: 675–678.

57. Srinivasan, M. and Rajendren, S.(2012). Current Concepts in Herbal Medicine Research Int. J. Modern Biol. Med, vol.2,pp:46-51.
58. Stephen, P. J. (1994). Current Protocols in Immunology Measurement of Basic Immunologic Characteristics of Human Mononuclear Cells. Contributed by National Institute of Allergy and Infectious Diseases Bethesda, Maryland, John Wiley & Sons, Inc.
59. Sytar O., Klaudia Bruckova, Elena Hunkova, Marek Zivcak, Kiessoun Konate, and Marian Brestic. The application of multiplex fluorimetric sensor for the analysis of flavonoids content in the medicinal herbs family Asteraceae, Lamiaceae, Rosaceae. Biol Res. 2015; 48(1): 5
60. Takır S, Altun IH, Sezgi B, Süzgeç-Selçuk S, Mat A and Uydeş-Doğan BS.(2015). Vasorelaxant and blood pressure lowering effects of alchemilla vulgaris: A comparative study of methanol and aqueous extracts. Pharmacogn Mag.No; 11(41): 163–169.
61. Teng F, Zeng YY, Huang XY, Yang Z, Yao ML, Song B and Li L.(2008) Effect of icariin on intermediate and advanced activation of murine T lymphocytes in vitro. Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi. ;24(11):1059-61.
62. Wenhuan Ding, Tao Yang, and Shuge Tian.(2014). Effect of different growth stages of *Ziziphora clinopodioides* Lam. On its chemical composition. Pharmacogn Mag.2014;10:S1–5.
63. Wilasrusmee, C.; Siddiqui, J. and Kitur, S.(2002).*In vitro* immunomodulatory effects of herbal products, Am. Surg.vol. 68,pp:441-445.
64. Wu, Z.Y.; and Raven, P.H. (1994). Verbenaceae through Solanaceae. Flora of China., vol. 17, pp: 300–332. Science Press, Beijing, and Missouri Botanical Garden Press, USA.
65. -Wu,H.; Guo, H. and Zhao,R.(2006).Effect of *Lycium barbarum* polysaccharides on the improvement of anti-oxidant ability and DNA damage in NIDDM rats. Journal of the Pharmaceutical Society of Japan, vol.126,pp:365-371.
66. Wu,H.; Guo, H. and Zhao.(2006).Effect of Lycium barbarum polysaccharides on the improvement of anti-oxidant ability and DNA damage in NIDDM rats. Journal of the Pharmaceutical Society of Japan, vol.126,pp:365-371.

67. Tao, X. and Lipsky, P.(2000).The Chinese anti-inflammatory and immunosuppressive herbal remedy Tripterygium wilfordi Hook. Clin North Am .vol.26,pp:29-50.

68. Powell JD, Ragheb JA, Kitagawa-Sakakida S & Schwartz RH (1998) Molecular regulation of interleukin-2 expression by CD28 co-stimulation and anergy. Immunol Rev 165, 287–300

69. Greene WC & Leonard WJ (1986) The human interleukin-2 receptor. Annu Rev Immunol 4, 69–96.

70. Nelson BH & Willerford DM (1998) Biology of the interleukin-2 receptor. Adv Immunol 70, 1–81.

71. Waldmann TA, Goldman CK, Robb RJ, Depper JM, Leonard WJ, Sharrow SO, Bongiovanni K, Korsmeyer SJ & Greene WC (1984) Expression of interleukin 2 receptor on activated human B cells. J Exp Med 160, 1450–1466.

72. Constant SL & Bottomly K (1997) Induction of Th1 and Th2 CD4þ responses: the alternative approaches. Annu Rev Immunol 15, 297–322.

73. Ghoreschi K, Thomas P, Breit S, et al. (2003) Interleukin-4 therapy of psoriasis induces Th2 responses and improves human autoimmune disease. Nat Med 9, 40–46.

الخلاصة

اصبح اكتشاف عقاقير جديدة ومن مصادر طبيعية, يمكن ان تحفز الوظيفية المناعية هدفا هاما في الدراسات المناعية, وهدفت الدراسة الحالية تقييم بعض التاثيرات البايولوجيا للمركبات الفعالة للنوع البري لنبات رجل الاسد على الخلايا الليمفاوية. تم استخلاص الاجزاء الهوائية لنبات رجل الاسد المجفف في الكحول الايثانولي (50%) باستعمال طريقة التنقيع (Maceration), اجري الكشف عن المواد الفعالة في المستخلص حيث تم عزل وتنقية الفلافونيدات ثم فصلت باستعمال كروماتوغرافيا الطبقة الرقيقة (TLC) باستعمال المذيبات المحتلفة. استعملت تقنية جهاز الكروماتوغرافي السائل عالي الدقة (HPLC) حيث اثبتت النتائج وجود مركبات الفلافونويدات (9.8 ملغرام فلافونويد لكل غرام من النبات المجفف) على شكل الروتين وكما يحتوي على مركبي الكامفيرول والكورستينين , وتم التحقق من كل منهما نوعاً وكماً باستعمال المركبات القياسية وكان تركيزهما اقل من الروتين. وتضمن الجانب المناعي معامله مستخلص الفلافونويد مع الخلايا الليمفاوية والتي عزلت من اشحاص اصحاء لبيان التأثير السمي للفلافونويد على الخلايا الليمفاوية باستعمال صبغة MTT. سببت معاملة الفلافونويد مع

الخلايا الليمفاوية الطبيعية تاثيرا معنوياً من خلال تثبيط انتشار الخلايا اللمفاوية مع انخفاض في تركيز مجموعة الفلافونيدات المستخلصة مقارنة مع السيطرة (السيطرة هي خلايا لمفاوية غير معاملة والتي تنمو في وسط مناسب). ان التأثير المناعي للمركبات الفعالة (الفلافونويدات) تبين من خلال التغير في مستوى الانترلوكين 2 والانترلوكين 10 باستعمال تقنية ELISA, تسبب انخفاض في تراكيز الفلافونويدات المعاملة تناسب مع زيادة معنوية في كل من الانترلوكين 2 والانترلوكين 10 في 2- 4ساعات معاملة. هذه الدراسة كشفت اثار مركبات الفلافونويدات المستخلصة من نبات رجل الاسد على مستوى انتاج الانترلوكين 2 والانترلوكين 10 التي غيرت بواسطة التعبير الجيني للخلايا اللمفاوية الطبيعية المنتجة التي تحفز مسالك انتقال الاشارة لانتاج كميات كبيرة من الانترلوكين 2 والانترلوكين 10.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية العلوم

تقييم سمية فلافونويدات نبات رجل الاسد في خلايا الدم اللمفية الطبيعية

رسالة

مقدمة الى كلية العلوم- جامعة النهرين وهي جزء من متطلبات نيل

درجة الماجستير في علوم التقانة الاحيائية

من قبل

سيف لؤي العوسج

بكالوريوس تقانة احيائية (2013)

بإشراف

أ. د. خلود وهيب السامرائي

شوّال 1437 تموز 2016