

CYTOTOXIC EVALUATION OF *ALCHEMILLA VULGARIS* EXTRACT IN NORMAL BLOOD LYMPHOCYTES

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ABSTRACT

Objective: The main aim of this study is to investigate the cytotoxic effect of the *Alchemilla vulgaris* estimated flavonoids on lymphocytes by MTT assay. **Methodology:** The aerial parts of plants were 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 HPLC. Extracted *A.vulgaris* goes throw quantitative measurement to calculate the total flavonoids in the extract. *A.vulgaris* flavonoids contain rutin as major, quareciten and kaempferol, all of them were investigated qualitatively and quantitatively in the present study. The immunomodulation occur

by treated extracted flavonoids with Lymphocytes isolated from healthy people to measure the cytotoxic effect of extracted Flavonoids on normal lymphocytes by using MTT dye. The cytotoxic evaluation was read by using sandwich ELISA technique at 450nm. **Results:** This study indicate that the treatment of 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). **Conclusion:** Immunomodulation study pointed that *A.vulgaris* flavonoids suppress the normal lymphocytes proliferation using MTT assay. **Recommendation:** Further studies with purified phytoconstituents of *A.vulgaris* extract are needed to comprehend the complete mechanism of Immunomodulation.

KEYWORDS: *Alchemilla vulgaris*; Immunomodulation; Flavonoids; MTT assay.

I. 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.

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.^[1]

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.^[2]

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.^[3]

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.^[4]

Aims of study

Investigation of the cytotoxic effect of the *Alchemilla vulgaris* total flavonoids on lymphocytes by MTT assay.

II. METHODOLOGY

2.1 Extraction of Flavonoids from *Alchemilla vulgaris*

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 50co for 4 hours, then the petroleum ether (40-60co) 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 40°C. The dried residue was weighted then resolved in 30 ml 50% ethanol. The obtained extract represented the total flavonoids.^[5]

2.2 Chemical Detection of Active Compounds in the *A. vulgaris* extract

2.2.1 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.^[6]

2.2.2 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.^[7]

2.2.3 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.^[8]

2.2.4 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.^[8]

2.2.5 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.^[8]

2.2.6 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.^[8]

2.2.7 Detection of glycosides

About 3ml from the extract was hydrolyzed with HCl for 4 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.^[9]

2.3 Determination of Total Flavonoids in the Extract

2.3.1 Quantitative Determination

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 redissolved 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% AlCl_3 in 50% ethanol was added, shaken 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.^[10]

2.3.2 Qualitative Determination

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. For better result the mobile phase that were choose was n-Hexane: Ethanol: Glacial acetic acid (31:14:5). The mobile system able to separate different flavonoids was selected and used then for application the column chromatography and preparative TLC chromatograph was done and compared with the standard solutions.^[11]

2.3.2 Quantitative determination

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. 5 μm 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.^[12] *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.

2.4 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.

2.4.1 Isolation of lymphocytes from whole blood

Three ml 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.^[13]

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 that 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:

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

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

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

2.4.3 Measurement of the Viable Lymphocytes by MTT Assay (14)

1-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.

2- 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.

3- 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. 4-The plate was incubated in a CO2 incubator for 24 hours at 37oC. 5-Finally, 50 µl of MTT dye (2mg/ml) was added to all wells, and then incubated for further 4 hours.

6-The medium was removed gently by fine gauge needle after centrifugation. 7-The MTT-formazan crystals which formed only by live cells were dissolved with100µl DMSO added to all wells. 8-Absorbance at 620 nm was recorded immediately by ELISA reader.

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

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

III. RESULTS AND DISCUSSION

3.1 Plant active component extraction

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.

3.2 Chemical Detection of active compounds in the plant extract

By using different chemical reagents, the result of detection classes of secondary metabolites 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 areal 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 absence of this active compound.

Alchemilla spp contains Polyphenols, flavonoids and tannins acid. These compounds are responsible for biological activities of the plant.^[15] While other study worked on three different species of *Alchemilla* (*A.faeroensis*, *A.alpina* and *A.vulgaris*) taking from Iceland, showed that there was no flavonoids compounds were detected in three *Alchemilla* species.^[16]

3.3 Determination of Total Flavonoids

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

3.3.1 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).

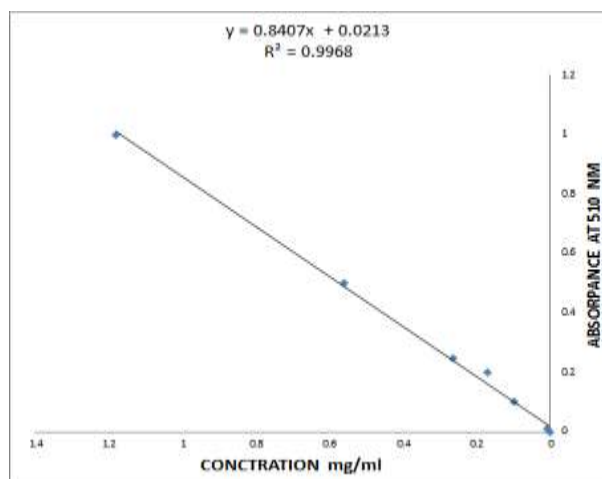


Figure (3-1): Standard curve for rutin as determined spectrophotometrically at 510 nm.

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

One hundred grams of dried *A.vulgaris* contains 1.83g of total flavonoids, the extracted flavonoids play major role in the antioxidant, antimicrobial and neutrophil-modulating activities of this plant.^[17] Another study worked on *A.vulgaris* concluded that 145mg of total flavonoids in 6g of this dried plant.^[18]

3.3.2 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), Quercetin (Q), Kaempferol (K), Luteoline (L) Rutin (R).

A.vulgaris collected from north-eastern black sea region of Turkey contain Rutin, Isoquercetin, Kaempferol and Quercitrin as flavonoids compounds by using TLC chromatogram.^[19]

Another study showed that spots of methanolic extracted *Alchemilla xanthochlora* on TLC chromatogram indicated the presence of Terpenes, tannins, polyphenols and coumarins.^[20]

3.3.3 HPLC Analysis

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

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

B. Quercetin, with retention time (2.587) minutes, figure (3-6) in comparison with Quercetin, standard (2.535) figure (3-4).

Retention 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:

Total flavonoid (mg) in 1g dried extract.

$$= \frac{\text{Peak area of extracts}}{\text{Peak area of standard}} \times \text{Standard solution concentration} \times \text{total volume of extract}$$

So

♦ Rutin

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

♦ Quercetin

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

One gram of dried extract contains

The rest quantities 0.088mg may be suggested as Luteolin 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 is found to be the major flavonoids constituents of the extract.

These results are in agreement with.^[17] They revealed that *A.vulgaris* contains 1.057g of rutin in each 100g of dried *A.vulgaris* extract.^[17] Also other research worked on the leaves *Alchemilla mollis* methanol extract, found that the *A.mollis* contains 0.98mg flavonoids in each gram of dried *A.mollis* plant.^[21]

Our HPLC result is in accordance with study by^[19] which also illustrated that Rutin as the major flavonoid constituents of the *A.vulgaris* followed by Quareciten.

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

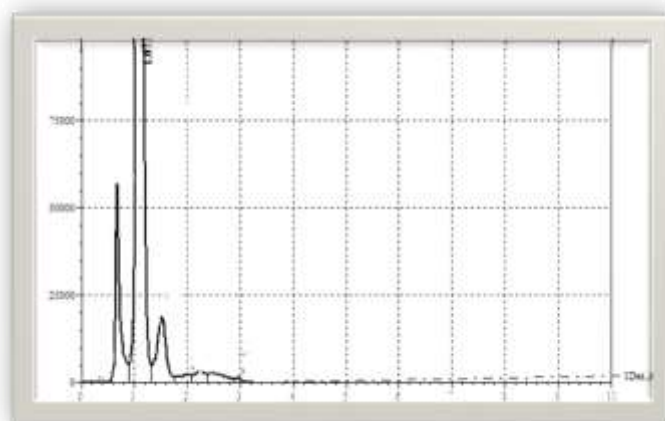


Figure (3-3): HPLC analysis of Rutin standard.

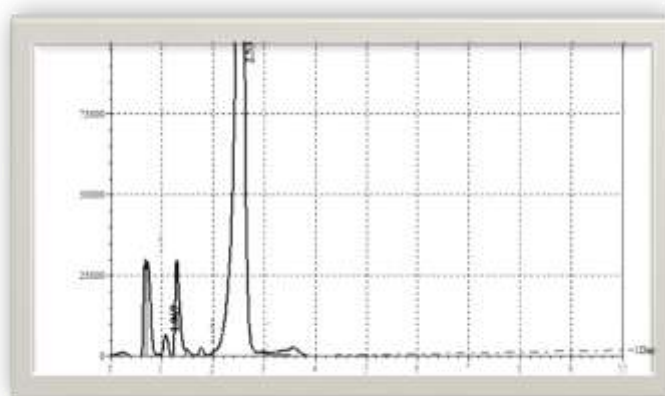


Figure (3-4) HPLC analysis of Quareciten standard.

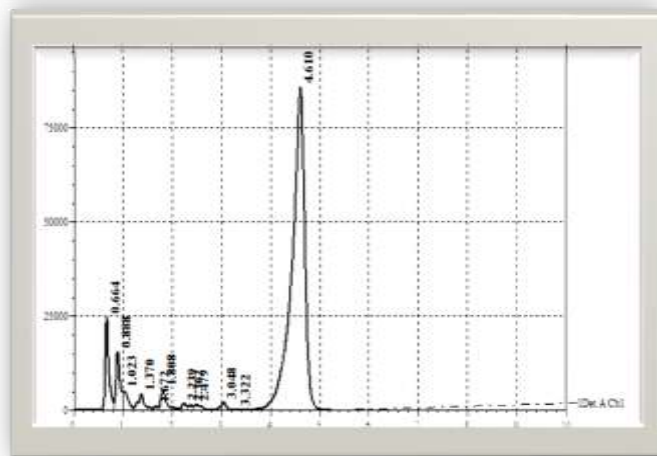


Figure (3-5) HPLC analysis of Kaempferol standard.

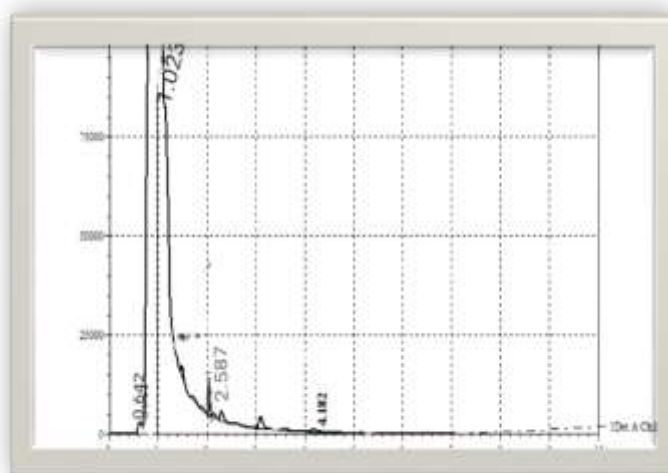


Figure [3-6]: HPLC analysis of the *A.vulgaris* dried areal parts flavonoid extract.

3.4 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 method. 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 with treatment of lymphocytes using different concentration of extracted flavonoids, a significant differences 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- κ B System. The aglycon part of these flavonoids possessed inhibitory effects on human normal lymphocytes.^[22]

Immunomodulatory therapy represented an important field in the treatment of infection and is more actually, 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 to be responsible of these activities.^[23] Another 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. The

effect of herbal products on host defense against pathogens and tumor were directly correlated with their ability to stimulate lymphocytes proliferation.^[24]

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.^[25] 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 this 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.^[26] T lymphocytes play a central role in adaptive immunity, *A.vulgaris* components are able to activate T cells. At the same time, the percentage of cells in G0/G1 phase was increased, 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, for this reason the plant active components had been used as immune stimulant or immune adjuvant.^[4,24]

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