International Baccalaureate

BIOLOGY

Extended Essay

The analysis of the possible effect of different concentrations of *Valeriana alliariifolia* (heliotrope - kitty plant) extract on cell migration

Candidate Number: 001129-0098

Word count: 3837

Background Information:

Plant diversity is quite diverse in some parts of the world, which is important both for the environment and for its potential health benefits. Many plants have been used as a healing tool for centuries. I have family members who are working as medical doctors and some of their family elders helped people as healers and used such plants for healing purposes in those ancient times. This information has reached our generation. The basis of producing some of the drugs used today is actually based on these old medical records. Based on this information, in these days we are living due to the current COVID-19 pandemic, we understand that health is very important and I wanted to do research on the potential therapeutical use of a plant which is less known, by analyzing its anti-inflammatory effect.

Many plants have been used for health purposes due to their biological content for years. Research on this field have confirmed these health-related properties of many plant-derived compounds (1). For example, *Camellia sinensis* (green tea), which is one of the best-known plants in this field, has been consumed as herbal tea for centuries and is good with their compounds for conditions such as autoimmune diseases, atherosclerosis and cancer (2,3). In this study I want to focus on the anti-inflammatory effect of a less known plant, *Valeriana alliariifolia,* which has a common name as helliotrope-kittyplant.

In the worldwide, the genus Valeriana (Caprifoliaceae) is represented by more than 350 species. The main components of valerian are known however its chemical composition varies with season (4). Many of iridoids, a component of valerian, have been studied for their antispasmodic, sedative, antimycobacterial, antiviral, cytotoxic and anxiolytic effects. *Valeriana alliariifolia,* (Fig-1) one of the species of valeriana, root infusions are used as sedatives and antispasmodics in traditional medicine by local people in East Anatolia (4,5). There is no information regarding the possible effects of *Valeriana alliariifolia* on inflammation in the literature.

Figure 1- *Valeriana alliariifolia* (5).

Inflammation is an important immune response mechanism. The immune system involves important components that allows a living organism to discriminate between "self" and "nonself". The immune response is basically divided into two as; innate and adaptive immune response. The innate immune response is the initial response and an essential part of the immune system which is activated once an invader tries to enter an organism. The innate and adaptive system is in communication with each other and innate response protects us until the adaptive immune system takes control by generating a memory of the invader to protect us against future attacks (6,7).

Inflammation, a mechanism of innate immune system, is a normal response to infection or other abnormal structures/signals in the organism. The main function of inflammation is to limit the infection and prevent it from spreading other parts of the body. However, inflammatory reaction needs to stop once the pathogen is eliminated from the body by the immune cells and damaged body tissues are repaired. If inflammation is not controlled, the immune system will be on high alert which can cause damage to the body's other organs and tissues and may contribute to serious problems (7,8). Thus, the anti-inflammatory effect is an important property for the molecules that may be considered for therapeutical applications especially for infectious, autoimmune and autoinflammatory diseases.

Cells of the immune system, known as white blood cells, are mainly functioning during inflammation and eliminating infection. The early arrival, 'migration', of these cells to the infected tissue is an important process of inflammation and causes dilation of the blood vessels which allow for the rapid transfer of other helper immune cells (9). Proinflammatory signals triggered by microbial structures/molecules (or aseptic conditions/abnormal structures in the organism such as atherosclerosis) from the site of affected tissue induce cell migration and the responsible cells are targeted to these inflammatory sites in order to function in immune defense and promote tissue healing (10). However, this inflammatory response is always like a double-edged sword, the increase in cell migration or in their function may contribute to tissue destruction during some infections and inflammatory diseases.

Therefore, in order to evaluate the anti-inflammatory properties of a candidate treatment molecule, it is important to investigate the effect of that molecule on cell migration. Determining the migratory capacity of cells is important for novel strategies not only in drug development and treatment but also to understand developmental stages of an organism or in cancer diagnosis and prognosis (11). There are different types of cell migration assays such as filter assay and wound healing assay. Wound healing assay in other words scratch assay, is a standard and inexpensive way of testing cell migration especially on adherent cells. Under experimental conditions a scratch is created in the area where the cells are dense, and migration is triggered similar to the way cells migrate to that area to heal when a wound occurs in a living organism (12).

So, in my research I wanted to evaluate the potential anti-inflammatory properties of different concentrations of *Valeriana alliariifolia* plant extract by performing cell migration analyzes in *in-vitro* conditions.

Research Question:

How does different concentrations (0, 0.08, 0.125, 0.25, 0.50 and 0.75 ul/ml) of *Valeriana alliariifolia* (helliotrope-kittyplant) extract affect cell migration efficiency of cells by using wound healing assay?

The control of inflammation is an important process in immune system functions. *Valeriana alliariifolia* due to its possible effect on inflammation, may be considered as a valuable candidate for further investigations in drug development by regulating immune response. The use of these kinds of plants for therapy among the local people is most commonly by consuming them in the form of 'herbal teas'. For this reason, by using the leaves of this plant, I aimed to analyze its anti-inflammatory effect which is important for immune response, in a cell- based system by performing cell migration analyzes.

Hypothesis:

The main hypothesis of this research is; *Valeriana alliariifolia* have anti-inflammatory properties and potential in therapy of inflammation related diseases. On the basis of *Valeriana alliariifolia's* use in traditional medicine by local people and the genus *Valeriana* components' known sedative, antimycobacterial, antiviral, cytotoxic properties reported in literature, I expect to see a lower rate of cell migration in cell plates which are incubated with non-toxic concentrations (0,08 ul/ml) of *Valeriana alliariifolia* extract.

PLANNING

	Name of Variable	Method of Management and/or Measurement
Independent variable	Different concentrations of plant extract (*Valeriana alliariifolia)* 0, 0.08, 0.125, 0.25, 0.50 and 0.75 ul/ml ± 0.001ul/ml Note that equal intervals in concentration could not be provided because the toxicity analyses restricted us in a certain value range.	*Valeriana alliariifolia* extract in cell culture medium RPMI
Dependent variable	Cell migration	Cell migration analysis: wound healing assay
Controlled variables	Cell culture conditions	Growth of cell culture in a controlled environment, incubator (constant heat, humidity)
Uncontrolled variables	Possible contamination of cell culture	Care about cell culture laboratory rules

Materials:

- Methanol 50 ± 0.1 ml
- *Valeriana alliariifolia* extract 2.000 ± 0.001 mg
- SW 982 cell line (human synovial cells) $1x10^6$ cells/ml
- Dimethyl sulfoxide (DMSO) 50 ± 0.1 ml
- Cell culture medium;
  - Roswell Park Memorial Institute (RPMI)1640 Medium 100 ± 1.000 ml
  - Heat inactivated Fetal Bovine Serum (FBS) (10% v/v in RPMI) 10 ± 0.1 ml
  - Penicillin / Streptomycin 10.000 units/mL of penicillin, 10.000 µg/mL of streptomycin (1% v/v in RPMI)
  - L-Glutamine 200 mM (1% v/v in RPMI).
- Trypsin 50 ± 0.1 ml
- MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) 5 ± 0.1 ml
- Cell culture plate (x10)
- Plastic microtubes (x100)
- Micropipette (x1) and pipette tips (x100)
- Microplate (96 well) (x2)
- Balloon (x2)
- Rotavapor (x1)
- Incubator, laminar flow (x1)
- Spectrophotometer (x1)
- Hemacytometer (x1)
- Microscope(x1)

Procedure:

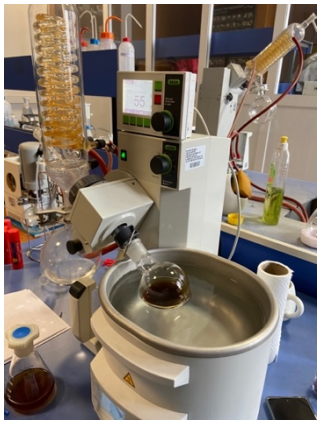


Figure 2- Rotavapor, the device used evaporation

Preparation of the *Valeriana alliariifolia* extract

1. Weigh 1 g of powdered *Valeriana alliariifolia* leafs and add 50 ml of hot distilled water.
2. Cool the mixture for 30 min then filter and evaporate to dryness in the rotavapor. (see Fig. 2).
3. Take all the extract in the balloon into vials.
4. Lyophilize the extracts using rotavapor for 48 hours after freezing at -20 °C.
5. Dissolve a portion of the lyophilized extracts in DMSO (0,5%, 1%, 2%, 4% w/v) and another portion in cell culture media (RPMI Medium, 0,25 mg/ml, 0,5 mg/ml, 1 mg/ml, 2 mg/ml, 5 mg/ml,10 mg/ml) * to use on cell cultures (Figure 3).

* According to the toxicity test (MTT assay-explained below) to be performed on the cells, it will be decided which one (RPMI or DMSO) will be used in the cell migration experiment.

Figure 3- Extracts of *Valeriana alliariifolia* dissolved in RPMI and DMSO ready to use on cell culture experiments.

Cell Culture

1. Culture (passage) SW982 cell lines (fibroblast like synoviocytes) (Figure 4) in every 3 days with RPMI (Roswell Park Memorial Institute) 1640 (Gibco) medium.
2. To passage cells, throw the medium of cells, add 5 ml of Trypsin and incubate at room temperature for 5 min to detach cells.

3. Add 5 ml of RPMI on detached cells and transfer the total volume to a separate falcon tube, centrifuge at 1000 rpm for 5 min.
4. Resuspend the cell pellet (precipitate) in 10 ml RPMI and transfer to a cell culture flask.
5. Incubate the cells in the incubator (37°C and 5% CO_2).
6. Use these cells for toxicity analysis of the extracts of *Valeriana alliariifolia* dissolved in RPMI and DMSO and for cell migration analysis.

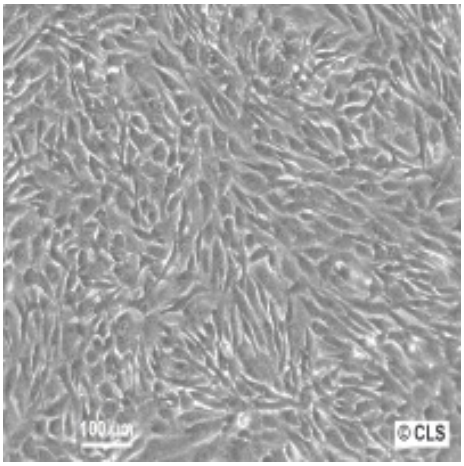


Figure 4- The microscopy picture of SW982 cells. Detailed properties of the cells are as follows; human cells with a mixed morphology isolated from joint, synovium (obtained from ATCC).

Toxicity analysis of extracts of *Valeriana alliariifolia*

Preparing the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay

1. Prepare SW982 cells and extracts of *Valeriana alliariifolia* with different concentrations as explained above to add different wells (in DMSO; control, 0,5%, 1%, 2%, 4% and in RPMI; control, 0,25 mg/ml, 0,5 mg/ml, 1 mg/ml, 2 mg/ml, 5 mg/ml,10 mg/ml) in 96-well plates containing a final volume of 100 µl/well.
2. Do not add any extract on the control wells (control wells will contain untreated cells).
3. Incubate cells for 24 hours in a 37°C, 5% CO_2 incubator.
4. For a 96-well plate, add 140 µl MTT working solution to each well (Figure 5).
5. Incubate 4 hours at 37°C, protected from light.

6. Add 100 µl solubilization solution to each well to dissolve formazan crystals.
7. Mix to ensure complete solubilization.
8. Record absorbance at 570 nm using spectrophotometer.

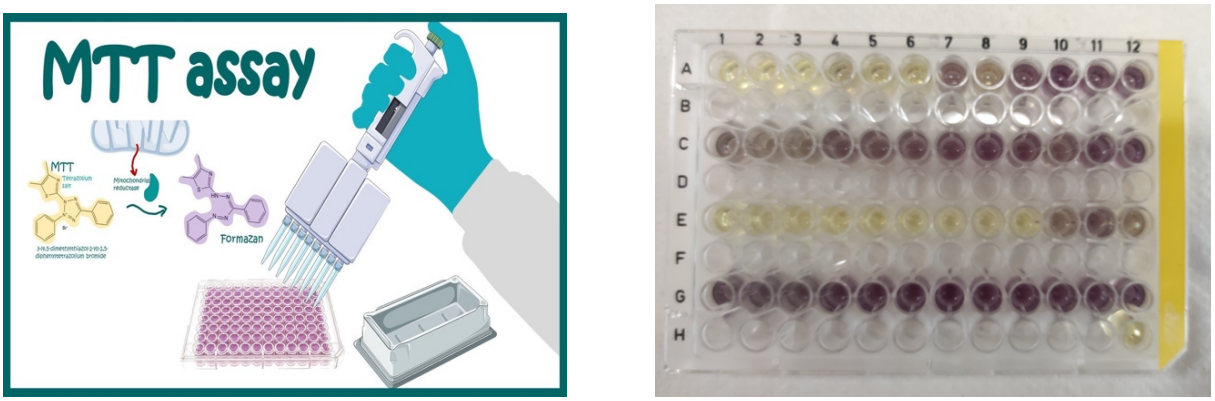


Figure 5- A; The principle of MTT assay, B; result of MTT assay in 96-well plate

Cell Migration Analysis

Wound healing assay

1. Detach SW982 cells from tissue culture dish, by incubating with 5 ml Tripsin for 5 minutes at room temperature.
2. Centrifuge the detached cells at 1000 rpm for 5 minutes. Resuspend the pellet with cell culture medium.
3. Count cells using hemacytometer under the microscope.
4. Prepare 6-well culture plate with 2 mL cell culture medium added to each well.
5. Put SW982 cells into each well of 6-well tissue culture plate at a density of $5x10^5$ cells/ml that after 24h growth, they reach 70-80% confluence.
6. At confluence scrape cell layer in a straight line using a 1 mm pipette tip. Keep tip perpendicular to the bottom of the well. After scratch, gently wash cell monolayer to remove detached cells, then replenish with fresh medium which has *Valeriana*

alliariifolia extract (control, 0,75 ul/ml, 0,50 ul/ml, 0,25 ul/ml, 0,125 ul/ml, 0,08 ul/ml in RPMI) (Figure 6).

7. Image and take pictures of the gap using phase contrast microscope on 4x and 10x magnification. In order to image the same spot each time put a sign on the plate to the region where you take picture.
8. Place 6-well culture plate in incubator, and image the same spot on phase-contrast microscope every 6 hours until cells migrate to meet in the middle for 24 hours.
9. Repeat this experiment for five times to obtain five trial data.

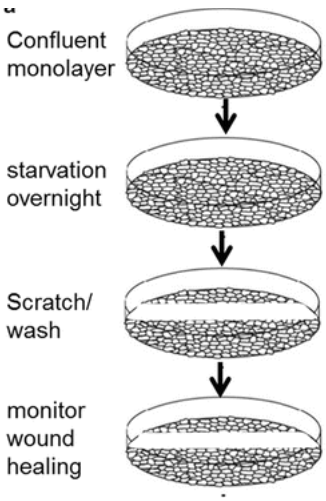


Figure 6- Demonstration of wound healing assay.

Justification:

The leafs of the plant; *Valeriana alliariifolia* were dried because these kinds of plants for therapy is consumed most commonly in the form of herbal teas (1,2,3) and used for the cell culture experiments.

SW982 cells are human synovial sarcoma cell line and known as fibroblast like synoviocytes. These cells are known to express inflammation related genes so can be used as cell culture model for functional inflammation assays. SW982 cells are adherent cells, and this property is important for their use in cell migration experiments.

In order to apply extracts of *Valeriana alliariifolia* on the SW982 cells and do cell migration experiments first we need to decide for the optimum concentration and solution Dimethyl sulfoxide (DMSO) and Roswell Park Memorial Institute (RPMI)1640 Medium, that is not toxic for these cells. RPMI and DMSO are commonly used as solvents for cell culture experiments which are suitable for a variety of mammalian cells. However, their solubility and toxicity on cells may vary depending on the solute. Therefore, it is necessary to perform a toxicity test to decide on the solvent and concentration. For this purpose, I used MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay and calculated cell death in different conditions (solvent and concentration) as explained in the previous 'Procedure' section. According to the toxicity test (MTT assay-explained below) to be performed on the cells, it will be decided which one (RPMI or DMSO) will be used in the cell migration experiment.

MTT is a cell permeable yellow tetrazolium salt which is used to measure cell viability, proliferation, and cytotoxicity. The principle of MTT assay is that only viable cells have active metabolism and water-soluble MTT is reduced in the mitochondria of these cells by succinate dehydrogenase to insoluble purple formazan crystals (Figure 5). The amount of formazan produced can be measured spectrophotometrically which is directly proportional to the number of viable cells in the culture. Dead cells are unable to convert MTT because of their loss in metabolic activity, that results in no visible color change (13).

Determining the cell migration capacity of the treated cells is an important parameter in order to understand inflammatory properties of molecules. So, I decided to do cell migration analysis by using *Valeriana alliariifolia* extracts treated SW982 cells. There are different types of cell migration assays. I decided to use 'wound healing assay' which is a standard and inexpensive way of testing cell migration especially on adherent cells. In wound healing assay, a cell-free area is created in a confluent monolayer by scratching the cell surface with a pipet

tip as demonstrated in figure 6. This way a cell-free gap is created which induces the cells to migrate into the gap. Then the collective cell migration and the area of the gap can be visualized and calculated under the microscope (12).

Risk Assessment and Ethical Considerations:

Basic safety rules given below were followed in the laboratory;

- Reading the biosafety rule book before the experiments, in order to know; the location of safety equipment and what to do in case of lab accidents.
- Dress lab coat, googles and gloves for protection,
- No drinking and eating in the laboratory,
- Dispose of lab waste properly- chemical waste were collected in glass containers, biological waste (cells) was collected in detergent filled containers and sent to waste service of the University.

A commercially available cell line (SW982 cells) was used for the experiments. Ethical approval is not required for cell lines. Cell culture experiments were done under the laminar flow in the cell culture room of the laboratory. This way the investigation was safe both for the cells (prevents contamination) and for the researcher.

RESULTS, ANALYSIS and CONCLUSION

MTT data

Raw Data Table

Absorbance (Optical Density-OD)	Different concentrations of *Valeriana alliariifolia* dissolved in DMSO				
	2 mg/ml	**1 mg/ml**	**0.5 mg/ml**	**0.25 mg/ml**	**Control**
Trial 1	2,19	19,59	61,68	104,88	97,01
Trial 2	0,73	20,87	80,90	88,41	99,02
Trial 3	7,32	8,24	77,89	90,97	96,28
Trial 4	3,26	12,52	78,78	87,15	103,23
Trial 5	2,26	15,85	63,76	86,98	98,66
**The values are rounded to have 2 decimal places.					

Table 1: Absorbance data obtained in different plant extract concentrations dissolved in DMSO. OD is directly proportional to cell viability.

Absorbance (Optical Density-OD)	Different concentrations of *Valeriana alliariifolia* dissolved in RPMI						
	10 mg/ml	**5 mg/ml**	**2 mg/ml**	**1 mg/ml**	**0,5 mg/ml**	**0,25 mg/ml**	**Control**
Trial 1	1,65	0,91	1,83	28,74	76,51	91,89	97,01
Trial 2	1,09	1,09	1,28	23,06	80,72	94,26	99,02
Trial 3	0,18	0,92	2,19	25,56	75,41	93,53	96,27
Trial 4	0,96	1,01	1,96	29,05	78,24	94,05	103,23
Trial 5	0,69	0,92	2,45	24,54	19,46	92,15	98,66
**The values are rounded to have 2 decimal places.							

Table 2: Absorbance data obtained in different plant extract concentrations dissolved in RPMI. OD is directly proportional to cell viability.

MTT Data Processing and Graph

The amount of formazan produced was measured spectrophotometrically which is directly proportional to the number of viable cells in the culture. Dead cells are unable to convert MTT because of their loss in metabolic activity, that results in no visible color change.

As the solubility of the extract is higher in RPMI (see figure 3) and MTT analysis showed that the extract dissolved in RPMI resulted in less cell death, I decided to use the extract dissolved in RPMI in the cell migration experiments (Figure 7).

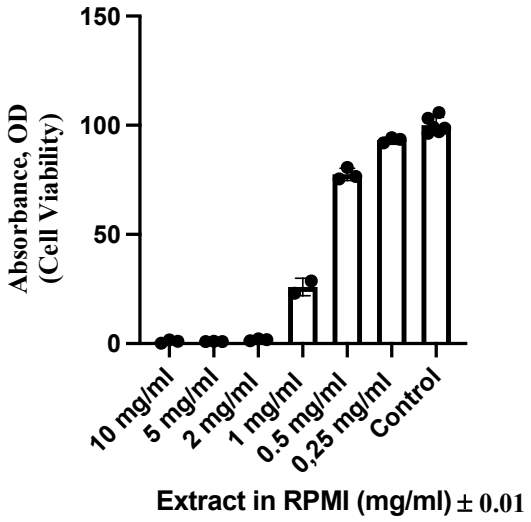
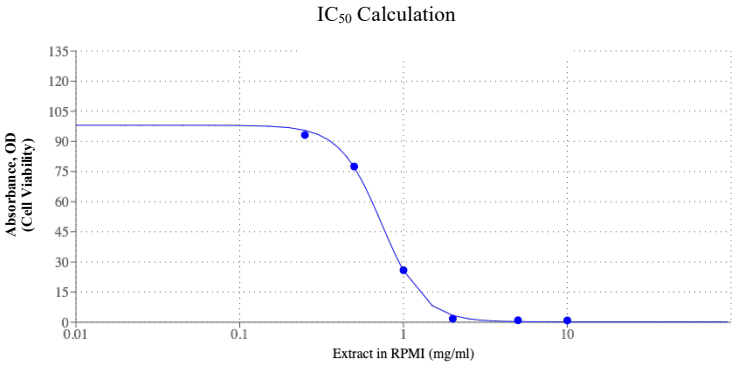


Figure 7- Graphical representation of MTT results.

IC_{50} is a quantitative measure of how much of a specific substance is required to effect biological component by 50%. For our experiment the IC_{50} value of a plant extract represents how much of it is required for cell death of 50% of the cells which is a useful measure for us to decide optimum concentration for the following cell migration experiments. IC_{50} is calculated for the extract dissolved in RPMI as it was better in terms of cell death rate and solubility of the extract in RPMI. IC_{50} was calculated as 0.7348 for the extract dissolved in RPMI incubated with SW982 cells (Figure 8).



Parameter	Value
IC_{50}	0.7348
Equation	$Y = 0.1686 + \frac{98.0372 - 0.1686}{1 + (\frac{X}{0.7348})^{3.38}}$
Equation Form	$Y = Min + \frac{Max - Min}{1 + (\frac{X}{IC_{50}})^{Hill\ coefficient}}$

Figure 8- Graphical representation and calculation of IC_{50}.

Wound Healing Assay Data

The cell-free gap created which induces the cells to migrate into the gap was imaged for the wound healing assay analysis (Figure 9).

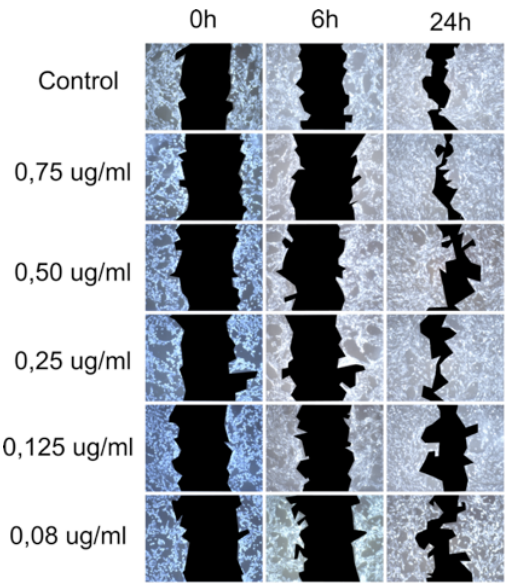


Figure 9- Microscope image (4X magnification) of the wound area in cells incubated with different concentrations of plant extract dissolved in RPMI.

**Note that equal intervals in concentration could not be provided because the toxicity analyses (MTT assay) restricted us in a certain value range.

Wound Healing Assay Data Processing and Graph

I took pictures of the gap using phase contrast microscope on 4x and 10x magnification and imaged the same spot on microscope every 6 hours until cells migrate to meet in the middle for 24 hours. The collective cell migration (wound healing) and the area of the gap was visualized and calculated in percentage unit under the microscope using microscope software and the following graph was formed (Figure 10).

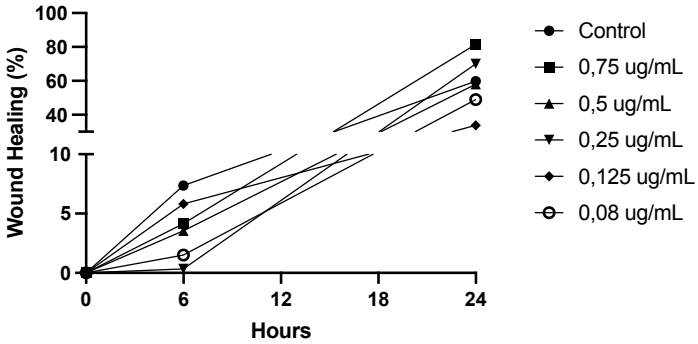


Figure 10- Graphical representation of wound area of cells incubated with different concentrations of *Valeriana alliariifolia* extract dissolved in RPMI.

DISCUSSION and EVALUATION

Discussion:

Many plants have been used for health purposes due to effects of their bioactive compounds. So, I wanted to do a research on the potential therapeutical use of a plant, *Valeriana alliariifolia* which is less known, by analyzing its anti-inflammatory effect.

Inflammation, the primer mechanism of innate immune system, is a normal response to abnormal structures/signals in the organism. The main function of inflammation is to limit the infection and prevent it from spreading other parts of the body. However, as we see in other physiological events, the balance is important. Inflammatory reaction needs to stop once the pathogen is eliminated from the body by the immune cells and damaged body tissues are repaired. Un-controlled inflammation can cause damage to the body's other organs and tissues and may contribute to serious problems (7,8). Thus, for the bioactive compounds, which may be considered as drug candidate, the anti-inflammatory effect is an important property especially for therapeutical applications of infectious, autoimmune and autoinflammatory diseases.

In the light of these information; the basis of this research was to understand the anti-inflammatory properties of *Valeriana alliariifolia* and its potential in therapy of infectious diseases.

The results of the cell migration assay, an important indicator of inflammation, demonstrated that *Valeriana alliariifolia* inhibits cell migration of SW982 cells in lower concentrations. I did not observe a change in wound area in higher concentrations when compared to control cells. This result may be due to a possible effect of the same extract on cell division which can also help cells to fill the gap at wound area at higher concentrations. Although the preliminary data that I obtained so far supports our hypothesis, the research

question should further invastigated by different cell migration analysis procedures and in additional cells such as monocytes.

Possible applications of this research may be use of *Valeriana alliariifolia* leaves as herbal tea for its anti-inflammatory effect following its toxicology research in *in-vivo* conditions. As the control of inflammation is an important process in immune system functions; *Valeriana alliariifolia* may be considered as a candidate for drug development research following the identification of bioactive compounds and use this information with the additional functional research on its anti-inflammatory effect for drug development.

Evaluation:

Strengths	**Reason it's believed to be a strength**
To do MTT assay by using different concentrations of extracts dissolved in different solvents.	An analysis used to determine the optimum concentration of the extract which is not toxic to cells.
To do cell migration assay in order to examine anti-inflammatory properties of a molecule	Cell migration is an important indicator of inflammation.

Limitations	**Effect of Limitation on the result of investigation**	**Suggested improvement and why it will improve the investigation**
Cell type used in the experiments (SW982 cells)	SW982 cells are known as fibroblast like synoviocytes which express inflammation related genes so can be used for inflammation assays. However, it is not a cell line of the immune system cells.	Other cell lines of cells which are mainly functioning in the immune system can be used to support the data. For example, THP-1 cells which is a human monocytic cell line can be used to improve the research.
Cell migration assay (wound healing)	Wound healing assay is a standard and inexpensive way of testing cell migration especially on adherent cells however wound area size is also affected by cell division which may change the results.	The results of wound healing assay can be supported by other cell migration methods such as 'Filter assay' which quantitatively analyses the migrating cells in two different compartments.

Only one functional assay used in the study design	Cell migration is one of the mechanisms that is important in inflammation. There are also other mechanisms such as cytokine (proteins important in signaling) secretion, caspase activation or phagocytosis which give information about inflammation.	The results can be further supported by other functional assays such as gene expression analysis of cytokines or phagocytosis assay.

Conclusion:

Migration of responsible cells to the infected tissue is an important process of inflammation and causes dilation of the blood vessels which allow for transport of other immune cells. Therefore, investigating the effect of a molecule on cell migration is a common approach to evaluate its possible anti-inflammatory properties.

The data I obtained by cell migration analysis, wound healing assay, showed that extracts of *Valeriana alliariifolia* dissolved in RPMI inhibits cell migration of SW982 cells in lower concentrations (0.125ug/ml, 0.08ug/ml). The results obtained in higher concentrations (0.25 ug/ml, 0.50 ug/ml, 0.75 ug/ml) was similar to those obtained in control SW982 cells. As can be seen from the results that I obtained at concentrations 0.125ug/ml and 0.08ug/ml (Figure 10) supported my hypothesis may be considered as a promising data for the anti-inflammatory properties *Valeriana alliariifolia*.

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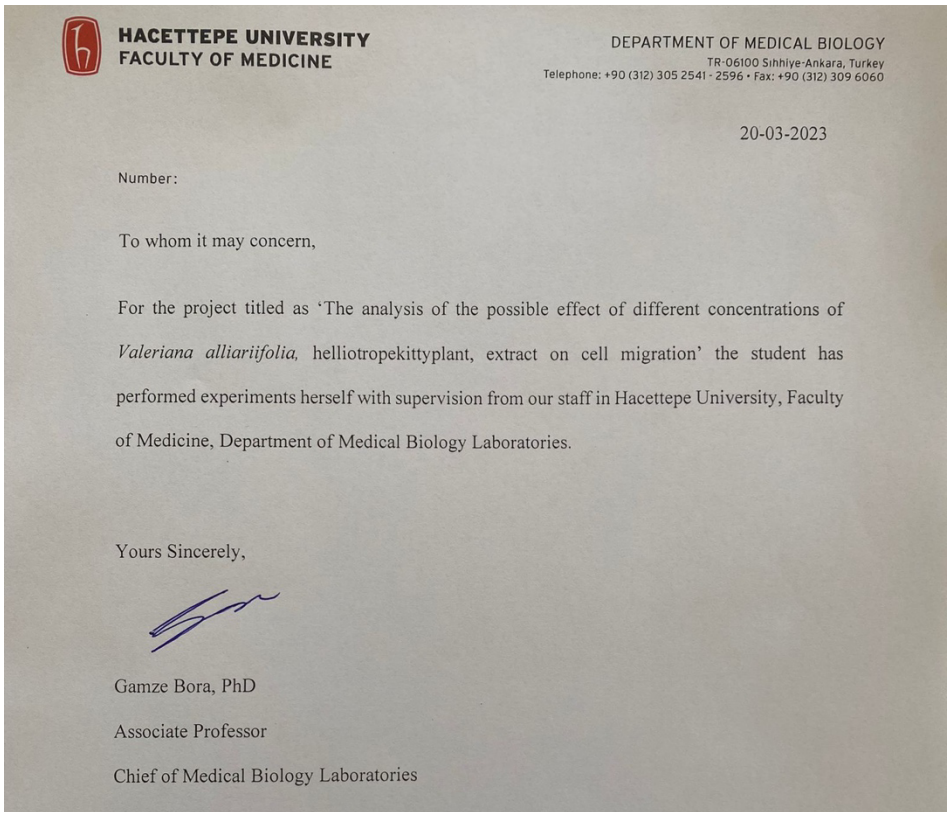
Appendix



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Number:

To whom it may concern,

For the project titled as 'The analysis of the possible effect of different concentrations of *Valeriana alliariifolia,* helliotropekittyplant, extract on cell migration' the student has performed experiments herself with supervision from our staff in Hacettepe University, Faculty of Medicine, Department of Medical Biology Laboratories.

Yours Sincerely,

Gamze Bora, PhD
Associate Professor
Chief of Medical Biology Laboratories