# DETERMINATION OF APOPTOTIC EFFECTS OF CLINOPTILOLITE ON HUMAN T LYMPHOCYTES

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**by**
**Mehmet Emin USLU**

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**İZMİR**

We approve the thesis of **Mehmet Emin USLU**

_____

**Assist. Prof. Dr. Ayten NALBANT**
Supervisor

_____

**Prof. Dr. Semra ÜLKÜ**
Co-Supervisor

_____

**Prof. Dr. Semra Koçtürk**
Committee Member

_____

**Assoc. Prof. Dr. Kemal Korkmaz**
Committee Member

_____

**Assist. Prof. Dr Gülşah Şanlı**
Committee Member

**09 0ctober 2008**

_____

**Prof. Dr. Semra ÜLKÜ**
Head of the Biotechnology and Bioengineering Department

_____

**Prof. Dr. Hasan BÖKE**
Dean of the Graduated School of Engineering and Sciences

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# ABSTRACT


## DETERMINATION OF APOPTOTIC EFFECTS CLINOPTILOLITE ON HUMAN T LYMPHOCYTES

Zeolites are defined as aluminum silicates that have made up of oxygen, aluminum and silicon. $SiO_4$ and $Alo_4$ tetrahedrals are the smallest units that give the specific shape to the molecule. There are more than 40 types of natural and over 150 types of synthetic zeolites occurs and those zeolites are used in agriculture, animal husbandry, architecture, pharmaceuticals and metallurgy. Recent years in literature it was shown that these zeolites can have regulatory effects on immune system. Also it was shown that they can influence the development of cancer and have a role on the expression of tumor suppressor genes. However there is no evidence can be found on how these molecules affect the function of specific cell types in the molecular level and also the mechanism of this effect.

In this study the apoptotic effects of clinoptilolite which is a natural zeolite found in Gördes region of Turkey, on human T lymphocytes were studied. T cells were chosen in this study because they are the main players in the immune system. These cells can establish immune regulation and organize immune response. As a result, they are important in pathology and therapy. Peripheral blood mono nuclear cells (PBMCs) in which T cells can be found were isolated from the healthy donors blood by Ficoll Hypaque Gradient Method and then these cells were incubated with clinoptilolite in the RPMI 1640 media. Apoptosis were measured in FacsArray after appropriate immunofluorescent labeling and by agarose electrophoresis technique after DNA fragmentation assay done.

# ÖZET


## KLİNOPTİLOLİTİN İNSAN T LENFOSİTLERİ ÜZERİNDEKİ APOPTOTİK ETKİLERİNİN BELİRLENMESİ

Zeolitler alkali ve toprak alkali kristal yapıya sahip sulu alüminyum silikatlar olarak tanımlanırlar. Oksijen, alüminyum ve silisyumdan oluşan kristalin yapının en küçük yapı birimi $SiO_4$ ve $AlO_4$ dört yüzlüsüdür. Bugün, tarım ve hayvancılık, kirlilik kontrolü, enerji uygulamaları, zirai kullanım, madencilik-metalürji, konstrüksiyon ve yapı elemanı olarak, sağlık uygulamaları (diyaliz, diş sağlığı, ameliyat ve tıbbi ilaç yapımı) gibi alanlarda kullanılan 40'dan fazla doğal ve 150'den fazla yapay zeolit türü bilinmektedir. Literatürde son yıllarda yapılan çalışmalar, zeolitlerin bağışıklık sistemi üzerinde düzenleyici etkisi olduğuna dair deliller sunmuştur. Ayrıca yapılan diğer çalışmalarda, klinoptilolitin kanserin gelişimini etkilediğini ve tümör baskılayıcı genlerin ekspresyonunda rol oynadığını göstermiştir. Fakat bu moleküllerin belli hücre fonksiyonlarını moleküler düzeyde nasıl etkilediği ve etkinin mekanizması konularında deliller gösterilememiştir.

Bu çalışmada Türkiye'nin Gördes bölgesinde çıkarılmış doğal bir zeolit türü olan klinoptilolitin insan T lenfositleri üzerindeki apoptotik etkilerinin belirlenmesi çalışılmıştır. T hücreleri immün regülâsyonu ve yanıt organizasyonunu sağlayan ana unsur olmaları nedeni ile bu çalışma için seçilmişlerdir. T hücrelerininde içinde bulunduğu periferal mono nükleer kan hücreleri (PBMCs) sağlıklı donörlerin kanlarından Ficoll Hypaque Gradient tekniği ile izole edilmiştir. Daha sonra bu hücreler RPMI 1640 besi yeri içerisinde klinoptilolit ile inkübe edilmiştir. Apoptoz sonuçları gerekli immünoflorasan boyamaları yapıldıktan sonra FACS Array cihazı ve DNA fragmentasyon analizi yapıldıktan sonra agaroz jel elektroforez tekniği ile gösterilmiştir.

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# CHAPTER 1

# INTRODUCTION

## 1.1. T Lymphocytes

T lymphocytes are the main players in the immune system. They can establish immune regulation and organize immune response. As a result, they are important in pathology and therapy. T lymphocytes are derived from hemopoietic stem cells in bone marrow. After their maturation in the thymus T lymphocytes become $CD4^+$ or $CD8^+$ T cells. While these naïve T cells circulate through peripheral blood stream they can get into lymphatic system. During their circulation in lymphatic system they can be activated in the lymph nodes where they can interact with the appropriate antigens via antigen presenting cells. It was shown that silicates can activate T cells and cause apoptosis via antigen presenting cells (Abbas and Lichtman 2003, Aikoh, et al. 1998, Hishumura, et al. 2006, Tomokuni, et al. 1997, Otsuki, et al. 1998, Otsuki, et al. 2000, Otsuki, et al. 2002).

## 1.2. Antigen Presenting Cells

Dendritic cells, B cells, monocytes and macrophages are the types of antigen presenting cells (APC). Antigen presenting cells can endocytose any extracellular protein antigen from the environment into vesicles and processed them in order to present them to T cells. Antigen processing is a biological process that prepares antigens for presentation to T lymphocytes. Proteins are endocytosed and degraded by acid-dependent proteases in endosomes. The MHC class II protein in the rough ER has its peptide binding cleft blocked by a trimer (the invariant chain) to prevent it from binding peptides. The invariant chain also facilitates MHC class II's export from the ER in a vesicle. This vesicle fuses with a late endosome containing the endocytosed, degraded proteins. It is then broken down in stages, leaving only a small fragment called CLIP which still blocks the peptide binding cleft. An MHC class II-like structure, HLA-DM, removes CLIP and replaces it with a peptide from the endosome. The stable

MHC class-II is then presented on the cell surface and then presents the antigen to T cells. T cells and monocytes are found in PBMC. Monocytes are important, because they are found in the blood stream and can take role in both conventional and unconventional antigen presentation by their MHC receptors. $CD4^+$ T cells' T cell receptors (TCR) can interact with MHC II receptors which are expressed on the antigen presenting cells membrane. On the other hand $CD8^+$ T cells' TCR's can interact with MHC I receptors which are expressed on all nucleated cells (Abbas and Lichtman 2003).

There are two types of antigen presenting pathways by which T cells can be activated as shown in figure 1. In conventional antigen presentation peptides are processed and presented to T cells. The other type of antigen presentation is unconventional antigen presentation and superantigens are the key elements. Superantigens are a class of highly potent immuno-stimulatory molecules. These molecules possess the unique ability to interact simultaneously with MHC class II molecules and T-cell receptors, forming a trimolecular complex that induces profound T cell proliferation. Unlike conventional antigens, superantigens remain unprocessed by antigen presenting cells instead binding directly to APCs on the outside of MHC class II molecules. Almost all superantigens interact exclusively with the $V_\beta$ region of the T cell receptor resulting in the stimulation of up to 10% of resting T cells (Abbas and Lichtman 2003, Baker, et al. 2004, Krakauer, et al. 2005). After presentation of antigens, T cells can respond to this stimulus in three different ways. First of all they can show immune response. Secondly, T cells can not give any response to that antigen because of the absence of co-stimulatory interaction between the antigen presenting cell and T cell. Lastly, T cells can directly go into apoptosis if they are repeatedly stimulated by persistent antigens.

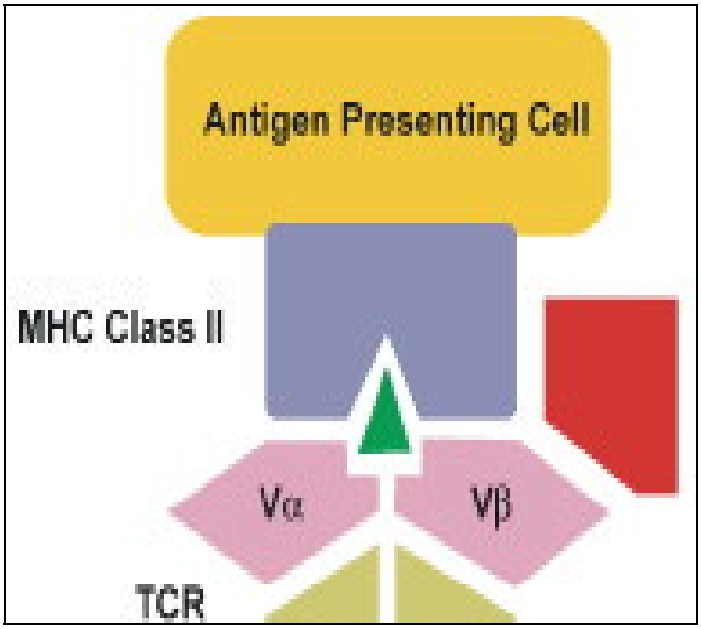


Figure 1.Types of antigen presentation. Schematic diagram showing the binding of superantigen (red) and conventional antigen (green) with major histocompatibility complex (MHC class II) and T cell receptor (TCR) molecules. Two types of antigen presentation, conventional and unconventional. Conventional: Antigens, which are endocytosed from the environment, processed and presented via MHC molecule of antigen presenting cells. Unconventional: Unprocessed superantigens can bind to the outside region of both antigen presenting cells MHC molecule and T cell receptor and those two pathways can activate T cells (Source: Solanki L.S, et al. 2008).

Aikoh et.al. (1998) showed that silicates; zeolites can be told as silicates as a result of Si ion content, have the ability to act as superantigen in T cell activation and apoptosis via unconventional antigen presentation pathway. Aikoh et al. (1998) and Hyodoh et al. (2005) showed that in silicolosis patients, silicates have an ability to induce apoptosis on T cells via stimulating the extrinsic or intrinsic pathways of apoptosis. Zeolites are kind of silicates and may cause apoptosis. During literature search it was seen that investigations only made on silicates especially on asbestos and the cell line that are studied, mouse fibrosarcoma, small cell carcinoma, human pancreas carcinoma cells and normal diploid fibroblasts. There were no any investigations carried on zeolite-T cell interaction and the effects of zeolites on T cells.

## 1.3. Apoptosis

There are three types of cell death. Autophagy, apoptosis are the types of programmed cell death, and necrosis is the cell death, which is a form of cell-death that results from acute tissue injury. Apoptosis is a naturally occurring energy-dependent process of cell death. In this process specialized proteases (caspases) and endonucleases (e.g.CAD) carry out the ordered degradation of the cell. Apoptosis can be characterized by detachment of cells from extracellular matrix, changes in membrane lipid distribution, nuclear condensation, DNA cleavage, and fragmentation, plasma membrane blebbing.

To detect apoptosis, annexin-V / 7-Aminoactinomycin-D (7AAD) labeling or DNA fragmentation assay will be used. Radiolabeled annexinV which is a human protein used to detect early apoptosis. This protein has a high affinity for the membrane aminophospholipid which is phosphatidiylserine. Phosphatidiylserine normally found in the inner part of cell membrane. As apoptosis starts this aminophospholipid redistributed onto the cell surface by the help of floppase enzyme. As it change the location annexinV can bind to it, therefore annexinV is a sensitive marker for early phases of apoptosis (Blankenberg, et al. 2003). 7-ADD is G-C base-specific DNA probe. In living cells 7 AAD can not pass through the nuclear envelope however as a result of hole formations on nuclear envelope this probe can pass through membrane and bind to G-C bases, consequently 7-AAD is a useful marker for the detection late apoptosis of cells (Schmid, et al. 1992). The DNA fragmentation can be used to see the products of apoptosis which forms from endonuclease cleavage. As a result of this cleavage, one of main the characteristic of apoptosis “DNA ladder” forms. Each band in the ladder separated in size by approximately 180 base pair. This assay involves extraction of DNA from a lysed cell homogenate and the DNA can be visualized by agarose gel electrophoresis (Elmore 2007).

There are two major pathways to apoptosis, the intrinsic (mitochondrial) and extrinsic (death receptor) pathways, shown in figure 2. The first one is extrinsic pathway which involves binding of ligands to death inducing membrane receptors (TNFR, TRAMP, TRAIL, and CD95L). These receptors induce other intracellular pathways until DNA cleavage occurs. The second pathway is intrinsic pathway. In this type mitochondrial membrane becomes permeable as a result of intra or extracellular

stimuli. As a result several proteins are released into the cytoplasm, such as cytochrome-c. This protein triggers the apoptotic pathways and at the end DNA cleavage occurs (Elmore 2007).

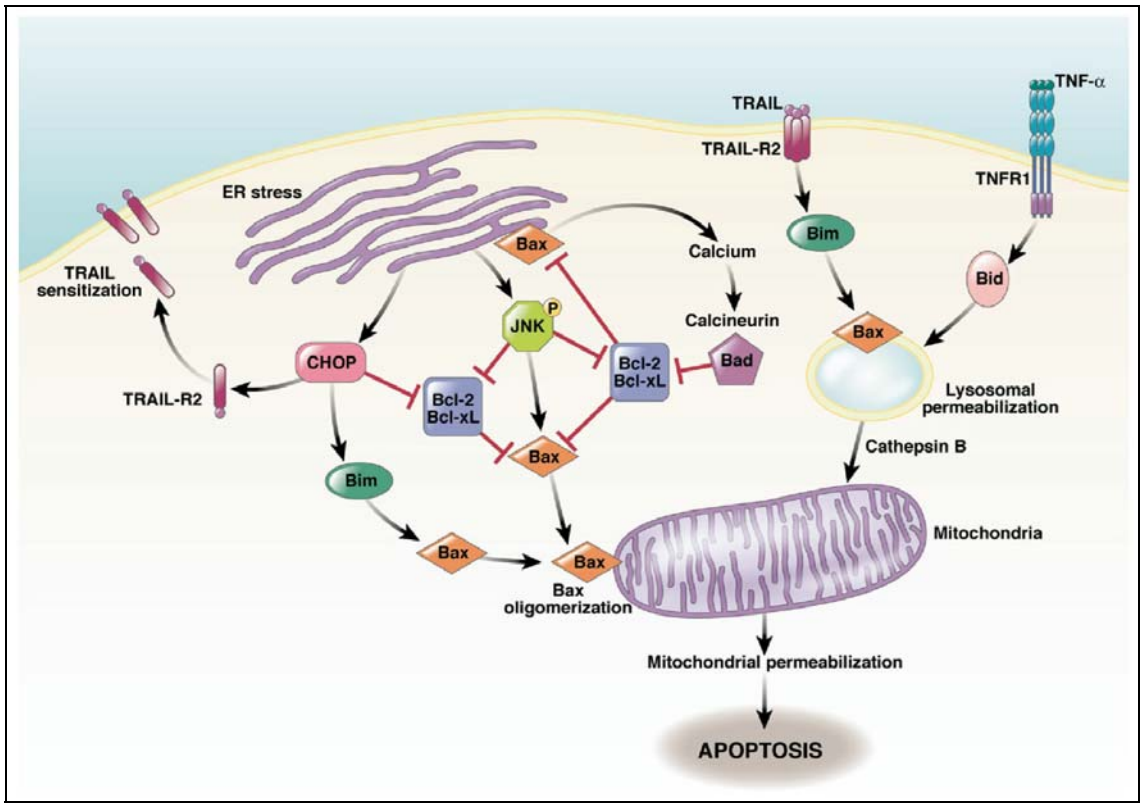


Figure 2.Pathways of apoptosis. Intrinsic and extrinsic pathways of apoptosis. Intrinsic pathway: Mitochondrial stress increase the permeability of mitochondria, as a result release of cytochrome-c into the cytoplasm occurs. This triggers caspase pathways and apoptosis occurs. Extrinsic pathway: Binding of ligands to death inducing receptors result in activation of caspase pathways and apoptosis occurs (Source: Malhi et. al, 2008).

Apoptosis of T lymphocytes is important because by this way immune system can eliminate unwanted and potentially dangerous T lymphocytes. Aikoh et al. (1998) carries out investigations on silicolosis patients and shows that these particles might cause apoptosis by Fas/FasL pathway, which is an extrinsic pathway.

In this research the effect of zeolites, which can be used as an antigen on T lymphocyte, apoptosis and the apoptotic pathway will be investigated. Zeolites can be used as antigens because they are silicates as a result of Si ion content. These particles can be act as superantigens and induce apoptosis of T cells.

## 1.4. Biological Effects of Zeolite

### 1.4.1. Characteristics

Zeolites are made up of $SiO_4$ and $AlO_4$ tetrahedrals as shown in figure-3 and the Si / Al ratio should be equal to 1 / 2. Those 3D aluminosilicate frameworks cause the developed system of micropores and channels which have a discrete size of 3 to 20 $^0$ A. (Kowalczyk, et al. 2004, Ören, et al. 2006, Doula, et al. 2006). Si atoms may exchange with Al atoms in the soil easily so the mineral gains a hydrophilic character (Rivera, et al. 2005, Deka, et al. 2002). As a result of aluminum ion's net negative charge, which is present in the zeolite framework, those channels and micropores are occupied by water molecules or by the extra framework cations such as Na, K, Ca, Mg, Fe, Sr, Br (Kowalczyk, et al. 2006, Doula, et al. 2006, Armaroli, et al. 2006, Kleiner, et al. 2001). The localization, number and sizes of these cations have an influence on the pore structure of our zeolites (Kowalczyk, et al. 2006). They also cause shape selectivity and molecular sieving effect of the channels (Bevilacqua, et al. 2006).

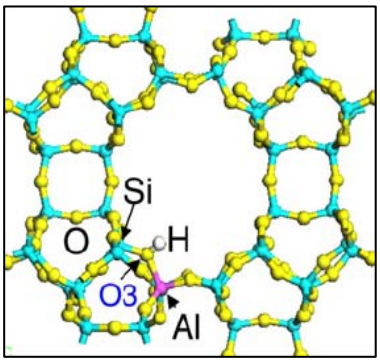


Figure 3.Structure of zeolite. Microporous structure and the cavities that occurs as a result of cations, which occupies inner structure of zeolites (Source: Oumi, et al. 2006).

These cations have the prime importance for the adsorption and ion exchange properties of zeolites. They can bind to the Al atoms loosely so they can be easily exchanged by other substances and those substances can be hold in the channel system until zeolite comes across a substance which has a high binding capacity or hold infinitely (Lomratsiri, et al. 2006). Those binding sites are called active sites and they

have also seen in the external surface and at the pore mouth of zeolite crystal. It is thought that these sites are responsible for unwanted non-selective catalysis (Armaroli, et al. 2006). More than 150 synthetic and 48 natural zeolites can be found. One of them is clinoptilolite. Clinoptilolite is a natural zeolite comprising a microporous arrangement of silica and alumina tetrahedral. It has the complex formula of $(Na,K,Ca)_{2-3}Al_3(Al,Si)_2Si_{13}O_{36}\cdot 12(H_2O)$. This mineral is abundant in Turkey. Dahm et. al (2004) showed that proteins adsorbed on the zeolite surface were shown to enter the endosomal pathway after phagocytosis and could be cleaved by the endosomal proteases.

Zeolites are the important molecules in human life as a result of their unique characteristics. Scientists start to investigate the effects of these unique characteristics of zeolites on biological systems and the direct effects of zeolites on these systems.

### 1.4.2. Effects of Zeolites on Immune Response

In 1984 Korkina et.al. showed that clinoptilolite particles cause hemolysis and macrophage toxicity (Kleiner, et al. 2001). Katic et al. (2006) showed that clinoptilolite can absorb the growth factors in cell media and inhibit the cell proliferation (Rivera, et al. 2005). These studies show that clinoptilolite, a kind of naturally occurring zeolite, has apoptotic, toxic and hemolysitic effects on living cells directly or indirectly. In addition to these investigations, it was found that silicates have the superantigen ability so that they can induce T cell apoptosis and may cause autoimmune diseases. Not only the T cells but also the alveolar epithelial and mesothelial cells can undergo apoptosis. Both intrinsic and extrinsic pathways may be triggered by silicate particles (Aikoh, et al.1998, Hishumura, et al. 2006, Tomokuni, et al. 1997, Otsuki, et al. 1998, Otsuki, et al. 2000, Otsuki, et al. 2002, Matthew, et al. 2004). Ueki et al. (1994) and Aikoh et al. (1998) showed that silica; silicates and aluminosilicates act as nonspecific immunostimulators (like superantigens) (Aikoh, et al. 1998, Pavelic, et al. 2001). Also Allison et al. (1966) showed that pro-inflammatory macrophages were activated by fibrinogen silicate particulate. Silicates are the substances of which have Si ions in their structure. Clinoptilolite is that kind of substance but there was no any investigations carried out on zeolites or clinoptilolite for their effect on T lymphocyte apoptosis. In this study T lymphocyte apoptosis via clinoptilolite will be investigated, and then the pathways that can take role in apoptosis will be enlightened. Therefore it can

hypothesized that clinoptilolite which is a natural occurring zeolite, can affect the T cell apoptosis.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1. Characterization of Clinoptilolite

Characterization of zeolites (clinoptilolite) which were come from Gördes / Turkey (ENLİ Mining) were done in the Chemical Engineering department of IYTE by a Master of Science student Ayten Top. Inductively Coupled Plasma Atomic Emission Spectrometer (ICP-AES) was used to determine the Si /Al ratio, pore size and ion content of clinoptilolite were determined. The zeolites that were used in this research have a size of 25 to 45 μm and have a dry weight of 88 %.

**Table 1:** Si/Al ratio and ion content of clinoptilolite determined by ICP-AES

| **Species (w/w %)** | **Original-Clinoptilolite** |
|---|---|
| **$SiO_2$** | **71.27** |
| **$Al_2O_3$** | **11.36** |
| **$Fe_2O_3$** | **11.36** |
| **$K_2O$** | **3.844** |
| **$Na_2O$** | **0.998** |
| **CaO** | **2.344** |
| **MgO** | **0.420** |
| **$H_2O$** | **14.22** |
| **Total** | **105.69** |

## 2.2. PBMC Isolation

Peripheral blood mononuclear cell (PBMC) will be separated by Ficoll Hypaque Density Gradient Separation method. Bloods were taken from healthy donors, diluted by 1X PBS and layered over ficoll separation solution. After centrifugation PBMC layer was collected from the gradient.

## 2.3 Conditional Media

RPMI 1640 media was treated with different doses of clinoptilolite to see the effect of Zeolite on growth media. Clinoptilolite was put into RPMI 1640 media between the range 1 to 100 mg/ml and treated for 24 to 96 hour at room temperature. After the treatment time finished clinoptilolite was separated from RPMI by centrifugation and filtration with 0, 22 μm filters. Clinoptilolite free RPMI was used in cell culture to detect if apoptosis occurs or not as a result of clinoptilolite treatment on RPMI

## 2.4 Cell Culture

Cell culture was done in triplicates. RPMI alone was used for negative control, for positive control 2 μM camptothecin, which is an apoptosis inducing agent, was used. The concentration of clinoptilolite that was used in the culture was between the ranges of 1 to 300 mg/ml. Incubation of cultures were held in 5% $C0_2$ at $37^0$C.

## 2.5 Immunoflouresence Labeling

Detection of apoptosis has been carried out by Annexin-V and 7 AAD labeling. After designed incubation time reached cells were harvested and freed from RPMI by centrifugation. Cells were than labeled and incubated for 20 min. at $4^0$C in dark. Apoptotic cells will be acquired by Flow Cytometry (BD FacsArray)

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## 2.6 DNA Fragmentation Assay

DNA fragmentation protocol was held to detect DNA ladders which form as a result of apoptosis. After incubation the cells were lysed with cold NP-40 lysis buffer for 5 min. (1% NP-40 in 20 mM EDTA, 50 mMTris-HCl, pH 7.5; 10 µl per $10^6$ cells, minimum 50 µ1). Lysed cells were centrifuged and were brought to 1% SDS. RNAse were added (final concentration 5 µg/ µ1) and incubated for 2 hour. at $56^0$C., than proteinase K (final concentration 2.5 µg/ µ1) were added to the homogenate and incubated for at least 2 hour at $37^0$C. After addition of ½ volume of 10 M ammonium acetate, DNA was precipitated with 2.5 V of cold absolute ethanol. Centrifugation was done at maximum speed for 10 min and pellets were washed with %70 EtOH. Aspirate the excess EtOH, dissolve the pellet in 1X TE buffer and separated by electrophoresis in 1.7 % agarose gel (Herrmann, et al.. 1994).

# CHAPTER 3

# RESULTS AND DISCUSSION

## 3.1 Effects of Clinoptilolite on RPMI 1640 Media

Main characteristics of clinoptilolite were its absorption and ion exchange capacity. These materials were used as an antigen in this research so whether this substance has an effect on the media or not should be investigated. Clinoptilolite can absorb the essential ions; amino acids and vitamins that are found in the RPMI 1640 media or clinoptilolite can exchange its ions with the surrounding molecules. These absorption or ion exchange of molecules in RPMI 1640 can change the pH of the media or the media can become an unsuitable place for cell growth.

Also clinoptilolite can absorb the essential vitamins, amino acids or ions in the media, which can affect the growth of cells. To detect this, the pre-treated media and un-treated media were used in cell culture and the viability of the cells was investigated.

In figure 4 RPMI 1640 media was treated with 1, 25, 50 and 100 mg/ml clinoptilolite for 24 hour at room temperature. The PBMC's were than cultured and incubated with this pretreated RPMI for 96 hour. After the 7AAD labeling data's were collected from FacsArray software. It was seen that there was a 6% of apoptosis occurs on the negative control culture. When the clinoptilolite treatment data's investigated it was seen that the percentage of apoptosis were increased only one percent. This will means that none of the doses that were used in this treatment can not make any drastic changes on RPMI's structure that will end apoptosis stimulation on 96 hour culture incubation.

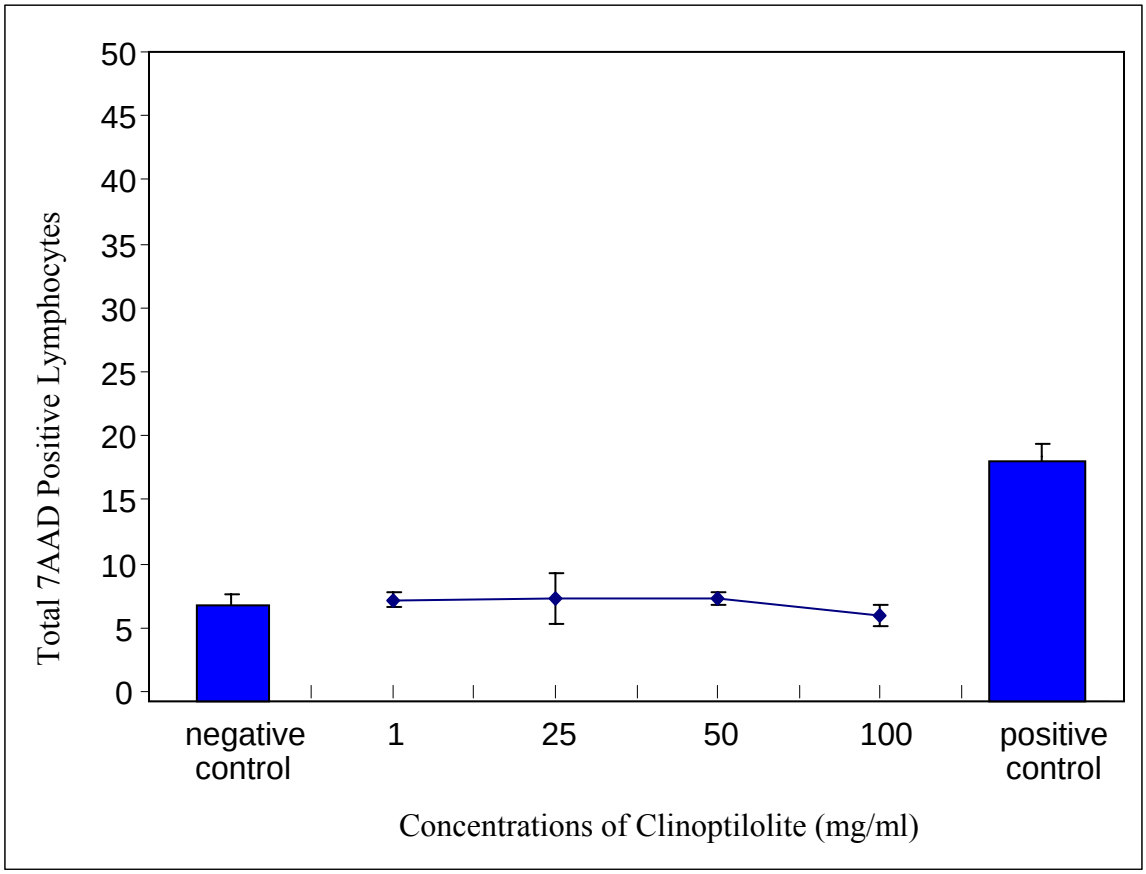


Figure 4. Effect of clinoptilolite treated RPMI on lymphocyte apoptosis. For positive control 2µM camptothecin was used, RPMI alone was used as negative control. 1, 25, 50, 100 mg/ml clinoptilolite treated with RPMI for 24 hour at room temperature. In 96 hour cell culture those treated clinoptilolite free RMPIs were used to detect the effect on apoptosis.

After investigating the effects of different doses on RPMI 1640 media the different treatment times with fixed dose should be investigated. In figures 5 and 6, the data's were show the effects of 50 mg/ml clinoptilolite treatment on RPMI 1640 media at 24, 48, 72, 96 and 120 hour time periods. Each pretreated media were used in PBMC cultures. In figure 5 the culture time is 72 hour however culture time was 96 hour on figure 6.

At the results of 72 hour incubation it was seen that 4 percent of PBMCs were gone into apoptotic cycle at negative control. When the treatment time data's analyzed it was seen that as the treatment time increases, the percentage of apoptosis also increases respectively. However difference between the percentage of apoptosis on negative control and the percentage of apoptosis on the maximum treatment that has the highest apoptotic value, is only 2 percent. The results were similar in 96 hour incubation

experiment. Both the negative controls and treatment times' apoptosis percentages increased. This is the result of increase in the incubation time. However the difference between the negative control's result and treatment times' were not bigger than 2 percent.

Therefore, it can be said that at room temperature, clinoptilolite can not do any drastic changes neither on the contents nor the pH of RPMI 1640 media to induce apoptosis.

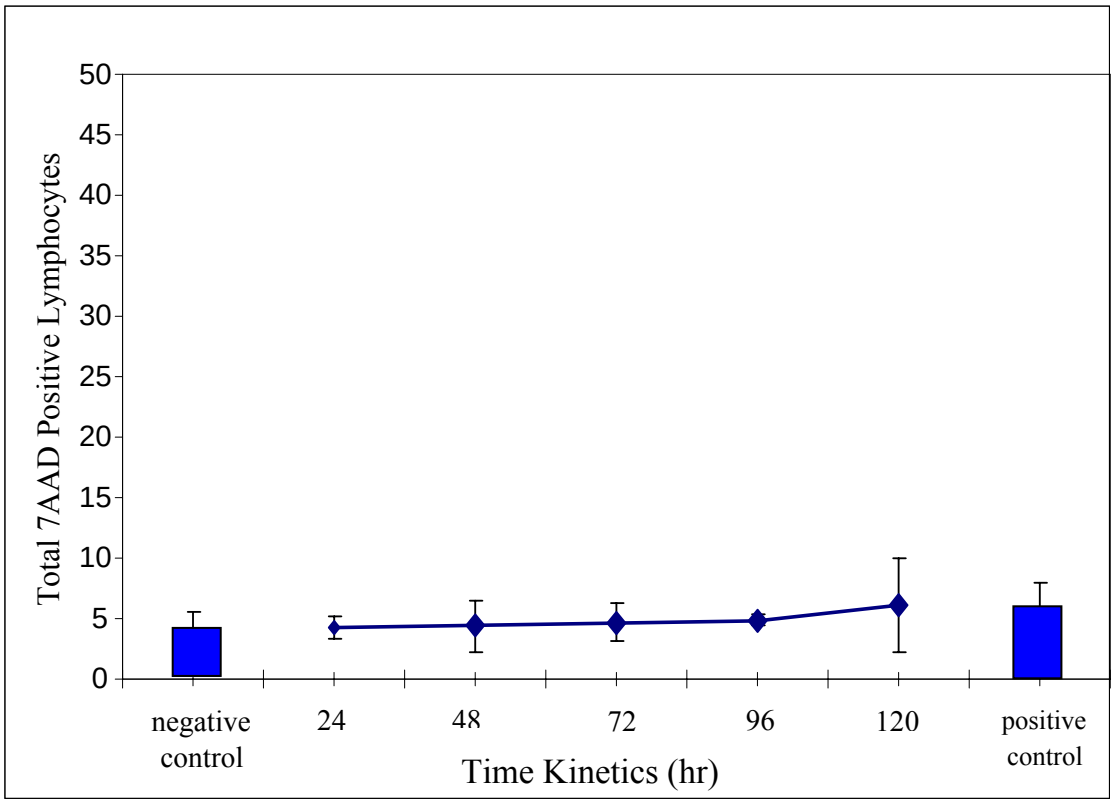


Figure 5.Effect of clinoptilolite treated RPMI on lymphocyte apoptosis. For positive control 2µM camptothecin was used, RPMI alone was used as negative control. 50 mg/ml clinoptilolite treated with RPMI for 24, 48, 72, 96 and 120 hours at room temperature. In 72 hour cell culture those treated clinoptilolite free RMPIs were used to detect the effect on apoptosis.

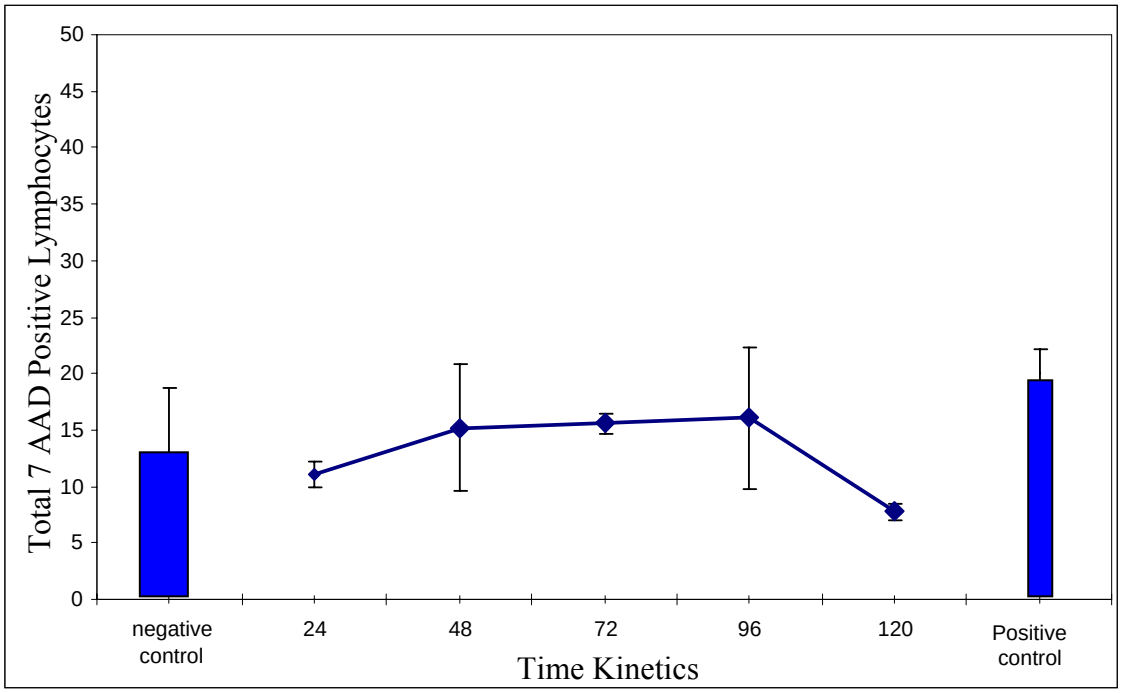


Figure 6. Effect of clinoptilolite treated RPMI on lymphocyte apoptosis. For positive control 2μM camptothecin was used, RPMI alone was used as negative control. 50 mg/ml clinoptilolite treated with RPMI for 24, 48, 72, 96 and 120 hours at room temperature. In 96 hour cell culture those treated clinoptilolite free RMPIs were used to detect the effect on apoptosis.

## 3.2 Effects of Clinoptilolite on PBMCs Apoptosis

### 3.2.1 Immunoflouresence Labeling

Three different experiments were held for the detection of apoptosis on T cells via clinoptilolite. In each experiment different doses and ranges were investigated. The time period was fixed to 24 hour.

In figure 7 the doses were 15, 20, 25, 30, 35, 40 and 50 mg/ml and the time of incubation was 24 hour. RPMI and clinoptilolite mixture were held together for 2 hours before adding to culture. Mixture was vortexed in every 20 min. It was also vortexed before adding to the culture and the stimulant was taken before the clinoptilolite was settled. In the data it was seen that negative control has an apoptotic percentage of 11 and positive control has a value of 65%. The maximum apoptotic percentage of the dose was 20.

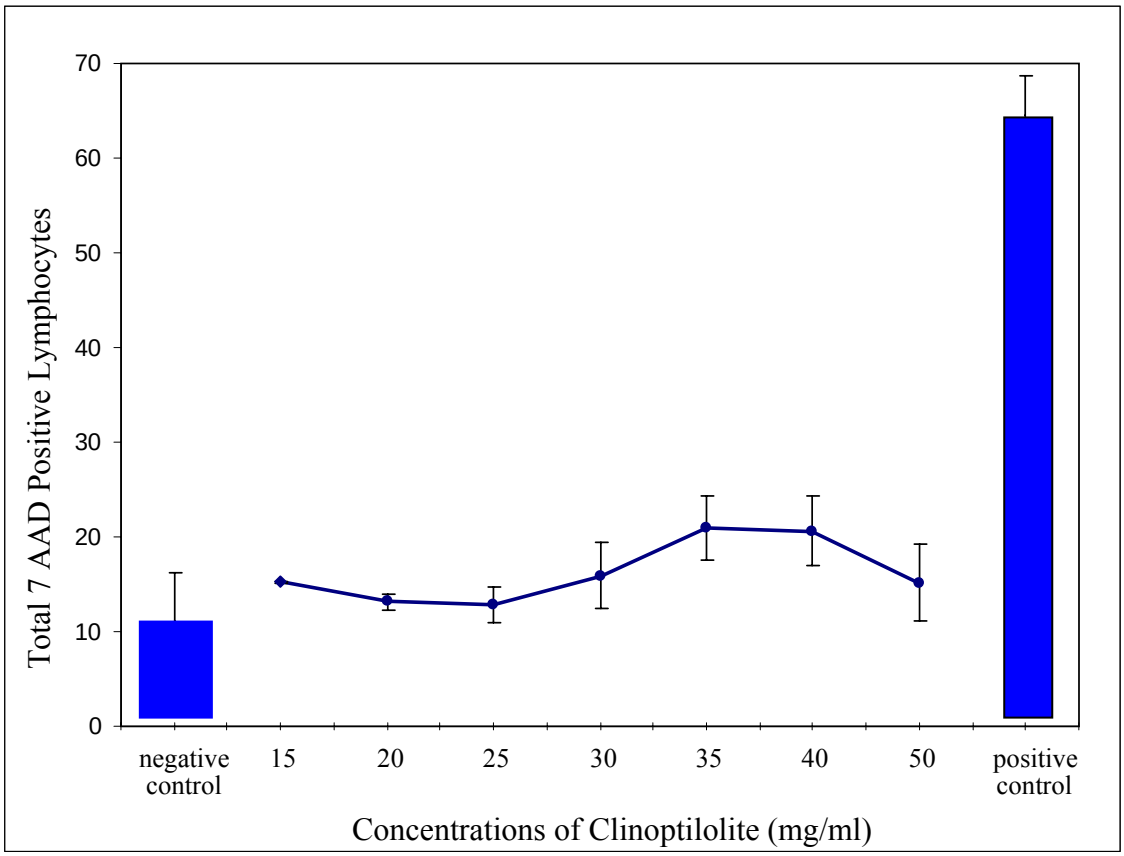


Figure 7.Effect of clinoptilolite on lymphocyte apoptosis. Positive control 2µM camptothecin was used, RPMI alone was used as negative control.15, 20, 25, 30, 35, 40 and 50 mg / ml clinoptilolite were directly added into the cell culture. 24 hour cell was used to detect the clinoptilolite effect on apoptosis.

In figure 8 the doses that were used, were 1, 5, 10, 15, 20, 25, 35 and 50 mg/ml. Incubation time was 24 hour. In the data it was seen that negative control has an apoptotic percentage of 5 and positive control has a value of 55%. The maximum apoptotic percentage of the dose was 15.

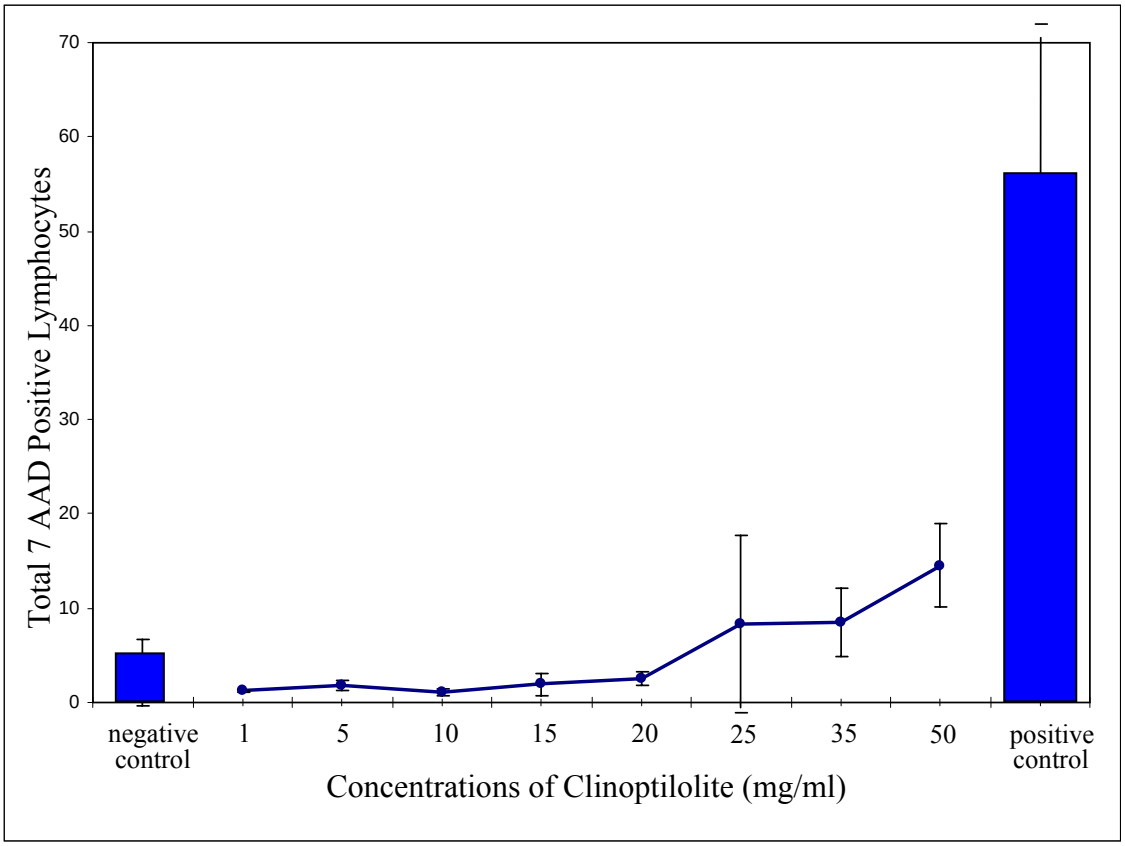


Figure 8.Effect of clinoptilolite on lymphocyte apoptosis. For positive control 2 µM camptothecin was used, RPMI alone was used as negative control. 1, 5, 10, 15, 20, 25, 35 and 50 mg/ml clinoptilolite were directly added into the cell culture. 24 hour cell was used to detect the clinoptilolite effect on apoptosis.

### 3.2.2 DNA Fragmentation Assay

In figure 9 the result of 24 hour cell culture were shown. After 24 hour cell culture the PBMCs was harvested and lysed. The wells were loaded with 150 ng fragmented DNA. The doses were 10 mg/ml, 30 mg/ml, 50 mg/ml, 70 mg/ml and 100 mg/ml. Marker was arranged between 100 bp to 1000 bp and increased by 100 bp. Except the 70 mg/ml and 100 mg/ml samples, the other samples have show the fragmented DNA pattern. Positive control has the highest intensity; this means that highest apoptotic events occur in this sample. Negative control, 10; 30 and 50 mg/ml doses have a same intensity. This is the result of background effect. The cells go into apoptosis without any antigenic stimulation.

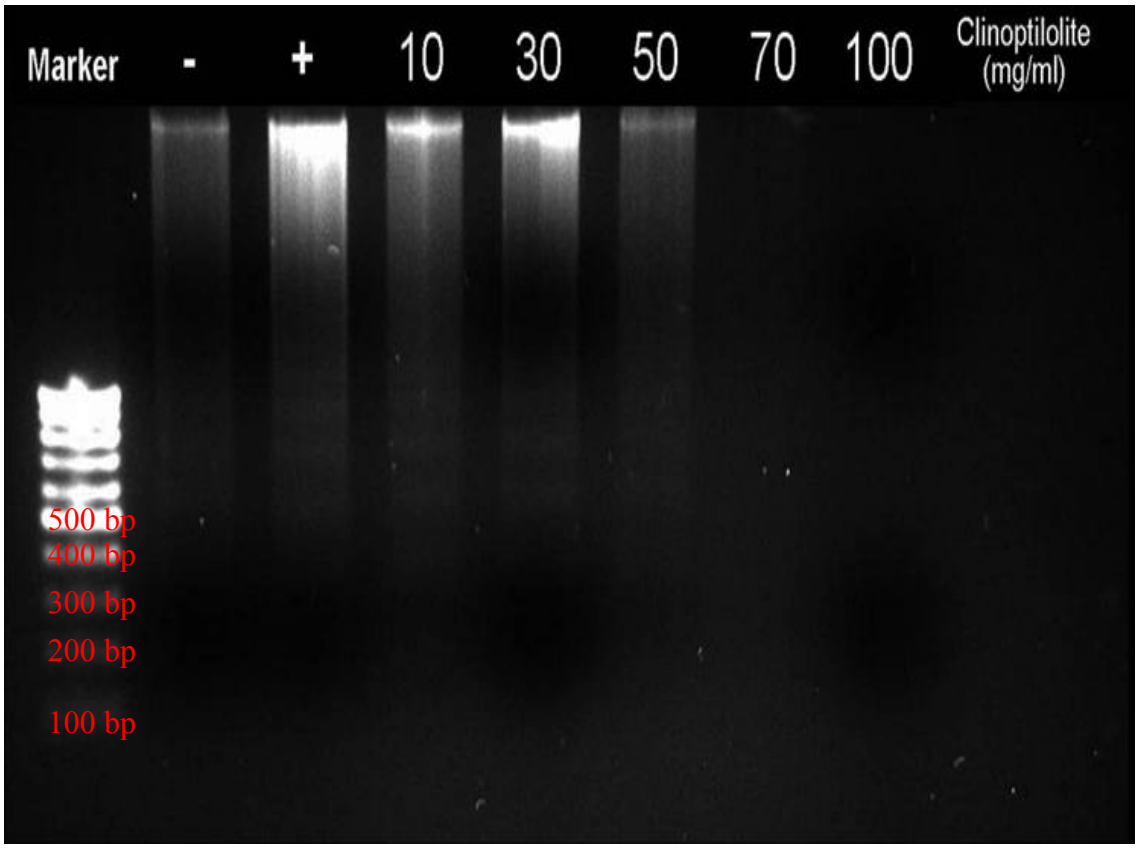


Figure 9.Agarose gel electrophoresis picture of DNA laddering formed by clinoptilolite in lymphocytes. For negative control RPMI alone was used, for positive control 2 µM camptothecin was used. 10, 30, 50, 70, 100 mg/ml clinoptilolites were added directly into the culture. 24 hour incubation was held. DNA ladders were isolated by DNA fragmentation assay, in each well 150 ng DNA added.

In figure 10 the result of 48 hour cell culture were shown. After 48 hour cell culture the PBMC's were harvested and lysed. The wells were loaded with 150 ng fragmented DNA. The doses were 10 mg/ml, 30 mg/ml, 50 mg/ml, 70 mg/ml and 100 mg/ml. Marker was arranged between 100 bp to 1000 bp and increased by 100 bp. Except the 70 mg/ml and 100 mg/ml samples, the other samples have show the fragmented DNA pattern. The intensities were close to each other. The negative control has shown the apoptosis occurred without any stimulant. As the intensities were close it can be said that the other samples have the fragmentation as a result of this background effect.

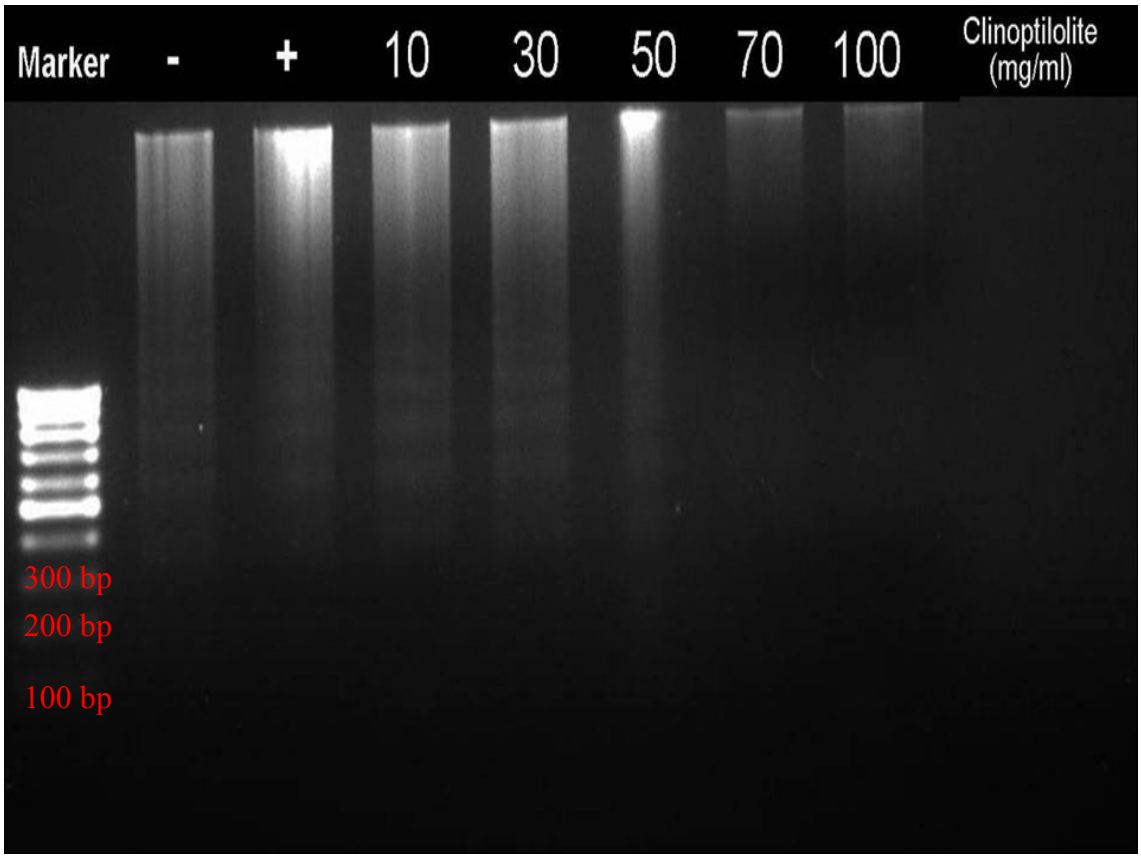


Figure 10.Agarose gel electrophoresis picture of DNA laddering formed by clinoptilolite in lymphocytes. For negative control RPMI alone was used, for positive control 2 µM camptothecin was used. 10, 30, 50, 70, 100 mg/ml clinoptilolites were added directly into the culture. 48 hour incubation was held. DNA ladders were isolated by DNA fragmentation assay, in each well 150 ng DNA added.

In figure 11, the result of 72 hour cell culture was shown. After 72 hour cell culture the PBMC's were harvested and lysed. The wells were loaded with 150 ng fragmented DNA. The doses were 10 mg/ml, 30 mg/ml, 50 mg/ml, 70 mg/ml and 100 mg/ml. Marker was arranged between 100 bp to 1000 bp and increased by 100 bp. Except the positive control the other samples can not show any fragmented DNA pattern.

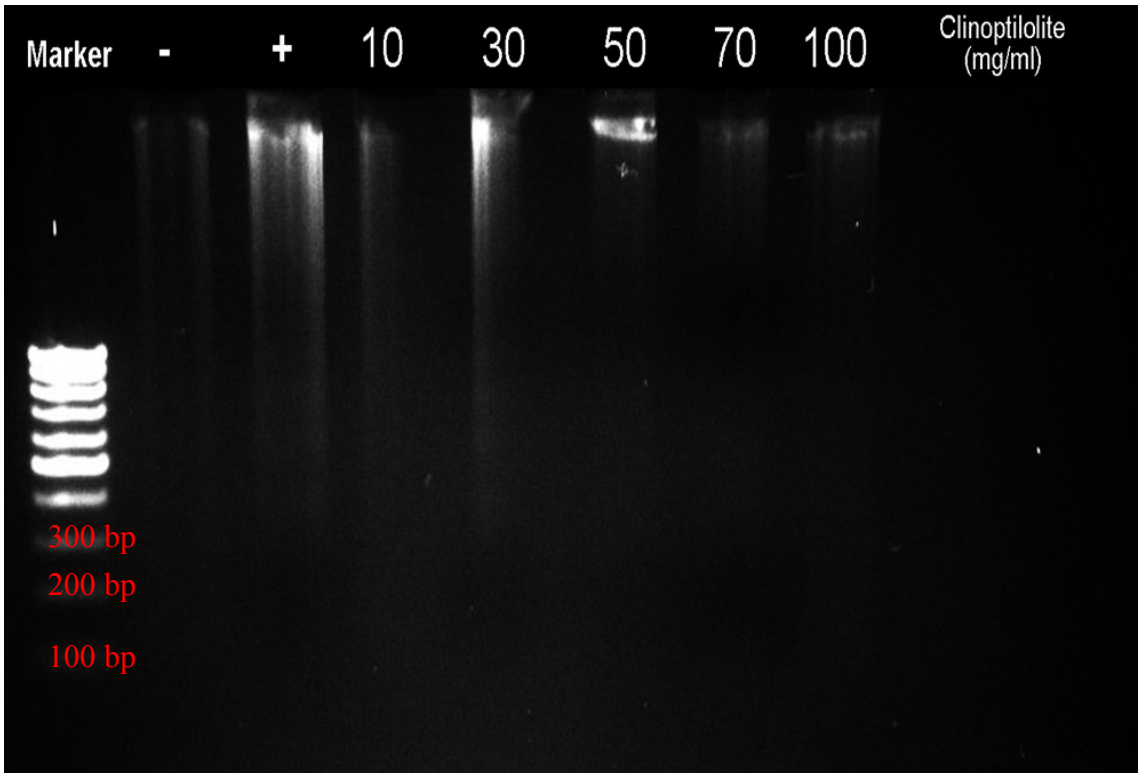


Figure 11.Agarose gel electrophoresis picture of DNA laddering formed by clinoptilolite in lymphocytes. For negative control RPMI alone was used, for positive control 2 µM camptothecin was used. 10, 30, 50, 70, 100 mg/ml clinoptilolites were added directly into the culture. 72 hour incubation was held. DNA ladders were isolated by DNA fragmentation assay, in each well 150 ng DNA added.

In figure 12, the result of 96 hour cell culture was shown. After 96 hour cell culture the PBMC's were harvested and lysed. The wells were loaded with 150 ng fragmented DNA. The doses were 10 mg/ml, 30 mg/ml, 50 mg/ml, 70 mg/ml and 100 mg/ml. Marker was arranged between 100 bp to 1000 bp and increased by 100 bp. Except the negative control the other samples can not show any fragmented DNA pattern.

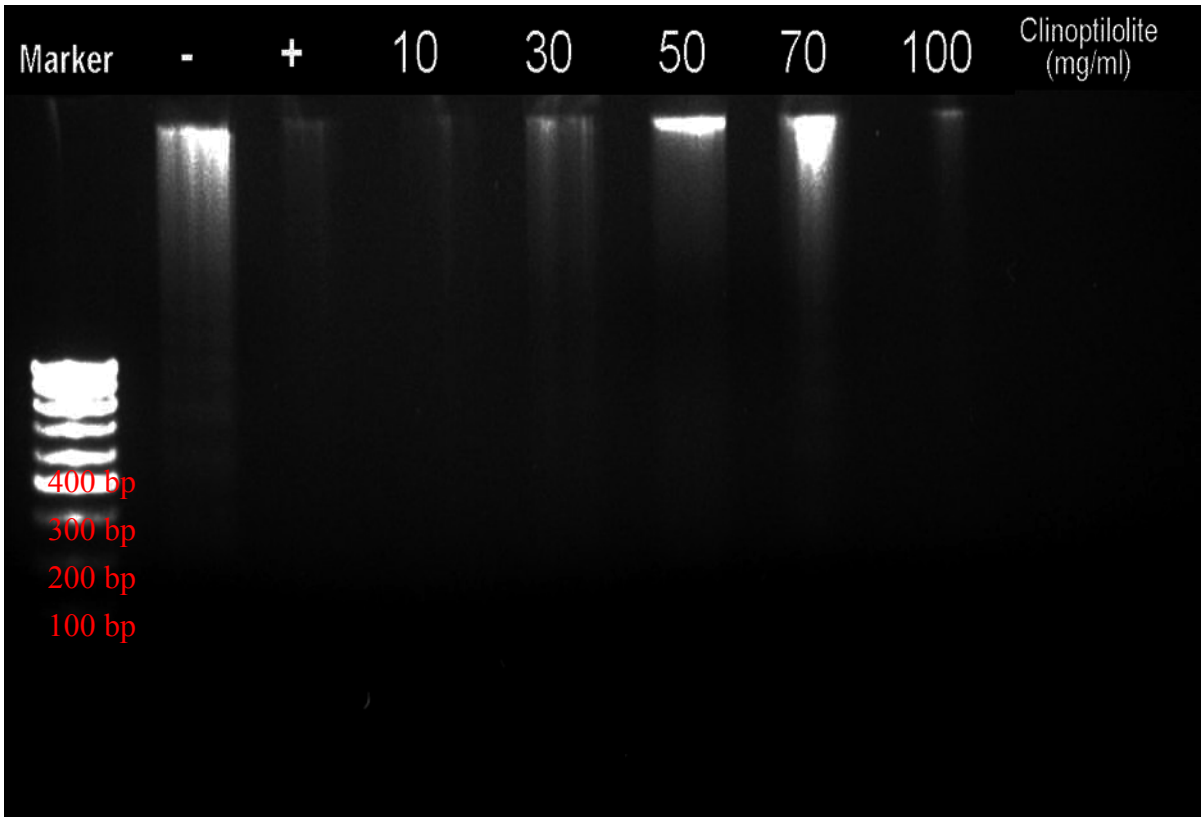


Figure 12.Agarose gel electrophoresis picture of DNA laddering formed by clinoptilolite in lymphocytes. For negative control RPMI alone was used, for positive control 2 µM camptothecin was used. 10, 30, 50, 70, 100 mg/ml clinoptilolites were added directly into the culture. 96 hour incubation was held. DNA ladders were isolated by DNA fragmentation assay, in each well 150 ng DNA added.

Same experiments were done with wider concentration of clinoptilolite with a range of 1 to 300 mg/ml and incubation time was held 72 to 96 hours. No DNA laddering as a result of apoptosis can be seen in those cultures either (Data not shown).

# CHAPTER 4

# CONCLUSION AND FUTURE PERSPECTIVES

In this research the effect of clinoptilolite on T lymphocyte apoptosis were investigated. From these results it was said that the clinoptilolite has no effect on RPMI 1640 media. Although clinoptilolite has an absorption and ion exchange capacity the buffer systems in the media were protect the changes that will occur.

By the help of the immunofluorescence labeling it was shown that at 24 hour time and at used doses clinoptilolite between the particle sizes 24 to 45 μm can not induce apoptosis. However as a result of big particle size of the used clinoptilolite (25 to 45 μm) high doses can not be investigated on FacsArray system. The clumps that were occurred stuck the system. Also as a result of high mass, the clinoptilolite easily settles by gravitational force. This will prevent the investigator to put desired doses into the culture.

The DNA fragmentation results were also parallel to the immunofluorescent labeling. Same problems were also seen on this experimental set up. The one can not be sure if absorption capacity of clinoptilolite has any effect on lysed cells and fragmented DNA. But it can be said that there was no induction of apoptosis on investigated cultures.

In the future the experiments can be held with smaller particle sized zeolites to find out the effect.

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