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EFFECTS OF NIGELLA SATIVA EXTRACTS ON THE CYTOKINE PRODUCTION OF ANTIGEN-STIMULATED PBMC OF ALLERGIC SUBJECTS

by

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by

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in

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I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science.

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November 2009

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M. S. Thesis – Biology Department
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Supervisor: Prof. Dr. Fatih ÖZKARAGÖZ

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ABSTRACT

Nigella sativa (Ranunculaceae family) seeds, commonly known as black seed or black cumin have been used for thousands of years as a protective and curative remedy in Eastern countries. Many studies have been conducted so far on the effects of Nigella sativa seed extracts on various body systems. Some immunopotentiating properties of Nigella sativa were shown however the real mechanism of action on allergic diseases is still unclear.

This in-vitro study was undertaken to evaluate the effects of hyrophilic and oil extracts of *Nigella sativa* on the cytokine production of antigen-stimulated peripheral blood mononuclear cells on allergic subjects. In this study 2.10^6 cell/ 2ml PBMC cultures isolated from allergic subjects were stimulated with 1 µg/ml house dust mite antigen. 0,05 % - 0.01 % for oil extract and 0.05 % - 0.01 % for hydrophilic extract were applied on the culture together with antigen. The amounts of IL-4, IL-5 and IF-γ amounts were measured by means of ELISA in the culture supernatants at the 3rd and 5th day of incubation. It was shown that both the oil and hydrophilic extracts of *N.sativa* prevents the IL-5 production which is one of the main cytokines taking role in the allergic reaction pathway. Also extracts had decreasing effects on IFN-γ concentration gave the idea that *N.sativa* might have an immunosuppressive effect. Further studies will provide a better understanding for the effects of *N.*sativa on allergy.

Keywords: *Nigella sativa*, black cumin, Allergy, T_H1-T_H2 balance

NİGELLA SATİVA ÖZÜTÜNÜN, ALERJİK HASTALARDAN ELDE EDİLİP, ANTİJENLE UYARILAN PERİFERAL KAN MONONÜKLEER HÜCRELERİNDEKİ SİTOKİN ÜRETİMİNE ETKİSİ

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ÖZ

Çörek otu olarak da bilinen *Nigella sativa* (Ranunculaceae ailesi) tohumları, Doğu ülkerlerinde binlerce yıldır koruyucu ve tedavi edici olarak kullanılıyor. *N. sativa'*nın pek çok sistem üzerindeki etkileri bu zamana kadar yapılan çeşitli araştırmalarda gösterilmiştir. Bağışıklık sistemi üzerine etkileri üzerine yapılan çalışmalar olsa da allerjik hastalıklar üzerinde nasıl bir etkisi olduğu kesin olarak keşfedilememiştir.

Bu *in vitro* çalışma *Nigella sativa* tohumlarının hidrofilik ve yağ özütlerinin, antijenle uyarılmış peripheral mononükleer kan hücrelerindeki sitokin üretimi üzerine etkilerini incelemek üzere yapıldı. Bu çalışmada alerjik bireylerden alınan peripheral mononükleer kan hücreleri 2.10^6 hücre/ 2ml yoğunluğunda kültür edildi ve 1 µg/ml ev akar antijeniyle uyarıldı. Yağ özütü % 0,05 ve % 0.01 luk konsantrasyonlarda ve hidrofilik özüt de % 0.05 ve % 0.01 lik konsantrasyonlarda kültüre uygulandı. ELISA yöntemiyle 3. ve 5. günlerde toplanan supernatantlardan IL-4, IL-5 ve IF-γ miktarları ölçüldü. Sonuçta hem yağ hem de hidrofilik özütün, allerjik reaksiyonlarda görev alan en önemli sitokinlerden biri olan IL-5 miktarında azalmaya sebep olduğu tespit edildi. Özütlerin aynı zamanda IFN-γ üzerindeki azaltıcı etkisi, *N.sativa'* nın bağışıklık sistemi baskılayıcı bir etkisi olabileceğini düşündürüyor. İlerki çalışmalar *N.sativa'* nın alerji üzerindeki etkileri konusunda çok daha net bir fikir verecek.

Anahtar Kelimeler: *Nigella sativa*, çörek otu, alerji, T_H1-T_H2 dengesi

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LIST OF SYSMBOLS AND ABBREVIATIONS

SYMBOL/ABBREVIATION	
ddw	double distilled water
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
IFN-γ (g)	interferon gamma
Ig E	immunoglobulin E
IL-4	interleukin 4
IL-5	interleukin 5
LDH	lactate dehydrogenase
PBMC	peripheral blood mononuclear cells
RT	room temperature
T_H1	T helper cell 1
T_H2	T helper cell 2

INTRODUCTION

1.1 THE IMMUNE SYSTEM

The immune system is the defense mechanism of the body. There are several layers of defense in which an organism or a substance come across with when it enters the body**.** First layer is the physical barriers that prevent microbes from entering the body. If a pathogen breaks these barriers, the innate immune system provides an immediate, but non-specific response. If it evades the innate response, the adaptive immune system takes the mission to fight against pathogen. It recognizes and eliminates the pathogen in several ways. The recognition of pathogen is recorded in the form of an immunological memory. This memory allows the adaptive immune system to give faster and stronger attacks each time this pathogen is encountered. (Alberts *et al*, 2001)

1.1.1 Elements of The Immune System

The immune system fulfills the defense of body with white blood cells (leukocytes). There are five types of leukocytes; neutrophils, basophils, eosinophils, lymphocytes and monocytes. All the elements of leukocytes derive from the same progenitor, the hematopoietic stem cells in the bone marrow and then they migrate all over the body together with the blood and lymphatic system (Figure 1.1).

Basophils, eosinophils, neutrophils and monocytes derive from the myeloid stem cell. They are carried on blood and enter the tissues only in presence of infection or inflammation. Neutrophils are the most abundant leukocytes (40-75 %) and in the case They circulate in the blood stream for few days and then migrate into the tissues where they differentiate into macrophages and dendritic cells. Macrophages are phagocytes, they engulf and digest the pathogens and also cellular debris. After digesting the pathogen, macrophages can present it to the helper T cells. Dendritic cells can be found in blood in immature state and in tissues in contact with the external environment in mature state. Immature dendritic cells are phagocytic, ingesting antigens. When they

encounter a pathogen, they mature and migrate into the lymphoid tissues where they present the antigen to the T helper cells and activate antigen-specific T lymphocytes. Mast cells arise from precursors in bone marrow circulates in blood in an immature form and mature in tissues. They play a key role important in inflammations and allergic responses, triggering a local inflammatory response to antigen by releasing granules containing histamine, seratonin, heparin and various hormonal mediators (Figure 1.2) (Janeway *et al*, 2007)

Lymphocytes are derived from the common lymphoid progenitor. There are two types of lymphocytes according to their appearance. Large, granular lymphocytes are natural killer cells and small lymphocytes are B and T lymphocytes. Natural killer cells are part of the innate immune system and don't have antigen-specific receptors. These cells circulate in the blood with distinctive cytotoxic granules and activate in response to interferons. They distinguish and kill the infected cells and tumors, and are important in the innate immune defense against intracellular pathogens. B cells and T cells are major elements of adaptive immune system. They both recognize specific antigens during the antigen presentation process. Once the invader is identified, cells generate different responses. When B cells are activated, they differentiate into plasma cells that secrete large quantities of antibodies. When T cells are activated, helper T cells differentiate in to cells, producing cytokines to activate other cells like B cells and macrophages, whereas another class of T cells called cytotoxic T cells, produce toxic granules which kill cells infected with pathogens. (Figure 1.3) (Janeway *et al*, 2007)

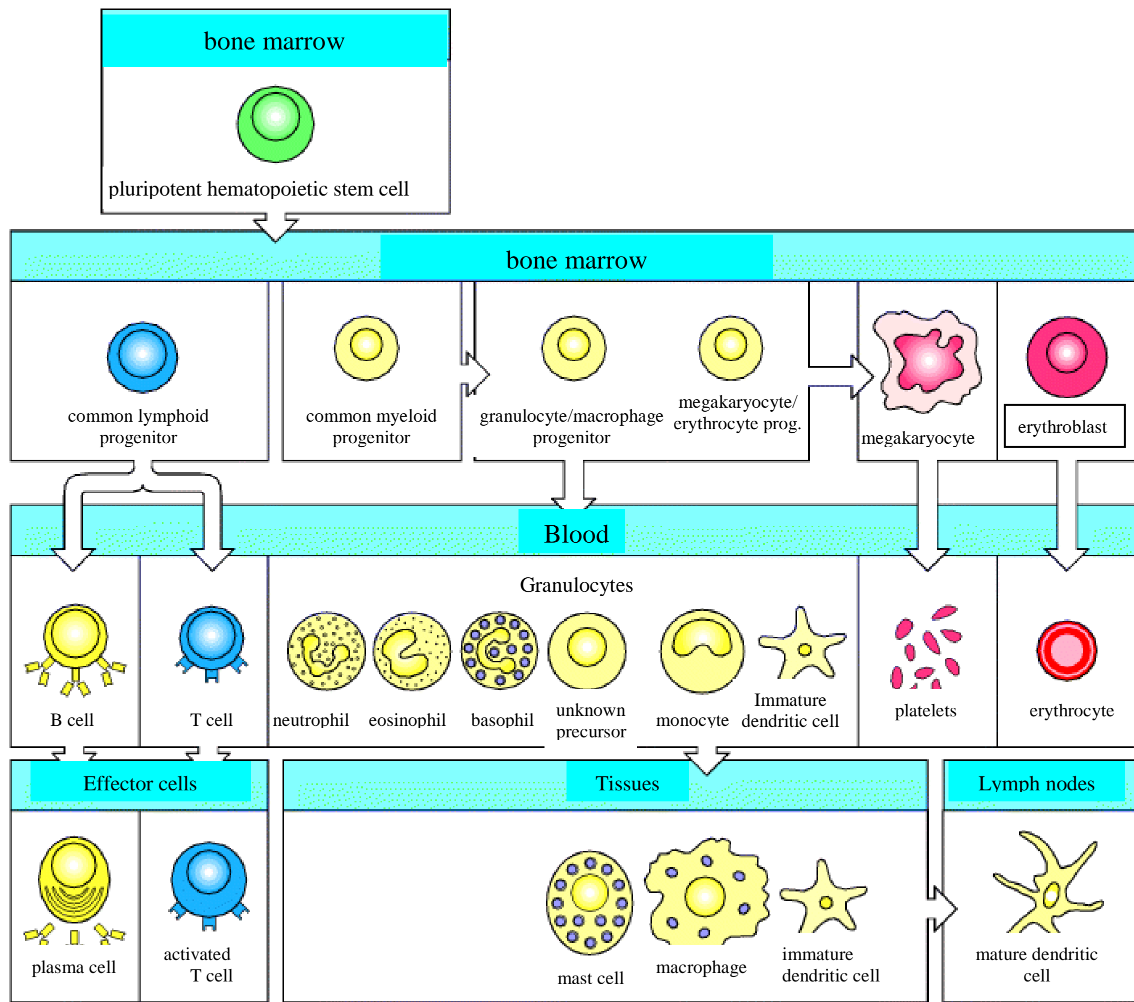


Figure 1.1 The cellular elements of blood. (Janeway *et al*, 2007)

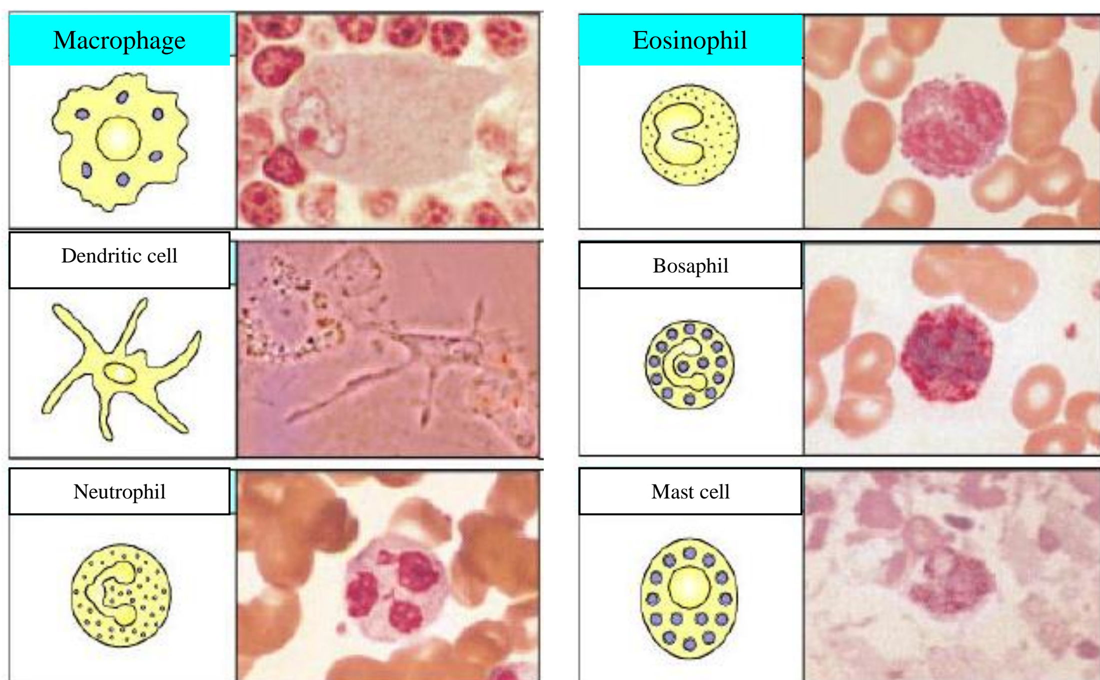


Figure 1.2 Myeloid cells in innate and adaptive immunity**.** (Janeway *et al*, 2007)

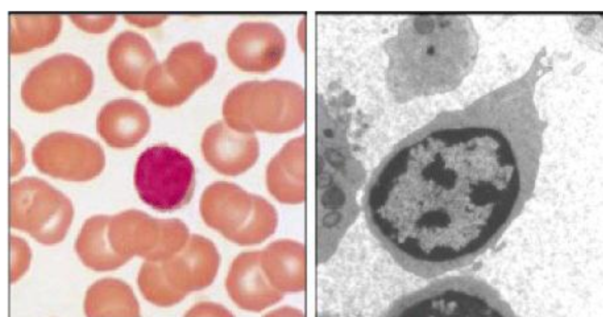


Figure 1.3 Lymphocytes. Light micrograph (left) and transmission electron micrograph of a small lymphocyte (right) are presented. (Janeway *et al*, 2007)

1.1.2 How The Immune System Works

Innate immune system components are the first preventive group that meets microorganisms when they penetrate the epithelial surfaces of the body. Phagocytic macrophages recognize and capture the bacteria with its surface receptors binding to the bacterial surfaces. This binding between the bacterial molecules and the surface receptors trigger the engulf of bacterium and also induce the secretion of biologically active molecules. Activated macrophages secrete cytokines, affecting the behavior of

other cells that have receptors for these proteins. They also release chemokines that attract neutrophils and monocytes from the bloodstream. The cytokines and chemokines released by macrophages in response to bacterial constituents initiate inflammation. The permeability of the blood vessels increase, resulting the increased local blood flow and the leakage of fluid, the heat, redness, and swelling. The main cell types seen in an inflammatory response in its initial phases are neutrophils. They are recruited into the inflamed, infected tissue in large numbers. Like macrophages, they have surface receptors for common bacterial constituents and complement, and they are the principal cells that engulf and destroy the invading micro-organisms. After the influx of neutrophils to the infection site, monocytes rapidly differentiate into macrophages. Later in the infection, antigens that have been drained from the site of infection activate the lymphocytes so that the innate immune response provides the activation of adaptive immune response. Macrophages that have phagocytosed bacteria and become activated also activate T lymphocytes. However, the cells that specialize in presenting antigen to T lymphocytes and initiating adaptive immunity are the dendritic cells. (Mayer, 2006)

1.1.2.1 The Adaptive Immunity

There are two major types of adaptive immune responses;

(a) T Cell- mediated immunity
(b) Humoral immunity

These immune responses differ according to the effector cells that are activated, pathogens they react and the location of invasion.

Effector T cells are divided into three functional classes that detect different peptide antigens from different types of pathogen; Cytotoxic T cells, TH1 cells and TH2 cells. Cytotoxic T cells kill the cells that are infected with the intracellular pathogens that multiply in the cytoplasm. The antigens of these pathogens are carried to the cell surface by MHC class I molecules and presented to CD8 T cells which later differentiate into cytotoxic T cells. MHC class II molecules carry the antigens of pathogens multiplying in intracellular vesicles, and ingested extracellular bacteria and toxins, and present to CD4 T cells. These cells can differentiate into TH1 and TH2 effector T cells. Pathogens that accumulate in large numbers inside macrophage and dendritic cell vesicles tend to stimulate the differentiation to TH1 cells, whereas

extracellular antigens tend to stimulate the production of TH2 cells. TH1 cells activate the microbicidal properties of macrophages, and induce B cells to make IgG antibodies that are very effective at opsonizing extracellular pathogens for uptake by phagocytic cells. TH2 cells initiate the humoral immune response by activating naive antigen-specific B cells to produce IgM antibodies. (Janeway *et al*, 2007)

1.1.2.1.1 The initiation of adaptive immunity

The adaptive immune response initiates when a pathogen is ingested by an immature dendritic cell in the infected tissue. These specialized phagocytic cells are resident in most tissues. All tissue-resident dendritic cells migrate through the lymph to the regional lymph nodes where they interact with recirculating naive lymphocytes. (Schlinger *et al*, 2000)

The immature dendritic cell carries receptors on its surface that recognize common features of many pathogens, such as bacterial cell wall proteoglycans. As with macrophages and neutrophils, binding of a bacterium to these receptors stimulates the dendritic cell to engulf the pathogen and degrade it intracellularly. However the function of dendritic cells is not primarily to destroy pathogens but to carry pathogen antigens to peripheral lymphoid organs and present them to T lymphocytes. When a dendritic cell takes up a pathogen in infected tissue, it becomes activated, and travels to a nearby lymph node. On activation, the dendritic cell matures into a highly effective antigen-presenting cell (APC) and undergoes changes that enable it to activate pathogen-specific lymphocytes that it encounters in the lymph node (Figure 1.4). Activated dendritic cells secrete cytokines that influence both innate and adaptive immune responses, making these cells essential gatekeepers that determine how the immune system responds to the presence of infectious agents. (Thery and Amigorena, 2001)

There are three cell types of antigen-presenting cells. Dendritic cells are the major antigen presenting cells in initiation of adaptive immune responses. Macrophages can also mediate innate immune responses directly and make a crucial contribution to the effector phase of the adaptive immune response. B cells contribute to adaptive immunity by presenting peptides from antigens they have ingested and by secreting antibody. (Thery and Amigorena, 2001)

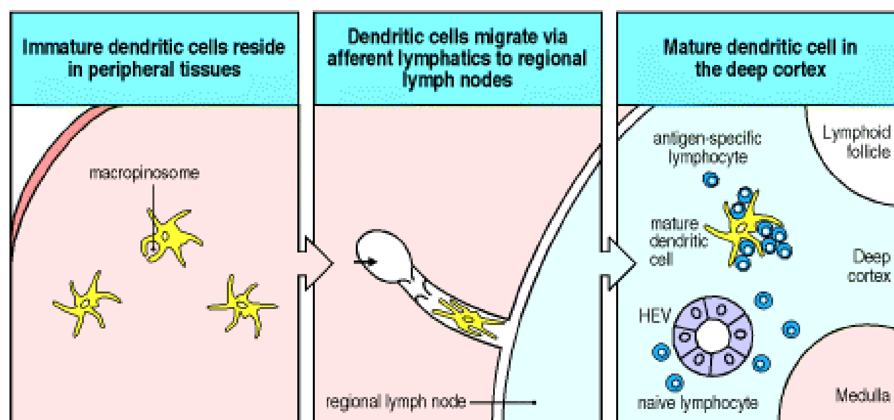


Figure 1.4 Dendritic cells initiate adaptive immune responses. (Janeway *et al*, 2007)

There are three cell types of antigen-presenting cells. Dendritic cells are the major antigen presenting cells in initiation of adaptive immune responses. Macrophages can also mediate innate immune responses directly and make a crucial contribution to the effector phase of the adaptive immune response. B cells contribute to adaptive immunity by presenting peptides from antigens they have ingested and by secreting antibody. (Thery and Amigorena, 2001)

Naive lymphocytes and activated antigen-presenting cells come together in the lymphoid tissues. Antigens are presented to the naive recirculating lymphocytes as they migrate through the lymphoid tissue. When the lymphocyte recognizes its specific antigen, it stops migrating and enlarges. The chromatin in its nucleus becomes less dense, nucleoli appear, the volume of both the nucleus and the cytoplasm increases, and new RNAs and proteins are synthesized. Within a few hours it turns to be a lymphoblast. The lymphoblasts begin to divide, normally duplicating themselves two to four times every 24 hours for 3 to 5 days, so that one naive lymphocyte gives rise to a clone of around 1000 daughter cells of identical specificity. These then differentiate into effector cells. (Picker and Butcher, 1992) If the differentiated effector cells are B cells, they secrete antibody, if effector cells are T cells, they destroy the infected cells or activate other cells of the immune system. After a naive lymphocyte has been activated, it takes 4 to 5 days before clonal expansion is complete and the lymphocytes have differentiated into effector cells. That is why adaptive immune responses occur only after a delay of several days. Effector cells have only a limited life-span and, once antigen is removed, most of them undergo apoptosis. However, some persist after the

antigen has been eliminated. These cells are known as memory cells and form the basis of immunological memory. This memory ensures a more rapid and effective response with a shorter lag phase, a higher level and affinity of antibodies to the antigen on a second encounter with the pathogen providing a permanent protective immunity. (Alberts *et al*, 2001 and Janeway *et al*, 2007)

1.1.2.1.2 Interaction between T cells and antigen presenting cells

Naive T cells circulate continuously from the bloodstream to the lymphoid organs and back to the blood crossing through the high endothelial venules (HEV), making contact with thousands of antigen-presenting cells in the lymphoid tissues. Antigen presenting cells bind naive T cells very efficiently through interactions between LFA-1, CD2, and ICAM-3 on the T cell, and ICAM-1, ICAM-2, LFA-3, and DC-SIGN on the antigen presenting cell (Figure 1.5). This transient binding provides time for T cells to sample large numbers of MHC molecules on each antigen-presenting cell, as only one naive T cell in 10^4-10^6 is likely to be specific for a particular antigen. When a naive T cell recognizes its peptide:MHC ligand, T-cell receptor induces a conformational change in LFA-1. This conformational change provides great increase in affinity for ICAM-1 and ICAM-2 and stabilizes the association between the antigen-specific T cell and the antigen-presenting cell. This association can stand for several days, during the naive T cell proliferates and differentiate into armed effector T cells. (Madri and Graesser, 2000; Ganpule *et al*, 1997; Gunzer *et al*, 2000)

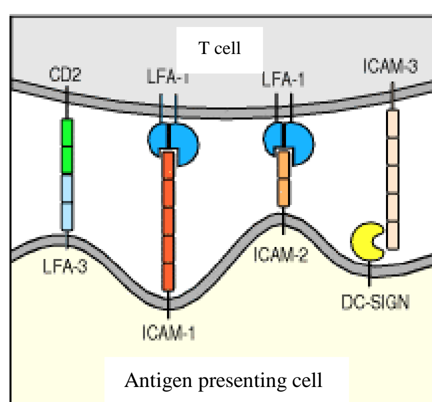


Figure 1.5 Cell-surface molecules provide the interactions between lymphocytes and antigen-presenting cells**.** (Janeway *et al*, 2007)

Armed effector *T* cells are activated when their antigen-specific receptors and either the CD4 or CD8 co-receptors bind to peptide:MHC complexes. However the clonal proliferation of antigen-specific naive T cells needs a second co-stimulatory signal which is delivered by the same antigen-presenting cell (Figure 1.6). The best-characterized co-stimulatory molecules are the glycoproteins **B7.1** (CD80) and **B7.2** (CD86). The B7 molecules are found exclusively on the surfaces of cells that can stimulate T-cell proliferation. The receptor for B7 molecules on the T cell is CD28. Ligation of CD28 by B7 molecules or by anti-CD28 antibodies co-stimulates the clonal expansion of naive T cells, whereas anti-B7 antibodies, which inhibit the binding of B7 molecules to CD28, inhibit T-cell responses. (Wang *et al.*, 2000)

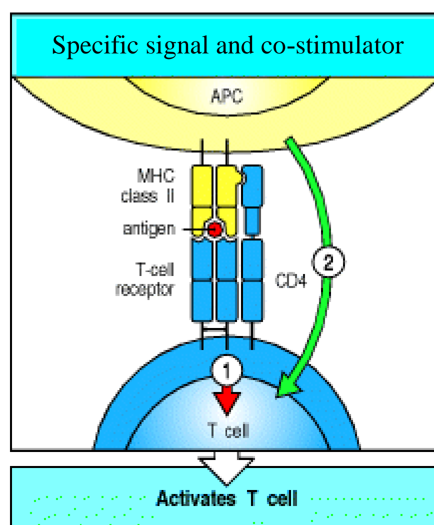


Figure 1.6 Activation of naive T cells requires two independent signals. (Janeway *et al*, 2007)

Once a naive T cell is activated, it produces a number of proteins that contribute to the co-stimulatory signal of antigen presenting cell. One such protein is **CD40 ligand**, which binds to CD40 on antigen-presenting cells. Binding of CD40 ligand by CD40 transmits activating signals to the T cell and also activates the antigen-presenting cell to express B7 molecules, thus stimulating further T-cell proliferation. CD40 and CD40 ligand belong to the TNF family of receptors and ligands and have a central role in the effector function of fully differentiated T cells. (Wang *et al.*, 2000)

Activated T cells also produce CD28-related proteins which modifies the co stimulatory signal as the Tcell response develops. One of those proteins is **CTLA-4** (CD152), an additional receptor for B7 molecules. It delivers an inhibitory signal to the activated T cell, making the activated progeny less sensitive to stimulation by the antigen-presenting cell and limiting the amount of an autocrine T-cell growth factor, interleukin-2 (IL-2) that is produced. Thus, binding of CTLA-4 to B7 molecules is essential for limiting the proliferative response of activated T cells to antigen and B7. (Wang *et al*, 2000)

A third CD28-related protein which is induced on activated T cells, enhances T-cell responses; this inducible co-stimulator (ICOS) binds a ligand known as LICOS, which is produced on activated dendritic cells, monocytes, and B cells, but its contribution to immune responses has not yet been clearly defined. Although it resembles CD28 in driving T-cell growth, it differs from CD28 in not inducing IL-2; instead it induces IL-10. (Cerdan *et al,* 1995)

Naive T cells can live for many years without dividing. These small resting cells have condensed chromatin and a scanty cytoplasm and synthesize little RNA or protein. For the activation, they must reenter the cell cycle and divide rapidly to produce the large numbers of progeny that will differentiate into armed effector T cells. Their proliferation and differentiation are driven by a cytokine called interleukin-2 (IL-2), which is produced by the activated T cell itself. (Picker and Butcher, 1992)

When the T cell encounters with its specific antigen of the T cell enter into the G_1 phase of the cell cycle; at the same time, it also induces the synthesis of IL-2 along with the gamma (γ) chain of the IL-2 receptor. The IL-2 receptor has three chains: alpha (α), beta (β) and gamma (γ) (Figure 1.7). In the resting T cell, this receptor is composed of alpha (α) and beta (β) chains which binds IL-2 with moderate affinity. In this case resting T cells respond only to very high concentrations of IL-2. However when the gamma (γ) chain associates with the alpha (α) and beta (β) chains, it creates a receptor with a much higher affinity to IL-2. This allows T cell to respond even to very low concentrations of IL-2. IL-2 which binds to its receptor with high affinity, promotes progression through the rest of the cell cycle. T cells activated in this way can divide two to three times a day for several days, allowing one cell to give rise to a clone composed of thousands of progeny that all bear the same receptor for antigen. IL-2 also promotes the differentiation of these cells into armed effector T cells. (O’Garra, 2000)

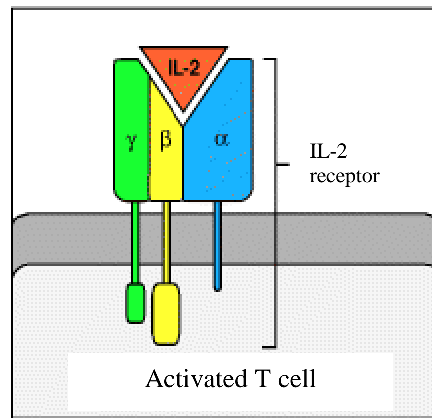


Figure 1.7 High-affinity IL-2 receptors are three-chain structures that are produced only on activated T cells. (Janeway *et al*, 2007)

1.1.2.1.3 T_H1 - T_H2 decision

Naive CD4 T cells can differentiate upon activation into either TH1 or TH2 cells, which differ in the cytokines they produce and thus in their function. The decision on which fate the progeny of a naive CD4 T cell will follow is made during the clonal expansion that takes place after the first encounter with antigen. (Stout and Bottomly, 1989)

The factors that determine whether a proliferating CD4 T cell will differentiate into a TH1 or a TH2 cell are not fully understood. The cytokines produced (principally IFN-γ, IL-12, and IL-4), the co-stimulators used to drive the response, and the nature of the peptide:MHC ligand, all have an effect. In particular, because the decision to differentiate into TH1 versus TH2 cells occurs early in the immune response, the cytokines produced in response to pathogens by cells of the innate immune system play an important part in the subsequent adaptive response. (Stout and Bottomly, 1989).

The selection of differentiation into TH1 or TH2 cells is very important because if TH1 cells are produced this will lead to cell-mediated immune response, whereas the prodominant production of TH2 cells will provide humoral immune response. A striking example for the consequences of different responses to an infection is observed in leprosy, a disease caused by infection with *Mycobacterium leprae*. It grows in macrophage vesicles, and effective host defense requires macrophage activation by TH1 cells. In patients with tuberculoid leprosy, TH1 cells are preferentially induced, few live

bacteria are found, little antibody is produced, and, although skin and peripheral nerves are damaged by the inflammatory responses associated with macrophage activation, the disease progresses slowly and the patient usually survives. However, when T_H2 cells are preferentially induced, the main response is humoral, the antibodies produced cannot reach the intracellular bacteria, and the patients develop lepromatous leprosy, in which *M. leprae* grows abundantly in macrophages, causing gross tissue destruction that is eventually fatal. (Moser and Murphy, 2000; O'Garra and Robinson, 2004)

The mechanisms that control CD4 T-cell differentiation are not yet fully understood; however, cytokines present during the initial phases of T-cell activation seem to influence the choice of differentiation. Experiments *in vitro* have shown that naive CD4 T cells initially stimulated in the presence of IL-12 and IFN-γ tend to develop into T_H1 cells in part because IFN-γ inhibits the proliferation of T_H2 cells. As IL- 12, produced by dendritic cells and macrophages, and IFN-γ, produced by NK cells and CD8 T cells, CD4 T-cell responses in these infections tend to be dominated by T_H1 cells. By contrast, CD4 T cells activated in the presence of IL-4, especially when IL-6 is also present, tend to differentiate into T_H2 cells. This is because IL-4 and IL-6 promote the differentiation of T_H2 cells, and IL-4 or IL-10, either alone or in combination, can also inhibit the generation of T_H1 cells. (Maggi, 1998)

The two subsets of CD4 T cells T_H1 and T_H2 have very different functions: T_H2 cells are the most effective activators of B cells whereas T_H1 cells are crucial for activating macrophages. These two CD4 T-cell subsets also regulate each other; once one subset becomes dominant, it is often hard to shift the response to the other subset. The overall effect is that certain responses are dominated by either humoral (T_H2) or cell-mediated (T_H1) immunity. (Moser and Murphy, 2000)

The amount and exact sequence of the antigenic peptide that initiates the response is another factor that has influence in the choice of CD4 T differentiation. High amounts of antigen on the surface of antigen-presenting cells tend to stimulate T_H1 cell responses, whereas low amounts tends to elicit T_H2 cell responses. Moreover, peptides that interact strongly with the T-cell receptor tend to stimulate T_H1-like responses, whereas peptides that bind weakly tend to stimulate T_H2-like responses. (Stout and Bottomly, 1989)

1.1.2.1.4 Cell-mediated immunity (T_H1 type response)

When the choice of CD4 T-cell differentiation is in the T_H1 direction, cell-mediated immune response is given to the specific antigen. The main action of T_H1 cells is the macrophage activation. Macrophages require two signals for activation. One of these is provided by IFN-γ. Other signal is to sensitize the macrophage to respond to IFN- γ. T_H1 cells can deliver both signals. IFN-γ is the most characteristic cytokine produced by armed T_H1 cells on interacting with their specific target cells, whereas the CD40 ligand expressed by the T_H1 cell delivers the sensitizing signal by contacting CD40 on the macrophage. T_H2 cells are inefficient in activating the macrophages because they produce IL-10, a cytokine that can deactivate macrophages, and they do not produce IFN-γ. T_H1 cells activate infected macrophages through cell contact and the secretion of IFN- γ. This converts the macrophage into a potent antimicrobial effector cell. Activated macrophages fuse their lysosomes more efficiently to phagosomes, exposing microbes to a variety of microbicidal lysosomal enzymes. Activated macrophages also make oxygen radicals and nitric oxide (NO), both of which have potent antimicrobial activity, as well as synthesizing antimicrobial peptides and proteases that can be released to attack extracellular parasites. Macrophage activation is controlled by mechanisms that control IFN-γ synthesis by activated effector T cells. (Paulnock, 1992; Stetson *et al*, 2004)

1.1.2.1.5 Humoral Immune response (T_H2 type response)

Many of the bacteria that cause infectious disease multiply in the extracellular spaces of the body, and most intracellular pathogens spread by moving from cell to cell through the extracellular fluids. The extracellular spaces are protected by the humoral immune response, in which antibodies produced by B cells cause the destruction of extracellular microorganisms and prevent the spread of intracellular infections. The activation of B cells and their differentiation into antibody-secreting plasma cells is triggered by antigen and usually requires T_H2 class of CD4 T cells, but a subset of T_H1 cells can also help in B-cell activation. (Galli, 2000)

Antibodies secreted by B cells may act in three main ways. Antibodies that bind to the pathogens can prevent the entry of pathogens to cells. This process is called neutralization. It is also important in preventing bacterial toxins from entering cells. The other way of act in antibodies is opsonization, coating the surface of pathogen to

enhance the uptake of pathogen by phagocytic cells. Alternatively, antibodies binding to the surface of a pathogen can activate the proteins of the complement system, results in complement proteins being bound to the pathogen surface, and these opsonize the pathogen by binding complement receptors on phagocytes. (Janeway *et al*, 2007)

1.2 ALLERGY

The term allergy was originally defined by Clemens Von Pirquet as "an altered capacity of the body to react to a foreign substance," which was an extremely broad definition that including all immunological reactions. Allergy is now defined as “disease following a response by the immune system to an otherwise innocuous antigen”. Allergy is one of a class of immune system responses that are termed hypersensitivity reactions. These are harmful immune responses that produce tissue injury and may cause serious disease. (Romagnani, 2000)

Allergy occurs in the individuals who elicit IgE responses to innocuous antigen (allergen). The allergen triggers the activation of IgE-binding mast cells in the exposed tissue, leading to a series of responses that are characteristic of allergy. There are circumstances in which IgE is involved in protective immunity, especially in response to parasitic worms, which are prevalent in less developed countries. However in more than 25 % of the populations in the industrialized countries, IgE responses to innocuous antigens predominate and allergy is an important cause of disease. (Romagnani, 2000)

IgE is produced by plasma cells located in lymph nodes, at the sites of allergic reactions. IgE is located predominantly in tissues, where it is tightly bound to the mast-cell surface through the high-affinity IgE receptor known as **FcεRI**. Antigen binding to IgE cross-links these receptors and this causes the release of chemical mediators from the mast cells. Basophils and activated eosinophils also express FcεRI; they can therefore display surface-bound IgE. The factors that lead to an antibody response dominated by IgE are still being worked out. There are certain antigens and routes of antigen presentation to the immune system that favor the production of IgE. CD4 TH2 cells can switch the antibody isotype from IgM to IgE. Antigens that selectively evoke TH2 cells that drive an IgE response are known as allergens. (Romagnani, 2000)

The antigens that elicit IgE responses are mostly very small proteins and glycoproteins that have molecular masses of 5-80 kDa which can easily be inhaled while respiration. We inhale many different proteins that do not induce IgE production;

this raises the question of what is unusual about the proteins that are common allergens. There isn't a clear answer for this question however there are some general principles of allergens. Most allergens are relatively small, highly soluble proteins that are carried on pollen grains or mite feces. Allergens are typically presented to the immune system at very low doses. Presenting an antigen transmucosally and at very low doses is seem to be an efficient way of inducing TH2-driven IgE responses. IgE antibody production requires TH2 cells that produce interleukin-4 (IL-4) and IL-13 and it can be inhibited by TH1 cells that produce interferon-γ (IFN-γ). Only some of those who are exposed to these substances produce IgE antibodies against them. (Lambrecht *et al*, 2000)

There are several evidences suggesting that IgE is important for the host defense against parasites. Proteolytic enzymes are secreted by many parasites break down connective tissue and allow the parasite access to host tissues. This process gives the idea that these proteolytic enzymes are particularly active at promoting TH2 responses. Many examples of allergens that are enzymes support this idea. Der p 1 is the major allergen in the feces of the house dust mite (*Dermatophagoides pteronyssimus*) (Fig.1.8). It is a cysteine protease homologous to papain. The proteolytic activity of Der p 1 on certain receptor proteins on B cells and T cells may also promote the allergenicity of Der p 1. It has been shown to cleave the α subunit of the IL-2 receptor, CD25, from T cells which will provide the loss of IL-2 receptor activity. This might interfere with the maintenance of TH1 cells, leading to a TH2 cells bias. (Cookson, 1999) Another example is the protease papain, derived from the papaya fruit. It is used as a meat tenderizer and causes allergy in workers preparing the enzyme. Another example of allergy is the asthma caused by inhalation of the bacterial enzyme subtilisin, the 'biological' component of some laundry detergents. Injection of enzymatically active papain (but not of inactivated papain) into mice stimulates an IgE response. (Romagnani, 2000)

However not all allergens are enzymes for example, two allergens identified from filarial worms are enzyme inhibitors. Many protein allergens derived from plants have been identified and sequenced, but their functions are currently obscure. Thus, there seems to be no systematic association between enzymatic activity and allergenicity (Romagnani, 2000).

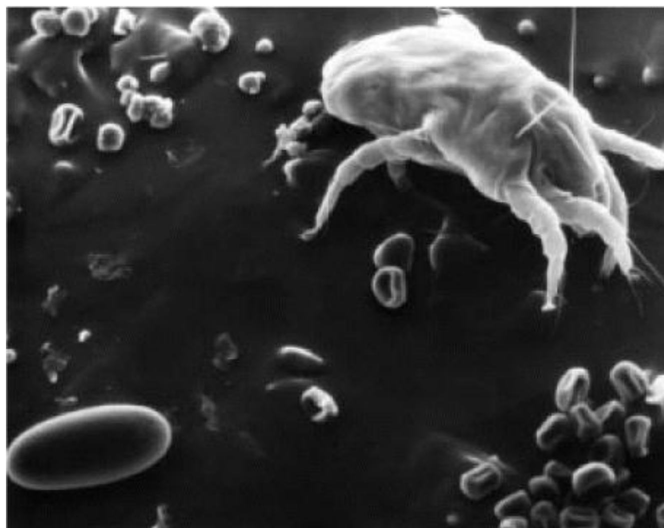


Figure 1.8 Scanning electron micrograph of *D. pteronyssimus.* (Janeway *et al*, 2007)

1.2.1 IgE Production

IgE antibodies are important in host defense against parasitic infections and this defense system is distributed mainly at the sites of entry of parasites under the skin, under the epithelial surfaces of the airways and in the sub mucosa of the gut. The fate of a naive CD4 T cell responding to a peptide presented by a dendritic cell is determined by the cytokines it is exposed to before and during this response, and by the intrinsic properties of the antigen, antigen dose, and route of presentation. Cells of the innate and adaptive immune systems at these sites are specialized to secrete predominantly cytokines that drive TH2 responses. The dendritic cells at these sites are migrate to regional lymph nodes after taking the antigen where their interaction with naive CD4 T cells drives the T cells to become TH2 cells, which secrete IL-4 and IL-10. However it is not known how myeloid dendritic cells induce this differentiation. One possibility is that they express a particular set of cytokines and co-stimulatory molecules yet to be characterized. Another is that they activate a specialized subset of CD4 T cells that produce abundant IL-4 that can induce CD4 T cells to differentiate into TH2 cells following stimulation by antigen. These in turn induce B cells to produce IgE . (Lambrecht *et al*, 2000; Garraud *et al*, 1995)

Induction of B cells to produce IgE is promoted by two separate signals, both of which are provided by TH2 cells. The first signal is provided by the cytokines IL-4 or

IL-13, interacting with receptors on the B-cell surface. These transduce their signal by activation of the Janus family tyrosine kinases JAK1 and JAK3 which ultimately lead to phosphorylation of the transcriptional regulator STAT6. Mice lacking functional IL-4, IL-13, or STAT6 all show impaired TH2 responses and IgE switching, demonstrating the key importance of these signaling pathways. The second signal for IgE class switching is a co-stimulatory interaction between CD40 ligand on the T-cell surface with CD40 on the B-cell surface. This interaction is essential for all antibody class switching. (Lambrecht *et al*, 2000)

The IgE response, once initiated, can be further amplified by basophils, mast cells, and eosinophils, which can also drive IgE production. All three cell types express FcεRI. When these specialized granulocytes are activated by antigen cross-linking of their FcεRI-bound IgE, they can express cell-surface CD40L and secrete IL-4; like TH2 cells, therefore, they can drive class switching and IgE production by B cells. Blocking this amplification process is a goal of allergy therapy. (Romagnani, 2000)

1.2.2 Genetic and environmental factors in allergy

40% of people in Western populations elicit IgE responses to a wide variety of common environmental allergens which seems to be controlled by several genetic factors. Atopic individuals have higher total levels of IgE in the circulation and higher levels of eosinophils than their normal counterparts. They are more susceptible to allergic diseases such as hay fever and asthma. Studies of atopic families have identified regions on chromosomes 11q and 5q that appear to be important in determining atopy; candidate genes that could affect IgE responses are present in these regions. The candidate gene on chromosome 11 encodes the β subunit of the high-affinity IgE receptor, whereas on chromosome 5 there is a cluster of tightly linked genes that includes those for IL-3, IL-4, IL-5, IL-9, IL-12, IL-13, and granulocyte-macrophage colony-stimulating factor (GM-CSF). These cytokines are important in IgE isotype switching, eosinophil survival, and mast-cell proliferation. It is also suggested that the susceptibility to asthma, rhinitis, and eczema, can be determined by different genes in different populations. (Yazdanbakshsh, 2002)

However there have been recent increases in the prevalence of allergic disorders that cannot be explained only with the genetic factors. It is increasing in economically advanced regions which may be explained by the environmental factors. There are four

main environmental factors that may affect the incidence of allergy. These factors are changes in exposure to infectious disease in early childhood, allergen levels, environmental pollution and dietary changes. Alterations in exposure to microbial pathogens in early childhood seem to be the most reasonable explanation at present for the increase in atopic allergy. It is possible that infection by an organism that evokes a T_H1 immune response early in life might reduce the likelihood of T_H2 responses later in life and vice versa. Exposure to environmental pollution was thought to increase the atopy and asthma however children from the city of Halle in the former East Germany, which has severe air pollution, had a lower prevalence of atopy and asthma than an ethnically matched population from Munich, exposed to much cleaner air. (Valenta, 2002) While it is clear that allergy is related to allergen exposure, there is no evidence that the rising prevalence of allergy is due to any systematic change in allergen exposure. Nor is there any evidence that changes in diet can explain the increase in allergy in economically advanced populations. (Yazdanbakshsh, 2002)

It has been observed that various immunological and autoimmune diseases are much less in developing countries than the industrialized countries. According to these observations David P. Strachan proposed the hygiene hypothesis. This hypothesis states that insufficient stimulation of T_H1 arm of immune system leads to a over reactive T_H2 arm, which will cause the allergic diseases. In another way of expression individuals living in too sterile conditions will not be exposed to enough pathogens which will result with the attack of immune system on harmless antigens, triggering an immune response called allergy. (Adkinson *et al*, 2002)

1.2.3 Treatment of allergy

There are two common methods used for the treatment of allergy; one is desensitization and the other is blocking the effector pathways. In desensitization the aim is to shift the antibody response from IgE dominated response toward IgG dominated response which will bind to the allergen and thus prevent it from activating IgE-mediated effector pathways. Patients are injected with increasing doses of allergen, starting with tiny amounts. This injection schedule gradually diverts the IgE-dominated response, driven by T_H2 cells, to one driven by T_H1 cells, with the consequent downregulation of IgE production. A potential complication of the desensitization approach is the risk of inducing IgE-mediated allergic responses. (Valenta, 2002)

Another approach to desensitization is vaccination with peptides derived from common allergen. This procedure induces T-cell anergy, including down regulation of cytokine production and reduced expression of the CD3:T-cell receptor complex. A major difficulty with this approach is that an individual's responses to peptides are restricted by their MHC class II alleles; therefore, patients with different MHC class II molecules respond to different allergen-derived peptides. As the human population is outbred and expresses a wide variety of MHC class II alleles, the number of peptides required to treat all allergic individuals might be very large. (Barnes, 1999)

Another vaccination strategy that shows promise in experimental models of allergy is the use of oligodeoxynucleotides rich in unmethylated cytosine guanine dinucleotides (CpG) as adjuvants for desensitization regimes. These oligonucleotides mimic bacterial DNA sequences known as CpG motifs and strongly promote TH1 responses. (Kline, 2000)

The signaling pathways that enhance the IgE response in allergic disease are also potential targets for therapy. Inhibitors of IL-4, IL-5, and IL-13 would be predicted to reduce IgE responses, but redundancy between some of the activities of these cytokines might make this approach difficult to implement in practice. A second approach to manipulating the response is to give cytokines that promote TH1-type responses. IFN-γ, IFN-α, IL-10, IL-12, and TGF-β have been shown to reduce IL-4-stimulated IgE synthesis *in vitro*, and IFN-γ and IFN-α have been shown to reduce IgE synthesis *in vivo*. (Kline, 2000)

Another target for therapeutic intervention might be the high-affinity IgE receptor. An effective competitor for IgE at this receptor could prevent the binding of IgE to the surfaces of mast cells, basophils, and eosinophils. Candidate competitors include humanized anti-IgE monoclonal antibodies, which bind to IgE and block its binding to the receptor. Yet another approach would be to block the recruitment of eosinophils to sites of allergic inflammation. (Kline, 2000)

The mainstays of therapy at present, however, are drugs that treat the symptoms of allergic disease and limit the inflammatory response. Anaphylactic reactions are treated with epinephrine, which stimulates the reformation of endothelial tight junctions, promotes the relaxation of constricted bronchial smooth muscle, and also stimulates the heart. Inhaled bronchodilators that act on β-adrenergic receptors to relax

constricted muscle are also used to relieve acute asthma attacks. Antihistamines that block the histamine H_1 receptor reduce the urticaria that follows histamine release from mast cells and eosinophils. Relevant H_1 receptors include those on blood vessels that cause increased permeability of the vessel wall, and those on unmyelinated nerve fibers that are thought to mediate the itching sensation. In chronic allergic disease it is extremely important to treat and prevent the chronic inflammatory tissue injury. Topical or systemic corticosteroids are used to suppress the chronic inflammatory changes seen in asthma, rhinitis, and eczema. However, what is really needed is a means of converting the T-cell response to the allergenic peptide antigen from predominantly T_H2 to predominantly T_H1. (Valenta, 2002)

1.3 NIGELLA SATIVA

Plants are invaluable sources of new drugs. There is an enormous historical legacy regarding the use of plant preparations in folk medicine. Scientific studies on plants have provided the discovery of many valuable drugs. *Nigella sativa* L. (Ranunculaceae family) seeds, commonly known as blackseed or black cumin, have been used for thousands of years as a spice and food preservative, as well as a protective and curative remedy for numerous disorders (Figure 1.12). Traditionally, there is a common Islamic belief that blackseed is a panacea for all ailments, but cannot prevent aging or death. Blackseed is also identified as the curative black cumin in the Holy Bible and is described as *Melanthion* by Hippocrates and Dioscorides and as *Gith* by Pliny. (Medicina *et al.* 1994) Many studies have been conducted, particularly during the last two decades, on the effect of *N. sativa* L. Seed extracts on various body systems *in vitro* or *in vivo*. The pharmacological investigations of the seed extracts reveal a broad spectrum of activities including immunopotentiation and antihistaminic, antidiabetic, anti-hypertensive, anti-inflammatory, and antimicrobial activities. Many of these activities have been attributed to the quinone constituents of the seed. (Ali and Blunden, 2003; Gilani *et al,* 2004)

Kingdom:	Plantae
Division:	Magnoliophyta
Class:	Magnoliopsida
Order:	Ranunculales
Family:	Ranunculaceae
Genus:	*Nigella*
Species:	*N. sativa*

Figure 1.9 *Nigella sativa*, scientific classification of *Nigella sativa*

1.3.1 Phytochemistry Of *Nigella sativa*

The seeds of *Nigella sativa* contain a yellowish volatile oil (0.5-1.6 %), a fixed oil (35.6-41.6 %), proteins (22.7 %), aminoacids, reducing sugars, mucilage, alkaloids, organic acis, tannins, resins, toxic glucoside, metarbin, glycosidal saponins, melanthin resmebling helloborin, melanthigenin, ash, arabic acid and also contents possesing nutritional valur like fats, crude fiber, minerals and vitamins. *N. sativa* seeds yield esters of fatty acids; eg. Palmitic acid, oleic acid, linoleic acid and dehydrostearic acid, higher terpenoids, aliphatic alcohols and α-β-unsaturated hydroxy ketones. Free sterols, steryl esters, steryl glucosides and acylated steryl glucosides were isolated from the seed oil. A novel alkaloid, nigellicin, an isoquioline alkaloid, nigellimine and an indizole alkaloid, nigellidine, were also isolated. The seeds also contain lipase, phytosterols, an β- sitosterol. (Ghosheh *et al*, 1999)

The active constituent of the seeds are the volatile oil and the crystalline active principle, nigellone, is the only constituent of the carbonyl fraction of oil. Pharmacologically active constituents of the volatile oil are thymoquinone, dithymoquinone, thymohydroquinone and thymol. Water stres influences the yield and composition of essential oil. The content of thymoquinone was highest (57.78%) when water was withheld for 12 days. (Ghosheh *et al*, 1999)

In a recent study, *N. sativa* seed oil was found to contain higher amounts of total lipids when extracted with N-Hexane and a mixture of chloroform / methanol. Major

fatty acids were linoleic acid, palmitic acid, oleic acid and stearic acid. (Ramadan and Morsel, 2002)

1.3.2 The Pharmacological Properties Of *Nigella sativa*

Many studies have been conducted, particularly during the past two decades, on the effect of *N. sativa* seed extract or its active compound(s) (thymoquinone) on various body systems *in vivo* or *in vitro*. Several studies has shown that it has antioxidant, antiinflammatory and analgesic effects, anticarcinogenic and mutagenic activities, antihepato and nephrotoxic, respiratory and immunological actions, antidiabetic effects, effects on cardiovascular system and blood, antiulcer, antimicrobial, antiparasitic actions. (Gilani *et al,* 2004)

1.3.2.1 Respiratory and immunological effects of Nigella sativa

It has been reported that intravenous administration of the volatile oil of *N. sativa* dose dependently increases the respiratory rate and intratracheal pressure of guinea-pigs However, thymoquinone increased the intratracheal pressure without significantly affecting respiratory rate. Based on the effects of certain antagonists, the actions of the volatile oil were ascribed to histaminergic and muscarinic mechanisms. These authors suggested that *N. Sativa* volatile oil might be a potentially useful respiratory stimulant if thymoquinone were removed from it. This experimental work in guinea-pigs supports the folkloric use of the plant in asthmatic patients. (Tahir *et al*, 1993)

In Pakistan, the effects of a crude extract of *N. sativa* seeds was studied on isolated rabbit jejunum and guinea-pig tracheal preparations. The extract was found to cause a dose-dependent relaxation of spontaneous contractions in the rabbit jejunum, and inhibition of KCl-induced contractions. These actions were similar to those produced by verapamil, a calcium channel antagonist. In the tracheal preparation, the extract antagonized the contractions induced by histamine, carbachol and KCl. The spasmolytic and bronchodilator actions observed were suggested to be mediated via calcium channels. The above pharmacological activities of the petroleum ether fraction of the extract were about 10 times higher than those of the crude extract. (Gilani *et al*, 2001)

In an experiment carried out on rat peritoneal mast cells *in vitro*, it has been shown that nigellone, a carbonyl polymer of thymoquinone isolated from *N. sativa*

seeds, was highly effective in inhibiting histamine release. (Chakravarty, 1993) This was in confirmation of an earlier report that suggested that thymoquinone and thymohydroquinone possess significant antihistaminic effects. (Marozzi *et al.* 1970) El-Kadi and Kandil (1987) were probably the first to show that *N. sativa* seeds have immuno-potentiating properties in human T cells *in vitro*. This was confirmed by Haq *et al*, who showed that *N. sativa* seeds activate T-lymphocytes to secrete the interleukin, IL-3, and increased IL-1β production indicating a stimulatory effect on macrophages either through a direct effect or via IL-1β. (Haq *et al.*, 1995) In further experiments the same authors purified the proteins in the whole *N. sativa* seeds, and showed that some proteins have suppressive and others stimulatory properties in lymphocyte cultures. (Haq *et al.*, 1999) The proteins were also effective in the production of cytokines (e.g. IL-1β). These results were somewhat different from those of Swamy and Tan who reported that, using mouse spleenocytes, *N. sativa* extract, *per se*, has no immunomodulatory activity. In the presence of optimal doses of mitogen, however, there was a significant potentiation of the immune response, the mechanism of which is unclear. (Swamy and Tan, 2000) Another study claimed that *N. sativa* oil doesn't affect the T-helper 1 and T-helper 2 type cytokine production from the splenic mononuclear cells in allergen sensitized mice. According to this study, BALB/c mice were given 0.3 ml of *N. sativa* oil by oro-eosophageal cannula once a day for a month, mice were sensitized by intraperitoneal injections of ovalbumin in the third week of study and the splenic mononuclear cells of mice were cultured with ovalbumin or Concavalin A. According to the measurements of IL-4, IL-10 and IFN-γ from supernatants, it's claimed that Nigella sativa oils doesn't seem to have an immunomodulatory effect on T_H1 and T_H2 cell responsiveness to allergen stimulation. (Büyüköztürk *et al*, 2005)

El Gazzar, investigated that thymoquinone suppresses in vitro production of IL-5 and IL-13 by mast cells in response to lipopolysaccharide stimulation in rat mast cells. (El Gazzar, 2007)

A latest study has shown the in vitro effects of *N. sativa* on the stimulated peripheral blood mononuclear cells by measuring tryptophan degradation and neopterin production. According to this study, *N. sativa* seeds extracts significantly suppressed both tryptophan degradation and neopterin formation which demonstrates and inhibitory influence on the activated T-cells and macrophages. (Winkler *et al.*, 2008)

CHAPTER 2

MATERIALS & METHODS

Donors: PBMC donors were characterized for allergic status in Hill Alerji Merkezi (kind regards to Prof. Dr. Fatih Özkaragöz). Allergic subjects had high allergicity against house dust mite (*Dermatophagoides pteronyssinus*). Nonallergic subjects gave no allergic response to these allergens..

Antigens: House dust mite antigen (*Dermatophagoides pteronyssinus*) was used in several concentrations

ELISA plates: 96 well Interleukin 4 (IL-4), Interleukin 5 (IL-5) and Interferron gamma (IF-γ) ELISA plates (Beckman Coulter, Istanbul, Türkiye) were used.

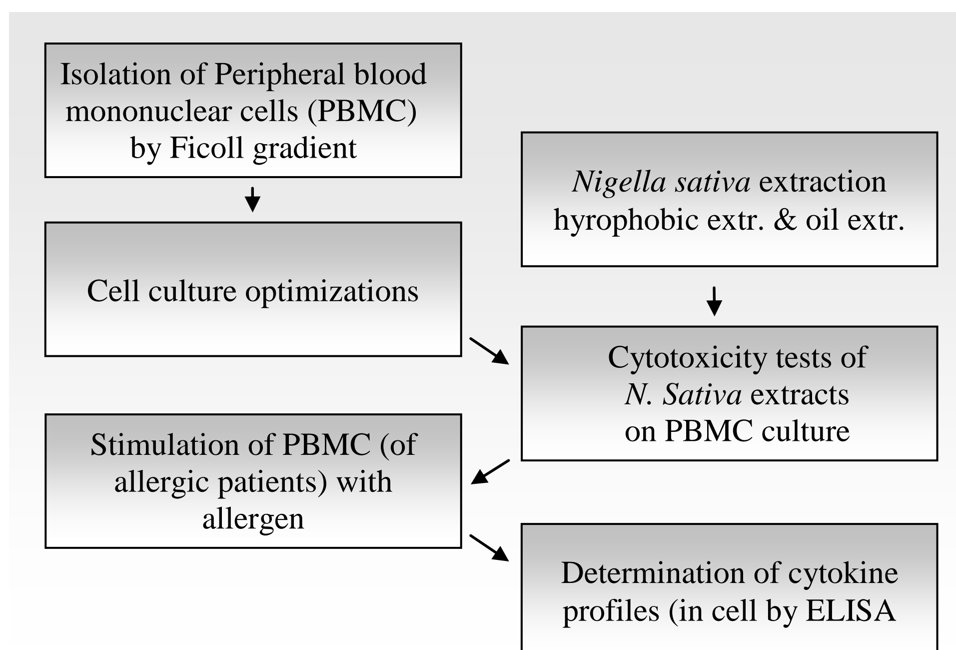


Table 2.1 Methodology of the experiment.

2.1 ISOLATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS

- 5 ml human blood was collected to a sterile 15 ml tube, containing 20U/ml heparin.
- Heparinized blood sample was diluted with PBS (at 37 C) to 10 ml total volume (1:1 blood/PBS ratio).
- 5 ml Ficoll-Paque Plus (at room temperature) was filled to another sterile 15 ml tube.
- 10 ml diluted cell suspension was gently poured on top of 5 ml of Ficoll-Paque Plus, taking care not to disturb the interface between two liquids (1:1:1 blood / PBS / Ficoll ratio) (Figure 2.1)
- The mixture was centrifuged for 20-25 minutes at 2000 rpm (with the break in "off" position) at room temperature (RT).
- At the interface mononuclear cells formed a thin white layer. The white blood cells at the interface were transferred to a new 15 ml tube using a sterile Pasteur pipette. Cells were taken together with a mixture of plasma and separation medium. The better, the less separation medium is taken up.
- At least equal volume of PBS was added on cell suspension and mixed up.
- Mixture was centrifuged at 1700 rpm for 10 minutes.
- The supernatant was discarded, and cell pellet was resuspended firmly tapping the tube.
- 5-10 ml RPMI-1640 was added on pellet and mixed up and down.
- Cell suspension was centrifuged at 1200 rpm for 10 minutes.
- Supernatant was discarded.
- Pellet was resuspended in 2-3 ml RPMI 1640 (including 10% FBS + 100 µg/ml penicillin-streptomycin)
- Cells were counted by 1:1 cell suspension / trypan blue solution under a light microscope using Thoma Lam and plated on 24 or 96 well plates at certain number of cells.

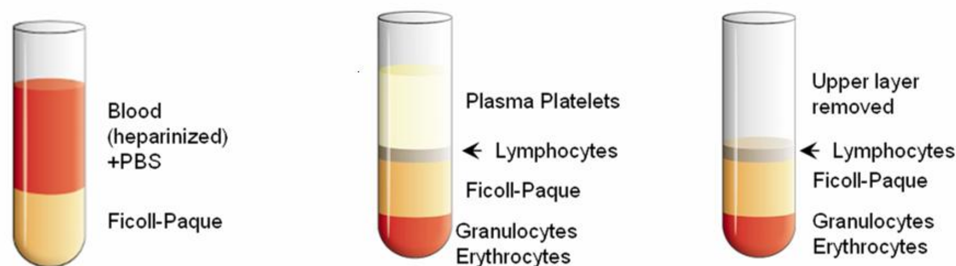


Figure 2.1 Isolation of PBMC by Ficoll-Paqua gradient

2.2 CELL CULTURE OPTIMIZATIONS

- Different cell concentrations were tried for the optimization of PBMC cultures, first at 25 cm^2 flasks, then in 6 well plates and finally in 24 well plates.
- Optimized cell culture condition was defined as 2.10^6 cell / 2 ml RMPI-1640 medium containing 10% FBS and 100µg/ml penicilin-streptomycin incubated in a humidified incubator with 5% CO_2 at 37ºC for 5 days.

2.3 NIGELLA SATIVA EXTRACTION

2.3.1 Extraction of hyrophilic portion of *Nigella sativa*

- 20 g *N. sativa* seed was washed with distilled water, dried at RT and pounded with mortor.
- 80 ml 60% methanol solution was added on paste and mixture was shaked for 5-10 minutes with magnetic stirrer.
- The mixture was filtered through a Wattman paper and elute was collected in another beaker
- Previous two steps were repeated for twice. In the first repeat 60 ml of 60% methanol and in the second repeat about 40 ml of 60% methanol was poured on paste, shaked for 5-10 minutes and then filtered.
- The total elute collected was filtered once more through a Wattman paper.
- 60% methanol was removed from the hydrophilic extract with vacuum rotary , at 40 C, under very low pressure.
- When 60% methanol was removed, about 1 ml of dark brown, waxy hydrophilic extract remained in the beaker

- The hydrophilic extract was dissolved with double distilled water in 1/5 ratio (extract/ddw)
- A final centrifugation was done at 2000 rpm for 5-10 minutes to get rid of undissolved particles, supernatant was taken to a new tube.
- The hyrophilic extract was sterilized by filtering through a 0.40 micron filter**.**
- Dissolved extract was stored at -20 C in 1 ml aliquots.

2.3.2 Extraction of hyrophobic (oil) portion of *Nigella sativa*

- 20 g of *Nigella sativa* seed was washed with distilled water, dried at RT and pounded with mortor.
- 80 ml pure hexane was added on paste and mixture was shaked for about 2 hours with magnetic stirrer.
- The mixture was filtered through a Wattman paper twice and elute was collected in another beaker
- The pure hexane was removed from the hydrophobic extract with vacuum rotary, at 40 C, under very low pressure.
- When pure hexane was removed, about 5 ml of oil extract remained in the beaker
- The hydrophobic extract was dissolved with DMSO (Dimethyl sulfoxide) in 1/1 ratio (extract /DMSO)
- A final centrifugation was done at 2000 rpm for 5-10 minutes to get rid of undissolved particles. Supernatant was taken to a new tube.
- The oil extract was sterilized by filtering through a 0.40 micron filter**.**
- Dissolved extract was stored at -20 C in 1 ml aliquots.

2.4 CYTOTOXICITY ASSAY

Cytotoxicity Detection Kit^{PLUS} (LDH) (Roche) was used for the cytotoxicity analysis of *N. Sativa* extracts on PBMC culture.

This method relies on the cell death measured by quantifying the plasma membrane damage. When the plasma membrane is damaged Lactate Dehydrogenase (LDH), which is a stable cytoplasmic enzyme present in all cells, is rapidly released to the cytoplasm. The cytotoxicity is detected colorimetrically by measuring the LDH acitivity released from damaged cells.

The principle of test is that cell culture is incubated with the reaction mixture containing the Diaphorase/NAD+ mixture as catalyst and Iodotetrazolium chloride (INT) and sodium lactate as dye solution from the kit. The LDH activity is determined in an enzymatic test; In the first step NAD^+ is reduced to $NADH/H^+$ by the LDH-catalyzed conversion of lactate to pyruvate. In the second step the catalyst (diaphorase) transfers H/H^+ from $NADH/H^+$ to the tetrazolium salt INT which is reduced to formazan (Figure 2.2).

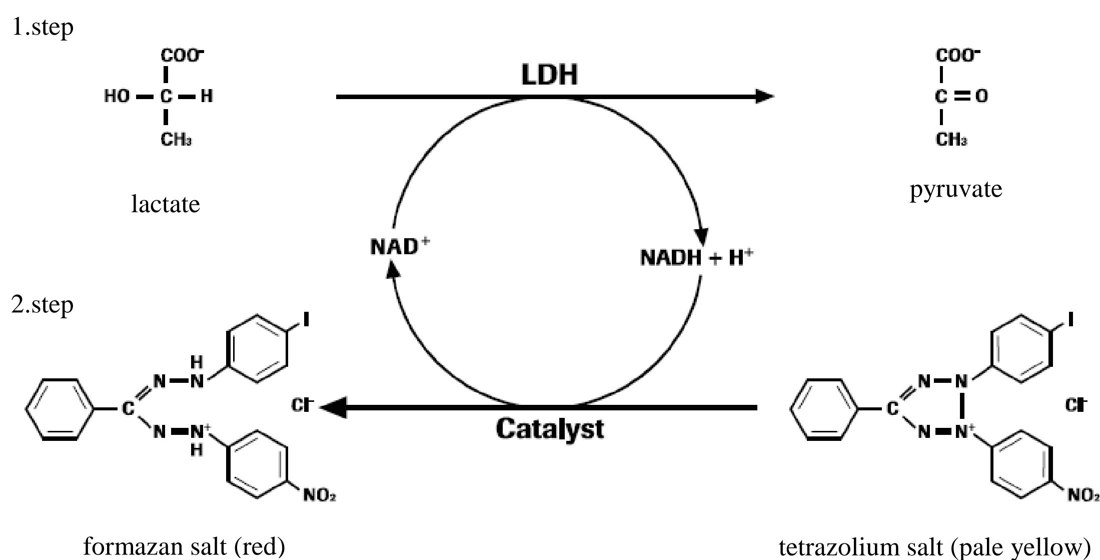


Figure 2.2 Assay of released LDH. In the first step, released lactate dehydrogenase (LDH) reduces NAD+ to $NADH^+/H^+$ by oxidizing lactate to pyruvate. In the second enzymatic reaction 2 hydrogens are transferred from $NADH^+/H^+$ to the yellow tetrazolium salt INT (2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium chloride) by a catalyst.

- PBMC was isolated and cultured in 96 well flat bottomed plates in concentration of 2.10^5 cells/ 200ml RPMI-1640 medium containing 10% FBS in each well.
- Before applying the extracts on PBMC cultures were incubated for 24h in a humidified incubator with 5% CO_2 and 37 C.
- Second day different dilutions of both hydrophobic and hydrophilic extracts were applied on PBMC cultures. For each day a separate 96 well plate culture was prepared in same conditions.
- After application of extracts each day cytotoxicity level was measured.

- To measure cytotoxicity, after resuspending the cultures with a micropippette, 100 µl of culture was transferred to a new well in 96 well-plate and 100 µl of reaction mix (the Diaphorase/NAD+ mixture as catalyst and Iodotetrazolium chloride (INT) and sodium lactate as dye solution) was added on each well.
- The plate was incubated at 37 C for up to 30 min.
- 50 µl of Stop solution was added on each reaction mix to stop LDH activity
- The absorbances of the samples at 490 nm were measured using an ELISA reader. (The reference wavelength should be more than 600nm)

2.5 STIMULATION OF PBMC CULTURES

Isolated peripheral blood mononuclear cells were cultured at 2.10^6 cells/2 ml in RPMI-1640 medium containing 10% FBS at 24 well flat bottomed flasks. After 1 day incubation of cells in a humidified incubator with 5% CO_2 and 37ºC, different concentrations (0.005 µg/ml, 0.02 µg/ml, 1 µg/ml) of house dust mite antigen was added with different concentrations (0.15 % and 0.10 %) of *Nigella sativa* extracts to the cell suspensions. Cultures were incubated at 37 C, 5% CO_2 for 5 days. At end of 5 days the cell suspensions were transferred to tubes and centrifuged at 2000 rpm for 5 minutes. Supernatants were saved for ELISA, in which IL-4, IL-5 and IF-γ concentrations were measured.

2.6 DETERMINATION OF CYTOKINE CONCENTRATIONS BY ELISA

Culture supernatants which were collected after 5 day incubation, were measured for IL-4, IL-5 and IFN-γ amounts by means of ELISA.

Assay procedure;

- 100 µl standard or sample was added to each well and incubated 2.5 hours at RT or over night at 40 C.
- 100 µl prepared biotin antibody was added to each well and incubated 1 hour at RT.
- 100 µl prepared Streptavidin solutions added and incubated 45 minutes at RT.
- 100 µl TMB One-Step Substrate Reagent was added to each well and incubated 30 minutes at RT.
- 50 µl Stop Solution was added to each well and absorbance is read at 450 nm immediately.

CHAPTER 3

RESULTS

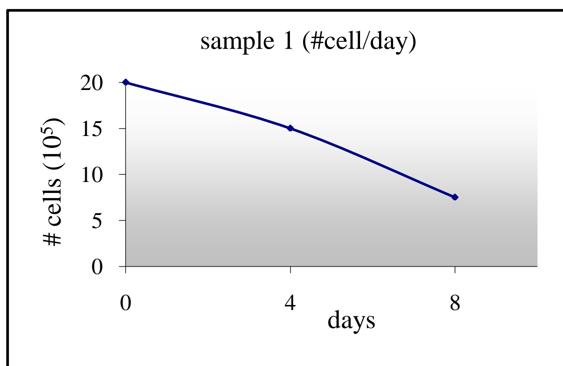
3.1 CELL CULTURE OPTIMIZATIONS

- Growth curve of PBMC cultured in 2.10^6 cell / 1ml in 24 well flasks:

Peripheral blood mononuclear cells isolated from three different individual's blood were cultured at 2.10^6 cells/1 ml in RPMI-1640 medium containing 10% FBS at 24 well flat bottomed flasks and incubated in a humidified incubator with 5% CO_2 and 37ºC for 8 days. At the 4th day medium was refreshed and cell numbers were counted at 4th and 8th day. (Figure 3.1)

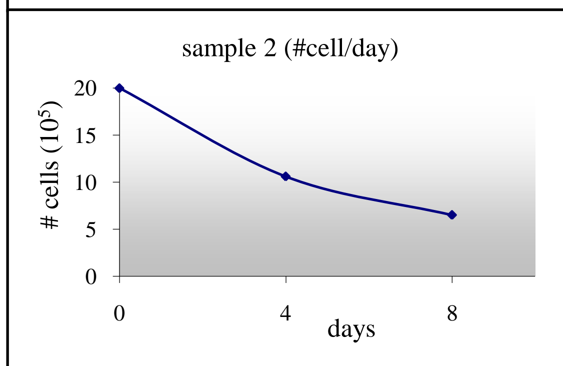
Sample 1

day	# cell (x10^5)
0	20
4	15
8	7,5



Sample 2

day	# cell (x10^5)
0	20
4	10,6
8	6,5



Sample 3

day	# cell (x10^5)
0	20
4	8
8	6

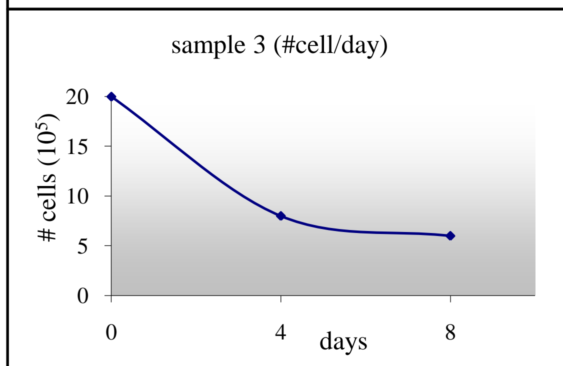


Figure 3.1 Cell growth curves of three PBMC samples that were cultured in 2.10^6 cell / 1ml medium in 24 well flasks and incubated at 37 C, 5% CO_2 for 8 days. At the 4th day mediums were refreshed.

- Growth curve of PBMC cultured in 2.10^6 cell / 2ml in 24 well flasks:

Isolated peripheral blood mononuclear cells were cultured at 2.10^6 cells / 2 ml in RPMI-1640 medium containing 10% FBS at 24 well flat bottomed flasks and incubated in a humidified incubator with 5% CO_2 at 37 C. Three experiment sets were prepared; one set was prepared for every 3 days medium refreshment, one set was prepared for every 5 days medium refreshment, and another set was prepared for every 7 days medium refreshment.

First set of PBMC culture's medium was refreshed in every three days. Cell growth curve for this set is shown in Figure 3.2

Day	**3.1 (#cell/2ml)**	**3.2 (#cell/2ml)**
0	$2,00.10^6$	$2,00.\ 10^6$
3	$1,36.\ 10^6$	$1,68.\ 10^6$
6	$1,74.\ 10^6$	$1,68.\ 10^6$
9	$1,64.\ 10^6$	$1,84.\ 10^6$
12	$1,20.\ 10^6$	$2,28.\ 10^6$

Table 3.1 Cell numbers of PBMC cultured in 2.10^6 cell / 2ml medium in 24 well flasks incubated at 37 C, 5% CO_2 for 12 days. Every 3 days medium was refreshed.

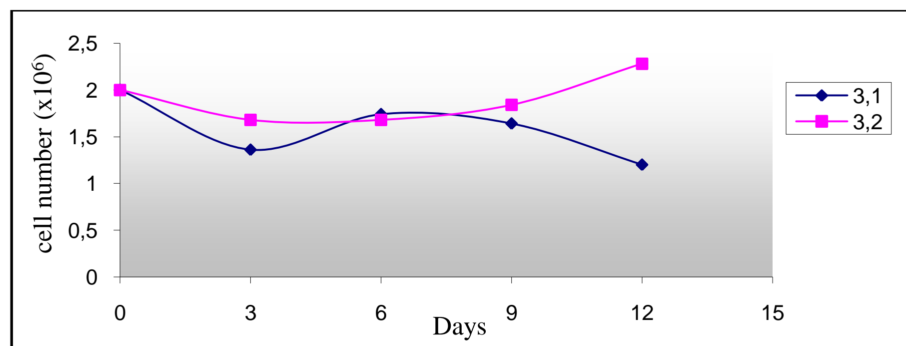


Figure 3.2 Cell growth curve of PBMC cultured in 2.10^6 cell / 2ml medium in 24 well flasks incubated at 37 C, 5% CO_2 for 12 days. Every 3 days medium was refreshed.

Second set of PBMC culture's medium was refreshed in every five days. Cell growth curve for this set is stated in Figure 3.3.

Day	**5.1 (#cell/2ml)**	**5.2 (#cell/2ml)**
0	$2,00.10^6$	$2,00.10^6$
5	$1,38.10^6$	$0,94.10^6$
10	$0,88.10^6$	$1,00.10^6$
15	$0,44.10^6$	$0,40.10^6$

Table 3.2 Cell numbers of PBMC cultured in 2.10^6 cell / 2ml medium in 24 well flasks incubated at 37ºC, 5% CO_2 for 15 days. Every 5 days medium was refreshed.

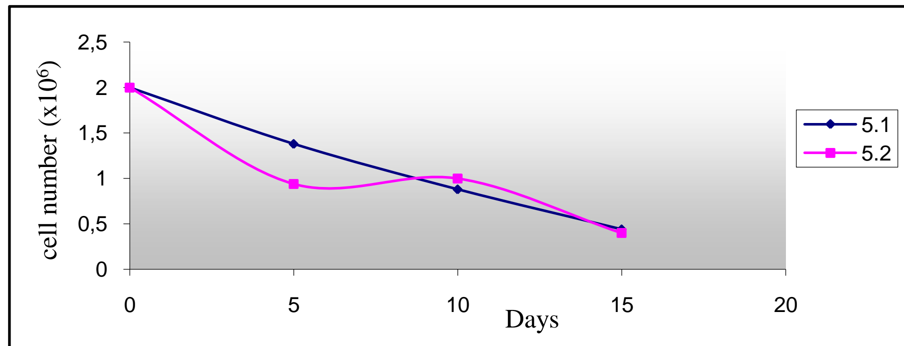


Figure 3.3 Cell growth curve of PBMC cultured in 2.10^6 cell / 2ml medium in 24 well flasks incubated at 37ºC, 5% CO_2 for 15 days. Every 5 days medium was refreshed.

Third set of PBMC culture's medium was refreshed in every seven days. Cell growth curve for this set is stated in Figure 3.4.

Day	**7.1 (#cell/2ml)**	**7.2 (#cell/2ml)**	**7.3* (#cell/2ml)**
0	$2,00.10^6$	$2,00.10^6$	$2,00.10^6$
7	$0,80.10^6$	$0,76.10^6$	$2,60.10^6$
14	$0,72.10^6$	$0,64.10^6$	$1,76.10^6$

Table 3.3 Cell numbers of PBMC cultured in 2.10^6 cell / 2ml medium in 24 well flasks incubated at 37 C, 5% CO_2 for 14 days. Every 7 days medium was refreshed. *7.3 => PBMC isolated with heparin

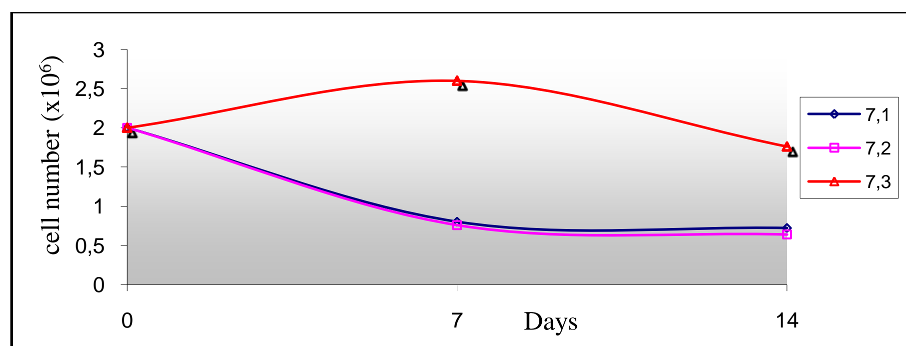


Figure 3.4 Cell growth curve of PBMC cultured in 2.10^6 cell / 2ml medium in 24 well flasks incubated at 37 C, 5% CO_2 for 14 days. Every 7 days medium was refreshed.

According to these trials, it was decided that isolated peripheral blood mononuclear cells will be cultured at 2.10^6 cells / 2 ml in RPMI-1640 medium containing 10% FBS at 24 well flat bottomed flasks and incubated in a humidified incubator with 5% CO_2 at 37 C for 5 days without refreshing the medium.

3.2 CYTOTOXICITY RESULTS

3.2.1 Cytotoxicity assay for oil extract, hydrophilic extract and DMSO

For the cytotoxicity analysis several dilutions of oil extract, hydrophilic extract and DMSO -in which oil is dissolved- were applied on $2x10^5$ cells / 200 µl PBMC culture (Table 3.1). Cultures were incubated in flat bottomed 96 well plates for 5 days in a humidified incubator with 5% CO_2 at 37ºC. Each day cytotoxicity analysis were done.

Oil extract was dissolved in DMSO in 1:1 ratio. The various amounts of oil extracts applied on PBMC culture are shown in the Table 3.1. The cytotoxicity results of oil extracts are presented in Figure 3.5. This cytotoxicity assay, colorimetrically measures the cell death relying on the lactate dehyrogenase secreted by damaged cells. The higher OD measured at 460 nm has the higher amount of LDH in the supernatants of the cell cultures, showing the higher amount of cell death. However this assay doesn't give a clear idea about the cytotoxicity levels of different concentrations of *N.sativa* extracts because even the cell cultures started with the same number of cells, according to cytotoxic effects of extracts cells die. Although it is thought that higher amount of LDH will show higher cytotoxicity, lower number of cell will release lower amount of LDH, which contradicts with the first hypothetical assumption. A much more clear idea about the cytotoxic effects of extracts came from the cell numbers counted at the end of 5th day (Table 3.4) and also the morphology of cells observed under light microscope. (Figure 3.6) These results presented that oil extract of *N. sativa* has high cytotoxic effects on blood mononuclear cells. Only 0.05 % oil extract seem to be applicable on the PBMC culture, higher concentrations kill the cells.

Oil extract (µl)	DMSO (µl)	Total(µl) (oil+DMSO)	Percentage (%)
10	10	20	5 %
5	5	10	2.5 %
2	2	4	1 %
0.5	0.5	1	0.25 %
0.1	0.1	0.2	0.05 %

Table 3.4 The percentages of oil extract dissolved in DMSO, applied on PBMC culture for the cytotoxicity test.

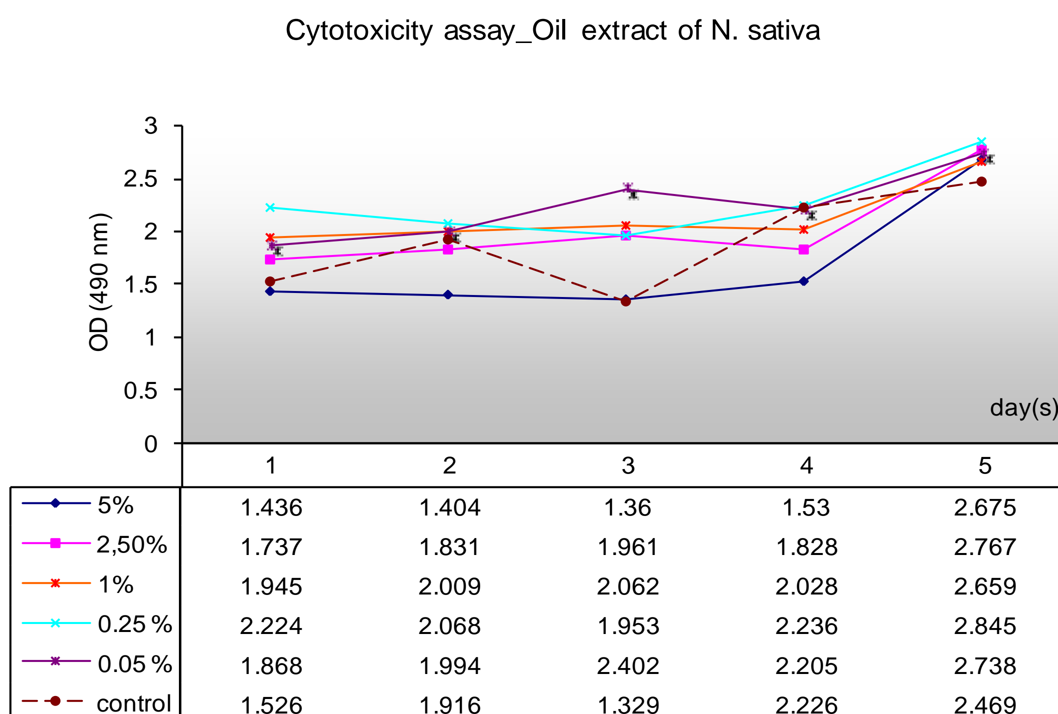


Figure 3.5 The cytotoxicity results of oil extracts on PBMC culture. More LDH in the culture gives a higher absorbance in 490 nm. However according to this graph it is hard to compare the cytotoxicities of different percentages, because the LDH half life is 9 h. Less cell, there will be less LDH secretion. Among other percentages 0.05 % gives the highest peak which can be explained with the higher number of cells**.**

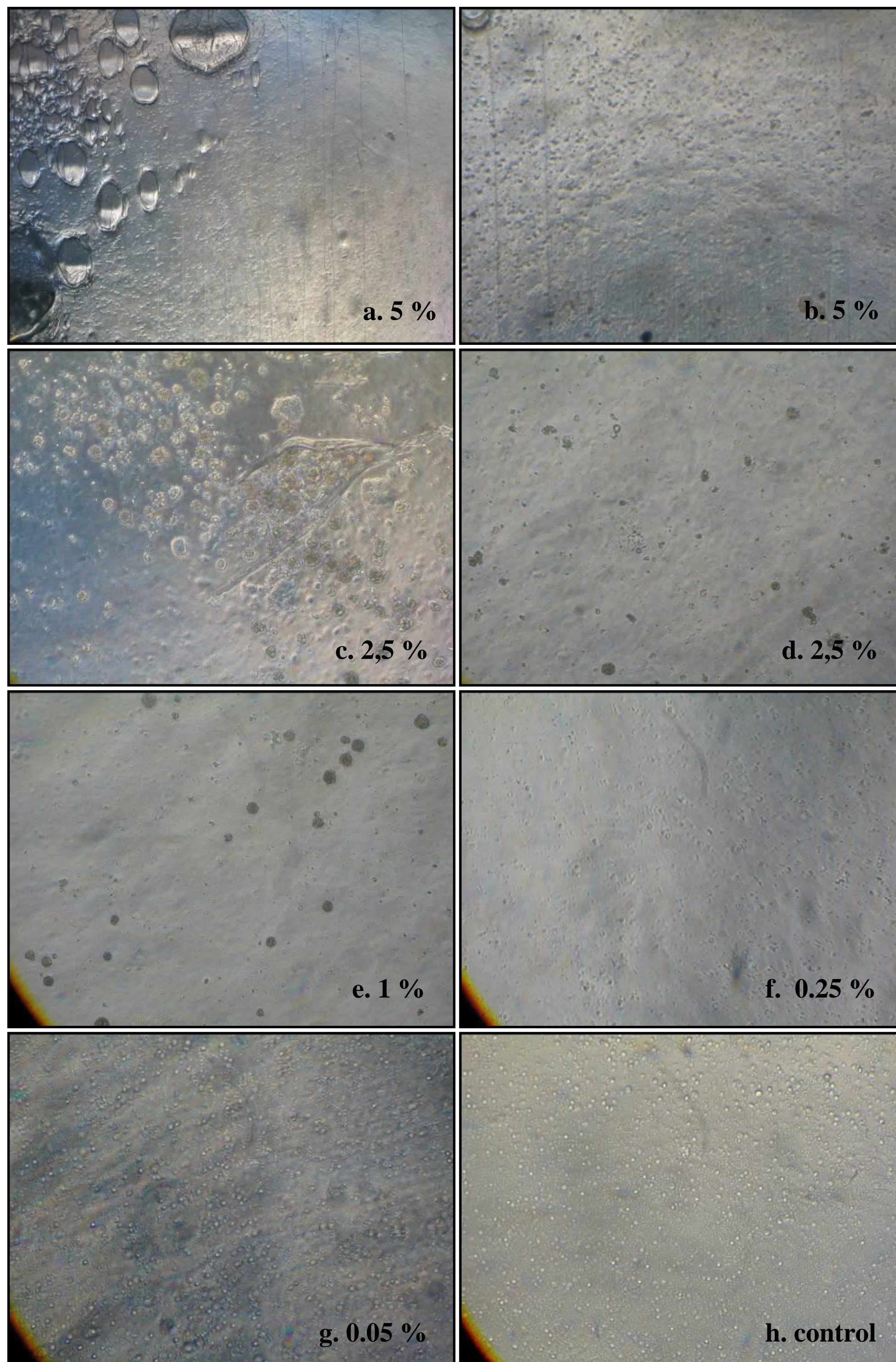
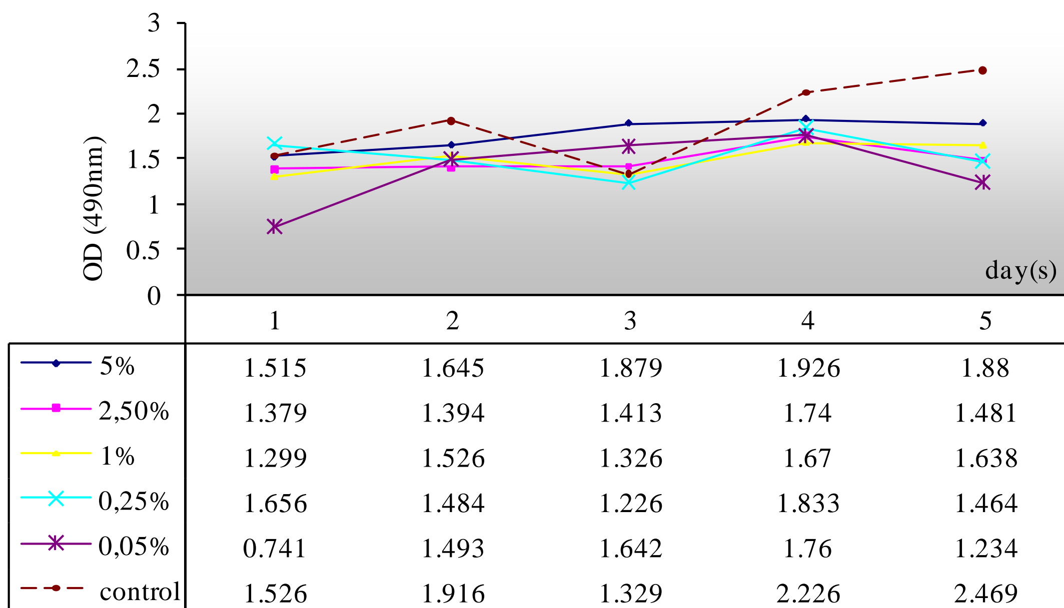


Figure 3.6 Light microscope photographs of $2x10^5$ cells / 200 µl PBMC cultures with several dilutions of *N.sativa* oil extracts, taken in the 5th day of culture in 20x magnification. **a,b** 5% oil extract **c,d** 2,5 % oil extract **e** 1% oil extract **f** 0.25% oil extract **g** 0.05% oil extract **h** control. The bubbles observed on the photographs were the undissolved oil particles. However the high cytotoxic effects of oil extracts showed that there were dissolved portions of the oil extract which affected the cell culture.

DMSO was used as a reagent for dissolving the oil portion of *N. sativa* extract. To eliminate the cytotoxic effects leading from DMSO, various concentrations of DMSO were applied on PBMC culture. (Table 3.5) The cytotoxicity results of several DMSO proportions applied on PBMC culture are presented in Figure 3.7. According to the cytotoxicity assay 0.05 % DMSO had the lowest cytotoxicity but it is a question mark why the control, which doesn't contain any DMSO, had the highest LDH amount at the end of 5th day. However even the highest amount of DMSO applied on PBMC culture doesn't have as much cytotoxic effect as the lowest amount of oil extract according to the number of cells at the end of 5th day in culture (Table 3.4) and the morphologies of the cells. (Figure 3.8)

DMSO (μl)	Percentage (%)
10	5 %
5	2.5 %
2	1 %
0.5	0.25 %
0.1	0.05 %

Table 3.5 The percentages of DMSO, applied on PBMC culture for the cytotoxicity test.



	1	2	3	4	5
5%	1.515	1.645	1.879	1.926	1.88
2,50%	1.379	1.394	1.413	1.74	1.481
1%	1.299	1.526	1.326	1.67	1.638
0,25%	1.656	1.484	1.226	1.833	1.464
0,05%	0.741	1.493	1.642	1.76	1.234
control	1.526	1.916	1.329	2.226	2.469

Figure 3.7 The cytotoxicity results of DMSO on PBMC culture. More LDH in the culture gives a higher absorbance in 490 nm. According to this graph as 0.05 % has the lowest cytotoxicity however it is a question mark why the control, which doesn't contain any DMSO has the highest LDH amount at the end of 5th day

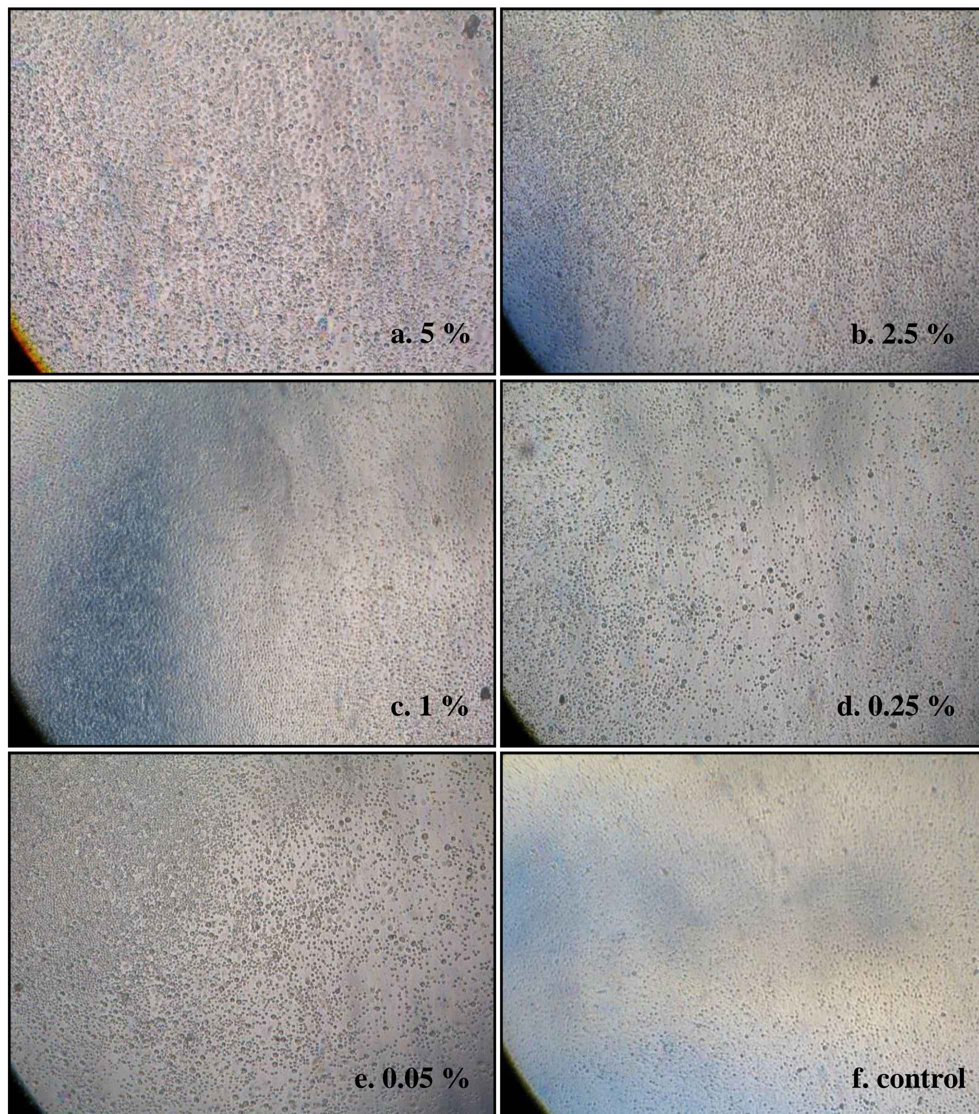
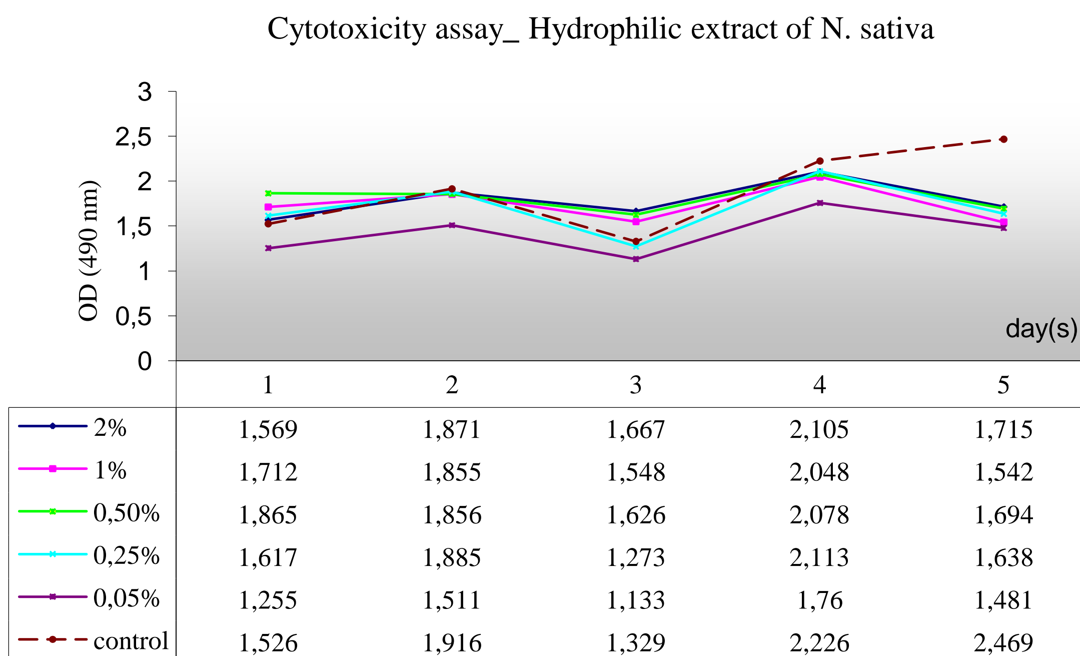


Figure 3.8 Light microscope photographs of $2x10^5$ cells / 200 µl PBMC cultures with several dilutions of *N.sativa* oil extracts, taken in the 5th day of culture in 10x magnification. **a.** 5% DMSO **b.** 2,5% DMSO **c.** 1% DMSO **d.** 0.25% DMSO **e.** 0.05% DMSO **f.** control.

Hydrophilic extract of *N.*sativa was dissolved in double distilled water in 1:4 ratios. Several amounts of hydrophilic extracts were applied on PBMC culture. (Table 3.6) The cytotoxicity assay results of hydrophilic extracts applied on PBMC culture are shown in Figure 3.9. However it doesn't give a clear idea about the cytotoxic effects of hydrophilic extract because even the cell cultures started with the same number of cells, according to cytotoxic effects of extracts, cells die. Although it is thought that higher amount of LDH will show higher cytotoxicity, lower number of cell will release lower amount of LDH, which contradicts with the first assumption. A much more clear idea about the cytotoxic effects of extracts comes from the cell numbers counted at the end of 5th day (Table 3.7) and also the morphology of cells observed under light microscope (Figure 3.10). These results presented that hydrophilic extract of *N.sativa* has high cytotoxicity on blood mononuclear cells. Only 0.05 % oil extract seem to be applicable on the PBMC culture, higher concentrations kill the cells in high number.

Hyrophilic extract (µl)	Total amout	Percentage (%)
4	20	2 %
2	10	1 %
1	5	0.5 %
0.5	2.5	0.25 %
0.2	1	0.10 %

Table 3.6 The percentages of hydrophilic extract dissolved in distilled water, applied on PBMC culture for the cytotoxicity test



	1	2	3	4	5
2%	1,569	1,871	1,667	2,105	1,715
1%	1,712	1,855	1,548	2,048	1,542
0,50%	1,865	1,856	1,626	2,078	1,694
0,25%	1,617	1,885	1,273	2,113	1,638
0,05%	1,255	1,511	1,133	1,76	1,481
control	1,526	1,916	1,329	2,226	2,469

Figure 3.9 The cytotoxicity results of hydrophilic extracts on PBMC culture. More LDH in the culture gives a higher absorbance in 490 nm. However according to this graph it is hard to compare the cytotoxicities of different percentages, because the LDH half life is 9 h and there is less cell, there will be less LDH secretion. Among other percentages 0.05 % gave the minimum peak which can be explained with the less degradation of cells**;** however it is a question mark why the control, which doesn't contain any extract, had the highest LDH amount at the end of 5th day.

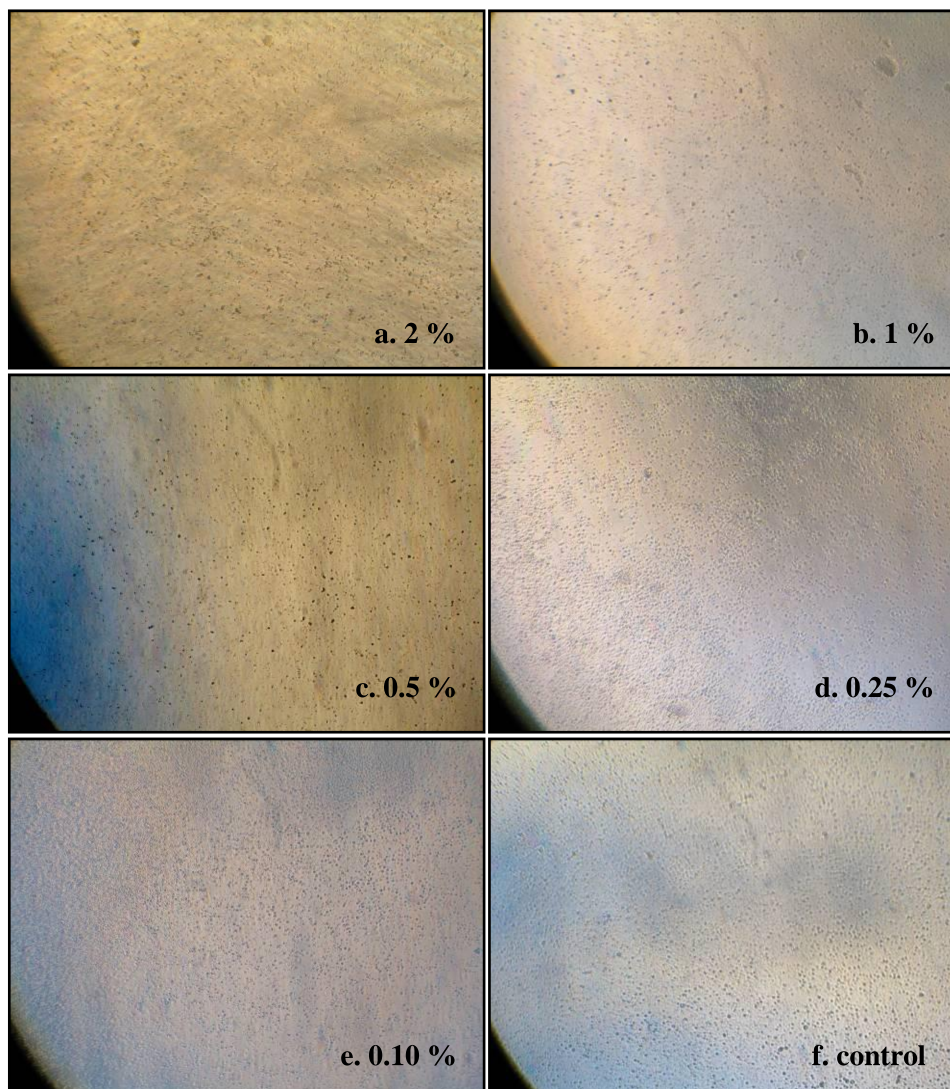


Figure 3.10 Light microscope photographs of $2x10^5$ cells/200 µl PBMC cultures with several dilutions of *N.sativa* hydrophilic extracts, taken in the 5th day of culture in 10x magnification. **a** 2 % hydrophilic extract **b** 1 % oil extract **c** 0.5 % oil extract **d** 0.25 % oil extract **e** 0.10 % oil extract **f** control. Cytotoxic effect was observed significantly in the higher concentrations of hydrophilic extract. Only the 0.10 % hydrophilic extract seemed to be applicable on the PBMC culture.

The morphologies of the cells and the cell number of cultures in 5. day of incubation (started with 200.000 cell/200µl) gave us more clue about the cytotoxic effect of the *N.sativa* extracts and DMSO (Table 3.4). DMSO didn't seem to be cytotoxic when it is less than 1%. Both the oil and hydrophilic extracts are highly cytotoxic.

After these results another cytotoxicity assay is done for the ranges between 0.25 % and 0.05 % both for the oil and hydrophilic extracts.

# cell/ 200µl	**Oil extract**	**DMSO**	**Hydrophilic extract**	**Control**
5%	-	150.000	-	
2,5%	-	130.000	-	
1%	-	220.000	-	300.000
0,25%	60.000	270.000	40.000	
0,05%	200.000	400.000	350.000	

Table 3.7 Cell numbers of the PBMC culture at the end of 5 day incubation with different amounts of oil extract, DMSO and hydrophilic extract of *N. sativa*.

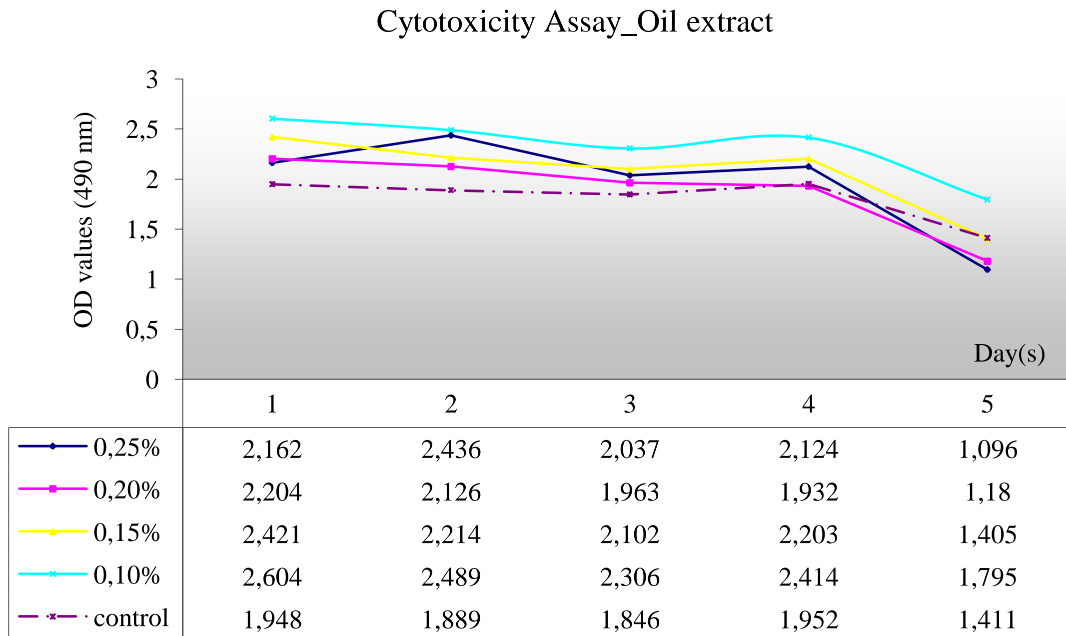
3.2.2 Cytotoxicity assay for 0.25 % - 0.05 % oil and hydrophilic extracts

For the cytotoxicity analysis several dilutions of oil and hydrophilic extracts ranging between 0.25% - 0.05% were applied on $2x10^5$ cells/200 µl PBMC culture (Table 3.8, Table 3.9). Culture was incubated in flat bottomed 96 well plates for 5 days in a humidified incubator with 5% CO_2 and 37ºC. Each day, cytotoxicity was measured and cell numbers are counted.

Oil extract was dissolved in DMSO in 1:1 ratio. The results of cytotoxicity assay and cell growth curves are stated in Figure 3.11 and Figure 3.12. According to these results 0,10 % and 0,05 % and less dilutions of oil extract of *Nigella sativa* were decided to be used with the antigenic stimulation of PBMC culture.

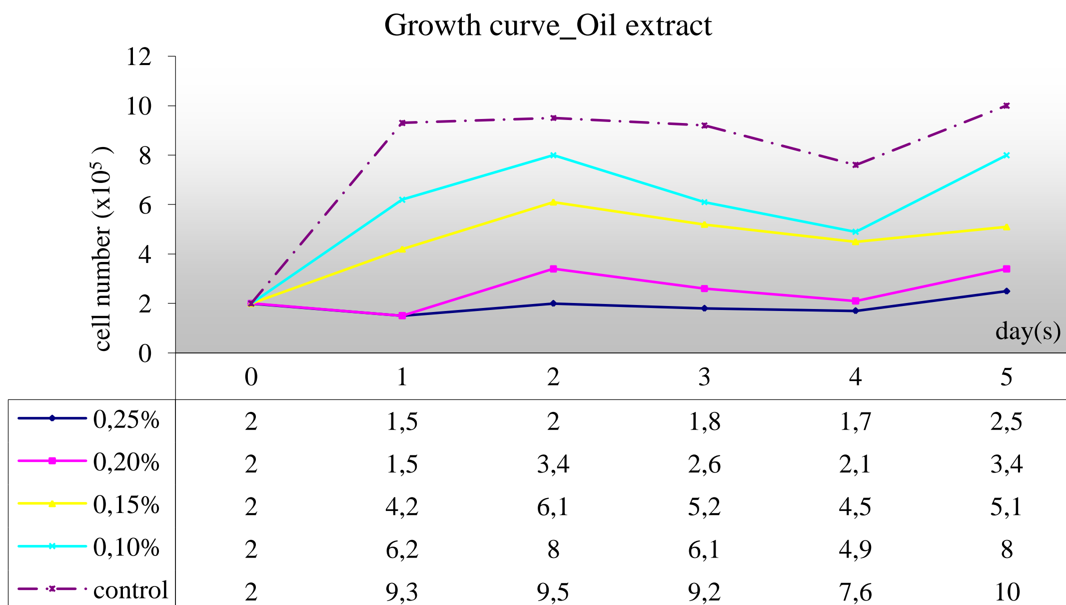
Oil extract (µl)	DMSO (µl)	Total(µl) (oil+DMSO)	Percentage (%)
0.5	0.5	1	0.25 %
0.4	0.4	0.8	0.20 %
0.3	0.3	0.6	0.15 %
0.2	0.2	0.4	0.10 %

Table 3.8 The percentages of oil extract dissolved in DMSO, applied on PBMC culture for the second cytotoxicity test.



	1	2	3	4	5
0,25%	2,162	2,436	2,037	2,124	1,096
0,20%	2,204	2,126	1,963	1,932	1,18
0,15%	2,421	2,214	2,102	2,203	1,405
0,10%	2,604	2,489	2,306	2,414	1,795
control	1,948	1,889	1,846	1,952	1,411

Figure 3.11 The cytotoxicity results of oil extract (0.25% -0.10 %) on PBMC culture. More LDH in the culture would be expected to give a higher absorbance in 490 nm.. Among other percentages 0.05 % gave the highest peak which may be explained with the higher number of cells secreting more LDH**.** Control seemed to have less LDH secretion even it had more cells at the end of 5 days, which means there was less cell degradation in control sample.



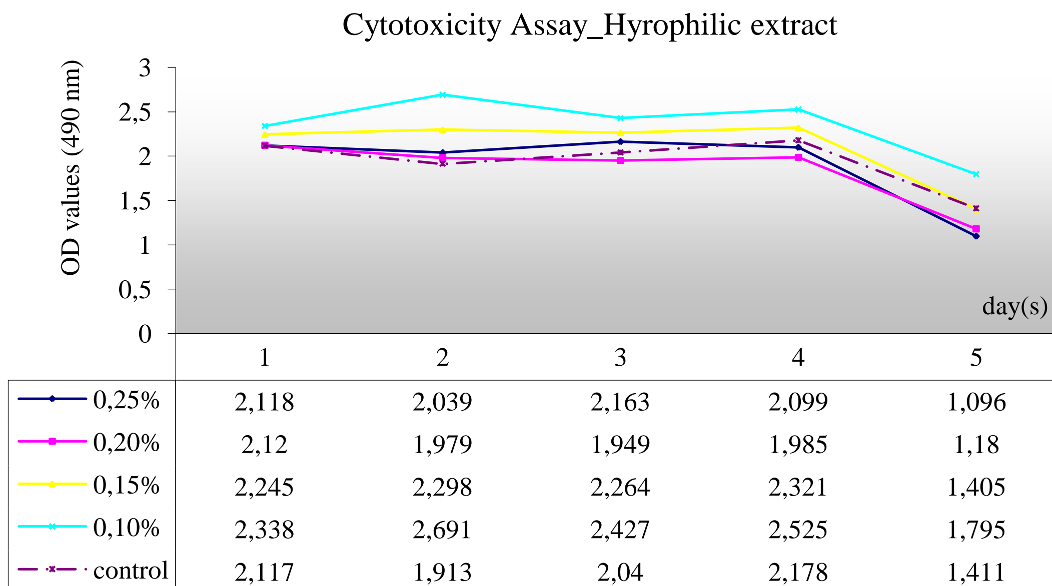
	0	1	2	3	4	5
0,25%	2	1,5	2	1,8	1,7	2,5
0,20%	2	1,5	3,4	2,6	2,1	3,4
0,15%	2	4,2	6,1	5,2	4,5	5,1
0,10%	2	6,2	8	6,1	4,9	8
control	2	9,3	9,5	9,2	7,6	10

Figure 3.12 The cell growth curves of PBMC culture treated with different concentrations of *N.sativa* oil extracts within 5 days. Culture with 0.10 % oil extract had the highest number of cells at the end of 5 day.

Hyrophilic extract was dissolved in double distilled water in 1:4 ratios. The results of cytotoxicity assay and cell growth curves are stated in Figure 3.13 and Figure 3.14. According to these results 0,10 % and 0,05 % and less dilutions of hydrophilic extract of *N. sativa* were decided to be used with the antigenic stimulation of PBMC culture

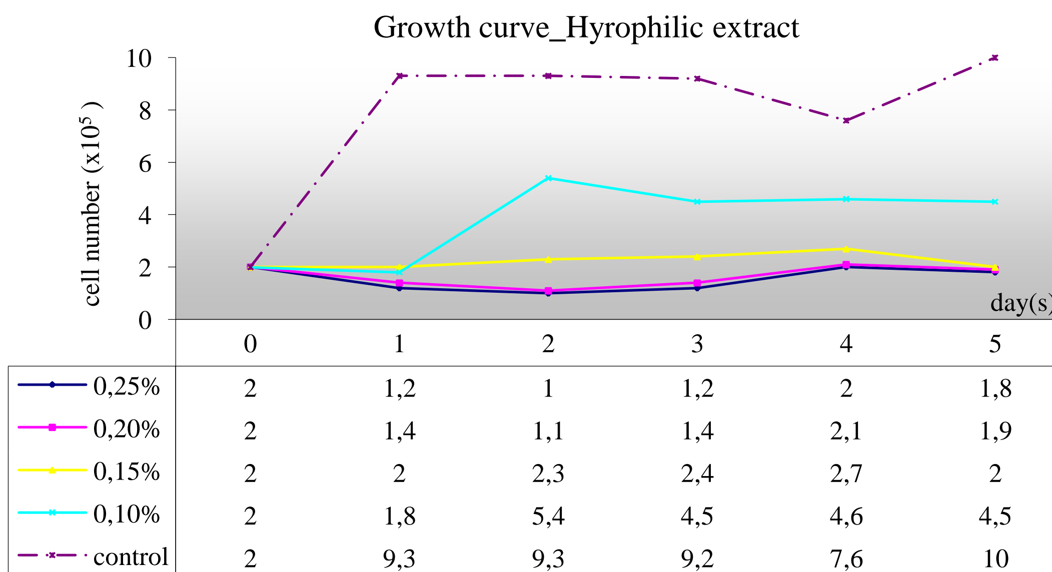
Hyrophilic extract (µl)	Total (µl) (extr+dw)	Percentage (%)
0.5	2.5	0.25 %
0.4	2	0.20 %
0.3	1.5	0.15 %
0.2	1	0.10 %

Table 3.9 The percentages of hydrophilic extract dissolved in distilled water, applied on PBMC culture for the second cytotoxicity test



	1	2	3	4	5
0,25%	2,118	2,039	2,163	2,099	1,096
0,20%	2,12	1,979	1,949	1,985	1,18
0,15%	2,245	2,298	2,264	2,321	1,405
0,10%	2,338	2,691	2,427	2,525	1,795
control	2,117	1,913	2,04	2,178	1,411

Figure 3.13 The cytotoxicity results of hydrophilic extract (0.25% -0.10 %) on PBMC culture. More LDH in the culture would be expected to give a higher absorbance in 490 nm.. Among other percentages 0.05 % gave the highest peak which may be explained with the higher number of cells secreting more LDH**.** Control seemed to have less LDH secretion even it had more cells at the end of 5 days, which means there was less cell degradation in control sample.



	0	1	2	3	4	5
0,25%	2	1,2	1	1,2	2	1,8
0,20%	2	1,4	1,1	1,4	2,1	1,9
0,15%	2	2	2,3	2,4	2,7	2
0,10%	2	1,8	5,4	4,5	4,6	4,5
control	2	9,3	9,3	9,2	7,6	10

Figure 3.14 The cell growth curve of PBMC culture treated with hydrophilic extract of *Nigella sativa* (0.25-0.10 %). Even the 0.10 % hydrophilic extract had the lowest cytotoxicity, still it seemed quite cytotoxic, that number of cells in control group was two times more than the cell number in 0.10% hydrophilic extract culture.

3.3 STIMULATION OF PBMC WTH ALLERGEN AND EXTRACTS

3.3.1 Determining the antigen concentration for antigenic stimulation

For defining the concentration of antigen, PBMC was isolated from an allergic subject known to have high allergicity against house dust mite *Dermatophagoides pteronyssinus*). For the antigenic stimulation 3 concentrations of house dust mite antigen were tried in reference to literature (Table 3.10) The PBMC culture was stimulated with house dust mite antigen in the absence of *Nigella sativa* extracts.

2.10^6 cells/2 ml in RPMI 1640 medium (with penicillin-streptomycin) containing 10 % FBS, is incubated at 37 C, 5% CO_2 for 5 days.

Supernatants of cell cultures were collected on 3rd and 5th day of incubation for the IL-4 and IFN-γ detection by means of ELISA.

Well	House dust mite
1	0.005 µg/ml
2	0.02 µg/ml
3	1 µg/ml
4	No antigen

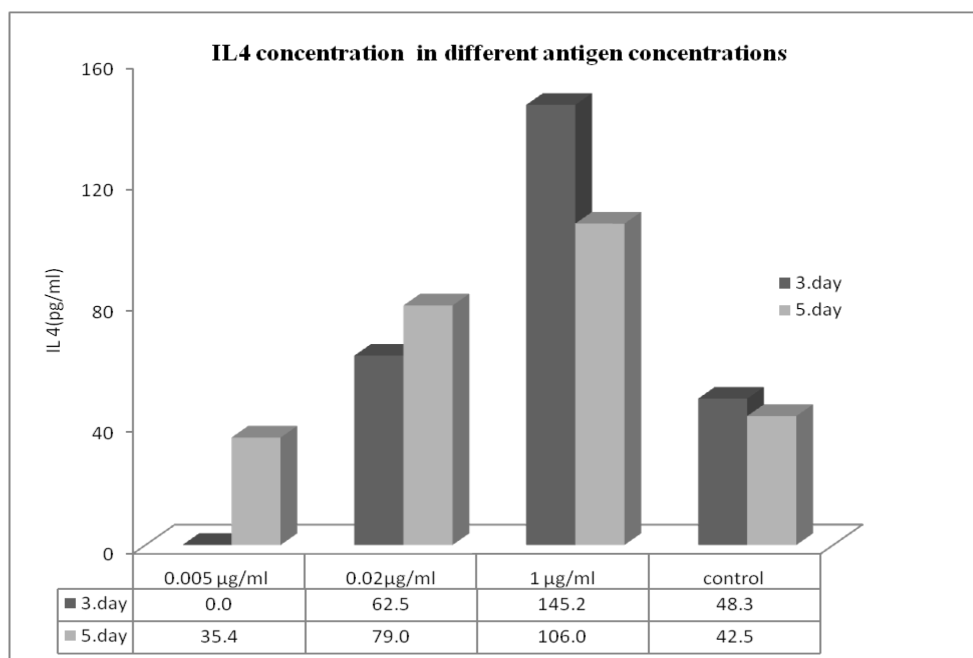
Table 3.10 Three concentration of house dust mite antigen applied on PBMC culture in the absence of *Nigella sativa* extracts

The concentration of IL-4 and IFN-γ in the PBMC culture supernatants were measured by ELISA method. It was observed that the amount of IL-4 production increased directly proportional to the amount of antigen used for stimulation of PBMC culture, whereas the amount of IFN-γ decreased inversely proportional to the amount of antigen and also IL-4. (Figure 3.15 and Figure 3.16)

These results indicated that the increasing amount of house dust mite antigen used, increased the IL-4 produced in response which is the sign of allergic reaction in the culture. In the lower concentrations of antigen stimulation (0.005 µg/ml, 0.02 µg/ml) the amount of IL-4 secreted was increasing in the 5th day, however when the amount of antigen was increased, IL-4 production had shown to be higher in the 3rd day, then

decreasing in the 5th day (Figure 3.15). On the other hand, the amount of IFN-γ decreased with the increasing amount of IL-4 which shows that T_H2 driven response inhibits the T_H1 driven response because IFN-γ drives the shift to T_H1 direction whereas IL-4 shifts the response to T_H2 direction and they supress eachother (Figure 3.17)

The next step to do after this trial was stimulating the PBMC culture both with antigen and *Nigella sativa* extracts, trying to shift this T_H1-T_H2 balance on the T_H1 direction.



	0.005 µg/ml	0.02µg/ml	1 µg/ml	control
3.day	0.0	62.5	145.2	48.3
5.day	35.4	79.0	106.0	42.5

Figure 3.15 Amounts of IL-4 measured by means of ELISA in the 3rd day and 5th day of incubation of PBMC culture after antigenic stimulation. 0.005µg/ml, 0.02µg/ml and 1 µg/ml of house dust mite antigens were applied on culture. It was observed that with the increasing amount of antigen more IL-4 was secreted.

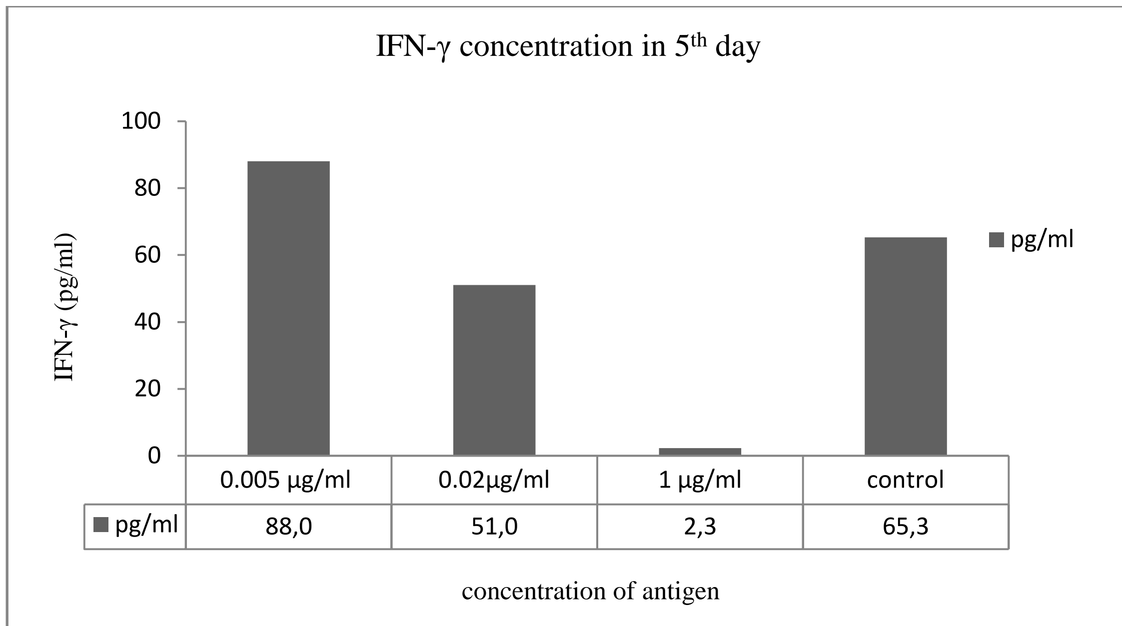


Figure 3.16 Amounts of IFN-γ measured by means of ELISA after 5 days incubation of PBMC culture after antigenic stimulation. IFN-γ had already seemed to be existing in the supernatant of control group, however it was clearly seen that with the increasing concentration of antigen, allergic reaction took place and amount of IFN-γ decreased.

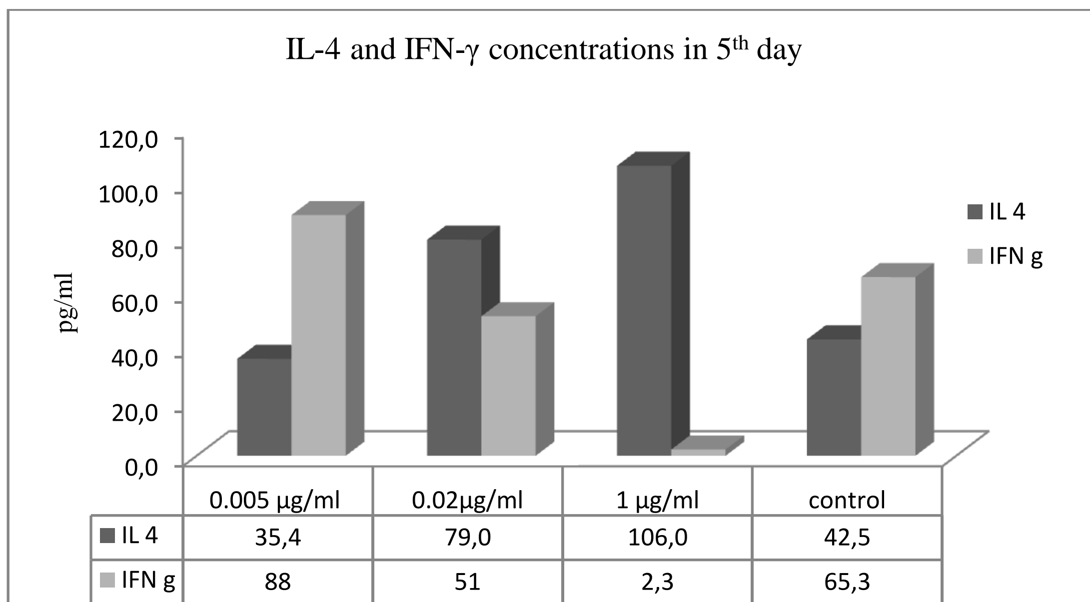


Figure 3.17 Comparison of IL-4 and IFN-γ measured by means of ELISA after 5 days incubation of PBMC culture after antigenic stimulation. The amount of IFN-γ decreasing with the increasing amount of IL-4 showed that T_H2 driven response inhibits the T_H1 driven response.

3.3.2 Antigenic stimulation of PBMC in presence of *N. Sativa* extracts

PBMC was isolated from two allergic individuals known to have high allergicity against house dust mite (*Dermatophagoides pteronyssinus*) and a non-allergic individual to be used as a control group. Cultures were stimulated with 1 µg/ml house dust mite antigen. 0.05% and 0.01% oil and hydrophilic extracts of *N.sativa* were applied on cultures together with the antigen. 2.10^6 cells/2 ml in RPMI 1640 medium (+penicillin-streptomycin) containing 10% FBS, is incubated at 37 C, 5% CO_2 for 5 days. Supernatants of cell cultures were collected on 3rd and 5th day of incubation for the IL-4, IL-5 and IFN-γ detection by means of ELISA. Results are listed in Table 3.11.

pg/ml	IL-4 3.day	IL-4 5.day	IL-5 3.day	IL-5 5.day	IFN-γ 3.day	IFN-γ 5.day
Control (no antigen)	0	0	7	0	0	0
Control (+antigen)	0	0	19	0	49	183
Control (A+0.05% oil)	15	0	0	0	0	0
Control (A+0.01% oil)	9	0	1	0	10	18
Control (A+0.05% hydr.)	6	1	5	1	0	0
Control (A+0.01% hydr.)	1	1	0	0	13	53
Allergic 1 (no antigen)	7	5	2	25	82	0
Allergic 1 (+antigen)	10	7	86	299	52	243
Allergic 1 (A+0.05% oil)	12	8	22	30	0	0
Allergic 1 (A+0.01% oil)	19	0	86	238	56	144
Allergic 1 (A+0.05% hydr.)	5	0	2	24	0	0
Allergic 1 (A+0.01% hydr.)	0	0	65	231	214	474
Allergic 2 (no antigen)	1	4	0	11	0	0
Allergic 2 (+antigen)	5	4	57	313	0	10
Allergic 2 (A+0.05% oil)	7	7	18	69	0	0
Allergic 2 (A+0.01% oil)	7	0	11	46	0	0
Allergic 2 (A+0.05% hydr.)	7	0	20	30	0	0
Allergic 2 (A+0.01% hydr.)	6	0	31	125	0	0

Table 3.11 IL-4, IL-5 and IFN-γ concentrations in allergic and non-allergic individuals' PBMC culture supernatants. PBMC cultures were stimulated with house dust mite antigen in the presence of 0.05% and 0.01% oil and hydrophilic extracts of *N.sativa*.

3.3.2.1 Interleukin 4 results

Peripheral blood mononuclear cells, that were isolated from two allergic and one non-allergic individual, were stimulated with house dust mite antigen together with the two concentrations of oil and hydrophilic extracts of *N.sativa* and incubated for 5 days. At the 3rd and 5th day culture supernatants were collected for the IL-4 measurement.

Figure 3.18, Figure 3.19 and Figure 3.20 shows the concentrations of IL-4 measured in 3rd and 5th day of incubation in non-allergic and allergic individuals. However these results don't give a clear idea about the effect of *N.sativa* extracts over IL-4 production. IL-4 cytokines are known to induce T_H2 cells which in response produce more IL-4 and providing an allergic immune response. So non-allergic individual were not expected to produce any IL-4, on the other hand allergic individuals were expected to have very high concentrations of IL-4 in the positive control, where cells were stimulated only with antigen. However according to the results of IL-4 ELISA there isn't a significant difference between the allergic and non-allergic individuals. Allergic individuals which were expected to show high IL-4 production when they were stimulated with antigen, didn't nearly produce any IL-4 according to these results. Even in some cases non-allergic individual seemed to produce more IL-4 than the allergic individuals. (Figure 3. 21, Figure 3.22)

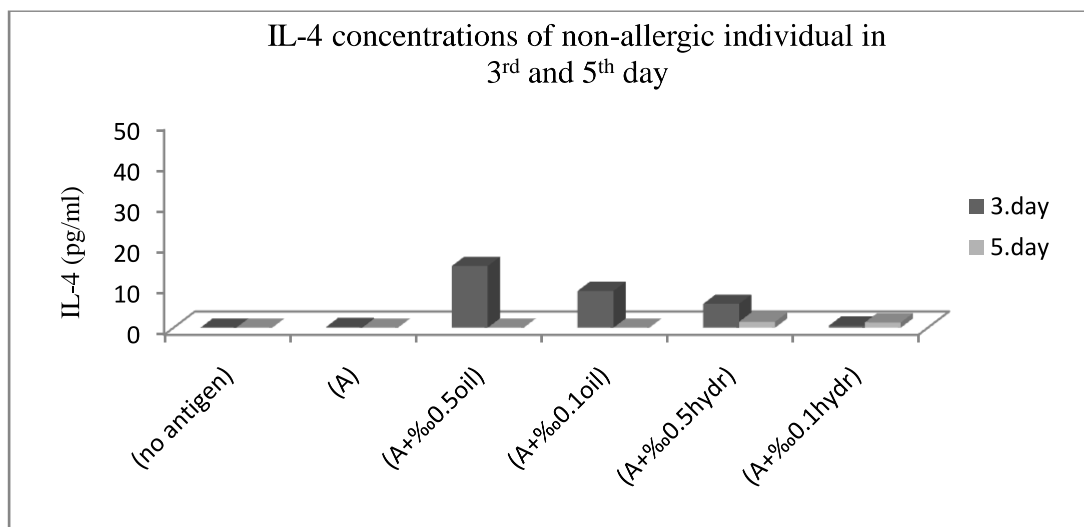


Figure 3.18 Non-allergic individual's IL-4 concentrations measured at 3rd and 5th day of incubation. There wasn't any IL-4 production, when it was stimulated with allergen, as it was expected, however when the extracts were applied together with antigen, it produced IL-4 in low amounts.

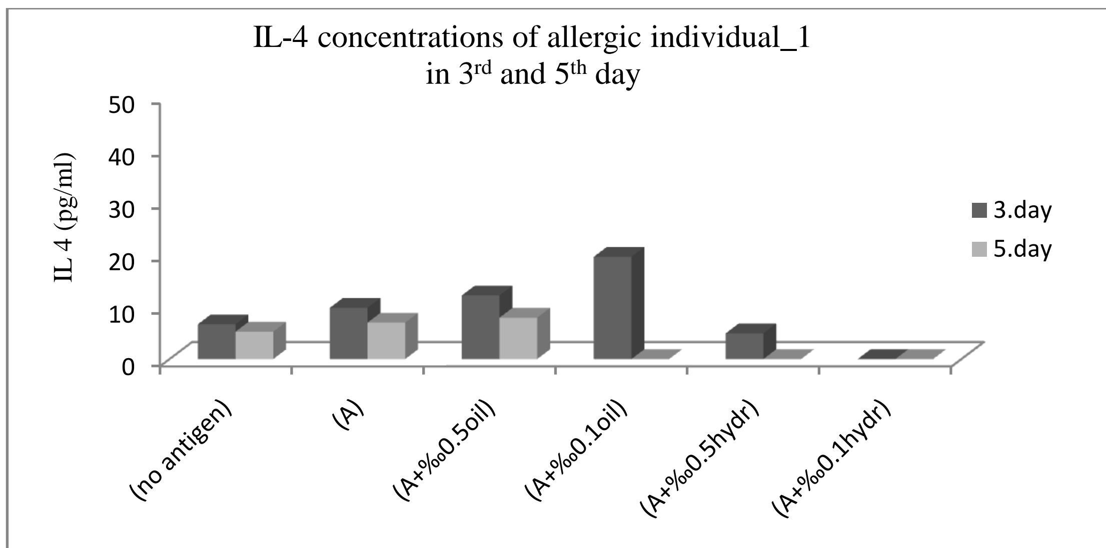


Figure 3.19 First allergic individual's IL-4 concentrations measured at 3rd and 5th day of incubation. There wasn't a significant difference in the IL-4 concentrations, when there was no allergen or when it was stimulated with the allergen, however it was expected to have a high concentration of IL-4 when stimulated with allergen. IL-4 seemed to disappear in the presence of hydrophilic extract.

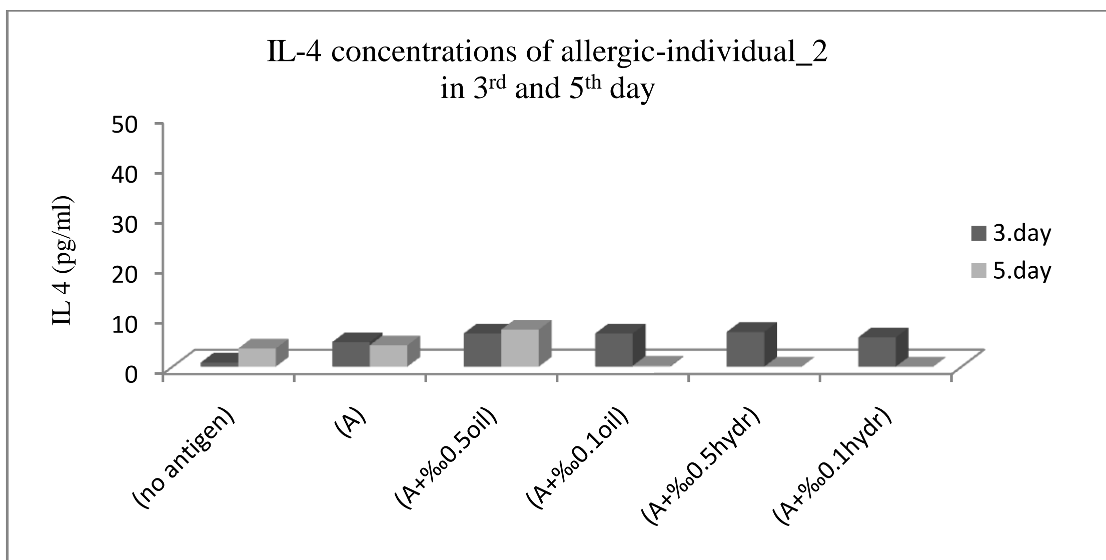


Figure 3.20 Second allergic individual's IL-4 concentrations measured at 3rd and 5th day of incubation. There wasn't a significant difference in the IL-4 concentrations in none of situations, however at least it was expected to have a high IL-4 concentration when stimulated with allergen. IL-4 concentrations measured were very low.

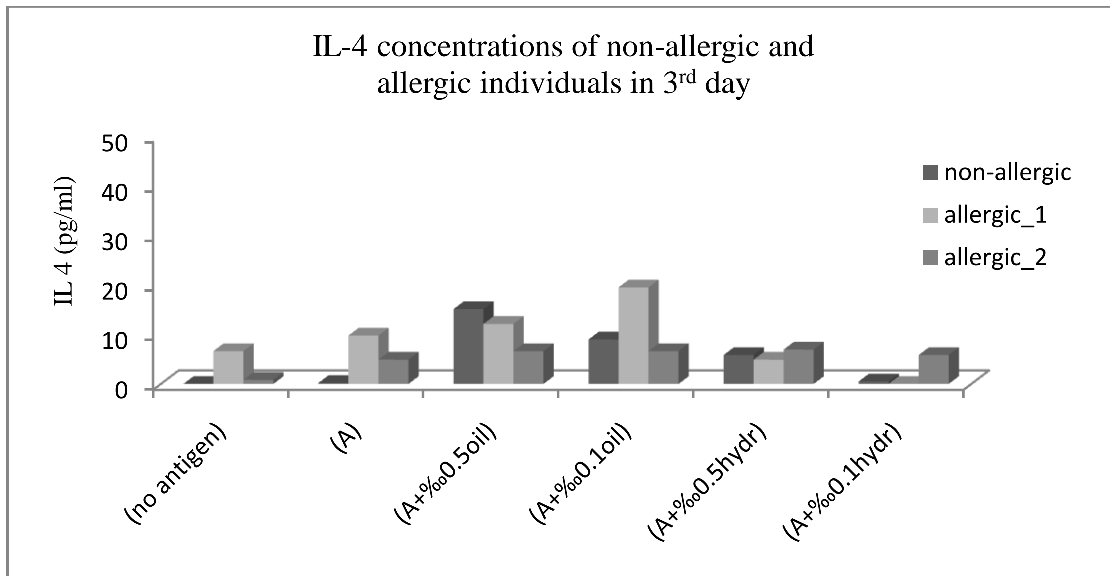


Figure 3.21 Comparison of IL-4 concentrations measured in non-allergic and allergic individuals in the 3rd day of incubation. In most cases there wasn't a significant difference between the allergic and non-allergic individuals, even in some cases IL-4 measured in non-allergic individual was higher than allergic individuals.

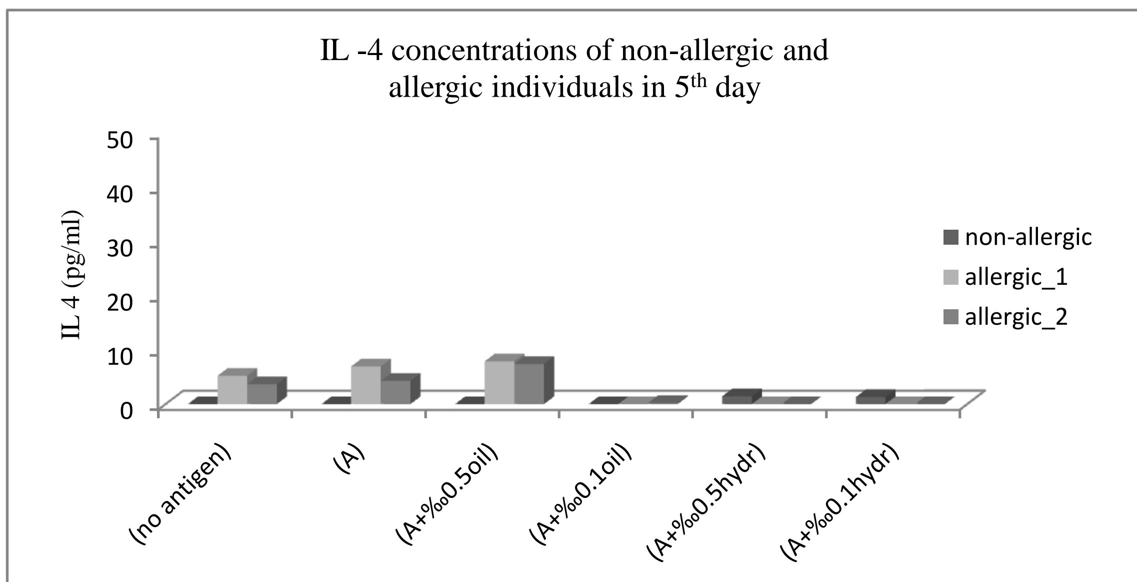


Figure 3.22 Comparison of IL-4 concentrations measured in non-allergic and allergic individuals in the 5th day of incubation. There wasn't any significant difference between the allergic and non-allergic individuals. Amounts of IL-4 measured were too low.

3.3.2.2 Interleukin 5 results

Peripheral blood mononuclear cells, that were isolated from two allergic and one non-allergic individual, were stimulated with house dust mite antigen together with the two concentrations of oil and hydrophilic extracts of *N.sativa* and incubated for 5 days. At the 3rd and 5th day culture supernatants were collected for the IL-5 measurement.

Figure 3.23, Figure 3.24 and Figure 3.25 shows the IL-4 concentrations produced in 3rd and 5th day of incubation in non-allergic and allergic individuals. These results give clues about the effects of *N.sativa* extracts on allergic reactions. IL-5 is a cytokine produced in the T_H2 allergic pathway. High amount of IL-5 is sign of an allergic immune response within cells. Without antigen stimulation non-allergic individual didn't have any IL-5 production. When it was stimulated with antigen, a very low amount of IL-5 was produced and then diminished. When it was stimulated with antigen in the presence of extracts no IL-5 production was observed (Figure 3.23)

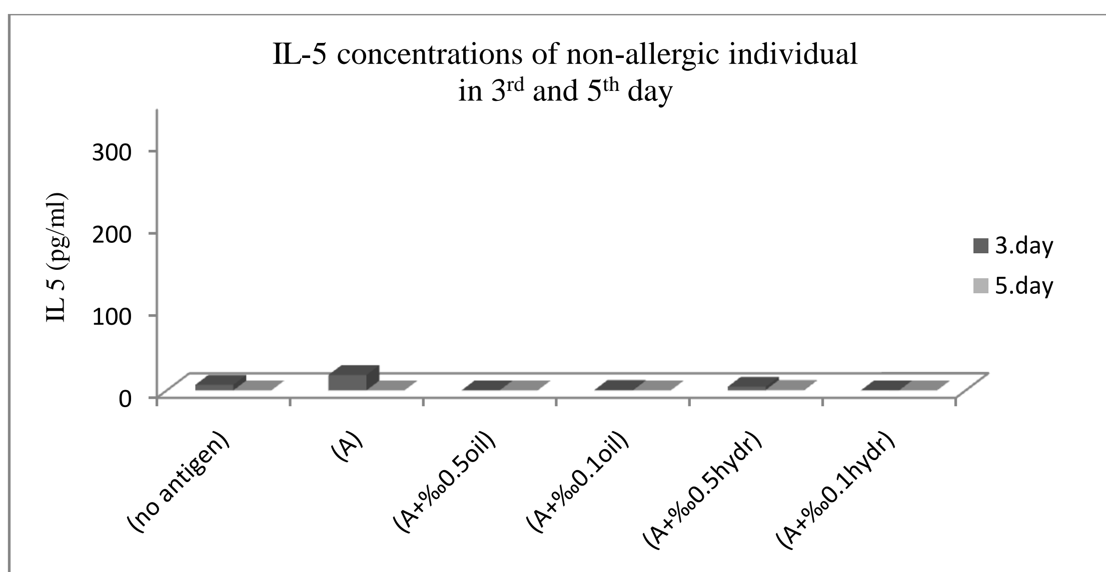


Figure 3.23 Non-allergic individual's IL-5 concentrations measured at 3rd and 5th day of incubation. Without antigen stimulation non-allergic individual didn't produce any IL-5 production. When it was stimulated with antigen a very low amount of IL-5 was produced and then diminished. When it was stimulated with antigen in the presence of extracts no IL-5 production was observed

IL-5 measurements in 5th day were higher than the 3rd day measurements. There was a very low amount of IL-5 (25 pg/ml) in the allergic individual without antigen stimulation, however with the antigenic stimulation IL-5 production increased about 12 fold (299 pg/ml) in the 5th day. When the extracts were applied together with antigen on PBMC culture, there was a visible decrease in the IL-5 production. Both the 0.1 ‰ oil and 0.1 ‰ hydrophilic extracts had a similar decreasing effect on IL-5 production but 0.5 ‰ oil and 0.5 ‰ hydrophilic extracts were seem to have a very tragic effect on the IL-5 concentration; the IL-5 concentrations receded to the values of negative control where there was no antigen stimulation. (Figure 3.24)

The second allergic individual also showed a similar pattern on the IL-5 production. The uprising IL-5 production with the antigenic stimulation, decreased tragically in the presence of both oil and hydrophilic extracts (Figure 3.25). But in this individual not only 0.5 ‰ but also 0.1 ‰ oil and hydrophilic extracts had very strong decresing effects on the IL-5 production when compared to first allergic individual (Figure 3.26, Figure 3.27).

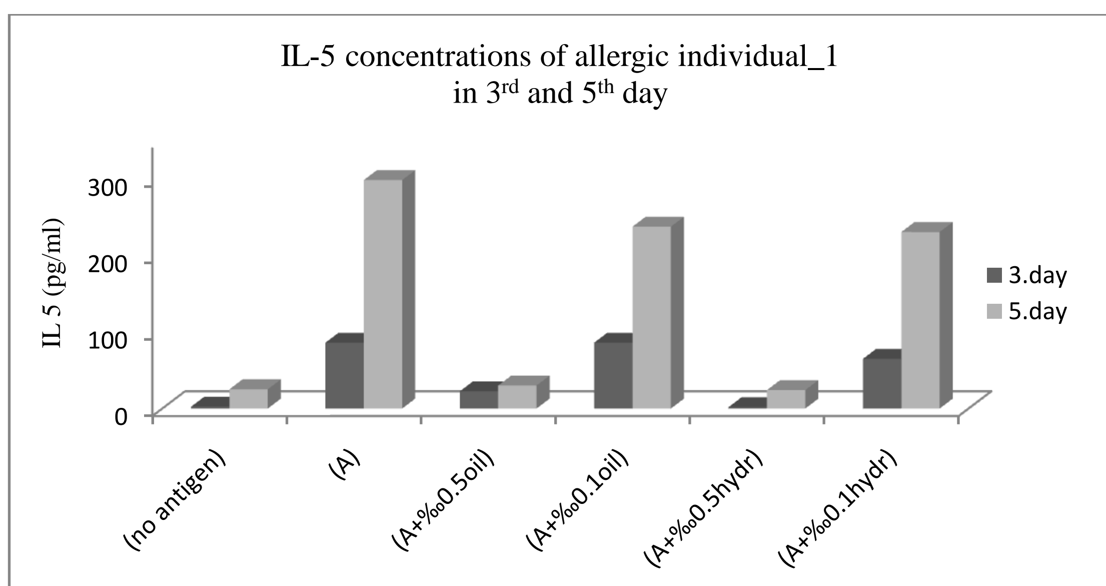


Figure 3.24 First allergic individual's IL-5 concentrations measured at 3rd and 5th day of incubation. The IL-5 concentration uprising with the antigen stimulation decreased in the presence of *N.sativa* extracts. Especially 0.5 ‰ oil and 0.5 ‰ hydrophilic extracts were seemed to have a very tragic effect on the IL-5 concentration.

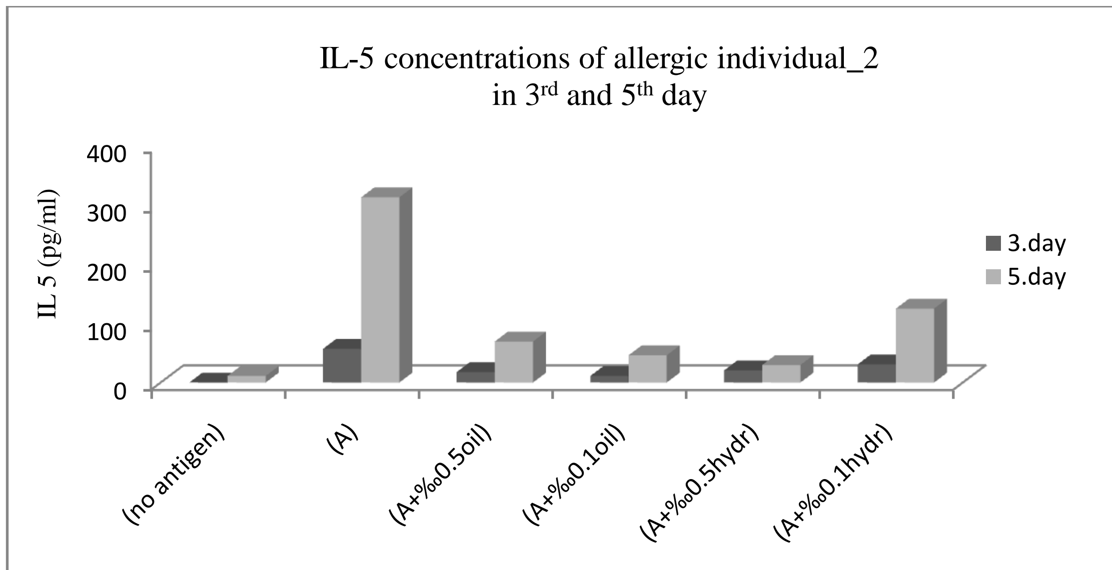


Figure 3.25 Second allergic individual's IL-5 concentrations measured at 3rd and 5th day of incubation. The IL-5 concentration uprising with the antigen stimulation decreased tragically in the presence of *N.sativa* extracts.

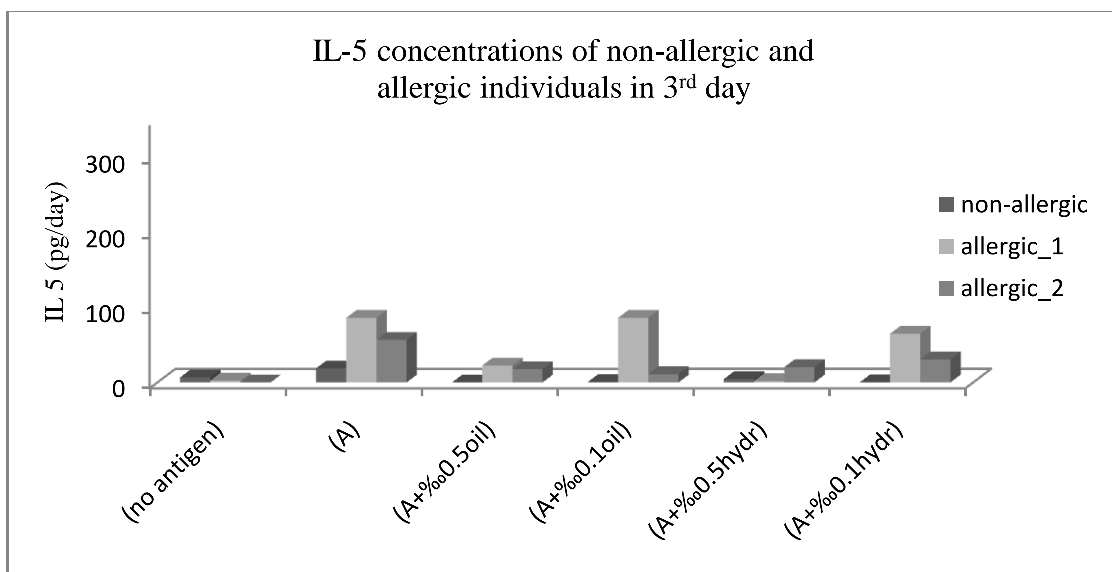


Figure 3.26 Comparison of IL-5 concentrations measured in non-allergic and allergic individuals in the 3rd day of incubation. At the 3rd day 0.5 ‰ oil and 0.5 ‰ hydrophilic extracts were seemed to have a very tragic effect on the IL-5 concentration in both of the allergic individuals. However for the first allergic individual 0.1 ‰ oil and 0.1 ‰ hydrophilic extracts didn't seem to be as effective as it is for the second one, but still had a decreasing effect in IL-5 concentration.

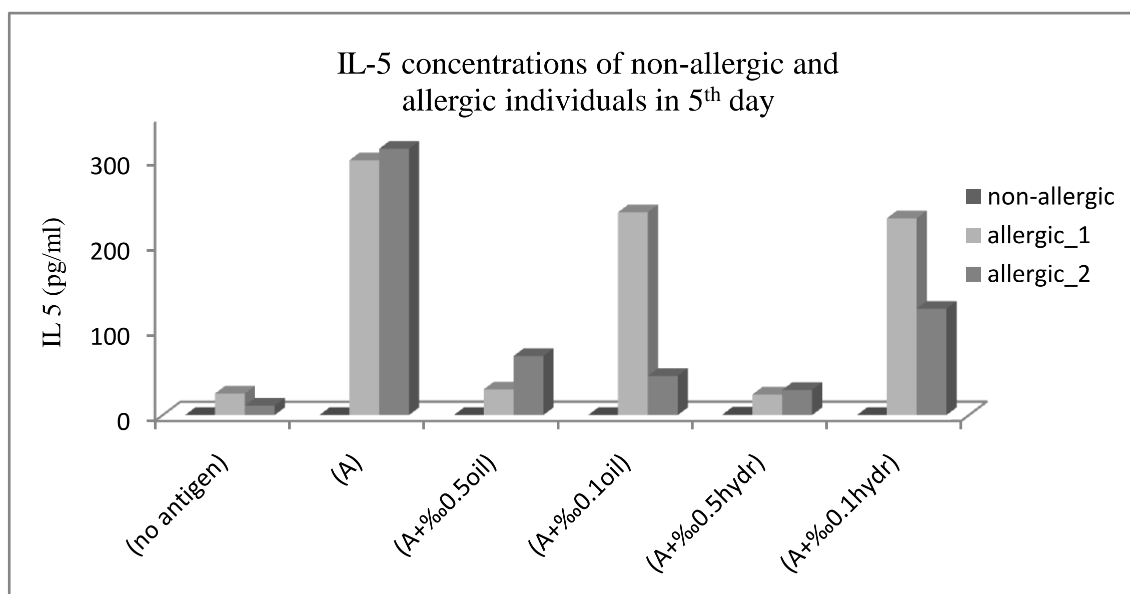


Figure 3.27 Comparison of IL-5 concentrations measured in non-allergic and allergic individuals in the 5th day of incubation. 0.5 ‰ oil and 0.5 ‰ hydrophilic extracts had a very tragic effect on the IL-5 concentration in both allergic individuals. However for the first allergic individual 0.1 ‰ oil and 0.1 ‰ hydrophilic extracts didn't seem to be as effective as it is for the second one, but still had a decreasing effect in IL-5 concentration.

3.3.2.3 Interferon Gamma results

Peripheral blood mononuclear cells, that were isolated from two allergic and one non-allergic individual, were stimulated with house dust mite antigen together with the two concentrations of oil and hydrophilic extracts of *N.sativa* and incubated for 5 days. At the 3rd and 5th day culture supernatants were collected for the IFN-γ measurement. Figure 3.28, Figure 3.29 and Figure 3.30 shows the IFN-γ concentrations produced in 3rd and 5th day of incubation in non-allergic and allergic individuals. In the non-allergic individual in the absence of antigen, IFN-γ was not produced. When the culture was stimulated with antigen high amount of IFN-γ was produced which is a sign of T_H1 type immune response. However when the cells were stimulated with antigen in the presence of *N.sativa* extracts, a dramatic decrease occurred in the IFN-γ concentration (Figure 3.28) In the first allergic individual even without the antigen stimulation, there was a high amount of IFN-γ produced in the 3rd day (82pg/ml) but at the day of 5 IFN-γ disappeared. When it was stimulated with antigen, IFN-γ concentration up rised to 243 pg/ml at the 5th day. It is hard to generalize the effects of *N.sativa* extracts on IFN-γ

production. In the presence of 0.5‰ oil and hydrophilic extract IFN-γ vanished. 0.1 ‰ oil extract had a decreasing effect in the IFN-γ concentration; however 0.1 ‰ hydrophilic extract had a controversial effect on the IFN-γ production. It increased the IFN-γ concentration two fold higher than the IFN-γ amount observed in only antigen stimulation (Figure 3.29). In the second allergic individual IFN-γ couldn't be detected in any case (Figure 3.30). Comparing the allergic_1 and non-allergic individuals' IFN-γ amounts measured both showed a similar behavior instead of the 0.1‰ hydrophilic extract increasing the IFN-γ concentration (Figure 3.31).

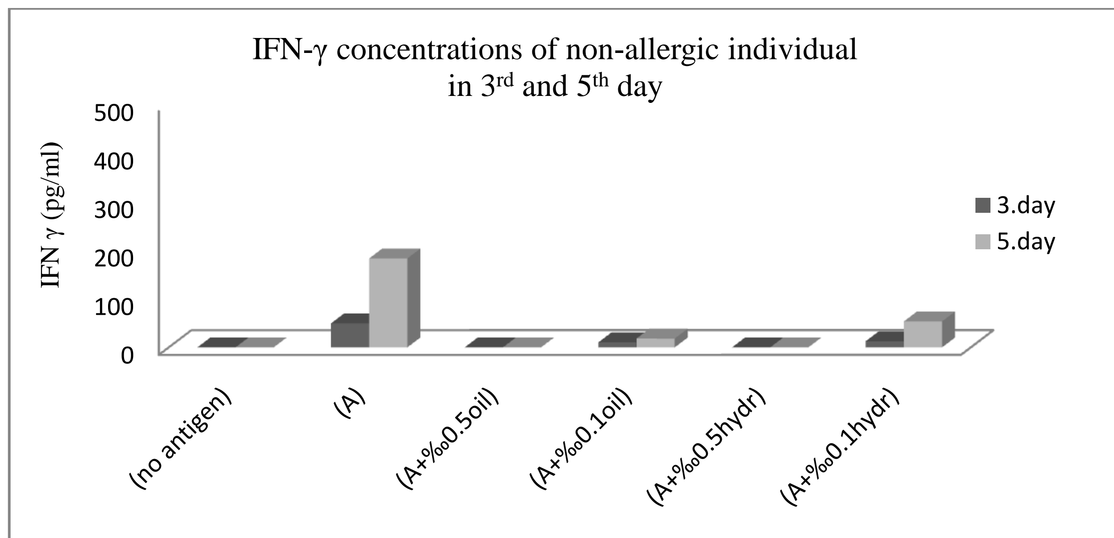


Figure 3.28 Non-allergic individual's IFN-γ concentrations measured at 3rd and 5th day of incubation. When the culture was stimulated with antigen, high amount of IFN-γ was produced which is a sign of T_H1 type immune response. However when the cells were stimulated with antigen in the presence of *N.sativa* extracts, a dramatic decrease occurred in the IFN-γ concentration.

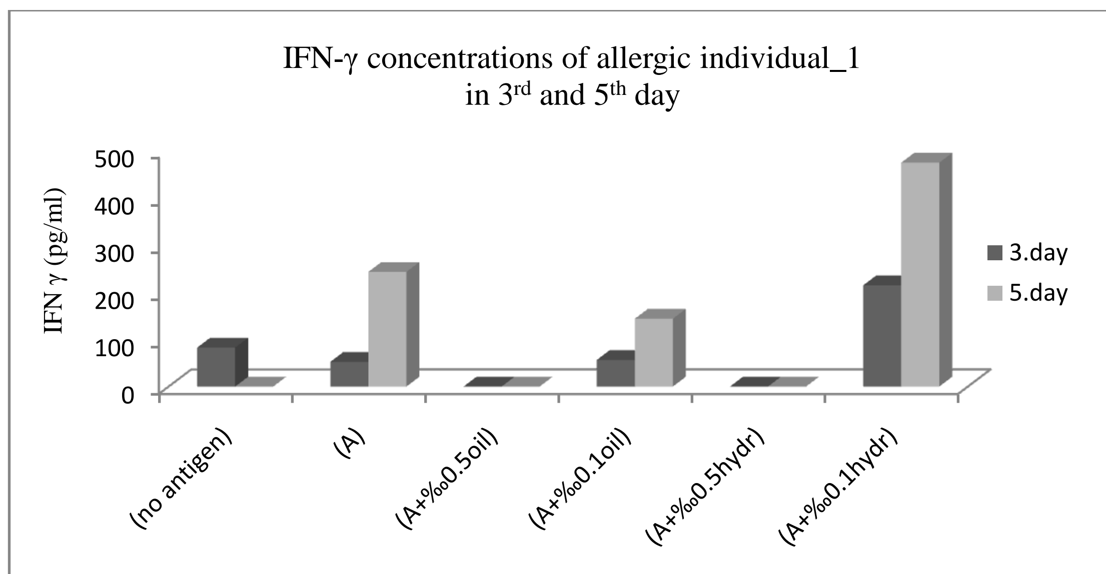


Figure 3.29 First allergic individual's IFN-γ concentrations measured at 3rd and 5th day of incubation. In the first allergic individual even without the antigen stimulation, there was a high amount of IFN-γ produced in the 3rd day (82pg/ml) but at the day of 5 IFN-γ disappeared. In the presence of 0.5‰ oil and hydrophilic extract IFN-γ vanished. 0.1 ‰ oil extract had a decreasing effect in the IFN-γ concentration; however 0.1 ‰ hydrophilic extract had a controversial effect on the IFN-γ production. IFN-γ increased in the presence of 0.1 ‰ hydrophilic extract.

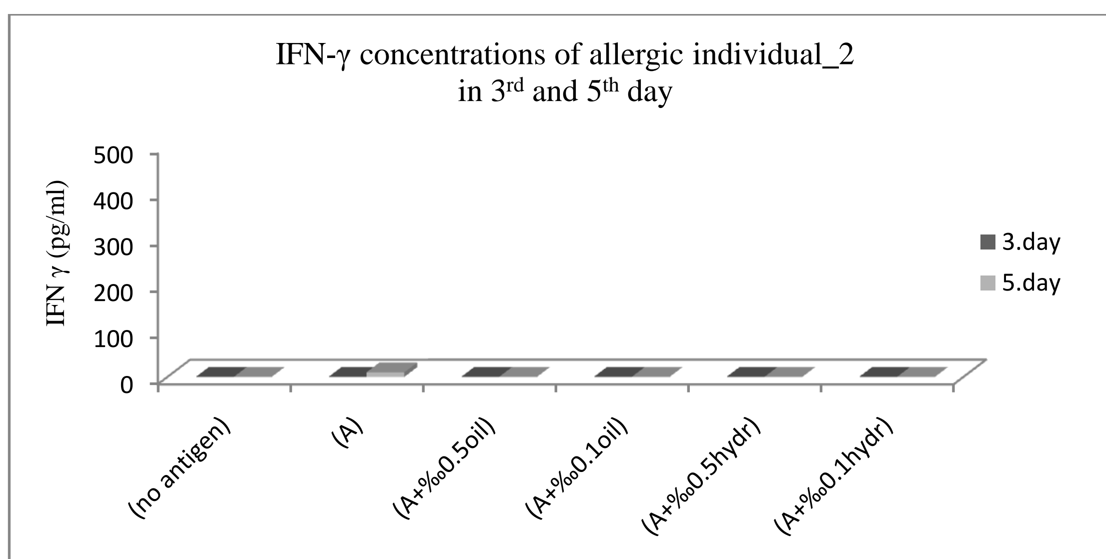


Figure 3.30 Second allergic individual's IFN-γ concentrations measured at 3rd and 5th day of incubation. In the second allergic individual IFN-γ couldn't be detected in any case

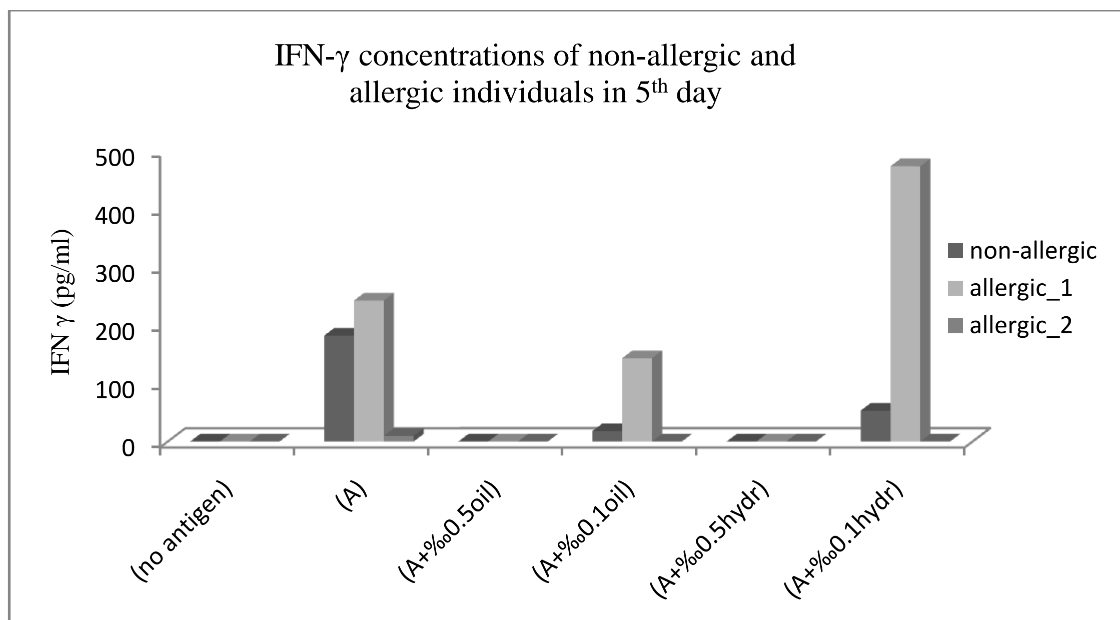


Figure 3.31 Comparison of IFN-γ concentrations measured in non-allergic and allergic individuals in the 5th day of incubation. Both the allergic_1 and non-allergic individuals' IFN-γ amounts showed a similar behavior instead of the 0.1‰ hydrophilic extract increasing the IFN-γ concentration

3.3.2.4 Comparison of IL-4, IL-5 and IFN-γ concentrations in allergic and non-allergic individuals

Non-allergic individual reacted by producing both IL-5 and IFN-γ against antigen stimulation. At the 3rd day IL-5 is less than half of IFN-γ, at the 5th day IL5 vanished totally while IFN-γ amount up rised to 4 fold. Addition of oil and hydrophilic extracts seemed to repress the immune response that IFN-γ in the medium also vanished (Figure 3.32, Figure 3.33).

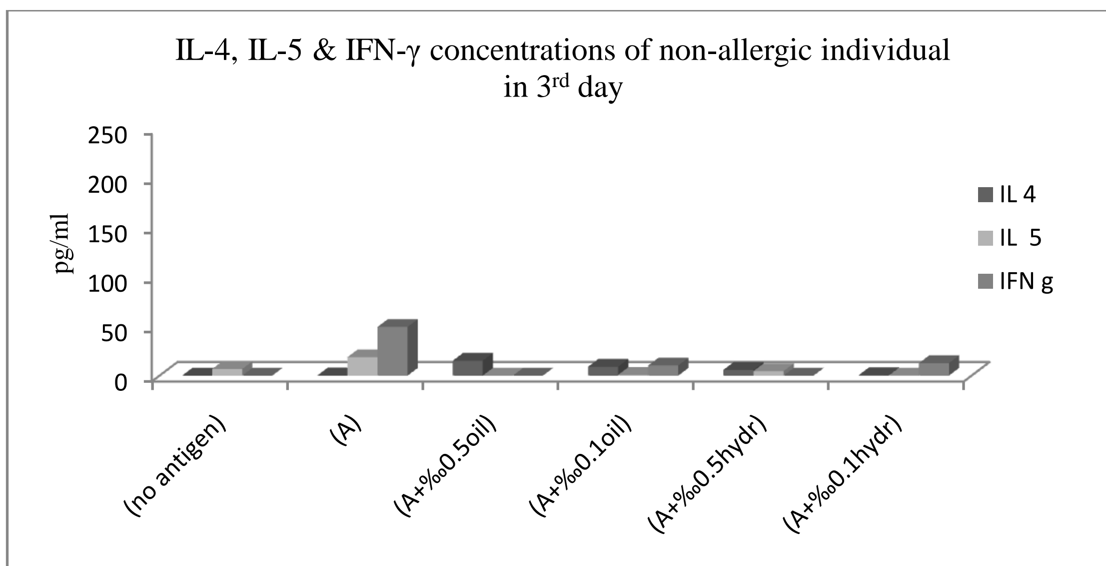


Figure 3.32 Non-allergic individual's IL-4, IL-5 and IFN-γ concentrations measured at 3rd day of incubation.

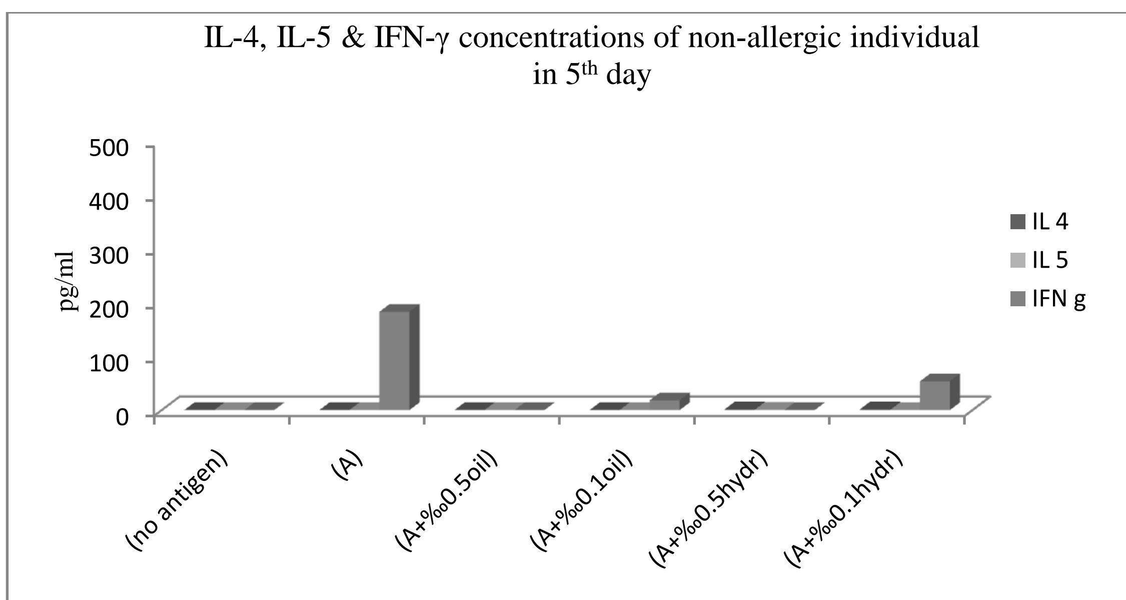


Figure 3.33 Non-allergic individual's IL-4, IL-5 and IFN-γ concentrations measured at 5th day of incubation.

In the first allergic individual, IFN-γ was already present in the medium without antigen stimulation, after stimulation with antigen IL-5 is produced and amount of IFN-γ lowered some, at the 5th day IL5 increased to 3 fold however IFN-γ also followed the IL-5 and increased about 3 fold but still lower than IL-5. When the 0.5 ‰ oil extract

and 0.5 ‰ hydrophilic extract were applied together with antigen both the IL-5 and IFN-γ decreased drammatically in the 3rd and 5th day. However 0.1 ‰ oil and 0.1 ‰ hydrophilic extracts caused a slight decrease in the amount of IL-5. IFN-γ decreased slightly in 0.1 ‰ oil extract, while it increased to 4 fold in 0.1 ‰ hydrophilic extract. (Figure 3.34, Figure 3.35)

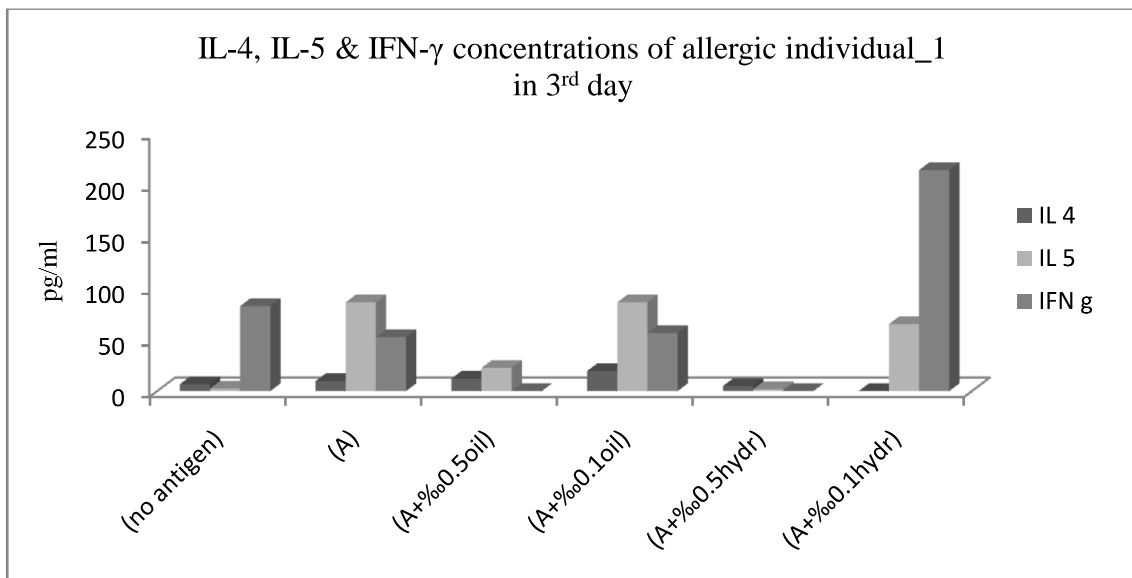


Figure 3.34 First allergic individual's IL-4, IL-5 and IFN-γ concentrations measured at 3rd day of incubation.

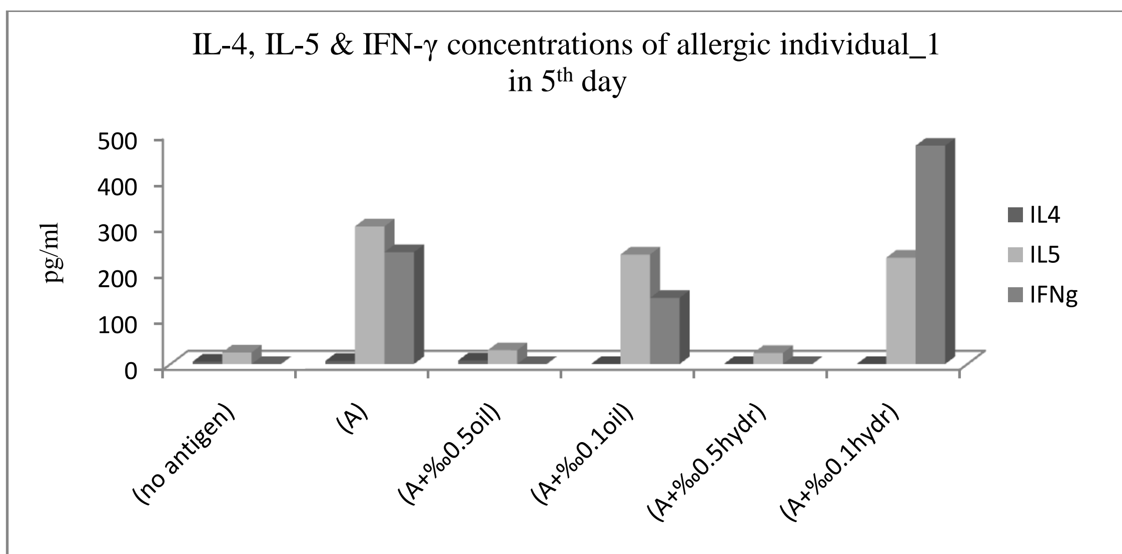


Figure 3.35 First allergic individual's IL-4, IL-5 and IFN-γ concentrations measured at 5th day of incubation

In the second allergic individual only IL-5 could be measured. High amounts of IL-5 produced with the antigenic stimulation decreased sharply in the presence of *N.sativa* extracts. (Figure 3.36, Figure 3.37)

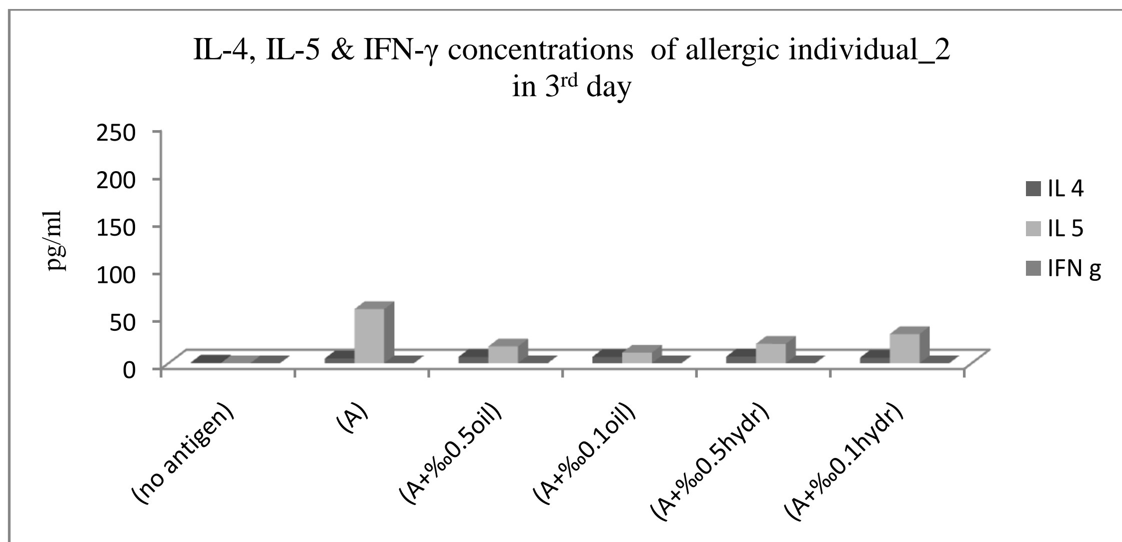


Figure 3.36 Second allergic individual's IL-4, IL-5 and IFN-γ concentrations measured at 3rd day of incubation

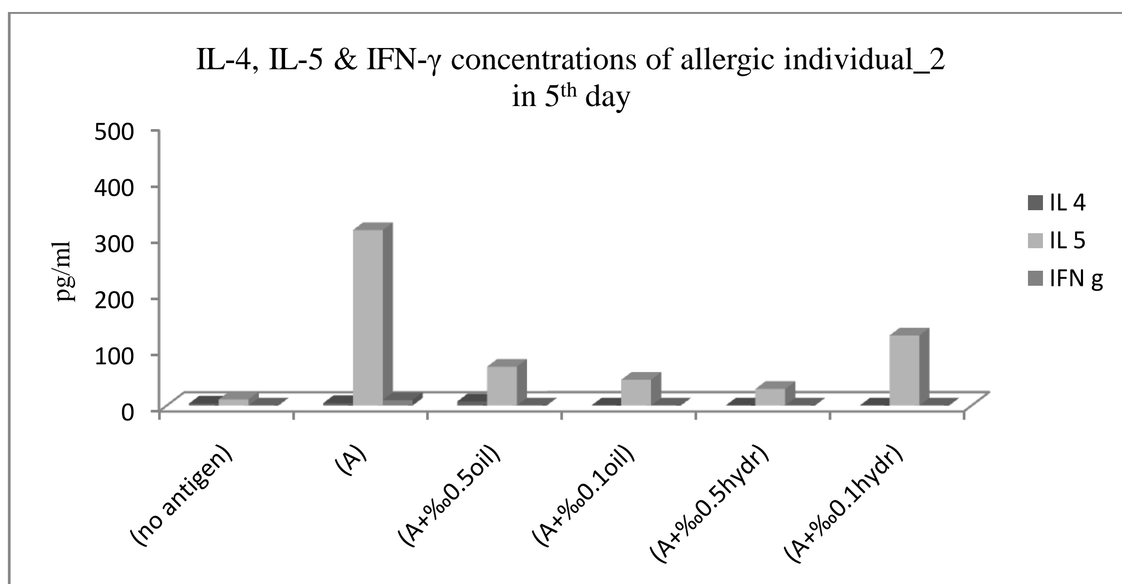


Figure 3.37 Second allergic individual's IL-4, IL-5 and IFN-γ concentrations measured at 5th day of incubation.

CHAPTER 4

DISCUSSION AND CONCLUSION

We already know how the immune system reacts when it comes across with an antigen, what kind of responses it can give. We also know what happens when the immune system gives an allergic reaction. Allergic reactions occur as a result of the production of specific IgE antibody to common, innocuous antigens, triggering a T_H2 type response. Naive allergen-specific T cells differentiate into T_H2 cells in the presence of IL-4, which seems to be derived from a specialized subset of T cells. T_H2 cells produce IL-4 and IL-13, which drive B cells to produce IgE. The specific IgE produced in response to the allergen, binds to the high affinity receptor for IgE on mast cells, basophils, and activated eosinophils. Once IgE is produced in response to an allergen, reexposure to the allergen triggers an allergic response.

However we still don't know why some individuals are allergic and others are not, why some individuals give an allergic immune response (T_H2 type response) to some harmless substances (allergens) while others give a T_H1 type response or sometimes doesn't give an immunological response even. What makes the antigen presenting cells trigger the T_H2 type response to innococuous substances but not a T_H1 type in some individuals, how this decision is made? (Figure 4.1) Why the incidence of allergy has increased this much in about 30 years. The answers of these questions are still a mystery for us.

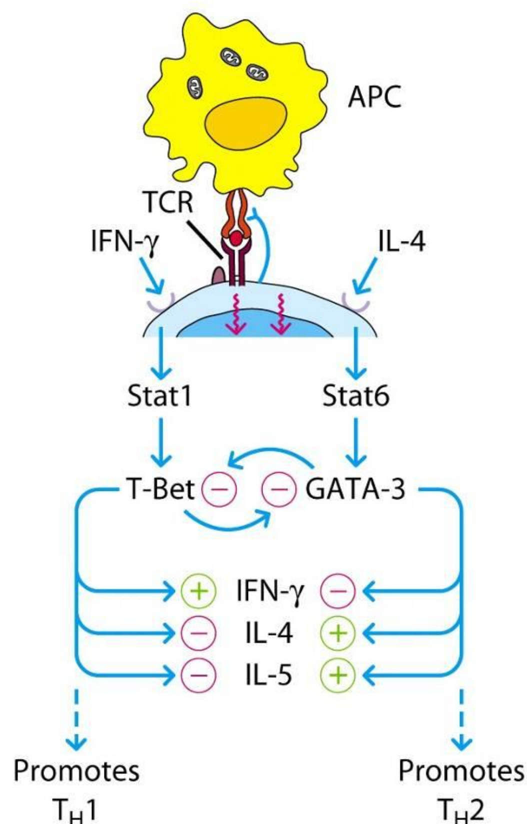


Figure 4.1 Interaction of antigen presenting cell with the T helper cells. APC provides the immune response to be in T_H1 direction or T_H2, however the exact mechanism providing this selection is not known still.

Plants are invaluable sources of new drugs. Scientific studies on plants leads to the discovery of many valuable drugs.

Nigella sativa (Ranunculaceae family) seeds, commonly known as black seed or black cumin have been used for thousands of years as a spice, as a protective and curative remedy in Eastern countries. The effects of *N. sativa* seed extracts have been studied on various body systems, In particular the black seed oil extract has been examined and shown to have bronchodilatory, hypotensive, antibacterial, antifungal, analgesic, anti-inflammatory, and immunopotentiating activities.

N. sativa seeds contain 36-38% fixed oils, proteins, alkaloids, saponin and 0.4-2.5 % essential oil. The fixed oil is composed mainly of unsaturated fatty acids. The major part of essential oil is thymoquinone which has been shown to

have various effects in vitro and in vivo by numerous studies. *N.sativa* oil was demonstrated to modify leukotriene synthesis and inhibit histamin release. (Ali and Blunden, 2003)

Some immunopotentiating properties of *N. sativa* were also shown however the exact mechanism of its action on allergic diseases is still unclear. This *in vitro* study is undertaken to evaluate the effects of hyrophilic and oil extracts of *N. sativa* on allergy.

As a first step, the PBMC culture was tried to be optimized. For the isolation of PBMC, blood was taken from the individuals. Density gradient centrifugation (Ficoll-paque) which allows monocytes, lymphocytes and basophils to remain at the blood-media interface, while other blood cells pass through to form a pellet, was used as isolation method. It was observed that a variable number of platelets are retained at the interface, together with the mononuclear cells. Both EDTA and heparin was tried as anticoagulant during the mononuclear cell isolation. Heparin was observed to have a more effective result in avoiding the platelets.

T-cell activation requires the interaction of T-cell with antigen presenting cell, so cells should be cultured in close proximity to one another, allowing interaction. On the other hand too many cells may lead to overcrowding which will cause the restriction of gas and nutrient exchange. To find the optimized number of cells to be incubated for an optimized number of day several trials were done. Finally 2.10^6 cell in 2 ml RPMI (10% FBS) medium, which is approximately the same concentration of mononuclear cells found in the human blood, was decided to be used for the antigenic stimulation.

In this study, two extracts of *N. sativa* was used; hydrophilic portion which was extracted by 60 % methionine and oil extract which was extracted by pure hexane. Hydrophilic extract was dissolved and diluted with double distilled water. Oil extract was dissolved with dimethyl sulfoxide (DMSO) which is an important polaraprotic solvent that dissolves both polar and apolar compounds. DMSO is miscible, as well as water, in a wide range of organic solvents. However it couldn’t dissolve the oil extract 100 %. Oil particules are observed on the upper layer of cell culture when the oil extract was applied on cell culture (Figure 3.6). But the high cytotoxic effect of oil extract on mononuclear cells gave the idea that there was still a dissolved portion in the oil extract causing the high cytotoxicity, because DMSO, in which oil extract is dissolved, didn’t show such a cytotoxic effect on mononuclear cells.

Several cytotoxicity assays were applied on PBMC culture with extracts ranging concentrations between 5 % and 0.5 ‰. Both the hydrophilic and the oil extract were found to be quite cytotoxic for the cells. More than the cytotoxicity assays relying on the lactate dehydrogenase (LDH) enzyme secreted into environment in the presence of cell damage, the cell counts and the cell morphologies under light microscope gave a clearer idea about the toxic properties of extracts. Extracts applied over 0.25 % were highly cytotoxic that cells couldn't survive. 0.10 %, 0.05 % and 0.01 % were applied on cell culture. They were still cytotoxic, but there seemed enough number of cells for the antigenic stimulation. Our aim was to use as much extract as possible on PBMC culture but also to have enough intensity of cells to provide cell to cell contact.

The first antigenic stimulation of PBMC was done to define the effect of antigen concentration on cytokine production in the absence of *N. sativa* extracts. Three concentrations of antigen were applied on PBMC culture. IL-4 and IFN-γ concentrations were measured at 3rd and 5th day of culture. These results provided the first information about the IL-4 and IFN-γ behaviors. First of all, it could be commented according to the control group that both IL-4 and IFN-γ were seemed to be readily exist in the medium in similar amounts to each other –IFN-γ was a little bit more- even in the absence of an antigen. Probably according to the antigen presented to T_0 cells, the immune response direction shifts to T_H1 or T_H2, with the help of cytokines IFN-γ or IL-4. Secondly in the higher concentration of antigenic stimulation (1µg/ml), the IL-4 produced in response increased and also the IL-4 measured in 3rd day was higher than 5th day. When the antigen is given to cells, antigen presenting cells are matching it one by one with the naive T lymphocytes, as soon it finds the exact T lymphocytes, it gives an allergic (T_H2 type) response, increasing the IL-4 production. When the antigen amount increase, more antigen presenting cells search for the matching T lymphocyte and that shortens the lag time till allergic response is given. Another result was that, in an allergic response with the increasing amount of IL-4 produced, significantly IFN-γ was decreasing, which shows that T_H2 driven response inhibits the T_H1 driven response because IFN-γ drives the shift to T_H1 direction whereas IL-4 shifts the response to T_H2 direction and they suppress each other (Figure 3.17)

Taking these results into consideration, finally the PBMC culture was stimulated with antigen in the presence of *N.sativa* extracts. Two allergic individuals and a non-allergic individual who were tested for house dust mite antigen were used as the PBMC

donors. Cells were cultured in concentration of 2.10^6 cell/2ml. At the second day 1µg/ml of antigen was added on cultures together with the four different extracts (0.5 ‰ and 0.1 ‰ oil extract and 0.5 ‰ and 0.1 ‰ hydrophilic extracts). At the 3rd and 5th day supernatants were collected for IL-4, IL-5 and IFN-γ detection by means of ELISA. Within the ELISA results, IL-4 didn't give a clear idea about the effect of *N. sativa* extracts on IL-4 production. IL-4 cytokines are known to induce T_H2 cells which in response produce more IL-4 and providing an allergic reaction within cells. So non-allergic individual was not expected to produce any IL-4, on the other hand allergic individuals were expected to have very high concentrations of IL-4 in the positive control, where cells were stimulated only with antigen. However according to the results allergic individuals nearly didn't produce any IL-4 at all when they were stimulated with antigen. There wasn't a significant difference between the allergic and non-allergic individuals, also the IL-4 measured in all of the samples were very low which gives the idea that this IL-4 measurement wasn't a successful one to comment on the effects of *N. sativa* extracts on IL-4 production. The reason for this result might be the instability of IL-4, because before the ELISA, supernatants were kept on -20 C for more than 2 weeks, which might have caused the loss of IL-4. According to the literature for the maximum release of IL-4 and other cytokines, Phytohaemagglutinin (PHA) is used. PHA is a lectin used as a mitogen to trigger cell division in T lymphocytes and affect the cell membrane permeability to proteins. PHA was not used in this study, which might have affected the concentration of IL-4. However, taking into consideration the results of the first antigenic stimulation in which IL-4 was measured well, not using PHA didn't seem to be the only reason for very little amount of IL-4 at least.

IL-5 ELISA results were the most informative and enchanting results about the effects of *N. sativa* extracts on allergic reactions. IL-5 is a cytokine produced in the T_H2 allergic pathway. High amounts of IL-5 found in the supernatant are the sign of an allergic immune response within cells. IL-5 measurements in 5th day were higher than the 3rd day measurements in general. Without antigen stimulation non-allergic individual didn't have any IL-5 production. When it was stimulated with antigen a very low amount of IL-5 was produced and then diminished. When it was stimulated with antigen in the presence of extracts no IL-5 production was observed. In the first allergic individual there was a very low amount of IL-5 (25 pg/ml) without antigen stimulation,

however with the antigenic stimulation IL-5 production increased about 12 fold (299 pg/ml). When the extracts were applied together with antigen on PBMC culture there was a sharp decrease in the IL-5 concentrations. In the first allergic individual 0.5 ‰ oil and 0.5 ‰ hydrophilic extracts were seem to have a more consuming effect on the IL-5 concentration than 0.1 ‰ oil and 0.1 ‰ hydrophilic extracts; the IL-5 concentrations lowered to the values of negative control where there is no antigen stimulation. (Figure 3.24) This result gave the logical idea that more concentrated extracts have more immunosuppressive effect. The second allergic individual also showed a similar pattern on the IL-5 production. The uprising IL-5 production with the antigenic stimulation, decreased sharply in the presence of both oil and hydrophilic extracts (Figure 3.25). But in this individual 0.1 ‰ oil and hydrophilic extracts were also as effective as the 0.5 ‰ oil and hydrophilic extracts. (Figure 3.26, Figure 3.27).

IFN-γ results were informative for the first allergic individual and non-allergic individual but not for the second allergic individual, in which IFN-γ couldn't be measured in any cases. In the non-allergic individual in the absence of antigen, IFN-γ was not produced. When the culture was stimulated with antigen high amount of IFN-γ was produced which is a sign of T_H1 type immune response. However when the cells were stimulated with antigen in the presence of *N. sativa* extracts, a sharp decrease occurred in the IFN-γ concentration (Figure 3.28) which might give an idea about the immunosuppressive effect of *N. sativa* on both T_H1 and T_H2 type responses. In the first allergic individual even without the antigen stimulation, there was a high amount of IFN-γ produced in the 3rd day (82pg/ml) but at the day 5th day IFN-γ disappeared. When it was stimulated with antigen IFN-γ concentration up rised to 243 pg/ml. It is hard to generalize the effects of *N.sativa* extracts on IFN-γ production. In the presence of 0.5 ‰ oil and hydrophilic extract, IFN-γ vanishes. 0.1 ‰ oil extract had a decreasing effect in the IFN-γ concentration; however 0.1 ‰ hydrophilic extract had a controversial effect on the IFN-γ production. It increased the IFN-γ concentration two fold higher than the IFN-γ amount observed in only antigen stimulation (Figure 3.29). Comparing the allergic and non-allergic individuals' IFN-γ amounts measured, both showed a similar behavior instead of the 0.1‰ hydrophilic extract increasing the IFN-γ concentration (Figure 3.31).

As a conclusion these results are still not enough to define the exact effect of *N. sativa* on allergic reactions, but we can clearly say that *N. sativa* at least has an effective

immunosuppressive effect. It is preventing the IL-5 production which is one of the main cytokines taking role in the allergic reaction pathway. In high amounts (0.5 ‰) of extracts it also prevents the IFN-γ release however in low amount of 0.1‰ hydrophilic extract while IL-5 decreases IFN-γ seem to increase, which may be the sign for the shift of the immune response from T_H2 type to T_H1 type. But yet it is early to claim that it shifts the T_H2 type allergic response to T_H1 type immune response. These studies should be repeated in higher number of allergic and non-allergic individuals and also in the presence of different kinds of antigens to be able to generalize the effect of *N.sativa.* Then the further steps will be trying to find the exact substance in *Nigella sativa* which provides this effect and how, in which stage of the T_H2 pathway does it effect. If we can find the answers of these questions, that will give us so important clues about the mechanism of T_H1- T_H2 balance, and how we can shift this balance into the T_H1 direction. Knowing this mechanism, taking precautions to eliminate the shift to T_H2 direction in allergic subjects, can provide a decisive cure for allergy. So that *Nigella sativa* or the effective substance in *N. sativa* can be a natural cure for allergy.

REFERENCES

Adkinson, N.F., P.A. Eggleston, D. Eney, E.O. Goldstein, K.C. Schuberth, J.R. Bacon, R.G. Hamilton, M.E. Weiss, H. Arshad, C.L. Meinert, J. Tonascia, and B. Wheeler. A controlled trial of immunotherapy for asthma in allergic children *N. Engl. J. Med.* 336: 324-331.1997

Alberts, B., D. Bray, J. Lewis, M. Raff, K. Roberts, and J.D. Watson, *Molecular Biology of the Cell* , 4rd edn. New York, Garland Publishing, 2001

Ali B.H., G. Blunden, Pharmacological and toxicological properties of *Nigella sativa*. *Phytother Res* 2003. 17: 299-305. 2003

Barnes, P.J. Therapeutic strategies for allergic diseases *Nature.* 402: B31-B38. 1999

Büyüköztürk, S., A. Gelincik, F. Özşeker, S. Genç, F.O. Şavran, B. Kıran, G. Yıllar, S. Erden, F. Aydın, B. Çolakoğu, M. Dal, H. Özer and A. Bilir. *Nigella sativa* (black seed) oil doesn't affect the T-helper 1 and T-helper 2 type cytokin eproduction form splenic mononuclear cells in allergen sensitized mice. *Journal of Ethno-pharmacology.* 100:295-298. 2005

Cerdan, C., Y. Martin, M. Courcoul, C. Mawas, F. Birg, and D. Olive. CD28 costimulation regulates long-term expression of the three genes (alpha, beta, gamma) encoding the high-affinity IL2 receptor *Res. Immunol.* 146:164-16. 1995

Chakravarty N. Inhibition of histamine release from mast cells by nigellone. *Ann Allergy.* **70**: 237–242. 1993

Cookson, W. The alliance of genes and environment in asthma and allergy *Nature* . 402: B5-B11. 1999

El Gazzar, M.A. Thymoquinone suppresses *in vitro* production of IL-5 and IL-13 by mast cells in response to lipopolysaccharide stimulation. *Inflamm.res.* 56: 345-351. 2007

El-Tahir K.E., M.M. Ashour, M.M. Al-Harbi. The respiratory effects of the volatile oil of the black seed (*Nigella sativa*) in guinea pigs: elucidation of the mechanism(s) of action. *Gen Pharmacol.* 24: 1115–1122. 1993

Galli, S.J. Allergy *Curr. Biol.* 10: R93-R95. 2000

Ganpule, G., R. Knorr, J.M. Miller, C.P. Carron, and M.L. Dustin. Low affinity of cell surface lymphocyte functionassociated antigen-1 (LFA-1) generates selectivity for cell-cell interactions *J. Immunol.* 159: 2685-2692. 1997

Garraud, O., C. Nkenfou, J.E. Bradley, F.B. Perler, and T.B. Nutman. Identification of recombinant filarial proteins capable of inducing polyclonal and antigen-specific IgE and IgG4 antibodies *J. Immunol.* 155: 1316-1325. 1995

Ghosheh, O.A., A.A. Houdi and P.A. Crooks. High Performance Liquid Chromatography of the pharmacologically active quinines and related compounds in the oil of the black seed (*Nigella sativa*). *J. Pharm. Biomed. Ana.* 19:757-762. 1999

Gilani A.H, N. Aziz, I.M. Khurram, K.S. Chaudary, A. Iqbal. Bronchodilator, spasmolytic and calcium antagonist activities of *Nigella sativa* seeds (Kalonji). *J Pak Med Assoc*. **51**: 115–120. 2001

Gilani, A.H., Q. Jabeen and M.A. Ulah Khan. A review of Medicinal USes and Pharmocological Activities of *Nigella sativa*. *Pak. J. Biol. Sci.* 7 (4):441-451. 2004

Gunzer, M., A. Schafer, S. Borgmann, S. Grabbe, K.S. Zanker, E.B. Brocker, E. Kampgen, and P. Friedl. Antigen presentation in extracellular matrix: interactions of T cells with dendritic cells are dynamic, short lived, and sequential *Immunity*. 13: 323-332. 2000

Haq A, M. Abdullatif , P. Lobo, K.S.A. Khabar, K.V. Sheth, S.T. Al-Sedairy. *Nigella sativa*: effect on human lymphocytes and polymorphonuclear leukocyte phagocytic activity. *Immunopharmacology.* **30**: 147–155. 1995

Haq A, P. Lobo, M. Al-Tufail, N. Rama, S.T. Al-Sedairy. Immunomodulatory effect of *Nigella sativa* proteins fractionated by ion exchange chromatography. *Int J Immunopharmacol*. **21**: 283–295. 1999

Janeway, C., P. Travers, M. Walport, M. Schlomchik. *Immunobiology*. 6th edition. Garland Publishing. 2007

Kline, J.N. Effects of CpG DNA on Th1/Th2 balance in asthma *Curr. Top. Microbiol. Immunol.* 247: 211-225. 2000

Lambrecht B.N., M. De Veerman, A.J. Coyle, J.C. Gutierrez-Ramos, K. Thielemans, and R.A. Pauwels. Myeloid dendritic cells induce T_H2 responses to inhaled antigen, leading to eosinophilic airway inflammation *J. Clin. Invest.* 106: 551-559. 2000

Madri J.A. and D. Graesser. Cell migration in the immune system: the evolving interrelated roles of adhesion molecules and proteinases *Dev. Immunol*. 7: 103-116. 2000

Maggi, E. The TH1/TH2 paradigm in allergy. *Immunotechnology.* 3: 233-24. 1998

Marozzi F.J., A.B. Koclalski, M.H. Malone. Studies on the antihistaminic effects of thymoquinone, thymohydroquinone and quercitin. *Drug Res.* **20**: 1574–1577. 1970

Mayer, Gene. Immunology - Innate (non-specific) Immunity. *Microbiology and Immunology On-Line Textbook*. USC School of Medicine, 2006

Medinica, R., S. Mukerjee, T. Huschart, W. Corbitt. Immunomodulatory and anticancer activity of *Nigella sativa* plant extract in humans. *Proceedings of the American Association for Cancer Research Annual Meeting*. p A2865. 1994

Moser, M., M. Murphy. Dendritic cell regulation of TH1-TH2 development. *Nature*. 1:199-205. 2000

O'Garra, A., D. Robinson. Development and function of T helper 1 Cells. *Advances in immunology*. Vol. 83:133-153. 2004

O'Garra, A. and N. Arai. The molecular basis of T helper 1 and T helper 2 cell differentiation *Trends Cell Biol.*.10: 542-550. 2000

Paulnock, D.M., Macrophage activation by T cells *Curr. Opin. Immunol.* 4: 344-349. 1992

Picker, K.J. and E.C. Butcher. Physiological and molecular mechanisms of lymphocyte homing *Annu. Rev. Immunol.* 10: 561-591. 1992

Ramadan, M.F., J.T. Morsel. Characterization of Phospholipid Composition of Black cumin (*Nigella sativa*) seed oil. *Nahrun.* 46:240-244. 2002

Romagnani, S. The role of lymphocytes in allergic disease *J. Allergy Clin. Immunol.* 105: 399-408. 2000

Schlienger, K., N. Craighead, K.P. Lee, B.L. Levine, and C.H. June. Efficient priming of protein antigen-specific human CD4(+) T cells by monocyte-derived dendritic cells *Blood*. 96: 3490-3498. 2000

Shakib, F., O. Schulz, and H. Sewell. A mite subversive: cleavage of CD23 and CD25 by Der p 1 enhances allergenicity *Immunol. Today.* 19: 313-316. 1998

Stetson, D., D. Voehringer, J. Grogan, M. Xu, L. Reinhardt, S. Scheu, B. Kelly, R. Locksley. Th2 cells: Orchestrating barrier immunity. *Advances in immunology*. 83:163-180. 2004

Stout, R. and K. Bottomly. Antigen-specific activation of effector macrophages by interferon-γ producing (TH1) Tcell clones: failure of IL-4 producing (TH2) T-cell clones to activate effector functions in macrophages *J. Immunol.* 142: 760. 1989

Swamy S.M.K., B.K.H. Tan. Cytotoxic and immunopotentiating effects of ethanolic extract of *Nigella sativa* L. seeds. *J Ethnopharmacol*. **70**: 1–7. 2000

Thery,C. and S. Amigorena. The cell biology of antigen presentation in dendritic cells *Curr. Opin. Immunol.* 13: 45-51. 2001

Valenta, R. The future of antigen specific immunotherapy of allergy. *Nature.* 2:446-453. 2002

Wang, S., G. Zhu, A.I. Chapoval, H. Dong, K. Tamada, J. Ni, and L. Chen. Costimulation of T cells by B7-H2, a B7- like molecule that binds ICOS *Blood.* 96: 2808-2813. 2000

Winkler, C., K. Schroecksnadel, M. Ledochowski, H. Schennach, B. Houcher and D. Fuchs. *In vitro* Effects of *Nigella sativa* Seeds Extracts on Stimulated Peripheral Blood Mononuclear Cells. *Pteridines.* 19:101-106. 2008

Yazdanbakshsh, M., . Allergy, Paratsites, and the Hygiene Hypothesis. *Science*. 296,490. 2002