Beyza Servet GÖNCÜ

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ANTI ALLERGIC EFFECTS OF PHENOLIC COMPOUNDS OF *NIGELLA SATIVA* EXTRACT

by

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Beyza Servet GÖNCÜ

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APPROVAL PAGE

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

Asist. Prof. Sevim IŞIK
Head of Department

This is to certify that I have read this thesis and that in my opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

Prof. Dr. Fatih ÖZKARAGÖZ
Supervisor

Assist. Prof. Sevim IŞIK
Coadvisor

Examining Committee Members

Prof. Dr. Fatih ÖZKARAGÖZ …….

Prof. Fahrettin GÜCİN …….

Asist. Prof. M. Fatih ABASIYANIK …….

Asist. Prof. Lokman ALPSOY …….

It is approved that this thesis has been written in compliance with the formatting rules laid down by the Graduate Institute of Sciences and Engineering.

Assoc. Prof. Nurullah ARSLAN
Director

ANTI ALLERGIC EFFECTS OF PHENOLIC COMPOUNDS OF *NIGELLA SATIVA* EXTRACT

Beyza Servet GÖNCÜ

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Thesis Supervisor: Prof. Dr. Fatih ÖZKARAGÖZ

ABSTRACT

The term allergy is used to describe a response, within the body, to a substance, which is not necessarily harmful in itself, but results in an immune response and a reaction that causes symptoms and disease in a predisposed person, which in turn can cause inconvenience, or a great deal of misery.

Allergic reactions are caused by substances in the environment known as allergens. Almost anything can be an allergen for someone. Allergens contain protein, which is often regarded as a constituent of the food we eat. In fact it is an organic compound, containing hydrogen, oxygen and nitrogen, which form an important part of living organisms.

Nowadays, there are many drugs available as antihistamine and anti-allergic agents, however, they have undesirable side effects and adverse reactions, such as drowsiness, headache, gastrointestinal tract disturbance, fatigue, and dry mouth. Since the modern medicines have some limited use, the traditional herbs are now becoming the promising approach for the treatment of allergies. Analyzing phenolic compounds present in biological fluids and in animal and human organs is a delicate task with very important implications for our health.

In conclusion, this study will let us to determine more effective methods for allergy. In this study, we try to explain relationship between APCs (antigen presenting cells) and lymphocytes. According to our hypothesis, allergic inflammation by the immune system to be introduced to the different allergens can be prevented alternatively.

Keywords: Allergy, lymphocytes, allergic inflammation, phenolic compounds

NIGELLA SATIVA (ÇÖREK OTU)'DAN ELDE EDİLEN FENOLİK BİLEŞİKLERİN ANTİALLERJİK ETKİSİ

Beyza Servet GÖNCÜ

Yüksek Lisans Tezi: Biyoloji

Tez Yöneticisi: Prof. Dr. Fatih ÖZKARAGÖZ

ÖZ

Alerji terimi vücut içerisindeki kendi için zararlı olmayan bir cevabı tanımlamak için kullanılır. Sonucunda immun cevap oluşturan ve yatkınlığı olan kişilerde semptomlara yol açan maddeler, büyük rahatsızlıklara sebep olabilirler.

Alerjik reaksiyonlar alerjen adı verilen maddeler sebebiyle meydana gelmektedir. Neredeyse herşey herhangi biri için alerjen olabilir. Alerjenler protein içerirler, genelikle yediğimiz yiyecekler ile alınmaktadır. Ayrıca bir organik blieşiktir; hidrojen, oksijen ve nitrojen içeren ve canlı organizmalar için yaşamsal bir yapıdır.

Günümüzde; bir çok antihistamin ve anti-alerjik yapıda ilaç mevcuttur. Fakat bu ilaçların birçok yan etkileride mevcuttur, baş ağrısı, mide bulantısı, ağız kuruluğu, baş dönmesi, gastrointestinal rahatsızlıklar gibi. Modern tıp limitli kullanım alanlarına sahiptir. Bu sebeple geleneksel tedavi yöntemleri gittikçe yaygınlaşmaktadır ve alerji tedavisi için ümit vaadetmektedir.

Biyolojik yapılarda bulunan fenolik bileşiklerin analizi ile insan ve hayvanlarda tedaviye yönelik çalışmaların artması sağlığımız açısından çok büyük önem taşımaktadır.

Sonuç olarak bu çalışma sayesinde alerji için çok daha etkili bir tedavi yöntemini açıklamaya çalışacağız. Bu araştırmada antijen sunan hücreler (APCs) ve lenfositler arasındaki ilişkiyi açıklamaya çalışacağız. Hipotezimize göre, immün sistemin alerjik inflamasyonunu farklı alerjenler ile birlikte olumlu şekilde etkileyebileceğiz.

Anahtar Kelimeler: Allerji, lenfositler, allerjik inflamasyon, fenolik bileşikler.

This thesis is dedicated to those that don't believe in themselves

or

non-believers in somebody trying to change.

I started this journey with uncertainity

but

I knew what I liked, what I was capable of and where I wanted to be in ten years.

I needed change, and I needed a challenge.

Have faith

Change is always good in the end...

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LIST OF SYMBOLS AND ABBREVIATIONS

SYMBOL/ABBREVIATION

RT	Room temperature
Der f	*Dermatophagoides farinea*
Der p	*Dermatophagoides pteronyssinus*
Ag	Antigen
AU	Allergen unit
IL	Interleukin
DC	Dentritic cell
TLR	Toll-like receptor
CD	clusters of differentiation
SIT	Specific immunotherapy
APCs	Antigen Presenting Cells
Ns	*Nigella sativa*
cDNA	Complementary Deoxyribonucleic acid
RNA	Ribonucleic acid
GF/PET	Glass fiber/polyethyleneterephtalate
PBMC	Peripheral Blood Mononuclear Cell
LDH	Lactate dehydrogenase
MP	Microplate
ELISA	Enzyme-linked immunosorbent assay
WST-1	Tetrazolium salt
SAV-HRP	Streptavidin
O.D.	Optical density
Hu	Human
RT-PCR	Reverse Transcription Polymerase Chain Reaction
TBE	Tris/Borate/EDTA
EDTA	Ethylenediaminetetraacetic acid
CUPRAC	Cupric Ion Reducing Antioxidant Capacity
HPLC	High-performance liquid chromatography
min	minutes
h	hours
s	second

CHAPTER 1

1. INTRODUCTION

1.1. Immune System

The Immune system includes barriers and cells called monocytes, macrophages, dendritic, and langerhans cells. These cells attract, bind, engulf, neutralize, and signal other cells of the immune system about the antigenic material. Monocytes circulate in the body and mature into macrophages, which can take residence in particular tissues of the host. The macrophages engulf an antigenic materials through a process of phagocytosis, to destroy it the antigen inside the cell. Other cells of this system, such as dendritic, langerhans, and macrophages, are termed antigen presenting cells (APCs), due to specific signaling molecules presented on their surfaces, alarming other cells of the immune system to the type of antigenic material encountered (Pascoe, 2009).

The interaction of the immune system is critical for elucidate immune response to a new encountered antigens (Leggat et al. 2008). Allergic reactions occur when an individual produced IgE antibody in response to an innocuous antigen (Janeway, Immunobiology 6th edition). The incidence of allergic diseases is increasing at an alarming rate, exceptionally in last decade. Most patients can be treated with conventional pharmacotherapy on an "as-needed" symptomatic basis whereat allergen immunotherapy represents a useful treatment IgE-mediated disease (James and Durham, 2008). It is important that allergen immunotherapy is the only treatment that can actually change a patient's immune system back toward normal.

1.1.1. Allergic Inflammation

Allergic diseases include a number of hypersensitivity disorders which may be classified into two main catagories; the acute response (anaphylaxis) and chronic response (exposure to the allergen or antigen). Both phases result from IgE reacting with an antigen (table 1.1). The development of allergic inflammation which is caused by an immune response to allergens driven by regulatory cells (Incorvaia et al., 2008).

1.1.1.1. Acute Response

Anaphylaxis is an acute-onset, potentially fatal systemic allergic reaction. It is usually triggered by an agent such as an insect sting, medication or food, through a mechanism involving IgE and the high-affinity IgE receptor on mast cells or basophils. Sensitization to allergen triggers suggested by the history needs to be confirmed by skin testing and measurement of allergen-specific IgE. Injection of epinephrine is life saving (Simons, 2008).

1.1.1.2. Chronic Response

The IgE induced inflammation is an activity like the inflammatory functions of complement system. The mechanism initiating the inflammation is different in each response. In the IgE system, antigen cross link IgE antibody on the mast cell surface to degranulate the mast cells. Then the cell releases its granule contents and begins synthesizing a series of secondary mediators (figure 1.1). The secondary mediators include cytokines like IL-4, IL5. IL4 promotes IgE isotype switch and IL5 activates eosinophils.

Table 1.1. IgE reactions

Term	Definition
Atopy	An inherited predisposition to respond to antigens with IgE antibodies
Atopic	An individual who responds to antigens with IgE antibodies
Anaphylaxis	Initially implies a hyperresponse to a toxin challenge
Allergen	Antigen that induces an IgE response
Sensitization	Immunization with or exposure to antigen that results in an IgE response

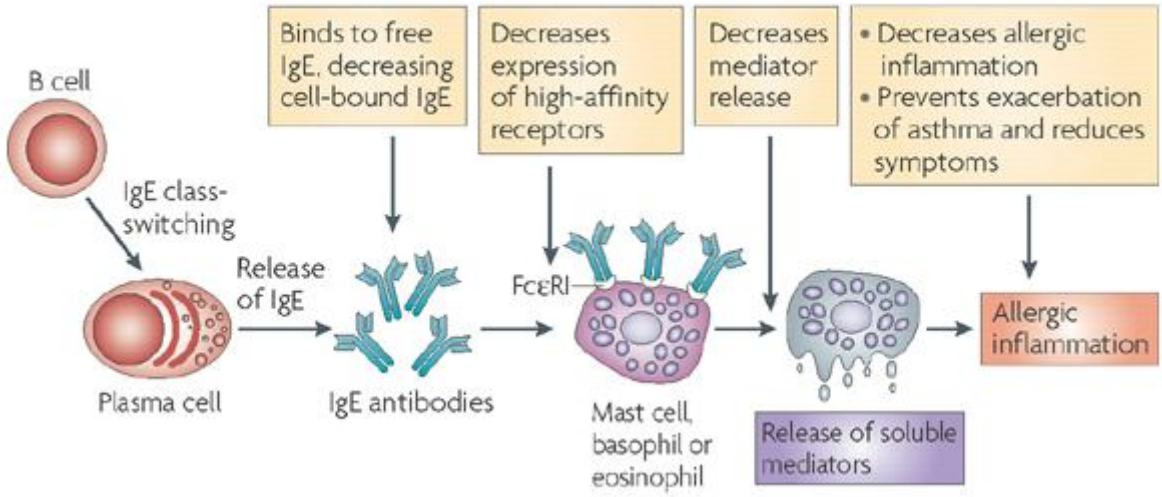

Figure 1.1. Function of IgE antibody (Holgate and Polosa,2008).

1.1.2. APCs

APCs and dentritic cells (DCs) are the most involved in allergic airway inflammation, showing a conspicuous role in presenting the allergens to T cells and influencing the proliferation of Th1 or Th2 cell types (Incorvaia et al., 2008). In allergic disease, immunity, toward the Th2 type results by allergen exposure (Leggat et al., 2008).

1.1.3. T cells

Cell-mediated immunity occurs through a process of T cells and APCs from the innate system. APCs display antigenic markers on their surface to communicate and sensitize T cells to the foreign substance (figure 1.2). T cells have a variable receptor system, termed clusters of differentiation (CD), allowing an estimated 10^{18} potentially different receptor forms (Pascoe, 2009). CD4+ T cells coordinate the immune response, stimulating the cell differentiation and proliferation of cytotoxic CD8+ T and B cells (Randolph, 2005). Memory T cells help post a defense for future encounters of similar antigenic organisms and suppressor T cells limit the proliferation of CD8+ T cells as a protective negative feedback mechanism (Pascoe, 2009).

CD4 T , Th1 and Th2 cells, are different from each other and their pattern of cytokine production and their functions. Nowadays at least 4 distinct CD4 T-cell subsets have been shown to exist, Th1, Th2, Th17, and iTreg cells. Mossman and Coffman (1986) recognized the Th1 and Th2 phenotypes. Th1 cells being regarded as critical for immunity to intracellular microorganisms and Th2 cells for immunity to many extracellular pathogens, including helminthes (Jinfang and Williams, 2008).

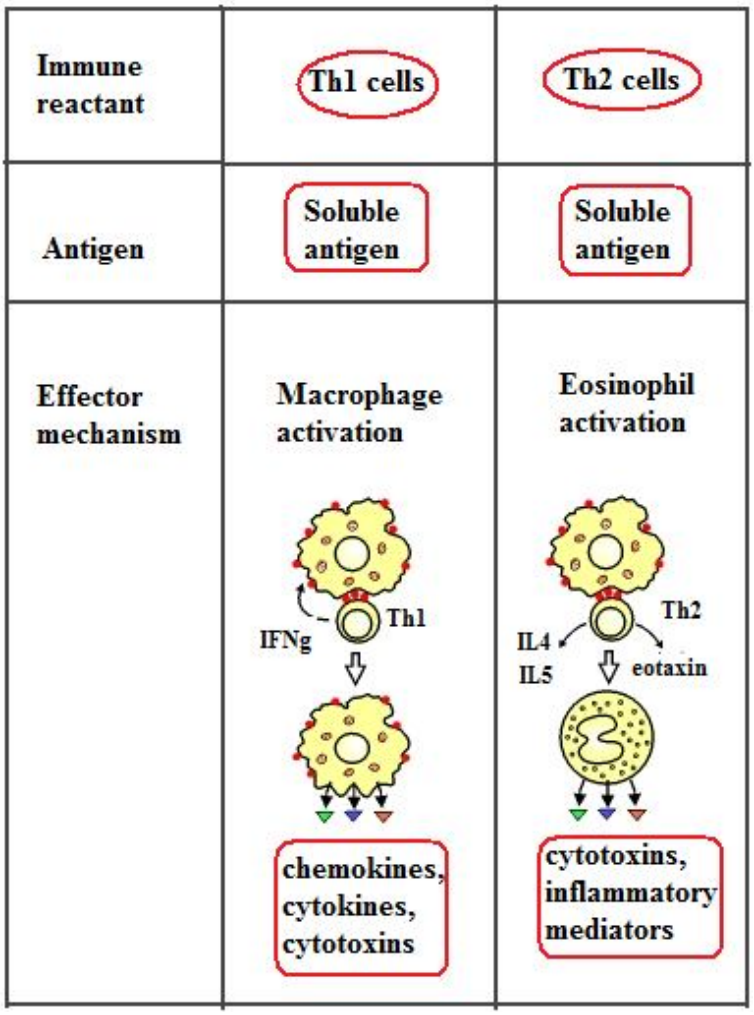

Figure 1.2. Hypersensitivity reactions of Th cells.

1.1.3.1. Th1 Cells

Th1 cells mediate immune responses against intracellular pathogens. In humans, they play a particularly important role in resistance to mycobacterial infections. Th1 cells are also responsible for the induction of some autoimmune diseases. Their principal cytokine products are IFNγ, lymphotoxin α (LTα), and IL-2 (Jinfang and Williams, 2008). IFNγ produced by Th1 cells is important in activating macrophages to increase their microbicidal activity (Suzuki et al., 1988).

In the initiation of Th1 responses, APCs, particularly activated DCs, stimulate naive CD4 T cells possessing cognate T-cell receptors. APCs that produce large amounts of IL-12 as a result of their activation promote Th1 cell differentiation by acting on both NK cells and T cells (figure 1.3). IL-12 activates NK cells to produce IFNγ (Trinchieri et

al., 2007). Thus, collaboration between IFNγ and IL-12 induces full Th1 differentiation (Murphy et al. 2002). Antigen-independent cytokine production is important for amplifying Th1 responses by recruiting other preexisting Th1 cells (Jinfang et al., 2008).

1.1.3.2. Th2 Cells

Th2 cells produce a characteristic coordinated array of cytokines and chemokines that maintain and amplify the polarization of the response and stimulate class switching of B cells to IgE production (Janeway, Immunobiology 6th edition). Th2 cells produce IL-4, IL-5, IL-9, IL-10, IL-13 and IL-25. IL-4 is the positive feedback cytokine for Th2 cell differentiation and is the major mediator of IgE class switching in B cells. Healthy individuals have very low serum concentrations of IgE, and absent allergen-specific IgE. Allergic individuals have a high IgE levels (James and Durham, 2008). IL-5 plays a critical role in induce eosinophil differentiation and their growth. Both IL-4 and IL-2 are required for Th2 differentiation *in vitro*. They are important in the induction and persistence of asthma and other allergic diseases (figure 1.2). The IL-4 production only occurs when cells receive low strength signals (Jinfang and Williams, 2008).

In summary; the IL4 function; It promotes IgE production with IL10, induces expression of adhesion molecules by endothelial cells, inhibits Th1 cell development, promotes Th2 cell development, also induces mucus production from mucosal glands and up-regulates expression of low affinity IgE receptors.

Th2 cytokines have multiple and overlapping effects on cells involved in allergic inflammation. It includes eosinophils, mast cells and basophils (James and Durham, 2008).

The major allergen in the feces of the house dust mite which is responsible for allergy. It is a cysteine protease known as Der p 1. This enzyme has been found to cleave occludin, a protein component of intercellular tight juctions.

Proteolytic enzymes from pollens disrupt tight junctions (figure 1.3). Moreover, Der p 1 cleave α-1-anti-trypsin, inhibiting its ability to protect the respiratory tract against serine proteases Der p 3 and Der p 9. This may disrupt the protease–antiprotease balance in

mucosal tissues, enhancing the activity of both endogenous and exogenous proteases and leading to tissue damage and immune response. The cysteine protease activity of several mite allergens (Der p 1, Der f 1) may directly impair innate defense mechanisms in the lung by degrading and inactivating lung surfactant proteins (SPs)-A and -D. SP-A and SP-D have multiple innate immune functions, including bacterial agglutination and modulation of leukocyte functions. (M. Willis et al., 2010).

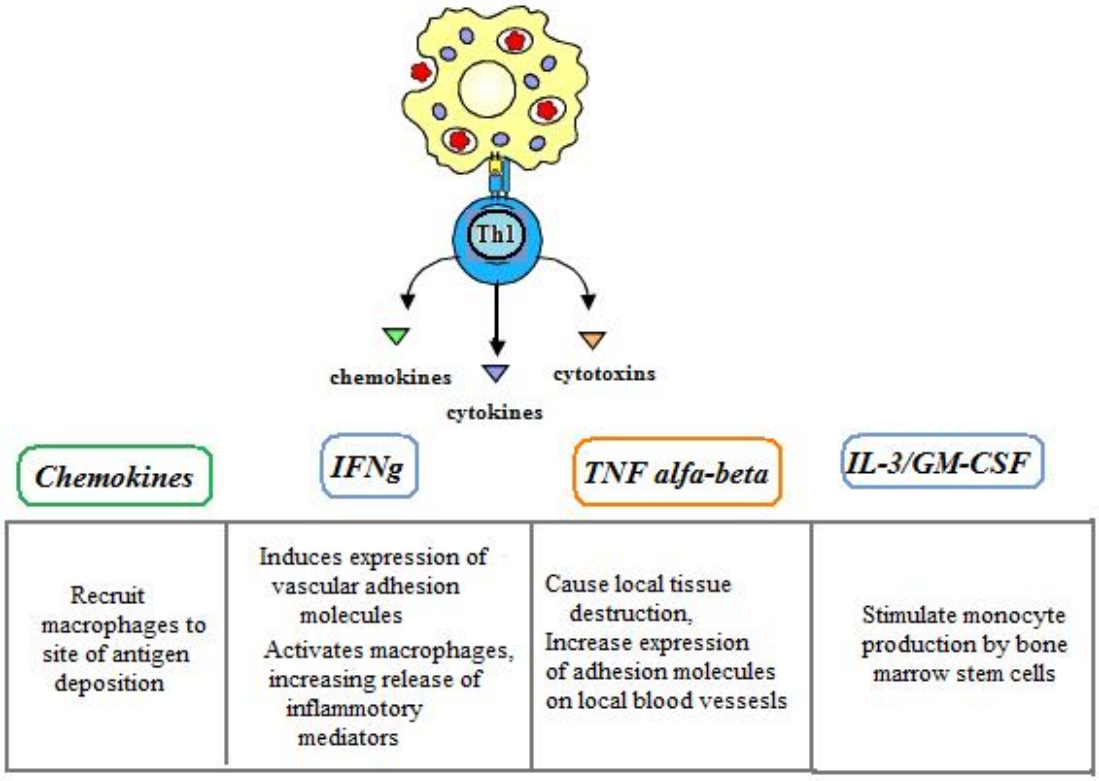

Figure 1.3. Hypersensitivity response is directed by chemokines and cytokines released by Th1 cells stimulated by antigen.

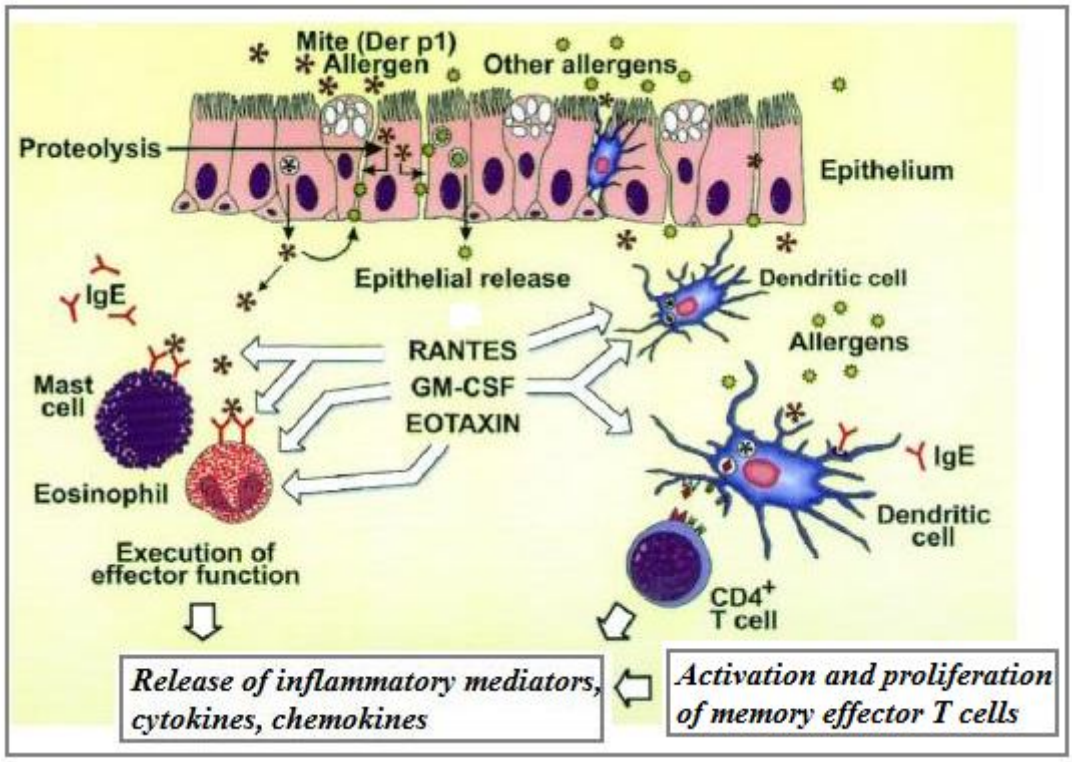

Figure 1.4. Summary of how Der p 1 and other antigens might cross the bronchial epithelium

1.1.3.3. Th1/Th2 Balance

Immune responses to allergens have both inflammatory and regulatory components (Akdis et al., 2004). The balance between these components determines either clinical tolerance or allergic inflammation (Larche, 2007).

It is well known that allergic subjects have an imbalance between Th1 and Th2 type responses in favour of the latter, which is thought to be due to the genetic background and the environmental factors. This leads to the production of greater amounts, compared to non-allergic subjects, of cytokines (such as IL-4 and IL-5) facilitating the allergic response (Incorvaia et al., 2008).

Studies showing beneficial effect of immunotherapy (IT) have demonstrated down regulation of Th2 responses in peripheral blood, and/or increased Th1 responses in the tissues. Increased Th1/Th2 rate accompanied by induction of IgG initially led to the conclusion that the effect of SIT was achieved through the induction of protective Th1 responses which antagonized Th2 immunity (Larche, 2007).

Shifting the balance to a Th1 phenotype is considered to be a standard for demonstrating a favorable anti-allergic response.

1.2. *Nigella sativa*

Nigella sativa (*Ns*), belongs to the Ranunculaceae family and is especially grown in East Mediterranean countries (Nergiz and Otles ,1993). It was first identified and described by Linnaeus in 1753 (Jansen, 1981). The detailed taxonomy of the plant was described by Muschler (Muschler, 1912). The plant is known to all Arabian and Islamic countries and carries various colloquial names.

There are losts of *in vitro* or *in vivo* experiments for *Ns* seed oil. This seed oil has been reported to possess antitumor activity (Worthen et al., 1998), anti-oxidant activity (Burits and Bucar, 2000), anti-inflammatory activity (Houghton et al., 1995), anti-bacterial activity (Morsi, 2000) and a stimulatory effect on the immune system (Salem and Hossain, 2000).

Treatment of rats with an aqueous extract of the seeds resulted in significant inhibitions of experimentally-induced inflammations and pain but not fever (Afrozul et al.,1999). *In vitro* exposure of the methanolic extract of *Ns* on Dalton's Lymphoma ascites cells and sarcoma cells to seeds resulted in significant cytotoxicity in the cells (Salomi et al., 1991).

In other experiments, the Methanolic extract succeeded in protecting mice against cisplatin-induced decreases in hemoglobin and leukocytes counts (Nair et al.,1991). Studies in vitro showed the potential inhibitory effect of the methanolic extract of *Ns* in suppressing the growth of *Bacillus subtilis, Escherichia coli, Streptococcus foecalis, Staphylococcus aureus*, *Pseudomonas aeroginosa*, and *Candida albicans* (Mashhadian et al., 2005).

The *in vitro* effects of the soluble fractions of *Ns* seeds on human peripheral blood mononuclear cells (PBMC) response to different mitogens, the components did not show any consequential stimulatory effect on the PBMC responses to the T cell mitogens

phytohemagglutinin, or concanavalin-A. However the components expressed stimulatory effect on the PBMC response to pooled allogenic cells (Haq et al. 1995).

The stimulatory effects of *Ns* on the cellular immunity are dependent on the nature of the immune response (Salem,2005). Even though *in vitro* treatment of human PBMC with the soluble fractions of *Ns* seeds had no effect on the bacterial phagocytosis or killing activities of these cells when cultured with Staphylococcus aureus (Haq et al. 1995).

Unfortunately, very few studies have addressed the possible toxicity of N. sativa seeds and their components. In an earlier study, aqueous extract of the seeds of *Ns* was administered orally to male Sprague–Dawley rats for 14 days, and the possible toxicity was measured in the levels of the key hepatic enzymes, and histopathological changes (Tennekoon et al. 1991).

CHAPTER 2

MATERIAL and METHODS

2.1. MATERIALS

2.1.1. General Reagents

All laboratory chemicals were analytical grade from Sigma Biosciences Chemical Company (USA), Invitrogen (CA), Bender MedSystems (USA)Merck (Germany), Applichem (Germany) and Fluka (Germany).

Table 2.1: List of general chemical reagents and their brands.

PBS	Biochrom AG
FBS	Biochrom AG
RPMI	Biochrom AG
Ficoll	Biochrom AG
Follin Ciocalteu Reagent	Sigma
MeOH	Sigma
Cu(II) chloride	Sigma
Neocuproin	Sigma
Ammonium Acetate Buffer (NH_4Ac)	Sigma
NaOH	Sigma
Na_2CO_3	Sigma
$CuSO_4$	Sigma
$NaKC_4H_4O_6$	Sigma
Ascorbic Acid	Sigma
Gallic Acid	Sigma
Catechin	Sigma
Chlorogenic Acid	Sigma
Epicatechin	Sigma

Caffeic Acid	Sigma
p-coumaric Acid	Sigma
Ferulic Acid	Sigma

2.1.2. Equipment

Table 2.2: List of equipments and their brands and models

Autoclave	CERTO CLAW A-4050 Traun, Austria
Balance	Sartorius, Wender Landstrasse 94-108 D-37075 Goettingen,Germany
Centrifuge	Hettich, Mikro 22
Vortex	IKA LABOTECHNIK
Water Purification System	Millipore
Water Bath	Nuve
Inverted Light Microscope	Zeiss
Fluorescent Microscope	Zeiss
Laminar Flow Hood	Esco, Kotterman
CO_2 Incubator	Thermo, Sanko
Ultrasonic Bath	UTS
Microfilter (1.0/0.45 μm) GF/PET	Szhleicher & Schuell
Chromatographic Column	Hamilton HxSil C18
Vacuum Pump (oil free)	Buchi
Vacuum Controller V850/855	Buchi
Vacuum Module Easy Vac	Buchi
Rotavapor	Buchi
Recirculating Chiller B-740	Buchi
Pipettor	Reddot Hirschmann Laborgerate
Centrifuge Tubes	Falcon
Micropipettes	Nichiryo
Serological Sterile, Plastic Pipets (2 mL, 5 mL, 10 mL)	Grenier
Syringe	Hayat Sırınga
Electrophoresis Equipment	Bio-Rad Sub Cell, GT
Power Supplies	Bio-Rad Power PAC

Thermocyclers	TECHNE TC-512
Transilluminator	Bio-Rad GelDoc 2000
PCR Machine	Techne
Fluorometer	Qubit, Invitrogen
Trypan blue	Sigma
Vacuette	Grenier
Vacutainer	Grenier
Holder	Grenier

2.1.3. *Ns* Extraction and Analysis

2.1.4. *Ns*

Ns seeds were purchased from an herbal shop in Istanbul.

2.1.4.1. Extraction Chemical

%60 MeOH was used and purchased from Sigma Aldrich.

2.1.4.2. Analysis Chemicals

2.1.4.2.1. CUPRAC Chemicals

Cu(II) chloride, neocuproin, ammonium acetate buffer (NH_4Ac), were used and purchased from Sigma Aldrich

2.1.4.2.2. Folin-Ciocalteu Chemicals

NaOH, $NaKC_4H_4O_6$, Folin-Ciocalteu Reagent were used and purchased from Sigma Aldrich.

2.1.4.2.3. HPLC Chemicals

Epicatechin, catechin, chlorogenic acid, caffeic acid ferrulic acid and p-coumaric acid were used and purchased from Sigma Aldrich.

2.1.3. Subjects

Four subjects (age median:32) were recruited from the Fatih University students and Hill Allergy Clinic in Istanbul. Two subjects had a history of severe dust allergy, positive skin tests reaction to *Dermatophagoides pteronyssinus* and *Dermatophagoides farinea*. The other two subjects had no history with allergy, negative skin test.

2.1.4. Antigens

Dermatophagoides pteronyssinus and *Dermatophagoides farinea* were purchased from Albio (Turkey, Istanbul).

2.1.5. Cell Type

Culture samples were collected from peripheral blood mononuclear cells. The PBMCs were isolated from heparinized blood samples.

2.1.6.1. Cell Culture

RPMI 1640 Medium, essential amino acids, fetal bovine serum (FBS), ficoll, heparin, and PBS were purchased from Biochrom (Berlin, Germany). Cell culture plates: 24-well and 96-well flat-bottomed culture plates were purchased from Greiner Bio-One Corp. (Germany).

2.1.6. Cytotoxicity Assay Kit

Roche Cytotoxicity Detection Kit (LDH) was used and the components are as follows: Catalyst and Dye solution. The kit purchased from Roche (Germany).

2.1.7. Proliferation Assay Kit

Roche Cell Proliferation Reagent WST-1 Kit was used and purchased from Roche (Germany).

2.1.8. ELISA (Enzyme-linked immunosorbent Assay) Kit

Invitrogen Immunoassay Kits were used for measurements of Human IL4, IL5 and IFNγ quantities.

2.1.9. RNA Isolation and Quantification Kits

2.1.9.1. RNA Isolation

Qiagen RNeasy Mini Kit was used and the components are as follows: Buffer RLT, Buffer RW1, Buffer RPE (concantrate) and RNase-Free Water.

2.1.9.2. RNA Quantification Kits

RNA quantification was done using Qubit fluorometer (Invitrogen) and its quantification kit components. Quant-it dsRNA BR Assay Kits Components are as follows: Working solution, Standard #1 Standard #2 and assay range is 2–1000 ng with sample starting concentration range: 100pg/µl-1µg/µl.

2.1.10. cDNA Synthesis

Qiagen Quantitect Reverse Transcription cDNA Synthesis Kit was used and the components are as follows: gDNA, quantiscript-reverse transcriptase, quantiscript-rt buffer 5x, rt primer mix**.**

2.1.11. Polymerase Chain Reaction (PCR)

2.1.9.1.Primers

Table 2.3: Base sequences of primers.

Primer	**Sequence 5' to 3' (Forward)**	**Sequence 5' to 3' (Reverse)**
IL4	ATGGGTCTCACCTCCCAACTGC	TTCCTGTCGAGCCGTTTCAG
IL5	ATGAGGATGCTTCTGCATTTG	CTATTATCCACTCGGTGTTCA
IFNγ	AGTTATATCTTGGCTTTTCA	ACCGAATAATTAGTCAGCTT

2.1.9.2.PCR Chemicals and consumables

Table 2.4: PCR kit components (QIAGEN)

10x QIAGEN PCR Buffer	Tris-Cl, KCl, $(NH_4)_2SO_4$, 15 mM $MgCl_2$; at pH 8.7 (20°C)
MgCl2	25 mM
dNTP mix	10 mM of each dGTP, dATP, dCTP, dTTP
Taq DNA Polymerase	5U/µl
Primers	10pmol/reaction

2.1.9.3. Electrophoresis and Documentation

2.1.9.2.1. Agarose Gel Electrophoresis Chemicals and Buffer

Table 2.5: Agarose Gel Electrophoresis chemicals, buffers and their components and composition concentrations.

10XTBE	For 1 Liter: 108g Tris base, 55g Boric acid,40mls 0.5M EDTA (pH 8.0), autoclave for 20 min
6XLoading Dye	10 mM Tris-HCl (pH 7.6), 0.03% bromophenol blue, 0.03% xylene cyanol FF, 60% glycerol, 60 mM EDTA.
GeneRuler™ 100 bp DNA Ladder	100 µl (0.5µg/µl) 100 bp sized DNA fragments in 10mM Tris-HCl (pH 7.6), 1mM EDTA.
Etbr Staining Chemicals	0.5 µg/ml EtBr.

2.1.9.2.2. Agarose Gel Visualization and Documentation

Bio-Rad (Italy) Gel Doc 2000 was used for gel visualizations. Bio-Rad Quantity One Programme (Italy) was used for imaging and analysis of gels (1-D).

2.2. METHODS

2.2.1 Extraction of Phenolic Portion from *Nigella sativa*

Ns were extracted with aqueous 60 % methanol. 10 g -amounts of the seed materials were extracted in stoppered flasks placed in an ultrasonic bath first with 50 ml of solvent for 60 min, then with 30 ml of solvent for 45 min, and finally with 20 ml of solvent for 15 min, the overall extraction taking 120min. The three extracts were combined, brought to a final volume of 100 ml with aqueous 60% methanol. The methanol was removed vacuum rotary at 40^0C until dryness. Then this portion was dissolved with 10ml deionized water. The extract was filtered through a GF/PET (glass fiber/polyethyleneterephtalate) 1.0/0.45 µm microfilter, and prepared for PBMC culture.

2.2.2 Isolation of PBMCs

The PBMCs were isolated from heparinized blood samples by density gradient centrifugation with Ficoll. First, whole peripheral blood was collected into a heparinized syringe. Using a wide centrifuge tube, blood was mixed with PBS onto an equal quantity. Taking another centrifuge tube filled with Ficoll an equal quantity with blood and PBS mix. Blood was slowly poured onto Ficoll, taking care not to disturb the interface between the blood and Ficoll.

Tubes were centrifuged at 2500rpm for 25min at RT. After centrifugation, there are four different layers appear (see figure 2.1).

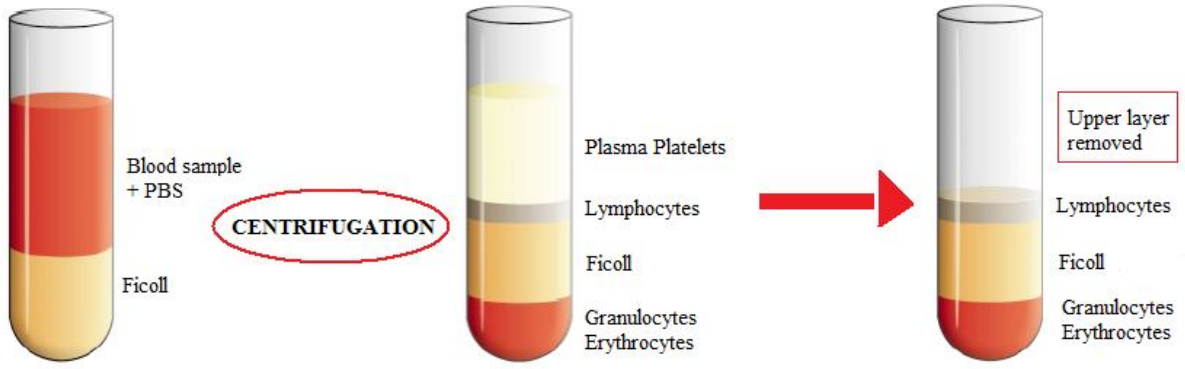

Figure 2.1. Isolations of PBMC populations with Ficoll density gradient centrifugation

Upper layer was removed then the lower layer was transferred into another empty falcon and 10ml PBS mixed was with it. Tubes were centrifuged at 1700rpm for 7min. Then supernatant was discarded and the cell pellet was resuspended by firmly tapping the bottom of the tube. Tube was refilled with RPMI 1640 medium, centrifuged at 1700rpm for 7min. Then supernatant was discarded and the cell pellet resuspended as before. Volume was brought up to 5ml with complete culture medium (RPMI-1640 supplemented with 10% FBS in the tube). The tube mixed gently and using a sterile pipet tip 10µl sample was removed into tube. Then 20 µl trypan blue solution was mixed with sample into the tube and mix loaded into haemocytometer. The cells were counted and calculated. The cell suspension was adjusted to $1x10^6$ cell / ml equality (Larche 2000).

2.2.2.1. Allergen Induced Cell Reaction

The PBMCs were seeded as the $1x10^6$ cell / ml equality and incubated in the presence of 10µg/mL (~755AU/mL) of *Dermatophagoides pteronyssinus* and *Dermatophagoides farinea* mix at 37°C and 5% CO_2 for 6 days (Larche 2000).

2.2.2.2. Adding Extract

The PBMCs were seeded as $1x10^6$ cell / ml equality and incubated with hydrophilic extract at 0.1% concentration at 37°C and 5% CO_2 for 6 days. This proportion estimated with cytotoxicity tests.

2.2.3 Cytotoxicity Tests

The cells were removed from the culture medium prior to the determination of LDH activity by centrifugation at about 250 × *g* for 10 min. Supernatant (100 µl/well) was removed carefully and transfered into 96-well flat bottom microplate (MP). Reaction mixture (described below) was added (100 µl to each well) and incubated for 30 min at RT. The MP was protected from light during this incubation period. The samples absorbances were measured at 490 nm with ELISA reader.

2.2.3.1.Preparation of Working Solutions

The lyophilisate (bottle 1, blue cap) was dissolved in 1 ml double distilled water for 10 min and mixed. The dye solution (bottle 2, red cap) was ready to use. The reaction mixture was mixed 250 µl of bottle 1 with 11.25 ml of bottle 2.

2.2.4 Proliferation Assay

Cells were cultured in microplates (tissue culture grade, 96 wells, flat bottom) in a final volume of 100 µl/well culture medium in a humidified atmosphere (37°C, 5% CO2). Cell Proliferation Reagent WST-1 was added in a 10 µl/well volume. Cells were incubated for 4h in a humidified atmosphere (37°C, 5% CO2). The samples absorbances were measured at 420 nm with ELISA reader.

2.2.5 Quantitation of Cytokines

2.2.5.1. Dilution of Wash Buffer

The 25x Wash Buffer concentrate was diluted 1/24 volume with deionized water. It was labeled as Wash Buffer.

2.2.5.2. Preparing SAV-HRP

Streptavidin-HRP was diluted with 1 ml of Diluent for each 8-well strip used in the assay. It was labeled as Streptavidin-HRP Working Solution. The Streptavidin HRP dilutions as described in the following dilution table 2.6.

Table 2.6: Dilutions of SAV-HRP

8-well strips	Volume of SAV-HRP concentrate	Volume of Diluent
2	20µl solution	2ml
4	40µl solution	4ml
6	60µl solution	6ml
8	80µl solution	8ml

2.2.5.3. ELISA IL5

Standard (7,500 pg/ml) was reconstituted with Standard Diluent Buffer and mixed gently and incubated 10 min at RT. The reconstituted standard was added 0.100 ml in a tube containing 0.900 ml Standard Diluent Buffer. It was mixed and labeled as 750 pg/ml Hu IL-5. Standard Diluent Buffer were added in a 0.300 ml/tube volume and each 6 tubes were labeled 375, 187.5, 93.7, 46.8, 23.4 and 11.7 pg/ml Hu IL-5. The standards serial dilutions were described in the following dilution figure 2.3. The numbers of 8-well strips were determined for the assay. The Standard Diluent Buffer was added in a 100 µl to the zero standard wells. One well was reserved for chromogen blank. Standards and samples were added in a 100 µl/well volume. The plate was covered with plate cover and incubated for 2h at RT. The solutions were aspirated from wells and discarded the liquid. Biotinylated anti-IL-5 (Biotin Conjugate) solutions were pipetted in a 100 µl/well volume (except the chromogen blank).

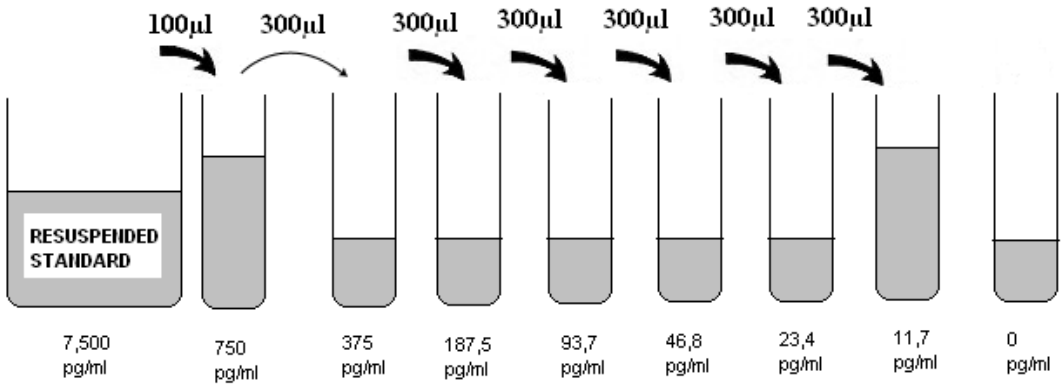

Figure 2.3.: Dilutions of the standards

The plate was tapped gently to mix. The plate was incubated for 30 min at RT. The solutions were aspirated from wells and discarded the liquid. The wells were washed 4 times. Streptavidin-HRP Working Solutions were added in a 100 µl/well volume (except the chromogen blank). The plate was incubated for 30 min at RT. The solutions were aspirated from wells and discarded the liquid. The wells were washed 4 times. Stabilized Chromogen was added in a 100 µl/well volume. The liquid in the wells began to turn blue. The plate was incubated for 30 min at RT and in the dark. Stop Solution were added in a 100 µl/well volume. The plate was tapped gently to mix. The solution in the wells changed from blue to yellow. The absorbances of each well were measured at 450 nm with ELISA reader. Curve fitting software was used to generate the standard curve. The concentrations of samples and controls were read from the standard curve.

2.2.5.4. ELISA IFNγ

Standard (5000 pg/ml) was reconstituted with Standard Diluent Buffer and mixed gently and incubated 10 min at RT. The reconstituted standard was added 0.100 ml in a tube containing 0.400 ml Standard Diluent Buffer. It was mixed and labeled as 1000 pg/ml Hu IFN-γ. Standard Diluent Buffer were added in a 0.150 ml/tube volume and each 6 tubes were labeled 500, 250, 125, 62.5, 31.2 and 15.6 pg/ml Hu IFN-γ. The standards serial dilutions were described in the following dilution table 2.7.

Table 2.7. Human IFNγ Standards serial dilutions.

Standard	Add	Into
1000 pg/ml		
500 pg/ml	0,150 ml of the 1000 pg/ml std	0,150 ml of the Diluent Buffer
250 pg/ml	0,150 ml of the 500 pg/ml std	0,150 ml of the Diluent Buffer
125 pg/ml	0,150 ml of the 250 pg/ml std	0,150 ml of the Diluent Buffer
62,5 pg/ml	0,150 ml of the 125 pg/ml std	0,150 ml of the Diluent Buffer
31,2 pg/ml	0,150 ml of the 62,5 pg/ml std	0,150 ml of the Diluent Buffer
15,6 pg/ml	0,150 ml of the 31,2 pg/ml std	0,150 ml of the Diluent Buffer
0 pg/ml	0,150 ml of the Diluent Buffer	An empty tube

The numbers of 8-well strips were determined for the assay. The Incubation Buffer was added in a 500 µl to the zero wells. One well was reserved for chromogen blank. Standards and samples were added in a 50 µl/well volume. Biotinylated anti- IFN-γ (Biotin Conjugate) solutions were pipetted in a 50 µl/well volume (except the chromogen blank). The plate was tapped gently to mix. The plate was incubated for 1h and 30 min at RT. The solutions were aspirated from wells and discarded the liquid. The wells were washed 4 times. Streptavidin-HRP Working Solutions were added in a 100 µl/well volume (except the chromogen blank). The plate was incubated for 45 min at RT. The solutions were aspirated from wells and discarded the liquid. The wells were washed 4 times. Stabilized Chromogen was added in a 100 µl/well volume. The liquid in the wells began to turn blue. The plate was incubated for 30 min at RT and in the dark. Stop Solution were added in a 100 µl/well volume. The plate was tapped gently to mix. The solution in the wells changed from blue to yellow. The absorbances of each well were measured at 450 nm with ELISA reader. Curve fitting software was used to generate the standard curve. The concentrations of samples and controls were read from the standard curve.

2.2.5.6. ELISA IL4

Standard (10,000 pg/ml) was reconstituted with Standard Diluent Buffer and mixed gently and incubated 10 min at RT. The reconstituted standard was added 0.050 ml in a tube containing 0.950 ml Standard Diluent Buffer. It was mixed and labeled as 500 pg/ml Hu IL-4. Standard Diluent Buffer were added in a 0.300 ml/tube volume and each 6 tubes were labeled 250, 125, 62.5, 31.2, 15.6, and 7.8 pg/ml Hu IL-4. The standards serial dilutions were described in the following dilution table 2.8.

Table 2.8: Dilution of Hu IL-4 Standard.

Standard	Add	Into
500 pg/ml		
250 pg/ml	0,300 ml of the 500 pg/ml std	0,300 ml of the Diluent Buffer
125 pg/ml	0,300 ml of the 250 pg/ml std	0,300 ml of the Diluent Buffer
62,5 pg/ml	0,300 ml of the 125 pg/ml std	0,300 ml of the Diluent Buffer
31,2 pg/ml	0,300 ml of the 62,5 pg/ml std	0,300 ml of the Diluent Buffer
15,6 pg/ml	0,300 ml of the 31,2 pg/ml std	0,300 ml of the Diluent Buffer
7,8 pg/ml	0,300 ml of the 15,6 pg/ml std	0,300 ml of the Diluent Buffer
0 pg/ml	0,150 ml of the Diluent Buffer	An empty tube

The numbers of 8-well strips were determined for the assay. The Standard Diluent Buffer was added in a 100 µl to the zero standard wells. One well was reserved for chromogen blank. Standards and samples were added in a 100 µl/well volume. Biotinylated anti-IL-4 (Biotin Conjugate) solutions were pipetted in a 50 µl/well volume (except the chromogen blank). The plate was tapped gently to mix. The plate was incubated for 2h at RT. The solutions were aspirated from wells and discarded the liquid. The wells were washed 4 times. Streptavidin-HRP Working Solutions were added in a 100 µl/well volume (except the chromogen blank). The plate was incubated for 30 min at RT. The solutions were aspirated from wells and discarded the liquid. The wells were washed 4 times. Stabilized Chromogen

was added in a 100 µl/well volume. The liquid in the wells began to turn blue. The plate was incubated for 30 min at RT and in the dark. Stop Solution were added in a 100 µl/well volume. The plate was tapped gently to mix. The solution in the wells changed from blue to yellow. The absorbances of each well were measured at 450 nm with ELISA reader. Curve fitting software was used to generate the standard curve. The concentrations of samples and controls were read from the standard curve.

2.2.6. RNA Isolation

Cells were cultured in suspension ($2x10^6$ cells). Cells were collected and centrifuged for 5 min at 300 x g in a centrifuge tube. Supernatant was removed by aspiration. The cells were disrupted by adding Buffer RLT. Buffer RLT was added the appropriate volume (see Table 2.9) and vortex to mix for 1 min.

Table 2.9: Volumes of Buffer RLT for Lysing Pelleted Cells

Number of pelleted cells	Volume of buffer RLT (µl)
$<5x10^6$	350
$5x10^6 – 1x10^7$	600

Ethanol (70%) was added 1 volume to the homogenized lysate, and mixed well by pipetting. The sample was transfered up to 700 µl, into an RNeasy spin column placed in a 2 ml collection tube. The lid was closed gently and centrifuged for 15s at 8000 x g. Buffer RW1 added in 700 µl to the RNeasy spin column. The lid was closed gently and centrifuged for 15s at 8000 x g. Buffer RPE was added in 500 µl to RNeasy spin column. The spin column membrane was centrifuged for 15s at 8000 x g to wash. Buffer RPE was added in 500 µl to the RNeasy spin column. The lid was closed gently and centrifuged for 2min at 8000 x g. the RNeasy spin column was placed in a new 1.5 ml collection tube. RNase-free water was added 30 µl directly into the spin column membrane and centrifuged for 1min at 8000 x g to elute the RNA.

2.2.7. RNA Quantification

For the RNA quantification sample tubes were set up as follows and with the components as prescribed in Table 2.10.

Quant-iT Working Solution was prepared by diluting the Quant-iT reagent 1:200 in Quant-iT buffer. 200 µl of Working Solution was required for each sample and standard. Assay Tubes were prepared according to the table below.

Table 2.10: RNA Quantification Kit ingredients and amounts (µl) required for assay.

	Standard Assay Tubes	**User Sample Assay Tubes**
Volume of Working Solution to add	190 µl	180-199 µl
Volume of Standard to add	10 µl	—
Volume of Sample to add	—	1–20 µl
Total Volume in each Assay Tube	200 µl	200 µl

All tubes were vortexed for 5s and incubated for 2min at RT. Tubes were read in Qubit fluorometer. To determine the concentration of the original samples, the instrument read values were multiplied by the dilution factor. Alternatively, Calculate sample concentration can be chosen to have the Qubit fluorometer perform this multiplication.

2.2.8. cDNA Synthesis

Template RNA was thawed on ice. gDNA wipeout buffer, quantiscript reverse transcriptase, quantiscript rt buffer, rt primer mix, and rnase-free water were thawed at RT (15–25°c). The genomic DNA elimination reaction was prepared on ice according to table 2.11 and mixed well and then stored on ice.

Table 2.11: Genomic DNA elimination reaction components

component	**volume/reaction**	**final concentration**
g DNA wipeout buffer,7x	2 µl	1x
template RNA (10pg-1µg)	variable	
RNase-free water	variable	
total volume	14 µl	

Reaction mixture was incubated for 2 min at 42°c and then placed on ice. Then, the reverse-transcription master mix was prepared on ice, according to table 2.12.

Table 2.12: Reverse-transcription reaction components

component	volume/reaction	final concentration
quantiscript-reverse transcriptase	1 µl	
quantiscript-rt buffer,5x	4µl	1x
rt primer mix	1 µl	
entire genomic DNA elimination reaction	14 µl	
total	20 µl	

Template RNA was added (14 µl) to each tube containing reverse-transcription master mix. It was mixed and then stored on ice. Master mix was incubated for 15min at 42°C and incubated for 3min at 95°C to inactivate quantiscript reverse transcriptase. Then it was stored at -20°C.

2.2.9. Reverse Transcription Polymerase Chain Reaction (RT-PCR)

RT-PCR utilizes a pair of primers, which are complementary to a defined sequence on each of the two strands of the cDNA. These primers were extended by cDNA polymerase and a copy of the strand was made after each cycle, leading to logarithmic amplification (see Figure 2.4.

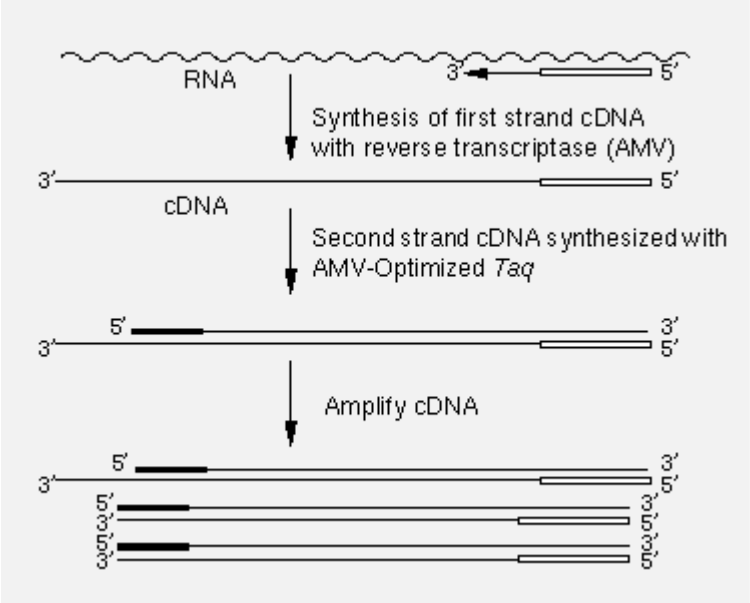

Figure 2.4: RT-PCR

PCR reactions were performed in reaction mixture of 28µl containing the components in Table 2.13.

Table 2.13: PCR solutions and their initial final concentrations with final volumes.

Reagent	**Initial Concentration**	**Final Concentration**	**Final Volume**
Taq Buffer	10X	1X	2.5 µl
dNTP	2 mM	0.2 µM	1.5 µl
$MgCl_2$	25 mM	2 mM	2.5 µl
Primers (x2)	12,5 pmol/µl	12,5 pmol	2 µl
ddH2O	-	-	16.8 µl
Taq DNA Polymerase	5 U/µl	1 U	0.2 µl
cDNA			2.5 µl
Total Reaction Volume			28 µl

The denaturation of the dsDNA at 94°C 1min, so that the two strands separated and the primers bound again at lower temperatures and began a new reaction. Then, the temperature decreased until it reaches the annealing temperature (see table 2.14).

Table 2.14: PCR procedure of Primers

Primers	Denaturation / time	Annealing temp./ time	Extension / time	Number of cycle
IL5	94 C 1min	59 C 1min	72 C 1min	36
IFNγ	94 C 1min	55 C 2min	72 C 1min	35
IL4	94 C 1min	65 C 2min	72 C 1min	37

The final step of PCR amplification is cDNA extension from the primers. This was done with thermostable Taq DNA polymerase, usually at 72°C, the temperature at which the enzyme works optimally. The length of the incubation at each temperature, the temperature alterations, and the number of cycles were controlled by a programmable thermal cycler.

2.2.9.1. Agarose Gel Electrophoresis

PCR products were resolved on 2% agarose gels. Gel is prepared adding 1.6 gram of powdered agarose gel into 80ml of 0.5 x TBE buffer solution and it is boiled until the agarose is completely dissolved in the buffer solution. Four µl of Ethidium Bromide was added when the boiled solutions began to cool down and reach approximately 55ºC. Solution was mixed homogenously by making hand-shaking. It was directly poured into horizontal agarose gel platform and 20 wells were placed one side of the gel. Gels were let to solidify for at least 10 minutes, to confirm full polymerization 45 min.

2.2.9.2. Loading and visualization of the gels

14 µl of PCR product was mixed with 2 µl bromophenolblue as loading dye/buffer. 16 µl PCR mixes were then loaded in each slot with appropriate micropipettes. 3µl of a 100 bp DNA Ladder (MBI Fermentas, USA) was mixed with 1 µl bromophenolblue. Then 4 µl of this mix was put into usually the first slot as a molecular size marker. The gel was run at 120 V in 0.5X TBE buffer for 45 min. The gel was placed in Gel Doc 2000 (Bio-Rad, Italy) apparatus and the bands were detected under UV transilluminator.

2.2.10. Folin Ciocalteu Method

Folin-Ciocalteu's phenol reagent was diluted at a volume ratio of 1:3 with distilled water prior to use. Lowry A solution was prepared from sodium carbonate such that the strength (w/v) of Na_2CO_3 in 0.1 M NaOH solution was 2%. Lowry B solution was prepared from copper (II) sulfate such that the strength (w/v) of $CuSO_4$ in 1% sodium potassium tartrate ($NaKC_4H_4O_6$) solution was 0.5%. Lowry C solution was prepared by freshly mixing 50 ml Lowry A with 1 ml Lowry B. *Nigella sativa* extract was mixed with H_2O. An aliquot of 2.5 ml Lowry C solution was added, and the mixtures were incubated for 10 min at RT. After that, 0.25 ml Folin reagent was added, and they were incubated for 30 more min at RT to get blue color. The absorbances of these extracts against a reagent blank were measured at 750 nm. Gallic acid was used as the reference standard.

2.2.11. CUPRAC (Cupric Ion Reducing Antioxidant Capacity)

The CUPRAC method as described by Apak et al. (2004), was applied as follows: Each mixture comprised of 1 ml of $1.0x10^{-2}$ M copper(II) chloride, 1 ml of $7.5x10^{-3}$ M neocuproine solution and 1 ml of 1 M ammonium acetate buffer at pH 7.0 was prepared, *Ns* extract and distilled water were added, and they were mixed (total volume: 4.0 ml). These final mixtures in test tubes were incubated for 30 min at RT. At the end of this period, the absorbances at 450 nm were measured against a reagent blank. Trolox was used as a reference standard.

2.2.12. Chromatographic Analysis of *Nigella sativa* Extracts (HPLC)

High-performance liquid chromatography (HPLC) is a form of liquid chromatography to separate compounds that are dissolved in solution. HPLC instruments consist of a reservoir of mobile phase, a pump, an injector, a separation column, and a detector (Figure 2.5).

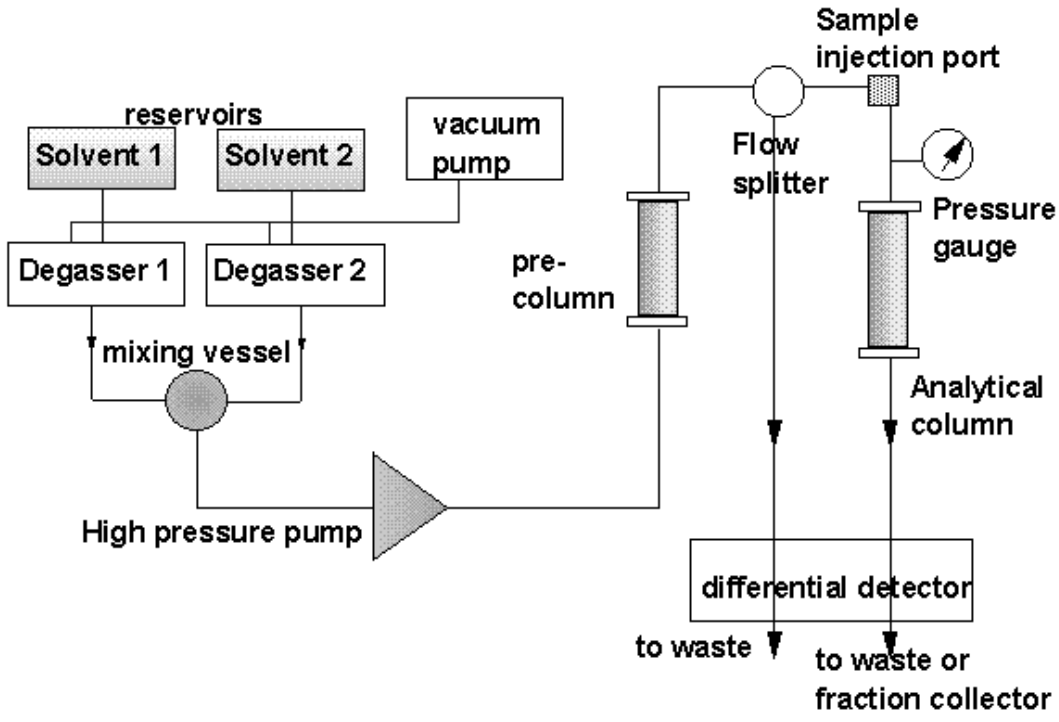

Figure 2.5: Schematic of an HPLC instrument

The HPLC method developed for phenolic constituents:

Gradient elution system was applied, using a binary solvent mobile phase comprised of methanol (A) and % 0.2 *o*-H_3PO_4(v/v)−aqueous solution (B).

8min 7% (A), slope (0.0);

8–13min to 30% (A), slope (−4.0);

13–48min to 66% (A), slope (1.0);

48–55 min to 75% (A), slope (−4.0).

The flow rate was 1 ml min^{-1}, and the detection wavelength was 280 nm.

CHAPTER 3

RESULTS

3.1. GROWTH CURVE ANALYSIS

In this study peripheral blood mononuclear cells isolated from heparinized peripheral blood. The cell developments in two different conditions were described by plotting a growth curve. First condition was cells alone in the medium, second condition was cells interact with antigen Ag Der p 1 and Der f 1 (5µg/ml/each). Growth curves were drawn for 5 days. All wells started with an equal cell number. Time-course dependency of cell growth curve is given in figure 3.1.

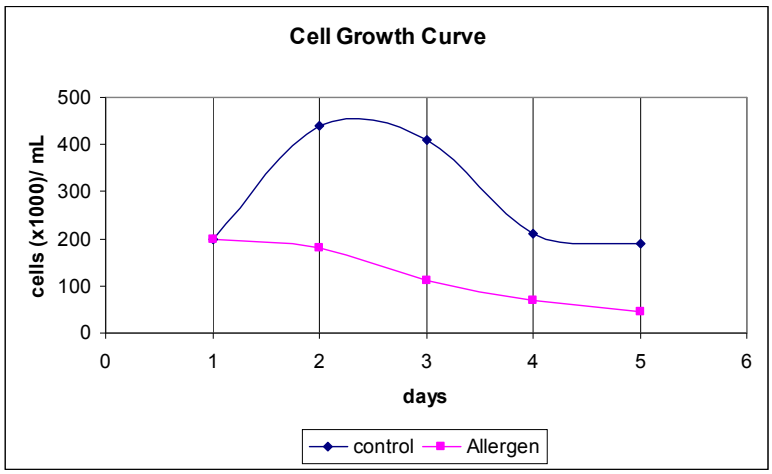

Figure 3.1: Cumulative doubling time of PBMCs

3.2. DETERMINING THE NON-TOXIC AND PROLIFERATIVE CONCENTRATION OF NIGELLA SATIVA HYDROPHILIC EXTRACT

Cell culture repeated as described previously. Cells cultured in 96 well plate (flat bottom) and cell suspension was adjusted $2x10^5$ cell / 200µl for each well. During 24h the cells kept at 37°C and 5% CO_2. After 24 hours, relevant concentrations were added into the

wells. Both (toxicity and proliferative) of values found and six different concentrations were followed for 3 days with their controls. Each concentrations were studied at duplicated wells. In here, Control means cells alone in their medium. Cell culture toxicity test were done at day 3, 4 and 5.

3.2.1. Cytoxicity Test Results of Hydrophilic Extract

First of all, working solutions prepared as described previously. Then cells were collected into centrifuge tubes and labeled their specifications. Cells were centrifuged at 250×*g* for 10 min. The absorbance value was measured. Supernatant was retrieved 100µl from each tube and added to a new sterile 96 well plate (flat bottom). Reaction mixture was added 100 µl to each well and incubated for 30 min at RT. The absorbance was measured at 490nm with ELISA reader. Reference wavelength was 690nm. During the three days, this process was repeated same way. Considering the average of the two values, the results are (Figure 3.2);

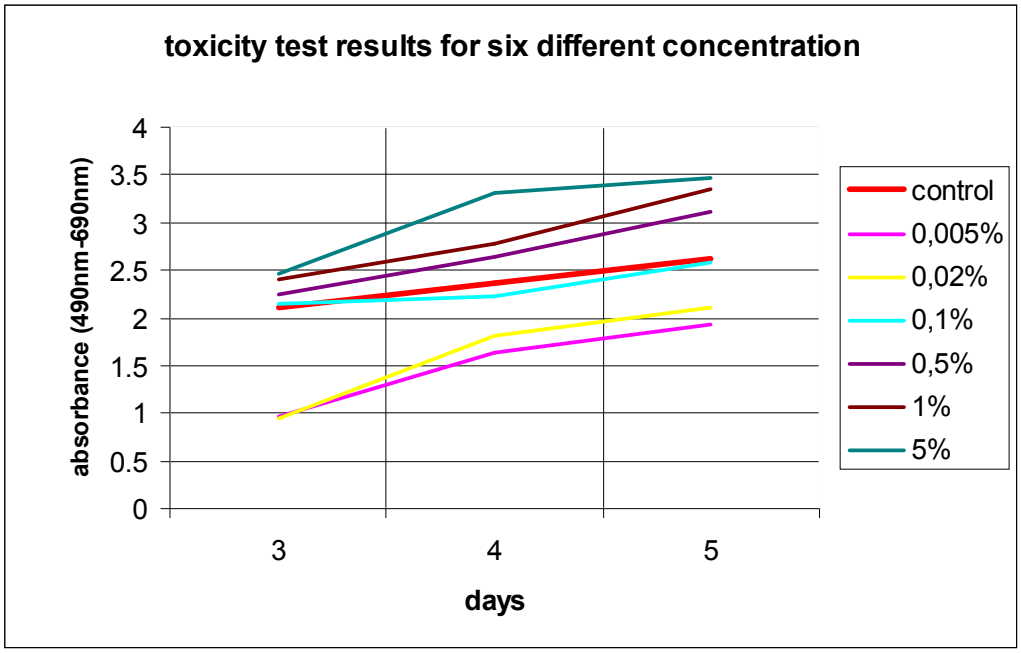

Figure 3.2: Cytoxicity test Results average for 6 different concentration

3.2.2. Proliferation Assay Result

WST-1 was added 20 µl/well into 96 wells (flat bottom) with cultured cells. The cells incubated for 4h at 37°C, 5% CO_2 and shaked for 1 min on a shaker. The absorbance was measured at 420 nm. The reference wavelength was 690 nm. Considering the average of the 7 values, the results are (Figure 3.3);

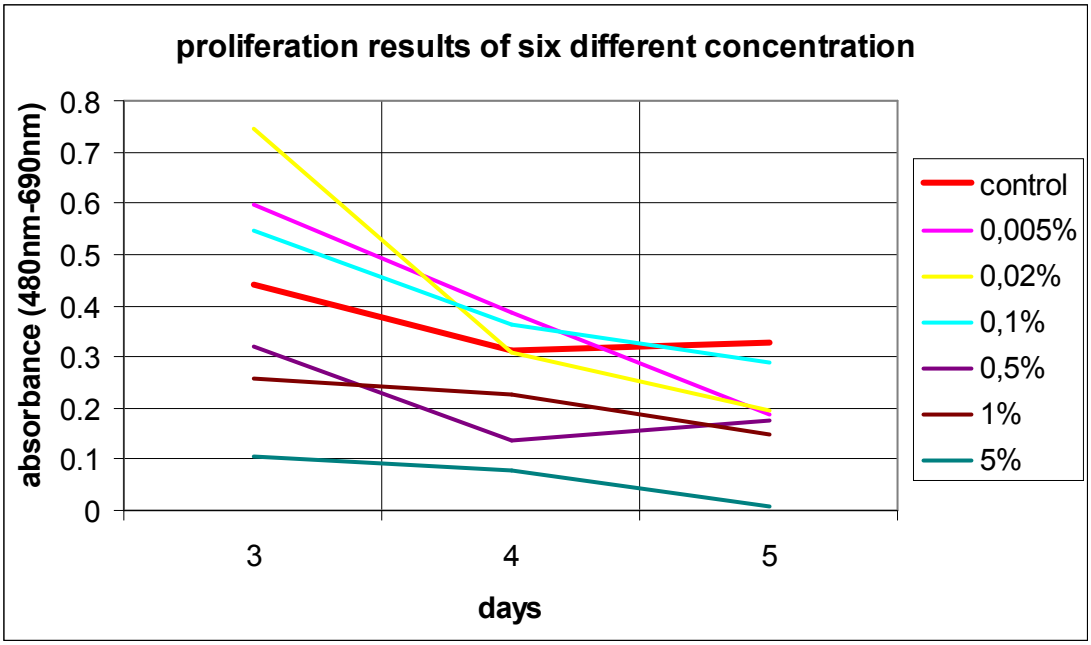

Figure 3.3: Proliferation Assay Results average for 6 different concentrations

3.3. ELISAs

Cell culture repeated as described previously. Cells were cultured in 24 well plate and adjusted cell suspension $2x10^6$ cell / 2ml for each well. During 24 hours the cells kept at 37°C and 5% CO_2. After 24 hours, 0,1% extract concentrations and 10µg/mL (755AU/mL) antigen (Ag Der p 1 and Der f 1) were added into the wells. In here, there were four different well type for each 5 day. Then every day supernatants and cells were collected into different tubes and kept at -20°C for ELISA and other experiments. We had two different groups in our work. Both groups had one allergy patient and one non-allergic person. Prepared wash buffer, SAV-HRP as described previously (see chapter 2).

3.3.1. Hu-IL5 ELISA

Made standard dilution on the microwell plate as described previously. Samples and sample diluent buffer were added into the wells and incubated for 2h. Biotin-conjugate was added to all wells and incubated for 30 min at RT. Wells were washed four times. SAV-HRP was added and incubated 30 min. The solution was aspirated from wells and discarded the liquid. Wells were washed four times. Stabilized Chromogen was added 100 µl to each well and incubated for 30 min at RT and in the dark. Stop solution was added. The absorbance was measured at 450 nm. Our reference wavelength was 620nm. Microsoft office excel was used for generate the standard curve. Considering the standard curve, our sample concentration results are;

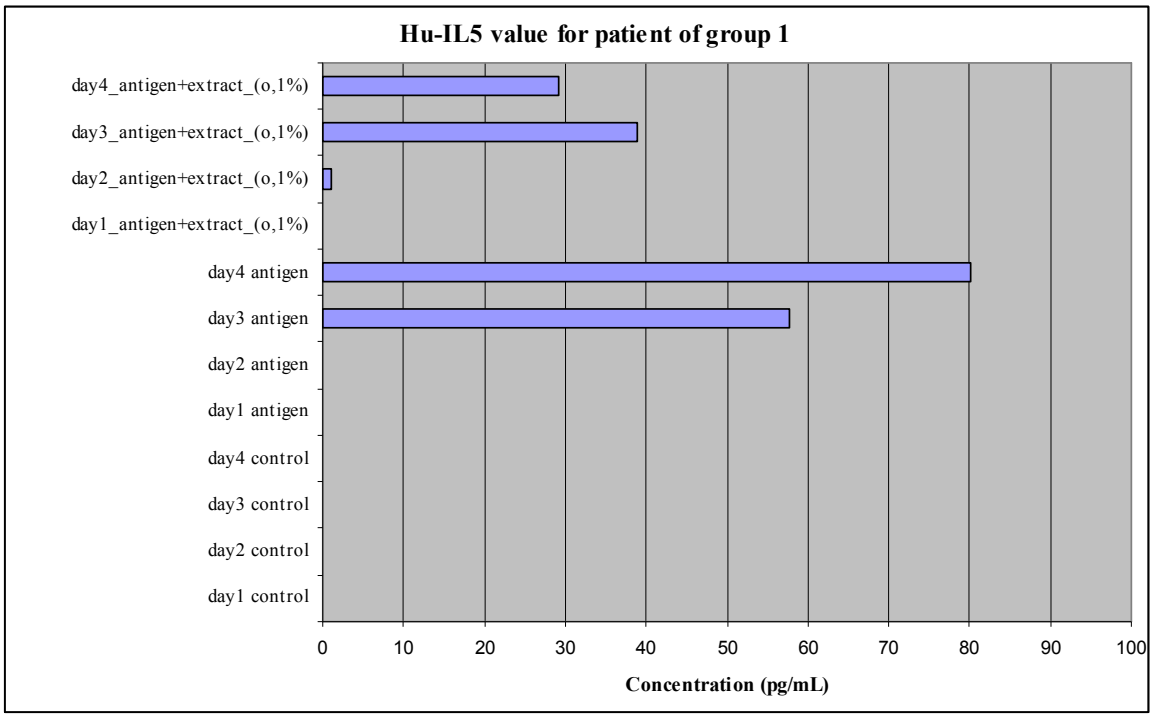

Figure 3.4: Hu-IL5 value for patient group 1

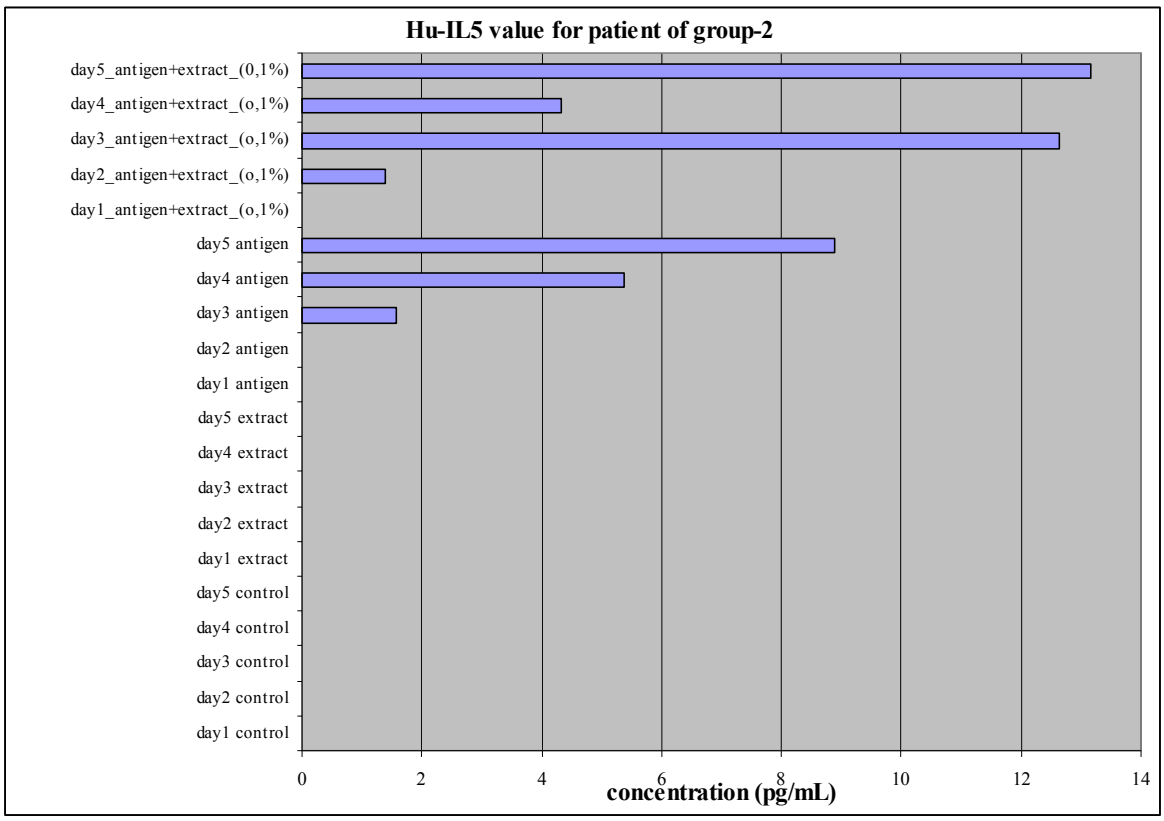

Figure 3.5: Hu-IL5 value for patient group 2

In our non-allergic group we had two way; control group and extract group. Cells didn't release IL5 in both groups.

3.3.2. Hu-IFNγ ELISA

Made standard dilution on the microwell plate as described previously. Samples and sample diluent buffer were added into the wells and incubated for 2h. Biotin-conjugate was added to all wells and incubated for 30 min at RT. Wells were washed four times. SAV-HRP was added and incubated 30 min. The solution was aspirated from wells and discarded the liquid. Wells were washed four times. Stabilized Chromogen was added 100 µl to each well and incubated for 30 minutes at RT and in the dark. Stop solution was added. The absorbance was measured at 450 nm. Our reference wavelength was 620nm. Microsoft office excel was used for generate the standard curve. Considering the standard curve, our sample concentration results are;

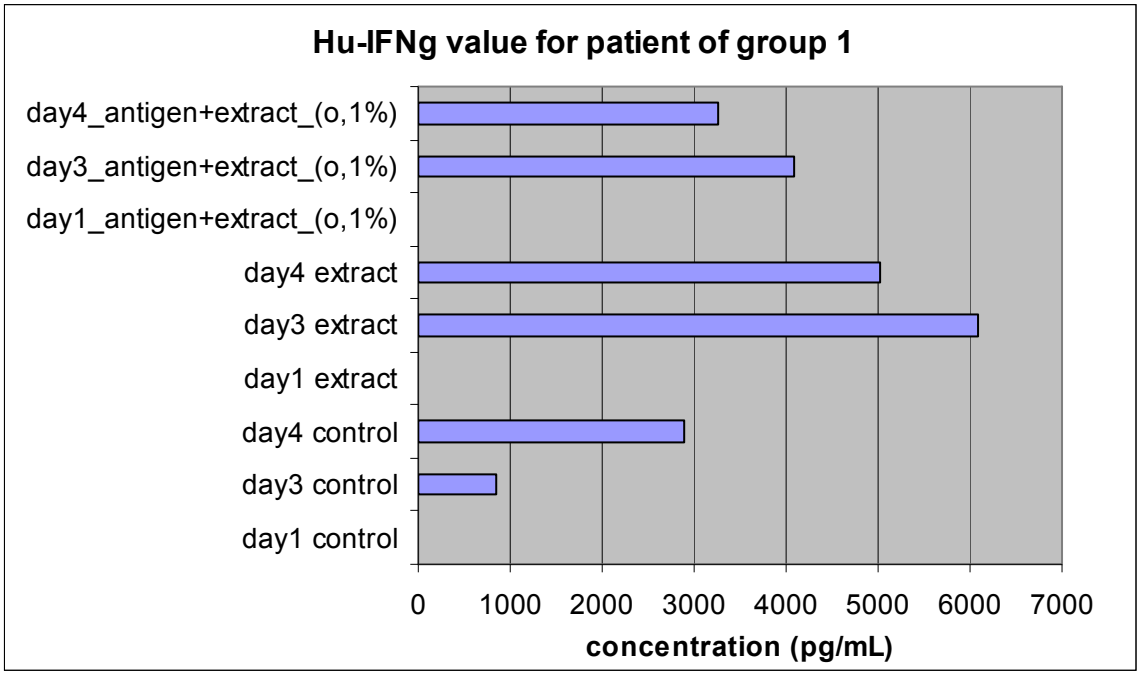

Figure 3.6: Hu-IFNγ value for patient group 1

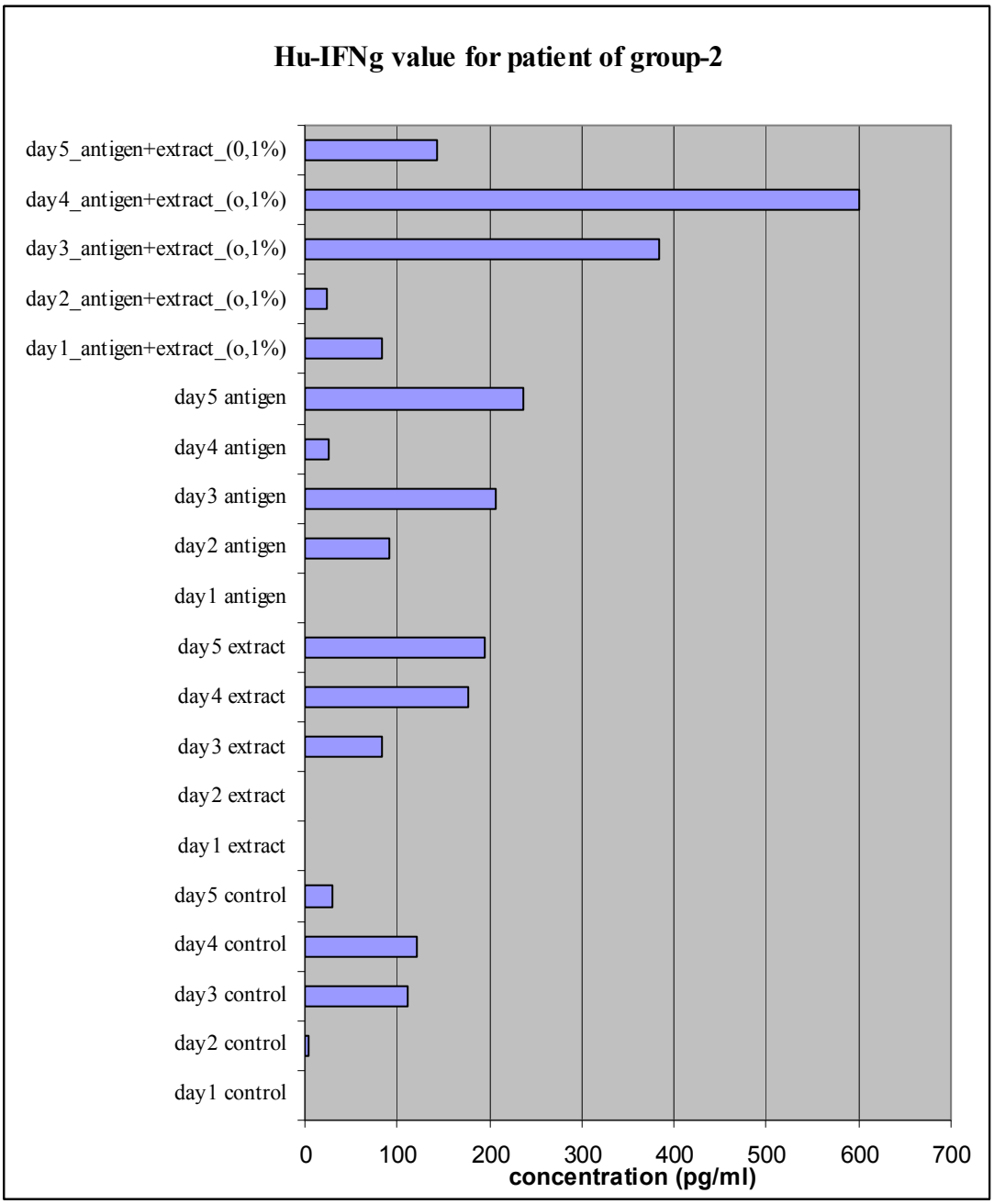

Figure 3.7: Hu-IFNγ value for patient group 2

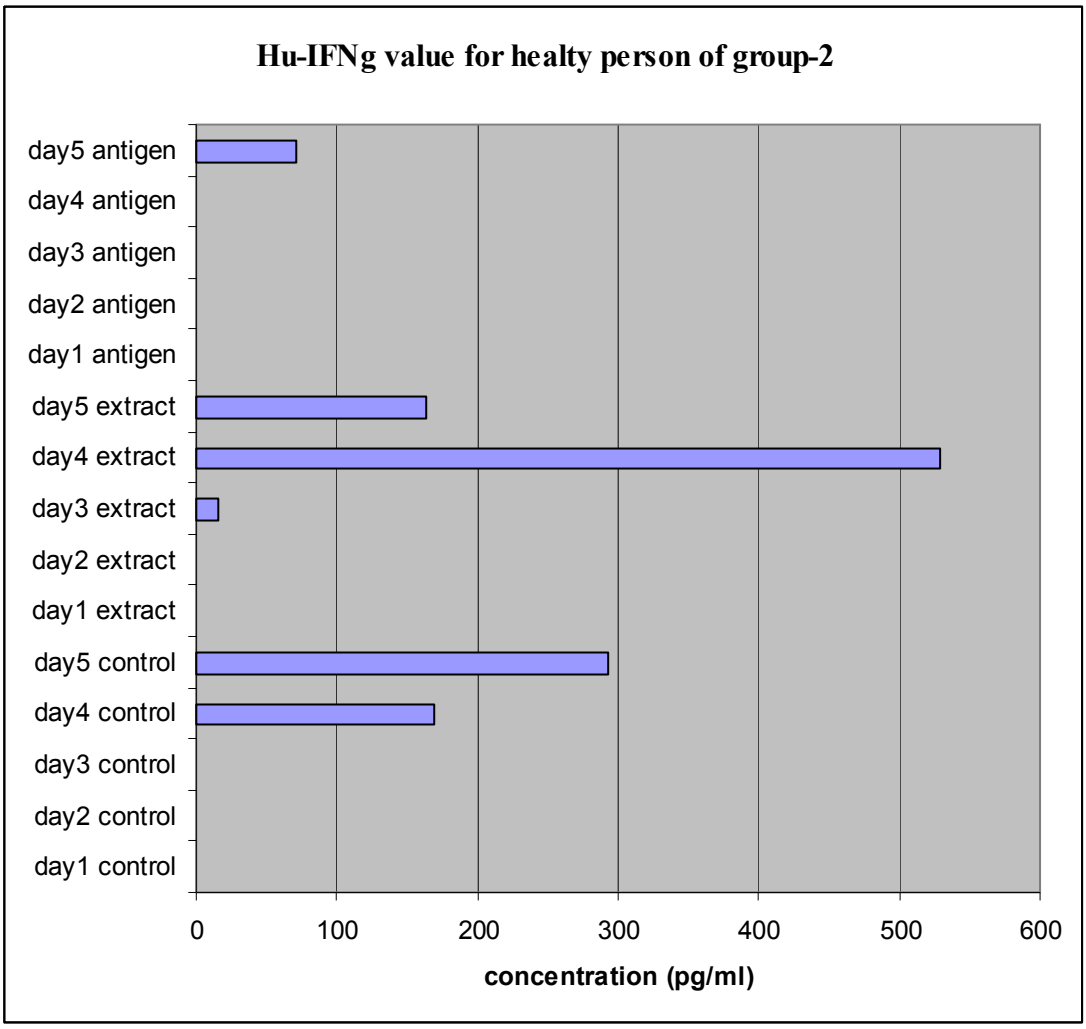

Figure 3.8: Hu-IFNγ value for non-allergic person group 2

In our non-allergic group 2; control group and extract group cells releases IFNγ in both groups.

3.3.3. Hu-IL4 ELISA

Made standard dilution on the microwell plate as described previously. Samples and sample diluent buffer were added into the wells and incubated for 2h. Biotin-conjugate was added to all wells and incubated for 30 min at RT. Wells were washed four times. SAV-HRP was added and incubated 30 min. The solution aspirated from wells and discarded the liquid. Wells were washed four times. Stabilized Chromogen was added 100 µl to each well and incubated for 30 min at RT and in the dark. Stop solution was added. The absorbance was measured at 450 nm. Our reference wavelength was 620nm. Microsoft office excel was used for generate the standard curve. Considering the standard curve, our sample concentration results are;

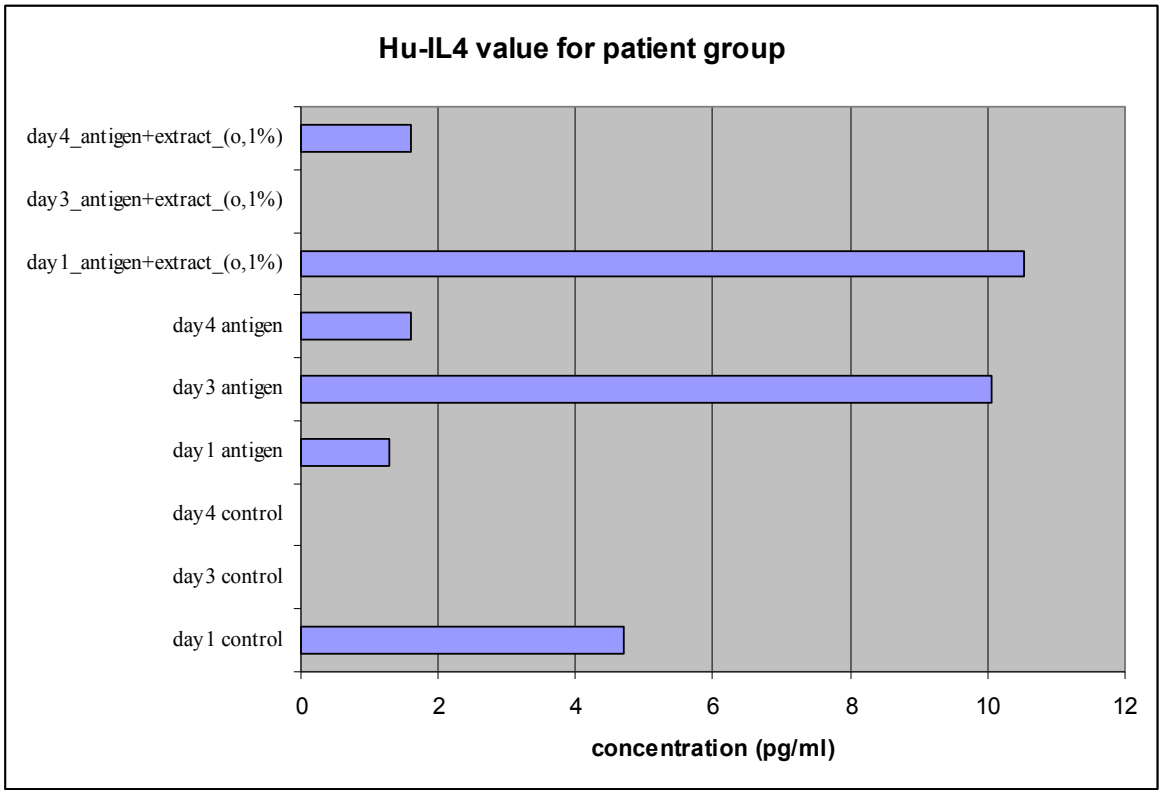

Figure 3.9: Hu-IL4 value for patient group

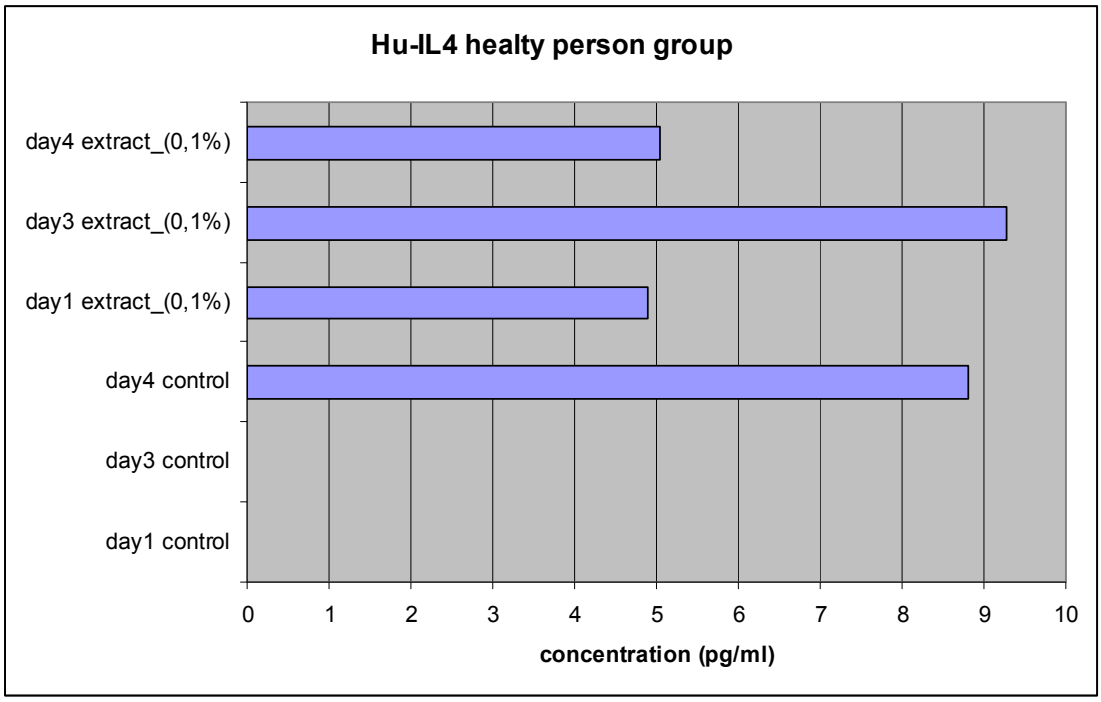

Figure 3.10: Hu-IL4 value for non-allergic group

3.4. EXPRESSION ASSAYs

The cells were cultured and stored at -20°C. Then cells were thawed. Their RNAs were isolated as described previously. Qubit fluorometer was used for determining RNA concentration values.

cDNAs were synthesized from template RNA as described previously. Then cells were stored at -20°C. PCR reactions were performed in reaction mixture as described previously. In here, we were used 3 different primers for our RT-PCRs. The length of the incubation at each temperature, the temperature alterations, and the number of cycles were controlled by a programmable thermal cycler.

PCR products were resolved on 2% agarose gels. PCR product was mixed with bromophenolblue as loading dye/buffer. The gel was run at 120 V in 0.5X TBE buffer for 45 min. The bands were detected under UV transilluminator. The expression results are;

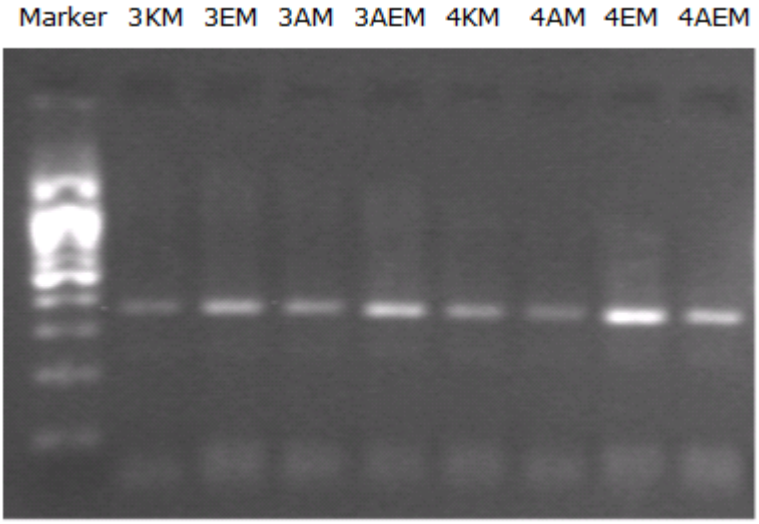

Figure 3.11. Hu-IFNγ Expression Results for patient group-1

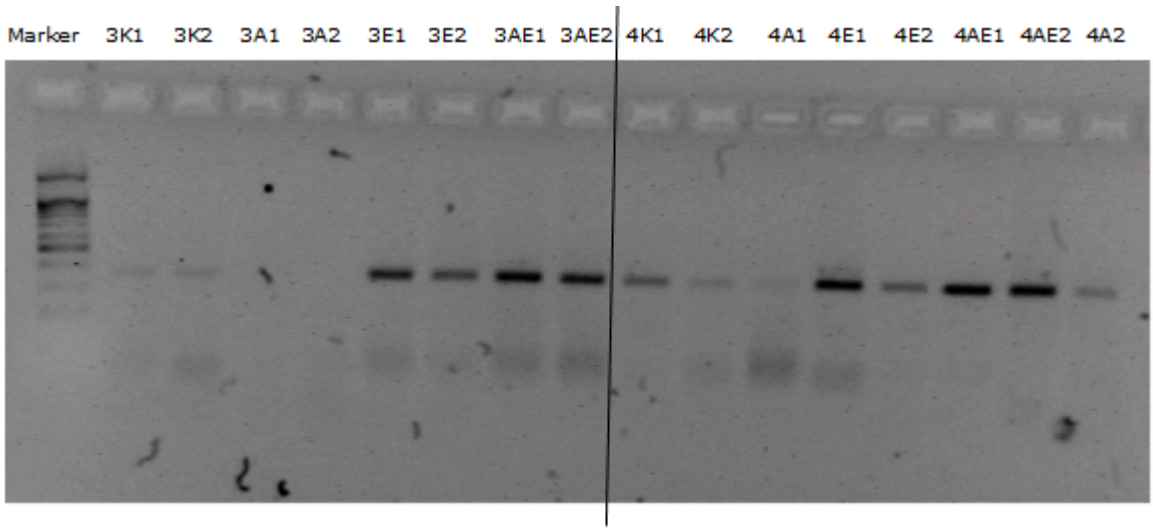

Figure 3.12. Hu-IFNγ Expression Results for patient group-2

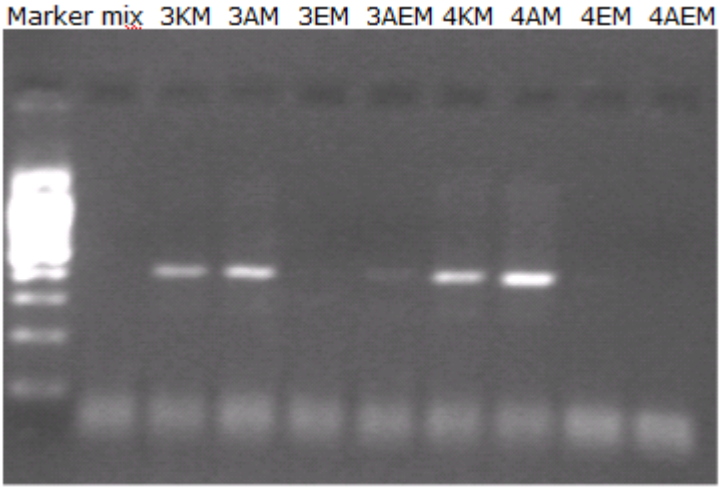

Figure 3.13. Hu-IL5 Expression Results for patient group-1

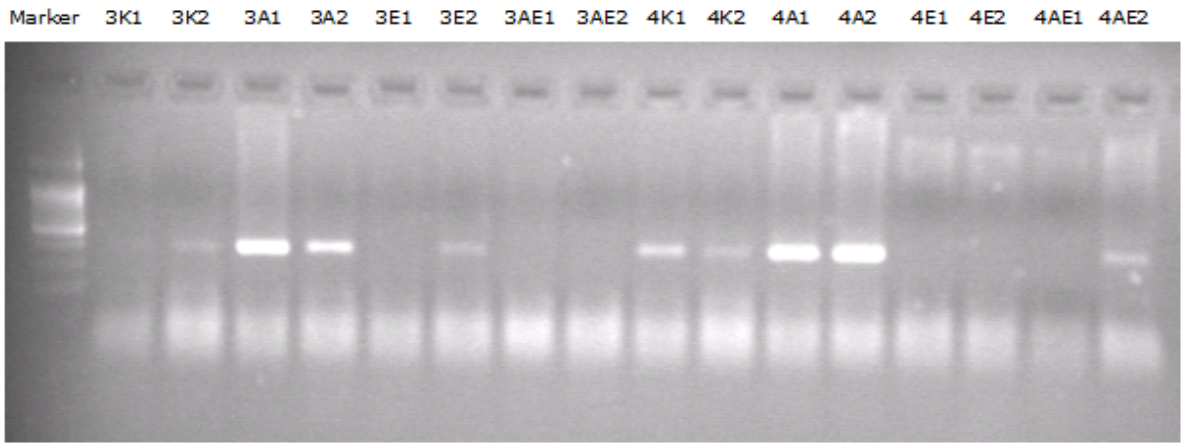

Figure 3.14.Hu-IL5 Expression Results for patient group-2

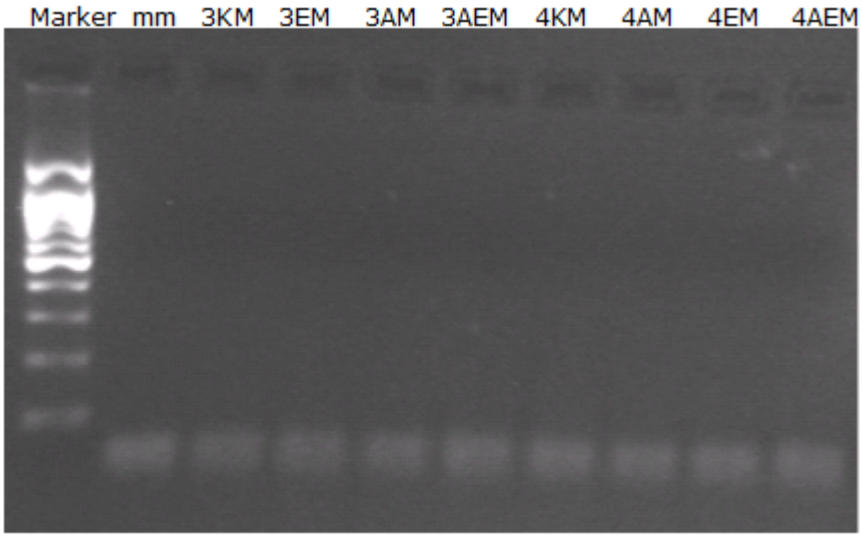

Figure 3.15. Hu-IL4 Expression Results for patient group-1

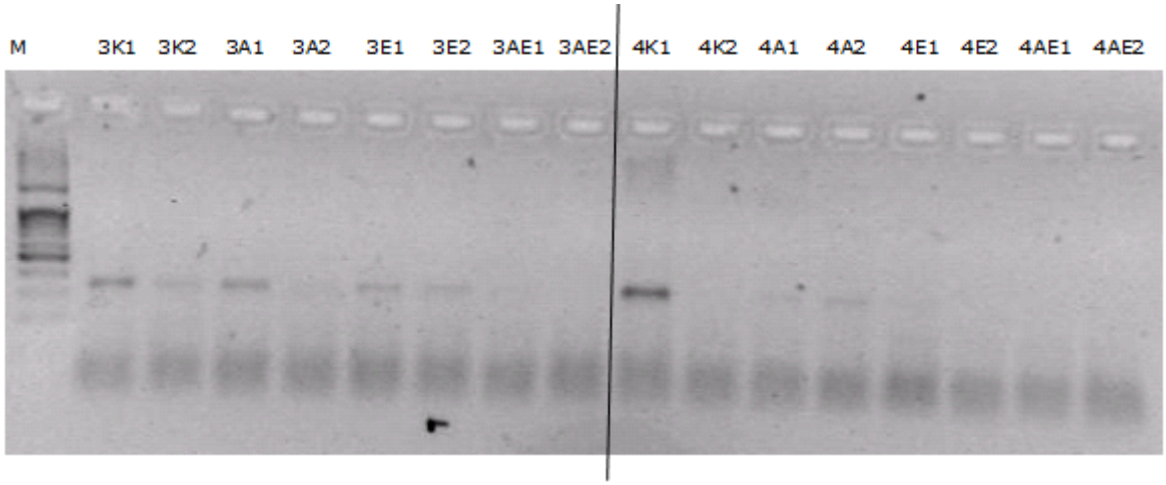

Figure 3.16.Hu-IL4 Expression Results for patient group-2;

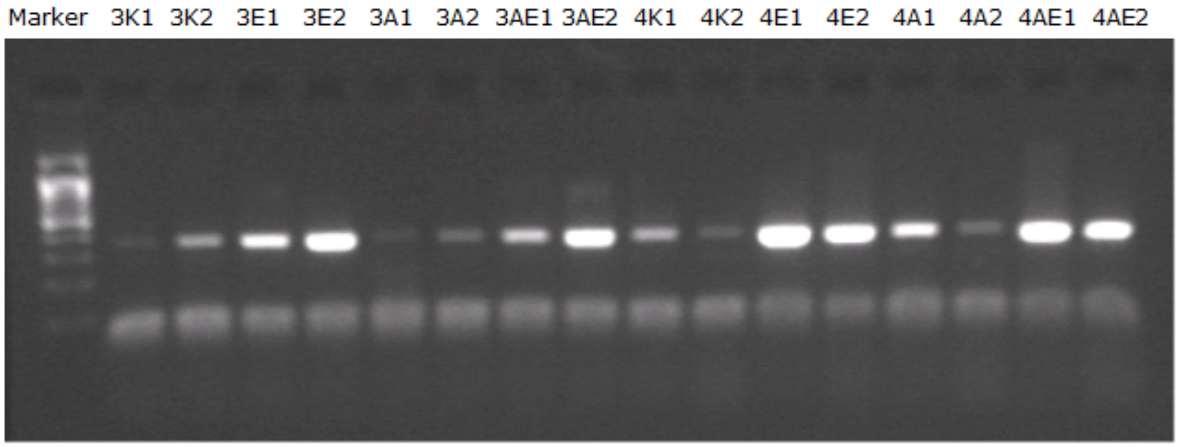

Figure 3.17.Hu-IFNγ Expression Results for healthy person of group-1;

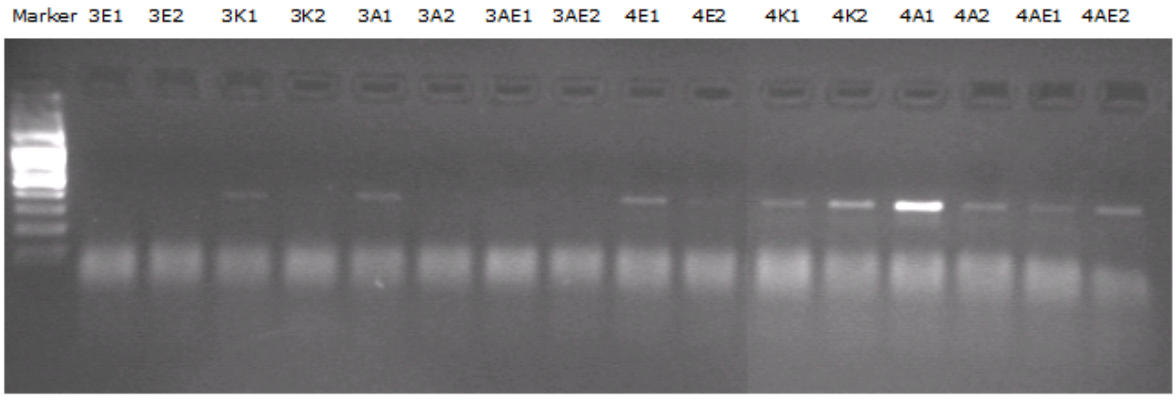

Figure 3.18.Hu-IL5 Expression Results for healthy person of group

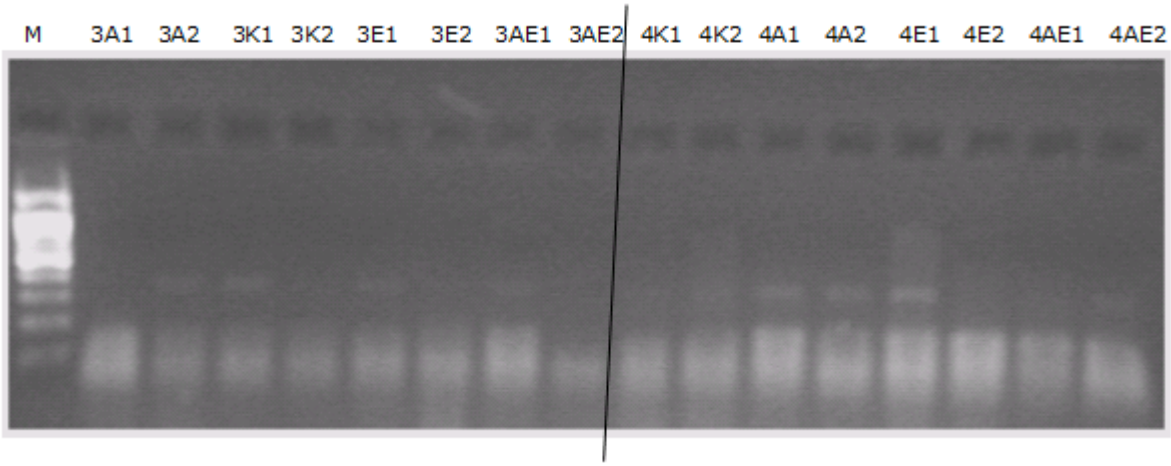

Figure 3.19.Hu-IL4 Expression Results for healthy person group

3.5. CHEMICAL ANALYSIS RESULT

3.5.1. Folin Ciocalteu Results

Absorbance values were used for total phenolic compounds quantity. The results are;

0,1:9 dilution from *Ns* extract → used o,6 ml → A_{660}

Each amount was duplicated.

1. 0,6 ml A_{660} = 0,431
2. 0,6 ml A_{660} = 0,387

0,1:9 dilution from *Ns* extract → used 0,8 ml → A_{660}

Each amount was duplicated.

1. 0,8 ml A_{660} = 0,501
2. 0,8 ml A_{660} = 0,490

0,1:9 dilution from *Ns* extract → used 1 ml → A_{660}

1 ml A_{660} = 0,602

The absorbance average was calculated for each quantity.

For 0,6 ml → A_{avg} = 0,409

For 0,8 ml → A_{avg} = 0,4955

(For Folin Ciocalteu A_{trolox}= 3254 trolox)

A_{avg} / A_{trolox} **x** dilution factor **x** total volume/amount used **x** amount of total extract/amount of solvent = Total phenolic compounds quantity

for 0,6 ml ;

0,409/3254 x 9/0,1 x 4,75/0,8 x 5/5 = 0,089 mmol Tr/g

for 0,8 ml ;

0,4955/3254 x 9/0,1 x 4,75/0,8 x 5/5 = 0,8137 mmol Tr/g

for 1 ml;

0,602/3254 x 9/0,1 x 4,75/1 x 5/5 = 0,079 mmol Tr/g

3.5.2. CUPRAC Results

Absorbance values were used for total phenolic compounds quantity. The results are;

0,1:9 dilution from *Ns* extract → used o,5 ml → A_{450}

Each amount was duplicated.

1. 0,5 ml A_{450} = 0,315
2. 0,5 ml A_{450} = 0,332

0,1:9 dilution from *Ns* extract → used 1 ml → A_{450}

Each amount was duplicated.

1. 1 ml A_{450} = 0,571
2. 1 ml A_{450} = 0,562

The absorbance average was calculated for each quantity.

For 0,5 ml → A_{avg} = 0,3235

For 1 ml → A_{avg} = 0,5665

(For CUPRAC A_{trolox}= 16200 trolox)

A_{avg} / A_{trolox} **x** dilution factor **x** total volume/amount used **x** amount of total extract/amount of solvent = Total antioxidant capacity

0,5 ml için;

0,3235/16200 x 9/0,1 x 4/0,5 x 5/5 = 0,0144 mmol Tr/g

1 ml için;

0,5665/16200 x 9/0,1 x 4/1 x 5/5 = 0,0126 mmol Tr/g

3.5.3. HPLC Result

The analyses were carried out using a Hamilton HxSil C18 (250mm×4.6mm, 5µm particle size) chromatographic column. The HPLC method was developed for phenolic constituents: Gradient elution system was applied, using a binary solvent mobile phase comprised of methanol (A) and % 0.2 *o*-H_3PO_4(v/v)−aqueous solution (B).

8min 7% (A), slope (0.0);

8–13min to 30% (A), slope (−4.0);

13–48min to 66% (A), slope (1.0);

48–55 min to 75% (A), slope (−4.0).

The flow rate was 1 ml min^{-1}, and the detection wavelength was 280 nm. Chromatogram of a synthetic mixture of the tested antioxidants showed below (Figure 3.20.);

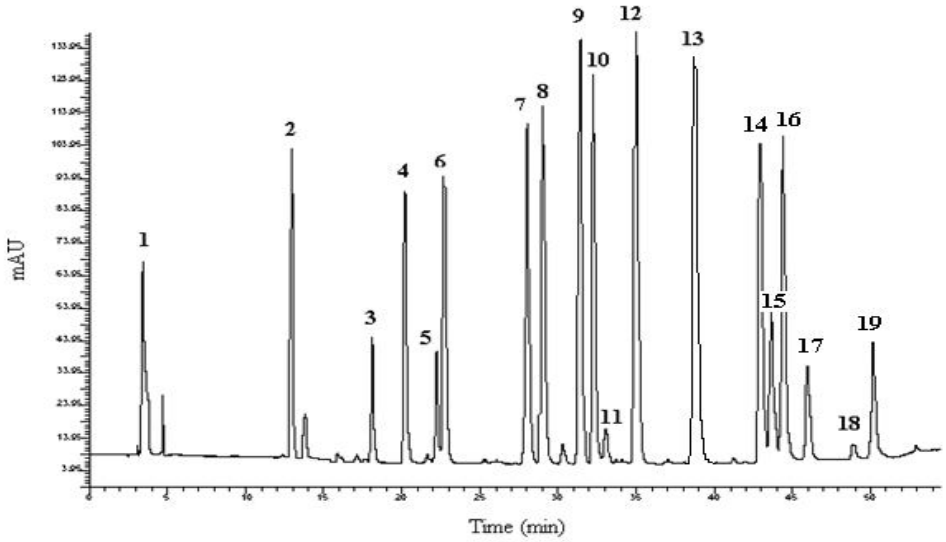

Figure 3.20. Chromatogram base-line of standarts. (1: Ascorbic acid; 2: gallic acid; 3: catechin; 4: chlorogenic acid; 5: epicatechin; 6: caffeic acid; 7: *p*-coumaric acid; 8: ferulic acid + sinapic acid; 9: naringin; 10: rosmarinic acid; 11: hesperidin; 12: rutin + isoquercitrin; 13: myricetin; 14: naringenin; 15: hesperetin; 16: quercetin; 17: luteolin; 18: kaempferol; 19: apigenin.).

Chromatogram of *Ns* hydrophilic extract showed in the graph below (Figure 3.21.);

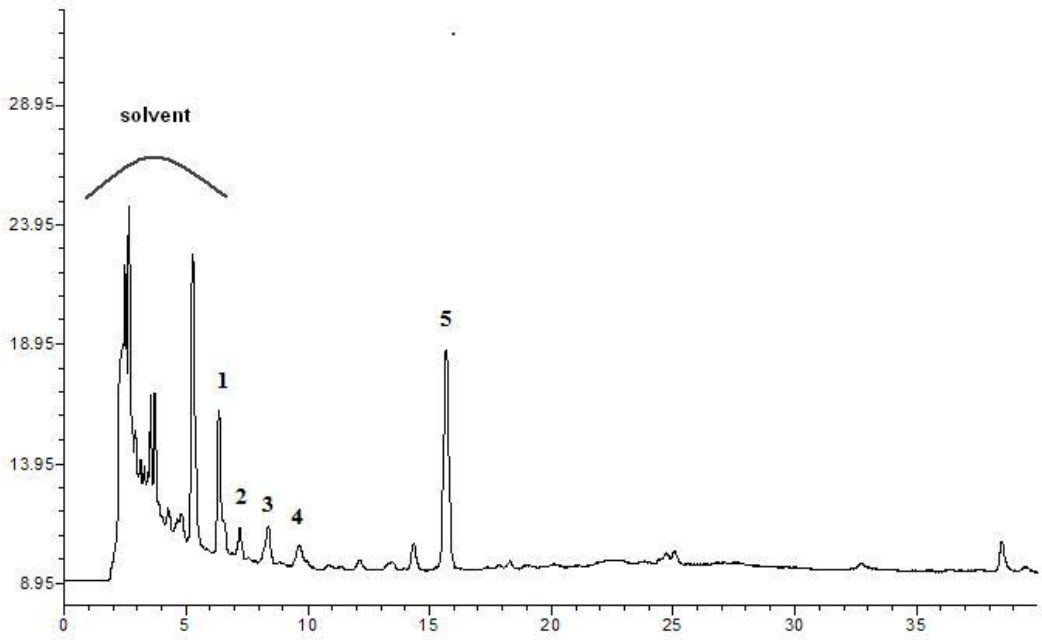

Table 3.22: HPLC Results for *Ns* hyrophilic extract (catechin (1) , chlorogenic acid (2), epicatechin (3), caffeic acid (4), *p*-coumaric acid (5)).

CHAPTER 4

DISCUSSION

We used a cell culture system where we can identify the effects of *Ns* on the immune system. Firstly we determined the non-toxic concentration of our *Ns* hydrophilic extract. We were used six different concentrations and also measured the proliferative effect of these concentrations. According to our results; 0,1% concentration was one of the non-toxic (figure 3.2) and proliferative value (figure 3.3), depending on its control value (see).

After those results, we were calculated the cytokine levels of our cells with ELISA experiments. We were used two patients and two healthy persons blood for this process. Then we were cultured the cells with extract (0,1% concentration) and antigen (10µg/ml) and every day collected the supernatants. First results for IL5-IL4-IFNγ ELISAs experiments; we were measured the cytokine quantities.

According to our IL5 ELISA results; in figure 3.4 that result belong to the patient group 1. We were used three way, first we were added the antigen and extract together. Second we were added the only antigen. Third, cells alone in their medium. Control gruop didn't release IL5 which means there was no contamination or antigen exposure. In antigen group third and fourt days of the culture cells releases IL5 which means antigen exposure causes allergic inflammation also Th2 type response was activated. In figure 3.5 that result belong to second patient group. We were used 4 way, first we were added the antigen and extract together. Second we were added the only antigen. Third we were added the only extract. Fourth cells alone in their medium. Control gruop didn't release IL5 which means

there was no contamination or antigen exposure. In extract group cells didn't releases IL5 which means there was no allergic effect of extract. In antigen group third, fourt and fifth days of the culture cells releases IL5 which means antigen exposure causes allergic inflammation also Th2 type response activated. Also we were measured non-allergic persons IL5 value. In our non-allergic group we had two way; control group and extract group. Cells didn't release IL5 in both groups. In control gruop there was no contamination or antigen exposure. In extract group there was no allergic effect of extract.

Both antigen and extract group third days of the culture, cells releases IL5 less than the antigen groups IL5 value which means Th2 type response changes its way to Th1 type response. In fifth days Th2 type response was activated again because our extract degradate.

According to our IFNγ ELISA results; in figure 3.6; that results belong to the patient group1. We were used three way, first we were added the antigen and extract together. Second we were added the only extract. Third cells alone in their medium. Control gruop release IFNgama which means there were maybe contamination or something we dont know dragged our cells TH1 type response. We didn't want to see that normally. In extract group third and fourth days of the culture cell releases IFNγ which means extract has non supressive effect on cells without allergen exposure. In antigen and extract group third and fourth days of the culture, cells releases IFNγ which means cells Th1 type response was activated. In figure 3.7; that result belong to the patient group 2. We were used four way, first we were added the antigen and extract together. Second we were added the only antigen. Third we were added the only antigen. Fourth cells alone in their medium. Control group releases IFNγ which means there were maybe contamination or something we dont know dragged our cells TH1 type response. We didn't want to see that normally. In antigen group cells releases IFNγ against the antigen. In extract group third, fourth and fifth days of the culture, cell releases IFNγ which means extract has non supressive effect on cells without allergen exposure. In antigen and extract group third,

fourth and fifth days of the culture, cells releases too high IFNγ which means Th1 type response was activated well. In figure 3.8; that result belong to the healty person group. We were used three way, first cells alone in their medium. Second we were added the extract. Third we were added the antigen only. Control gruop releases IFNγ. In extract group cells releases IFNγ too high than the control group which means oue extract has non-supressive effect on cells for releasing IFNγ.

According to our IL4 ELISA results; in figure 3.9 and 3.10; those results belong to the patient and non-allergic group. We were used three way, first we were added the antigen and extract together. Second we were added the only antigen. Third cells alone in their medium. Our IL4 values is too low when we compared the litreture and according to those results we choose ignore these results. In literature they were usually used stimulator for increase IL4 value.

After these results, we used the cultured cells for cytokine expressions. We used the same patients and healthy person blood for this. Then we cultured the cells again with extract (0,1% concentration) and antigen (10μg/ml). Then we were collected the cells for this experiment. We were isolated their RNA and then synthesied cDNA with the same RNA concentration. Then we were applied RT-PCR.

According to our IFNγ in figure 3.11; we have third and the fourth days of the culture cells expression results. That was belong to the patient group 1. This results show us, our extract has non supressive effect on cells without allergen exposure. When we compared third and the fourth days extract results, we can see significant effect on releasing IFNγ. In figure 3.12; we were duplicated the samples. We have third and the fourth days of culture cells expression results. That was belong to the patient group 2. This results show us, our extract has non supressive effect on cells without allergen exposure. Also allergen and extract were applied together and we can see significant effect on releasing IFNγ. In figure 3.17; we were duplicated the samples again. We have third and the fourth days of the culture cells expression results. That was belong to the healty person

group. In extract group, there were significant effect of *Ns* extract on IFNγ synthesis which means our extract has non supressive effect on cells without allergen exposure.

According to our IL5 expression results; we have third and the fourth days of culture cells expression results (figure 3.13). That was belong to the patient group 1. Third and fourth day antigen results; we exposured the cells to the allergen and this was a proof that antigen values was enough for allergic response. Also third and fourth day antigen+extract cells result; show us IL5 release supressed with extract. In figure 3.14; We have third and the fourth days of culture cells expression results and duplicated the samples in here too. That was belong to the patient group 2. This result show us, third and fourth day antigen results; we exposured the cells to the allergen which means antigen value that we use, is enough for allergic response. Also antigen+extract cells result; show us IL5 releases supressed with extract in both days. In figure 3.18; that was belong to the healty person group. Antigen group of the fourth day cell result, cells effected with antigen exposure. This was not what we expected but in the third day, it seems Th2 type response supressed with extract.

According to our IL4 expression results; our extract has no significant effect on cells, for IL4. Also we didn't see any effect with ELISA either (figure 3.15, 3.16, 3.19)

According to our first results (ELISAs result) we investigated that IL5-IL4-IFNγ Expressions (Table 4.5-4.6-4.7); then we showed the cytokine expressions (see chapter 3). We used the Quantity One 1D analyses programme for comparing our bands.

The total phenolic content of this extract; 0,08137 mmol GA/g. The total antioxidant capacity of *Ns* extract by CUPRAC assay was 0,0144 mmol Tr/g. As we can see here, the folin value is higher than the CUPRAC value of this extract. The CUPRAC assay is probably the most consistent method of total antioxidant measurement in relation to Folin reagent-responsive total phenolic content because the major antioxidant capacity of fruits and vegetables is a direct outcome of their polyphenol content. Thus Folin reagent-responsive polyphenols/ascorbic acid should constitute most of the observed

antioxidant capacity of herbal extracts, necessitating a close correlation of any antioxidant assay with total phenolic content in order to be realistic and successful, as shown in this study. Because the Folin method works for the compounds which are antioxidant and non-antioxidant as well; but the CUPRAC method only works for the compound which are antioxidants.

Results were intriguing in the sense that our initial hypothesis was to find a protective role for NS on the immune system to protect from allergies. But the controlled design of our study showed that not only NS protects from allergies specific to the allergen but also has a separate direct non-specific effect on the immune system to revert the immunologic status to a regulatory Th1 type which is considered a curative effect. This may mean that NS could be used, not only as a protective agent, but also a powerful drug with no significant side effects.

Our study explored a unique feature of the NS extract. Here we have shown that the NS extract shifts the allergic pathway from the inflammatory Th2 type to a regulatory Th1 type, which may cure the disease process not just alleviate the symptoms temporarily. Later we explored the main chemical in NS responsible for this beneficial effect. It was to our surprise that the chemical compound was not in the oil but rather in the hydrophilic component of NS such as catechin, chlorogenic acid, epicatechin, caffeic acid, p-coumaric acid.

CHAPTER 5

CONCLUSION

Common anti-allergic drugs in market are far from stopping the cause of allergic disorders. Many chronic allergic patients find themselves taking many drugs, some with considerable side effect, and no hope of being able to stay off medication. Herbal Pharmacopoeia on the other hand is an alternative approach taken by some patients in many parts of the world including ours and the rest of the western countries.

None of the currently available drugs has curative effect on allergies. Drugs only stop the symptoms but not the cause of the disease. Allergen immunotherapy is the only treatment that satisfies this goal and stops the cause which is the allergic inflammation. But the main disadvantage of immunotherapy is its complexity and difficulty in administration.

The main objective of our study was to explore an easier alternative to immunotherapy. A simpler treatment approach that will shift the allergic pathway from the inflammatory Th2 type to a regulatory Th1 type, hence cure the disease not just alleviate the symptoms temporarily.

Ns has been used as ancient remedy for allergic diseases among others and still being used today. These patients have mixed outcomes since there are no reports outlining clearly its main chemical or specific function on the immune system.

We used a cell culture system where we can identify the effects of *Ns* on the immune system. Results were intriguing in the sense that our initial hypothesis was to find a protective role for *Ns* on the immune system to protect from allergies. But the controlled design of our study showed that not only *Ns* protects from allergies specific to the allergen

but also has a separate direct non-specific effect on the immune system to revert the immunologic status to a regulatory Th1 type which is considered a curative effect. This may mean that *Ns* could be used, not only as a protective agent, but also a powerful drug with no significant side effects.

Later we explored the main chemical in *Ns* responsible for this beneficial effect. It was to our surprise that the chemical compound was not in the oil but rather in the hydrophilic component of *Ns* such as catechin, chlorogenic acid, epicatechin, caffeic acid, p-coumaric acid. Currently we are exploring the details of these compounds and their effects which is the subject of our next research.

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