

# **Studies on Honey Proteins and Peptides and Their Role in Modulation of Innate/Adaptive Immune Response**

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Ph. D Work Seminar

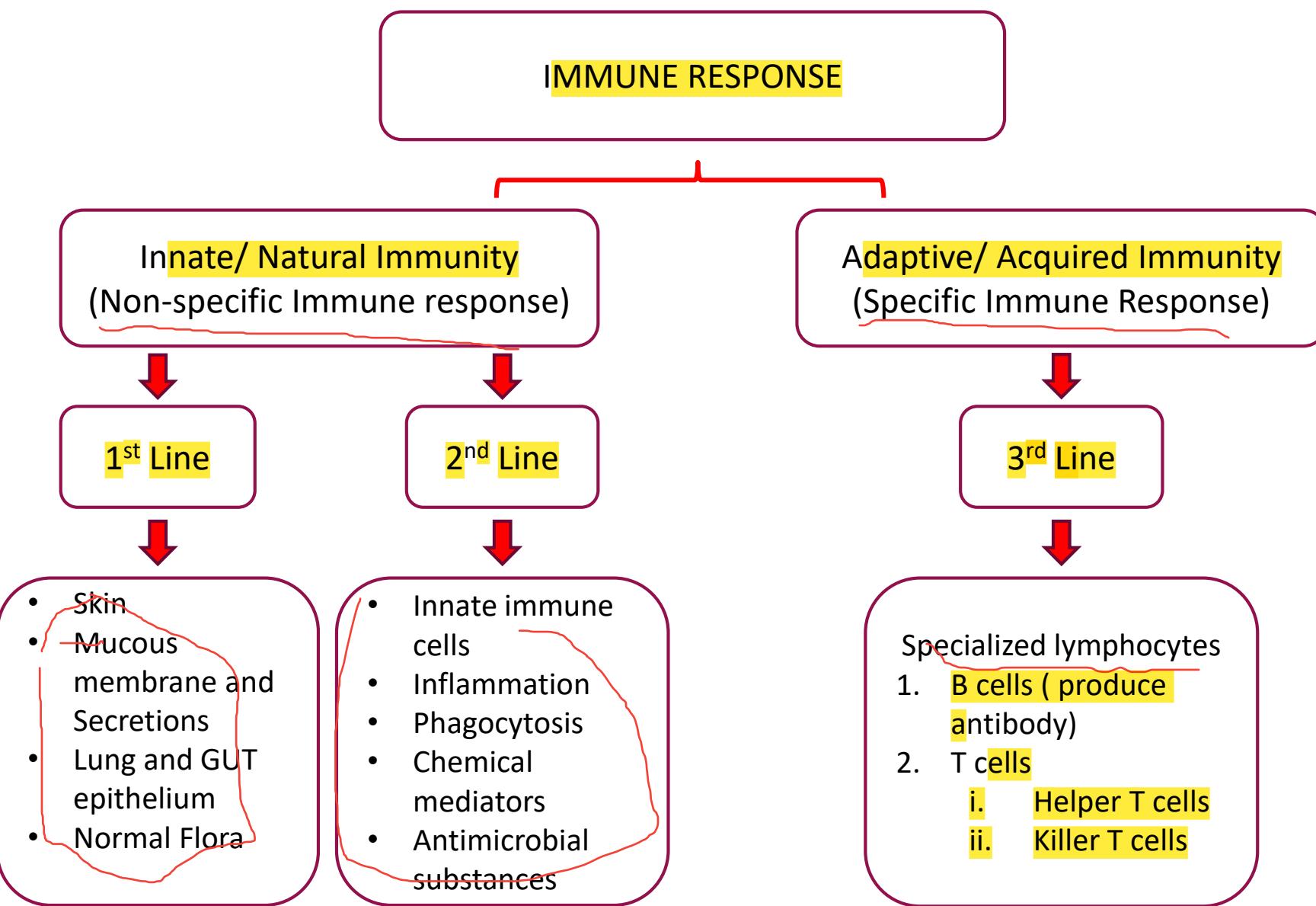
BY

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# Immune System

Immune system is a defense system of an organism, comprise of different organs, cells and other molecules, which enables us to resist against infections. Immune system is capable to distinguish between self and foreign particles and has ability to detect an extensive variety of pathogens comprising bacteria, parasites, viruses, fungi and various allergens.

## IMMUNE RESPONSE

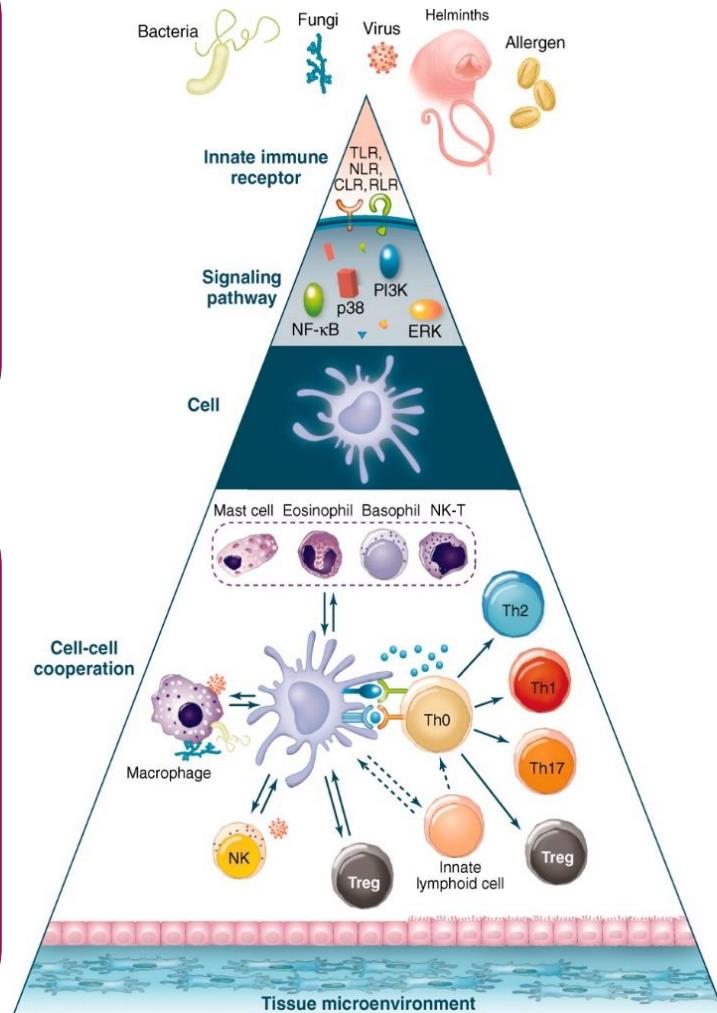


# Innate Immune Response

## Physical Barriers

Intact skin and mucus membrane (secrete mucus and other antimicrobial peptides) together with epithelial lining of lungs and gut (secrete mucin and other glycoproteins) serve as first line of defense against microorganisms.

**Pathogen and Damage Recognition**  
PAMPs and DAMPs are pathogen and damage associated molecular pattern which are recognized by host PRRs. These receptors mainly involve phagocytic receptors, chemotactic receptors and signaling receptors.



## Inflammation

Inflammation is a complex process involving a multifactorial arrangement of cellular and chemical signals to mediate the action. Chemotaxis, cellular and chemical mediators play important role.

## Phagocytosis

Phagocytosis (endocytosis) is the engulfment of solid particles (usually larger than  $0.5\mu\text{m}$ ) including bacteria, cell debris, dust particle and any particulate antigen. Main players of phagocytosis are white blood cells include neutrophils, macrophages, monocytes, mast cells and NK cells.

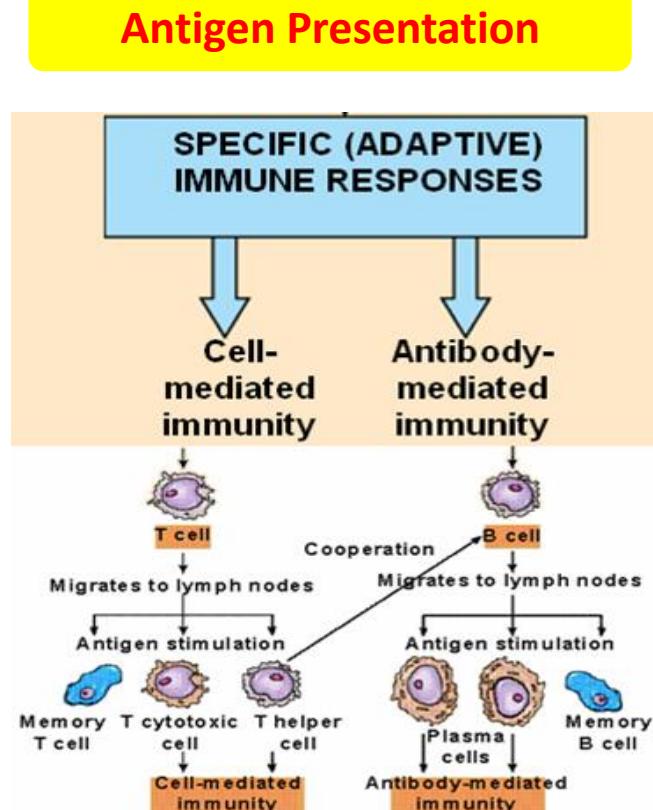
# Adaptive Immune Response

Compared to innate immunity, adaptive immune response is acquired upon exposure to foreign particles including microbes. When innate immune system is unable to remove the invading particles/microbes it calls adaptive immune system for help. It is a slow but highly specific immune response which also generates memory.

## Cell-Mediated Immune Response

It involves the activation of phagocytes, antigen-specific cytotoxic T-lymphocytes, and the release of various cytokines in response to an antigen. It rests on direct communications between T lymphocytes and cells bearing the antigen that the T cells identify.

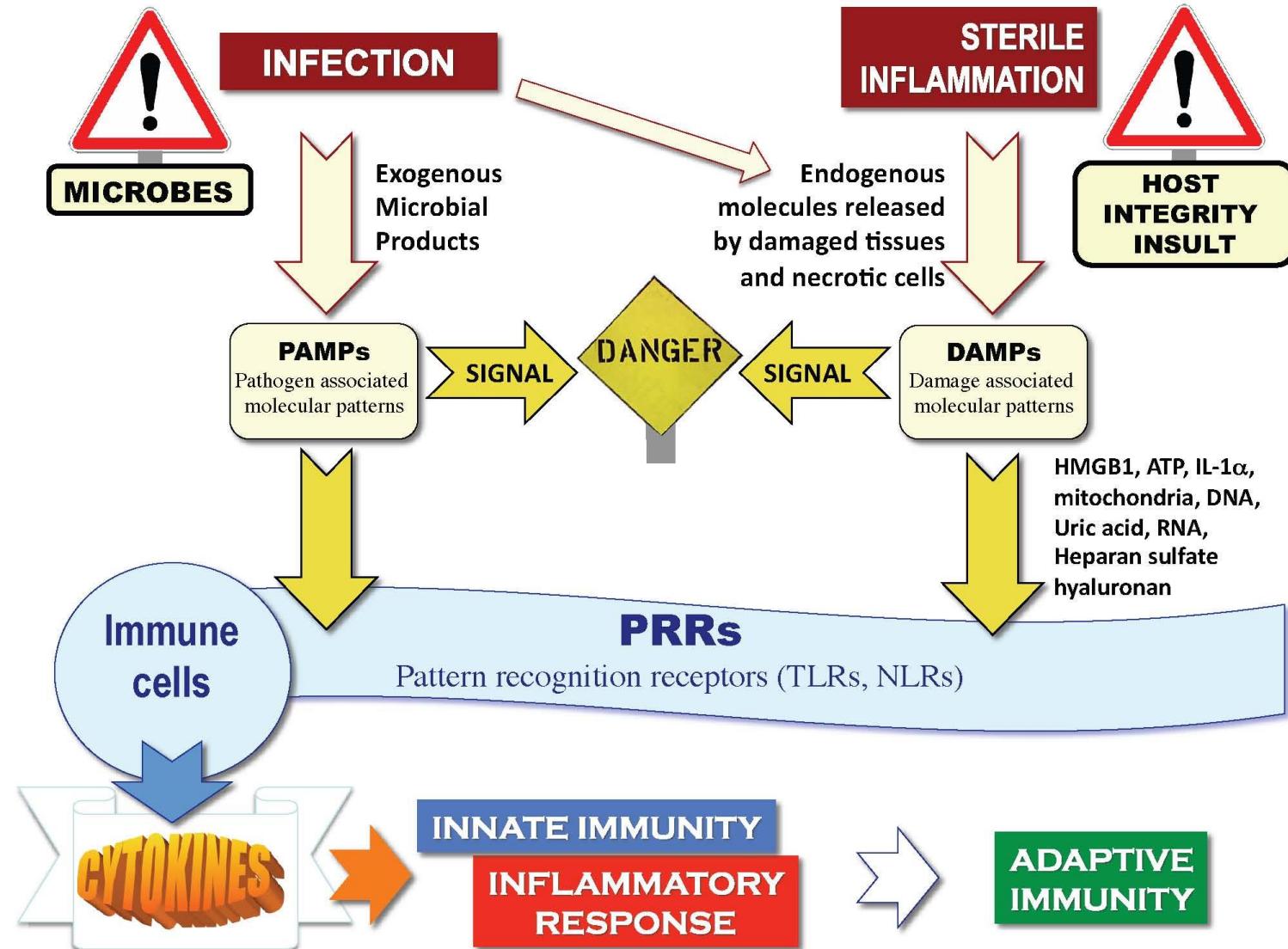
- CD8+ Cytotoxic T cells (Tc)
- CD4+ helper T cells (Th)



## Humoral Immune Response

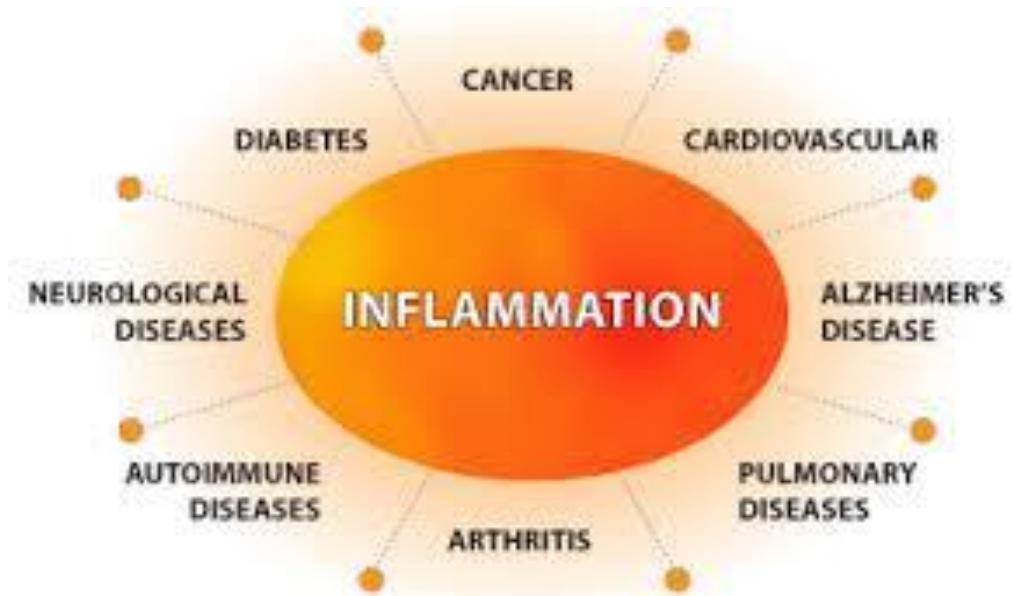
It is mainly comprised of secretory components found in extracellular fluids such as antibodies, complement proteins and certain antimicrobial peptides. These antibodies perform three main functions:

- Neutralization
- Opsonization
- Complement Fixation



# Inflammation:

Inflammation is the immediate response of the body's white blood cells against any foreign organisms, such as bacteria, viruses, fungi, etc. Chemotaxis and phagocytosis are the major events in inflammation.



# Mediators of Inflammation and Immune Response

## A. Cytokines

- **Interleukin 1 (IL-1)**
- Interleukin 6 (IL-6)
- Interleukin 8 (IL-8)
- **IL-2**
- **Tumor Necroting Factor (TNF- $\alpha$ )**

## B. Phagocyte Products

- Cationic Proteins
- Neutral Proteases
- **Oxygen derived free radicals**

## C. Vasoactive Amines

- Histamin (mast cell and platelets)
- Serotonin from platelets

## D. Nitric Oxide

## E. Plasma Protein System

- Kinin system
- Complement System
- Clotting/ Fibrinolysis System

## F. Eicosanoids (Arachidonic Acid)

- Prostaglandins
- Leukotriens

## G. Platelet Activating Factors

# Current Anti-inflammatory Therapy

## Steroidal Anti-inflammatory Drugs

Adverse effects involving disturbance of gastrointestinal (GI), cardiovascular, nervous, musculoskeletal, endocrine and metabolic functions

## Nonsteroidal Anti-inflammatory Drugs

Adverse effects include cardiovascular disorder, gastrointestinal disorder, hypersensitivities, also associated with kidney disorders

Herbal Medicines comprise of the elements belonging to various classes of phytochemicals.

**However**, herbal remedies beside their efficacy, are reported to have serious side effects, adverse effects including direct toxic effects, allergic reactions, bleeding, cardiovascular instability, effects from contaminants, and interactions with drugs and other herbs

**Therefore, there is still need of the new anti-inflammatory drug which in addition with its improved efficacy, give broader safety index.**

# Honey

Allah Says in Al-Quran

*"And your Lord inspired to the bee, "Take for yourself among the mountains, houses, and among the trees and [in] that which they construct. Then eat from all the fruits and follow the ways of your Lord laid down [for you]."* There emerges from their bellies a drink, varying in colors, in which there is healing for people. Indeed in that is a sign for a people who give thought." [Sura Al-Nehl, 16:68-69]

# Honey

Honey is a sweet, amber-colored, thick sugary liquid synthesized by the honey bees *Apis mellifera*, via collecting nectar from a large variety of flowers.

Nectar is transformed into honey through the process of **regurgitation**, in the stomach of bee using its digestive enzymes and then evaporation.

Honey is an important natural and traditional medicine which has been using for an ancient time to treat many diseases including bacterial infections (additionally associated with multidrug resistant bacterial strains), burns, cancers, cardiovascular diseases, inflammatory disorders, etc.



# Composition of Honey

Honey (Nutritional value per 100 g)	
Component	Average
Carbohydrates	82.4 g
Fructose	38.5 g
Glucose	31 g
Sucrose	1 g
Other sugars	11.7 g
Dietary fiber	0.2 g
Fat	0 g
<b>Protein</b>	<b>0.3 g</b>
Water	17.1 g
Riboflavin (Vit. B2)	0.038 mg
Niacin (Vit. B3)	0.121 mg
Pantothenic acid (Vit. B5)	0.068 mg
Pyridoxine (Vit. B6)	0.024 mg
Folate (Vit. B9)	0.002 mg
Vitamin C	0.5 mg
Calcium	6 mg
Iron	0.42 mg
Magnesium	2 mg
Phosphorus	4 mg
Potassium	52 mg
Sodium	4 mg
Zinc	0.22 mg

# Aims and Objectives:

The current studies were aimed to explore new therapeutic uses of dietary proteins of honey. Honey proteins could be a useful tool for the treatment of various inflammatory disorder as it is known to have immunoregulatory potential.

Objectives of the current studies are given below:

- Isolation and purification of honey proteins.
- Effect of honey protein fractions on various mediators of innate and adaptive immune response.
- Qualitative analysis of honey proteins by SDS-PAGE with Comassie brilliant blue R250 and glycoprotein staining.
- Effect of honey proteins on expression profile of genes related to innate and adaptive immune response using Real time PCR Array with data analysis and interpretations.
- Characterization of honey proteins through in-gel tryptic digestion and mass spectrometry using MALDI-TOF.
- Identification of proteins by analyzing mass data through Mascot and Expasy Find MOD servers.

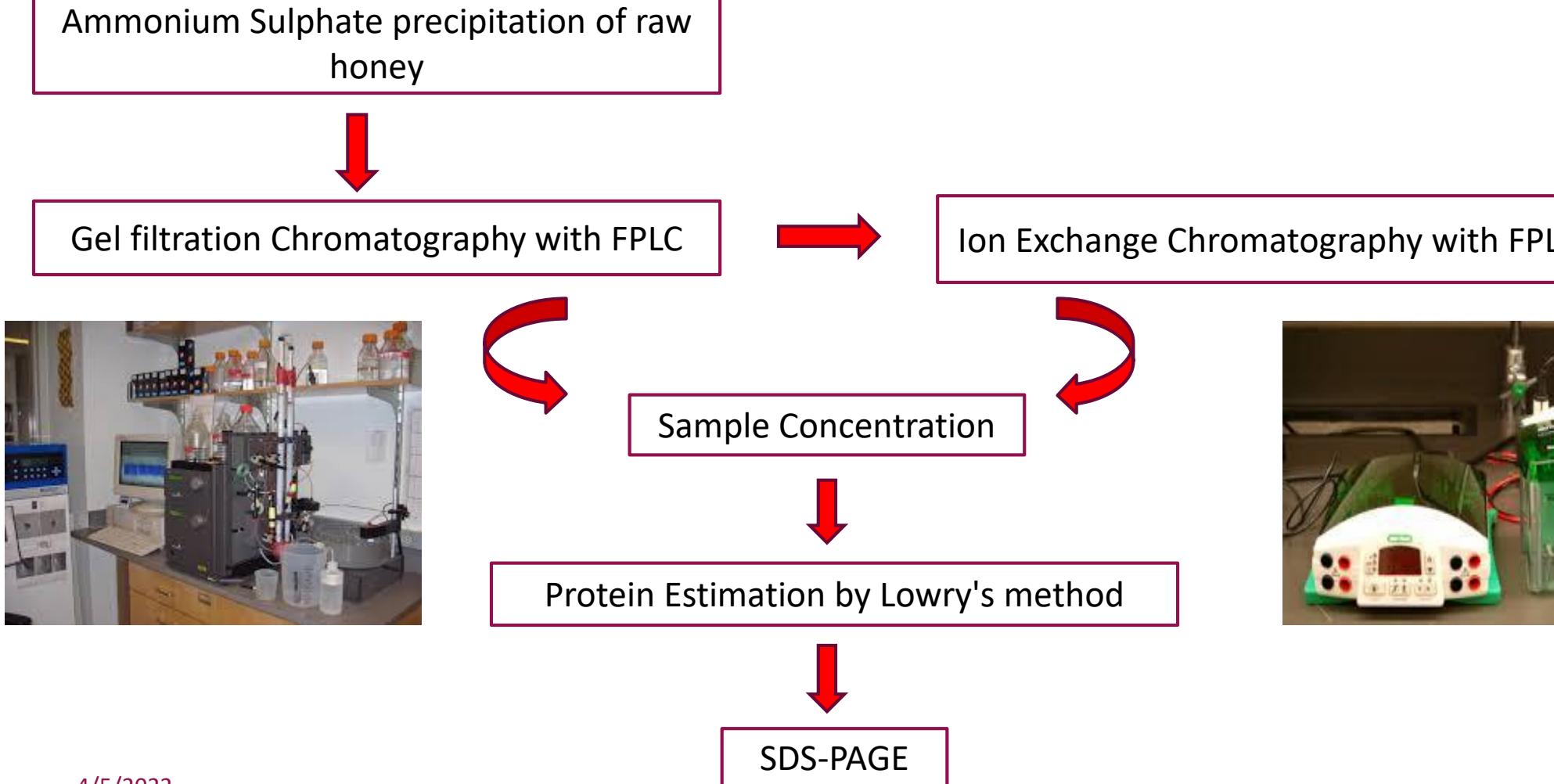
# Methodology:

Honey protein  
extraction and  
Purification

Immunomodulatory  
Activities and Gene  
Expression Profiling

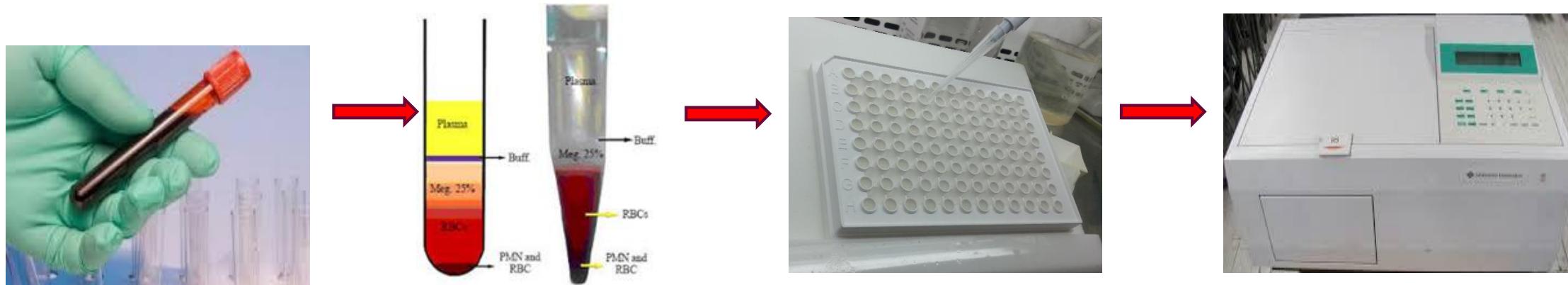
Honey Proteins  
Characterization  
using Mass  
Spectrometry

# Honey protein Extraction and Purification

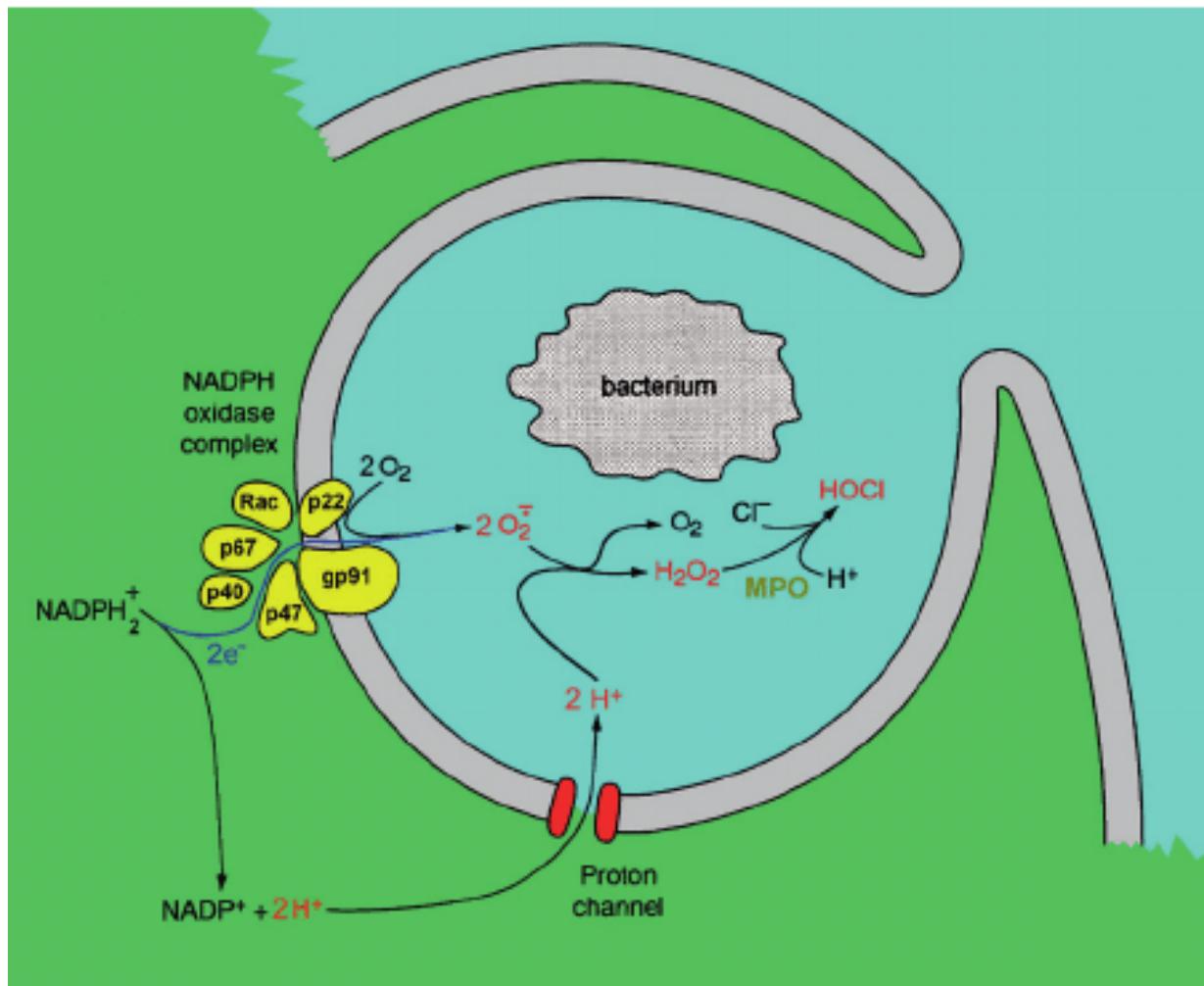
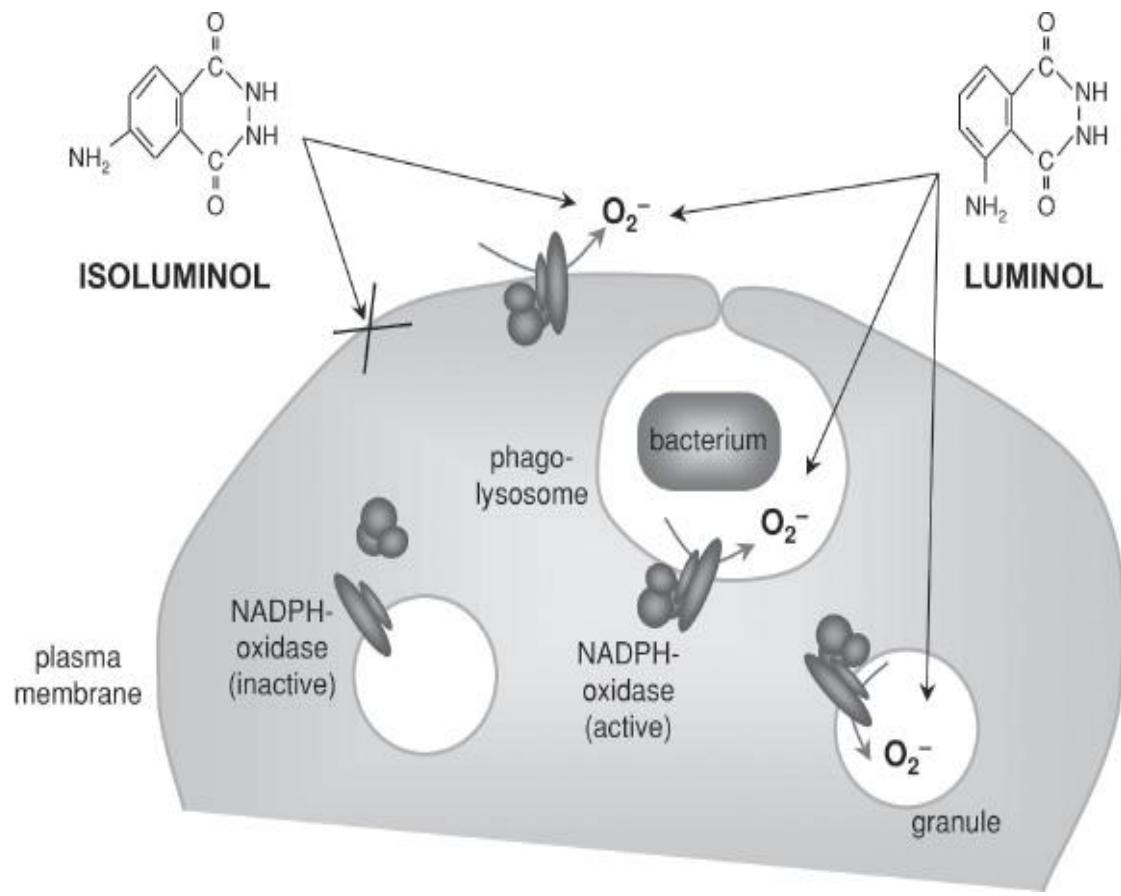


# Immunomodulatory Activities

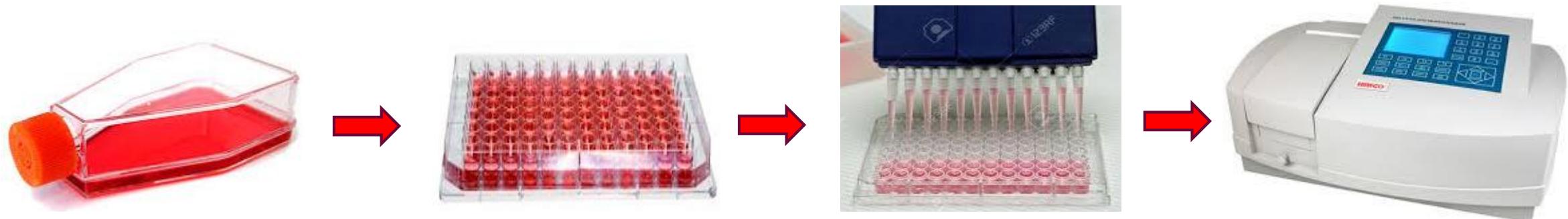
# Oxidative Burst Assay by Chemiluminescence Measurement



Neutrophils were isolated and cultured in the presence of GFC fractionated honey proteins. Cells were activated with different activators (SOZ/ PMA/ fMLP). Luminol was used as luminescent probe (SOZ – Serum Opsonized zymosan, PMA – Phorbol 12-myristate 13-acetate, fMLP –



# Nitric Oxide Assay



J774.2 mouse macrophage cells were cultured in presence of 10% FBS and 20 mM L-glutamin in DMEM medium

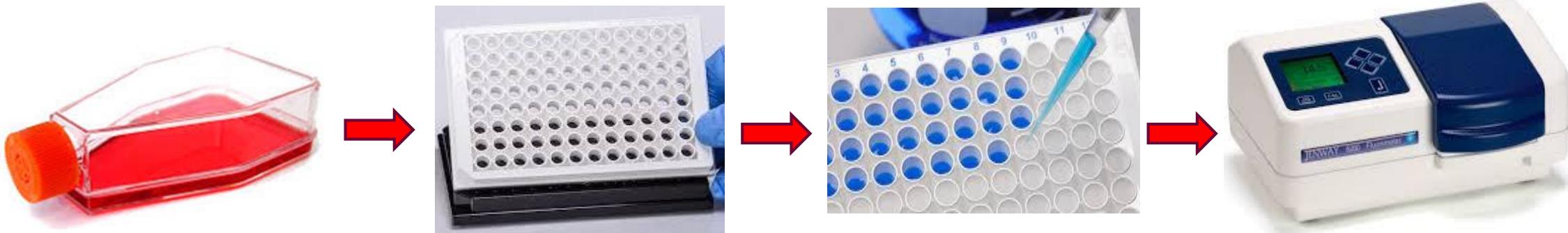
Cells were cultured in flat bottom clear 96-well plate. Cells were activated with 30  $\mu$ g/mL LPS and added with GFC fractionated honey protein peaks. Incubated for 48 h.

Supernatants were transferred to another plate and greiss reagents (A and B) were added in equal amount. Covered with foil for 5-10 min.

Absorbance was recorded at 540 nm

Concentration of NO was calculated using standard plot of sodium nitrate.

# Phagocytosis Assay



J774.2 mouse macrophage cells were cultured in presence of 10% FBS and 20 mM L-glutamin in DMEM medium

Cells were cultured for 24 h alone and then added with honey proteins and carboxylated modified latex beads as 100 beads/cell and. Further incubated for 24 h.

Supernatants were removed and cells were washed with PBS thrice. 1X trypan blue was added for 1 minute and then quickly removed.

Fluorescence was recorded with excitation maxima a 575-610 nm.

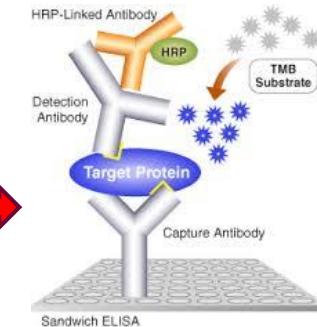
# ELISA for Cytokines

For  
TNF- $\alpha$   
and  
IL-1 $\beta$ :



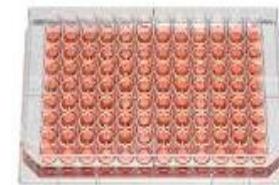
THP-1 human monocyte  
cells were cultured

Cells were differentiated  
with PMA and activated  
with LPS for 4h. Honey  
proteins were also added



Absorbance was recorded  
with ELISA reader

For  
IL-2

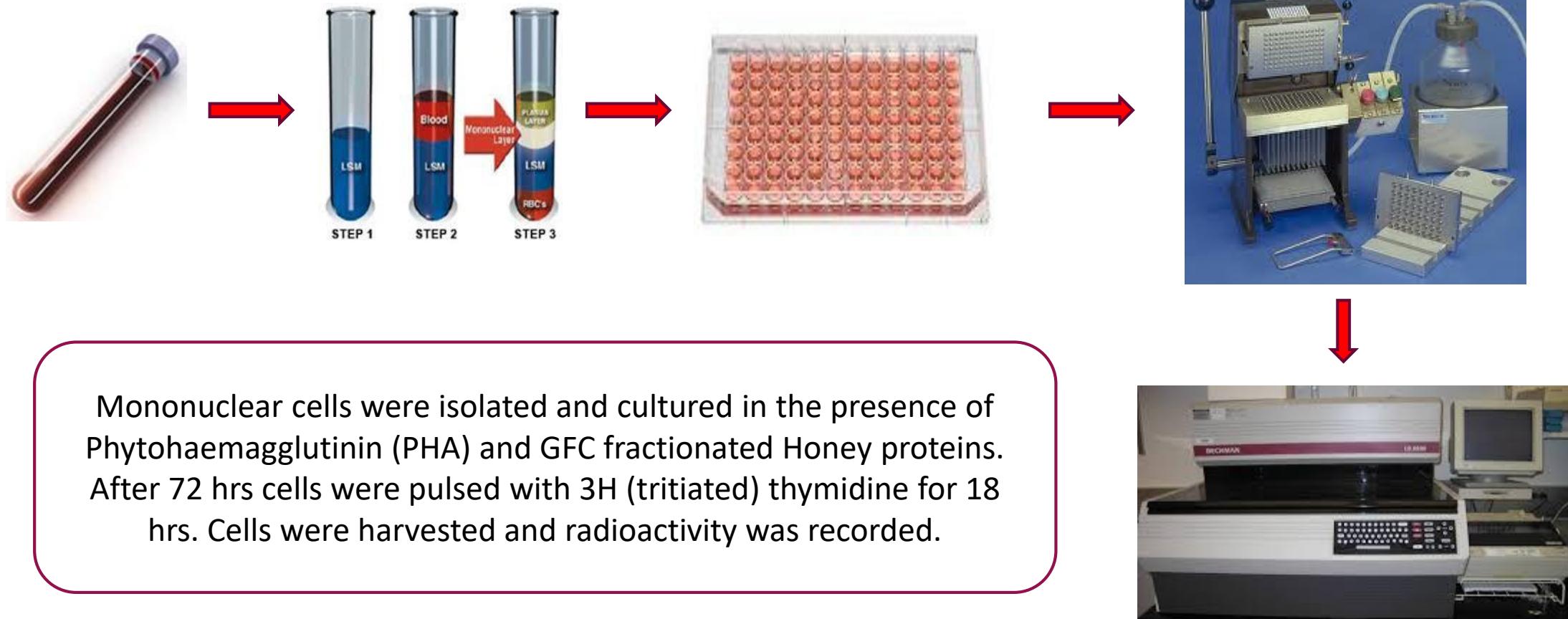


Fresh human  
blood from  
healthy donor

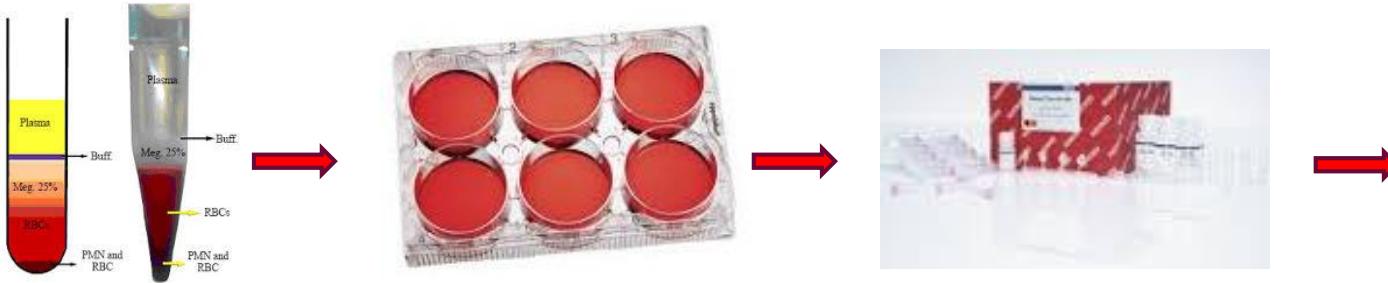
Isolation of  
lymphocytes

Lymphocytes were cultured  
and activated with PMA and  
PHA in presence of honey  
protein for 24h

# T-Cell Proliferation Assay



# REAL Time PCR for Multigene Profiling



Neutrophils were isolated and preincubated in the presence of honey protein GFC-P2 and incubated for 10 min, then added with 100 ng/mL different activators [PMA (50 ng/mL), fMLP(100 nM) and LPS (1  $\mu$ g/mL)] and incubated for 30 min. RNA was extracted using kit method and processed for cDNA synthesis.

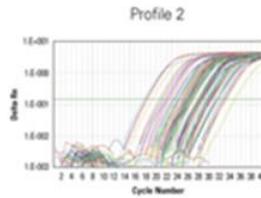
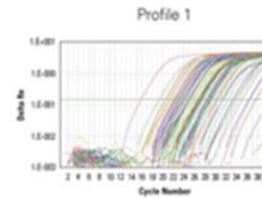
## 1. Convert Total RNA to cDNA.



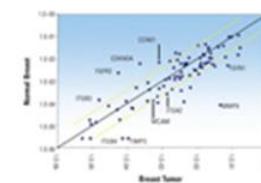
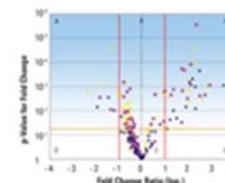
## 2. Add cDNA to RT<sup>2</sup> qPCR Master Mix & Aliquot Mixture Across PCR Array.



## 3. Run in Your Real-Time PCR Instrument.



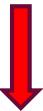
## 4. Data Analysis.



# Protein Characterization

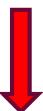
## Protein Deglycosylation

Five deglycosylases were used to remove sugar from the honey proteins



## Mobility Shift Assay

SDS PAGE was performed to analyze mobility shift and comassie and fluorescent staining were used.



## In Gel Digestion of proteins

Bands were cut and after alkylation and denaturation digested with porcine trypsin into peptides



## Data Analysis

Data was analyzed using Mascot search tool

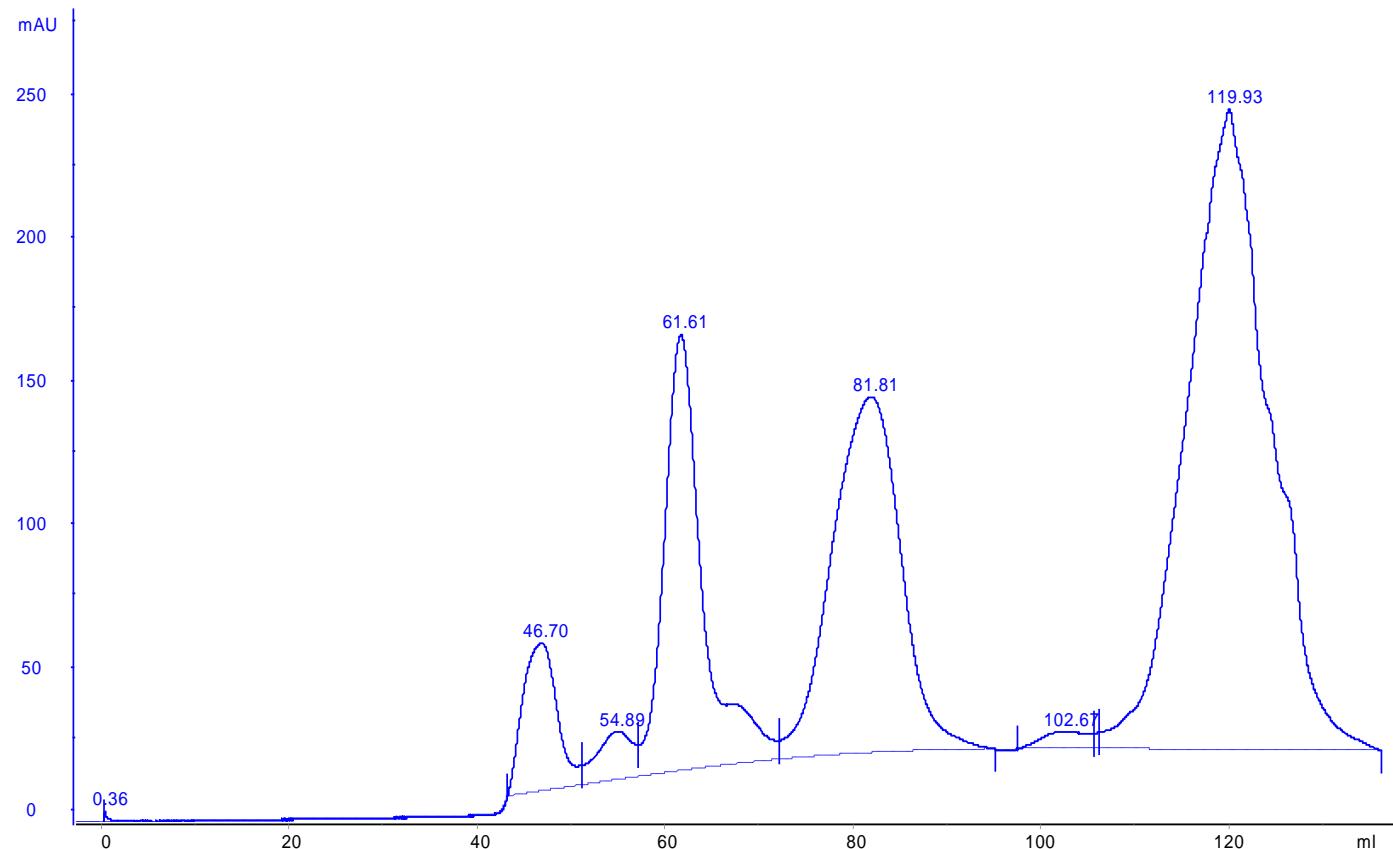


## MALDI-ToF Mass Spectrometry analysis

MALDI was performed with positive ion mode

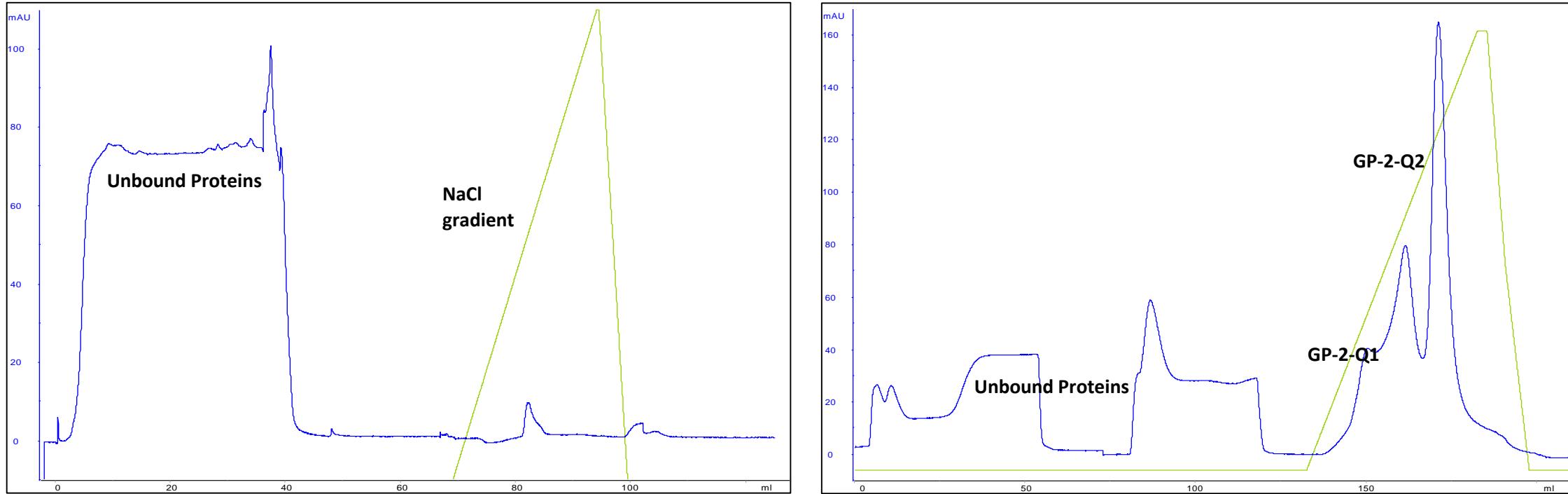
# RESULTS

# Gel filtration Chromatography



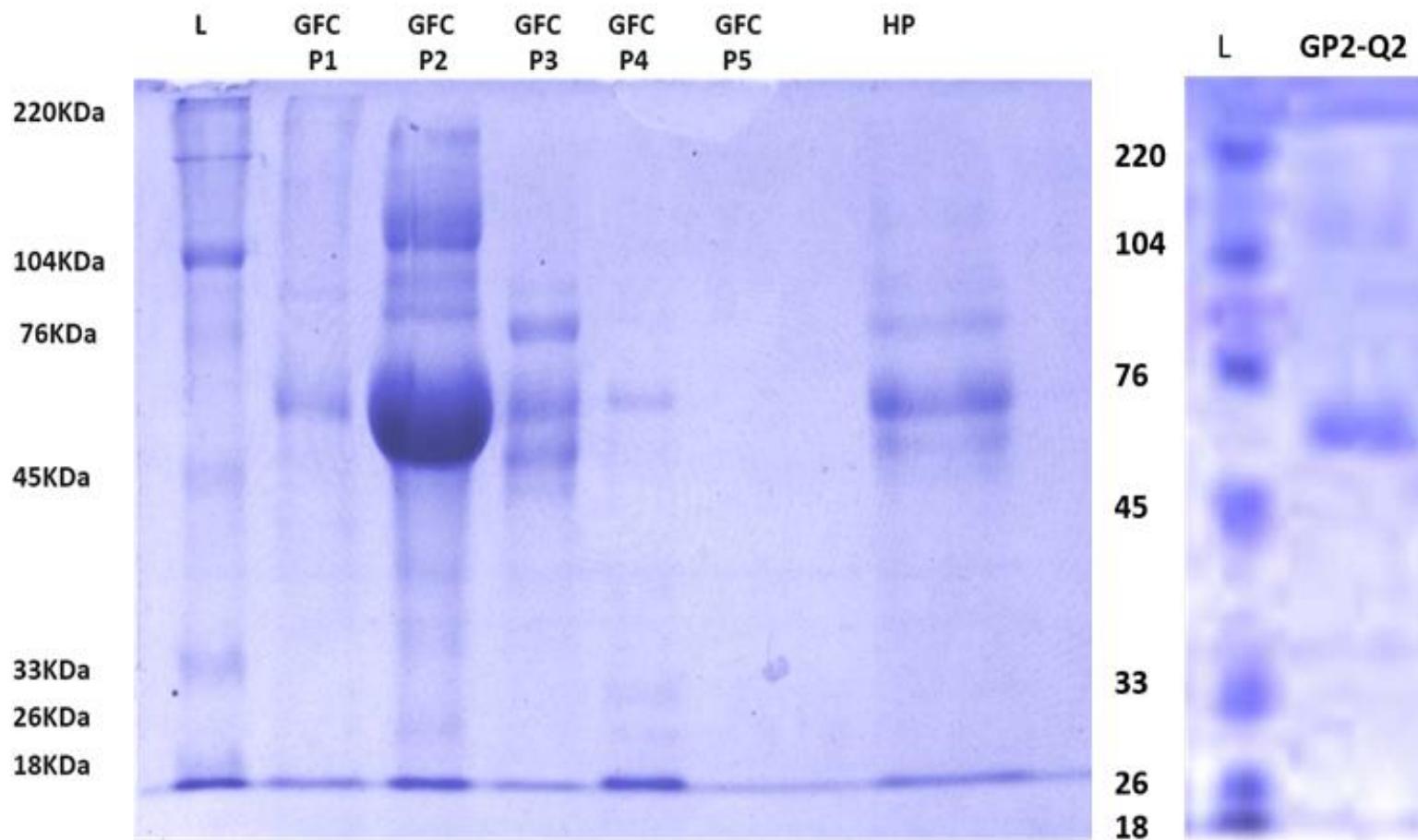
- **Figure-3.1:** Chromatogram of Honey Proteins Separated by Gel Filtration Column (GFC) using Akta FPLC System. Gel filtration peak 1 is designated as GFC-P1 and so on.

# Ion Exchange Chromatography

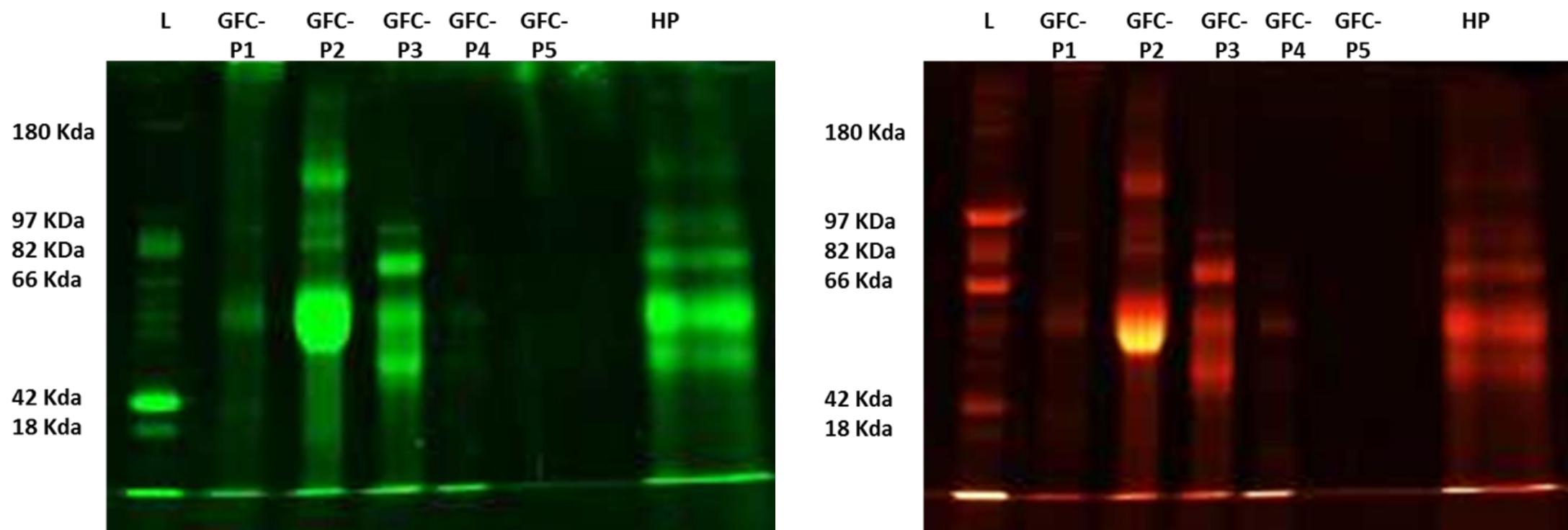


- **Figure-3.2:** Ion exchange chromatography of GP-2 using FPLC system. (A) Cation exchange chromatography via SP sepharose column. (B) Anion exchange chromatography through Quaternary ammonium column.

# SDS-PAGE

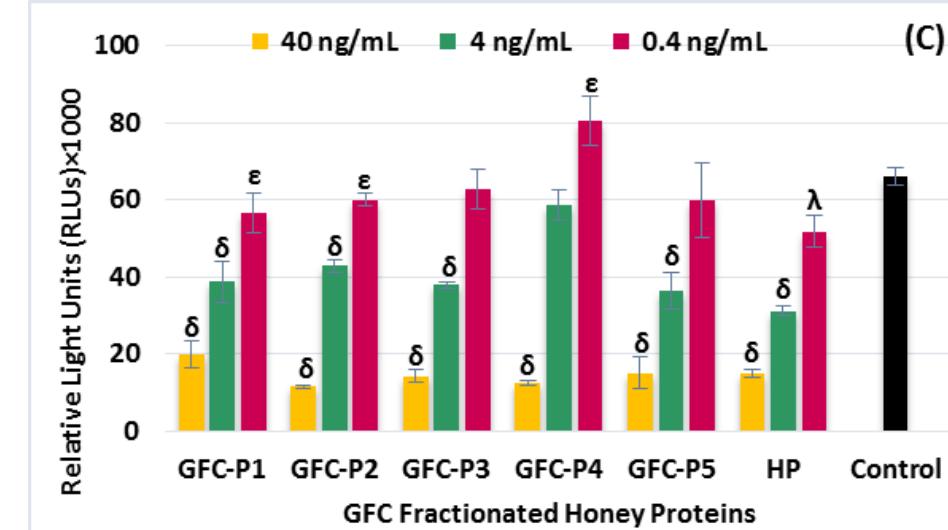
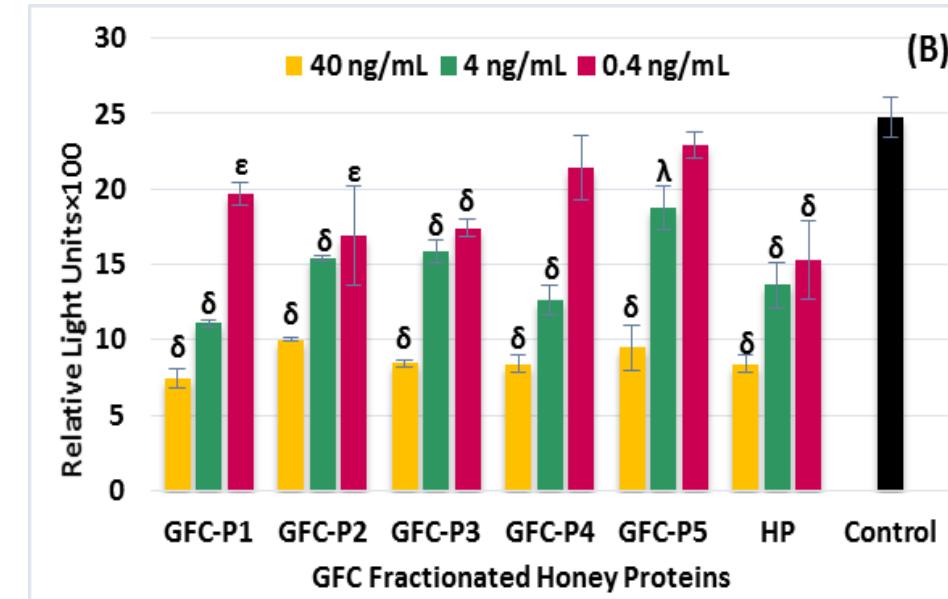
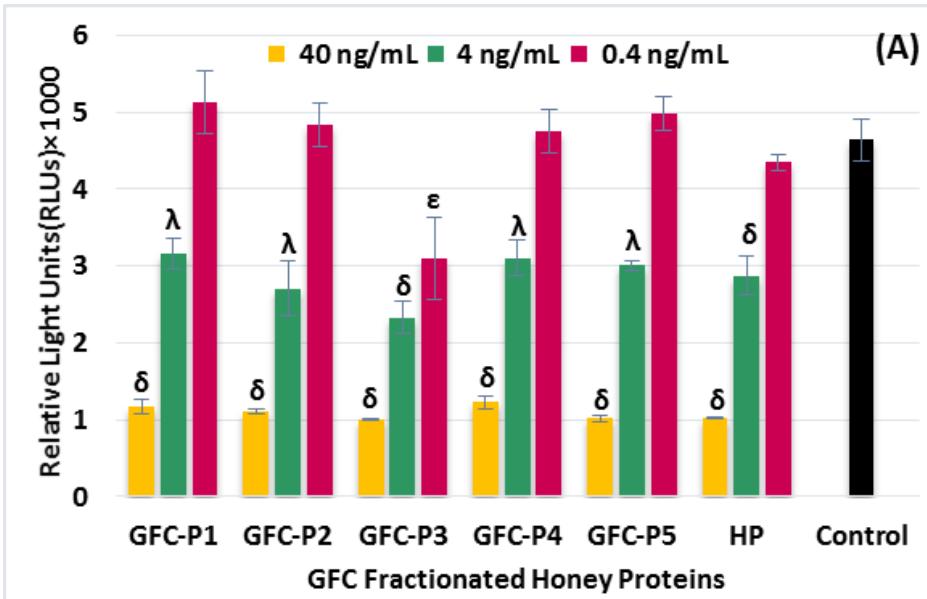


- **Figure 3.4:** SDS-PAGE for honey proteins determination



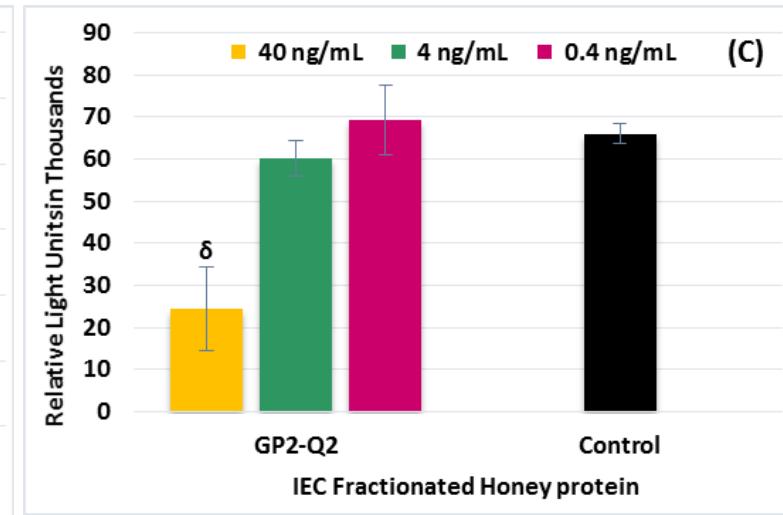
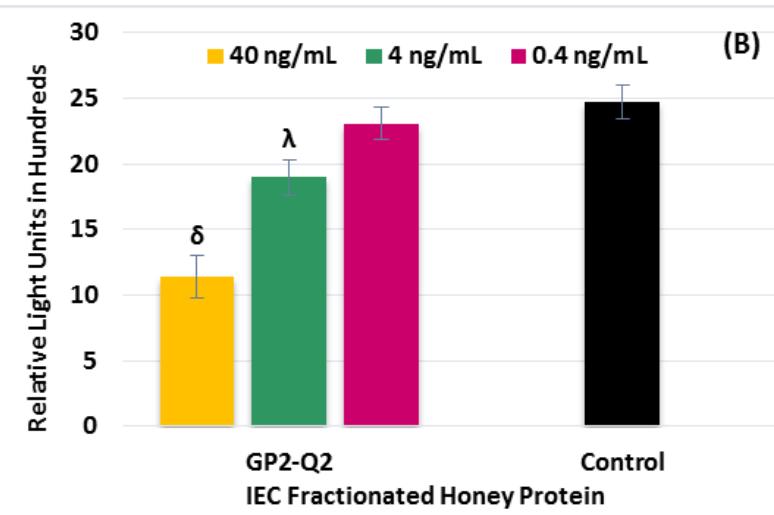
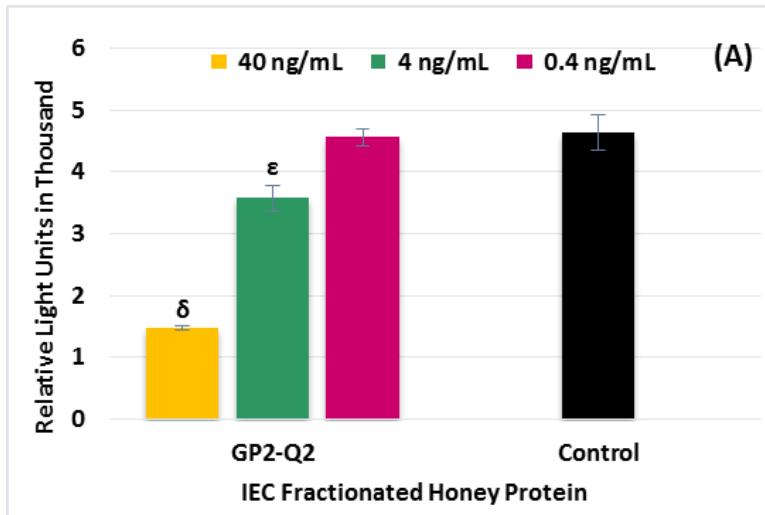
• **Figure 3.5:** Fluorescent staining of SDS-PAGE gels.

# Oxidative Burst Assay- Continue.....



- Effect of GFC fractionated honey protein fractions on ROS production by PMNLs stimulated with different activators. (A) Serum opsonized Zymosan (SOZ) was used with final concentration of 250 µg/ml, (B) fMLP was used at 100 nM, and (C) PMA was used at 200 ng/mL. ROS was detected using luminal probe.

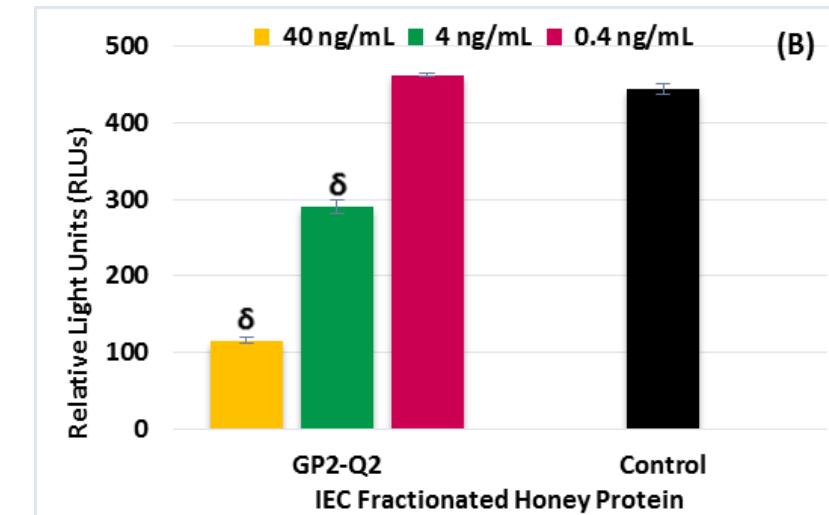
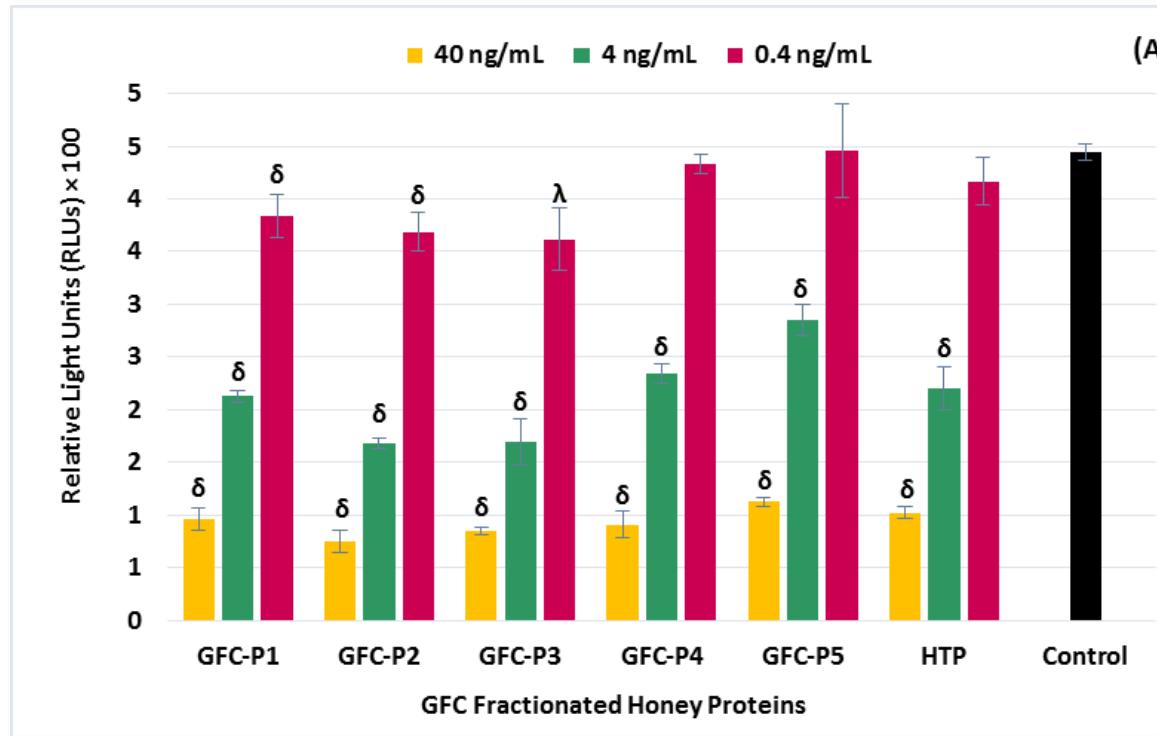
# Oxidative Burst Assay- Continue....



- Effect of ion exchange fractionated honey protein sample on ROS production by neutrophils. Cells were activated with different activators. (A) Serum opsonized Zymosan (SOZ) was used with final concentration of 250 µg/ml, (B) fMLP was used at 100 nM, and (C) PMA was used at 200 ng/mL

S. No	Sample Code	IC <sub>50</sub> ±SD FOR ROS (in ng/mL) using human PMNLs		
		SOZ	fMLP	PMA
1	GFC-P1	13.3 ± 1	2.8 ± 0.2	8.8 ± 0.8
2	GFC-P2	12.4 ± 0.1	14.6 ± 0.8	5.5 ± 0.3
3	GFC-P3	6.6 ± 0.1	12.6 ± 3.8	6.4 ± 0.6
4	GFC-P4	14.7 ± 1.1	5.7 ± 1.6	14.2 ± 1.1
5	GFC-P5	12.2 ± 0.2	23.3 ± 1.5	4.9 ± 0.9
6	HP	11.3 ± 2	9.2 ± 0.8	3.2 ± 0.5
7	GP2Q2	21.4 ± 1.1	23.4 ± 1.2	22.4 ± 3.3

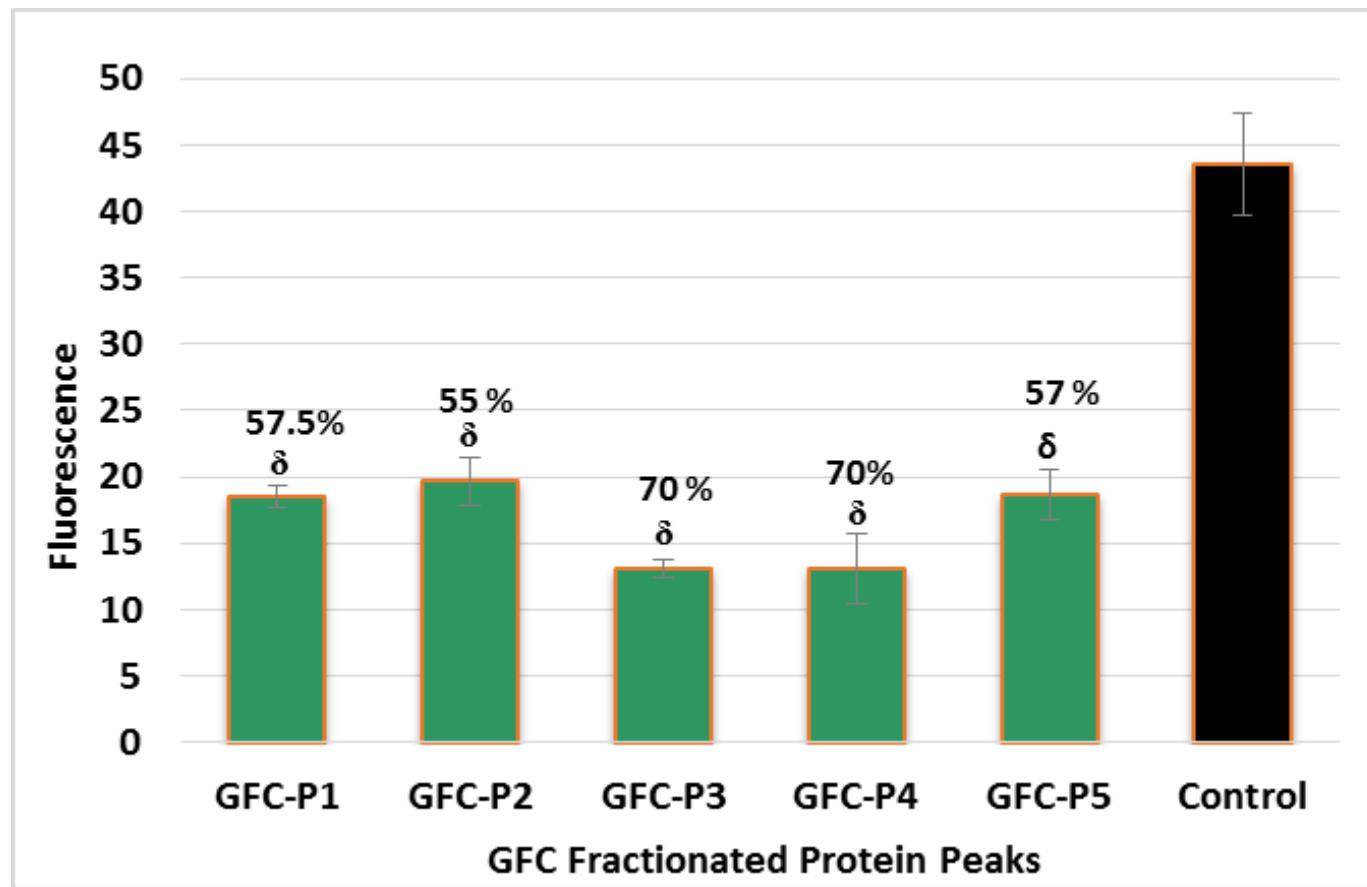
# Oxidative burst Assay



- **Figure 3.8:** Chemiluminescence assay- effect of honey proteins fractions on production of ROS by mouse peritoneal macrophages ( $2 \times 10^6$  cells/mL), stimulated with SOZ (250  $\mu$ g/mL) and detected with luminal probe (1.8  $\mu$ g/mL).

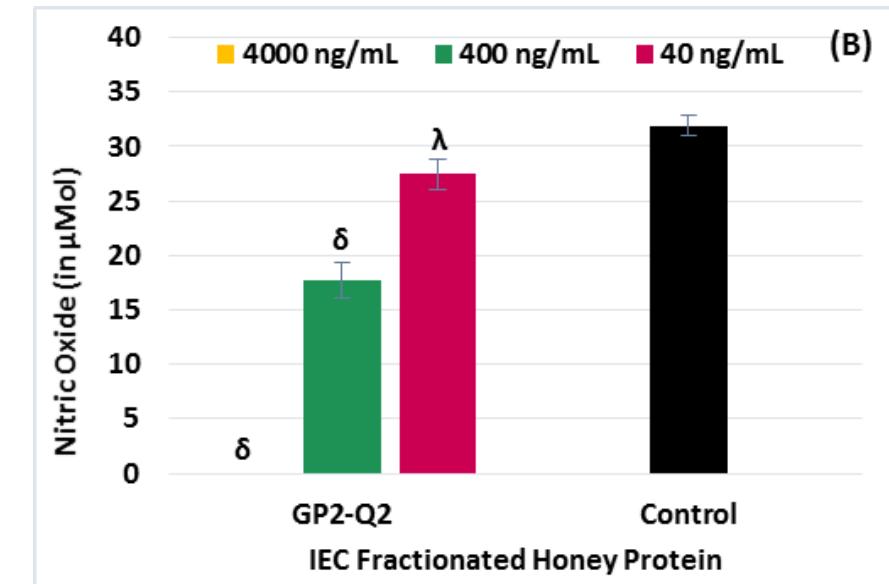
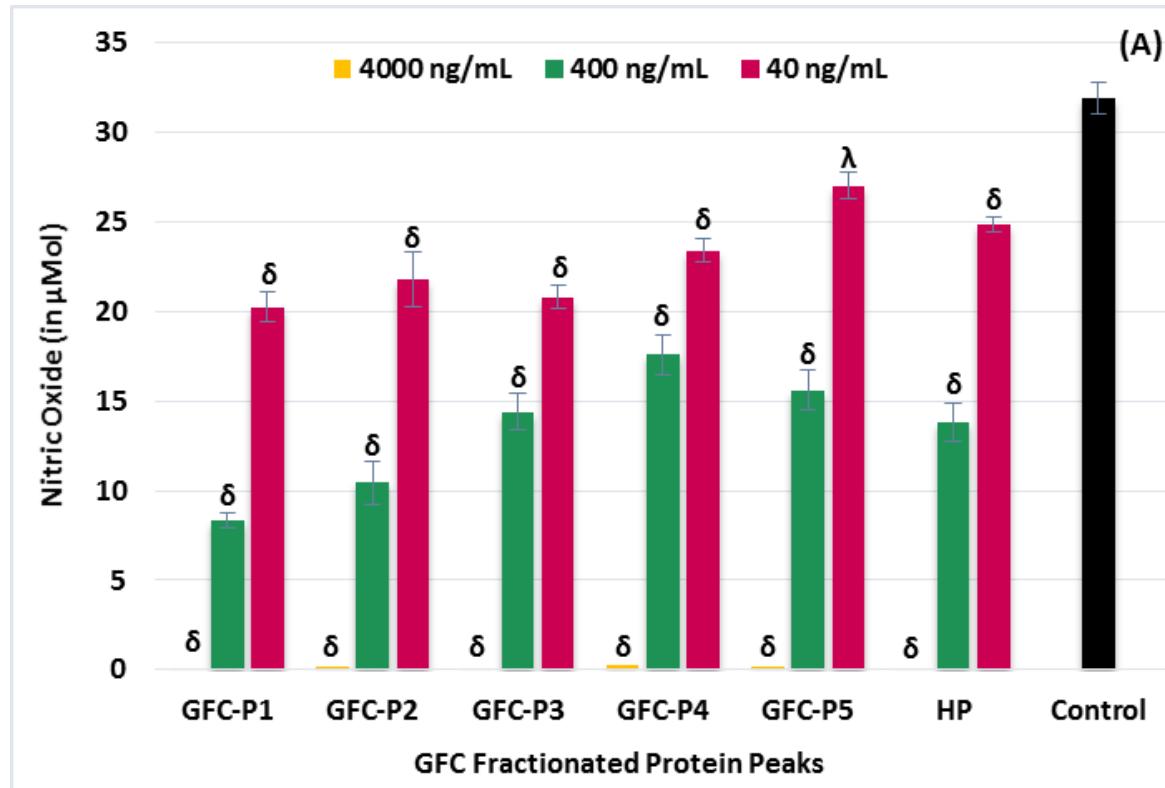
S. No	Sample Code	$IC_{50} \pm SD$ FOR ROS (in ng/ml)
.		<b>Peritoneal</b>
		<b>Macrophages</b>
<b>1</b>	<b>GFC-P1</b>	<b><math>3.5 \pm 0.3</math></b>
<b>2</b>	<b>GFC-P2</b>	<b><math>2.1 \pm 0.1</math></b>
<b>3</b>	<b>GFC-P3</b>	<b><math>2.2 \pm 0.6</math></b>
<b>4</b>	<b>GFC-P4</b>	<b><math>4.8 \pm 0.6</math></b>
<b>5</b>	<b>GFC-P5</b>	<b><math>9.2 \pm 1.0</math></b>
<b>6</b>	<b>HP</b>	<b><math>4.5 \pm 0.4</math></b>
<b>7</b>	<b>GP2Q2</b>	<b><math>9.8 \pm 0.8</math></b>

# Phagocytosis Assay:



- Effect of honey protein fractions on carboxylated modified latex beads phagocytosis

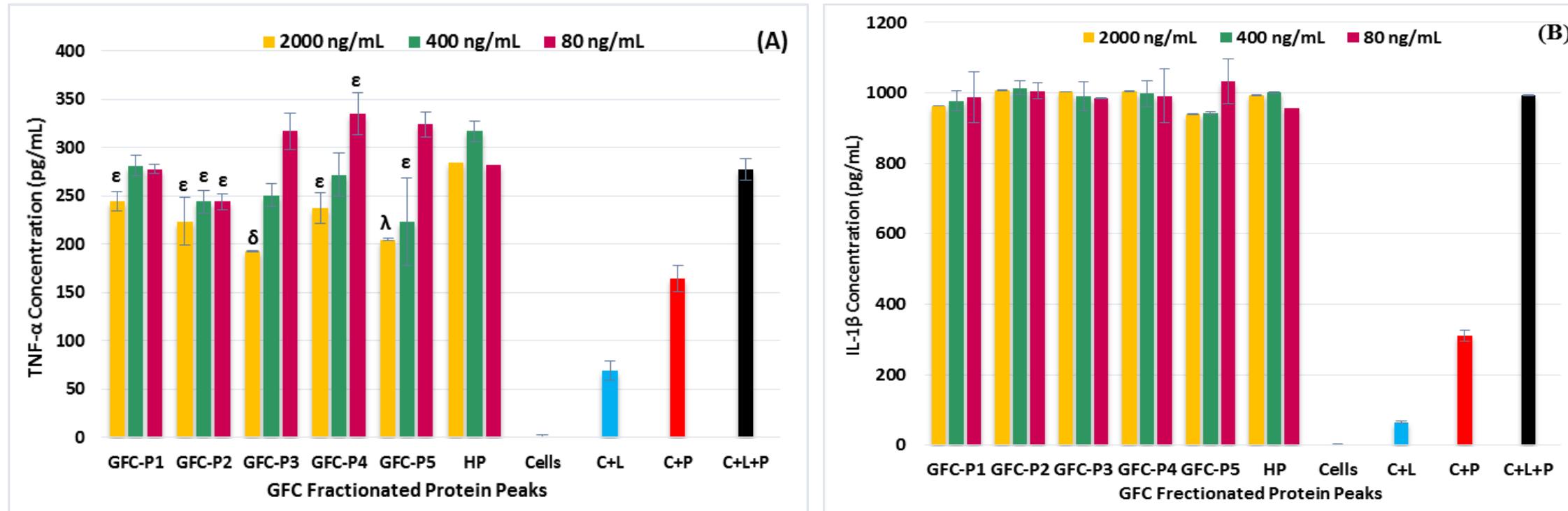
# Nitric oxide Assay:



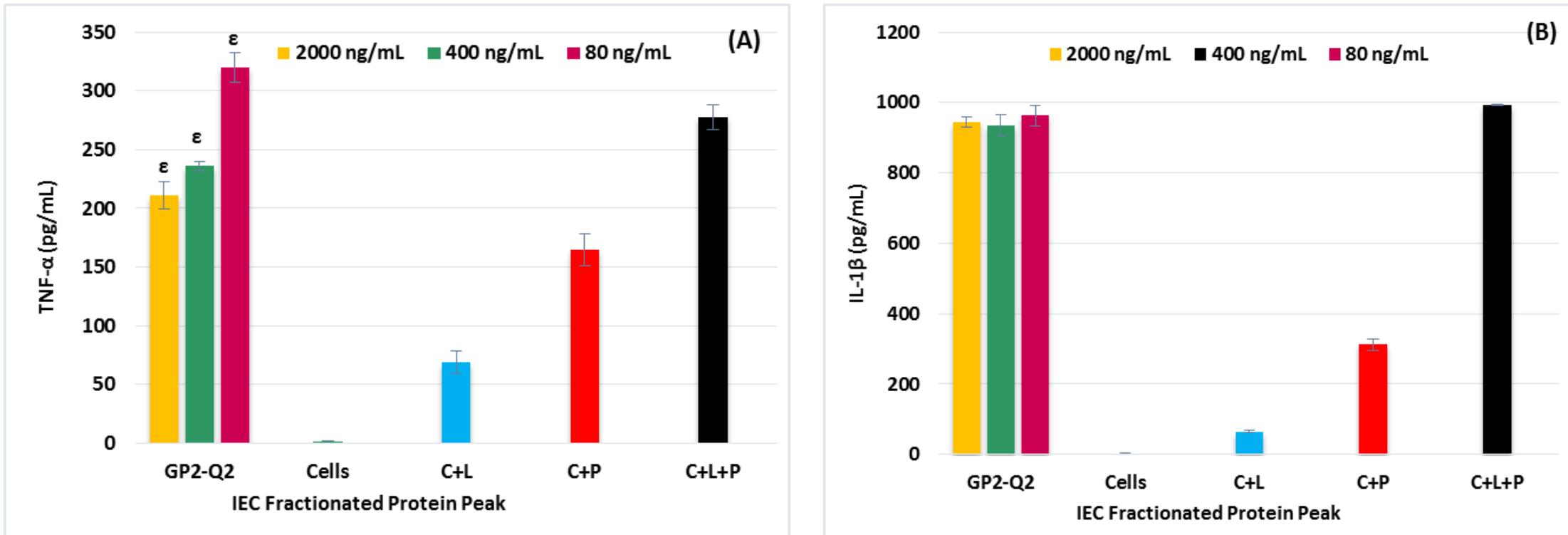
- Figure 3.10: Effect of GFC fractionated honey protein on nitric oxide production of J774.2 mouse macrophages.

S. No	Sample Code	$IC_{50} \pm SD$ for Nitric Oxide (in ng/ml)
1	GFC-P1	$96.2 \pm 4.7$
2	GFC-P2	$114.7 \pm 2.3$
3	GFC-P3	$175.4 \pm 21$
4	GFC-P4	$452 \pm 3.6$
5	GFC-P5	$417.2 \pm 24.2$
6	HP	$228.4 \pm 25$
7	GP2Q2	$462.5 \pm 30$

# Pro-inflammatory Cytokines:

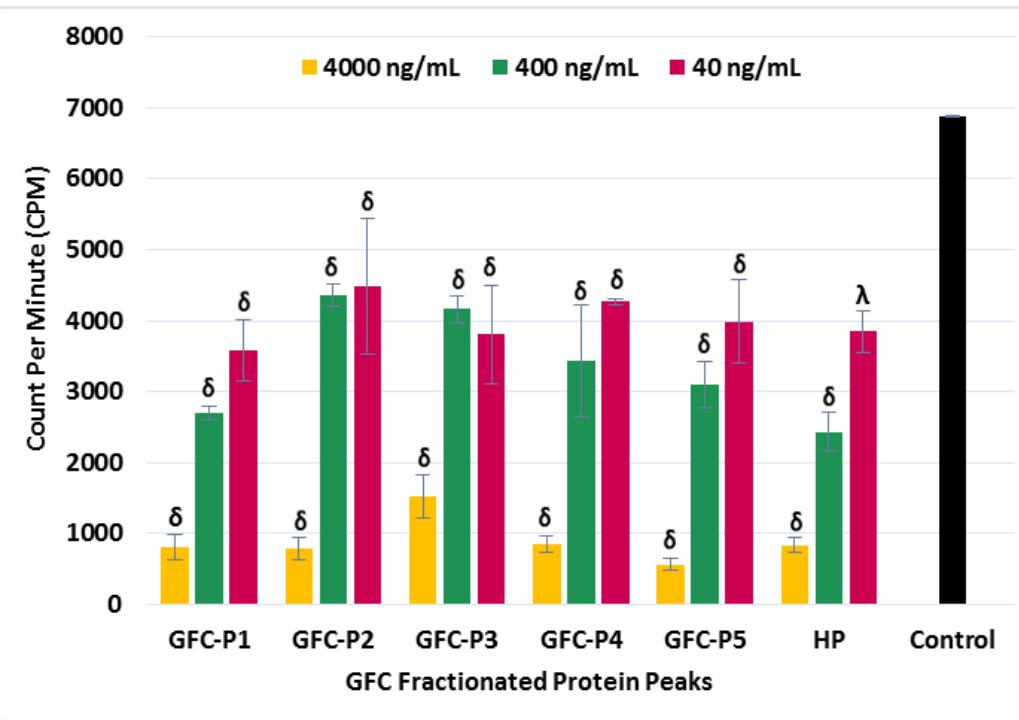


- Figure 3.11: Effect of GFC fractionated honey proteins on pro-inflammatory cytokines production include (A) TNF- $\alpha$  and (B) IL-1 $\beta$ .



- **Figure 3.12:** Effect of IEC fractionated GP2-Q2 on the production of TNF- $\alpha$  (A) and IL-1 $\beta$  (B) using three different concentrations.

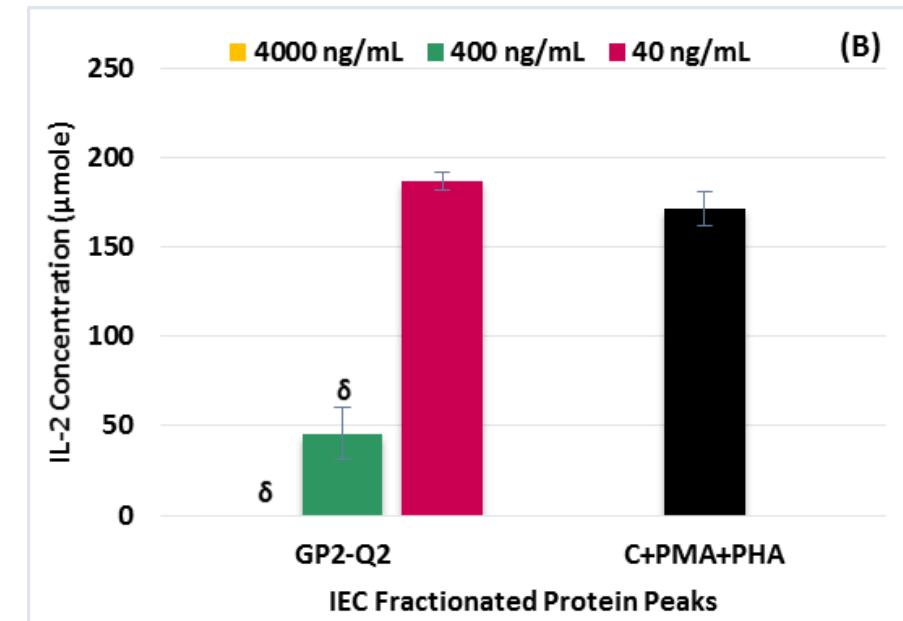
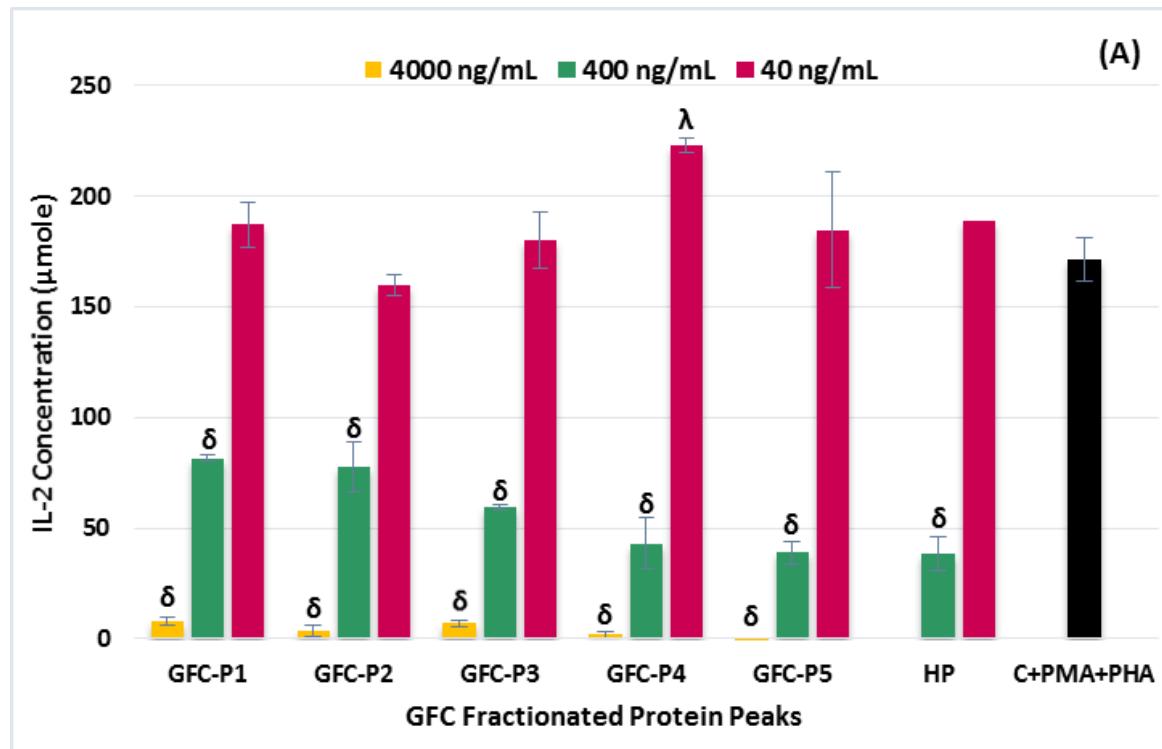
# T-cell Proliferation Assay:



S#	Sample Codes	IC <sub>50</sub> in ng/mL
1	GFC-P1	76.3 ± 21.2
2	GFC-P2	776 ± 129.8
3	GFC-P3	782.7 ± 108
4	GFC-P4	498.4 ± 139
5	GFC-P5	215.4 ± 56.3
6	HP	153.6 ± 13

- Figure 3.13: Effect of fractionated honey proteins on proliferation of T-lymphocytes. Cells were activated with 5 µg/mL of PHA.

# IL-2 Assay:



- **Figure 3.14:** Effect of GFC fractionated honey proteins (A), and ion exchange fractionated protein (B), on IL-2 production by PMBCs. Cells were stimulated with PMA (20 ng/mL) and PHA (7.5  $\mu$ g/mL).

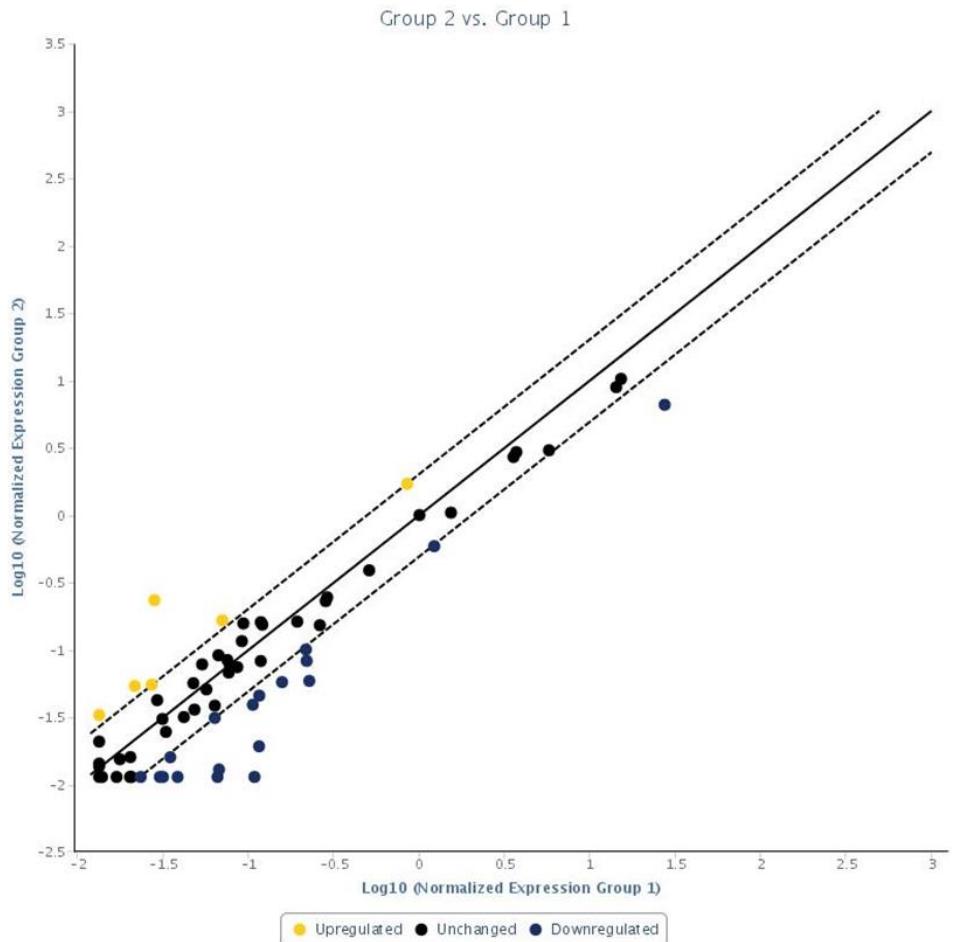
# Multi gene Profiling by Real Time PCR Array

PCR was performed in 96-well array coated with:

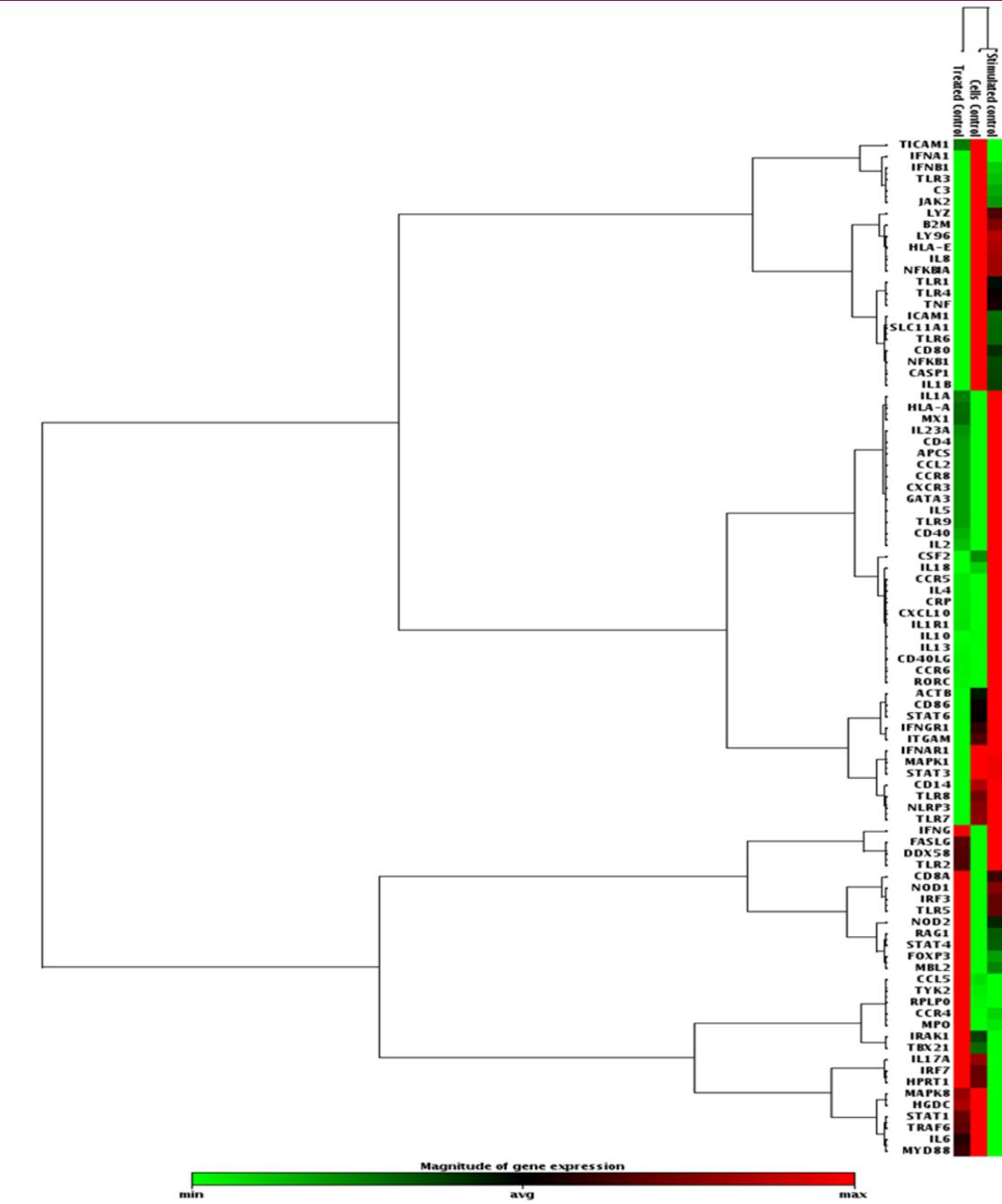
- 84 gene primer of innate and adaptive immune response
- 3 were reverse transcription control
- 1 was genomic DNA control
- 3 were positive PCR control
- 5 were house keeping genes control

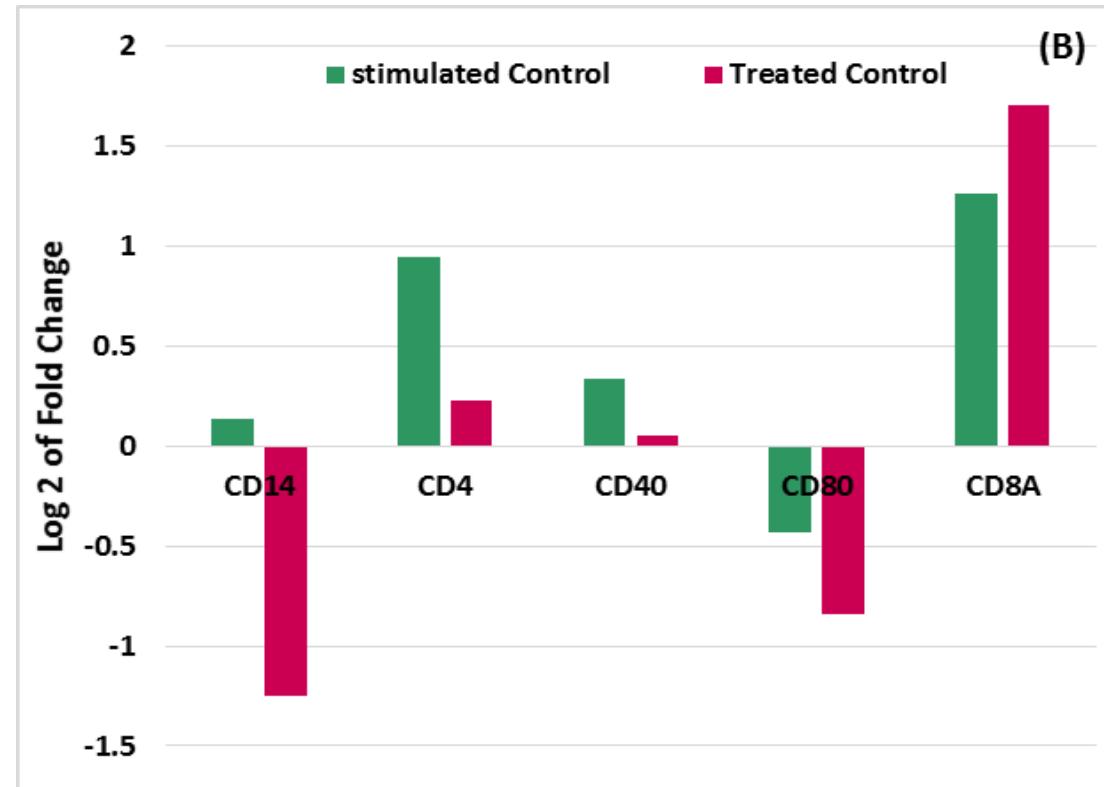
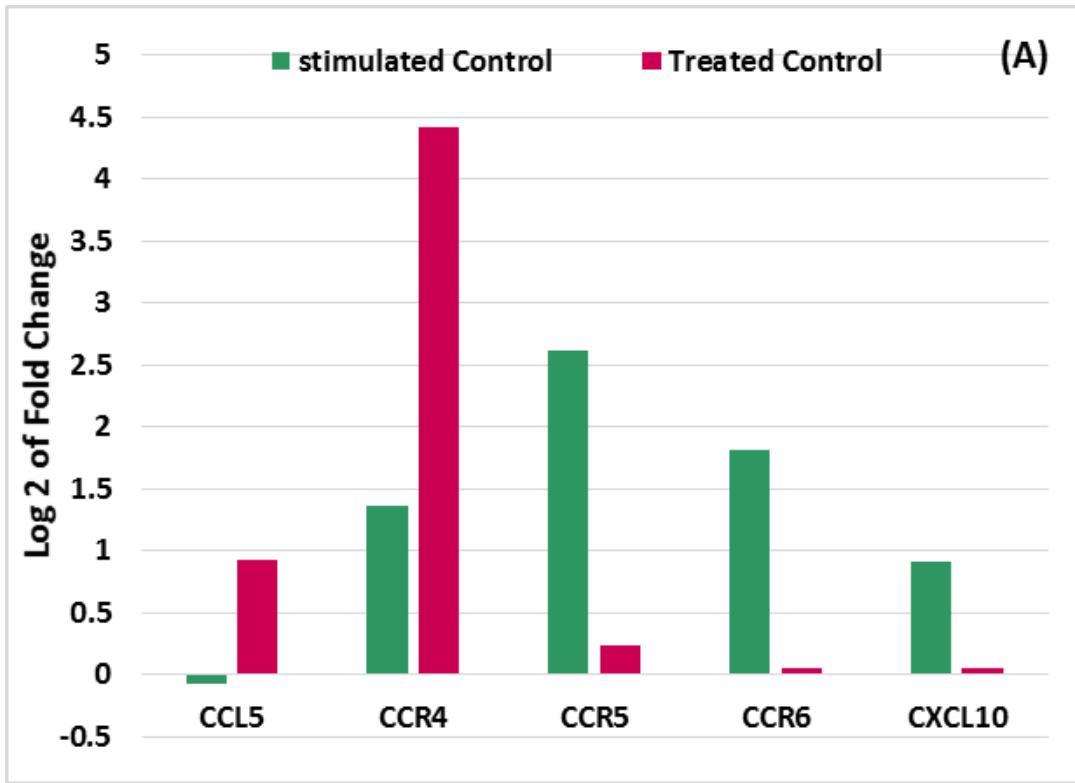
House keeping genes were:

- β-actin
- β-2 macroglobulin
- Glyceraldehydes-3-phosphate dehydrogenase
- Hypoxanthine phosphoribosyltransferase 1
- Ribosomal protein

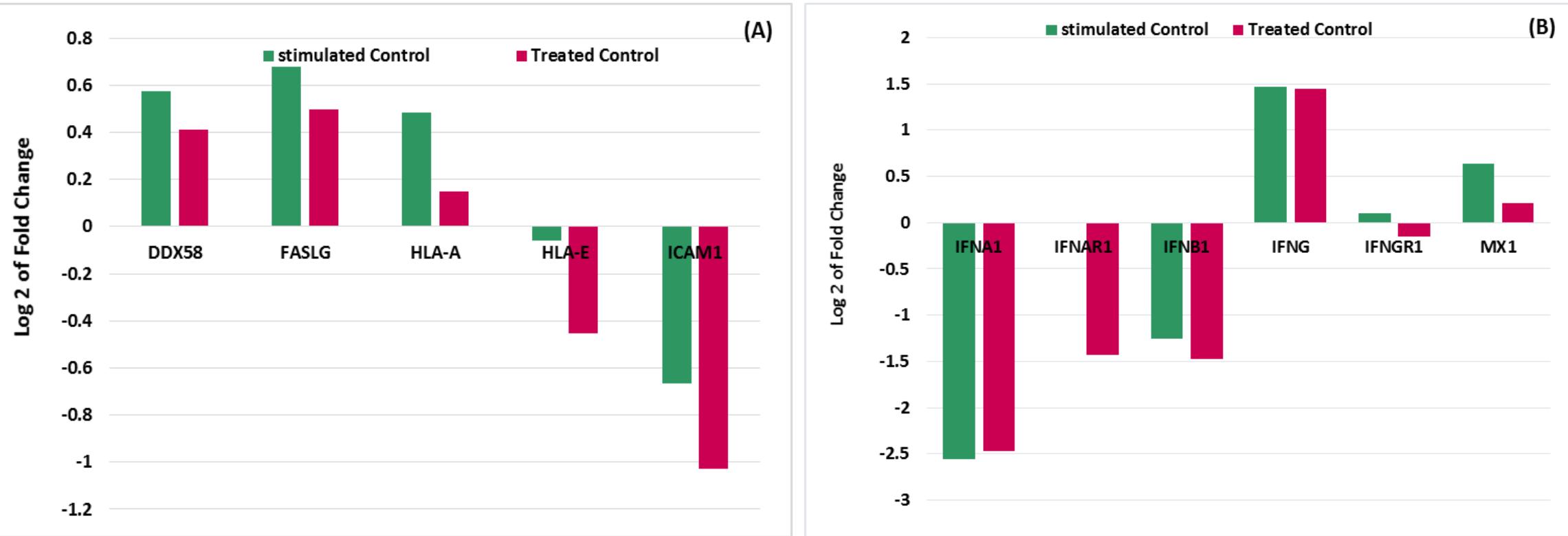


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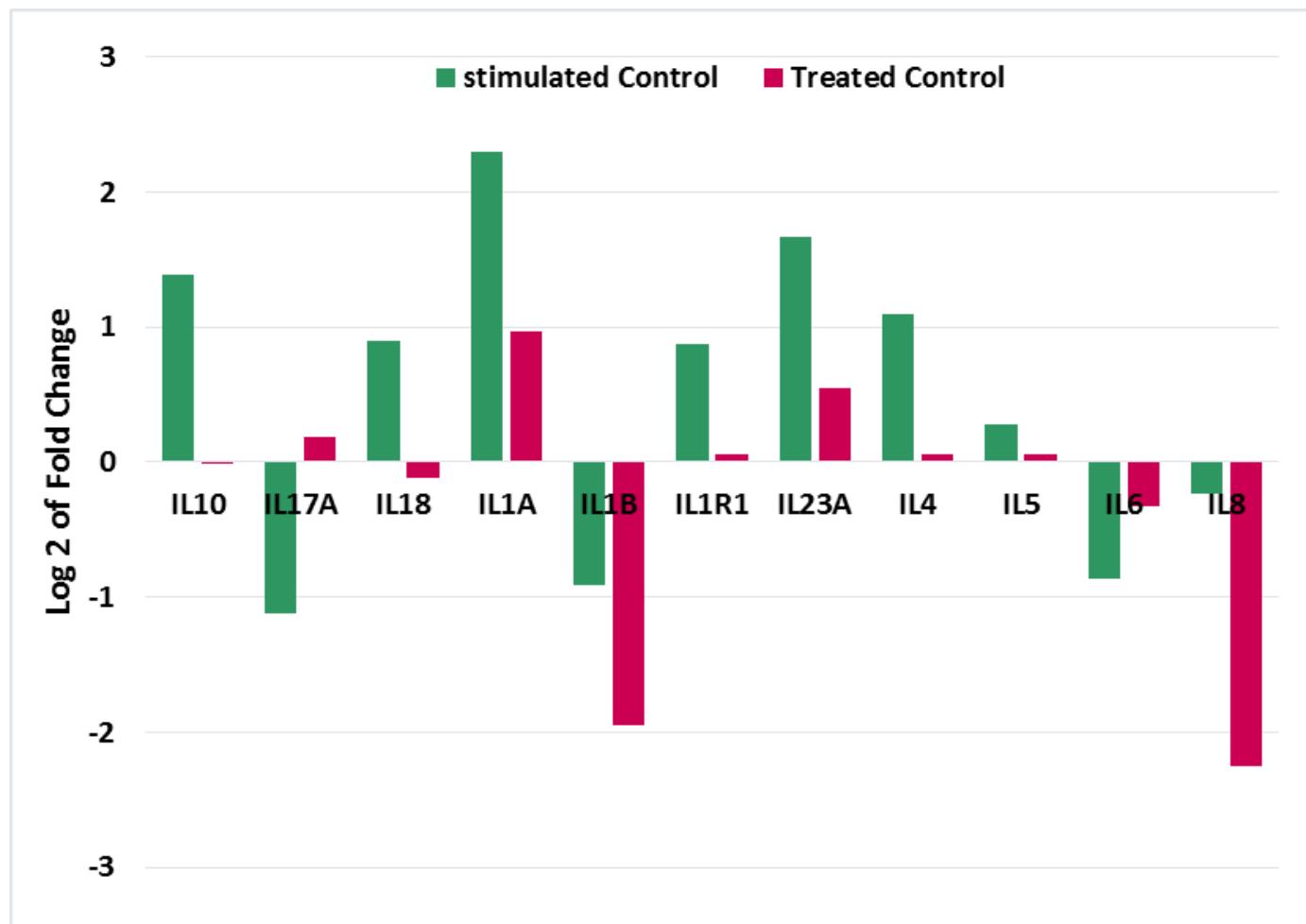




- **Figure-3.18:** Gene expression profiling for different genes related to chemokines ligand and receptors (A) and Cluster of differentiation molecules (B).

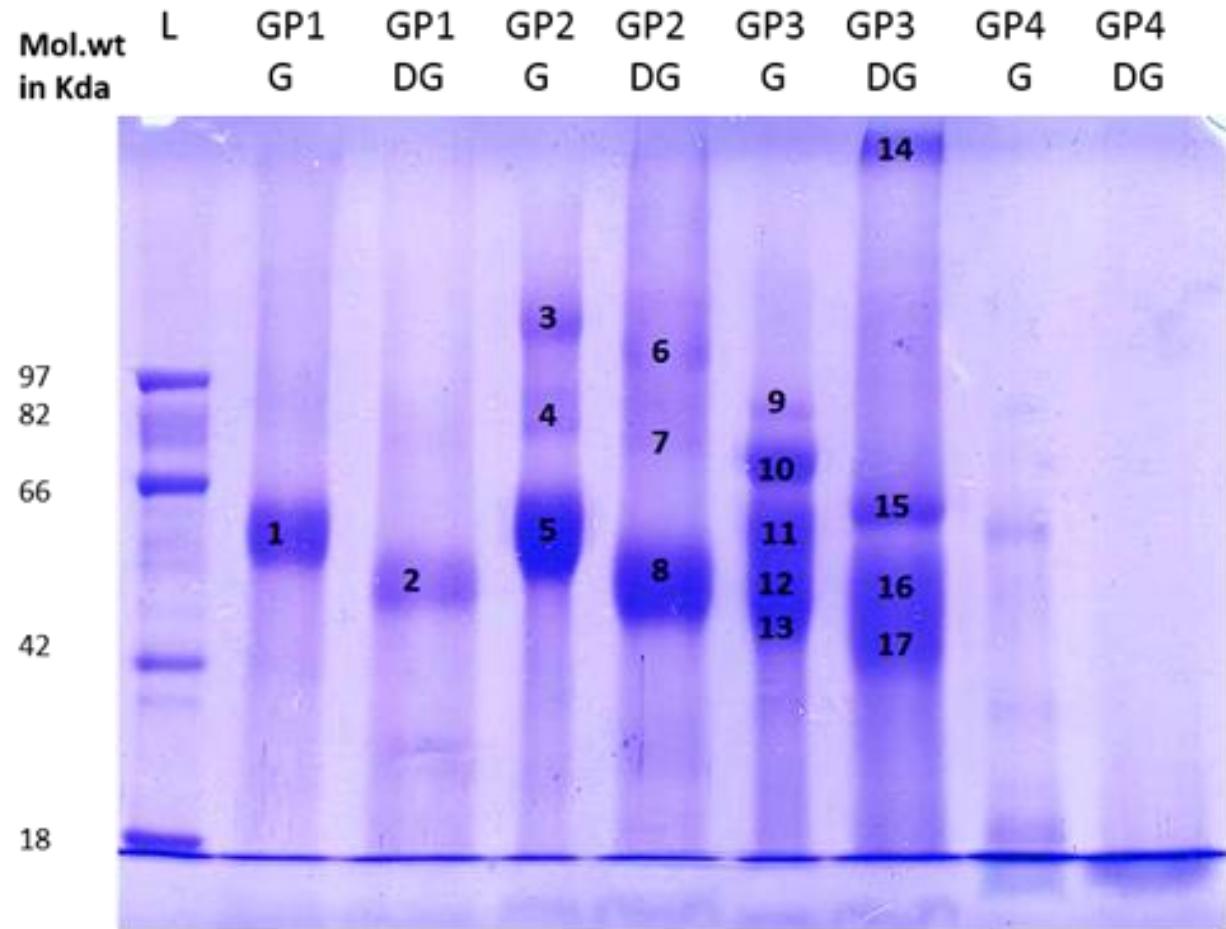


- **Figure 3.19:** Gene expression analysis cell adhesion molecules (A) and interferons genes (B).



- **Figure 3.20:** Gene expression profile of interleukin genes before and after honey proteins treatment.

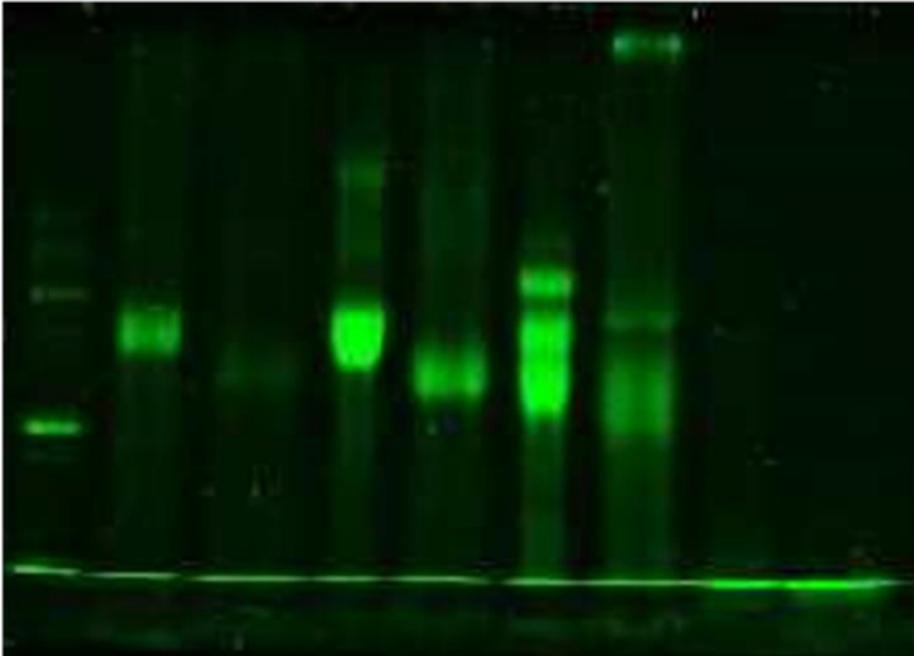
# Mobility Shift Assay:



# Glycoprotein Staining

Mol.wt  
in Kda

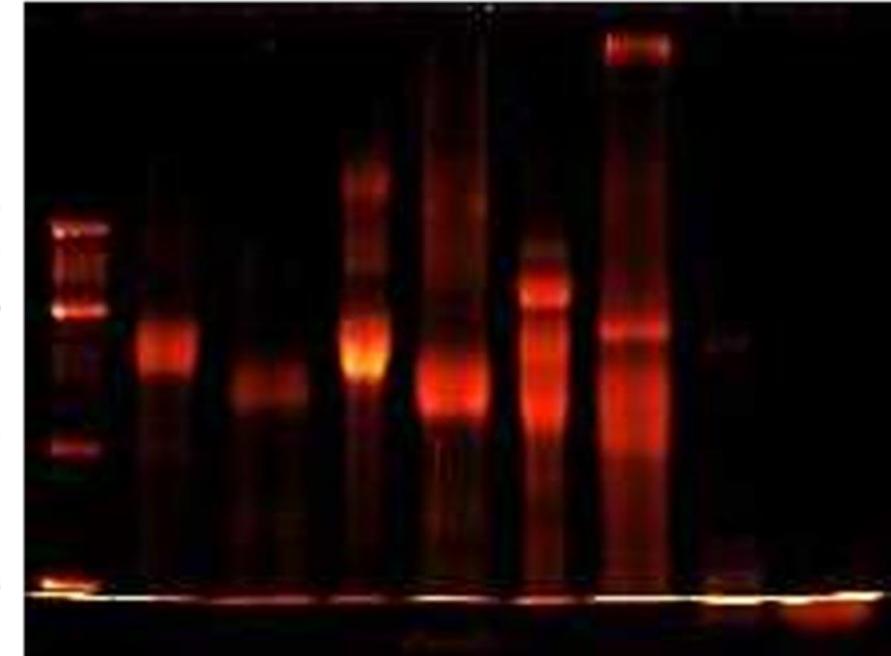
97  
82  
66  
42  
18



- Pro-Q Emerald Staining for glycoproteins

Mol.wt  
in Kda

97  
82  
66  
42  
18



- Sypro Ruby Staining for total proteins

# Mascot Results of GFC Fractionated Honey Proteins after MALDI-ToF MS

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Band #	Codes	Accession#	Protein Name	Source	Mascot Score	Mol. Wt.	ppm
1	NG-P1	Q548D6	Major royal jelly protein 1	Apis mellifera	66	49311	40
2	NDG-P1	Q548D6	Major royal jelly protein 1	Apis mellifera	45	49311	150
3	NG-P2-1	-	-	-	-	-	-
4	NG-P2-2	B2D0J4	Venom dipeptidyl peptidase 4	Apis mellifera	9	87882	50
5	NG-P2-3	Q548D6	Major royal jelly protein 1	Apis mellifera	77	49311	50
6	NDG-P2-1	-	-	-	-	-	-
7	NDG-P2-2	-	-	-	-	-	-
8	NDG-P2-3	Q548D6	Major royal jelly protein 1	Apis mellifera	58	49311	130
9	NG-P3-1	-	-	-	-	-	-
10	NG-P3-2	Q17058	Alpha-glucosidase	Apis mellifera	40	65694	155
11	NG-P3-3	-	-	-	-	-	-
12	NG-P3-4	Q17060	Major royal jelly protein 3	Apis mellifera	52	61966	80
13	NG-P3-5	-	-	-	-	-	-
14	NDG-P3-1	-	-	-	-	-	-
15	NDG-P3-2	-	-	-	-	-	-
16	NDG-P3-3	Q566B0	Bursicon (an insect hormone)	Apis mellifera	24	17616	130
17	NDG-P3-4	Q548D6	Major royal jelly protein 1	Apis mellifera	43	49311	40

# Conclusion:

- These studies indicated that honey contain different types of proteins which are all heavily glycosylated.
- Honey proteins showed potent inhibition on ROS, nitric oxide and phagocytosis of latex particles.
- Honey proteins also showed variable effects on TNF- $\alpha$  production while did not produce any effect on IL-1 $\beta$  production.
- These proteins significantly inhibited the production of IL-2 cytokines and proliferation of T-lymphocytes which indicated their role in adaptive immune response.
- Honey proteins (GFC-P2) greatly interfere with the genes of innate and adaptive immune response through up and down regulation of many important genes.
- MALDI-ToF mass spectrorum were analyzed with MASCOT Searching tool revealed that MRJP-1 is the major component in all GFC fractionated peaks and hence the most abundant protein of honey.
- It is indicated that MRJP-1 is the most immunologically active component of protein portion.
- Band # 10 and 12 in GFC-P3 were also identified as alpha glucosidase and MRJP-3 respectively.

# Publication:

JOURNAL OF  
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Article

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## Characterization of Immunomodulatory Activities of Honey Glycoproteins and Glycopeptides

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### Supporting Information

**ABSTRACT:** Recent evidence suggests an important role for natural honey in modulating immune response. To identify active components responsible, this study investigated the immunomodulatory properties of glycoproteins and glycopeptides fractionated from *Ziziphus* honey. Honey proteins/peptides were fractionated by size exclusion chromatography into five peaks

Thanks!



