**Ministry of Higher Education and Scientific Research**
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**College of Medicine**

# Immunogenetic study of Foxp3 gene on purified human peripheral blood mononuclear cells stimulated with Pseudomonas antigen and treated by propolis extracts

**A Thesis**
**Submitted to the Council of the College of Medicine, University of Babylon, in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in science/medical Microbiology**

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وزارة التعليم العالي والبحث العلمي
جامـــــعـــــــــة بابـــل
كـــــلـيـة الـــــطـــب

# دراسة مناعية ووراثية للجين Foxp3 في خلايا الدم البشري أحادية النواة المحفزة بالمستضد البكتيري للزوائف الزنجارية والمعالجة بمستخلص العكبر

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**بإشراف**

**الأستاذ الدكتور** — **محـمـد عـــبـد كاظـــم الســـــعدي**

**الأستاذ المساعد الدكتور** — **قيـــــصر نعمـــــة مظلـــوم**

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**بِسْمِ اللَّـهِ الرَّحْمَـٰنِ الرَّحِيمِ**

وَأَوْحَىٰ رَبُّكَ إِلَى ٱلنَّحْلِ أَنِ ٱتَّخِذِي مِنَ ٱلْجِبَالِ بُيُوتًا وَمِنَ ٱلشَّجَرِ وَمِمَّا يَعْرِشُونَ (٦٨) ثُمَّ كُلِي مِن كُلِّ ٱلثَّمَرَٰتِ فَٱسْلُكِي سُبُلَ رَبِّكِ ذُلُلًا يَخْرُجُ مِنۢ بُطُونِهَا شَرَابٌ مُّخْتَلِفٌ أَلْوَٰنُهُۥ فِيهِ شِفَآءٌ لِّلنَّاسِ إِنَّ فِي ذَٰلِكَ لَأَيَةً لِّقَوْمٍ يَتَفَكَّرُونَ

**صدقَ اللهُ العليُ العظيم**

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Farah

# *Dedication*

*In this life, many dreams have been shattered and many wishes have vanished…*

*To everyone who dreamed of writing a doctoral thesis and was unable to achieve and obtain his dream …*
*I dedicate this in my thesis…*
*And to the my best friend Dr.Ali AL-Habboubi…*

*Farah*

## Supervision Certification

We certify that this thesis entitled "Immunogenetic study of Foxp3 gene on purified human peripheral blood mononuclear cells stimulated with Pseudomonas antigen and treated by propolis extracts" was prepared under our supervision at the college of Medicine, University of Babylon, as a partial requirement for the degree of doctor of philosophy in Microbiology, and this work has never been published anywhere….

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In view of the available recommendation, I forward this thesis for debate by the examining committee.

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# ألخلاصــة

الدراسة الحالية هي دراسة تحليلية عشوائية تهدف الى دراسة فاعلية العكبر كمحَور مناعي ضد بكتريا الزوائف الزنجارية المقاومة لمعظم المضادات الحيوية وتأثيره على خلايا الدم البشري أحادية النواة، حيث تضمنت الدراسة (٢٠ شخص نصفهم ذكور والاخر إناث) تم تقسيم كل عينة الى أربعة مجاميع المجموعة الاولى خلايا لمفية أحادية النواة معالجة بالمستضد البكتيري للزوائف الزنجارية بعد ١٦ ساعة من الحضانة، أما الثانية خلايا لمفية أحادية النواة معالجة بالمستضد البكتيري للزوائف الزنجارية و المستخلص الكحولي للعكبربتركيز ٥مايكروغرام بالمل لمدة ٤٨ ساعة أما المجموعة الثالثة تكونت من خلايا لمفية أحادية النواة معالجة بالمستضد البكتيري للزوائف الزنجارية والمستخلص المائي للعكبر بتركيز ٥مايكروغرام بالمل لمدة ٤٨ ساعة هذه المجاميع الثلاث مثلت مجموعة السيطرة الموجبة.بينما تكونت المجموعة الرابعة من خلايا لمفية أحادية النواة فقط بتركيز($١*١٠^{٦}$) والتي مثلت مجموعة السيطرة السالبة و تم عزل الخلايا اللمفية الأحادية النواة من الدم بطريقة الطرد المركزي المتدرج الكثافة لكل عينة وتنميته بطريقة الزرع الخلوي خارج الجسم وتحت ظروف خاصة حيث خضعت جميع العينات بعد الزرع لفحص الحيوية الخلوية بصبغة التريبان الزرقاء بعد مرور ٤٨ ساعة ، تم فحص حيوية الخلايا بصبغة التريبان الزرقاء وتم جمع الزرع الخلوي لكل المجاميع الأربعة لتقيم فاعلية مادة العكبر وتأثيرها على الخلايا اللمفية المعالجة بالمستضد البكتيري ولغرض حساب العدد الكلي الحي من الخلايا ،قياس تركيز انترفيرون كاما، انترلوكين ١٧ أ، انترلوكين ٢٣ونسبة التعبير الجيني للجين Foxp3 لكل المجاميع.

أشارت النتائج الى زيادة معنوية في تركيز الانترفيرون كاما في المجموعة الإولى (٦٣,٤٠ بيكوغرام/ مل) مقارنة بمجموعة السيطرة بينما إنخفاضا معنويا كبير وملحوظ في تركيزه في المجموعتين الثانية والثالثة مقارنة بالمجموعة الاولى ( ٥٠,٠ بيكوغرام/ مل ،٥٢,٨٥ بيكوغرام/ مل على التوالي) كما وأظهرت النتائج لهذا السايتوكين عدم وجود فرق معنوي بين المستخلص الكحولي والمائي للعكبر في تأثيره على الخلايا المناعية وأيضا عدم وجود فرق معنوي في تأثيره على مجموعة الذكور والإناث رغم الزيادة الطفيفة لهذا السايتوكين في مجاميع الاناث.

فيما يخص نتائج تركيز الانترلوكين ١٧ اشارت الى زيادة معنوية في المجموعة الاولى (١٦٤,٠٧ بيكوغرام/مل) مقارنة بمجموعة السيطرة الرابعة بينما اظهرت المجموعة الثانية انخفاظا معنويا كبير في تركيز الانترلوكين١٧ (١٠٤,٨٤ بيكوغرام/مل) مقارنة بالمجموعة الاولى والثالثة (١٦٤,٠٧بيكوغرام/مل

، ١٣٠,٦١ بيكوغرام/مل على التوالي) بينما اظهرت المجموعة الثالثة إنخفاظا غير معنوي في تركيز هذا السايتوكين (١٣٠,٦١ بيكوغرام/مل) مقارنة بالمجموعة الاولى,أيضا أظهرت وجود فرق معنوي في تأثير التحوير المناعي لمستخلص العكبر الكحولي مقارنة بالمستخلص المائي في تأثيره على تركيز هذا السايتوكين في الخلايا اللمفية المعالجة بمستضد الزوائف الزنجارية.

فيما يخص تركيز الانترلوكين ١٧ في الجنسين أظهرت النتائج وجود فرق معنوي في المجموعة الرابعة ( مجموعة السيطرة السالبة) بارتفاع ملحوظ في تركيزه عند الإناث ( ١٤٧,٩ بيكوغرام/مل)مقارنة ب(١٢٩,١٣ بيكوغرام/مل) في الذكور .بينما كانت الفروق غير معنوية لبقية المجاميع بين الذكور والاناث.

فيما يخص الانترلوكين٢٣ أظهرت النتائج وجود زيادة معنوية كبيرة في تركيزه في المجموعة الاولى (٤٩١,٨٢بيكوغرام/مل) مقارنة بمجموعة السيطرة الرابعة (٣١٨,٨٤بيكوغرام/مل).أيضا أشارت النتائج الى وجود انخفاض معنوي في المجموعة الثانية لتأثير المستخلص الكحولي للعكبر(٤٠٢,٨٤بيكوغرام /مل) والمستخلص المائي في المجموعة الثالثة (٣٩٥,٣٥بيكوغرام/مل) وبتأثير مشابه تقريبا مقارنة بالمجموعة الأولى (٣١٨,٨٤بيكوغرام/مل) وكذلك عدم وجود فرق معنوي بين تأثير المستخلص الكحولي والمستخلص المائي للعكبرعلى هذا السايتوكين. كما وبينت النتائج وجود فروق معنوية فيما يخص الجنسين في المجاميع الاولى والثالثة والرابعة بزيادة معنوية في مجاميع الذكور (٥٤٩,٧بيكوغرام/مل ,٤٥٩,٤٢بيكوغرام/مل,٣٧١,٣٨بيكوغرام/مل على التوالي) مقارنة بالاناث (٤٤٧,٨٤ بيكوغرام/مل ,٣٨٠,٨٤بيكوغرام/مل ,٣٢٨,٥٢بيكوغرام/مل) على التوالي. وعدم وجود فرق معنوي في تأثير مستخلص العكبر الكحولي في المجموعة الثانية بين الذكور والاناث.

فيما يخص نتائج التعبير الجيني للجينFoxp3 أظهرت نتائج البحث وجود انخفاض معنوي في نسبة التعبير الجيني لهذا الجين في المجموعة الاولى(١,٣٥) مقارنة بمجموعة السيطرة الرابعة (٢,٢٥) ,كما واشارت النتائج الى وجود ارتفاعا معنويا كبيرا في التعبير الجيني في المجموعة الثانية (٤,٨٠) والتي أظهرت تأثيراً معنويا كبير للمستخلص الكحولي للعكبر كذلك المجموعة الثالثة أظهرت نتائج معنوية لتأثير المستخلص المائي للعكبر على التعبير الجيني لل Foxp3 (٣,٠٥) مقارنة بمجموعة السيطرة (٢,٢٥) كما وأشارت الى وجود فرق معنوي في تأثير المستخلص الكحولي للعكبر بزيادة ملحوظة على التعبير الجيني مقارنة بالمستخلص المائي.وفيما يخص الجنس أظهرت النتائج عدم وجود فروق معنوية بين الذكور والاناث لكل المجاميع في التعبير الجيني لجين Foxp3.

أما فيما يخص العلاقة المترابطة بين المعايير المناعية للسايتوكينات الثلاث والتعبير الجيني في مجموعة الذكور,فقد أظهرت النتائج وجود فروق إحصائية معنوية وعلاقة عكسية بين مستوى ال IFN-γ و التعبير الجيني لجين Foxp3في المجموعة الثالثة (مجموعة المستخلص المائي للعكبر).

كذلك وأظهرت النتائج وجود علاقة عكسية معنوية بين مستوى IL-23 والتعبير الجيني لجين Foxp3في المجموعة الاولى والثانية (مجموعة المستضد البكتيري و المستخلص المائي للعكبر على التوالي).

فيما يخص العلاقة المترابطة بين المعايير المناعية للسايتوكينات الثلاث والتعبير الجيني في مجموعة الاناث,فقد أظهرت النتائج وجود فروق إحصائية معنوية وعلاقة عكسية بين مستوى IL-17Aوالتعبير الجيني لل Foxp3في مجموعة المستخلص الكحولي فقط,أما المجاميع الثلاثة الأخرى بينت النتائج عدم وجود فرق معنوي بين السايتوكينات والتعبير الجيني لل Foxp3.

يستنتج من خلال هذه الدراسة إن العكبر كمحَّور مناعي ضد بكتريا الزوائف الزنجارية المقاومة للمضادات الحيوية وكذلك مضاد التهابي للأمراض المناعية المرتبطة بالإمراضية المناعية للزوائف الزنجارية من خلال خفض مستوى عوامل المناعة الخلوية اضافة الى تعزيز وزيادة التعبير الجيني للخلايا التائية المنظمة للتوازن المناعي والاستجابة المناعية خلال وبعد الاصابة ببكتريا الزوائف الزنجارية.

# SUMMARY

This experimental analytical randomized controlled trail (parallel group) study aims to evaluate the immune modulatory activity of propolis against antibiotic resistance *Pseudomonas aeruginosa* in peripheral blood mononuclear cells (PBMCs) stimulated with *Pseudomonas aeruginosa* antigen *invitro*. It included 10 human males and 10 females, each sample from these volunteer evenly divided into four groups. Groups I was PBMCs ($1*10^6$cells/ml) treated with *pseudomonas aeruginosa* Ag, and group II PBMCs was treated with *Pseudomonas aeruginosa* Ag plus ethanol extracted propolis (EEP) 5µg/ml, while group III was PBMCs treated with *Pseudomonas aeruginosa* Ag and water extracted propolis (WEP) 5µg/ml, all these groups were serve as positive control while the group IV was PBMCs only that serve as negative control group. PBMCs were isolated by density gradient medium and all the groups were cultured with RPMI1640 medium under special condition. After 48 hrs. cell cultures samples were collected from all groups to detect the PBMCs viability by trypan blue dye and to estimate the concentration of immunological markers, interferon-γ (IFN-γ), interleukin-17A (IL-17A) and interleukin 23(IL-23) by enzyme linked immunosorbent assay technique. Additionally, the genetic parameter were done to detect the level of Foxp3 mRNA gene expression by real time PCR. The results indicated a significant increase in the median value of IFN-gamma in group I(63.40pg/ml) as compared with group IV (60.0pg/ml) the differences between them were significant at ($p<0.05$). The results also pointed to a significant decreasing in the concentration of this cytokine in group II and III as compared with the positive control group I (50.00 pg/ml, 52.85pg/ml,63.40pg/ml, respectively). Also the result shown a non significant differences between EEP and WEP at ($p>0.05$). In contrast to

the gender the results showed a non-significant correlation between gender and IFN-γ.

Also the results pointed to a significant elevation in IL-17A concentration in group I(164.07pg/ml) in comparison to group IV negative control(140.23pg/ml) ,also there was significantly high decreasing in IL-17A level in group II(104.84 pg/ml) in comparison to group I and III(164.07pg/ml,130.61 pg/ml, respectively) while group III shown a non-significant decreasing(130.61pg/ml) at ($p > 0.05$). Also the result shown a significant differences between EEP and WEP immunomodulation at ($p < 0.05$) with EEP has more activity on PBMCs treated with *pseudomonas aeruginosa* Ag than WEP. Regarding gender the group IV shown significant increasing in IL-17A in females (147.9pg/ml) as compared to males (129.13 pg/ml),while the other groups shown a non-significant differences.

IL-23 concentration results revealed a significant increase in group I(491.82 pg/ml) as compared with group IV (318.84 pg/mL) in addition to a significantly high decreasing in the median value of IL-23 levels in group II EEP stimulated and III WEP stimulated PBMCs with nearly the same effects as compared with group I (318.84 pg/ml) at ($p < 0.05$), also pointed a non- significant differences between EEP and WEP effect on IL-23. Regarding gender the results shown significant elevation in group I,III,IV in males groups (549.7pg/ml, 459.42pg/l, 371.38pg/ml, respectively) as compared to females groups (447.84pg/ml, 380.84pg/ml, 328.52pg/ml, respectively), and shown a non-significant differences in the EEP effect in the group II between males and females.

In concern to the genetic parameter results of Foxp3 gene expression , this study revealed a significant decreasing in the Foxp3 mRNA gene expression

levels in group I(1.35) as compared to group IV negative control (2.25). In addition the results pointed a significantly high elevation in Foxp3 mRNA gene expression levels in group II(4.80) with EEP effect and III(3.05) with WEP effect as compared to group IV(2.25). On the other hand, revealed significant differences between EEP and WEP effect on PBMCs stimulated with gram negative *pseudomonas aeruginosa* Ag with EEP has more effect than WEP at ($p < 0.05$).

Regarding the correlation between the immunological cytokines markers and Foxp3 gene expression in males the results had been shown a significant negative correlation between IFN-γ level and Foxp3 expression in the WEP group, also the results showed statistically significant negative correlation between IL-23 level and Foxp3 in the Ag only stimulated group and EEP group.

While the results of correlation in females showed a significant negative correlation between IL-17A and EEP group only.

It is concluded that propolis extracts have immunomodulator role against *Pseudomonas aeruginosa* antigen and pathogen induced immune-pathogenesis via modulation and decreasing the levels of cellular immunity elements and increasing the activity of T regulatory cells via acting on Foxp3 gene expression during and after infection with *Pseudomonas aeruginosa*.

## *1.0 Introduction:*

The global spread of so-called high-risk clones of multidrug -resistant or extensively drug resistant (MDR/XDR) *Pseudomonas aeruginosa* has become a public health concern in recent years. It causes severe infections, especially in health-care settings and in immunocompromised patients, It has an exceptional capacity for being selected and spreading antimicrobial resistance in vivo, and the effective worldwide spread of so-called "high-risk" clones of *P. aeruginosa* presents a challenge to global public health that requires immediate attention and management (Horcajada *et al*.,2019). *Pseudomonas aeruginosa* is a common pathogen in nosocomial and ventilator-associated pneumonia, abscess, cystic fibrosis (CF), soft tissue, and urinary tract infections, meningitis, corneal infection, and also conjunctival erythema in immunocompromised patients, it can also cause catheter-associated and chronic lung infections (Chatterjee *et al.*,2016), offered its ability to develop biofilm on medical devices and exploit the host's altered normal flora as a result of the use of broad-spectrum antibiotics (Francesca *et al.*,2020).

The lack of therapeutic alternatives means that infections caused by these antibiotic-resistant bacteria pose a considerable threat regarding morbidity and mortality worldwide. In 2017, the World Health Organization classified carbapenem-resistant *P. aeruginosa* as a "critical" infection for which new antibiotics are required (Tacconelli *et al.*,2018).

Medicinal plants are plants that contain substances that could be used for therapeutic purposes or which are precursors for the synthesis of useful drugs (Abolaji *et al.*, 2007). To reduce the emergence of resistance and adverse drug reactions, folk medicine offers a valuable and underutilized

resource for the discovery and creation of potential new medicines against microbial infections. Additionally, the use of medicinal plants provides opportunities for developed countries because they are much more inexpensive, safe, and available (Nigussie *et al*.,2021). Plants represent an inestimable source of anti-pseudomonal agents. An example of these compounds is propolis. Since ancient times propolis has been used by humans for health and food protection. Because of its broad range of biological and pharmacological properties, interest in this natural product has increased in recent years (Atanas *et al*., 2015).

Propolis is a resinous substance obtained by bees from buds and plant secretions and mixed with salivary gland products and wax (Grecka *et al*., 2019). Propolis is made up of about 50% resin and vegetable balsam, 30% wax, 10% essential and aromatic oils, 5% pollen, and 5% other minerals and vitamins (Ibrahim, 2011; Ahangari *et al.,* 2018). Flavanone, 3-Hydroxyflavone, Chrysin, Apigenin, Caffeic acid, Quercetin, Galangin, Kaempferol, and Ferulic acid were found in Iraqi propolis, according to the chemical analysis. Propolis has also been used to improve health and prevent diseases like autoimmune disease, atherosclerosis, heart disease, diabetes and cancer (Grecka *et al*.,2019).

## *1.1. Aim of the study:*

This study aimed to illustrate the modulatory activity of propolis against selected antigens separated from drug –resistant *Pseudomonas aeruginosa*. This aim will be assessed through the following parameters:

1- Extraction of propolis by ethanol and water.
2- Extraction of somatic antigen of *pseudomonas aeruginosa*.
3- Isolation of peripheral blood mononuclear cells(PBMCs) from healthy volunteers .
4- Evaluation of immune responses in PBMCs cell culture for Ag, EEP, WEP stimulated groups, and control by the following parameters:
   A- Measuring the levels of interferon- gamma (IFN-γ), (interleukin-17A) and interleukin-23(IL-23) in PBMC cell culture.
   B- Measuring the Foxp3 gene expression level in PBMC cell culture by qRT- PCR and detection the immunomodulatory effect of propolis on PBMCs stimulated with MDR-*pseudomonas aeruginosa* antigen (*in vitro*) .
   C- Detection of the correlation between propolis effect on Foxp3 and interferon- gamma (IFN-γ), (interleukin-17A) and interleukin-23(IL-23) in males and females.

## *1.2. Literatures Review:*

### *1.2.1 Pseudomonas aeruginosa:*

*Pseudomonas aeruginosa* is a Gram-negative, aerobic rod-shaped bacterium of the bacterial family Pseudomonadaceae, *P. aeruginosa* is a heterotrophic, motile, bacterium, non-fermenting aerobic that takes its energy from oxidation rather than from the fermentation of carbohydrates. Even though that it is capable of using more than 75 different organic compounds. *P. aeruginosa* tolerates high concentrations of salt, weak antiseptics, dyes and many commonly used antibiotics. It is a major cause of nosocomial infections, including respiratory, soft tissue, urinary tract, skin, surgical, and bloodstream infections (Ramphal *et al.*,2018).

*P. aeruginosa* envelope which is like that of another Gram-negative bacterial envelope, contain three layers: the inner(cytoplasmic membrane), the peptidoglycan layer(PG), and the outer membrane. The latter is composed of phospholipids, proteins, and lipopolysaccharides (LPS). The LPS of *P. aeruginosa* is less toxic than LPS of other Gram-negative rods and it is in most strains of *P. aeruginosa* contains heptose, 2-keto-3-deoxyoctonic acid and hydroxy fatty acids, in addition to a side-chain and core polysaccharides. It is one of the most important opportunistic pathogens in humans that can cause a wide range of infections in both immunocompetent and immunocompromised hosts (Kroken *et al*., 2018; Stephen and Marvin, 2019).

This bacterium has a high antibiotic resistance due to the lower outer membrane permeability and multiple drug efflux pumps developments. Various secreted and cell-associated antigens of *P.aeruginosa* have been manipulated for use in vaccine production. The primary pseudomonas

antigen is an extracellular mucous membrane (slime), which is a mucoid membrane composed primarily of alginate. High M.W. alginate contents tend to contain preserved epitopes, while low M.W alginate contents contain preserved and unique epitopes. Due to its high antigenic nature, surface exposed antigens including O antigen (O-specific LPS polysaccharides) or H flagellar antigens were used for serotyping (Bassetti *et al*., 2018; Ahmed, *et al*.,2020). According to the chemical structures of repeating units of O specific polysaccharide there are 31 chemotypes of *p. aeruginosa* were recognized. The main structural proteins of the outer membrane represented by three types: porins, lipoproteins and heatlabile proteins such as (OmpA) (Saleh *et al*.,2013).

Rocha *et al*.,(2019) pointed out that *P. aeruginosa* has two porins that permit the passage of general substrates. OprD is a specific porin that allows small molecules of less than 200 Da to pass, while OprF porin, which is a member of the OmpA family of outer membrane proteins, has a wide exclusion limit. It is responsible for the permeation and passage of molecules in the range of 200 and 3,000 Dalton. In addition, OprB porin is the man conduit for saccharides.

In addition to LPS, *P.aeruginosa* produces phospholipase, leukocidin, and many other enzymes (Saade *et al*.,2020). This bacterium produces a variety of virulence factors, including exotoxins (exotoxin A, exotoxin S, exoT, exoU, and cytotoxin), elastase and alkaline protease, also it can produce the non-fluorescent pyocyanin bluish pigment during growth and the fluorescent green pigment pyoverdin. All of these virulence factors can cause a wide range of infections, but rarely cause serious illness in healthy individuals without a predisposing factor (Rocha *et al*.,2019).

### *1.2.2. Pseudomonas aeruginosa pathogenicity:*

Pathogenesis in this bacterium is performed by multiple virulence factors that mediate adherence and disrupt the signaling pathways of host cells, while targeting the extracellular matrix. *P. aeruginosa* stands out as a unique and threatening bacterial infection that is capable of causing extreme invasive diseases and of resisting immune defenses that cause chronic infections that are usually impossible to eradicate. The subsequent tissues invasion and spread of *P. aeruginosa* is likely related to the many virulence factors it produces. These factors play a key role in the motility and adherence of the host epithelium, such as LPS, flagellum, and Type III secretion systems, which injects the toxic effector protein into the host cells cytosol and are accompanied by increased mortality rates, also exotoxin A, proteases, Type IV Pili, quorum sensing, biofilm formation, alginate, and type VI secretion systems are major virulence factors acting in different manners in the immune system (Chevalier *et al*., 2017; Skariyachan *et al*.,2018).

*P. aeruginosa* uses exotoxin A as a virulence factor to inactivate eukaryotic elongation factor 2(EF-2) in the host cell, such as the action of diphtheria toxin, hence eukaryotes cannot synthesize proteins and necrotize (Eman, *et al*., 2017). The single unsheathed polar flagellum of *P. aeruginosa* is mainly responsible for the swimming motility of this bacterium (Cassin *et al*., 2019). However, its role in virulence goes beyond simple motility. Flagellar proteins were shown to play important roles in attachment, invasion, biofilm formation of pseudomonas and mediation of inflammatory reactions. Type IV pili of *P. aeruginosa* has a role in the adhesion to many cell types and this is likely to be important in attachment to specific tissues

(tissue tropism), initiation of non-opsonic phagocytosis which is mediated by phagocytes receptors that recognize corresponding adhesions on bacterial surfaces and also the initiation of biofilm formation (Kumar *et al*., 2018) .

Cafora *et al*.,(2019) was indicated a direct correlation between the adherence of *P. aeruginosa* and the presence of glycosphingolipids on host cells, thus demonstrating the specific role of this glycosphingolipids as a bacterial receptors. In particular, *P. aeruginosa* pili, bind to glycosphingolipids in host epithelial cell membranes, ganglio-N-tetraosylceramide (asialo-GM1), pili-and asialo-GM1 interactions, is mediated by internalization of *P. aeruginosa* in host epithelial cells. In addition, these pili also mediate the twitching motility, a factor found to be important in biofilm formation in vitro (Huang *et al*., 2019). ExoT and ExoS are bi-functional cytotoxins that possess both Rho GTPase-activating protein and ADP ribosyltransferase activities. These proteins can inhibit phagocytosis by interrupting actin cytoskeletal rearrangement, signal transduction, and focal adhesions (Barbieri & Sun, 2004). In addition, ExoU is a phospholipase that directly contributes to acute cytotoxicity towards macrophages and epithelial cells, while ExoY is an adenylate cyclase that affects cytoskeleton reorganization and intracellular cAMP levels (Kostylev *et al*.,2019).

*P. aeruginosa* produces several of secreted proteases, including alkaline protease, metalloendopeptidase LasA and zinc metalloprotease (elastase) LasB, These proteases work in a concerted way to destroy host tissues and therefore play a crucial role both in acute lung infection and in burn wound infections(Martinez-Garcia and de Lorenzo, 2019). *P. aeruginosa* is one of the five leading causes of nosocomial bacteremia and often leads to sepsis. It

may cause infection in almost any part of the body, although it does not usually cause infection in a healthy host. People most susceptible to *P. aeruginosa* infections are those whose mucous membranes or skin have been compromised in such a way that they no longer serve as physical barriers to infections (e.g. in burn patients). Neutropenic or otherwise immunocompromising predisposes patients to infection with many different organisms, of which *P. aeruginosa* is one. The unique lung environment that occurs in patients with cystic fibrosis promotes chronic infection with this organism, in which this bacterium has a distinctive mucoid phenotype due to the alginate production that surrounds microcolonies of this organism. It has a tremendous ability to cause both chronic infections in the lungs of patients with CF and acute nosocomial pneumonia in hospitalized patients with cardiovascular disease, diabetes, or cancer, and in particular patients with mechanical respirator are likely to develop pneumonia or bacteremia due to *P. aeruginosa* (Mielko *et al*.,2019).

Another important virulence factor of this pathogen is biofilm formation, the secreted extracellular matrix mainly consists of proteins, nucleic acids, lipids, and exopolysaccharides (EPS), EPS are predominantly characterized by the presence of alginate. The alginate participates in the structuring of the biofilm and the overexpression of alginate was shown to protect *P. aeruginosa* from phagocytosis and host responses (Yang *et al*.,2012). Like most bacteria, *P. aeruginosa* can develop two distinct lifestyles, planktonic and sessile cells. The planktonic state is encountered when *P. aeruginosa* evolves freely in a liquid suspension, whereas on natural or synthetic surfaces, *P. aeruginosa* can form sticky clusters in permanent rearrangements characterized by the secretion of an adhesive and protective

matrix defined as biofilm, this set of bacterial community adherent to a surface appears as an adaptive response to an environment more or less unsuited to growth in planktonic form (Okshevsky and Meyer,2015).

Also, extracellular DNA (eDNA) is an important component of the biofilm matrix. It has been established that eDNA plays roles in bacterial adhesion and in the structural stability of biofilms by maintaining coherent cell alignments interestingly, its contribution to antimicrobial resistance has also been proposed as eDNA, a highly anionic polymer, is believed to bind cationic antibiotics, such as aminoglycosides and antimicrobial peptides (Hazan *et al*.,2016).

### *1.2.3. P. aeruginosa antimicrobial Resistance :*

A general definition of antimicrobial resistance is the ability of an organism to resist the action of antimicrobial agents to which it was previously susceptible and *P. aeruginosa* is often resistant to many types of antibiotics and therapeutic agents, making it difficult to treat during infection, *P. aeruginosa* may be a particularly difficult organism to treat once an infection has been established as it is intrinsically resistant to many of the available antibiotics.

The genome of *P. aeruginosa* contains the largest known resistance gene on the island (Balasubramanian *et al*., 2013; Khattab *et al*., 2015). The important reason for antimicrobial resistance was the impermeability that belonging to the outer membrane lipoprotein (oprL gene) involved in the efflux transport systems and affecting cell permeability (Santajit and Indrawattana, 2016). Three mechanisms in which *P. aeruginosa* resists the action of antibiotics have been studied. The outer membrane of this bacterium restricts the penetration of antibiotics and the efficient removal of

antibiotic molecules by efflux systems before acting on their targets, and *P. aeruginosa* has the genetic ability to modify and inactivate antibiotics. This bacterium may become resistant by mutational changes in antibiotic targets(Abreu *et al*.,2017). As a result, *P. aeruginosa* has now achieved the status of superbug.

Kritsotaki *et al*.,(2017) reported that the intrinsic resistance arises from the combination of slow uptake and the secondary mechanisms that benefit from slow uptake, including degrading enzymes such as periplasmic β-lactamase and, in particular, multidrug efflux systems. At least four antibiotic efflux systems are present including MexAB-OprM , MexXY-OprM, MexCD-OprI, and MexEF-oprN elsewhere. Multidrug efflux pumps mediated resistance to many classes of antibiotics. a large number of drug efflux systems contained in the *P. aeruginosa* genome (Thaden *et al*., 2017) which have been categorized into five superfamilies, including the small multidrug resistance family, the resistance-nodulation-cell division family, the multidrug and toxic compound extrusion family, the ATP-binding cassette family, and the major facilitator superfamily (Kollef *et al*.,2014; Pachori *et al*., 2019).

Beta- lactam antibiotics are known to interfere with the synthesis of bacterial peptidoglycan cell walls. This class includes cephalosporin, monobactam and carbapenem. Resistance to beta-lactam is mediated by the action of beta-lactamases, the enzymes that destroy the amide bond of the beta-lactam ring, which causes the antimicrobial to be ineffective. This inactivation of the drug related to the expression of endogenous beta-lactamases or to the high expression of acquired beta lactamases. There are four main types of beta-lactamases identified in *P. aeruginos*: A-D. Classes

A, C and D inactivate b-lactams via the catalytic activity of serine residues, whereas class B or metallo-b-lactamases (MBLs) require zinc for their action (Raineri *et al*.,2014).

All of these mechanisms are inducible, and their regulation is mediated by the environment encountered by this organism. Endogenous beta lactamase production such as AmpC beta lactamase (chromosomal cephalosporinase) in this bacterium may be induced by a number of beta lactams such as narrow spectrum cephalosporin, benzyl penicillin, and imipenem. *P. aeruginosa* is naturally sensitive to carboxypenicillins, aztreonam and ceftazidime However, resistance can be acquired by gene mutation, which leads to the hyper-production of AmpC beta-lactamase(Nathwani *et al*.,2014)

Regarding the resistance of aminoglycosides, this class of antibiotics is an inhibitor of microbial protein synthesis acting by binding to the bacterial 30S ribosomal subunit and disrupts the initiation of protein synthesis. In pseudomonas, resistance to this antibiotic is mediated by transferable aminoglycoside modifying enzymes (AMEs), active efflux low outer membrane permeability and seldom target modification(Bialvaei and Samadi,2015).

Fluoroquinolones resistance develops by a chromosomal gene encoding DNA gyrase or topoisomerase 1V mutation or by active drug transport out of the cell. Topoisomerase 1V mutation may occur in gyrA/ gyrB genes within the QRDR (quinolone-resistant-determinative region) motif, which is regarded to be the active site of the enzyme. This results in the modified amino acid sequences of the A and B subunits, and therefore the modified

topoisomerase II with a low affinity to the quinolone molecules (Rello *et al*.,2014).

### *1.2.4. Immune response against P. aeruginosa:*

*P. aeruginosa* produces a number of molecules that have direct inhibitory or toxic effects on immune cells. A redox-active phenazine (pyocyanin) can trigger neutrophils apoptosis in vitro. Another secreted small molecules, rhamnolipid leading to neutrophils necrosis, bacterial mutants deficient in rhamnolipid molecule synthesis are rapidly cleared by the immune system . Pyocyanin and rhamnolipid production are regulated positively by quorum sensing signals and the expression of these molecules is greater when bacteria are in stationary phase or high-density growth conditions, such as biofilms (Moradali *et al*.,2017).

Numerous conserved microbial structures, commonly known as microbe-associated molecular patterns (MAMPs), have been involved in activating the hosts innate immune response to *P. aeruginosa*. MAMPs are sensed by a set of germ-line encoded receptors called pattern recognition receptors (PRRs) that include cell surface and endosomal Toll-like receptors (TLRs) and cytosolic Nod-like receptors (NLRs). MyD88, an adaptor molecule for almost all Toll-like receptors (TLRs), is required to control *P. aeruginosa* in lung infection (Mijares *et al*., 2011).

Both TLR4 and TLR5, which recognize LPS and flagellin respectively, can initiate protective responses to *P. aeruginosa* infection. Flagella not only serves primarily for motility in *P. aeruginosa*, but also assists attachment to the respiratory epithelium and also activation of TLR5, TLR5 is a type of pathogen recognition receptors that recognizes PAMPs and initiates innate immunity. Once the bacterium has been attached to the epithelium, the

flagellum is shed and with the help of pili, *P. aeruginosa* can penetrate the epithelium. Other TLRs expressed by macrophages, like TLR2 and TLR9, do not appear to mediate *P. aeruginosa* recognition (Hogarth, *et al*.,2019).

Dendritic cells (DCs) expressing specific receptor recognizing PAMPs. DC activation by pathogens leading to the induction of antigen-specific adaptive immune responses, therefore it bridging the innate and adaptive immune systems. Intra-tracheal dendritic cells modulate the balance between IL-12 and IL-10, suggesting that they play an important role in inflammatory responses to pulmonary *P. aeruginosa* infection (Biber *et al*.,2020).

Regarding adaptive immune response, activation of the adaptive immune responses is initiated shortly or simultaneously after innate immune response activation. The persistent pseudomonas infection can resist the released antibodies, activated chemoattracted and opsonized phagocytes, as well as the other components of the host responses (Garai *et al.*,2019).

Colonization with *P.aeruginosa* results in an antibody response that is not however protective. In contrast, cell -mediated immunity to this bacterium is important in host defenses. Attention has focused on the novel T helper 17 (Th17) the subset of Th cells. These produce IL-17 that plays a critical role in the generation and recruitment of neutrophils to sites of infection (Basu, *et al*.,2012).

Lymphoid DC which is called DC2 related to their ability to induce Th2 responses activate Th 17 to produce IL-17. The Th17 response was also recently addressed in CF as having the ability to stimulate neutrophil mobiliser G-CSF and the neutrophils chemoattractant IL-8, thus contributing to pulmonary pathology during chronic *P. aeruginosa* lung infection. The

rate of naive T-cells that differentiate into Th17 cells is nearly twice as high as in healthy subjects.

This amount is correlated with the severity of lung disease, especially caused by *P. aeruginosa,* which suggests that IL-17 and associated cytokines may serve as biomarkers for early disease detection and potential therapeutic targets. In addition, the Th2-Th17 axis in CF may predispose to the development of *P. aeruginosa* lung infection (Bayes *et al*.,2014).

B cell response plays an important role in the defense against *P. aeruginosa* acute pneumonia infection. Patients with persistent *P. aeruginosa* infection reported significantly higher levels of immunoglobulins, primarily IgA (Yonker *et al*.,2015). Significantly higher concentrations of IgG2 have been reported in sera of CF patient chronically infected with this bacterium, as well as higher mean level of circulating immune complexes (CIC) , IgM and IgG1–4, IgA (Mauch *et al*.,2017).

### *1.2.5.Treatment of P. aeruginosa :*

### *1.2.5.1. Conventional treatment of P. aeruginosa:*

The option of treatment depends entirely on the nature of antibiotic drug resistance and the genes involved in drug resistance.

*P. aeruginosa* has a wide range of intrinsic and adaptive antibiotic resistance mechanisms. Conventional commonly used antibiotics, including beta lactam and similar narrow-spectrum antibiotics, appear to be ineffective as this organism is capable of degrading the ring responsible for their activity(Gast Gurung *et al.*,2010).

Beta-lactams, ticarcillin piperacillin, meropenem, imipenem and doripenem are the most effective beta-lactams commonly used for the treatment of *Pseudomonas aeruginosa* (Lu *et al*.,2014).

The use of combination antimicrobial strategies (notably, those empirically designed), were more effective than monotherapy was for patients with *P. aeruginosa* infections, this was especially the case for critically ill and neutropenic patients. Additionally, several international consensus guidelines that addressed the optimal use of antimicrobial polymyxins recommended that the invasive infection of CR-PA should be treated with combination therapy (Tsuji *et al.*,2019).

Unfortunately, due to the increasing use of colistin in the treatment of CR-PA infection, *P. aeruginosa* strain of colistin-resistant (CoR) has been noticed. At this time, colistin-resistant *P. aeruginosa* (CoR-PA) has been documented only sporadically, but will most likely become a significant problem in the near future. The most effective combination of therapy was amikacin with aztreonam, followed by piperacillin/tazobactam with amikacin and meropenem. Additive reactions were identified when amikacin was supplemented with any of the antibiotic classes of beta-lactam (i.e. ceftolozane/tazobactam, piperacillin/tazobactam, ceftazidim, meropenem, aztreonam,or imipenem) (Pungcharoenkijkul *et al*.,2020).

### *1.2.5.2. Alternative treatment of P.aeruginosa:*.

This bacterium is resistant to almost all approved antimicrobial agents. and it tends to escape from the conventional antimicrobial inhibitory action, the Gram-negative pathogen, *P. aeruginosa*, present an even more complex

situation because it is more resistant to multiple drugs (Mulani *et al*.,2019). Considering this, new treatment that alternatives for infections caused by such pathogens were applied.

Grapefruit furocoumarins inhibit QS signaling (AHLs and AI-2) as well as biofilm formation in *P. aeruginosa*. These furocoumarins when purified cause 94% inhibition of the autoinducers (AHLs). For *P. aeruginosa*, biofilm inhibition was up 72%, also the 6-gingerol, isolated from fresh ginger oil, decreasing the generation of many virulence factors and lowering the pathogen's mortality rate (Kim *et al*.,2015). Another compounds affecting *P. aeruginosa* QS-regulated genes, are sulforaphane and erucin, two isothiocyanates were isolated from broccoli, inhibit the PAO1 *las* and *rhl* system as well as biofilm formation in *P. aeruginosa.*

The combination of apple vinegar and garlic extract is a new natural plant extract treatment for Pseudomonas. Garlic's therapeutic effect is possible due to its oil and water-soluble organosulfur compounds, which are responsible for garlic's characteristic odor and taste. Garlic's antibiotic activity is largely dependent on thiosulfinates. The over 100 phytotherapeutic sulfur compounds found in varying amounts in garlic, including allicin and thiosulfinates, have been credited with this wide spectrum of action (Hindi, 2013).

Vinegar (also known as acetic acid) has antibacterial properties. Vinegar is an acidic liquid made from the fermentation of an alcoholic beverage, mostly wine, and it has a high inhibitory activity against *P.aeruginosa* when combined with carlic extracts. Allicin is one component of garlic extract, is a volatile agent that can be effective against microorganisms via the gas phase by reacting with glutathione, shifting the cell reactivity to a more oxidized

state, and causing disulfide stress (Müller *et al*.,2016). Allicin has been identified as a cellular redox toxin in this regard. Drugs with several sites of action, such as allicin, are especially attractive because they prevent the development of resistance (Jana *et al*.,2017).

## *1.3. Propolis:*

Propolis, is a product of the vital activity of the honey bee, they collect resins from leaf bud and bark from plants that grow around of hives. It is thought to exhibit a broad spectrum of biological activities including antimicrobial, anti-diabetic, anti-inflammatory, antioxidant, immune-modulatory and antitumor activity (Bryan *et al*.,2011).

### *1.3.1. Antimicrobial Activity of Propolis:*

The antibacterial activity of the proppolis should be assessed at two levels. First, they are related to overt actions on the microorganism and the other with stimulation of the immune system, which stimulates the microorganisms' natural defenses (Przybyłek and Karpinski, 2019).

The chemical constitutes of Propolis allow it to predict effects on the permeability of the microorganism's cell membrane, disruption of the membrane potential and the development of adenosine triphosphate (ATP) and reduced bacterial mobility. In general the antimicrobial activity of the propolis in comparison to Gram negative bacteria is higher with Gram positive bacteria, this is clarified by the species specific structure of the Gram-negative bacteria's outer membrane and by the synthesis of hydrolytic enzymes that break down the bioactive ingredients of propolis (Sforcin, 2016). The high concentration of phenolic compunds in propolis mainly Artipillin C in ethanol extracted propolis (EEP) show high antimicrobial activity against methicillin resistant *Staphylococcus aurus* (MRSA) ,

artipillin C also showed antioxidant and antibacterial activity *against S. saprophyticus, Enterococcus faecalis* and *Listeria monocytogenes* (Veiga *et al*.,2017; Seibert, 2019). Cinnamic acid is an aromatic group of carboxylic acids widely present in the plant and its derivatives. They occurring in the green sections of plants and its flowers. Propolis is a rich source of esters and cinnamic acid as a material that is composed mainly of plant secretions. Many studies have reported the cinnamic acid's antimicrobial activity against Aeromonas spp., Vibrio spp., *P. aeruginosa, Streptococcus pyogenes, Salmonella enterica* serotype Typhimurium and *Mycobacterium tuberculosis*.

Vasconcelos *et al*.,(2018) showed that propolis constitutes improve the bactericidal activity of macrophages against *Salmonella typhimurium* which is the causative agent of typhoid fever in humans depending on its concentration. The main mechanism of action of Cinnamic acid is a bacterial cell membrane damaging, inhibiting cell division and biofilm formation and also has the ability of bacterial metabolic enzymes (ATPases) inhibition. Also, they have the activity in anti-quorum sensing. It is thought that flavonoids, phenolic and esters of phenolic acids are known to be responsible for the above mentioned activities of bee glue (Yilmaz *et al*.,2018).

Do Nascimento *et al*., (2019) pointed out that the most active flavonoids in propoli are galangin, that have highest percentage of activity as antibacterial against *Campylobacter jejuni* strains while the other flavonoid, quercetin has lower antibacterial activities against such bacteria.

Regarding anaerobic bacteria, Turkish propolis samples have shown high antibacterial activity against these bacteria that caused oral infections, thus

making standardized preparations of propolis useful for prevention and treatment of oral diseases. Bulgarian propolis showed high antibacterial activity against most anaerobic strains of different genera of bacteria in addition to oral pathogens, that's includes Clostridium, Propionibacterium and Bacteroides species (Kokoska *et al*.,2019).

Propolis extracts can act as a synergistics agents that increased the effects of antibiotic assigning antibacterial propolis related mainly to flavonoids or the synergisim between certain phenolic compounds (Nguyen *et al.* 2017). The studies of ( Dugler and Gonuz, 2004) observed that the (EEP) act on nucleic acids (DNA and RNA) and some bioactivities of bacterial metabolic enzymes. Also, (De marco *et al*.,2017) reported that propolis and bud poplar resins extract shown the ability to inhibit biofilm formation of *P. aeruginosa* and also influence the swarming and swimming motility.

Regarding propolis antibacterial activity against *S. aurus* the propolis extract effects bacterial adherence ability and inhibit bacterial quorum sensing , it shown the ability to impair bacterial biofilm formation and also the results have revealed EEP reduces violacein production by *S. aurus* (El-Guendouz *et al*.,2018). Phenols and flavonoids also activate bactericidal activity of macrophages against fungi like pathogenic yeast *Candida albicans* and also have effect on *paracoccidioides Brasiliensis*, which causes paracoccidioidomycosis, the main cause In Latin America systemic mycosis (wolska *et al*.,2019).

### *1.3.2. Anti -inflammatory activity of propolis :*

Inflammation is the dynamic biological reaction of the immune system to harmful stimuli such as bacteria, damaged cells and irradiation, inflammatory mediators including vasoactive amine, PAF,eicosanoids (PGE2 and leukotrienes) and cytokine release involving (TNF-α, IL-1) by activated macrophages and PBMC result in vessels dilation and relaxation of smooth muscles leading to increased local blood flow (Pai,*et al*.,2018). Cytoplasmic membrane phospholipids activate intracellular and extracellular phospholipases and these enzymes lead to activation of lipoxygenase (LOX) and cyclooxygenase (COX) which act on arachidonic acid (AA) metabolism. PGE2 which is derived from arachidonic acid that is stored as phosphoglycerates in the cell membranes converted by cyclooxygenase or lipoxygenase, when tissue damage, conversion through COX leads to the formation of PGE2 which participate mainly in the beginning and progression of the inflammatory reactions (Pasupuleti *et al*., 2017).

Propolis could inhibit cyclooxygenase and the subsequent synthesis of PGE2. This anti-inflammatory activity is related to the presence of flavonoid and cinnamic acid derivative (Araujo *et al*., 2011; Shahinozzaman *et al*., 2019).

EEP can down-regulate the expressions of TNF-α. This was significantly increase the inflammatory cells recruitment in variety of inflammatory models (Bueno-Silva, *et al*.,2016). CAPE significantly reduces the level of production and secretions of IL-8 that play important role in neutrophils recruitment and monocyte chemotactic protein(MCP)( Shahinozzaman *et al*.,2018).

### *1.3.3. Immunomodulatory effects of propolis:*

Mechanism of the immune system controlling is known as immunomodulation. The effect of propolis extracts could be attributed to the synergistic effects among its components, the main components among propolis are flavonoids (galangin and pinocembrin) have the ability to stimulate neutrophil chemotaxis and phagocytic activity(Wolsak *et al*.,2016).

Yuan *et al*.,(2012) was reported that bioactive propolis components also have been reported to prevent and alleviate pollen allergy in humans by inhibiting histamine releases and suppressing the levels of immunoglobulin E (Ohkuma *et al* .,2010), (Phenolic and flavonoids) have been recognized as significant anti-complementary compounds and have the ability to inhibit alternative and classical pathway of complement system (Taira *et al*.,2016). It is also effective in enhancing and modulating of the immune system. (CAPE) strongly inhibits mitogen-induced T-cell proliferation, lymphokines production and activation of NF-κB by induction of caspase-3 expression. Quercetin, hesperidin and other flavonoids have a strong inhibition effect in a concentration-dependent manner to DNA synthesis and the production of inflammatory cytokines (Tomaszewski *et al*,. 2019).

Erfani, *et al*.,(2014) was noted that the treatment with propolis extract leading to a decrease in the relative number of (CD4+CD25+FoxP3+) regulatory Tcells expressing IL-10 and TGF-β. These suggest that propolis have strong effect on lowering the Treg cells activity in which (IL- 10) is one of the immunosuppressive cytokines produced by Treg cells (Kusnul *et al*.,2017).

Shahinozzaman *et al*.,(2020) reported that cinamic acid stimulates lymphocytes proliferation and stimulates the production of antibodies by B lymphocytes and acts chemotactically on neutrophils. Such mechanisms can explain how innate and adaptive (humoral and cellular) immunity increases (Espindola *et al*., 2019).

Extract of particular propolis constitute (Artepillin C) can modulate the immune response leading to change in the ratio of (CD4+/CD8+) lymphocytes, as well as affect the total number of Th cells. Thus simultaneously they affect both cellular and humoral immunity(Franchin *et al*., 2018) .

In general, immunomodulatory activity of propolis represented by stimulation of antibody production by B cells, Inhibits and activate lymphocytes proliferation according to their concentration, decreasing serum level of allergen-specific IgE, inhibits degranulation of mast cells/basophils,enhances the phagocytic activity of monocytes/macrophages and neutrophils, stimulating spreading, distribution and motility of monocytes, neutrophils and macrophages and also enhances lytic function of NK cells (Wolska *et al*.,2019).

### *1.3.3.1. Immunomodulation effects on neutrophils, macrophages and monocytes :*

Propolis and its constituents, CAPE, artepillin C and cinnamic acids affect non-specific innate immunity through neutrophils modulation and activation of monocytes / macrophages (Chan *et al*.,2013). The polymorphonuclear neutrophil leukocytes are an essential component of non-specific immunity and are the predominant phagocytic cells present in peripheral blood. Monocytes / macrophages are the other immune system

effector cells that protect against microbial infection. Macrophages are phagocytic mononuclear cells derived from peripheral blood monocytes and reside in most tissues. They serve as a bridge between the innate and adaptive immune response by differentiating into cells in order to exert various functions after being stimulated by various stimuli, such as interferon-γ (IFN-γ). Monocytes/macrophages have three essential functions: phagocytosis, presentation of antigens to effective T cells and production of distinct inflammatory mediators (Orsatti *et al*.,2010).

Once bacteria enter the body as an antigen, neutrophils and monocytes/ macrophages can kill them. Phagocytosis is the principal mechanism in the innate immune system for the destruction of microorganisms within phagocytic cells. This complicated process has the following phases: chemotaxis of the phagocytes, adhesion of microorganisms to the phagocyte surface, endocytosis, and microorganism killing. Two pathways within phagocytic cells kill microbes, one is oxygen-dependent (reactive oxygen intermediates (ROI) and the other is oxygen-independent (including secretion of lysosomal enzymes including lysozyme and proteinases)(Tauffenberger and Magistretti, 2021).

Neutrophils are responsible as specialist phagocytes for destroying extracellular microorganisms, whereas monocytes / macrophages destroy pathogens-infected cells, including intracellular bacteria (e.g. salmonella), parasites and yeasts. Propolis can greatly improve the phagocytic activity of macrophages, the release of interferon-gamma (IFN-γ), IL-1β and interleukin-6 ( IL-6), and it can induce higher concentrations of many types of immune cells and specific immunomodulatory cytokines that are essential to maintaining homeostasis (Tao *et al*.,2014).

### *1.3.3.2. Immunomodulatory effects on the function of T and B lymphocytes:*

T and B cells are another significant group of mononuclear peripheral blood cells (PBMCs), which play a key role in the adaptive immune response. The ability of these cells to distinguish self from non-self lets them clear exogenous antigens (Wolska *et al.*,2019).

Specific types of antigens bind and stimulate different T cells and/or B cell populations which have different immune functions, However, bacteria will be phagocytized within the macrophages and then recognized by the major histocompatibility complex II (MHC II) and identified as peptide antigen. MHC II binds to T lymphocytes (Li *et al*.,2021).

The immunomodulatory effect of propolis in human resting PBMC is due primarily to its action on monocytes. T and B lymphocytes, however, are another major component of PBMC that may be affected by propolis influence. Some authors have documented that propolis activates the proliferation of lymphocytes, while others claim that propolis and its constituents, particularly flavonoids, are active immune-suppressants that inhibit lymphocyte proliferation.

The immunosuppressive bioactivity of CAPE on T cells linked to the fact that this phenolic component is a potent inhibitor of the early and late events in receptor-mediated T cell activation. In addition, they found that CAPE particularly inhibited both transcriptions of the gene IL-2 and expression of IL-2R (CD25) in stimulated human T cells. They proved that CAPE inhibited the activation of T-lymphocytes by targeting both NF-κB and NFAT and transcription factors.

Another explanation for propolis' inhibitory effects on lymphoproliferation arises from (Canovas *et al*.,2021) observations, they studied the signalling pathway of mitogen-activated protein (MAP) kinase for measuring the activation of mRNA expressions of extracellular-signal-regulated kinase (Erk-2), which regulate several transcription factors. In turn these regulate the regulation of essential genes of lymphocytes one of them IL-2 gene. (Erk-2) was strongly inhibited in propolis-stimulated PBMC, which clearly implies that one way of signaling elicited by propolis is mediated by (MAP kinase Erk-2) while Artipilin C have mitogenic action on B and T lymphocytes .

shang *et al*.,2020, noted that after supplementation with propolis, IL-6, C reactive protein (CRP), and TNF-α were significantly reduced. In vivo studies have shown that propolis has stimulated the proliferation of T lymphocytes and the secretion of IL-2, IL-4 and propolis extract can potentiate the humoral immune response by increasing the level of serum IgG and enhanced specific antibodies response ( Kalsum *et al*.,2017).

### *1.3.3.3. Immunomodulatory effect of propolis on basophil and mast cells functions:*

Basophiles are peripheral blood cells that present in a trace amounts and accounting for (0-1 %) of the total white blood cells while mast cells are present and distributed throughout certain epithelia and vascularized tissues.

The anti-allergic activity of propolis is represented by its effect on asthma ,the propolis treated patients have shown reduced incidence and the severity of nocturnal attacks and also shown improvement in ventilator functions that are related to decrease production and reduced level of PG, proinflammatory cytokines, histamin and interlukines, proplis inhibit the production of (IgE)

and TH2 cytokines that lead to inhibition of airway hyper responsiveness (AHR) and also inhibit collagen and inflammatory cells deposition in the lung (Shinmie *et al.*,2010).

Kwon *et al.*,(2018) has reported that ethanol extracted propolis modulate the TH cells proliferation in the lung from TH2 to TH1and reduced histamine release from mast cells and inhibit mast cells\basophils degranulation, CAPE strongly decreased the total leukocytes, lymphocytes, macrophages and eosinophil counts and also reduced the infiltration of inflammatory cells within bronchiolar and perivascular regions.

## *1.4. Immunoadjuvant :*

Is a substance that stimulates, improves, potentiates or suppresses the main components of the immune system, including innate and adaptive immune responses. It increases the response to the vaccine and does not have any antigenic effects. Some of the adjuvants are endogenic (such as IL-1, histamine, and interferon). Their mode of action is either antigen-specific, affecting a specific immune response to a narrow group of antigens or non-specific mode of action, leading to the increased immune response to a variety of antigens. Adjuvants are considered to be immune modulators since they enhance the body's immune response. It performs one or more of the three main functions. First, they enhance antigen depot for slow release, second, they enhance antigen targeting of immune cells and stimulate phagocytosis, and third, they enhance and modulate the type of antigen-induced immune response. Adjuvants may also provide a danger signals that the immune system needs to respond to the antigens as it does to an active infection.

As a result, immunoadjuvants have a significant role to play in every aspect of the immune response (Kostinov *et al*.,2020). The immunomodulatory activity of propolis was considered as an alternative adjuvant in the treatment of various diseases. Propolis has been shown to improve the effectiveness of the associated vaccine when used as an adjuvant, boost its protective index, stimulate cell response, induce prolonged and high mucosal immunity, improve and stimulate phagocytic activity, increased safety of the vaccine, Increased leukocytic reactions, decreased concentration of the optimum dose and stimulates the non-specific immunity regardless of the type of vaccine preparation. These effects were correlated to the active ingredients (flavonoids contents) of propolis (Ouyang *et al*., 2010; Tao *et al.*, 2014).

It was commonly used as an adjuvant for vaccines because it could induce an earlier immune response and a longer duration of defense (Fan *et al*., 2015). Propolis is considered a safe immunostimulant and a potent vaccine adjuvant, its flavonoids constitute have potential activity as adjuvants, enhancing IgG, IL-4 and IFN-γ in serum. In the mouse model, propolis has been applied as an adjuvant to the inactivated swines herpesvirus type1 vaccine, induced increased humoral and cellular responses (Lopes *et al*.,2018).

Mojarab *et al*.,(2020) reported that propolis as an adjuvant gave a similar immune response (increasing IFN-γ levels), to Alum and Freund's adjuvant in mice vaccinated with an HIV-1 polytope vaccine candidate, with less risk of undesirable side effects.

## *1.5. Blood cells:*

Blood is a connective tissue made up of blood cells and plasma. The blood cells are made up of erythrocytes, leukocytes and thrombocytes. Peripheral blood mononuclear cells (PBMCs) are round-shaped blood cells, such as monocytes and lymphocytes. The lymphocyte population consists of CD4+ and CD8+ T-cells, B-cells and Natural Killer cells, CD14+monocytes, and basophils, neutrophils, eosinophils and dendritic cells (Al-Dulaimi *et al*.,2018).

### *1.5.1. Peripheral blood mononuclear cells (PBMCs):*

PBMCs are a diverse mixture of highly specialized immune cells that play a critical role in keeping our bodies healthy (Sambor *et al*.,2014). On average, the majority of PBMCs are lymphocytes (70-90% ). Lymphocytes play a key role in cell-mediated and humoral immune response, primarily associated with the T and B cells activation. PBMCs are essential tools for research in new drug toxicity analysis that provide the potential toxicity of new compounds to the human immune system.

The effects of drug toxicity on PBMCs cause various serious toxic side effects, including suppression and toxicity of the immune system. PBMCs are therefore also explored for the determination of the dose limit for new drug compounds which are critical tools for predictive studies. Similarly, normal PBMCs and diseased PBMCs are analyzed to determine which pathways or molecules play an important role in the diseases. In addition, mRNA expression ratios or receptors expression in normal and diseased PBMCs are used for biomarker research (Ahangari *et al*., 2015;Sen *et al*.,2018).

PBMCs have also been studied in immunotoxicity or cytotoxicity studies for drug researches and chemotherapy compounds. Many scientists are studying PBMCs in the fields of immunology, including autoimmune diseases, transplantation immunology, hematological malignancies and infectious diseases. In addition, the PBMC-based vaccine strategy provides for a more marked and durable protective immune response (Kumar, *et al.*,2017). Performing such experimentation in the PBMC require essential need for high recovery, viability and functionality of PBMCs for accurate assessments(Gill,2019).

### *1.5.2.Isolation of Peripheral Blood Mononuclear Cells:*

Two primary techniques separate PMNCs from entire peripheral blood are the use of gradient density centrifugation or by leukapheresis. Because cells have a specific density, the use of a density gradient centrifugation separates the main cells population, including lymphocytes, monocytes, granulocytes, and RBC throughout the density. gradient medium, The medium will have had a density of 1,077 g/ml allowing sufficient separation of PBMCs (density < 1,077 g/ml) from RBCs and granulocytes with density of ( > 1,077 g/ml). The layering of whole blood under or over a density medium without mixing the two layers then samples were centrifuged and this will disperse the cells according to their density (Jia *et al*.,2018). After centrifugation, the PBMC fractions appear as a thin white layer at the interface between the density gradient medium and plasma, making it easy to isolate the PBMC fraction. Leukapheresis is an automated device that separates the inflow of the whole blood from the target PBMCs fractions using elevated speed centrifugation while returning the outflow content, such as red blood cells, plasma, and granulocytes back to the donor. PBMCs

are an important component and a powerful tool for science and medical studies on human health and disease. Through efficient and effective processing and analysis of PBMCs, researchers and healthcare professionals can test immune responses, develop a greater understanding of the immune system, and apply their observations to treatments and medications for human diseases (Pourahmad and Salimi, 2015).

### *1.5.2.1 Primary cell culture:*

Primary cell culture is the disassociation of cells from a parental animal or plant tissue through enzymatic or mechanical measures and maintaining the growth of cells in a suitable substrate in glass or plastic containers under controlled environmental conditions. Primary cell culture could be classified into two based on the kind of cells used in culture.

• Anchorage Dependent or Adherent Cell : These cells require an attachment for growth. Adherent cells are usually derived from tissues of organs, for instance from kidney where the cells are immobile and embedded in connective tissue (Freshney, 2006).

• Anchorage Independent or Suspension Cells : These cells do not require an attachment for growth. In other words, these cells do not attach to the surface of culture vessel. All suspension cultures are derived from cells of the blood system, for instance, PBMCs is suspended cells. Cells derived from primary cultures have a limited life span. Cells cannot be held indefinitely due to several reasons. Increasing cell numbers in primary culture will lead to exhaustion of substrate and nutrients. Also, cellular activity will gradually increase the level of toxic metabolites in the culture inhibiting further cell growth.

At this stage, a secondary or a subculture has to be performed to ensure continuous cell growth (Ramos *et al*.,2014).

### *1.5.2.2 Secondary cell culture:*

when cells in adherent cultures occupy all available substrate or when cells in suspension cultures surpass the capacity of the medium to support further growth, cell proliferation begins to reduce or to entirely cease. In order to maintain optimal cell density for continued growth and to stimulate further proliferation, primary culture has to be sub-cultured. This process is known as secondary cell culture.

During the secondary cell culture, cells from primary culture are transferred to a new vessel with fresh growth medium. The process involves removing the previous growth media and disassociating adhered cells in adherent primary cultures. Secondary cell culturing is periodically required to provide cells with growing space and fresh nutrients, thereby, prolonging the life of cells and expanding a number of cells in the culture.

Secondary culturing a certain volume of a primary culture into an equal volume of fresh growth medium allows long-term maintenance of cell lines. Secondary culturing into a larger volume of fresh growth medium is practiced to increase the number of cells, for instance in industrial processes or scientific experiments (Luo,2019).

### *1.5.3.Characterization of Peripheral Blood Mononuclear Cells:*

Most PBMCs are present as naive or resting cells with no effector function. In the absence of a continuous T-cell immune response, the largest fraction of isolated PBMCs are present mainly as naive or memory T-cells. Naive T-cells have never encountered their cognate antigen before and are commonly characterized by the lack of activation markers such as CD25,

CD44 or CD69 and the absence of the CD45RO isoform memory marker. Recognition of the antigen by a naive T cell may result in activation of the cell (Ashraf and Khan 2003), which will then enter a program of differentiation and develop T cell effector functions .

In peripheral blood, the frequency of lymphocytes with the specificity of a single antigen is low, therefore polyclonal activators are used for in vitro stimulation as they can activate a significant portion of lymphocytes independently of their antigen specificity. The most common activator are carbohydrate-binding proteins that bind to a number of glycoproteins expressed on the lymphocytes plasma membrane and the other activator was mitogenic lectins (Sen *et al*.,2017).

Polyclonal activation of T cells is also obtained by antibodies that bind to CD3 specifically, alone or in combination with CD28. Impacts on the immune function of PBMCs are generally monitored by studying changes in the characterization of cytokine secretion profiles, lymphocyte proliferation, or gene expression alterations (Kleiveland,2015). Upon activation with polyclonal activators, PBMCs produce cytokines and up-regulate stimulation markers. Characterization of cytokine profile and changes in the expression of activation marker particularly in T cells may provide relevant information as to whether the response is in the direction of Th1, Th2, Th17 or regulatory T cells. Th1 cells have been identified as CD4+, CD69+ and CXCR3+ and Treg cells as CD4+, CD25+ and Foxp3+. Increased of IFN-γ and Il-10 production suggests increased levels of Th1 and Treg cells, while decreases in IL-17 support suppression of Th17 cells ( Golubovskaya and Wu,2016).

## *1.6.Activation of PBMCs:*

peripheral blood mononuclear cells can be activated in vitro by different methods, the most reliable one is phytohemagglutinin (PHA) activation that stimulates the (PBMCs) proliferation. Another methods including bacterial LPS, IL.2,Concanavalin A(Con A) and different cytokines(Wu, *et al*.,2016).

The response of such a diverse group of cells to different stimuli provides insight into their role in the disease and the development of treatment options.

Activation of T-cell is normally initiated mostly by the interaction of the cell surface receptor with its specific ligand molecule along with a co-stimulatory molecule (This binding event triggers inositol phospholipids rapid hydrolysis by phospholipase C (PLC) to diacylglycerol and inositol phosphates. Diacylglycerol is an allosteric protein kinase C(PKC) activator . PKC activation and inositol phosphates which trigger Ca++ release and mobilization result in a chain reaction of supplemental cellular reactions mediating T cell activation. Lipopolysaccharide (LPS) derived from gram-negative bacteria is a hazard signal recognized by PBMCs. Detection of LPS results in transcriptional reactions involving the expression of immunological and inflammatory response related genes that help to clear infection stimulated PBMCs that are macrophage precursors that may contribute to the production of cytokines leading to systemic inflammation (Dasu *et al*.,2010). Peripheral blood immunological cells serve as a surveillance body to detect invasive microbes(Miller *et al*.,2005).

LPS-induced immune responses mimic the complex immune response during sepsis. LPS bindig to its receptor complex resulted in the activation

and nuclear translocation of the interferon regulatory factor-3 and NF- κB, leading to the expression of TNF-α (Liu *et al*.,2017).

LPS can stimulate the release of interleukin 8 (IL-8, CXCL8, CXC ligand 8) and the other inflammatory cytokines in different cell types, resulting in acute inflammatory responses to pathogens (Singh *et al*.,2019).

The cellular receptor that is transducing the LPS signal has been defined as the Toll-like 4 receptor (TLR4). Binding of LPS to TLR4 results in the activation of NF-κB by recruitment and activation of MyD88, IL-1R kinase-(IRAK), NADPH oxidase (NOX) as well as TNFR associated factor 6 (TRAF-6), NF-κB plays a key role in controlling the transcription of genes related to innate immune response and inflammatory reactions, and its activation is regulated by the reactive oxygen species (ROS) in immune modulation in the monocytes (Steven *et al*.,2018).

## 2.1 Materials:

**2.1.1 Laboratory Instruments**: All instruments used in this study are documented in Table (2-1):

Table (2-1): Laboratory instruments and tools used in this study

| No. | Instruments | Company/ Origin |
|---|---|---|
| 1 | Autoclave | Memmert/Germany |
| 2 | Bensen burner | Cony/Germany |
| 3 | Centrifuge tubes | Cony/Germany |
| 5 | Conical flasks | Pyrex/ England |
| 4 | Cool ultra centrifuge | Sigma/USA |
| 6 | Cover slides | Citoglas/ China |
| 7 | Cylinders | Technico/ England |
| 8 | Deep freezer | Revco/ USA |
| 9 | Disposable petri-dishes | Jordan |
| 10 | Distillate water | GFL/ Germany |
| 11 | DNA ladder 1kb | Viogene /Taiwan |
| 12 | ELISA Printer | Epson/ Germany |
| 13 | ELISA Reader | Biotek-ELX800/ Germany |
| 14 | ELISA washer | Biotek-ELX 50/ Germany |
| 15 | Epindrof tubes | AFCO/Jordan |
| 16 | Face mask | Broche/ China |
| 17 | Gloves | Sidra/ China |
| 18 | Heparin- tubes | AFCO/ Jordan |
| 19 | Incubator | Memmert /Germany |
| 20 | Laminar cabinet Hood | Labogene/Denemark |
| 21 | Light microscope | Olympus /Japan |
| 22 | Loop wire | China |
| 23 | Microcentrifuge | Fisons /Engeland |
| 24 | Microfilter 0.22µm | Satorins membrane filter Gm, BH, W. |
| 25 | Micropipette | Dragon/China |

| | | |
|---|---|---|
| 26 | Oven (hot-air oven) | Memmert / Germany |
| 27 | Plain tubes | AFCO/ Jordan |
| 28 | Plastic containers | Afco/China |
| 29 | Plastic droppers | Afco/China |
| 30 | Powder grinder | Silver green /Germany |
| 31 | Red safe stain | iNtRON biotechnology/Korea |
| 32 | Refrigerator | Concord/ Lebanon |
| 33 | Screw cap bottles | Pyrex/ England |
| 34 | Sensitive electronic balance | Sartorius/ china |
| 35 | Shaking incubator | Memmert/Germany |
| 36 | Slides | Afco/ China |
| 37 | Swabs | Afco/ Jordan |
| 38 | Syringes 5ml, and 10ml | Sum bow/ China |
| 39 | Timer watch | Dragon/China |
| 40 | Tips | Afco/ Jordan |
| 41 | Vortex | Gemmy/Taiwan |
| 42 | Water bath | Tafesa/ Germany |
| 43 | 0.2ml and 1.5ml micro tube | Sigma /USA |

**2.1.2 Biological and Chemical materials:** All biological and chemical materials used in this study are documented in Table (2-2):

Table (2-2) the biological and chemical materials used in this study:

| **No.** | **Name of material** | **Company/ Origin** |
|---|---|---|
| 1 | Barium chloride,NaCl | BDH/Engeland |
| 2 | Blood agar base | Himedia / India |
| 3 | Brain-heart infusion agar | Oxoid/ England |
| 4 | Brain-heart infusion broth | Oxoid / England |
| 5 | Deionized water | Al-Jazeera/ Iraq |
| 6 | Diethyl ether | BDH/ England |
| 7 | Ethyl alcohol 95% and 96% | Crescent/ KSA |
| 8 | Fetal bovine serum | Gibco/ USA |
| 9 | Formalin 37% | BDH/ England |

| | | |
|---|---|---|
| 10 | Giemsa stain | Crescent/ KSA |
| 11 | Glycerol | Fluka chemika/ Switzerland |
| 12 | Gram stain set | Crescent/ KSA |
| 13 | Hepes buffer solution tablet | BDH/ Engeland |
| 14 | Hydrogen peroxide 3% | BDH/ England |
| 15 | Kilgler iron agar | Diffco-Michigan/USA |
| 16 | Liquied nitrogen | Iraq |
| 17 | Lymphoprep | Capricorn-GmbH/ Germany |
| 18 | MacConkey agar | Himedia/India |
| 19 | MacFarland tubes | BioMeriex/ France |
| 20 | Maganesium chloride, potassium sulphate | Merck/Germany |
| 21 | Methyl- red solution | BDH/ England |
| 22 | MR-VP broth | Diffco-Michigan/USA |
| 23 | Muller-Hinton agar | Oxoid / England |
| 24 | *N*-acetyl-*N*-*N*-*N*-trimrthyl ammonium bromide (cetramid) | Biolife/Italy |
| 25 | Normal saline | ADWIC/ Egypt |
| 26 | Nutrient agar | Himedia / India |
| 27 | Nutrient broth | Himedia / India |
| 28 | Pepton | Oxoid/Engeland |
| 29 | Phosphate buffer saline tablet | BDH/ England |
| 30 | Propolis products | Karbala / Iraq |
| 31 | RPMI 1640 compelete cell line medium | Capricorn--GmbH/ Germany |
| 32 | Simmon citrate agar | Diffco-Michigan/USA |
| 33 | Sterile urea, αnaphthol,KOH, Kovac′s reagent | Sigma /USA |
| 34 | Urea agar base | Diffco-Michigan/USA |
| 35 | WBC solution | Crescent / KSA |

**2.1.3 Antibiotic Disks**: All antibiotic discs that used in this study are documented in the table (2-3)**.**

Table (2-3) the antibiotic discs used through the study with their abbreviations and potency of each one

| No. | Antibiotic name | Abbreviation | Disc potency/ disc | Company/ Origin |
|---|---|---|---|---|
| **1** | Gentamicin | GEN | 10µg | HiMedia/India |
| **2** | Cefepime | CEP | 30µg | |
| **3** | Cefatazidime | CEF | 30µg | |
| **4** | Imipenem | IMP | 10µg | |
| **5** | Ciprofloxacin | CIP | 5µg | Bioanalyse, Turkey |

**2.1.4 Immunological kits:** Immunological kits that used in this study were documented in the table (2-4).

Table (2-4) ELISA kits used in this study with their origin.

| No. | Kit name | Company/ Origin |
|---|---|---|
| 1 | Interferon gamma (IFN-γ) | Boster / USA |
| **2** | Interleukin-17 (IL-17A) | |
| **3** | Interleukin-23 (IL-23) | |

## 2.2 Methods:

### 2.2.1 Preparation of Culture Media.

The culture media were prepared according to the method recommended by the manufacturing Companies mentioned in (table 2-2).

### 2.2.1.1 Nutrient Broth Medium.

This medium was prepared for primary isolation of bacteria and preparation of preservation media by dissolving 1.3gm of a medium base in 100 ml of D.W.

### 2.2.1.2 Brain-Heart Infusion(BHI) Broth.

The BHI medium was prepared by dissolving 3.7 gm of (BHI) in 100 ml of D.W and autoclaved at 121°C for 15 minutes then stored in the refrigerator until used.

### 2.2.1.3 Muller-Hinton Agar .

This medium was prepared by dissolving 3.8 grams in 100 ml of D.W and autoclaved 15 minutes at 121 ° C. This has been used for testing the resistance to antibiotics.

### 2.2.1.4 Blood agar.

Blood agar medium was prepared according to sources of manufacture by dissolving 40gm of blood agar base in 1000ml D.W. It was autoclaved for 15 minute at121ºC , and then cooled down to 50ºC, (5%) of fresh human blood was added. This medium was used for bacterial cultivation and to assess the capacity of bacteria to hemolysis blood cells .

### 2.2.1.5 Urea agar medium.

This medium was prepared by adding 15ml of urea solution that was sterilized by filtration to 100ml of urea agar base sterilized by autoclave for 15 minutes at 121ºC, and then cooled to 50ºC. The pH was set to 7.1, and the medium was dispersed into test tubes that were sterilized and allowed to

solidify in a slant fashion. This medium was used to asses the bacterial production of urease enzyme.

### 2.2.1.6 Semi solid medium :

This medium used for detection of bacterial motility. It was prepared by dissolving 0.5gm agar agar in 100ml of nutrient broth, and subsequently dispensed into 5ml sterile test tubes in each. (Collee *et al*., 1996).

### 2.2.1.7 pepton water.

This medium was used for detection of bacterial ability for carbohydrate utilization (MacFaddin,2000).

### 2.2.1.8 MacConkey agar.

It was used for differentiation of lactose fermenter bacteria and for isolation of Gram negative bacilli. It was prepared by dissolving 49grams of dehydrated medium in 1000 ml D.W.then autoclaving for 15 minutes at 121ºC ( Baron *et al*.,1994).

### 2.2.1.9 Methyl red-Vogas Proskauer medium.

This medium was used for assess the bacterial ability for partial or complete glucose fermentation (MacFaddin,2000).

### 2.2.1.10 Kligler′s Iron agar(KIA).

This medium was used to detect the ability of microorganism to produce ($H_2S$) and fermented sugar with producing acid and gas (MacFaddin,2000) .

### 2.2.1.11 Simmon Citrate agar.

This medium used as a differential medium to detect bacteria 's ability to utilize citrate as their sole source of carbon (MacFaddin,2000) .

### 2.1.1.12 Pseudomonas selective agar (Cetrimide agar).

This is a type of selective media which used for *P. aeuruginosa* isolation and detection. This contains ceramide (N-acetyl-N-N-trimethyl ammonium bromide) for inhibition of other microorganisms except pseudomonas. This medium was prepared by dissolving each of : 20gm Pepton, 10gm Potassium sulfate, 1.5gm Magnesium chloride, 0.3 cetrimide, 15gm Agar-Agar in 1000ml of distilled water, PH set at 7.2 and strelized by autoclave. (Cruickshank *et al*.,1975).

### 2.2.1.13 Preservative Medium Preparation .

This medium is used mainly for storage of bacterial isolates. It was prepared by mixing of 15 ml of glycerol solution with 85ml of nutrient broth media that prepared in section 2.2.1.1 and then autoclaved at 121°C for 15 minutes, then stored at -20°C for 6-8 months and monthly re-subcultures (Forbes, 2007).

## 2.2.2 Preparation of Solutions and Buffers.

### 2.2.2.1 Phosphate Buffer Solution (PBS).

The solution for the phosphate buffer was prepared by dissolving one tablet in 100 mL DW. And sterilized at 121 ° C for 15 minutes by autoclave in compliance with Manufacturer Company instructions and held at 4ºC. This solution was utilized to dilute and wash WBCs.

### 2.2.2.2 Hepes buffer solution.

Hepes buffer prepared by dissolving 2.382gm of Hepes in a little of distilled water, then completing the volume to 1000ml and regulating PH at (7.4), then it was strelized by milipore filter 0.22 µm. This bufferwas used in extraction of bacterial outer membrane (Carlone *et al*.,1986).

### 2.2.2.3 Phenol Red.

This solution was prepared by dissolve 0.05 grams of phenol red dye in 150 ml of ethyl alcohol (95%) and the volume was completed up to 750 ml by adding D.W. It was used as a pH indicator in media LAB fermentation (Forbes *et al*., 2007).

### 2.2.2.4 Oxidase Reagent.

This reagent was used to assess the bacterial ability oxidase enzyme production. This reagent was prepared by dissolving 1gram of tetramethyl-paraphyenylen diamine dihydrochloride in 100 milliliter of D.W. This solution was keeptin dark bottle and used immediately (MacFaddin, 2000).

### 2.2.2.5 Catalase Reagent.

Catalase reagent was used as a ready-to-use solution (3% H2O2) as an indicator for bubble production by mixing with bacterial colonies to ensure catalase enzyme production.

### 2.2.2.6 Normal saline solution.

Normal saline solution was prepared by dissolving 8.5grams of NaCl in a small volume of D.W., then adjusted the volume to 1000 ml, PH will ajusted at (7.2) and srtilized in autoclave at( 121ºC) for 15 minutes,( PH 7.3 pre and post autoclave checked) (MacFaddin,2000).

### 2.2.2.7 Vogas-Proskauer reagent.

It was prepared according to (collee *et.al*.,1996) and this reagent consist of two solutions:

**Solution A:**α-naphthol indicator, this indicator was prepared by dissolving (0.5)gram of α-naphthol in 100ml of absolute ethylic alcohol.

**Solution B:** KOH, it was prepared by dissolving 4grams of (KOH) in (10ml) of sterile distilled water, this reagent used for detection partial bacterial hydrolysis of glucose.

### 2.2.2.8 Kovac′s reagent.

The reagent was prepared by dissolving 0.5gram of (dimethylaminobenzaldehyde) in 15ml ethyl alcohol and 5ml of concentrated HCL. This reagnt was used for indole production detection (MacFaddin,2000).

### 2.2.2.9 MacFarland standard solution .

According to Baron *et al*. ,(1994) the tube solution No.0.5 was prepared by combining 0.05 ml of barium chloride with 9.95 ml of concentrated sulfunic acid , resulting in turbidity approximately was equivalent to $1.5 * 10^8$ cell / ml of bacterial cell density.

### 2.2.2.10 Urea solution.

Urea solution prepared by dissolving (20) grams of urea in a small volume of distilled water , and then completed the volume up to 100ml with sterile D.W and using millipore filter paper for sterilization and set PH at 8-10. It

was used for the detection of the urease positive bacteria in urease test (MacFaddin,2000).

### 2.2.2.11 Tris-EDTA Buffer Solution (TE buffer):

This solution was prepared by dissolving 0.05 mole Tris-OH and 0.001 mole EDTA in 800ml of distilled water. The pH was adjusted to 8 then completed to 1000 ml, then autoclaved at 121°C for 15 min., and stored at 4°C until being used (Sambrook and Rusell, 2001).

### 2.2.2.12 Red stain-agarose gel preparation:

**Preparation of agarose gel :**

The extracted RNA was confirmed by agarose gel electrophoresis (Robinson *et al.*, 2000) as following:

Agarose gel was prepared by dissolving 1.5gm of agarose powder in 100ml of 10X TBE buffer (pH:8) and allowed to cool to 50°C**.** After preparing 100 ml of agarose gel solution (concentration from (0.8~3%) in a 250 ml flask and mixing thoroughly, the flask was putting in the microwave, heat in until the solution is completely clear and on small floating particles are visible(about2~3minutes), five µl of Red SafeTM Nucleic Acid Staining Solution (20,000x) was added to the agarose solution and the flask Swirled gently to mix the solution and avoid forming bubbles. then while the agarose solution cools, it is poured into the gel tray until the comb teeth are immersed bout1/4~1/2 in to the agarose. Then the agarose cooled until solidified and the samples were loaded on the well of gel and perform electrphoresis. Finally the bands of RNA were detected under UV illumination.

## 2.2.3 Isolation and Identification of *pseudomona auruginosa.*

Specimens was taken according to the methods suggested by (Collee *et al*., 1996). One specimen was collected from 22 aged burned female, the swab was taken from the depth of burn, a dry swab must first be moistened with a little amount of (BHI) broth, the swab was transported to the laboratory without delay and it was immediately inoculated in nutrient and blood agar medium by streaking method and then the plates incubated under the aerobic condition at 37°C for 24hours. and lastly subculture on the same media to do further diagnostic tests (Alkhazraji *et al*., 2015).

### 2.2.3.1 Bacterial identification.

*P.aeruginosa* was isolated and identified morphologically and biochemically and then compared with their characteristics being reported in referential references, MacFaddin,(2000).

## 2.2.4 Stock culture.

The bacterial isolate was stored as a stock culture in the Brain-Heart Infusion broth(BHI) tube that was supplemented with (15%) glycerol at (-20)ºC and kept for 6 months (Collee *et.al*.,1996).

## 2.2.5 Antibiotic Susceptibility Test .

This test was done on Muller-Hinton agar medium using the antibiotic discs in the table (2-3) according to the Bauer *et al*.,(1966) approach. It was carried out by using a pure culture of the identified bacterial organism. The bacterial inoculum used in this test was prepared by adding bacterial growth from 5 colonies grown on a blood agar medium to (5 ml) of nutrient

broth . Then incubate the culture for (3-4hrs) to produce a standard bacterial suspension of turbidity equal to McFarland standard tube (0.5). A wooden swab from bacterial suspension was streaked on Muller-Hinton plate. The antibiotic discs have been located on the surface of the medium at evenly spaced intervals using flamed forceps. Incubation was usually overnight with an optimal time of (14 hours) at 37ºC. Antibiotics inhibition zones were measured. Zone sizes were compared to CLSI standard to determine the sensitivty or resistance of bacterial isolate to each antibiotic according to (CLSI, 2020).

## 2.2.6 Extraction of *Pseudomonas aeruginosa* LPS:

Extraction of pseudomonas (LPS) was carried out using the procedure of (Carlone *et al*.,1986) as the following:

1- Pure culture of *P.aeuruginosa* pure was prepared on nutrient agar.

2- Two to four colonies were transferred by wire loop to test tube contains 10ml of (BHI) broth.

3-Then , the tubes were incubated with shaker at 100rpm for (18-24)hrs. at 37ºC.

4- Ten ml of bacterial growth was centrifuged at 4ºC at 10000 rpm for 10 minutes.

5-The supernatant were decanted and the sediments was suspended in 1.5 of cold Hepes buffer solution 10(N)($N_2$-Hydroxyethylpiperazine-N-2-ethanesulfonic acid $C_8H_{18}N_2O_4S$ )molecular weight 238.31.

6-The bacterial cells suspension was centrifuged for 2 minutes at 18000 rpm at 4ºC.

7-The supernatant was decanted and the sediment was used immediately or stored at (-20ºC).

### 2.2.7 Preparation of Crude Propolis.

Propolis sample was collected from honey bees hives in Karbala City during the spring season of 2020. The propolis sample was cleaned and cut into small pieces and stored in a clean container for preparation of the two types of propolis extract, ethanol extracted propolis (EEP) and water extracted propolis(WEP).

#### 2.2.7.1 Preparation of ethanol extracted propolis (EEP).

To prepare (EEP), 10 grams of propolis mixed with 100 ml of 96% ethanol alcohol in dark-bottle and left for 10 days with shaking (4-5) times per day at 37°C. Then the supernatant was collected and the insoluble fractions were separated by filtration using filter paper and evaporate the ethanol by an oven at 45-50ºC. After that, the EEP was weighted and stored in a dark container and made a concentration of 5µg/ml of EEP to be use in this research after filtration by milipore filter paper of (0.22µm) (Hendi, 2011).

#### 2.2.7.2 Preparation of water extracted propolis (WEP).

One hundred ml of distilled water were added to10 grams of propolis that were put previously in liquid nitrogen and then grinding it and added to sterile D.W.in a dark glass which was left at 37ºC for 10 days with shaking two to three times daily with a shaker, then filtration was done first using

filter paper to get rid of the large particles, then the resultant liquid was filtered using a sterile millipore filter paper of (0.22µm). The filtered extract was evaporated in the oven at 45ºC .Then put in a clean and dark container in a cold place until use , 5µg/ml concentration of this extract was prepared to be used in this research (Contari,1987).

**2.2.8. Study Design**: Experimental analytical randomized controlled trail.

**A- Subject Criteria- Inclusion & Exclusion Criteria:**

Apparently 20 healthy subjects with age range(20-40)years, 10 males and 10 females. All subject were not suffering from any healt h problems and did not receive any drugs. The excluded criteria included any person who had an infection or disease.

**B-Ethical Approval:**

Volunteer were asked permission before taking any blood specimen. In addition, the study concept was accepted by the Research Ethical Committee at the College of The Medicine / University of Babylon.

## 2.2.9. Collection of Blood Samples.

Blood samples 5 ml were collected aseptically by vein puncture from apparently healthy male and female age range about(20-40 years), 5 ml of blood were defibrinated by putting in anticoagulant tubes to calculates differential WBCs count and to isolate peripheral blood mononuclear cells from whole blood cells by density gradient medium, the blood samples were

handled within one hour after blood draw to ensure good separation and also a high percentage of viability of isolated cells (Lewis, 2001).

## 2.2.10. Method of PBMCs separation by lymphosep.

This method was applied according to(Bøyum,1968).

1-The lymphosep bottle was firstly inverting gently to mixing thoroughly.

2- Five ml of lymphosep was transferred aseptically to a 15 ml centrifuge tube.

3- Using a balanced salt solution, the blood was diluted in 1:1 by mixing 5ml of blood with 5 ml of physiological saline or phosphate buffer solution.

4-The blood samples were kept at room temperature at15-20ºC prior to and during centrifugation.

5-Diluted blood layered carefully over 5ml of lymphosep in a 15 ml centrifuge tube making sharo blood-lymphosep interphase.

6-The tubes were centrifuged at 1800 rpm for 30 min. at (RT) this will precipitate erythrocyte and polynuclear leukocytes leaving mononuclear cells above lymphosep.

7-The top layer of clear plasma about 3mm above lymphoprep was discarded.

8-The lymphocyte layer and about half of the lymphoprep layer under it was aspirated and transferred to centrifuge tube containing about two times the volume of phosphate buffer saline.

9-The cells suspension was centrifuged at 800 rpm for 10 minutes at room temperature and the supernatant was aspirated and dsiscarded and the sedimented cells were washed to remove the lymphosep and reduce platelets.

10-The cells were washed again with phosphate buffer saline and resuspended in RPMI1640 complete medium at cell culture plate.

11-The cells number was determined by Neubauer chamber count in an optical microscope the cell concentration was adjusted to be $1x10^6$ cell/ml in RPMI complete medium.

## 2.2.11 Cell cultivation.

Peripheral blood mononuclear cells that were isolated from whole blood were re-suspended with density of $1x10^6$ /ml in RPMI 1640 complete medium supplemented with 5% of 100 U/ml penicillin, 100 mg/ml streptomycin and 10% of bovine fetal serum. The tested components were added to the culture medium in a final volume of 500µl per well . PBMCs firstly incubated for 16 hr after that PBMCs were divided in to four groups, group I stimulated with bacterial somatic antigen only ,and the group II of PBMCs stimulated with bacterial antigen plus ethanol extracted propolis while the third group was stimulated with bacterial antigen plus water extracted propolis and group IV was PBMCs without the tested components cultured in medium to serve as a negative control, all these groups were incubated in the cell culture plates at 37ºC with 5%$CO_2$ for 48hrs. Fig(2-1)(Flow chat).

## 2.2.12 PBMCs viability test.

Viability test was done according to the method described by manufacture instructions:

a) Viability of the isolated PBMCs needs to be monitored add 200 µl of cell suspension to 200 µl of 0.4% of trypan blue solution and incubate for 15 min. Cheque for viability of cells by trypan blue staining and score under a microscope using haemocytometer (cells taking up a blue stain are dead cells).

b) Calculate percentage viability as follows: Cell Viability = total Viable cells/ total cells x $100^{3}$. Cell suspension having more than 95% viability should be used for culture (Strober ,2015).

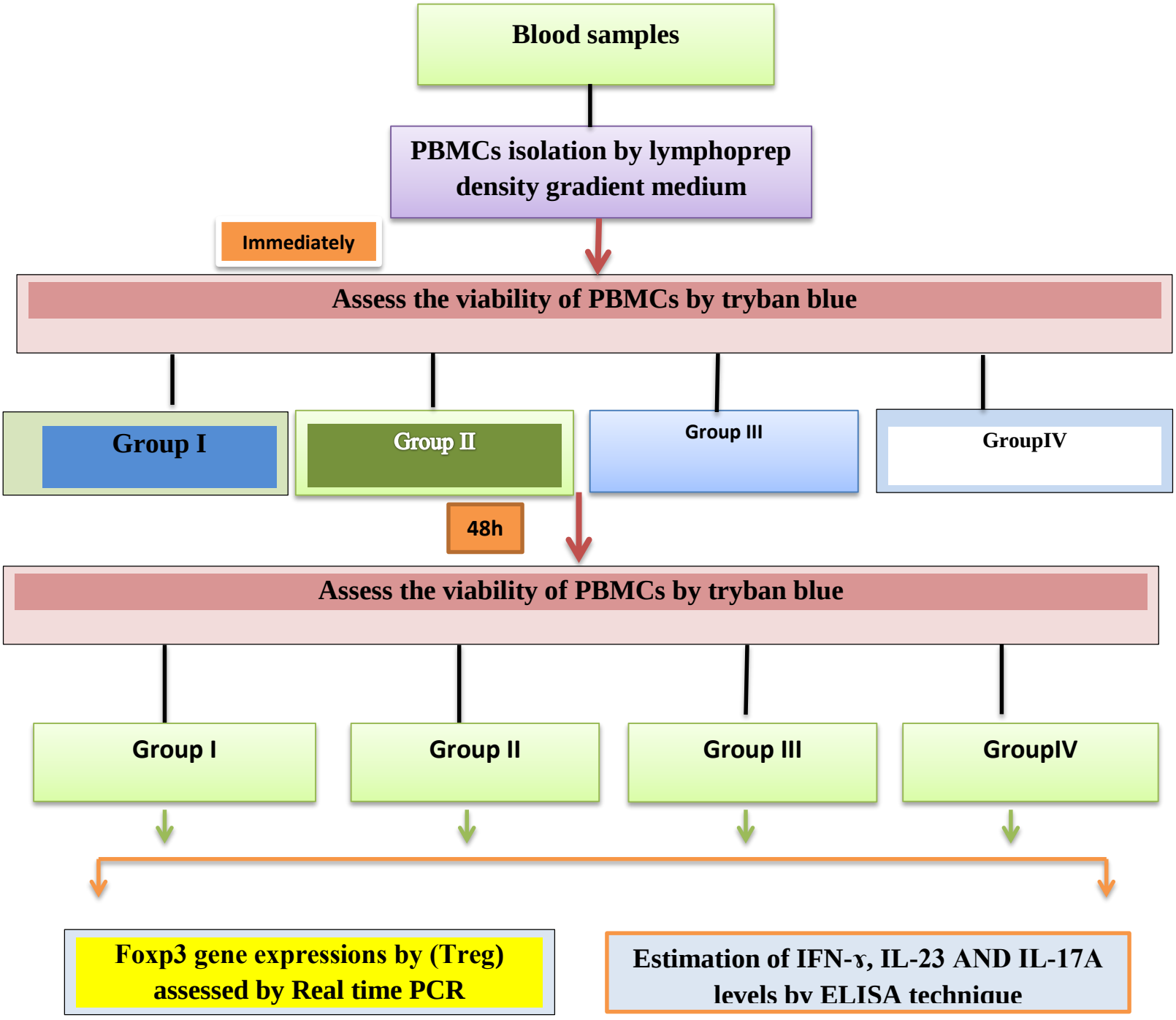



**Figure (2-1)Schematic flow chat of the study procedure**

## 2.2.13.Hematological Examinations:

### 2.2.13.1 Leukocytes Differential:

Differential TLCs test was done for all blood samples as the technique below (Hean, 1995):

**a)** Glass slide was cleaned and blood drop was putted on one end of the slide and by another clean slide with smooth margin gently prepare blood film and left until dried.

**b)** The smears were fixed by embedding with absolute ethanol alcohol.

**c)** The slides were left at room temperature until drying.

**d)** The slides were stained by Giemsa stain.

**e)** After the slides dried, under oil immersion lens, the slides were examined to observe the five different types of leukocytes.

**f)** Calculated hundred cells and calculate each type of these cells, in order to gate the percentage of each type of leukocytes.

## 2.2.14 Immunological Parameters:

### 2.2.14.1 Estimation of ( IFN-γ, IL-17 A, and IL-23) by ELISA:

### A-Principle:

The immunological parameters were included (IFN-γ , IL-17A and IL-23) cytokines. The principle of these testes has the same principle according to leaflets that provided with kits of manufacture Company (Boster Company).

## 2.2.15Genetic Parameters:

The RNA extraction of all samples was done using **(Direct-zol™ RNA MiniPrep,R2051, ZYMO RESEARCH / USA.** RNA was extracted from cell

culture of peripheral blood mononuclear cells after separation from a blood sample using lymphoprep .

**A-Principle**:

The Direct-zol™ RNA Mini Prep provides a streamlined method for the purification of up to 50 µg (per prep) of high-quality RNA directly from samples in TRI Reagent. Total RNA, including small RNAs (17-200 nt), is effectively isolated from a variety of sample sources including cell culture. Isolation of RNA by conventional phase separation was shown to selectively enrich for some species of miRNA, leading to bias in downstream analysis. The Direct-zol™ method assures unbiased recovery of small RNAs including miRNA. The procedure is easy and simply done by applying a prepared sample in TRI Reagent® directly to the Zymo-Spin™ IIC Column and then spinning, washing, and eluting the RNA. No phase separation, precipitation, or post-purification steps are necessary. The eluted RNA is high quality and suitable for subsequent molecular manipulation and analysis (including RT-PCR).

## B-Conversion of RNA to cDNA

Prime Script TM RT reagent Kit is designed to perform the reverse transcription optimized for real-time RT-PCR. It uses Prime Script TM RTase, which features excellent extendibility and makes fast, efficient cDNA template synthesis for Real Time PCR possible. The step experimental procedure is simple and suitable for high throughput analysis. This kit can be used in combination with Real Time PCR reagent, SYBR® Premix.

## Principle:

Prime Script TM RT reagent Kit makes fast and efficient synthesis of cDNA templates for Real Time PCR. This kit is best suited for two step real-time RT-PCR.

It includes Random 6 mers and Oligo dT Primer for use as reverse transcription primers. The reaction can be performed using mixture of these two primers, or the primer can be selected based on the purpose of the experiment. Furthermore, Gene Specific Primers can be used for specific gene detection. A standard curve must be generated for the quantitation of Real-Time RT-PCR. Dilution of total RNA or cDNA after reverse transcription is necessary because low concentrations are required for a viable standard curve. However, dilution with water or TE can narrow the range of the curve due to unstable dilution at low concentrations. Using EASY Dilution Solution (for Real Time PCR), for dilution causes the results to be accurate at lower concentrations and facilitates the creation of a wide-range standard curve.

< For 1 reaction >

| Reagents | Volumes |
|---|---|
| 1- 5 × Prime Script TM mix | 2 µl |
| 2- total RNA | 8 µl |
| 3- RNase Free dH2O | up to 10 µl |
| | Total 20 µl |

1. The reaction mixture was incubated under the following condition.

   37℃ , 15 minutes*3 (Reverse transcription)

   85℃ , 5 sec (Inactivation of reverse transcriptase with heat treatment)

   4℃

## C-Performing RT-PCR

### Product Description

KAPA SYBR FAST qPCR Master Mix (2X) is designed for high-performance real-time PCR. The kit contains a novel DNA polymerase—engineered via a process of molecular evolution—resulting in a unique enzyme specifically designed for real-time quantitative PCR (qPCR) using SYBR Green I dye chemistry. KAPA SYBR FAST DNA Polymerase has been engineered to perform optimally in stringent qPCR reaction conditions, exhibiting dramatic improvements in signal-to-noise ratio (fluorescence), quantification cycle (Cq), linearity, and sensitivity. The KAPA SYBR FAST DNA Polymerase and proprietary buffer system improve the amplification efficiency of difficult targets, including both GC- and ATrich templates.

### Master Mix Preparation

| Component | 20 µL (Final volume) | Final concentration |
|---|---|---|
| **KAPA SYBR FAST qPCR Master Mix (2X) Universal** | 10 Ml | 2x |
| **Forward primer** | 0.4 µL | 0.2Mm |
| **Reverse primer** | 0.4 µL | 0.2µM |
| **Nuclease-free water** | Up to 10 µL | |
| **Template DNA Sample Volume** | 2 µL | 1pg-100ng |

**Table (2-5):RT-PCRCycling Program**

| Genes | Primer sequence (5'-3') | PCR condition | Reference |
|---|---|---|---|
| ***Foxp3* –F** | **TGTGCTAGGGCGGTATGAGA** | 95 ºC 5min 1x | ***Kleine et al.,2010*** |
| ***Foxp3* -R** | **GCTGGGGTGCAACTATGGG** | 95 ºC 20 sec X 40<br>60 ºC 20 sec X 40<br>72 ºC 20 sec X 40 | |
| | | 72ºC 5min 1x | |

## 2.2.16.Statistics analysis :

The data were presented and analyzed using a statistical package for social science (SPSS version 25) and Microsoft Office Excel 2010. The normality of data distribution was tested by the Kolmogorov–Smirnov test. The data were represented as medians with 25% and 75% interquartile ranges (IQR) or means with standard deviations (SD). t-tests were used to compare two independent groups as appropriate with the confidence interval for *P* value was (0.05). Kruskal–Wallis (KW) tests were used to compare three or more independent groups where indicated. The correlation was evaluated using Spearman's correlation test.

## *3.1. Ethanole extracted propolis (EEP):*

The yield of this extraction was 2.36g of propolis extract that was dissolved in 15 ml of 96% ethanol to give a final stock concentration of $1.57*10^5$ mg/l. table (3-1). In order to prepare the required concentration of 5µg/ml of propolis, (Because a high dose of propolis affects the activation and/or survival of T cells and to avoid the sub-effect of propolis such as cell death). The stock solution was diluted by RPMI 1640 medium and estimate the effect of EEP on bacterial LPS as anti-inflammatory and PBMCs immunomodulator. This concentration of EEP was used because it is the best concentration that has less toxic effects and has less stimulation activity of cell apoptosis in cell cultures (Draganova-Filipova,*et.al*.,2010). Less concentration does not affect PBMCs and more concentration is toxic and induced apoptosis in greatest population of PBMCs and significant reduction in a cell line (Salih *et al*.,2010).

The extract had a clear reddish color. Additions of buffer to the ethanol extract of propolis caused sedimentation of the low water-soluble materials, which are very sticky. The applied method for the preparation of extracts of propolis allows obtaining EEP with a high content of bioactive compounds (Pobiega *et al*. 2019). The frequency of microbial genetic mutations is drastically enhanced especially among *P.aeruginosa*, resulting in an increased incidence of antibiotic resistance. Therefore, there is an increasing need to find other molecules, such as natural compounds, capable of killing or inhibiting microbial virulence factors likely without promoting resistance mechanisms, therefore focusing on propolis extracts in this study to estimate its effects on MDR-*P.aeruginosa.*

This conventional extraction has some disadvantages, mainly because it is a slow process that presents solvent residues in the final product (Walczak-

skierska, 2020), EEP has advantages over water extracted propolis in that has the greatest antioxidant ability with the concentration of ethanol (50-90%) due to its high flavonoids components incomparable with that of WEP. Another advantage is that EEP has high activity in inhibition of hayaluronidase enzyme that play a critical role in inflammation, hence EEP has more anti-inflammatory activity than WEP that has no anti-hyaluronidase activity. In this research this extracts exert high immunomodulation by decreasing proinflammatory cytokines and significantly increasing the expression of Foxp3 gene in PBMCs.

**Table (3-1): Comparsion between propolis extracts yields**

| Type of propolis extract | The yield in mg | The stock conc. |
|---|---|---|
| **Ethanol extracted propolis** | 2360 mg | $1.57*10^5$ mg/l |
| **Water extracted propolis** | 426.5 mg | $2.48*10^4$ mg/l |

### *3.2. Water extracted propolis (WEP):*

The yield of this extract was 426.5 mg of WEP that dissolved in 15ml of D.W. The final stock concentration of the WEP stock were $2.48*10^4$ mg/l. In order to achieve the required concentration of 5µg/ml of WEP, the stock was diluted with RBMI 1640 medium and then 0.1ml was added to the fourth group of PBMCs into cell culture plate that was labeled as WEP which contain PBMCs and LPS plus WEP as shown in table (3-2). According to (Kubiliene *et al*.,2015), the advantage of this extract is that has a less toxic effect on PBMCs viability and proliferation after 48hrs., Regarding the immunomodulatory action of WEP, it enhanced the innate

immunity, activating the initial steps of the immune response by upregulating TLR-2 and TLR-4 expression and pro-inflammatory cytokines (IL-1 and IL6) production by macrophages contributing to the recognition of microorganism and to lymphocytes activation by antigen-presenting cells and also increased hydrogen peroxide (H2O2) generation, favoring the microorganisms killing (Orsatti *et al.*, 2010).

Propolis derived flavonoids show the antibacterial activity by different mechanisms than those of conventional drugs, hence, bacteria or other pathogens cannot easily develop resistant because most of the natural compounds are not initially encoded by resistance gene so they could be of importance in the enhancement of antibacterial therapy especially against MDR-pathogens.

In this method most of the flavonoids, vitamins, amino acids and other water-soluble compounds were released and remain free of wax and resin. The addition of buffer (under cold conditions) allows quick precipitation of wax and resin. This method is very simple when compared to other methods and allowing the removal of the most of resin and wax present in propolis that makes it more applicable in cell culture and medical applications. In this research this extracts exert high immunomodulation by decreasing proinflammatory cytokines and significantly increasing the expression of Foxp3 gene in PBMCs.

## 3.3 Testing groups:

The twenty samples from males and females were cultured in RPMI1640 medium and then each sample were divided in to four groups, table (3-2).

**Table (3-2):The testing groups in this study**

| Testing groups | No. | Treatment |
|---|---|---|
| **Group I** | 20 | PBMCs+ *p.aeruginosa* Ag |
| **Group II** | 20 | PBMCs +EEP+ Ag |
| **Group III** | 20 | PBMCs +WEP+ Ag |
| **Group IV** | 20 | Control(PBMCs) only |

## *3.4. Peripheral blood mononuclear cells (PBMCs) isolation:*

The PBMCs were isolated by density gradient medium (lymphoprep). The principle of PBMC isolation protocols is the centrifugal separation of blood components against a high-density medium, PBMCs can be separated from other components of the blood, such as erythrocytes and granulocytes, due to their higher density, erythrocytes, granulocytes and dead cells will pass through the lymphosep layer, whereas lymphocytes and monocytes, based on their lower density, will accumulate at the plasma-gradient boundary. This approach is concordant with the method for isolation of PBMC, developed by (Boyum in 1968). This method of isolation is fast and easy incomparable to the other methods of PBMCs isolation, due to its uncomplicated and robust feasibility, density gradient centrifugation is now ubiquitously applied to isolate PBMCs.

To evaluate the quality of the separation, it was determined whether defined interphase with clearly delineated phase transitions was visible. Turbidity of the liquid may indicate suboptimal separation of the PBMCs. The PBMCs composed of monocytes and lymphocytes were isolated from the buffy coat at the interface between the plasma and red blood cells were cultured after washing twice with phosphate buffer saline. Aliquots of $1*10^6$ cells were incubated without antigen in 24-well tissue

culture plates at a final volume of 500µl per well at 37ºC in the presence of 5% CO2 after counting by hemocytometer by aseptic technique to prevent contamination of cell cultures according to (Absher,1973), the expected yield for mononuclear cells falls between 0.8 and $3.2*10^6$ cells/mL of blood, our result falls with this expected range at 1and $2*10^6$ cells/mL in order to achieve the final concentration of $1*10^6$ for all samples in each well the samples were diluted using RPMI medium. In order to confirm the quality of the PBMC isolation using lymphosep, the PBMC samples were subjected to an analysis of their yield and viability. This techniques had been shown a cell viability rating of 100% immediately after isolation had taken place and this result was achieved by trypan blue test to estimate the availability of PBMCs before and after propolis and Ag addition .

## *3.5. Viability of Peripheral blood mononuclear cells (PBMCs)* by trypan blue exclusion test:

The result of viability immediately after isolation was 100% (figure 3-1), and after culturing for 16hrs the viability was high for all samples with average values of 96% and viability were within expected ranges that agree with (Chen *et al*.,2020) that had (96.6%) viability result. The result of the viability of the first group (Ag) group after 48hrs. was(80%) while for EEP,WEP,and control groups were (78%),(83%),(88%), respectively. The viability of PBMCs after treatment with WEP was (83%) which is significantly more than that of EEP. This result is in agreement with (Chen *et al*.,2020) that has result of (84%) viability after 48hrs. of incubation, table (3-2).

The decrease in cells viability was greater in PBMCs treated with EEP+LPS than PBMCs treated with WEP+LPS. However both WEP and EEP reducing the viability of the cells when compared with the negative

control group. Propolis is a complex composition that includes numerous organic compounds, many of which are active and capable to interact with cells and influence their metabolic functions, and its cytotoxicity is dependent on the concentration ,when the high concentration of the propolis extracts promoted more cytotoxicity and induce cell apoptosis than the low concentration (Campocciaa *et al*.,2021), and the aqueous extract of propolis was less cytotoxic than EEP at the same concentration. The mechanism involved in propolis extracts induced cytotoxicity is still unknown. However, the propolis extract may act via a direct cytotoxic activity by alteration of biological membranes under the action of natural flavone molecules (Herrerias *et al*., 2010) or by DNA-binding of flavonoid aglycone or flavoloid glycosides compromising DNA duplication and leading to the cell cycle arrest (Wang *et al*., 2008). In conclusion, the cytotoxic activity of propolis extracts is a complex phenomenon depending not only on the nature o flavonoiedes components but also on the nature of the extract.

Trypan blue exclusion test for viability is based on the principle that living cells possess intact cell membranes that exclude certain dyes, such as trypan blue, or eosin whereas dead cells do not. In this test, a cell suspension is mixed with dye and then visually examined to determine whether cells take up or exclude the dye (Strober,2015). In this test, a viable cell will have a clear cytoplasm whereas a nonviable cell will have a blue cytoplasm. Figure (3-2).

**Table (3-3):The viability results by trypan blue test for each group after 48hrs.**

| Testing group | Description | Viability percentage after 48hrs |
|---|---|---|
| **Group I** | Ag | 80% |
| **Group II** | EEP+ Ag | 78% |
| **Group III** | WEP+ Ag | 83% |
| **Group IV** | Control | 88% |

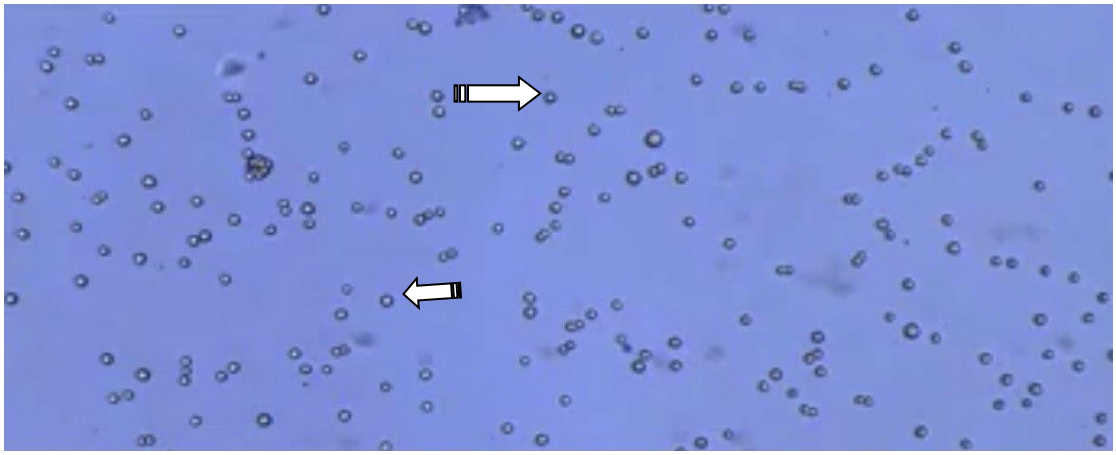


Figure (3-1) viability of PBMCs using trypan blue dye(40X power).

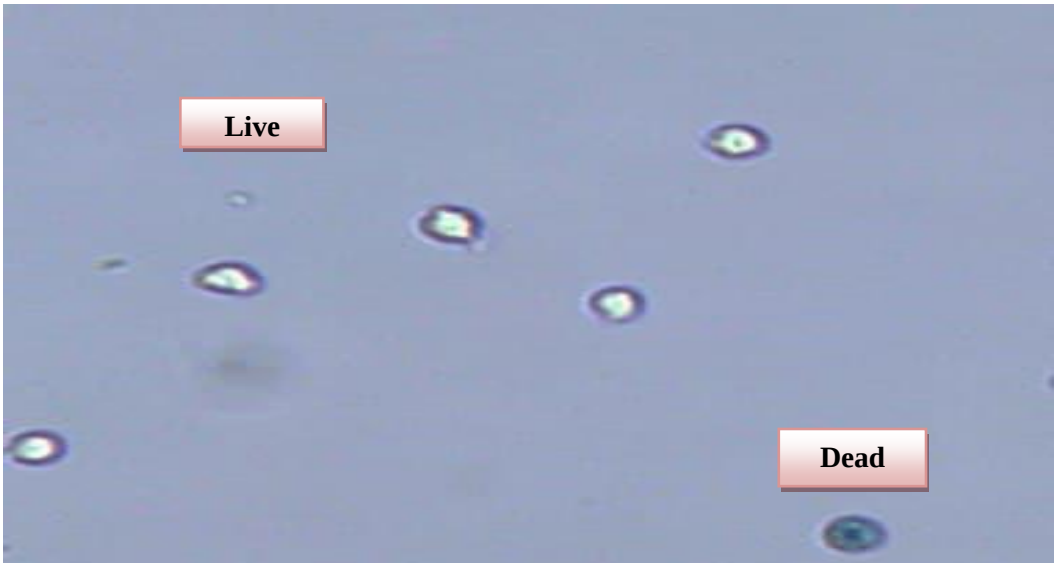



Figure (3-2): Trypan blue exclusion test (living and dead PBMCs) under inverted microscope at 40X power.

## *3.6. Immunological parameters:*

### *3.6.1. Cytokines:*

#### *3.6.1.1. Interferon Gamma (IFN- γ) levels:*

The IFN-γ concentration results pointed a significant elevation ($p<0.001$) in the median value of Ag group 63.40 (7.46) pg/ml as compared to control, (EEP+ Ag) and (WEP+ Ag) groups, 60.0 (3.99) pg/ml, 50.0 (6.07) pg/ml and 52.85 (4.88) pg/ml respectively, and also shown that the median concentration level of IFN-γ was high significantly in control in compared to (EEP+Ag) and (WEP+Ag) groups, ($p<0.001$), as in table (3-4).

Regarding gender, the results of the present study indicated non-significant association between median levels of IFN-γ and males and females, table (3-5). The results in this study were in agreement with (Singh *et al*.,2015; Blackwood *et al.*,2020) who pointed out that IFN-γ level was increased in response to *pseudomonas aeruginosa* Ag as an innate immunity against such infection in comparison to the control group. Also (Catharina *et al*.,2002) has reported that the IFN-γ level was increased significantly only 24 to 48 hrs. following stimulation with pseudomonas LPS.

Also, the same result for Ag stimulated group in comparison to EEP and WEP groups with($P<0.001$), this result was in agreement with (Silveira *et al*.,2021) who pointed that in comparison to Ag-stimulated peripheral blood macrophages, propolis extracts significantly reduced the development of proinflammatory mediators, especially IFN-γ as well as the LPS-induced activation of transcription factors involved in inflammatory signaling such as (Pdk1, Gsk3b, Nfkb1, and Elk1). This downregulation of IFN gamma production confirms the data obtained by

(Liberio *et al.*,2011) in a mouse model, they observed that geopropolis increased production of IL-4 and IL-10 and cytokines associated with a Th2 response, suggesting an anti-inflammatory activity.

**Table (3-4): Interferon- gamma levels (pg/ml) in testing groups**

| | *Study groups* | | | | **Total P value** |
|---|---|---|---|---|---|
| ***IFN-γ*** | ***Ag n = 20*** | ***EEP + Ag n=20*** | ***WEP+ Ag n=20*** | ***Control n = 20*** | |
| **Range** | 46.90 – 66.90 | 46.67 – 61.43 | 46.67– 57.62 | 49.52 – 62.62 | **< 0.001 † HS** |
| **Median (IQR)** | **63.40** (7.46) | **50.0** (6.07) | **52.85** (4.88) | **60.0** (3.99) | |
| **P1 C/Ag** | **0.043 S** | | | | |
| **P2 C/EEP** | **< 0.001 HS** | | | | |
| **P3 C/WEP** | **< 0.001 HS** | | | | |
| **P4 Ag/EEP** | **< 0.001 HS** | | | | |
| **P5 Ag/WEP** | **< 0.001 HS** | | | | |
| **P6 EEP/WEP** | **0.08 NS** | | | | |

IQR: inter-quartile range; †: Kruskal-Wallis test; ¥: HS: Highly significant at P ≤ 0.001; NS: not significant at P ≤ 0.05; P1: Control vs Ag; P2: Control vs EEP; P3: control vs WEP; P4: Ag vs EEP; P5: Ag vs WEP; P6: EEP VS WEP .

In our result, the level of this cytokine in Ag stimulated group was increased significantly in comparison to control group with (P<0.043), while the P value for control in comparison to the EEP and WEP groups was highly significant with(P <0.001), EEP and WEP groups had been shown highly decreased median level in IFN-γ in comparison with the control group, this result was in agreement with (Ningshi *et al.*,2017) who reported that the IFN-γ was inhibited by propolis. The propolis PBMCs treatment attenuated the LPS level and down-regulated the inflammatory markers expressions and reduced the inflammatory cytokines (Pahlavani *et al.*,2020). This action of propolis extracts points to its potential as a

natural anti-inflammatory agent and a source for the production of new alternative medicines.

**Table (3-5):Relation between IFN-γ levels and gender**

| IFN-γ | *Male* | *Female* | *P* |
|---|---|---|---|
| **Control** | | | |
| **Mean± SD** | **57.5** ± 4.56 | **60.44** ± 2.0 | **0.151 †** **NS** |
| **Range** | 49.52 - 62.38 | 56.43 - 62.62 | |
| **Ag** | | | |
| **Mean± SD** | **59.37** ± 7.04 | **63.85** ± 3.02 | **0.148 †** **NS** |
| **Range** | 46.90 -66.76 | 59.52-66.9 | |
| **EEP+ Ag** | | | |
| **Mean± SD** | **51.97** ± 5.03 | **50.64** ± 3.23 | **0.568 †** **NS** |
| **Range** | 46.67-61.43 | 46.90-55.71 | |
| **WEP+ Ag** | | | |
| **Mean± SD** | **51.71** ± 2.76 | **54.21** ± 3.27 | **0.149 †** **NS** |
| **Range** | 46.67 -54.67 | 50.19 -57.62 | |

† : Independent Samples Test; HS: Highly significant at P ≤ 0.001; NS: not significant at P ≤ 0.05;

Another comparison between EEP and WEP groups according to IFN-γ levels in this study had been shown to be non-significant with(P<0.08). That means no difference in the immunomodulation between ethanol extracted and water extracted propolis on the IFN-γ level on *pseudomonas aeruginosa* LPS stimulated PBMCs in vitro as shown in table(3-4).

Regarding gender, the result in this study was non-significant with(P ≤ 0.05) for all groups that mean the immune response in male and females volunteer in this study for IFN-γ cytokine was nearly the same although slightly increase in IFN- γ median level in female in comparison to male

in (control, Ag stimulated, and water extracted propolis groups) and also the propolis extracts with its two types has the same effect (NS) on the male and female PBMCs in vitro stimulation as shown in table (3-5).

In this study, propolis extracts show high immuno-modulating activity on IFN-γ cytokine as illustrated previously by decreasing the concentration of these cytokines both in EEP and WEP stimulated groups (table 3-4).

IFN-γ is a type 1 cytokine that is involved in both innate and acquired antimicrobial immune regulation (Touma *et al*.,2021). A series of complex interactions between accessory cells including macrophages and dendritic cells, as well as T lymphocytes and natural killer (NK) cells, regulates the expression of IFN-γ. A complex interaction between immune cell activity and IFN- γ through organized integration of signals from other cytokines and Pattern Recognition Receptors (PRRs) such as Interleukin (IL-4, Lipopolysaccharide, TNF-α and Type-I Interferons ) create a cascade of proinflammatory responses (Naglak *et al*., 2016).

This factor is produced by NK cells, which are innate cells that produce IFN-γ immediately upon stimulation (Kak *et al.*, 2018). IFN-γ also assists in the recruitment of leukocytes to infection sites by increasing the expression of adhesion molecules and chemokines and it was found to be effective in preventing B-cell differentiation (Day *et al*., 2017). Gamma interferon (IFN-γ) has been shown to stimulate cells and potentiate the effect of LPS. When IFN-γ binds to its receptor, Janus kinases (Jak1 and Jak2) in the cytoplasm become activated, phosphorylating specific tyrosine residues on signal transducer and activator of transcription (STAT)-1 molecules, STAT1α forms homodimers, translocates to the nucleus, and binds unique STAT-binding

DNA sequences when activated and phosphorylated leading to the transcription of a significant portion of IFN-γ induced genes.

Propolis flavonoids inhibit (STAT)-1 leading to inhibition of IFN-γ in propolis extracts stimulated groups, CAPE flavonoides of propolis inhibits this cytokine and increasing the production of anti-inflammatory cytokines mainly IL-10 and IL-4 together with inhibition of IL-2 and subsequent inhibition of T cells proliferation thus affect IFN-γ production from these cells. Also, propolis interrupts the interaction of the ligand (LPS) with the receptor complex (TLR4/MD2) and therefore it inhibited the activation of Toll-like receptor 4 (TLR4). TLR4 receptor is dysregulated in chronic inflammatory diseases (Pahlavani *et al*.,2020). Therefore, propolis may be effective in inflammatory diseases and the result demonstrated its activity in suppression the LPS induced inflammatory cytokines and its secretion by mechanism mentioned by (Bufalo *et al*.,2013) through suppression of LPS induced signaling pathways mainly NF-$_K$β and NO inhibition, suggesting its use as a natural source of safe anti-inflammatory drug.

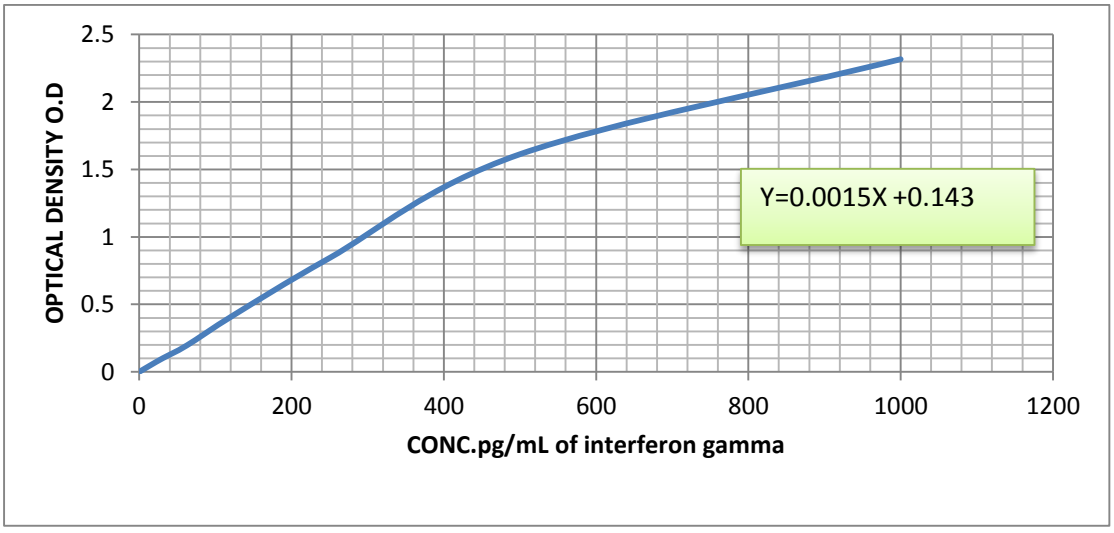



**Figure (3-3) standard curve of IFN-γ estimation**.

### *3.6.1.2. Interleukin-17A(IL-17A) levels:*

The median concentration of IL-17A was significantly higher in Ag only stimulated group 164.07(25.75) pg/ml as compared to control, EEP and WEP groups,140.23 (24.42) pg/ml, 104.84 (29.04) pg/ml, and 130.61(51.3) pg/ml respectively,($p<0.001$), and also the median concentration level of IL-17A were high significantly in control in compared to EEP, and WEP groups, ($p<0.001$), table (3-6). The results of the present study indicated a non-significant association between median levels of IL-17A and the gender, table (3-7).

**Table ( 3-6): IL-17 A level (pg/ml) in testing groups**

| | ***Study groups*** | | | | **Total P value** |
|---|---|---|---|---|---|
| ***IL-17A*** | ***Ag n = 20*** | ***EEP + Ag n=20*** | ***WEP+ Ag n=20*** | ***Control n = 20*** | |
| **Range** | **92.54– 194.85** | **67.15 – 159.46** | **103.3– 251.7** | **110.23– 166.38** | **< 0.001 † HS** |
| **Median (IQR)** | **164.07 (25.75)** | **104.84 (29.04)** | **130.61(51.3)** | **140.23 (24.42)** | |
| **P1 C/Ag** | **0.007 S** | | | | |
| **P2 C/EEP** | **< 0.001 HS** | | | | |
| **P3 C/WEP** | **0.532 NS** | | | | |
| **P4 Ag/EEP** | **< 0.001 HS** | | | | |
| **P5 Ag/WEP** | **0.205 NS** | | | | |
| **P6 EEP/WEP** | **0.003 S** | | | | |

IQR: inter-quartile range; †: Kruskal-Wallis test; ¥: HS: Highly significant at $P \leq 0.001$; NS: not significant at $P \leq 0.05$; P1: Control vs Ag; P2: Control vs EEP; P3: control vs WEP; P4: Ag vs EEP; P5: Ag vs WEP; P6: EEP VS WEP .

In Ag stimulated group IL.17A level was significantly increased in comparison to the control group ($p<0.007$), the results that in agreement with (Lore *et al.*,2016) who pointed out that IL.17A level was

significantly increased in response to *pseudomonas aeruginosa* infection. Also (Xu *et al*.,2014) had been reported that the IL.17A level was increased significantly after 48 hrs. following stimulation with pseudomonas Ag.

As the IL-17A pathway plays a significant and critical role in resistance and regulation of the inflammatory response to *P. aeruginosa* acute infection, IL-17 levels have been shown to play a role in the decline of lung function, and IL-17 generating CD4+ T cells have been increasingly identified in cystic fibrosis patients, among the possible cellular sources. As a consequence of these findings, it's possible that CF is an IL-17-mediated disorder.

In our results, the level of this cytokine in Ag stimulated group was increased significantly in comparison to the control group with ($P<0.007$), according to the researchers, this was linked to substantial increases in the proportion of CD4+ Th17 cells that expressed these cytokines in response to pseudomonas LPS according to (Blackwood, *et al*.,2020) which supports the significance of Th17 cell responses to these pathogens, while the P for Ag stimulated only in comparison to EEP group was highly significant with ($P <0.001$) and the median level of this cytokine in EEP group was significantly decreased, this was in agreement with (Missima *et al*.,2010; Tanaka *et al*.,2012; Laerte *et al.*,2020) who stated that propolis reduced the development of proinflammatory IL.17A from CD4+TH17 cells. The abnormal IL-17 development or overexpression in pseudomonas infection causes a range of pathological effects, such as asthma, pneumonitis, and the development or exacerbation of pulmonary fibrosis (Gurczynski *et al*.,2018).

As a result, these negative effects can clarify a more subtle mechanism of propolis' anti-inflammatory action. Propolis inhibited the

differentiation of IL-17-producing cells, and this effect was mediated by inhibiting IL-6-induced STAT3 phosphorylation (Okamoto *et al*.,2012).

IL-17A has the ability to act directly on epithelial cells, fibroblasts, and myofibroblasts, facilitating epithelial to mesenchymal transformation. IL-17A may also cause epithelial cells to upregulate mucus-producing gene products, aggravating asthma symptoms (Zhou *et al*.,2016).

**Table (3-7): Relation between IL-17A levels and the gender.**

| IL-17A | *Male* | *Female* | *P* |
|---|---|---|---|
| **Control** | | | |
| **Mean± SD** | **129.13** ± 16.04 | **147.92** ± 9.34 | **0.021 † S** |
| **Range** | 110.23 - 162.54 | 140.23- 166.38 | |
| **Ag** | | | |
| **Mean± SD** | **165.27** ± 20.6 | **152.47** ± 30.6 | **0.377 † NS** |
| **Range** | 131.77- 194.85 | 92.54- 189.46 | |
| **EEP+ Ag** | | | |
| **Mean± SD** | **111.65** ± 22.86 | **106.60 ± 24.27** | **0.695 † NS** |
| **Range** | 67.15- 137.15 | 88.69-159.46 | |
| **WEP+ Ag** | | | |
| **Mean± SD** | **144.92** ± 32.82 | **145.5** ± 51.53 | **0.980 † NS** |
| **Range** | 109.46-205.62 | 103.31-251.77 | |

† : Independent Samples Test; HS: Highly significant at P ≤ 0.001; NS: not significant at P ≤ 0.05;

As a result, propolis extract has been shown to have strong immunomodulatory effects on this cytokine, reducing their pathological effects following an acute early infection. Increased Th17 cell activity was linked to a lower number of T regulatory cells and higher levels of IL-21, IL-22, and IL-23, both of which contributed to autoimmunity pathogenesis in CF patients (Jacek *et al*.,2015).

In pseudomonas infection, propolis can play a role in balancing pro-inflammatory Th17 cells and regulatory T lymphocytes to prevent immune response exacerbation.

Regarding water extracted propolis group IL-17A level nearly the same as control IL-17A level that is a non-significant relationship with (P<0.532). Also, the result of Ag stimulated group versus the WEP group was non-significant at (P<0.205), this may be related to the lower amount of phenolic compounds in WEP in comparable with EEP. This agree with (Kubiliene *et al*.,2015) who was reported that WEP has fewer bioactive compounds and also its flavonoids contents are different according to the origin and climate, therefore there is no clear additive effect of WEP on IL-17A levels in LPS stimulated PBMCs in this study.

Finally, the results show that there is significant decrease in the EEP+ Ag group median IL-17A level in compared to the WEP+Ag group median level (104.48 for EEP vs130.61 for WEP group) at (P<0.003). This means that EEP has high additive and immunomodulatory effect on this cytokine and Ag stimulated PBNCs than WEP due to its high flavonoids, caffeic acid, and phenolic contents, while the results regarding gender appeared that the level of IL-17A was significantly higher in females control group than males control (P<0.021), this result agree with (Newcomb *et al*.,2015) who observed that interleukin-17A was significantly higher in females serum than males. This is related to estrogen and progesterone effects on proinflammatory cytokines mainly IL.17 and increased IL.17A mediated inflammation, As a result, women are more likely than men to develop Th17-mediated autoimmune disorders such as lupus and multiple sclerosis, as well as extreme asthma and allergic diseases ( Mohammad *et al*.,2018).

The mechanism by which estrogen affects this cytokine in females through signaling via the ER increased the expression of IL-23R and the development of IL-17A by Th17 cells. In the other groups, there were no major differences between males and females performance (AbdulHussaina *et al*.,2020).

*P.aeruginosa* is a conditioned pathogen and may cause severe or even fatal infection. Although great progress has been achieved in the antibacterial treatment, the wide application of broad-spectrum antibiotics increases the drug tolerance of bacteria and increased the emergence of MDR-PA. Therefore, a better alternative treatment is required to increase the immunity against MDR- bacterial infection.

Bee propolis and its constituents can function as a natural anti-inflammatory agent by modulating IL.17A immune responses. Given that propolis extract inhibit multiple signaling pathways in PBMCs produceing IL-17A involved in the LPS-activated inflammatory process.

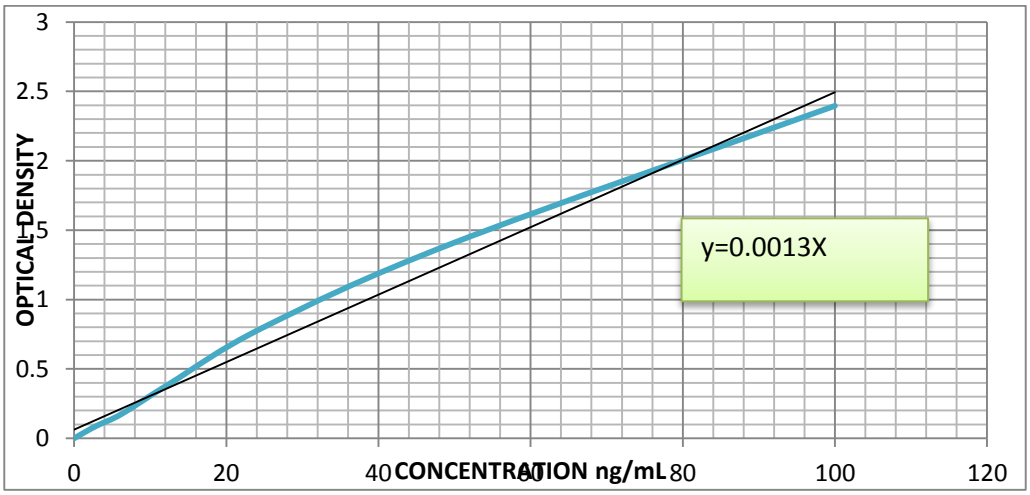



**Figure (3-4) standard curve of IL.17A estimation**.

### *3.6.1.3. Interleukin-23 (IL.23) levels:*

The median concentration of IL-23 was significantly higher in Ag only stimulated group 491.82(141.24) pg/ml as compared to the control, EEP+Ag, and WEP+Ag groups 318.84(69.22)pg/ml, 402.842(88.52) pg/ml, and 395.35(125.7) pg/ml respectively. and also show the median concentration level of IL-23 were significantly higher in EEP+Ag group in compared to control versus WEP+ Ag groups in compared to control . WEP+Ag group versus control($P<0.05$), Table (3-8).

The results of the present study indicated a significant association between median levels of IL-23 and the gender, where the mean of IL-23 increased in males compared to females in the control (371.38 ± 49.66) versus (328.52± 45.818), LPS (549.7 ± 73.32) versus (447.84 ± 74.73), and WEP+Ag (459.42 ± 71.53) versus (380.84 ± 62.12), but non-significant differences between male and female in EEP+Ag groups (404.53 ± 61.63) versus (395.98 ± 55.11),table (3-9).

In Ag only stimulated group IL.23 level was significantly highly increased in comparison to the control group ($P<0.001$), this result was in agreement with (Cui *et al*.,2018) who found out that in response to *pseudomonas aeruginosa* Ag, IL.23 levels were significantly elevated and induced . The production of IL-23 by peripheral blood mononuclear cells in response to bacterial lipopolysaccharides (LPS) by pseudomonas aeruginosa was also observed by (Louis *et al*., 2010).The pro-inflammatory cytokines IL-23 play a significant role in the activation of the immune response in the host defense against pathogens and the maintenance of mucosal barrier functions. The cytokine IL-23 belongs to the IL-12 family of cytokines.

The IL-23 receptor, which is made up of IL-12R 1 and IL-23R, has been identified as a functional IL-23 receptor. Antigen-presenting cells (APCs), such as dendritic cells (DCs), monocytes, and macrophages, express IL-23. IL-23 also increased CD4+ T cell IL-17 production and

created a new population of IL-17-producing CD4+ T cells (Th17 IL.23, a proinflammatory cytokine that has been shown to be a crucial cytokine for Th17 maintenance and expansion, was thought to control the differentiation of CD4+ naive T cells to Th17 cells (Cauli *et al*.,2015).

**Table ( 3-8): IL-23 level (pg/ml) in testing groups:**

| | *Study groups* | | | | Total P value |
|---|---|---|---|---|---|
| **IL-23** | ***Ag n = 20*** | ***EEP + Ag n=20*** | ***WEP+ Ag n=20*** | ***Control n = 20*** | |
| **Range** | 332.30– 630.0 | 315.54– 481.2 | 320.2 – 526.85 | 280.91– 445.7 | **< 0.001 †** **HS** |
| **Median (IQR)** | **491.82** (141.24) | **402.842**(88.52) | **395.35**(125.7) | **318.84**(69.22) | |
| **P1 C/Ag** | **< 0.001** **HS** | | | | |
| **P2 C/EEP** | **0.003** **S** | | | | |
| **P3 C/WEP** | **0.05** **S** | | | | |
| **P4 Ag/EEP** | **0.008** **S** | | | | |
| **P5 Ag/WEP** | **0.019** **S** | | | | |
| **P6 EEP/WEP** | **0.854** **NS** | | | | |

IQR: inter-quartile range; †: Kruskal-Wallis test; ¥: HS: Highly significant at P ≤ 0.001; NS: not significant at P ≤ 0.05; P1: Control vs Ag; P2: Control vs EEP; P3: control vs WEP; P4: Ag vs EEP; P5: Ag vs WEP; P6: EEP VS WEP .

Th17 transcription factor (RORt) is stabilized by IL-23, allowing it to function properly and release effector cytokines that mediate protection against extracellular bacteria and participate in barrier immunity (Tsukazaki and Kaito , 2020). According to experimental evidence. The pathological activation of IL-23 and IL-17 causes chronic inflammatory diseases, especially in CF patients (Cuthbert, *et al*.,2019).

The results in this study had been shown that there is significantly decrease in the median level of IL-23 in (EEP+Ag) and (WEP+Ag) groups in comparison to Ag only stimulated group (P<0.008),(P<0.019) respectively. These results are in agreement with the previous findings obtained by (Alqarni et al.,2019) who stated that all the propolis extracts suppressed the proinflammatory cytokines at LPS stimulated level, The anti-inflammatory effect of propolis extracts on PBMCs is mediated by inhibition of pro-inflammatory cytokines and metabolic reprogramming of LPS activity, implying an immunomodulatory effect, as IL.23 plays a key role in the pathogenesis of chronic inflammatory disease.

**Table ( 3-9): Relation between IL-23 levels and the gender.**

| *IL-23* | *Male* | *Female* | *P* |
|---|---|---|---|
| **Control** | | | |
| **Mean± SD** | **371.38** ± 49.66 | **328.52**± 45.818 | **0.041 † S** |
| **Range** | 295.87- 445.76 | 280.91- 410.43 | |
| **Ag** | | | |
| **Mean± SD** | **549.7** ± 73.32 | **447.84** ± 74.73 | **0.024 † S** |
| **Range** | 412.30 - 630.0 | 332.30- 555.67 | |
| **EEP+Ag** | | | |
| **Mean± SD** | **404.53** ± 61.63 | **395.98 ± 55.11** | **0.789 † NS** |
| **Range** | 319.35- 481.23 | 315.54 -480.24 | |
| **WEP+Ag** | | | |
| **Mean± SD** | **459.42** ± 71.53 | **380.84** ± 62.12 | **0.049 † S** |
| **Range** | 340.98-526.85 | 320.23-504.56 | |

† : Independent Samples Test; S: significant at P ≤ 0.05; NS: not significant at P >0.05;

Propolis acts on a distinct inflammatory pathway, primarily proinflammatory cytokines, and on the transcription factor (NF-κB) that regulates the expression of proinflammatory cytokines and reduced IL.2

productionthe which is responsible for the T cell differentiation and consequently reduced proinflammatory cytokines.

Propolis extracts and propolis compounds (e.g., caffeic acid, phenethyl ester, hesperidin and quercetin) strongly inhibit DNA synthesis and the development of inflammatory cytokines (i.e., IL-1, IL-12, IL-2, IL-17, IL-23, and IL-4) in Th1- and Th2-type T cells, while increasing the production of transforming growth factor-1 (TGF- β1), which suppress cells differentiation ( Bueno-Silva *et al.*,2017).

Conventional inflammatory cytokines mediate inflammation, which helps to kill invading microorganisms or damaged cells while also promoting tissue repair and regeneration (Karin & Clevers, 2016). However, uncontrolled inflammation causes significant macrophage activation and self-inflicted death, which then induces widespread neutrophil recruitment, resulting in extreme immunopathologies. In response to a variety of microbial and environmental signals, PBMCs polarize into different subpopulations with different effector functions. Toll-like receptor ligands, such as LPS or proinflammatory cytokines, affect such cells with the goal of removing their primary causes that disrupt homeostasis.

This phenotype is linked to high NO levels and proinflammatory cytokines, as well as LPS-induced development of pro-inflammatory mediators including IL-23, IFN gamma, and IL-17A. Propolis' ability to increase IL-10 development demonstrates its ability to control inflammation. After an inflammatory stimulus, many immune cells produce IL-10, which is essential for maintaining homeostasis by controlling both innate and adaptive immune responses (Ma *et al*., 2015; Taomingm *et al*.,2020).

IL-23 imbalance and increase are associated with autoimmune and cancerous diseases. It is preferentially acts on memory CD4(+) T cells. During the early innate immune response, beore IL-17 induction, IL-23 acts synergistically with IL-1β to facilitate early neutrophil (polymorphonuclear leukocyte PMN) recruitment, IL-23 also facilitated IL-17 development by lung γδT cells at later time points, which was greatly enhanced in the presence of IL-1β. These findings show that IL-23 regulates two distinct phases of neutrophil recruitment in response to *P. aeruginosa* infection: IL-17-independent early PMN emigration and IL-17-regulated later PMN emigration (Hanzel and Geert, 2019).

Propolis inhibits the cytokine IL-23, which suppresses Treg cells, resulting in increased Treg cell proliferation and reduced autoimmune disease effects. In addition, IL-23 is implicated in the development of airway inflammation in mucoid *P. aeruginosa* infection, suggesting that IL-23 may be a candidate for immunotherapy to treat inflammation in patients (Olszowiec-Chlebna *et al*.,2019).

These data convincingly show that propolis has a direct regulatory effect on basic functional properties of immune cells which may be regulated by the Erk2 MAP-kinase signal pathway. Thus, the bee product propolis can be regarded as a strong natural anti-inflammatory medicine influencing different types of immune-responses possibly through immunoregulatory T cells.

Regarding the difference between EEP and WEP effect on Ag stimulated PBMCs there is no significant result with ($P<0.890$). They had been shown approximately the same effect on this interleukin. While the result regarding gender, the level of IL.23 were significantly increased in males in compared to females (in control, Ag, and WEP+Ag groups)

while EEP+Ag group was given non-significant elevation (P<0.789) although there is increasing in the level of IL.23 in the male in compared to females. The significant difference among the three above groups was related to progesterone hormone that significantly down-regulated the secretion of the Th1 cytokines among them IL.23(Abdul Hussaina *et al*.,2020). This hormone level was high in females than males and may be responsible for this decreasing.

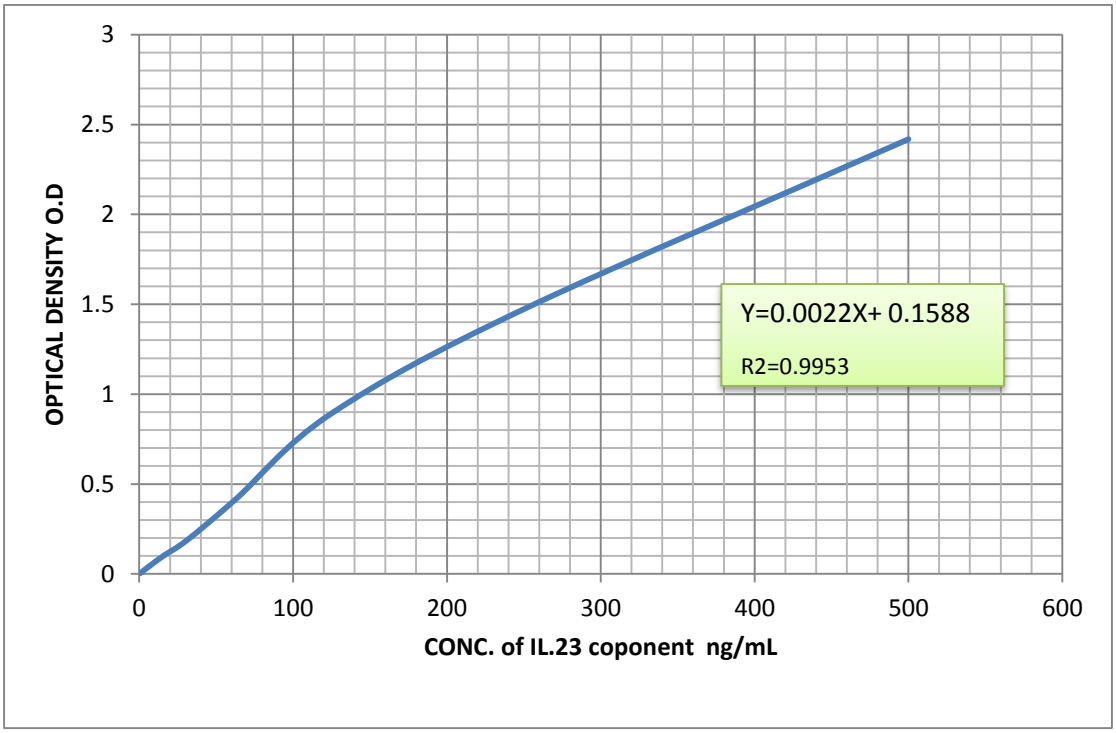



**Figure (3-5) standard curve of IL.23 estimation.**

## *3.7. Genetic parameters :*

### *3.7.1. Extraction of PBNCs RNA and cDNA synthesis:*

Total RNA was extracted from all subjects after treating samples with TRI Reagent using silica-membrane based spin column (sold phase) technology of extraction. The purity of the extracted RNA by this method was between (1.8−2.1), and the results for RNA purity were fall within

this range when measuring the RNA concentration and purity for each sample using Nano-drop, table (3-10), figure(3-6).

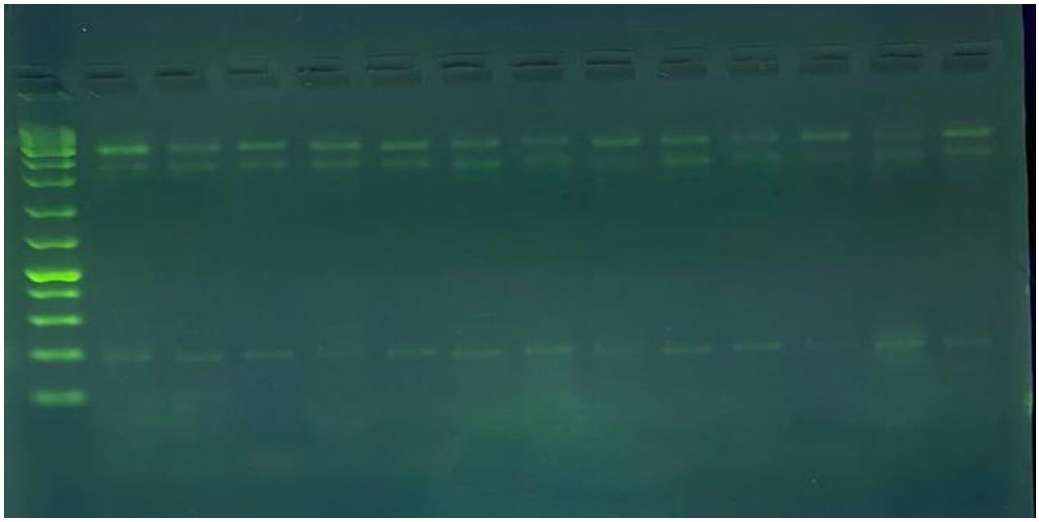


**Figure (3-6): Gelelectophoresis for extracted RNA with the red dye**

**Table(3-10):concentration and purity of RNA samples using Nano-drop**

| Sample | RNA Con. ng/µl | 260/280 |
|---|---|---|
| **Patients** | | |
| **1** | 12 | 1.40 |
| **2** | 22 | 1.50 |
| **3** | 14.3 | 1.20 |
| **4** | 18 | 2.10 |
| **5** | 15 | 2.40 |
| **6** | 28 | 1.40 |
| **7** | 23 | 1.5 |
| **8** | 31 | 2.1 |
| **9** | 23 | 1.3 |
| **10** | 23 | 1.5 |
| **11** | 11.8 | 1.6 |
| **12** | 14 | 1.7 |
| **13** | 8.1 | 1.6 |

| | | |
|---|---|---|
| **14** | 23 | 1.7 |
| **15** | 7 | 1.6 |
| **16** | 13 | 1.5 |
| **17** | 21 | 1.6 |
| **18** | 14 | 1.7 |
| **19** | 30 | 1.7 |
| **20** | 10 | 2.1 |
| **21** | 7.1 | 1.46 |
| **22** | 23 | 2.04 |
| **23** | 7.6 | 1.8 |
| **24** | 9 | 1.69 |
| **25** | 7.5 | 1.94 |
| **26** | 8.2 | 1.9 |
| **27** | 16 | 1.6 |
| **28** | 11.5 | 1.41 |
| **29** | 68.8 | 1.74 |
| **30** | 9.8 | 1.28 |
| **31** | 33 | 1.3 |
| **32** | 7.6 | 1.60 |
| **33** | 20.2 | 1.70 |
| **34** | 22 | 1.70 |
| **35** | 24 | 1.80 |
| **36** | 13 | 1.60 |
| **37** | 13 | 1.30 |
| **38** | 22 | 1.90 |
| **39** | 11 | 1.70 |
| **40** | 12 | 1.40 |
| **41** | 7 | 0.84 |
| **42** | 10.2 | 0.8 |
| **43** | 7.6 | 1.63 |
| **44** | 8.1 | 0.94 |
| **45** | 8.5 | 1.09 |
| **46** | 8.9 | 1.1 |
| **47** | 16 | 0.79 |

| | | |
|---|---|---|
| **48** | 23 | 0.85 |
| **49** | 25 | 0.84 |
| **50** | 7 | 1.5 |
| **51** | 7.1 | 1.4 |
| **52** | 10.3 | 1.77 |
| **53** | 7.7 | 1.1 7 |
| **54** | 14.2 | 1.2 2 |
| **55** | 12.9 | 2.09 |
| **56** | 18 | 1.44 |
| **57** | 8.5 | 1.52 |
| **58** | 12.5 | 1.91 |
| **59** | 24 | 1.86 |
| **60** | 9.8 | 2.01 |
| **Control** | | |
| **61** | 9.32 | 1.8 |
| **62** | 27.1 | 1.67 |
| **63** | 78.8 | 1.34 |
| **64** | 89.8 | 0.8 |
| **65** | 64 | 0.6 |
| **66** | 7.5 | 2.03 |
| **67** | 11 | 1.89 |
| **68** | 13.7 | 1.1 |
| **69** | 15.1 | 1.93 |
| **70** | 19.4 | 1.9 |
| **71** | 15 | 1.75 |
| **72** | 68 | 1.95 |
| **73** | 27 | 2.01 |
| **74** | 34.6 | 1.94 |
| **75** | 25.7 | 1.83 |
| **76** | 11.6 | 1.6 1 |
| **77** | 21 | 1.7 5 |
| **78** | 31 | 1.9 |
| **79** | 7.7 | 1.73 |
| **80** | 8.3 | 1.5 |

Complementary DNA (cDNA) was synthesized with oligo (dT) primer through a process called reverse transcription with 1 µg of RNA.

### *3.7.2. Foxp3 gene expression :*

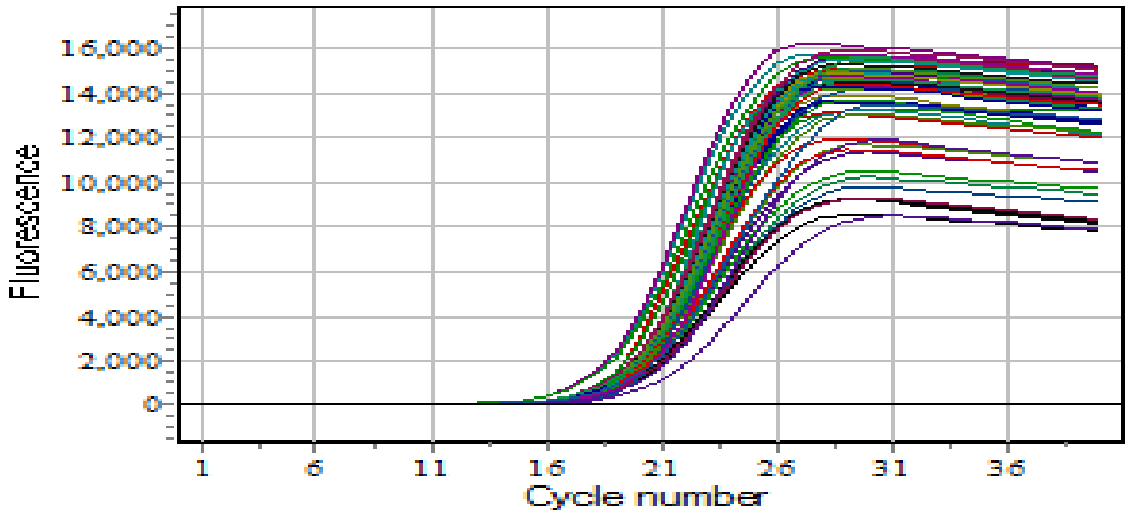
The relative expression of the FoxP3 gene in volunteer PBMCs samples was estimated depending on Livak Method (2^-ΔΔCT) which is based on the normalization of RT-qPCR (CT values) of tested genes with (GAPDH) as reference gene in control and treatment groups, table (3-11), figure (3-7) and (3-8) for gene expression of reference and target genes. The results of relative gene expression in the FoxP3 gene showed that the median concentration of Foxp3 gene expression level were significantly highly increased in (EEP+Ag) group 4.80 (2.78) as compared to control, Ag, and WEP+Ag groups, 2.25 (1.83), 1.35 (2.30), and 3.05 (2.32) respectively, and also the median concentration level of Foxp3 gene expression was significantly increased in WEP+Ag in compared to control and Ag groups. Finally, the median concentration of Foxp3 gene expression was significantly decreased in Ag versus the control group,($P \leq 0.05$). Table (3-12)

Also the results of present study indicated non-significant association between mean concentration of Foxp3 gene expression and the gender. Table (3-13). This study's findings agreed with those of (Benson *et al*.,2012), who found that acute responses to bacterial Ag resulted in a transient decrease in the frequency and an absolute number of Treg cells as well as Foxp3 gene expression. *Pseudomonas aeruginosa* Ag caused a partial loss of Treg cells, which are required for the initiation of a potent Th1 response and host defense against this pathogen because the increasing of Treg cells in such case resulted in a greatly enhanced susceptibility to this pathogen.

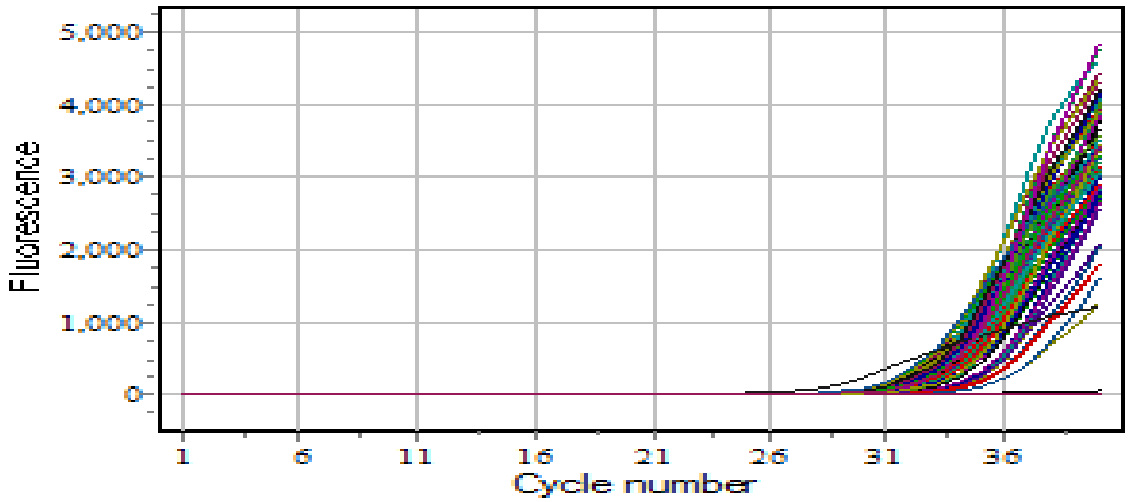
## Table (3-11): Relative Foxp3 gene expression and folding

| sample | Average CT TARGET GENE | Average CT REFERENCE GENE | ΔcT | ΔΔcT | Folding 2^-ΔΔCt |
|---|---|---|---|---|---|
| **Control 1** | 32.4 | 17.3 | 15.10 | -0.86 | 1.8 |
| **Control 2** | 32.9 | 17.5 | 15.40 | -0.56 | 1.5 |
| **Control 3** | 32.8 | 17.8 | 15.00 | -0.96 | 1.9 |
| **Control 4** | 32.6 | 17.6 | 15.00 | -0.96 | 1.9 |
| **Control 5** | 33 | 19.3 | 13.70 | -2.26 | 4.8 |
| **Control 6** | 32.8 | 17.5 | 15.30 | -0.66 | 1.6 |
| **Control 7** | 31.8 | 17.6 | 14.20 | -1.76 | 3.4 |
| **Control 8** | 32.3 | 18 | 14.30 | -1.66 | 3.2 |
| **Control 9** | 31.3 | 17.5 | 13.80 | -2.16 | 4.5 |
| **Control 10** | 32 | 17.5 | 14.50 | -1.46 | 2.8 |
| **Control 11** | 33 | 17.6 | 15.40 | -0.56 | 1.5 |
| **Control 12** | 31.8 | 17.8 | 14.00 | -1.96 | 3.9 |
| **Control 13** | 32.7 | 18.1 | 14.60 | -1.36 | 2.6 |
| **Control 14** | 33.3 | 17.6 | 15.70 | -0.26 | 1.2 |
| **Control 15** | 31.2 | 17.6 | 13.60 | -2.36 | 5.1 |
| **Control 16** | 33.4 | 17.5 | 15.90 | -0.06 | 1.0 |
| **Control 17** | 33.1 | 17.6 | 15.50 | -0.46 | 1.4 |
| **Control 18** | 31.1 | 17.9 | 13.20 | -2.76 | 6.8 |
| **Control 19** | 33.2 | 18 | 15.20 | -0.76 | 1.7 |
| **Control 20** | 32.5 | 18.2 | 14.30 | -1.66 | 3.2 |
| **Ag 1** | 35 | 18.1 | 16.90 | 0.94 | 0.5 |
| **Ag 2** | 33 | 16.9 | 16.10 | 0.14 | 0.9 |
| **Ag 3** | 31 | 17.4 | 13.60 | -2.36 | 5.1 |
| **Ag 4** | 33 | 18.6 | 14.40 | -1.56 | 2.9 |
| **Ag 5** | 35.6 | 18.1 | 17.50 | 1.54 | 0.3 |
| **Ag 6** | 36.3 | 18.8 | 17.50 | 1.54 | 0.3 |
| **Ag 7** | 31.5 | 17.6 | 13.90 | -2.06 | 4.2 |
| **Ag 8** | 31.6 | 17.5 | 14.10 | -1.86 | 3.6 |
| **Ag 9** | 36.1 | 16.6 | 19.50 | 3.54 | 0.1 |
| **Ag 10** | 36.6 | 18.4 | 18.20 | 2.24 | 0.2 |
| **Ag 11** | 33.1 | 18.3 | 14.80 | -1.16 | 2.2 |
| **Ag 12** | 33.2 | 17.4 | 15.80 | -0.16 | 1.1 |
| **Ag 13** | 33.1 | 17.5 | 15.60 | -0.36 | 1.3 |
| **Ag 14** | 34.1 | 18.6 | 15.50 | -0.46 | 1.4 |
| **Ag 15** | 33 | 17.5 | 15.50 | -0.46 | 1.4 |
| **Ag 16** | 31.5 | 17.5 | 14.00 | -1.96 | 3.9 |
| **Ag 17** | 32.8 | 17.6 | 15.20 | -0.76 | 1.7 |
| **Ag 18** | 33.1 | 17.5 | 15.60 | -0.36 | 1.3 |

| | | | | | |
|---|---|---|---|---|---|
| **Ag 19** | 31.9 | 17.5 | 14.40 | -1.56 | 2.9 |
| **Ag 20** | 33 | 17.4 | 15.60 | -0.36 | 1.3 |
| **EEP + Ag 1** | 32.1 | 18.1 | 14.00 | -1.96 | 3.9 |
| **EEP + Ag 2** | 32 | 18.7 | 13.30 | -2.66 | 6.3 |
| **EEP + Ag 3** | 31.9 | 18.9 | 13.00 | -2.96 | 7.8 |
| **EEP + Ag 4** | 31.6 | 18.4 | 13.20 | -2.76 | 6.8 |
| **EEP + Ag 5** | 30.9 | 17.3 | 13.60 | -2.36 | 5.1 |
| **EEP + Ag 6** | 31 | 17.6 | 13.40 | -2.56 | 5.9 |
| **EEP + Ag 7** | 30.4 | 17.1 | 13.30 | -2.66 | 6.3 |
| **EEP + Ag 8** | 31.4 | 17.5 | 13.90 | -2.06 | 4.2 |
| **EEP + Ag 9** | 31.6 | 17.7 | 13.90 | -2.06 | 4.2 |
| **EEP+ Ag 10** | 31.9 | 17.2 | 14.70 | -1.26 | 2.4 |
| **EEP+ Ag 11** | 31 | 17.3 | 13.70 | -2.26 | 4.8 |
| **EEP+ Ag 12** | 30.4 | 17.2 | 13.20 | -2.76 | 6.8 |
| **EEP+ Ag 13** | 30.5 | 17.5 | 13.00 | -2.96 | 7.8 |
| **EEP+ Ag 14** | 30.6 | 17.6 | 13.00 | -2.96 | 7.8 |
| **EEP+ Ag 15** | 30.9 | 16.9 | 14.00 | -1.96 | 3.9 |
| **EEP+ Ag 16** | 31 | 17.1 | 13.90 | -2.06 | 4.2 |
| **EEP+ Ag 17** | 31.3 | 17.6 | 13.70 | -2.26 | 4.8 |
| **EEP+ Ag 18** | 31.5 | 17.4 | 14.10 | -1.86 | 3.6 |
| **EEP+ Ag 19** | 31.9 | 17.5 | 14.40 | -1.56 | 2.9 |
| **EEP+ Ag 20** | 31.7 | 17.6 | 14.10 | -1.86 | 3.6 |
| **WEP+Ag 1** | 33 | 17.7 | 15.30 | -0.66 | 1.6 |
| **WEP+Ag 2** | 32.8 | 18.3 | 14.50 | -1.46 | 2.8 |
| **WEP+Ag 3** | 32.6 | 17.9 | 14.70 | -1.26 | 2.4 |
| **WEP+Ag 4** | 31.9 | 17.6 | 14.30 | -1.66 | 3.2 |
| **WEP+Ag 5** | 32.4 | 18.8 | 13.60 | -2.36 | 5.1 |
| **WEP+Ag 6** | 32.1 | 18.4 | 13.70 | -2.26 | 4.8 |
| **WEP+Ag 7** | 30 | 17 | 13.00 | -2.96 | 7.8 |
| **WEP+Ag 8** | 32.5 | 17.4 | 15.10 | -0.86 | 1.8 |
| **WEP+Ag 9** | 33.4 | 19 | 14.40 | -1.56 | 2.9 |
| **WEP+Ag 10** | 32.9 | 19.6 | 13.30 | -2.66 | 6.3 |
| **WEP+Ag 11** | 31.1 | 17.9 | 13.20 | -2.76 | 6.8 |
| **WEP+Ag 12** | 31.5 | 17.7 | 13.80 | -2.16 | 4.5 |
| **WEP+Ag 12** | 31.6 | 16.9 | 14.70 | -1.26 | 2.4 |
| **WEP+Ag 14** | 31.8 | 16.8 | 15.00 | -0.96 | 1.9 |
| **WEP+Ag 15** | 32.3 | 17.7 | 14.60 | -1.36 | 2.6 |
| **WEP+Ag 16** | 32 | 18 | 14.00 | -1.96 | 3.9 |
| **WEP+Ag 17** | 33.1 | 18.3 | 14.80 | -1.16 | 2.2 |
| **WEP+Ag 18** | 32.9 | 18.8 | 14.10 | -1.86 | 3.6 |
| **WEP+Ag 19** | 32.7 | 18 | 14.70 | -1.26 | 2.4 |
| **WEP+Ag 20** | 31.9 | 17.9 | 14.00 | -1.96 | 3.9 |




**Figure(3-7) Standard curve of reference gene expression (qRT-PCR).**




**Figure(3-8) Standard curve of FOXP3 gene expression (qRT-PCR).**

## Table ( 3-12): Foxp3 gene expression in testing groups

| | *Study groups* | | | | Total P value |
|---|---|---|---|---|---|
| **Foxp3 gene expression** | ***Ag n = 20*** | ***EEP + Ag n=20*** | ***WEP+ Ag n=20*** | ***Control n = 20*** | |
| **Range** | 0-1– 5..10 | 2.40-7.80 | 1.60-7.80 | 1.00-6.8 | **< 0.001 † HS** |
| **Median (IQR)** | **1.35(2.30)** | **4.80(2.78)** | **3.05(2.32)** | **2.25(1.83)** | |
| **P1 C/Ag** | **0.026 S** | | | | |
| **P2 C/EEP** | **< 0.001 HS** | | | | |
| **P3 C/WEP** | **0.74 NS** | | | | |
| **P4 Ag/EEP** | **< 0.001 HS** | | | | |
| **P5 Ag/WEP** | **0.001 S** | | | | |
| **P6 EEP/WEP** | **0.006 S** | | | | |

IQR: inter-quartile range; †: Kruskal-Wallis test; ¥: HS: Highly significant at P ≤ 0.001; NS: not significant at P ≤ 0.05; P1: Control vs Ag; P2: Control vs EEP; P3: control vs WEP; P4: Ag vs EEP; P5: Ag vs WEP; P6: EEP VS WEP .

Forkhead box P3 Protein (Foxp3) is a regulatory T cell transcription factor that plays an important function in the body's immune system balance. Foxp3+ regulatory T (Treg) cells have pleiotropic immune-regulatory functions that are important for immunological homeostasis, autoimmunity prevention and the regulation of pathogen-induced inflammatory responses. The transcription factor Foxp3 controls Treg cell development, differentiation and function, T-cell receptor (TCR) signaling plays central roles in Treg differentiation and Foxp3-mediated gene regulation. Differentiating Treg will have recognized their cognate antigens and received TCR signals before initiating Foxp3 transcription,

which is triggered by TCR-induced transcription factors including NFAT, AP-1 and NF-κB.

**Table ( 3-13): Relation between Foxp3 gene expression levels and gender**

| *Gene expression* | *Male* | *Female* | *P* |
|---|---|---|---|
| **Control** | | | |
| **Mean± SD** | **2.84** ± 1.92 | **2.74** ± 2.35 | **0.891 † NS** |
| **Range** | 1.00 - 6.80 | 1.50 - 4.80 | |
| **Ag** | | | |
| **Mean± SD** | **1.81** ± 1.93 | **1.85** ± 0.92 | **0.953 † NS** |
| **Range** | 0.10 - 5.10 | 1.10 - 3.90 | |
| **EEP** | | | |
| **Mean± SD** | **5.29** ± 1.62 | **5.02 ± 1.8** | **0.729 † NS** |
| **Range** | 2.40 - 7.80 | 2.90 -7.80 | |
| **WEP** | | | |
| **Mean± SD** | **3.87** ± 2.05 | **3.42** ± 1.47 | **0.580 † NS** |
| **Range** | 1.60-7.80 | 1.90 - 6.80 | |

† : Independent Samples Test; NS: not significant at P > 0.05;

Once expressed, Foxp3 seizes TCR signal induced transcriptional and epigenetic mechanisms through interacting with AML1/Runx1 and NFAT. Thus, Foxp3 modifies gene expression dynamics of TCR induced genes, which constitute cardinal mechanisms for Treg-mediated immune suppression.

The deficiency in the Foxp3 gene results in hyper-activation of CD4+ T cells, overproduction of pro-inflammatory cytokines, and massive multi-organ pathology. Regulatory T lymphocytes, (Tregs) that express, FOXP3 are involved in the beneficial attenuation of immune-pathology but are also implicated in the down-regulation of protective

responses to infection (Sanz-Rubio *et al*.,2020). FOXP3 has been shown to have a direct role in inducing immune-suppression and has been identified as a good marker for cells with a suppressor function. In humans these cells were first thought to be specifically CD4+ CD25 high naturally occurring Tregs, but more recent studies have shown this not to be the case and FOXP3 is also expressed in other cells (such as CD8+) with a suppressor function (Morgan *et al*.,2005).

Via Foxp3 induction, TGF-1β priming was able to promote Treg cell differentiation from non-regulatory CD4+CD25–Tcells in a concentration-dependent manner. Following TCR activation, T cells never showed any regulatory functions or major Foxp3 expression.

Freshly isolated CD4+CD25–CD45RBLOW cells, on the other hand, were unable to suppress CD4+ effector T cell proliferation while expressing low levels of Foxp3 mRNA and protein, but acquired regulatory activity and de novo Foxp3 expression after TGF-1 exposure. TGF-β1 can also induce the conversion of CD4+ T cells into Treg cells by inducing FOXP3 expression (Zhu *et al*.,2020), as well as increasing the expression of other important Treg cell markers including CD25, CD122, IL-2 and CTLA-4 (Zheng *et al*., 2014).In autoimmune diseases, in vivo Treg expansion appears to be a go od therapeutic option, and several studies have shown that treatments like IL-2 administration are successful (Sakaguchi *et al*., 2020). As a result finding compounds, of natural resources that modulate Treg function is critical for preventing autoimmune or pathogen induced inflammatory diseases.

Propolis is a natural product made by honey bees from various resinous plant secretions such as gums and resins, as well as from the

leaf buds of certain plants (Anjum *et al.*, 2019). The ethanolic extracts of green propolis are primarily composed of cinnamic acid derivatives, flavonoids, and caffeoylquinic acid derivatives (Tani *et al*., 2019).

In this study, the results have shown that the ethanolic extract of propolis has a significantly high immunomodulation effect on Foxp3 mRNA gene expression on PBMCs stimulated with bacterial antigen by increasing its level after 48 hours of post *pseudomonas aeruginosa* Ag induction in comparison to PBMCs treated with bacterial Ag only. Propolis increased Foxp3 expression in Tregs and promoted their expansion and activation. Propolis and its constituents have the potential to activate Tregs via Foxp3 expression. Furthermore, these findings would encourage the development of prevention and therapy for inflammation or immune diseases as well as pathogen-induced inflammatory responses with a focus on Treg expansion.

During bacterial infection, Foxp3 expression is needed to restore immune homeostasis. The capacity of honey propolis to increase TGF-β is investigated as a possible mechanism for honey propolis to affect Foxp3 (Kassim *et al*.,2012).

FOXP3 is the most accurate specific molecular marker of natural Tregs (nTregs) and is linked to CD4+CD25+ Tregs' immunosuppressive role. The expression of FOXP3 is required for the development and function of CD4+CD25+ Tregs. Tregs in the periphery, specifically CD4+CD25+ FOXP3+ Tregs, tightly regulate auto-reactive, B and T cell responses. FOXP3+ Tregs are the most well-known type of immune cell with the most powerful inhibitory mechanism and the broadest range of inhibitory targets. FOXP3+ Tregs avoid auto-reactive T cell activation, reduce the

incidence of autoimmune and allergic diseases, and have anti-inflammatory properties (Agarwal *et al*.,2014).

Furthermore, down regulation of FOXP3 expression can impair Tregs' ability to protect against infection and tumors (Khanand Ghazanfar, 2018). propolis and artepillin C have the potential as Treg activators via TNFR2 expression and may be useful for the prevention and/or therapy of autoimmune or inflammatory diseases. Tregs express the master transcription factor Foxp3 and upregulate the expression of CD25 and CTLA-4 on their surfaces in order to suppress the activation of effector CD4 T cells in a cell contact-dependent manner (Kelepouri *et al.*,2018).

Regarding the correlation between immunological markers and Foxp3 gene expression level in males the results have shown a significant positive correlation between Foxp3 gene expression and IFN-γ level in WEP stimulated group ($P<0.003$),while the correlation regarding this cytokine and Foxp3 gene expression in the other three groups were non-significant ($P>0.05$), also the result showed a significant negative correlation between IL-23 level and Foxp3 gene expression in Ag only stimulated group ($P<0.039$), and also a significant negative correlation between IL-23 level and Foxp3 gene expression in EEP stimulated group,($P<0.009$). while the results for WEP and Control groups were showed a non-significant correlation ($P>0.05$).Table(3-12).

Regarding IL-17A and Foxp3 gene expression the results of correlation were non-significant for all four groups ($P > 0.05$) as shown in table (3-14).

**Table(3-14):Correlations between the gene expression and the immunological markers levels in males**

| Characteristic | | *IFN-γ level* | | *IL-17a level* | | *IL-23 level* | |
|---|---|---|---|---|---|---|---|
| | | ***R*** | ***P*** | ***R*** | ***P*** | ***r*** | ***P*** |
| **Control** | **Gene expressions** | 0.487 | 0.268 | 0.304 | 0.394 | -0.230 | 0.523 |
| **Ag** | | -0.074 | 0.840 | -0.289 | 0.266 | -0.657 | 0.039 |
| **EEP** | | -0.012 | 0.973 | 0.090 | 0.848 | -0.768 | 0.009 |
| **WEP** | | -0.929 | 0.003 | 0.107 | 0.819 | -0.750 | 0.052 |

*r*: Spearman correlation coefficient

Regarding the correlation between immunological markers and the Foxp3 gene expression level in females, The correlation results for IFN-γ, and IL-23 with Foxp3 were a non-significant(P>0.05), while for IL-17A only EEP group shown a negative correlation between this cytokine and Foxp3 gene expression level(P<0.05), while the correlations for other three groups were a non-significant. Table(3-15).

**Table(3-15):Correlations between the gene expression and the immunological markers levels in female**s

| Characteristic | | *IFN-γ level* | | *IL-17a level* | | *IL-23 level* | |
|---|---|---|---|---|---|---|---|
| | | ***r*** | ***P*** | ***R*** | ***P*** | ***r*** | ***P*** |
| **Control** | **Gene expressions** | -0.393 | 0.383 | 0.377 | 0.283 | 0.394 | 0.260 |
| **Ag** | | 0.342 | 0.452 | -0.197 | 0.586 | - 0.455 | 0.186 |
| **EEP** | | -0.418 | 0.350 | -0.798 | 0.006 | -0.373 | 0.288 |
| **WEP** | | -0.571 | 0.180 | 0.043 | 0.907 | -0.305 | 0.392 |

*r*: Spearman correlation coefficient

Tregs with transcription factor Foxp3 play a pivotal role in controlling immune response mediated inflammation, the expression of Foxp3 mRNA was found to be significantly decreased in PBMCs stimulated with Ag as compared with healthy controls, while the level of IFN-γ in this group was highly increased in comparison to control group. IFN-γ is endowed with the exquisite ability to promote rapid acidification of phagolysosomes within infected macrophages. This low pH within the phagolysosome ameliorates RNS production and this cooperation leads to the elimination of the pathogen (Kak *et al*.,2018).

It had been discovered that only IFN-γ and TGF-β but not other Th1 and Th2 cytokines could characteristically induce the expression of Foxp3 in $CD4^{+}CD25^{-}$ T cells . This initial finding prompted to address the potential role of IFN-γ in the induction of $CD4^{+}CD25^{+}$ Treg cells. The $CD4^{+}CD25^{+}$ Treg network has been recognized recently as an important regulatory mechanism that keeps autoreactive T cells in check, IFN-γ plays an essential role in the self-regulatory mechanisms of the immune system in response to acute inflammation through the induction of transcription factor Foxp3 (Sumantri *et al*.,2020).

This decreae in IFN-γ level in propolis stimulated groups was related to an immunomodulatory effect of propolis on TH-1 cytokines which in turn reduce the ability of CD4+Foxp3+ regulatory T (Treg) cells to produce the proinflammatory cytokine IFN-γ. At the same time propolis up-regulates Foxp3 expression, can regulate inflammatory responses and undisputedly play a central role in protecting tissues from immune damage during chronic MDR-bacterial infections.

Also, the negative correlation between Foxp3 gene expression and IL-23 in Ag stimulated group was indicated that the Foxp3 Treg+ cells

responses are dysregulated during *pseudomonas aeruginosa* infection by shifting toward a TH2-TH17 dominated immune response. Th17 cells, their key cytokine IL-17, and IL-23 seem to play pivotal roles in MDR-*pseudomonas aeruginosa* infection thus increasing IL-23 production in Ag stimulated group. Th17 cell differentiation is induced by STAT3 and retinoid acid related-orphan nuclear receptor γt (RORγt) that work synergistically with one another (Yang *et al*.,2014).

The transcription factor forkhead box P3 (FOXP3) is the negative regulator of RORγt and maintains the tolerance of the organism to self-antigens by inducing the differentiation of Tregs via STAT6 and downregulating differentiation of Th17 cells. However, the Treg/Th17 balance is shifted in favor of Th17 in the presence of proinflammatory cytokines like IFN-γ (Kubra and Thomas,2019).

Regarding the correlation between immunological cytokines markers and Foxp3 gene expression in females the significant negative correlation between IL-17A and Foxp3 expression level in EEP stimulated group can be explained by the fact that the ethanol extracted immune-modulatory effects on Tregs leading to increse TGF-β production by these cells that may downregulate Th17 cell responses leading to decrease IL-17A, $CD4^{+}CD25^{+}Foxp3^{+}$ Treg cells negatively control the effector functions of diverse immune cells (Kyung *et al*.,2017).

The propolis mainly ethanolic extract inhibited the cytokines level by immunomodulation of the TH1/TH2 that decreasing the effect of effector Tcells while increasing the effect of Treg cells and increasing gene expression of Foxp3. This appears in the EEP group IL-23 level and its correlation with Foxp3 gene expression in males. These findings may have direct therapeutic implications for patients with *pseudomonas*

*aeruginosa* infection, because the numbers of Treg correlated positively with immue-pathogenesis of this bacterium suggesting that enhancing Treg numbers and\or function may represent a promising approaches to beneficially modulate immune response against MDR-*pseudomonas aeruginosa* infection.

The differences in the correlation between cytokines and Foxp3 gene expression among males and females could be related to the sex hormones effects as mentioned by (Singh and Bischoff, 2021) who provide evidence that sex hormones and gender influence both the number and phenotype of Tregs and the Treg expression of FoxP3 differentially in healthy men and women.

# Conclusions:

The results of the present study lead to the following conclusions:

1- The crude propolis could significantly modulate the immune response against MDR-*pseudomonas aeruginosa* induced immune-pathogenesis.
2- Propolis significantly decrease the concentration of (IFN-ɣ, IL-17A, and IL-23) pro-inflammatory cytokines .
3- The propolis extract could potentially increase the Foxp3 mRNA expression in purified blood mononuclear cells.
4- The controlling mechanisms through the regulatory T-cells are very important in managing the MDR-*Pseudomonas aeruginosa* pathogenicity.

# Recommendations

1- Using propolis as immuno-modulator can reducing the immunopathogenesis of MDR-bacterial infections.

2- The potential use of propolis extracts as immunomodulator in therapy against *P. aeruginosa* chronic infection because it is promising due to its biological properties as anti-inflammatory and antioxidant properties and its low toxicity.

3- Studying the immunodulation of propolis against fungal and viral infections in vivo.

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# List of Abbreviations

| Abbreviations | **Full Name** |
|---|---|
| Ag | Antigen |
| AMPs | Anti-microbial Peptides |
| ANOVA | Analysis Of Variance |
| AP | Alternate Pathway |
| APCs | Antigen Presenting Cells |
| ASRs | Antigen Specific Receptors |
| BHI | Brain Heart Infusion |
| CaCO3 | Calcium Carbonate |
| CDC | Center for Disease Control and Prevention |
| CIC | Circulating immune complex |
| CMI | Cell Mediated Immunity |
| COX | Cyclooxygenase |
| CR-PA | Carbapenem resistant Pseudomonas aeruginosa |
| CTC | Cytotoxic T-Cell |
| DC2 | Lymphoied dendritic cell |
| DCs | Dentric Cells |
| DNA | Deoxyribonucleic Acid |
| DTH | Delayed Type Hypersensitivity |
| DW | Distilled Water |
| EEP | Ethanolic Extract Propolis |
| ELISA | Enzyme Linked Immune Sorbent Assay |
| FasL | Fas Ligand |
| FOXP3 | Forkhead box p3 |
| HRP | Horse Radish Peroxidase |
| ICU | Intensive care unit |
| IFN-γ | Interferon Gamma |
| IgG | Immunoglobulin G |
| IgM | Immunoglobulin M |
| IL-17 | Interleukin-17 |
| IL-2 | Interleukin -2 |
| IL-23 | Interleukin-23 |

| | |
|---|---|
| KDa | Kilo Dalton |
| Kgs | Kilograms |
| LAK | Lymphokine Activated Killer Cells |
| LOX | Lipooxygenase |
| LPS | Lipopolysaccharide |
| M.W | Molecular weight |
| MAb | Monoclonal Antibody |
| MAC | Membrane Attach Complex |
| MAMPs | Microbial associated molecular patterns |
| MBL | Mannan binding lectin |
| MDR | Multi-drug resistance |
| Mg/dl | Milligram Per Deciliter |
| MHC | Major Histocompatibility |
| Mm | Millimeter |
| NADPH | Nicotinamide adenine di nucleotide phosphate |
| NF-κB | Nuclear Factor kappa-light-chain activated B-cells |
| Ng/mm | Nanogram Per Milliliter |
| NK | Natural Killer cell |
| NKT | Natural Killer T-Cell |
| NLR | Nod like receptor |
| NO | Nitric Oxide |
| OD | Optical Density |
| OMPs | Outer membrane proteins |
| PAMPs | Pathogen-Associated Molecular Patterns |
| PBMCs | Peripheral blood mononuclear cells |
| PBS | Phosphate Buffer Solution |
| PG | Peptidoglycan |
| Pg/mL | Pigogram Per Milliliter |
| $PGE_2$ | Prostaglandin |
| PRRs | Pathogen Recognition Receptors |
| RNA | Ribonucleic Acid |
| RNS | Reactive nitrogen species |
| ROS | Reactive Oxygen Species |
| SIgA | Secretory Immunoglobulin A |

| | |
|---|---|
| STAT-4 | Signal Transducer and Activator of Transcription |
| T3SS | Type three secretion system |
| TCR | T-Cell Receptors |
| Th-1 | T-helper 1 |
| TLCs | Total Leucocytes Count |
| TLRs | Toll Like Receptors |
| TNF | Tumor necrosis factor |
| TNF-α | Tumor Necrosis Factor Alpha |
| T-reg | T- Regulator |
| WHO | World Health Organization |

# CHAPTER ONE

# Introduction
# and
# Literatures Review

# CHAPTER TWO

# Materials and Methods

# CHAPTER THREE

# Results and Discussion

# Conclusions and Recommendations

# References

## References:


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