JCHR (2021) 11, 143-151

Journal of Chemical Health Risks

www.jchr.org

ORIGINAL ARTICLE

Immunomodulatory Effect of Propolis on IFN-γ, IL-17A and IL-23 Production in Human Peripheral Blood Mononuclear Cells Treated with *Pseudomonas aeruginosa* Ag

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(Received: 30 May 2021 Accepted: 31 August 2021)

KEYWORDS

Antibiotic resistance;
Pseudomonas aeruginosa
immunomodulatory;
Propolis;
IFN-γ;
IL-17A;
IL-23

ABSTRACT: Propolis has many potential effects on the immune response against *P. aeruginosa* both cellular and humoral immunity. Aim of this study is to verify the anti-Pseudomonas property of ethanol and water extracted propolis on PBMCs stimulated with *P. aeruginosa* Ag. In this study a total of (20) apparently healthy volunteers consist of (10) males and (10) females were included, their ages ranged from (20-40) years. Five mL of blood were taken from each included subject for evaluation of IFN-γ, IL-17A and IL-23 in peripheral blood mononuclear cells (PBMCs) that were isolated from blood using density gradient lymphoprep and stimulated with *pseudomonas aeruginosa* LPS in to four groups, LPS stimulated PBMCs, Ethanol extracted propolis EEP+LPS stimulated PBMCs, water extracted propolis WEP+LPS stimulated PBMCs and control (PBMCs) by ELISA technique after 48Hrs. The results showed that both the EEP and WEP could significantly inhibit pro-inflammatory cytokines production by human PBMC after stimulation with pseudomonas Ag. Propolis exhibits an anti-*pseudomonas aeruginosa* with the same effect with ethanol and water extracts.

INTRODUCTION

P. aeruginosa, in general, is a threat to global public health that must be studied and managed with urgency and determination [1]. *P. aeruginosa* is a common pathogen that caused nosocomial and ventilator-associated pneumonia, cystic fibrosis, soft tissue, meningitis, abscess, urinary tract infections, corneal infection, and also conjunctival erythema. Furthermore, in immunocompromised and hospitalized patients, it can cause catheter associated and chronic lung infections [2]. Provided its ability to develop a biofilm on medical equipment and exploit the host's altered normal flora as a result of broad-spectrum antibiotic administration [3].

Due to a lack of therapeutic options, infections caused by these antibiotic-resistant bacteria pose a significant morbidity and mortality risk worldwide. In these infections, inadequate therapy has a significant impact; indeed, the World Health Organization reported in 2017 that carbapenem-resistant *P. aeruginosa* was listed as a "critical" group for which new antibiotics were needed [4].

Plants that contain substances that could be used for therapeutic purposes or that are precursors for the synthesis of useful drugs are considered medicinal plants [5]. Folk medicine is a valuable and underutilized resource for the development of new potential medicines against microbial infections in order to reduce the emergence of resistance and also adverse drug reactions. Furthermore, use of medicinal plants provides the opportunity for developing countries because they may be less expensive, more accessible, and more readily

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DOI: 10.22034/jchr.2021.685425

available [6]. Plants are an unrivaled source of anti-pseudomonal agents. Propolis is an example of a plant compound. Propolis has been used by humans since ancient times to meet the needs of health and food preservation. Because of its broad range of biological and pharmacological properties, this natural product has gained popularity in recent years [7].

Propolis is a resinous material collected by bees from the buds and plant exudates and mixed with salivary gland and wax products [8]. Propolis is composed of approximately 50% resin and vegetable balsam, 30% wax, 10% aromatic oils, 5% pollen, and 5% other substances such as minerals and vitamins [9]. Flavanone, 3-Hydroxyflavone, O-coumaric acid, Chrysin, Quercetin, Caffeic acid, Galangin, Kaempferol, and Ferulic acid were discovered in Iraqi propolis through chemical analysis. Furthermore, propolis has long been used to improve health and prevent diseases such as diabetes, heart disease, atherosclerosis, and cancer. [8]. This study aimed to demonstrate propolis' immune adjuvant activity against MDR-*Pseudomonas aeruginosa in vitro.*

MATERIALS AND METHODS

In this study a total of (20) apparently healthy volunteers consist of (10) males and (10) females were included. Their ages ranged from (20-40) years. The information for each volunteer was noted included: name, sex and age. The blood samples were collected from the volunteers during (December 2019 to April 2020).

Included & excluded criteria

The enrollment standards of volunteers in this study comprised any person who has apparently healthy. The excluded criteria included any person who had infection or disease.

Samples preparation

Propolis sample collected from the hives of honey bees in Karbala City during spring season of 2020. Propolis sample cleaned and made as small pieces and stored inside a clean container to prepare two types of propolis extract, water extracted propolis (WEP) and ethanol extracted propolis

Study design

Case-Control study.

Methods

Sample processing

Ethanolic Extract of Propolis (EEP)

Ethanolic extract of the red propolis (EEP) were obtained using the methodology of Paviani et al. [9].

Water extracted propolis (WEP)

Water extracted propolis was prepared according to the methodology of Contari [10].

Pseudomonas aeruginosa outer membrane isolation

Extraction of pseudomonas (OMPs) was carried out using the procedure of Carlone et al.[11].

Blood samples processing

five ml of blood were defibrinated by putting in anticoagulant tubes contain heparin to isolate the mononuclear cells of peripheral blood from whole blood cells by density gradient medium, the blood samples were handled within one hour after blood drawing to ensure good separation and also high percentage of viability of isolated cells.

Isolation of PBMCs by lymphosep

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood using lymphoprep density gradient medium according to methodology of Boyum [12].

Culturing and Stimulation of PBMCs

After isolation of PBMCs from heparinized blood these cells were re-suspended at a final concentration of ($1 \times 10^{6)}$ cells ml^{-1} in RPMI 1640 complete medium and supplemented with 10% FBS and 5% penicillin and streptomycin and were culturing in 24 well tissue culture plate at 37ºC and 5% CO_2 for 16 hr. then each sample were divided in to four groups, Ag stimulated PBMCs as a positive control, ethanol extracted propolis and Ag

stimulated PBMCs, water extracted propolis and Ag stimulated PBMCs and the negative control group represented by PBMCs only. At each group, to 360 µl of isolated PBMCs in the first three groups 40µl of *pseudomonas aeruginosa* bacterial Ag were added , while 100µl of 5µg ml^{-1} EP was added to the second group and the same volume and concentration of WEP was added to stimulate the third group for 48hrs. at 37ºC and 5% CO_2.

ELISA technique for cytokines estimation

The immunological parameters included (IFN-γ, IL-17A and IL-23) cytokines. The method of these testes has the same procedure depending on the instructions provided with kits of manufacture Company (Elabscience Company).

Statistical analysis

Calculation of the comparative data through **S**oftware of Statistical Package for the Social Sciences (SPSS), version 26.0, to explain the differences of study parameters between the four groups. 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. Kruskal–Wallis (KW) tests were used to compare three or more independent groups where indicated. It is considered a significant difference when P value <0.05.

RESULTS

The IFN- γ concentration results pointed a significant elevation (p< 0.001) in the median value of Ag stimulated PBMCs group 63.40 (7.46) pg ml^{-1} as compared to control, (EEP+ Ag) and (WEP+ Ag) groups, 60.0 (3.99) pg ml^{-1} , 50.0 (6.07) pg ml^{-1} and 52.85 (4.88) pg ml^{-1} respectively, and also shown that the median concentration level of IFN-γ in EEP and WEP stimulated groups had been shown highly decreased median level in IFN- γ in comparison with Ag only stimulated PBMCs group and also this study indicated that the differences between EEP and WEP effects was non-significant,(P=0.08) as in Table 1 and Figure 1.

Table 1. Interferon-γ levels (pg ml^{-1}) in testing groups.

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

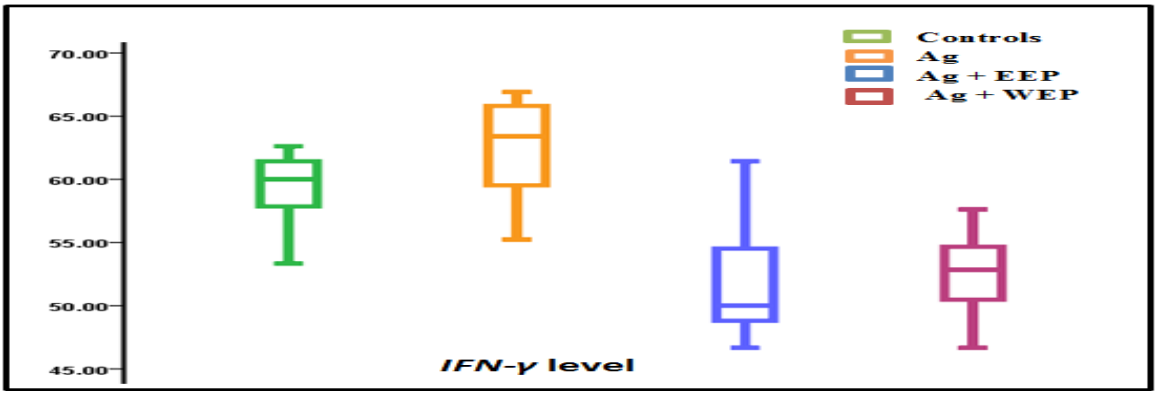

Figure 1. Distribution of groups with Ag, EEP+Ag, WEP+Ag and control groups according to the level of IFN-γ.

Regarding gender, the results of present study indicated non-significant association between median levels of IFN-γ and the gender, although the median level of IFN-γ increased in female compared to male.

Also this study detected that the median concentration of IL-17A was significantly higher in Ag only stimulated group 164.07(25.75) pg ml^{-1} as compared to control, EEP+Ag and WEP +Ag groups,140.23 (24.42) pg ml^{-1}, 104.84 (29.04) pg ml^{-1} and 130.61(51.3) pg ml^{-1} respectively, (p<0.001),and also the median concentration of IL-17A was significantly highly decreased in, EEP+Ag in comparison to Ag only stimulated PBMCs groups,((P<0.001), while non-significant decreasing in WEP+Ag stimulated groups in comparison to Ag only stimulated PBMCs groups(P=0.205). The results also had been shown that there is significant decreasing of (EEP+ Ag) group median of IL-17A level in compared to WEP+Ag group median level (104.48 for EEP vs130.61 for WEP group) at (P<0.003). Table 2 and Figure 2, while the results regarding gender appeared that the level of IL-17A was significantly higher in female control group than male control (P<0.021), and in the other groups, there were no major differences between male and female performance

Table 2. IL-17 a level (pg ml^{-1}) in testing groups.

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

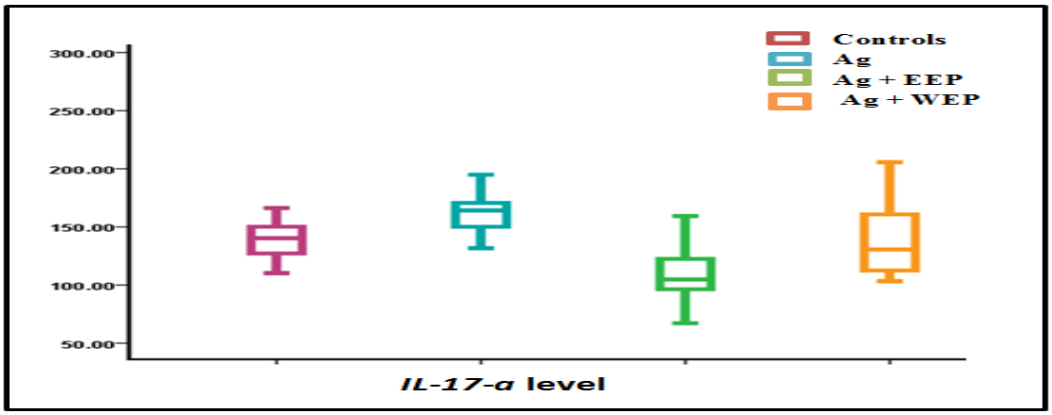

Figure 2. Distribution of groups with Ag, EEP+Ag, WEP+Ag and control according to the level of IL-17A.

The median concentration of IL-23 were significantly higher in Ag only stimulated group 491.82(141.24) pg ml^{-1} as compared to control, EEP+Ag and WEP+Ag groups 318.84(69.22)pg ml^{-1}, 402.842(88.52) pg ml^{-1} and 395.35(125.7) pg ml^{-1} respectively and also show the median concentration level of IL-23 were significantly decreased in EEP+Ag group and WEP+ Ag groups in compared to Ag only stimulated group(P<0.005), Table and Figure 3. Also the results of the present study indicated a significant association between median levels of IL-23 and the gender, were the mean of IL-23 increased in male compared to female in control Ag only, and WEP+Ag groups but non-significant differences between male and female in EEP+Ag groups.

Table 3. IL-23 level (pg ml^{-1}) in testing groups.

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

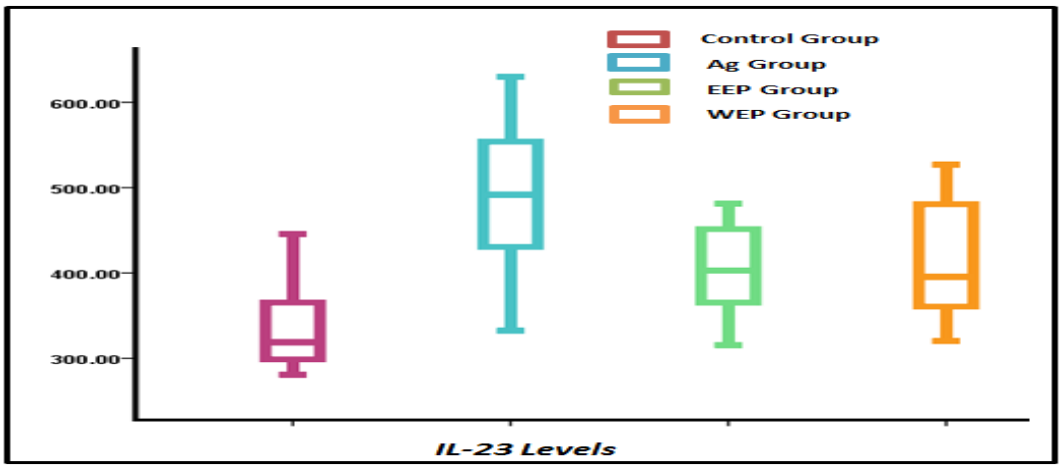

Figure 3. Distribution of groups with Ag, EEP+Ag, WEP+Ag and controls groups according to the level of IL-23.

DISCUSSION

Pseudomonas aeruginosa is known to be responsible for developing progressive diseases in immune-competent and immune-compromised patients. Lipopolysaccharides (LPS) on the outer membrane of *Pseudomonas aeruginosa* have the key function in stimulating the host's innate and adaptive immune response by releasing pro-inflammatory mediators through the NF-κB pathway. As a response to infection, the immune system will undergo an inflammatory processes [13]. There are many cellular and biochemical processes resulting from the tissues damage caused by these infection processes. There was a release of inflammatory mediators, such as pro inflammatory cytokines from peripheral blood mononuclear cells. These inflammatory mediators were assumed to be a two-edged sword, serving to fight infection while simultaneously causing damage to their own tissues. Previous research has demonstrated that propolis components have a direct regulatory effect on fundamental immune cell functions, particularly the production of proinflammatory cytokines by TH1 and TH2 cells [14]. For centuries, propolis has been utilized in traditional medicine. Propolis extracts contain phenolic chemicals that have been demonstrated to have immunomodulatory, anti-inflammatory, and anti-tumor properties. Propolis' anti-inflammatory properties have been attributed to a variety of mechanisms, including inhibition of eicosanoids and nitric oxide generation, antioxidant action, and angiogenesis [15]. The findings showed that the presence of propolis had distinct effects than the absence of propolis; it significantly reduced the levels of interferon-gamma, IL-17A, and IL-23 in propolis extract and Ag stimulated groups as compared to Ag alone, this result was in agreement with Pahlavani et al.,[16] who pointed out that The PBNCs treatment with

propolis reduced the levels of inflammatory cytokines and decreased the expression of inflammatory markers. Propolis extracts had a considerable immunomodulating action on IFN-γ cytokine in this study [17]. IFN-γ is a key player in cellular immunity, since it can coordinate a variety of defensive processes to boost immune responses to infections and malignancies. The induction of NF-κB by LPS was demonstrated to be enhanced considerably by IFN- γ. NFB (nuclear factor kappa B) is also a complex protein that controls DNA transcription. Interleukin 1, IL6, IL8, intercellular adhesion molecule 1, and interferon gamma are all transcription factors regulated by NFB. The NFB transcription factor can be thought of as a regulator of a number of proinflammatory mediators [18] . It's also found in almost all animal cells and is involved in cellular responses to stimuli, including microorganisms. *P.aeruginosa* in bodily tissue acts as an antigen, triggering both cellular and humoral immune responses. The presence of *P.aeruginosa* in body tissue acts as an antigen that triggers cellular and humoral immunologic response. In the presence of a lesion, the immune response is a complicated cellular interaction that triggers the body to preserve homeostasis and, in severe situations, can lead to a pathological state [19] . NF-κB controls the expression of cytokines, and its a ctivation causes an inflammatory response to persist be yond initial stimulation. Some components present in propolis extract such as flavonoids (quercetin, galangin and pinocembrin), caffeic acids, and cinnamic acid are thought to act on the microbial membrane or cell wall site, causing functional and structural damage. Flavonoids and phenolic acid esters are thought to be responsible for propolis' antibacterial properties. Other chemicals contained in propolis, such as steroids and salicylic acid, may have a synergistic effect on the ultimate antibacterial and antiinflammatory actions. CAPE in low concentrations decreases NF‑κB expression, lowering IFN-γ and all other proinflammatory cytokines like IL-17A and IL-23. Propolis also inhibits TNF-α expression mediated by NF-κB, implying that flavonoids and phenolic acids are a significant class of polyphenols and hence the most important class of pharmacologically active chemicals found in propolis and responsible for their biological activities [20-22] . Propolis action on IFN-γ following LPS stimulation plays a vital function in lowering inflammatory mediators such as prostaglandins and leukotrienes by decreasing nitric oxide production from macrophages and reducing (ROS) and (RNS), as well as inhibiting many cytokines. [23] . Overexpression of these mediators induces pathological acute or chronic inflammatory responses [24]. Hence propolis can be utilized as an anti-inflammatory drug in the treatment of chronic infections caused by antibiotic-resistant *pseudomonas aeruginosa.*

Interleukin-17 is a pro-inflammatory cytokine that actively encourages neutrophil recruitment and activation in the respiratory tract when infected with Pseudomonas aeruginosa by inducing the release of chemokines. This cytokine is most commonly found in acute infections caused by Pseudomonas aeruginosa [25] . However, IL-17A is thought to play a role in the pathogenesis of lung diseases like acute respiratory distress syndrome [26] . Previous researches suggested that targeting IL-17A to reduce pulmonary inflammation could be a therapeutic option in *P. aeruginosa* pneumonia. In this study the results indicated significant decreasing of IL-17A cytokines from PBMCs treated with *pseudomonas aeruginosa* LPS. Prior to the induction of IL-17, IL-23 collaborates with IL-1β to promote the recruitment of early neutrophil PMNCs. In response to *P. aeruginosa* infection, IL-23 regulates two distinct phases of neutrophil recruitment: early PMN emigration that is independent of IL-17 and later PMN emigration that is regulated by IL-17. Several studies have previously shown that airway interleukin-23 (IL-23) correlates with clinical findings and inflammation in people with cystic fibrosis (CF) [27, 28] . Under our experimental conditions, propolis extract inhibits IL-17 and IL-23 in a statistically significant way. Both the EEP and WEP propolis groups' mean clinical scores remained lower than those of the Ag and control groups. Artepillin C, a component of propolis extract, is one of the most active ingredients. Artepillin C is also known as a nuclear factor kappa B (NF-kB) inhibitor and has subsequent anti-inflammatory activity [29] . In human T cells, IL-17 expression is partially regulated by NF-kB [30] . Our findings agreed with those of Laerte et al. [31] , who found that propolis significantly inhibited IL-17. The

inhibition of NF-kB by propolis components could be related to the reduction of IL-17 and IL-23 production.

The fact that different propolis extracts (EEP and WEP) have different chemical contents may explain the differences in their effects. Numerous studies have shown that different extracts of propolis have different chemical compositions and biological activities. Finally, in terms of propolis's effect on gender, the different effects are related to hormonal effect mainly progesterone effect in the control group.

CONCLUSIONS

According to the findings of this study, propolis extracts are a potential anti-inflammatory agent that modulates the production of pro-inflammatory cytokines by PBMCs after Pseudomonas aeruginosa infection, and they have aided in modulating the immune response against highly resistant bacteria, primarily in chronic infection, to reduce pathogen-inducing inflammatory response.

ACKNOWLEDGEMENTS

The authors would like to thank department of microbiology- university of Babylon for financial support.

ETHICAL CONSIDERATION

Volunteers were asked permission prior to take any blood specimen. In addition, the study concept was accepted by the Research Ethical Committee at the College of Medicine / University of Babylon.

Conflict of interest

The authors declare no conflict of interest.

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