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Immune functionalization of outer membrane proteins (OMPs) for enteropathogenic E.coli in vivo

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Abstract--This study aimed to reveal the susceptibility of Outer Membrane Proteins (OMPs) to Enteropathogenic E.coli (EPEC) after linking it to an immunosuppressant (from natural sources) to stimulate the immune system of laboratory animals as a primitive attempt to initiate the manufacture of a vaccine against the above bacteria and reduce of the fatalities that occur in children under the age of five when injured Relying on some common methods, outer membrane proteins (OMPs) were separated from EPEC bacteria membrane in an amount of 200 mg / 20 L of culture medium. The molecular weight and purity of this protein were detected using Sodium Dodecyl Sulfate-Polyacrylamide gel (SDS-PAGE), as the outer membrane proteins OMP appeared in the form of a ladder containing successive bundles consisting of (9-13) bundles of polypeptides, whose molecular weight ranges between (15-125) kDa. The ability of EPEC bacteria, its outer membrane proteins (OMPs), the immune helper (olive oil), and the foreign protein complex with the immune helper to induce systemic immunity through changes in the antibody titer averages were studied. A significant increase in the titer of antibodies was observed in the treated mice (IgG and IgA) compared to the positive control group and those that appeared in the animals before treatment. Also, the changes in cellular kinetics were studied, which are interleukin-6 (IL-6), interleukin-8 (IL-8) and interferon-gamma (INF- γ). It was observed that there was a significant increase in the average concentration of the three parameters in the sera of animals inoculated with EPEC bacteria and the outer membrane proteins (OMPs) of the olive oil complex - membrane proteins. It was noticed from the results that there were significant differences at the level of

probability ($P \leq 0.005$) when compared with their concentrations in the control group.

Keywords---enteropathogenic *E. coli*, outer membrane proteins (OMPs), interferon.

Introduction

Enteropathogenic *E. coli* (EPEC) is one of the pathogens that cause the death of many children under five years of age as a result of diarrhea, especially in developing countries, and this bacteria is the basis for the emergence of summer epidemics that spread in infant centers and nurseries. The role of childbirth the infection of this type results in diarrhea of different severity that causes inflammation of the epithelial cells in the intestine, and the degeneration of the villi of the intestinal cells. (Ochoa and co., 2018). EPEC bacteria are characterized by multidrug resistance (MDR) (Laird, 2016) It is characterized by its high resistance to antibiotics due to its possession of resistance enzymes such as β -lactamases (which confer resistance to β -lactams), Enzymes that confer resistance to aminoglycosides, and quinolones. These bacteria also have other mechanisms that give them resistance to antibiotics, such as changing the permeability of the cell membrane, changing the target site, inhibiting protein synthesis, and the bacteria's possession of pumps efflux, It confers bacteria resistance to antibiotics such as groups of Macrolides, Novobiocin and Rifamcin (Kapoor et al., 2017). With these challenges for these bacteria, scientists have not yet succeeded in producing vaccines against EPEC bacteria, and current treatments are limited to antibiotics in addition to some other chemical drugs to strengthen the role of antibiotics, However, this treatment supports the emergence of new strains of colon bacteria that are resistant to antibiotics, in addition to the virulence factors that these bacteria possess (Scaletsky et al, 2012). Some research attempts have begun to work on the production of anti-adhesion agents that would prevent the binding of proteins that help bacteria to bind to the epithelial cells in the intestine, which in turn will help to eliminate bacteria without any harm to the body, and thus, Studies have begun to intensify on studying the complete genome sequence of bacteria, identifying virulence factors, their chemical composition, the possibility of modifying them and using them in developing vaccines against bacterial infection and immunizing the mother with them so that the contraceptives are transmitted to the child and provide protection against infection with the bacteria concerned (Harro). Et al, 2011). One of the strategies used in the manufacture of vaccines is the use of Outer Membrane Proteins (OMPs) to induce an immune response against pathogenic bacteria. The outer membrane proteins exposed on the surface of Gram-negative bacteria are rapidly recognized by the host's immune system as foreign particles, leading to the formation of an immune response against Osman's bacterial pathogens. and Marouf, 2014). Therefore, this study was designed to determine the immune response to EPEC outer membrane proteins after fusing them with a natural immune aid in laboratory animals.

Materials and Methods

Enteropathogenic E.coli

The above-mentioned bacteria were isolated from cases of diarrhea among children attending children's hospitals in Al-Diwaniyah city and diagnosed based on phenotypic and microscopic characteristics and using the biochemical and molecular test mentioned in the approved references (Collee et al. 1996, Macffadin, 2000)

Extraction of outer membrane proteins from EPEC bacteria

The method (Cho et al, 2015) was used to extract outer membrane proteins (OMPs) from EPEC bacteria, as follows:

1. 500 ml of Nutrient broth medium distributed in 5 ml tubes were inoculated with EPEC bacteria isolated from diarrheal cases, and incubated at 37°C for 18 hours.
2. Then the bacterial culture was discarded in a refrigerated centrifuge at a speed of 6000 rpm for 4 minutes.
3. The filtrate was neglected and the precipitate was suspended with 10 microliters of Tris-HCL Buffer twice.
4. The bacterial culture was centrifuged at a speed of 6000 rpm for 4 minutes.
5. The precipitate was collected and lysozyme enzyme was added to it at a concentration of 0.10 µl and 1 ml of EDTA. The precipitate was incubated at 37°C for 24 hours.
6. Sarcosinate was added at a concentration of 1%, then to dissolve the outer membrane proteins, the suspension was incubated at a temperature of 37 °C for two hours.
7. The exposure displayed the Ultrasonic Sonicator 30 times, each time having a duration of 30 seconds with an interval of 10 seconds between each time, and at a frequency of 20 vibrations per second.
8. 0.001 M of magnesium chloride (Mgcl₂) was added to the suspension to inhibit the action of EDTA, and 1 ml of DNase and RNase enzymes at a concentration of 300 µg was added to get rid of nucleic acids. The suspension was incubated at a temperature of 37°C for two hours.
9. Centrifuge at 5000 rpm for 30 minutes at a temperature of 4°C.
10. The sediment was discarded and the filtrate was placed in a centrifuge at 40,000 rpm for 30 minutes at 4°C.
11. The precipitate was suspended in a buffer containing 2 molarity of (Thiourea) and consisting of 2% (3-Cocamidopropyl-dimethylammonium), 1% propane sulfonate, 1% dithiothreitol, 1% pharmaceutically) in addition to 7 molarity of urea.
12. The suspension was exposed to a centrifuge at a speed of 100,000 rpm for 30 minutes, the precipitate was collected and washed with sterile distilled water three times.
13. The dialysis was performed on several exchanges using distilled water at a temperature of 4° C for 72 hours.
14. Centrifuge at a speed of 6000 rpm for an hour at a temperature of 4°C.

15. The filtrate contains outer membrane proteins (OMPs), which were lyophilized using a lyophilizer. The product was kept at -20 temperature until use.

Detection of purity of outer membrane proteins (OMPs) and determination of their molecular weight by SDS-PAGE (Sodium dodecyl sulfate polyacrylamide gel electrophoresis)

Gel migration technique (SDS-PAGE) was used to separate proteins on the basis of their molecular weight and to ensure that they are free from nucleic acids or lipids and other substances that hinder the purity of the extracted protein. The migration is done according to what was stated in Laemmli (1970) as follows:

1. Resolving gel was poured into a gel electrophoresis device until the line appearing on the glass dish, which can be determined by placing the comb before pouring and marking a line below it with a distance of 1 cm.
2. Air bubbles can be removed after pouring by using a layer of isopropanol or distilled water on the surface of the gel up to the level of the gel.
3. Leave the gel to harden 20-30 minutes.
4. Stacking gel is prepared by adding all solutions except APS and TEMED, which are added immediately before pouring. Bromophenol blue dye is also added to distinguish the stacking gel from the separating gel as the two are transparent in nature.
5. The isopropanol is dried using filter paper.
6. Add the Stacking gel, then add the comb into the space between the two slides and leave to solidify for 10 minutes.
7. The protein was mixed with the buffer solution of the sample in a ratio of 4:1. The mixture was heated in a water bath at a temperature of 37°C for 30 minutes.
8. The comb is carefully removed from inside the gel so as not to break the wells.
9. The gel is separated from the casting frame and the two plates or slides are fixed inside the gel device, so that the short plate is inward
10. The two plates are secured and placed inside the holder and closed in a certain way with a clamp. The holder is placed inside the Bio-Rad tank.
11. Fill the space inside the holder with 10X SDS running buffer (pH 8.3) Here the gel is ready for use and sample loading.
12. The sample is withdrawn using the micro-pipette, and then it is placed a few millimeters in the wells.
13. The power source is connected by closing the tank and connecting the positive and negative poles in their correct places. The current is set to 180 volts and left for 1 hour.
14. After the expiry time, the holder is taken out of the separation tank and the gel is removed from between the gel plates and the gel is placed in the kumasi bright dye solution for 30 minutes.

The gel is placed on a vibrator, in the de-staining solution for 8 hours or until the protein bands appear. The molecular weight of the separated proteins is roughly estimated in comparison with the molecular weights of the known proteins (Proteins Marker).

Immunization Program

The immunization program for this study was carried out according to Hamid and Jain (2008), as 25 white mice of 9-10 weeks of age and weight (20-22) g were bred, divided into five groups and the program was implemented as follows:

1. 2 ml of blood was withdrawn from each animal using a heart puncture method before immunization and placed in clean and sterile tubes that do not contain anticoagulant. The samples were centrifuged at 3000 rpm for 5 minutes, then the serum was separated using special pipettes (Micropipettes.) and placed in special collection tubes and kept in freezing until used in the estimation of immunoglobulins (IgG and (IgA) and cytokines (IL-6, IL-8 and IFN- γ).
2. The first group: the animals were injected peritoneally with 1 ml of (1×10^6 cells/ml) of EPEC bacteria.
3. The second group: the animals were injected into the peritoneum membrane with 1 ml of (50 μ g/ml) of the outer membrane proteins (OMPs).
4. The third group: the animals were injected into the peritoneum with 1 ml of pure olive oil
5. The fourth group: Injected in its peritoneum with 1 ml of mixture of membrane proteins (OMPs) with olive oil (0.5 ml of 50 μ g/ml of OMPs with 0.5 ml of pure olive oil).
6. Fifth group: the animals were injected in the same methods with 1 ml of physiological saline solution.

The program was implemented on days 0, 7, 21 and 28, then a dose was given on day 40 and on day 50 blood was drawn using the same method mentioned in point 1 to study the immune response by calculating systemic antibody concentrations (IgA and IgG) and cellular kinetics (IL-6, IL-8 and INF- γ).

Immune function tests

Determination of the concentration of specific antibodies (IgG and IgA) specific to EPEC bacteria

The ELISA technique was used to estimate the concentration of the above antibodies. The concentration of cellular kinetics (IL-6, IL-8 and (TNF- α) was also measured according to the manufacturer in animals immunized with EPEC bacteria antigens, outer membrane proteins (OMPs), olive oil and membrane protein complex with olive oil.

Results and Discussion

Bioseparation of outer membrane proteins (OMP) of EPEC

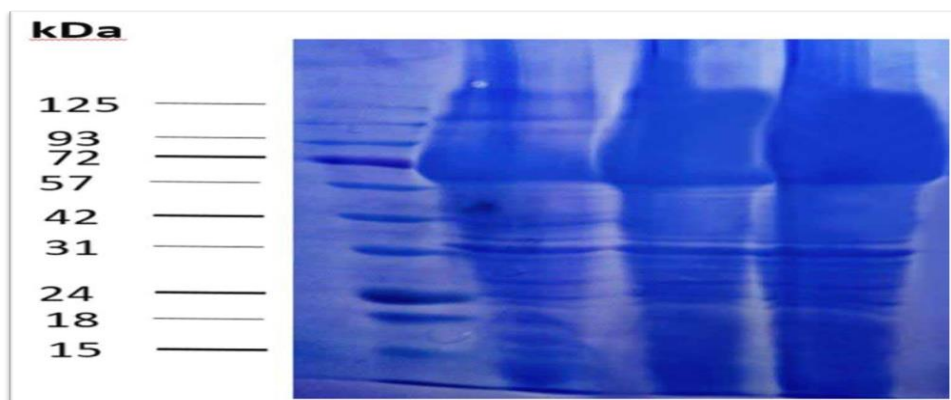
One isolate of pathogenic Escherichia coli (EPEC) was used for the purpose of isolating outer membrane proteins, This isolate was grown under standard conditions in Nutrient Broth liquid medium with PH equal to 7.2 and incubated for 24 hours at 37°C, as approximately 200 mg/20 liters of culture medium were obtained. This quantity depends on the efficiency of the isolation and the

conditions used in the insulation. Many methods have been used to extract membrane proteins, but not all of them give good quality proteins. The reason for the failure of some methods is due to the nature of the outer membrane proteins, which bind strongly with the layer of lipopolysaccharides. Which requires high accuracy to separate it from the outer membrane protein, as the addition of some substances can lead to disengagement between the two layers and at the same time a loss of the basic structure of outer membrane proteins. Therefore, extracting bacterial membrane proteins requires following the correct scientific methods, and the minute to obtain pure proteins gives accurate results when used in subsequent tests and to ensure the elimination of all other interferences such as glycolipids, phospholipids, and nucleic acids, which certainly affect the other tests planned in the study, (Hitchcock and Morrison 1984).As for the results obtained in this study, it became clear that the Laemmli method (1970) is a simplified method that requires and needs careful handling of the additives, but it gave OMP membrane proteins characterized by high purity and good quantity, which ensures its reliability in giving accurate results in subsequent tests.The outer membrane protein (OMP) family of Gram-negative bacteria includes proteins associated with essential physiological functions, virulence and multidrug resistance, and thus play an essential role in cell maintenance. These proteins are in the form of channels of porins that are selectively permeable to allow the passage of substances into and out of the cell,Many studies focused on understanding the nature of these proteins and the possibility of modifying the nature of the antibiotic resistance displayed by these proteins, especially in Salmonella typhi bacteria, as they were modified in different ways such as dilution or modification and were used to stimulate immunity against bacterial infections (Hamdi, 2001).

SDS-Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel (Coomassie brilliant blue R-250) was used to detect the molecular weight of the extracted outer membrane protein OMP and determine its purity. The method of dyeing with this dye is considered one of the sensitive methods in detecting protein, even if it is in the amount of one nanogram. The above dye is routinely used to stain pure proteins, where the protein bands appear colored and visible, as the outer membrane proteins OMP appeared in the form of a ladder containing successive bands consisting of (9-13) bundles of multiple peptides, whose molecular weight ranges between (15-125) Kilodaltons (Picture 1).As it appears in the picture, OMP proteins do not contain LPS, nucleic acids, DNA and RNA, where they were eliminated using nucleases enzymes, which were later eliminated, as these sequential treatments gave our protein pure. Proteins are separated using sodium dodecyl sulfate (SDS) gel electrophoresis based on isoelectric point (pI), molecular weight, electric charge, or a combination of these factors. The nature of the separation depends on the method of processing the sample and the nature of the gel used to date, this is the most common method for separating proteins and determining their purity, using polyacrylamide gels and solutions containing sodium dodecyl sulfate (SDS). This method maintains the protein chain in a denatured state after treating it with strong reducing agents to eliminate secondary and tertiary forms. (such as converting S-S disulfide bonds to SH) thus separating proteins according to their molecular weight. The proteins in the sample are covered with negatively charged

sodium dodecyl sulfate and transferred to the positively charged electrode through the acrylamide network in the gel. Small proteins migrate faster through this medium and thus the proteins are separated by size (usually measured in kilodaltons, kD, kiloDaltons.) The accuracy of the gel depends on the concentration of acrylamide - the higher the concentration of acrylamide the higher the fineness of proteins with higher molecular weights and proteins



Picture (1) Electrophoresis of sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) at 180 V for 1 hour, containing outer membrane proteins (OMPs), Stained with Coomassie brilliant blue R-250 Lane A = shows external protein bundles (OMPs) of gram-negative bacteria (Standard OMPs). Lanes B, C, D = outer membrane proteins (OMPs) extracted from three EPEC isolates isolated in the study.

Immunological study in laboratory animal

Concentration of IgA and IgG antibodies specific to EPEC types

Table (1) shows the changes in antibody titers in mice treated with EPEC bacteria, outer membrane proteins, olive oil, and the membrane protein complex with olive oil. The concentration of IgG antibody was 8.3 IU/ml when animals were treated with EPEC bacteria, and 16.1 IU/ml when treated with outer membrane proteins only, and it reached 18.20 IU/ml when animals were treated with olive oil only, but this rate increased to 85.12 IU/ml. Titers in the serum of animals treated with the complex of outer membrane proteins - olive oil, compared with the control group, where the concentration of IgG antibody was 6.3 IU/ml. The same applies to the IgA antibody whose concentration was 10.7 IU/ml, 11.2 IU/ml, 19.1 IU/ml and 80.2 IU/ml for the above three treatments, respectively, compared to the control group, which amounted to 6.6 IU/ml.

Table (1) average concentration of IgA and IgG immunoglobulins in laboratory animals treated with OMPs antigen and protein-adjuvant immune complex

concentration of antibodies (IU/ml)	Immunization antigens				
	control group	Membrane protein complex - olive	(Olive Oil)	outer membrane proteins	EPEC

		oil		(OMPs)	
IgG	4.3±6.3	11.3 ±85.12	3.9±18.20	4.2±16.1	3.3±8.3
IgA	3.5±6.6	9.4±80.2	5.8±19.1	3.8 ±15.95	±10.7 5.2

The significant increase in the concentration of antibodies appeared when the animals were treated with outer membrane proteins, and the increase in the antibody titer in mice treated with outer membrane proteins could be due to stimulating the generation of lymphocytes to produce antibodies. Babu et al (2017) indicated in his study the ability of outer membrane proteins extracted from *Klebsiella pneumonia* bacteria to extend the lifespan of lymphocytes invitro and in vivo, which increases the secretion of immunoglobulins that bind to the host cell membrane and immobilize the pathogen or it results in the phenomenon of opsonization, which leads to killing the pathogen with complement factors, as well as the protein works to increase the receptors of the crystallizable part on phagocytic cells, and this helps to stimulate the process of deliberate cellular killing on antibodies. Pham and McSorley (2015) reach to similar results when activating mice against infection with salmonella bacteria, as they noticed a significant increase in antibody titer and since the first month of activation. We also note that this significant rise increases when animals are treated with olive oil only and reaches its peak when used with membrane proteins (membrane proteins - olive oil) and the production of immune globulins, and then the elimination of the pathogen, and this result gives hope. It opens new horizons in the possibility of developing an effective vaccine against pathogenic *Escherichia coli* and other bacterial diarrheal pathogens, which is one of the most common diseases among children living in low- and middle-income countries. As there were few attempts that depended on using weakened bacteria as a vaccine for these pathogens, most of these attempts were unsuccessful, and perhaps the reason was the ability of the attenuated pathogen to revitalize or modify the components of the cell wall, including LPS and OMPs inside the host cells. As for mice treated with EPEC bacteria, they showed a decrease in antibody titer compared to mice treated with membrane proteins - olive oil. These results somewhat agreed with what Hélène et al, , 2007 found (when investigating antibodies in mice infected with EPEC bacteria, they noticed that it was not possible to diagnose antibodies during the first three months of infection, The researcher stated that not recording the antibody titer does not mean that it does not exist, but that its quantity may be limited or not detectable by ELISA testing in the early stages of infection. In any case, The mice immunized with EPEC recorded higher levels of antibodies than those recorded in the positive control group. The absence of antibodies in the sera of the above bacteria-infected animals may be due to the inactive effect of circulating antigens (Giulling et al, 2007). The low antibody titer in positive control mice may be due to the ability of bacteria and its wall components to inhibit lymphocyte proliferation, possibly by stimulating inhibitory T cells, directing cytotoxicity, and as a result, weakening the humoral response. It is also believed that the non-specific transformation of T lymphocytes may contribute to the destruction of the T lymphocyte dependent areas by the autotoxicity of the toxin produced by bacteria as well as may affect the balance of Th1 and Th2 cells. The researchers, Hansen, and Jackson (1990) indicated that the ability of macrophages to ingest and cellular killing, as well as the response to interferon in advanced cases of EPEC infection, is attenuated, and

thus leads to a decrease in the process of antigen presentation and antibody production.

Cellular Kinetics Concentration

Determination of the concentration of interleukins 6 and 8 IL-6, IL-8,

The results in Table (2) show a significant increase in the average concentration of IL-6 in sera of animals treated with EPEC bacteria by 42 pg/ml. When animals were treated with outer membrane proteins and olive oil separately, olive oil complex and membrane proteins, the concentrations of interleukin-6 were (42 pg/ml, 41.34 pg/ml and 185.3 pg/ml), respectively. It was noticed from the results that there were significant differences at the level of probability ($P \leq 0.005$) when compared with the concentration of interleukin-6 in the control group, which amounted to 6.3 pg/ml. It was noticed from the results that there were significant differences at the probability level ($P \leq 0.005$) when compared with the interleukin-6 concentration in the control group, which amounted to 6.3 pg/ml, and the average interleukin-6 concentration in the animals before dosing, which was 6.45 pg/ml. As for the interleukin-8 concentration, there was an increase in the average interleukin concentration by 50.3 pg/ml when the animals were treated with EPEC bacteria, and it reached (51.4) pg/ml when treated with outer membrane proteins (OMPs). Injecting the animals with the prescribed dose of olive oil led to an increase in the interleukin level - 8 (IL-8) to 57.7 pg/ml, As for the complex of olive oil and membrane proteins, it led to an increase in the concentration of interleukin-8 (200 pg / ml). It was noticed through the results that there were significant differences at the level of probability ($P \leq 0.005$) when compared with the concentration of interleukin-8 in the control group, which amounted to 7.81 pg/ml and the average concentration of interleukin-8 in the animals before the dose, which was 7.56 pg / ml (Table 2).

Estimation of the interferon-gamma- γ INF concentration

As for the concentration of interferon-gamma, it was noted from Table (2) also that there are significant differences at the same level of probability above compared to the control group, as it was 6.6 pg/ml, and its average concentration in the animals before the dose was (8.5) pg/ml. While the average interferon-gamma concentration when mice were treated with EPEC bacteria was 49 pg/ml and when treated with outer membrane proteins was 52.3 pg/ml. When mice were treated with olive oil and olive oil complex with outer membrane proteins, the gamma interferon concentrations were (40.7 pg/ml and 102.2 pg/ml), respectively.

Table (2) Mean concentrations of IL-6, IL-8 and IFN- γ in laboratory animals treated with OMPs antigen and protein complex – immunosuppressive

Interleukin concentration (Pg/ml)	Immunization antigens					
	control group	Membrane protein complex - olive oil	(Olive Oil)	Membrane protein complex - olive oil	EPEC	before dosing

IL-6	7.3±6.3	±185.3 13.4	41.34±6.3	12.2±43	±42 10.2	5.6±6.45
IL-8	7.2±7.81	9.3±200	8.6 ±57.7	11.3±51.4	±50.3 7.4	6.8±7.56
IFN- γ	6.5±6.6	12.2±102.2	6.2±40.7	52.3 ±38.	±49 11.1	8.9±8.5

This may be due to the ability of the outer membrane protein and the outer protein complex with olive oil to induce cellular immunity by stimulating the secretion of chemokines, which in turn work to selectively transfer and recruit white blood cells in immune response reactions, as occurs in the migration of eosinophils to sites of allergic inflammation. Allergic Inflammation) in the late stages of lymphocyte accumulation, This was confirmed by Mahajan and Galley (2011), who indicated that the hallmark of hypersensitivity reactions is the accumulation of eosinophils in tissues preceded by the accumulation of Th2 lymphocytes, and they concluded that delayed hypersensitivity reactions stimulate the production of attractant chemicals and reticulum endothelial cells. Cells) necessary for eosinophil migration, and eosinophil migration was inhibited by depletion of Th2 cells or with anti-IFN- α antibodies, This indicates the vital role of Th2 cells and γ -IFN- α in delayed hypersensitivity reactions. On the other hand, Mast cells determine T-cell-dependent neutrophil accumulation. In delayed hypersensitivity reactions mediated by the secretion of two types of immunomodulators, TNF- α and Macrophage inflammatory protein-2 (MIP-2). Hence, it is clear that olive oil is a good immune stimulator and represents a good, new, and effective means in the treatment and prevention of many diseases. In recent years, isolated substances from different sources have been used as immune modifiers to improve the immune response in a qualitative or non-qualitative manner, and some of these attempts have met Partial success in treating some types of cancer and some viral and bacterial diseases. Research is still ongoing in this therapeutic field, which includes the use of natural products as immune aids, as Al-Kaabi (2018) used Propolis as an immune aid with a compound of Lipopolysaccharides (LPS) of *Salmonella typhimurium*. In the results of his study, he concluded that Propolis is a good immune aid when combined with LPS, as it contributed to stimulating immunity in laboratory animals.

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