Original paper

Study of Immune Adjuvant Activity of Propolis against MRSA in Laboratory Animals

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Abstract

Background: Propolis is a natural resinous product that honeybees collect from several plants and mix it with bees wax and salivary enzymes that is also called bee glue. Propolis has many properties that make it is chief in the medicine including antibacterial, antiviral, antifungal, antitumor and immunodulatory properties.

Aim: The aim of this study was to evaluate immune adjuvant activity of propolis against Methicillin Resistant *Staphylococcus aureus* MRSA in *vivo*.

Materials and Methods: Twenty male white New Zealand rabbits included in this study, that divided into tow groups (10 rabbits at each one), group I received orally one milliliter of Ethanolic Exract Propolis (EEP) at 20% concentration per day for lasting 20 days, while group II have no received any thing as control group. After 10 days from last dose of adminstration of EEP, all animals injected in intraperitonial (IP) with six doses of 10^8 cfu/ ml of killed somatic antigen of *Staphylococcus aureus*. Blood samples were taken by heart puncture from all animals to detect the immunological parameters by ELISA technique.

Results: Results revealed to significantly increased the mean value of total leukocytes counts (TLCs) in group I (5705 cells/mm^3) as comparing with group II (4600 cells/mm^3), at level $p < 0.05$, as well as the results pointed to increased significantly the percentage of neutrophil cells in group I (42%) as compared with group II (34%), in contrast to lymphocytes results that show significantly decrease in group I (54%) as compared with group II (61%), in addition, monocytes, eosinophil and basophil were have no differences among the testing groups. Phagocytic activity result shows increased significantly in group I (55.47%) as compared with group II (43.94%). The results of complement component C3 revealed increased significantly in group I (69.76 pg/mL) as compared with group II (18.61 pg/mL), while the results of C4 concentration pointed to increase significantly in group I (4.20 pg/ml) as compared with group II (2.38 pg. /ml). IFN-γ concentration results revealed increased significantly in group I (22.18 pg/mL) as compared with group II (18.05 pg/mL. IL-2 results referred to increased significantly in group I (32.42 pg/mL) as compared with group II (24.27 pg/mL). On the other hand, the level of perforin was increased significantly in group I (0.23 ng/ mL) as compared with group II (0.12 ng/ mL).

Conclusion: It was concluded that propolis improve immune responses as adjuvant against MRSA antigen via increase the levels of cellular immunity elements.

Key words: Propolis, Adjuvant, Perforin, MRSA.

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Introduction

Propolis is a natural resinous product that honeybees collect from several plants and mix it with beeswax and salivary enzymes that is also called bee glue [1]. Bees use propolis on their hives as defense against killers and microorganisms, to repair harm, as a thermal isolator, and to build aseptic locals to avoid microbial infection [2,3]. Since ancient times, propolis has been used by humans to meet the wants of health and food preservation. In the last years the interest in this natural product has better due to its wide spectrum of biological and pharmacological properties [4]. Propolis is a lipophilic substantial that is firm and fragile when cold however elastic, soft, and very gummy when it heated. Possesses an agreeable aromatic odor and diverse color, including brown, green, and red, among others [5, 6]. The chemical composition of it, generally composed of 50% resin, 30% wax, 10% essential oils, 5% pollen, and 5% other elements which contain flavonoids, organic compounds like phenolic acids (cinammic and caffeic acid), minerals, fatty acids and esters of phenolic acids terpenes, and alcohols [7].

Propolis has many properties that make it is chief in the medicine including antibacterial, antiviral, antifungal, antitumor and immunodulatory properties. Also propolis used as adjuvant with inactive vaccines that stimulate the immune status [8]. Chemical analysis revealed that propolis have more than 300 constituents among them phenolic compounds such as flavonoids that is the major components in addition to cinammic acid derivatives that is play important role in the immunodulatory actions [9]. In 1995 [10] were stated that cinammic acid stimulate the proliferation of lymphocytes and inducing production of IL-2, so stated that propolis action on NK cells activity as well as stimulate action associated with IFN-γ. However, when used the propolis as adjuvant it has been shown to increase the activity of vaccine and also increasing protective index in addition to eliciting a higher antibody titer and enhance the cellular response, offering high activity of phagocytosis [11]. Propolis has an effect on humoral immunity by enhancing antibody production in rats immunized with bovine serum albumin. Artepillin that is one of the extracts of particular propolis, also modulate the immune response, as well as stimulating T-cells, IFN-γ, and IL-2 secretion. Activation of T-cells leads to series of events like activation of transmembrane signals and expression of cytokine genes. Cytokines are bound to specific receptors on the surface of target cells regulate the growth and differentiation of cells and thus optimize the immune responses [12]. In 2008, a group of research in China introduced the use of ethanolic extract of propolis (EEP) in the formulation of the protein subunit vaccine for entero-toxigenic *E. coli* ETEC-F4 to boost the immune system [13]. In 2012, *Klebsella pneumoniae* vaccine was formulated with EEP of Egyptian propolis, this vaccine was received by injection into white New Zealand rabbits which compulsory a lower amount of antigen to produce analogous levels of antibodies titer continued for longer period [8], higher level of IL-2, IFN-γ and produced better peripheral lymphocyte proliferation [14].

Furthermore, in 2011 [15] stated the extract propolis possess antioxidant capacity in vitro conditions. Flavonoids present in propolis are the major components which may reduce free radical formation and consequently may have a protective effect on serum lipids on oxidation [16]. The anti-inflammatory potential of the EEP was confirmed by inhibition of the hyaluronidase enzyme, which is degradation of hyaluronic acid which is an important component of articular cartilage and plays an important role in tissues' renovation. The degradation of hyaluronic acid by hyaluronidase enzyme may cause bone

loss, inflammation, and pain [17, 18]. The activity of propolis varies according to the pH of media and geographic station [19]. The presence of flavonoids and derivatives of caffeic acid is associated with the bactericidal activity [20]. Many researchers revealed that the inhibition of bacterial RNA-polymerase by the components of propolis was probably associated with the loss of their ability to bind to DNA. It is believed that antimicrobial and anti-inflammatory properties of propolis are mainly attributed to its flavonoid and phenolic compounds composition [21]. The aim of this study was to evaluate the adjuvant activity of propolis against methicillin resistance *Staphylococcus aureus* MRSA in *vivo*.

Materials and methods

Twenty male white New Zealand rabbits included in this study, that divided into tow groups (10 rabbits at each one), group I received orally one milliliter of 20% of ethanolic exract propolis (EEP) that prepared according to (20) per day for lasting 20 days, while group II have no received any thing as control group. After 10 days from last dose of adminstration of EEP, all animals injected in intraperitonial (IP) with six doses lasting for eleven days (0.1, 0.2, 0.3, 0.5, 0.7, 1.0 milliliter of 10^8 cfu/ ml of killed somatic antigen of *Staphylococcus aureus*, that were isolated and identified according to (22). Blood samples were taken by heart puncture from living animals to detect the immunological parameters (complement component C3 and C4; IFN-γ; IL-2 and perforin) by ELISA technique according to the procedure of Manufacture Company (Elabscience/China) that provided with each kit.

Statistical analysis

Obtained results were analyzed by SPSS (version 18) to measure mean, stander deviation (SD) and percentage. ANOVA test was used to test the significance of data. P value less than 0.05 was considered significant.

Results And Discussion

Total Leukocytes Count (TLCs):

The results of TLCs in laboratory animals indicated a significant increasing the mean value in group I (5705 cells/mm^3) as comparing with group II (4600 cells/mm^3) $p < 0.05$ as in table (1).

The obtained results in this study were agreement with [23] who stated, TLCs were increased in animals fed with propolis reflect to stimulate protein synthesis and enhanced cell mitosis that lead to stimulate body immune responses. TLCs give an overall picture to the immune system functions against pathogens.

Differential Leukocyte Count

The results of differential leukocyte count indicated an increasing significantly the percentage of neutrophil in group I (42%) as compared with group II(34%). On the other hand the result indicates to decrease significantly ($p< 0.05$) the percentage of lymphocytes in group I (54%) as compared with group II (61%), while the percentage of monocytes in group I was (3%), and in same time the results of eosinophil and basophil show no differences between animals in group I and group II as illustrate in table (2).

Table 1. the values of total leukocytes count (TLCs) among the testing animals

Testing groups	No.	Mean value Cells/mm^3	Standard deviation	P value
Group I*	10	5705	910.68	< 0.05
Group II**	10	4600	162.01	

Group I*: animals fed with propolis, Group II**: animals fed without propolis as control group.

Neutrophil cells are involved in an important innate immune function that is phagocytosis, therefore increased as a result to achieve this function in order to defense against pathogens. Neutrophils are very important component of innate immunity and the severe infections occur if they are few in their numbers in blood stream. The cytoplasm of neutrophil contains granules which involve in bactericidal action as a result of lysosome. These cells have receptors for IgG and don't display MHC II protein on their surface and therefore don't present antigen to T-helper cell that is contrast to macrophages that are also phagocytes but do present antigen to Th- cell [(24)]. Lymphocyte is of fundamental importance in the immune system because lymphocytes are the cells that determine the specificity of the immune response to infectious microorganisms. They are found in the circulation and also are concentrated in peripheral lymphoid organs and tissues, such as the lymph nodes and spleen. The two primary types of lymphocytes are B cells and T cells. Both originate from stem cells in the bone marrow and are initially similar in appearance. Most lymphocytes are short-lived, with an average life span of a week to a few months, but a few live for years, providing a long-lived T and B cells. These are account for immunologic memory, a more rapid vigorous response to a second encounter with the same pathogen or any antigen (Ag). Each lymphocyte bears receptors that bind to a specific antigen. The ability to respond to virtually any antigen comes from the enormous variety of lymphocyte populations that the body contains each of them with a receptor capable of recognizing Ag [(25)]. Once stimulated by binding to Ag, such as a component of a bacterium or virus, a lymphocyte multiplies into a clone of identical cells. Some of the cloned B cells differentiate into plasma cells that produce antibody molecules. These antibodies, once released into the blood and lymph, they bind to the target Ag and initiate its neutralization or destruction. Antibody production continues for several days or months, until the antigen has been overcome. Other B cells, the memory B cells, are stimulated to multiply but do not differentiate into plasma cells; they provide the immune system with long-lasting memory [(26)].

Phagocytic activity

The results of phagocytic activity indicate a significant increasing in group I (55.47%) as compared with group II (43.94%) as in table (3).

Table 3. The percentage of phagocytic activity among the testing groups

Testing groups	No.	Mean value %	Standard deviation	P value
Group I	10	55.47	2.37	$p < 0.05$
Group II	10	43.94	1.14	

Table 2. The percentage of differential leukocytes in testing groups

	Group I		Group II	
Leukocyte types	**%**	**SD**	**%**	**SD**
Neutrophil	**42**	1.03	**34**	0.73
Lymphocyte	**54**	1.39	**61**	0.75
Monocyte	**3**	1.11	**3**	0.35
Eosinophil	**1**	0.61	**1**	0.50
Basophil	**0**	0	**1**	0.66

SD= standard deviation

The increasing of phagocytic activity by propolis in present study agreement with [27, 11] who stated propolis can significantly enhance the phagocytic function. The increased phagocytic activity against *S. aureus* bacteria may be due to the presence of glucose which assists in the destroying action of macrophages [28]. Phagocytosis in mammalian immune cells is activated by attachment to pathogen-associated molecular patterns (PAMPs), which leads to NF-κB activation. Opsonins such as C3b and antibodies can act as attachment sites and aid phagocytosis of pathogens. Macrophages have three main functions, one of them is phagocytosis. These cells have Fc receptors that interact with Fc portion of immunoglobulin gamma (IgG), thereby enhancing the uptake of opsonized organism. Also, it has receptors for C3b [24].

Complement Component levels

A- C3 Component:

The results of complement component C3 indicated to increase significantly in the concentration of C3 in group I (69.76 pg/ml) as compared with group II (18.61 pg/mL) as in table (4).

B- C4 Component:

The obtained results of C4 concentration pointed to a highly significant increasing in group I (4.20 pg/ml) as compared with group II (2.38 pg. /ml), as in table (5).

The complement system is a part of the immune system that helps antibodies and phagocytic cells to clear pathogens. The complement system consists of a number of small proteins found in the blood that synthesized by the liver, and normally circulating as inactive proteins [24]. When stimulated by an antigen, proteases in the system cleave specific proteins to release cytokines. The end-result of this activation cascade is massive amplification of the response and activation of the cell-killing membrane attack complex. There are three biochemical pathways activate the complement system: the classical complement pathway, the alternative complement pathway, and the lectin pathway [29].The three pathways generate homologous variants of the protease C3-convertase. The classical pathway typically requires antigen: antibody complexes for activation (specific immune response), whereas the alternative pathway (AP) and lectin pathway can be activated by antigens without the presence of antibodies. In all three pathways, C3-convertase cleaves and activates component C3, creating C3a and C3b. C3b binds to the surface of pathogens, leading to greater internalization by phagocytic cells by opsonization. C3a is the precursor of an important cytokine and is usually rapidly cleaved by carboxypeptidase B. Both C3a and C5a have anaphylatoxin activity, as well as increasing vascular permeability and smooth muscle contraction [30]. C5b initiates the membrane attack pathway, which results in the membrane attack complex (MAC), consisting of C5b, C6, C7, C8, and polymeric C9. MAC is the cytolytic end product of the complement cascade; it forms a transmembrane channel, which causes osmotic lysis of the target cell [31, 24].

Table 4. The result of C3 component concentration among the testing groups

Testing groups	No.	Mean value pg/ml	Standard deviation	P value
Group I	10	69.76	10.66	$p < 0.05$
Group II	10	18.61	0.58	

Table 5. the result of C4 component concentration among the testing groups

Testing groups	No.	Mean value pg/ml	Standard deviation	P value
Group II	10	4.20	0.51	$P < 0.05$
Group II	10	2.38	0.31	

On the other hand, Complement component C4 is a central protein in the classical and lectin pathways within the complement system. During activation of complement, its major fragment C4b becomes covalently attached to the surface of pathogens and altered self-tissue, where it acts as an opsonin marking the surface for removal. Moreover, C4b provides a platform for assembly of the proteolytically active convertases that mediate downstream complement activation by cleavage of C3 and C5 [24, 32].

Cytokines

A. Interferon Gamma (IFN- γ) levels:

The IFN- γ concentration pointed to a significant elevation ($p< 0.05$) in the mean value in group I (22.18 pg/ml) as compared with group II (18.05 pg/ml) as in table (6).

The propolis effects, conducted in several laboratories indicated to increasing the secretion of many cytokines that regulate the immune system by treating with propolis that lead to association with inflammatory response or autoimmunity [33]. T-cells have several functions, which can be divided into two categories, regulatory and effectors. The regulatory mediated primarily by helper T-cells which secretes cytokines like IFN-γ that is activating the macrophage, the main mediators of delayed type hypersensitivity (DTH against intracellular infections. The effector functions are carried out by CD^{+8} T-cells which kill virus-infected cells and tumor cells [24]. CD^{+4} T-cells require signal transducer and activator of transcription STAT-4 beside T-cell receptors (TCRs) signalization to produce IFN-γ, while CD^{+8} T-cells need only TCRs activation [34, 35].

B. Interluekine-2 (IL-2) concentration:

The results indicated to a significantly arise of IL-2 value in group I (32.42 pg/ml) as compared with group II (24.27 pg/mL) as in table (7).

IL-2 is a member of cytokines that produced by activated CD+4 and CD+8 T-cells in addition to activated DCs, NK and NKT cells [36]. It is stimulates both helper and cytotoxic cells, in addition to it is conceder as T-cell growth factor [24]. IL-2 is produced primarily by CD^{+4} T-cells following antigen stimulation but also produced to a lesser extent by CD^{+8} T-cells, NK cells, activated DCs and mast cells [37]. Transcription of IL-2 requires two signals mediated by calcium and protein kinase C. The range of recognized actions of it has expanded with roles in inducing the differentiation of T-helper cells type 1 and 2. It has broad essential biological actions not only driving T-cell proliferation and modulation effector cell differentiation but also limiting the dangerous autoimmune reactions [38]. This cytokine plays important role in immune responses via promotes the generation, proliferation and differentiation of T-cells as well as enhances the activity of NK and promote antibodies production [39]. In the presence of high concentrations of interleukin-2, NK cells differentiate to lymphokine activated killer cells (LAK cells). LAK cells are cytotoxic and are more potent killers than the NK cells [40, 24].

Perforin levels

Perforin significantly increased ($p< 0.05$) in group I (0.23 ng/ mL) as compared with group II (0.12 ng/ mL) as in table (8).

Table 7. The result of Interluekin-2 concentration among the testing groups

Testing groups	No.	Mean value pg/mL	Standard deviation	P value
Group II	10	32.42	7.31	< 0.05
Group II	10	24.27	0.30	

Table 6. The results of interferon gamma IFN-γ concentration among the testing groups

Testing groups	No.	Mean value pg/mL	Standard deviation	P value
Group II	10	22.18	0.91	p < 0.05
Group II	10	18.05	0.28	

Table 8. The result of perforin levels among the testing groups

Testing groups	No.	Mean value ng/ml.	Standard deviation	P value
Group II	10	0.23	0.02	< 0.05
Group II	10	0.12	0.01	

Perforin studied in this work as a marker for the activity of cytotoxic cells. A cytotoxic response is concerned primarily with destroying the cells that infect with virus and tumor cells. In response to viral infections, CD^{+8} must recognize both viral antigens and class I molecules on surface of infected cells. In order to kill these cells, the cytotoxic T-cells (CTC) must be activated by production of IL-2 by CD^{+4} T-cells. The a activated T-helper cells produce cytokines such as IL-2 that stimulates the virus specific (CTC) to form a clone of activated CTC, and these latter kill a virus infected by inserting a specific protein called perforin and granzymes [41,24]. Perforin is a glycoprotein that weights 60-70 kilo Dalton KDa and consists of four domains, two (C-terminal and N-terminal) which are related to biological functions, and other two domains are located in perforin molecule center. CTC and NK cells are the main source of perforin as well as CD^{+4} cells are also able to express a few amount of it [40, 41]. The structure and function of perforin is similar to complement component 9 C9 that is formed transmembrane tubules and is capable of lysing non-specifically a variety of target cells. In order to become the perforin inactive form require to enzyme that is playing role in protecting it by keeping it in the glomerular form which called calretikulin that act as a chaperon protein [42]. Perforin polymerization and pore formation are accountable for transformation to active forms which able to incorporate into cell membrane [43]. NK cells produce IFN-γ that is activating macrophages to kill ingested bacteria as well as produce a perforin [24]. Perforin and FasL are the key molecules in lymphocyte mediated cytotoxicity with some accessory functions of TNF. FasL induce the receptor-mediated apoptosis, while the perforin mediated lysis does not require to these receptors but depend on the interaction with phosphorylcholine residues on the surface of target cells [44, 41]. Perforin is bind to target cells plasma membrane and oligomers in a Ca^{+2} dependent manners to create pores on target cell. These pores formed allow to passive diffusion of a pro-apoptotic proteases that called as granzymes into the target cell [45, 41]. Furthermore, they activate caspases (are a family of cysteine proteases that play essential roles in apoptosis programmed cell death, necrosis, and inflammation) that initiate a cell death program that named apoptosis that may occur in multicellular organisms. Biochemical events lead to characteristic cell changes (morphology) and death. These changes include blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, chromosomal DNA fragmentation, and global mRNA decay. In contrast to necrosis, which is a form of traumatic cell death that results from acute cellular injury, apoptosis is a highly regulated and controlled process that confers advantages during an organism's lifecycle. For example, the separation of fingers and toes in a developing human embryo occurs because cells between the digits undergo apoptosis. Unlike necrosis, apoptosis produces cell fragments called apoptotic bodies that

phagocytic cells are able to engulf and quickly remove before the contents of the cell can spill out onto surrounding cells and cause damage [46]. Perforin has been shown anti-tumor mediator that assist the growth and spread of tumor, and this achieved with study by [47] were stated the lung cancer is associated with decreased perforin and IFN-γ expression, possibly as a result of factors produced via cancer cells like PGE2.

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