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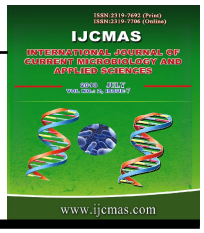

Original Research Article

Immunoadjuvant activity of honey against bacterial antigens: *In vivo* study

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A B S T R A C T

Honey is a natural biological product with a various medicinal activities. This study aimed to investigate the immunoadjuvant activity of orange honey (OH) to enhance the immune response against Gram negative and Gram positive bacteria. Two clinical isolates of *Pseudomonas aeruginosa and Staphylococcus aureus* were used as test bacterial antigens. Albino Swiss male rats were treated with OH, another group of animals were not treated with OH as control. Animals then immunized with somatic antigens for both isolates .Specific and nonspecific immune parameters were estimated. Significant increase ($p \leq 0.05$) in phagocytic index was measured for groups of animals treated with OH and immunized with both isolates compared with animals groups not treated with OH. The same results were estimated for IL-12 and complement component C3.Significant increase in concentration of IFN-γ was estimated in animals treated with OH and immunized with somatic antigen of *Staph. aureus*. Antibody titer was highly increase in animals treated with OH and immunized with somatic antigen of *P. aeruginosa.* Also, C4 was significant increase in the later animal group. The results provide a good support for the role of OH in the enhancement of specific and non specific immune response against bacterial infections.

Keywords

Honey; immuno-adjuvant activity; Gram positive bacteria; phagocytic index.

Introduction

Honey is the natural sweet substance, produced by honeybees from the nectar of plants or from secretions of living parts of plants, or excretions of plant-sucking insects on the living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store and leave in honeycombs to ripen and mature(Codex Alimentarius,2001). Honey is a supersaturated solution, mainly fructose (38%),glucose (31%), sucrose (1%), water (18%), other sugars (7%) plus amino acids, organic acids, enzymes, anti-oxidants, vitamins as well as minerals (Salih *et al*., 2009). Natural products are known to have biological activity, and many previous investigations for the effect of natural products on immune function have been carried out (Takeuchi *et al.*, 1993; Nakajima *et al*., 2002). Honey is rich with glucose and fructose, various vitamins, minerals and amino acids (Qiu *et al*., 1999; Vassya *et al*., 2005). The

medicinal usage of honey had been mentioned and it being prescribed by the physicians of many ancient races of people for a wide variety of ailments (Ransome, 1937). Haffejee and Moosa (1985) reported a clinical trial in which honey was used in place of glucose in a rehydration fluid (solution of electrolytes) given to infants and children admitted into hospital with gastroenteritis. The treatment with honey gave a statistically significant reduction in the duration of diarrhoea caused by bacterial infection .Honey has anti-inflammatory activity (Molan,1999). Moreover honey has a great ability to enhance the immune system by increasing the activity of immune cells, antibody, and cytokines production (Tonks *et al*., 2003; Al-Waili and Haq, 2004; Tonks *et al*., 2007). One of the most popular health problems now days is emerge of multi-drug resistant bacteria. Infections caused by resistant microorganisms often fail to respond to the standard treatment, resulting in prolonged illness and greater risk of death. The death rate for patients with serious infections treated in hospitals is about twice that in patients with infections caused by non-resistant bacteria (WHO, 2013). Many of multidrug resistant bacteria have also many strategies for immune escaping (Rich *et al*., 2003) that make them immune resistant in addition to the another characteristic feature drug resistant. This study aimed to study the immunoadjuvent effect of orange honey (OH) on the specific and nonspecific immune response against clinical isolates of *Pseudomonas aeruginosa* and *Staphylococcus aureus* .

Materials and Methods

Bacterial isolates

Clinical isolates of *Staph.aureus* and *P.aeruginosa* were provided from department of Medical Microbiology/ College of Medicine/ Babylon University. Both of them were clinically isolated from patients with urinary tract infection. The identification of bacteria was done according to the morphological, biochemical and cultural characteristics (Forbes *et al.,* 2007).These two clinical isolates were selected as they are commonest multidrug resistant Gram positive and Gram negative bacteria.

Bacterial antigens

Somatic antigens for both *S.aureus* and *P.aeruginosa* were prepared according to the methods described by Garvey *et al*., 1981; Stanislavsky and Lam (1997).

Preparation of orange Honey

Orange honey (OH) was a gift from Dr.M.T Kerim College of Agriculture /Kufa University-Iraq. OH was dissolved with distilled water to final concentration 10 mg/ml. This solution was sterilized by 0.22-mm filtration (Millipore, MA, USA) and then stored at 4^{o}C before use (Fukuda *et al*., 2009). OH was used as a drinking solution instead of water for animal groups three months before immunization with bacterial antigens. The OH treatment continued during immunization with bacterial antigens.

Animals

Albino Swiss male rats were caged for acclimatization for one month in the Animal House. Rat's weight was ranged 150-250g. The animals were kept on normal pellet diet, standard room temperature and normal diurnal rhythm.

Study design

A cohort study design was used in this study. Experimental rats were classified in to the following groups. Each group

consists of three animals: Group A-immunized with somatic antigens of *Ps.aeruginosa* with OH solution; Group B-immunized with somatic antigens of *Ps.aeruginosa* without OH solution; Group C- immunized with somatic antigens of *S.aureus* with OH solution;Group D- immunized with somatic antigens of *S.aureus* without OH solution.

Immunization Schedule

Experimental rats were injected intraperitonealy (IP) with somatic bacterial antigens the dose and time intervals were carried out according to (Caponi and Migliorini 1999). After seven days from the last dose of treatment, blood samples were collected via heart puncture (Lewis *et al*., 2001).Sera as well as anticoagulant whole blood was used.

Measurement of immune parameters

a) Phagocytic index

The phagocytic activity expressed by phagocytic index was estimated according to Furth *et al*., (1985).

b) IFN-Gamma

Interferon gamma (IFN- γ) was ELIZA estimated by using kit prepared by Koma Biotech, Korea.

c) IL-12:

Interleukin -12(IL-12) was also ELIZA estimated by using kit prepared by Koma Biotech, Korea.

d) Antibody titer

Antisomatic bacterial antibody titer for both *P.aeruginosa* and *S.aureus* was estimated by using tube agglutination method (Garvey *et al*.,1981).

e) Complement components concentration (C3,C4)

The concentration of complement components as a marker for both innate immunity and liver function test was estimated by using single radial immunoassay according to the instructions of manufacturer company (LTA, Italy).

Statistical Analysis

Statistical analysis was carried out using SPSS version 18. Continuous variables were presented as means and standard deviations. Independent sample t-test was used to compare means between two groups. A *p*-value of $\leq$ 0.05 was considered as significant.

Result and Discussion

Effect on the Phagocytic index

The results of phagocytic index revealed significant differences ($p \leq 0.05$).The index for group A was higher than group B. The same results were noted between group C and group D (Table 1).

Effect on the production of Interferon gamma (IFN-γ)

The results of the estimation of IFN-γ revealed significance increasing in the group A treated with OH and Somatic antigen of *Staph,aureus* in comparsion to control group D treated with antigen only. On the other hand, no significance increasing ($p \leq 0.05$)in the IFN-γ concentration between group A treated with OH and somatic antigen of

P.aeruginosa and group B treated with antigen only (Table 2).

Effect on the production of Interleukin 12(IL-12)

Significance increasing ($P \leq 0.05$) in the concentration of IL-12 was noted between group A and B as well as group C and group D (Table.3).

Effect on Antibody titer

The results of antibody titer expressed in figure (1a, 1b) revealed highest antibody titre against somatic antigen of *P.aeruginosa* in group A as compared to the group B, but no difference in the titer of anti staphylococci antibody in group C and D.

Effect on the Complement concentration

Complement component 3(C3)

The effect of OH on the production of C3 revealed a significant difference between A and B as well as between C and D (Table 4).

Complement component 4(C4):

Significant increasing in the concentration of C4 in the sera of animals in group A in comparison to that of group B. On the other hand, no significant difference in the concentration between group C and D (Table 5).

Discussion

One of the most health problem worldwide is the emergence of multidrug resistant bacteria which are difficult to treat with available antibiotics especially in immuno-compromized patients. *P.aeruginosa* and *Staph.aureus*; which are used in this work as indicators for the immunoadjuvant activity of OH, are the commonest multidrug resistant bacteria. These bacteria are responsible for healthcare-associated infections and prone to multidrug resistance (ECDC, 2013). Chen *et al*. (2008) explained that *P.aeruginosa* is considered as the most common naturally drug resistant bacteria. On the same manner *Staph. aureus* was the leading cause of hospital acquired and community acquired infections(Gordon and Lowy, 2008). Thus the enhancements of host immunity against drug resistant bacteria provide the effective solving for this problem and reduce the dependency on antibiotics.

Immune parameters used in this work represent both specific and specific immunity. Phagocytic index is a method to check the activity of neutrophile as an innate defense mechanism against microbial infections. The results of this study revealed significant increasing in phagocytic index when OH was used in combination with somatic pseudomonal and staphylococcal antigen as compared with control. This results is agreed with Fukuda *et al.* (2009) who indicated that jungle honey induces neutrophil to phagocytize the foreign materials. Also, honey triggers a chain of molecular events in the cell that stimulates the uptake of glucose and amino acids, and promotes cell growth (Tonks *et al.*, 2001). The honey glucose is important for activation of phagocytes by inducing of respiratory burst (Ryan and Majno 1977). Also, honey provides substrates for glycolysis, the major mechanism for energy production in

Table.1 Phagocytic index for animal groups treated with orange honey and immunized with bacterial antigens

Animal groups	Phagocytic index(%)(Mean ±SD)	P value
A(antigen of *Ps.aeruginosa* and orange honey)	11 ± 1.73	0.011*
B(antigen of *Ps.aeruginosa* only)	5.66 ± 1.15	
C(antigen of *S.aureus* and orange honey)	15 ± 3.6	0.05*
D(antigen of *S.aureus* only)	8 ± 1.73	

*significant

Table.2 Concentration of interfreron gamma (IFN- γ) for animal groups treated with orange honey and immunized with bacterial antigens

Animal groups	Mean Concentration of IFN- γ (pg/ml) ±SD	P value
A(antigen of *P.aeruginosa* and orange honey)	352 ± 7.76	0.088
B(antigen of *P.aeruginosa* only)	326 ± 19	
C(antigen of *S.aureus* and orange honey)	471 ± 13.52	0.001*
D(antigen of *S.aureus* only)	302 ± 7.81	

Table.3 Concentration of interleukin 12 (IL-12 for animal groups treated with orange honey and immunized with bacterial antigens

Animal groups	Mean Concentration of IL-12 (pg/ml) ± SD	P value
A(antigen of *Ps.aeruginosa* and orange honey)	178 ± 9.64	0.001*
B(antigen of *Ps.aeruginosa* only)	66 ± 11.35	
C(antigen of *S.aureus* and orange honey)	232 ± 25.7	0.015*
D(antigen of *S.aureus* only)	165 ± 11.53	

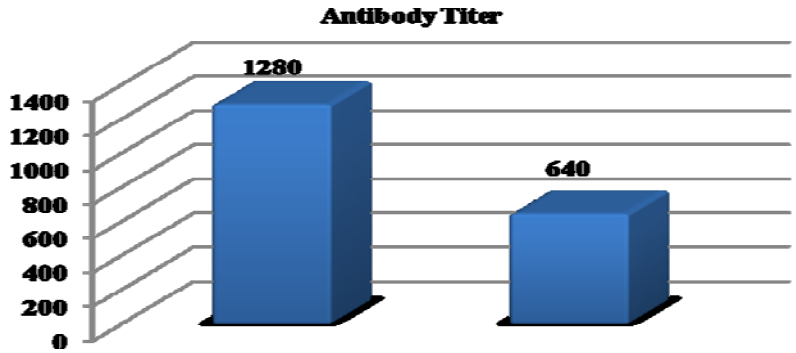
Table.4 Concentration of complement component 3(C3) for animal groups treated with orange honey and immunized with bacterial antigens

Animal groups	Mean Concentration of C3 (mg/dl)	P value
A(antigen of *P.aeruginosa* and orange honey) B(antigen of *P.aeruginosa* only)	198.3 ± 10.69 144.6 ± 12,5	0.005*
C(antigen of *Staph.aureus* and orange honey) D(antigen of *Staph.aureus* only)	212.6 ± 7.5 153.3 ± 7.63	0.001*

Table.5 Concentration of complement component 4(C4) for animal groups treated with orange honey and immunized with bacterial antigens

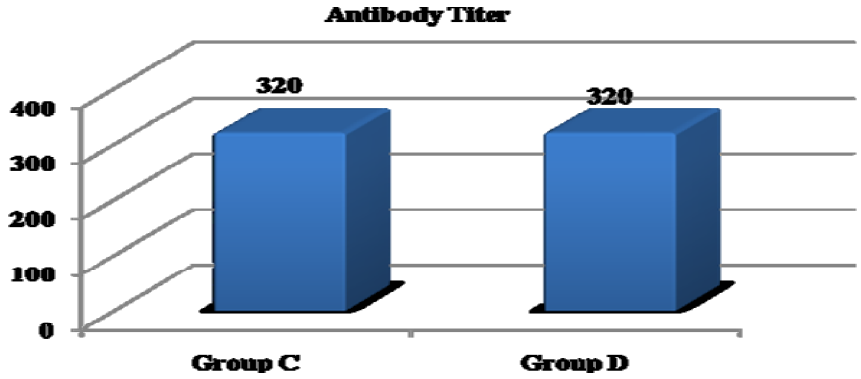
Animal groups	Mean of Concentration of C4 (mg/dl)	P value
A(antigen of *P.aeruginosa* and orange honey) B(antigen of *P.aeruginosa* only)	28.3 ± 4.04 16.6 ± 2.3	0.012*
C(antigen of *Staph.aureus* and orange honey) D(antigen of *Staph.aureus* only)	22.3 ± 3.05 19.3 ± 3.12	0.295

Figure.1a Anti-Pseudomonal antibody titer for animal groups



A: immunized with somatic antigens of *Ps.aeruginosa* with OH solution
B: -immunized with somatic antigens of *Ps.aeruginosa* without OH solution

Figure.1b Anti-Staphylococcal antibody titer for animal groups



C: immunized with somatic antigens of *Staph.aureus* with OH solution
D: immunized with somatic antigens of *Staph.aureus* without OH solution

the phagocytic cells. Additional to this nutritional optimization of the body's immune system ,honey enhances immunity through a bioactive effect, and activate phagocytes in blood, and has a mitiogenic effect for phagocytic cells and enhances the production reactive oxygen species with bactericidal effect (Tonks *et al.* (2001). The immunostimulation of phagocytic cells against drug resistant bacteria such as *Ps,aeruginosa* and *Staph aereus* is the target by many researchers ,since these bacteria are characterized by their ability to resist antibiotic and their behavior to intracellular living(Thomas *et al*., 2009,AlThamir *et al*., 2013).

Interferon gamma (IFN-γ) is a cytokine secreted by TH1 lymphocytes and has many potential activities .It able to induce bactericidal activity of macrophages and stimulate the expression of MHC system, and inhibits microbial proliferation(Delves *et al*., 2011; Doan *et al*., 2013).The results of the current study revealed a significant increase in IFN-γ in group C compared to group D which reflects the immuoadjuvant activity for OH to induce TH1 response that mediates the mechanism of eradication of intracellular infections including *Staph aereus*. Many previous studies confirmed the ability of honey to induce cytokines production (Tonks *et al*.,2001; Tonks *et al*.,2003). Salih *et al* (2009) confirmed the ability of honey to enhance the production of IFN- γ.

Interleukin 12(IL-12) is a cytokine secreted by dendritic cells ,B cells ,and T cells and has the ability to enhance the production of cytokines including IFN- γ and TNF- γ ,and activates the natural killer (NK) cells(Doan *et al*., 2013). The NK cells play a direct cytotoxic role against intracellular bacteria and malignant cells. The results of this study revealed the ability of OH to enhance significant increase in the concentration of IL-12 in group A and C compared to group B and D respectively .Honey increases the capacity of lymphocytes to secrete cytokines by increase their proliferating rate (Salih , 2008). The results of antibody titer revealed a significant increasing in the anti-pseudomonal antibody titer in group A as compared to group B.

This indicates the ability of OH to enhance the activity of B cells to respond and secrete antibodies required to act as opsonin, blocking, and complement fixation ending with the attack and damaging of *Ps.aeruginosa*. Oral intake of honey enhances antibody production against T dependent and T independent antigens (Al-Waili and Haq, 2004).

Complement system plays a vital role against bacterial infections and malignant cells directly by formation of membrane attack complex (MAC) or by their activity of some complement components to act as opsonin for phagocytic cells (Abbas *et al.*, 2012). The results of estimation of complement system revealed a significant increase in the concentration of C3 in group A and C as compared to group B and D respectively. This result illustrates the great effect of OH to enhance the immune response mediated by complement system. The component C3 is the corner stone for all the pathways of complement activation and it is necessary for classical and alternative pathways. Also, the results of C4, which is the marker of classical pathway, revealed the ability of OH to stimulate the significant increasing in the production of C4 in group A in comparison to group B. These results indicates the major role of OH to enhance the production of complement system during the infection with *P.aeruginosa*.

The complement components produced may be used for direct killing of *Ps.aeruginosa* or by opsonization of this pathogenic bacterium to be engulfed and destroyed by phagocytic cells. Honey has ability to enhance the haemolytic activity of complement system(Abdulrhman *et al.*, 2011).

From the results expressed above the OH has the ability to enhance the specific immune response type TH1 (IFN-γ, IL-12) as well as TH2 response (antibody production). Also, OH can increase non-specific innate immunity (phagocytosis and complement system).The results also indicate the immunoadjuvant activity of OH against Gram negative bacteria represented by *P.aeruginosa* and to a lesser extent Gram positive bacteria represented by *Staph.aureus*.

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