Antibacterial Activity of Different Types of Honey in Comparison to Ciprofloxacin Against Multidrug-Resistance Pseudomonas aeruginosa Isolated from Infected Burn

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الخلاصة

على الرغم من تطور المضادات الجرثومية وطرق متابعة الحروق، فأنُّ الخمج (Infection) يبقى المشكلة الاساسية في علاج الحروق، وبالأخص علاج الزوائف (*Pseudomonas aeruginosa*) ذات المقاومة المتعددة الذي يشكل تحدياً كبيراً يحتاج الى علاجات مبتكرة. إنُّ فشل العلاج بالمضادات الجرثومية كحالات خَمَج الحروق ، والاعراض الجانبية لأستخدام المضادات الجرثومية الموضعية (خاصة في الرُضّع و الاطفال و الحوامل وكبار السن) ، اضافة الى تأخُّر الالتئام الذي تسببهُ المطهرات ، كلُ ذلك يُشكل حافزاً قوياً لتجربة علاجات جديدة فعّالة وآمنة ، ولهذا الغرض فقد اجريت عدة دراسات حديثة على المنتجات الطبيعية ذات الفعّالية المضادة للجراثيم والتي تحسّن شفاء الحروق.

إستهدفت الدراسة تقصي فعالية انواع معينة من العسل خارج الجسم الحي ضد جراثيم الزوائف (*Pseudomonas aeruginos*a)، المعزولة من مرضى الحروق في مستشفى الصدر التعليمي / مدينة النجف الأشرف، مقارنة بالسيبروفلوكساسين المستخدم لعلاج خمج الحروق ، وقد إستخدِمت طريقة (Disk diffusion method) وطريقة (Agar dilution MICs assay) والتي أظهرت ان معدل التثبيط بلغت اقطارهُ بالملمتر (0.41 ±12.1 ,0.27 ± 11.25 ,0.29 ± 11.05) واقل تخفيف مثبط (١٠-٢٠%، ٢٠% ، ٢٠%) لكل من عسل السدر وعسل الاكاسيا وعسل اليوكاليبتوس على التوالي. لذا فان للعسل فاعلية جيدة كمضاد جرثومي ضد جميع العُزلات الجرثومية المفحوصة.

Abstract

Despite recent advances in antimicrobial chemotherapy and burns management, infection continues to be an important problem in the burns. Treatment of multi-drug resistant *Pseudomonas aeruginosa*, which causes burn infection, is a big challenge in clinics and needs novel strategies. Failure of the current treatment strategies to control many cases of burns infections, the local and systemic adverse effects that are produced by many topical antibiotics (especially in infants, children, pregnant ladies, and elderly people), and the delay of healing caused by many antiseptics, make a strong motive to find out a new, safe and effective products. Many recent studies were done to find out natural products that have antimicrobial properties, and enhance the healing process.

This study aimed to investigate the *in vitro* activity of different types of honey against *P. aeruginosa* in comparison to the ciprofloxacin one of the antibiotics used in treatment of burns infection by using disk diffusion method and agar dilution MICs assay.

The mean±SE inhibition zone in mm were (12.1± 0.41, 11.25 ± 0.27, 11.05 ± 0.29) and MICs {(20% - 10%), 20%, 20%} for Sider, Acacia, Eucalyptus respectively. So the conclusion was that all tested types of honey exerted a good in vitro antimicrobial activity against all tested isolates.

Key wards: *Pseudomonas aeruginos*a, Sider honey, Acacia honey, Eucalyptus honey, ciprofloxacin.

Introduction

Burns are among the main causes of death of humans in the world. The World Health Organization reports that over 90% of burns occur in developing or underdeveloped nations, where the mortality for large burns (over 40% total body surface area) approaches 100% [1]. As thermal injury removes or impairs the body's natural barrier to microbes, the cause of death in over 75% of these burned individuals is infection [2]. *P. aeruginosa* is one of the most prevalent opportunistic pathogens that infect burn wounds, and the mortality associated with a systemic infection is over 75% [3]. The exceptional virulence of this gram-negative bacterium is due to the production of numerous virulence factors, including toxins, lysins and proteases [4]. *P. aeruginosa* proliferates rapidly in burn wounds, and the infection can be divided into two phases [5,6]. In the first phase of infection, *P. aeruginosa* quickly colonizes the devascularized burnt tissue, which provides a warm, moist, nutrient-rich environment ideally suited for bacterial growth. *P. aeruginosa* proliferates quickly, forming biofilms in the hypodermis, specifically surrounding blood vessels [7]. Once a threshold concentration of *P. aeruginosa* is reached in the eschar (approximately 10^9 CFU/g tissue), *P. aeruginosa* spreads systemically through the bloodstream, causing bacteremia, which is the second stage of infection. *P. aeruginosa*-induced bacteremia is soon followed by multiple organ failure and eventually death [5,6]. Infections in clinical settings are of growing concern because of the increasing resistance of *P. aeruginosa* to antibiotics. One-third of *P. aeruginosa* clinical isolates are resistant to three or more antibiotics, including broad-spectrum cephalosporins and imipenem, which has been the "gold standard" [8,9]. Despite the growing resistance of *P. aeruginosa* and many other pathogens to current antimicrobials, very few new drugs are in advanced development or clinical trials [10]. Therefore, new antimicrobials that target this pathogen are urgently needed.

Honey has long been known to possess antibacterial properties and has an established usage as wound dressing [11], The topical application of honey has been reported to clear existing wound infection rapidly [12,13,14]. It acts against several bacteria such as *Pseudomonas,* staphylococci, streptococci and *Escherichia coli.* Even some antibiotic-resistant bacteria, such as methicillin-resistant *Staphylococcus aureu*s and vancomycin resistant Enterococci are reportedly sensitive [15,16]. Several properties are attributing to its antimicrobial effects such as low water content and thus facilitating an "osmotic effect"[11], low pH between 3.2 to 4.5 and this acidity is low enough to inhibit the growth of most microorganisms [17], production of hydrogen peroxide, as result of glucose oxidase activity which has a very potent bactericidal activity[18] and also other researchers found several still not fully characterized phytochemicals with antibacterial activity to be present in honey [17]. This study aimed to investigate the *in vitro* activity of different types of honey against *P. aeruginosa* in comparison to the ciprofloxacin antibiotic.

Material and Methods

Bacteria

During the period from January to April, 2010, thirty six, consecutive, non-duplicate samples were obtained from burn patients, in Al-Sadr Teaching Hospital, Najaf, Iraq. The samples were quickly cultured on MacConkey agar (Bioanalyse, Turkey) and incubated overnight at 37 °C. Any suspicious colony was then sub-cultured and purified. The isolates were preserved at 4 °C on nutrient agar slant and then

identified as a *P. aeruginosa* based on morphological and biochemical tests and then confirm the obtained results by using Api 20E system [19].

Antibiotic Susceptibility Test

Antibiotic susceptibility of *P. aeruginosa* isolates were studied against the carbenicillin 100 µg, piperacillin 100 µg , ticarcillin 75 µg , ceftazidime 30 µg, ceftriaxone 30 µg, cefotaxime 30 µg, cefepim 30 µg,imipenem 10 µg, Ciprofloxacin 5 µg, norfloxacin 10 µg, levofloxacin 5 µg, gentamicin 10 µg , amikacin 30 µg, tobramycin 10 µg (Bioanalyse, Turkey) by the disk diffusion technique on Muller-Hinton agar (Bioanalyse), using inhibition zone criteria recommended by the disk manufacturer and based on the method of Barry [20]. The selection of antibiotic disks was performed according to the guidelines recommended by CLSI [21]. *E. coli* ATCC 25922 was used as control to test the validity of antibiotic disks.

Antimicrobial Activity of Honey

The types of honey used in this study were Acacia honey (Langnese Honig, Germany) and the locally obtained Sider and Eucalyptus honey that were procured from Al-Mohana local beekeeper of (Iraq/Najaf). It did not contain any additives and had not been heated. To determine the antibacterial activity of the above mentioned types of honey alone or in combination with each other and with ciprofloxacin. Sterile 6 mm filter paper disks (Whatman No. 1) were placed on the inoculated Mueller-Hinton agar plates and immediately 15 µl sterilized portions of different types of 100 % (v/v) honey were added. Sterile filter paper was used as control. Allowing 1 hr at 4 °C for the honey to diffuse across the surface, then the plates were incubated upright at 37°C for 24 hrs. The inhibition zone was measured in millimeters and the assay was carried out three times for each type. At first, the individual inhibitory effects of the types of honey were determined [22]. The data obtained were used for determination of their combined effects; the different disk types were placed on the inoculated agar plates in such manner that the inhibitory circles would just touch each other tangentially. Finally, the diameters of inhibition zones produced due to individual and mutual effects of two agents were recorded on the same plate [23,24].

Minimum Inhibitory Concentrations (MIC) of the Honey

The agar dilution method was used to assess the antibacterial activity of these types of honey against these isolates. After filtration through Millipore filter, different concentrations of honey (v/v) were prepared in sterile Muller-Hinton agar at 56 °C to give final concentrations of 1%, 5%, 10%, 20%, 30% and 40 %. After bacterial inoculation the plates were incubated for 18-24 hrs at 37 °C. After incubation, the plates were observed for inhibition of growth. MIC was defined as the lowest concentration of the honey that yielded no growth. Plates of Mueller Hinton agar with and without honey were included to check the sterility of honey and the medium used in the test [25]. The experiments were performed in triplicate.

Statistical Analysis

Results were expressed as mean ± standard error. ANOVA tests were run at a confidence level of 95% when comparing two means. A p value less than 0.05 was considered statistically significant.

Result

Thirty different Gram-negative bacteria were isolated from thirty six clinical specimens. Twenty (66.7%) of isolates were identify as *P. aeruginosa* according to the cultural, morphological and biochemical tests. Whereas, the remainder ten (33.3%) of the isolates indicated the presence of different Gram-negative bacteria other than *P. aeruginosa*.

The antibiotic sensitivity among *P. aeruginosa* isolates varied according to the nature of the isolate and antibiotic. The percentage of resistant isolates to each antibiotic is shown in Figure (1). Present study showed that 85% of isolates considered as multidrug resistance, which mean resistance to three or more than three antibiotics from different classes (Figure 2).

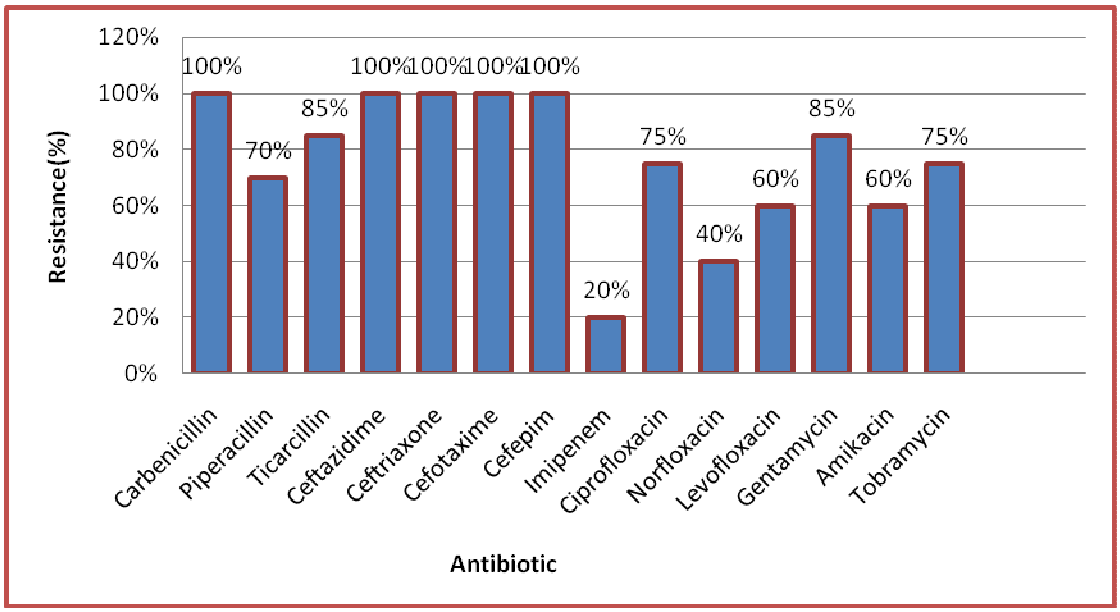

Figure (1): Antibiotics resistance of 20 *P. aeruginosa* isolated from burn wounds.

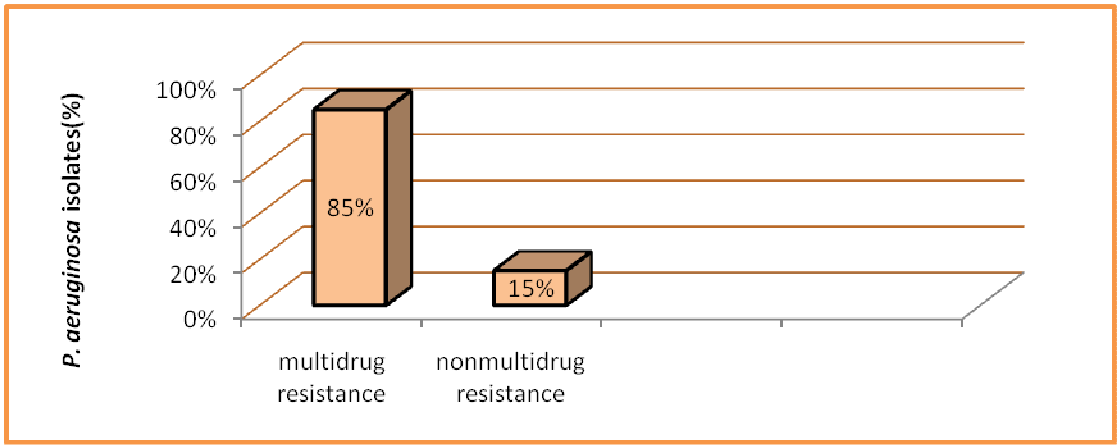

Figure (2): Distribution of multidrug resistance *P. aeruginosa* isolates (n: 20) obtained from burn wounds.

The present study showed that different types of honey have different mean inhibition zones ± standard error mean (M ± SEM); Sider honey, Acacia honey and

Eucalyptus honey have mean inhibition zone (12.1± 0.41, 11.25 ± 0.27, 11.05 ± 0.29) mm, respectively. While ciprofloxacin has mean inhibition zone (8.95 ±2.25) mm. So that, in term of M ± SEM, the Sider honey was the highest one followed by Eucalyptus honey, Acacia honey and ciprofloxacin (Figure 3).

Present results also showed there is no significant difference between honey types; furthermore, only Sider honey was significantly (P< 0.05) higher than ciprofloxacin in term of mean inhibition zone in mm (Table 1).

Disk diffusion method also reveal that the interaction between these agents with each other were indifference, which mean there is no additive, synergistic or antagonistic effect between them.

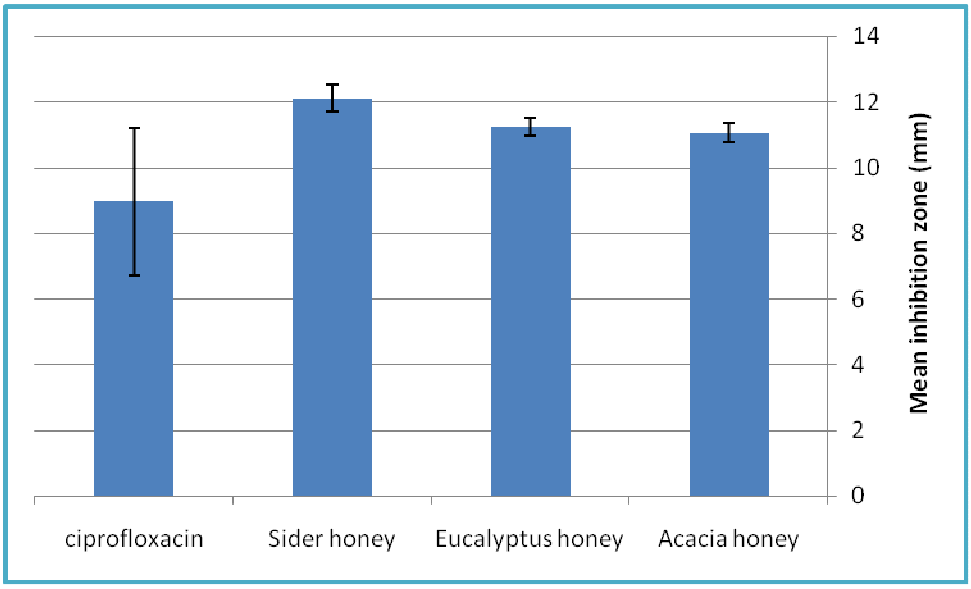

Figure (3): Error bar chart shows the difference in mean ±SEM values of inhibition zone of the five agents against *P. aeruginosa* after 24 hrs incubation.

Table (1): Multiple comparisons among different groups mean values inhibition zone in mm by using ANOVA test

Groups	Eucalyptus	Acacia	Sider
Ciprofloxacin	-2.1	-2.3	-3.95*
Sider	1.05	0.85	
Acacia	0.2		

*** p<0.05**

The results of present study also showed that MIC of honey revealed that all types of honey effectively induced inhibition of bacterial growth, even at 20% dilutions

(Table 2). At 10% dilutions Acacia and Eucalyptus honey have no effects on bacterial growth, while Sider honey inhibited 20% of isolates. At 5% dilutions all types of honey could not inhibit bacterial growth.

Tab.(2): Minimum inhibitory concentration of different types of honey against 20 *P. aeruginosa* isolates

Sider honey											
40%		30%		20%		10%		5%		1%	
S	R	S	R	S	R	S	R	S	R	S	R
20	0	20	0	20	0	4	16	0	20	0	20
Acacia honey											
40%		30%		20%		10%		5%		1%	
S	R	S	R	S	R	S	R	S	R	S	R
20	0	20	0	20	0	0	20	0	20	0	20
Eucalyptus honey											
40%		30%		20%		10%		5%		1%	
S	R	S	R	S	R	S	R	S	R	S	R
20	0	20	0	20	0	0	20	0	20	0	20

(S) sensitive, (R) resistance.

Discussion

Pseudomonas aeruginosa is plays a significant role in colonization and infection of patients admitted to hospitals and it cause a variety of systemic infections, particularly in victims of severe burns [26].

The results of present study revealed that *P. aeruginosa* was a common isolate representing (66.7%) of isolates cultures of infected burns which in agreement with the findings of Agnihotri *et al.* [27] and Abd-El Aal *et al.* [28] who showed that *P. aeruginosa* was the organism most frequently isolated, representing (59%) and (53.3%) of the isolates cultures of infected burns, respectively.

Many of the pharmaceuticals presently prescribed in hospitals have a long history of use as herbal remedies including quinine, belladonna, digitalis, emetine, strychnine and aspirin. Antimicrobial screening methods could provide the needed preliminary observations necessary to select among natural product, those with potentially useful properties for further chemical and pharmacological investigations [29].

There are many reports about antibacterial properties of natural honey, but these properties of honey are not unlimited such as other antibacterial agents.

In this study, both locally obtained honey and commercially honey has shown *in vitro* antibacterial activity against *P. aeruginosa* (isolated from burned patient) with little variation in the potency of their antibacterial activity. The mean inhibition zone of

Sider honey, Acacia honey and Eucalyptus honey were (12.1± 0.41, 11.25 ± 0.27, 11.05 ± 0.29) mm, respectively (Figure 3), which was higher than the result obtained by Osman *et al*. [30] who reported that bee honey zone was 8 mm against *P. aeruginosa* but lower than results obtained by Claudia *et al*. [31] and Abd-El Aal *et al*. [28] who showed that mean inhibition zone produced by honey when applied on isolated *P. aeruginosa* were 17.1± 0.1 mm and 18.2 ± 2.5 mm, respectively.

Acacia honey and Eucalyptus honey showed no significant difference in comparison with ciprofloxacin, while mean inhibition zone of Sider honey was significantly higher than that of ciprofloxacin. However, the antimicrobial activity of honey and its variability according to floral origin have been widely reported [32,33]and to different botanical and geographical origins, and also to bee-origin metabolism products [34,32,33]. Furthermore, honey constituents such as sugars, volatiles, beeswax, nectar, pollen and propolis have also been considered as responsible for the antimicrobial activity [35,34]. The variation in sensitivity is also attributable to differences in temperature, inoculum's size and the test method itself [36].

Present study showed that the MIC of all isolates of *P. aeruginosa* for both Eucalyptus honey and Acacia honey was 20% dilutions, while for Sider honey 80% of isolates inhibited by 20% dilutions MIC and the remainder isolates inhibited at 10 % dilutions (Table 2). i.e., Sider honey showed better activity with a MIC of 10% for 20% of isolates. These results were lower than results obtained by Subrahmanyam [25] and Nzeako and Hamdi [37] who showed that all the *P. aeruginosa* tested failed to grow at concentrations of 25% of Jambhul honey and 40% of six commercially honey in Muller-Hinton medium respectively, but higher than results obtained by Molan [38], Andargarchew *et al*. [39] and Mullai and Menon [40] who reported that MIC were 6% Manuka honey, 7.5% honey and 11% Khadikraft honey, respectively.

Mullai and Menon [40] also has reported that Manuka honey and Heather honey were have 20% MIC against *P. aeruginosa* which comparable with results obtained by this study. There is therefore a need for a microbiological assay of every honey sample in order to determine its activity before it can be used as an antimicrobial agent.

It is also clear that both antibiotic-sensitive isolates and antibiotic-resistant isolates were equally susceptible to these types of honey which agreed with the findings of Cooper *et al*. [11,16] and French *et al.* [41] and that may be attributed to physicochemical properties of honey that not act simultaneously (high content of reducing sugars, high viscosity, high osmotic pressure, low pH, low water activity, low protein content) and hydrogen peroxide libration [42,43].

We concluded that all types of honey inhibiting bacterial growth where the vast majority of these bacteria are multidrug resistance.

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