ORIGINAL ARTICLE

Disruption of *Pseudomonas aeruginosa* and *Staphylococcus aureus* biofilm by Manuka and Beri Honey

MUHAMMAD SHAHBAZ HUSSAIN[1], AZFAR SATTAR[2], ALIA BATOOL[3]

ABSTRACT

Aim: Formation and detection of the biofilm in microtiter plate and role of honey in prevention and disruption of this biofilm.

Methods: A six months experimental study was carried out at the of Microbiologydepartment university of Health Sciences (UHS) Lahore, Pakistan in which MDR clinical isolate of *Staphylococcus aureus* and *Pseudomonas aeruginosa* were used**.** Biofilm formation and detection was done by microtiter plate method. Two different honeys were used in order to see either they inhibits and disrupt the biofilm or not.

Results: Manuka and Beri honey inhibited the biofilm formation of *both organism* 20% and 30% (w/v) . Both honeys disrupted the established biofilm ofat concentration greater than 40% (w/v). It was clearfrom results that a higherconcentration of both honey was required to inhibit as well as disrupt established biofilm.

Conclusions: *Staphylococcus aureus*and *Pseudomonas aeruginosa* form strongly adherent biofilm. The conclusion of this study is that honeywhether local or from outside possesses good anti biofilm activity against *MDR organism.*

Keywords: *Staphylococcus aureus* ,*Pseudomonas aeruginosa*, Manuka honey, Beri honey, Biofilm, Anti-biofilm activity.

INTRODUCTION

A biofilm is an aggregate of microorganisms on biotic or abiotic surfaces by means of a bacterium initiated matrix[1]. Biofilm matrix is adynamic and immobilized microbial environment which is a very stable "dwelling house" and have high levels of resistance to antimicrobial agents. Planktonic cells (free floating, single cell phenotype) can cause serious systemic infection or shedding of the microorganism into the external environment [2]. Biofilms are held together and protected by extracellular polymeric substance (EPS) [3] **.**This EPS designfunctions bothstructural and protective .It assist the transport of metabolites, micronutrients, different enzymes, and waste products [4].

The components of the EPS are present in the matrix of biofilm communities [5]. These components of the EPS can reduce the penetration of antibioticsalong withhelping the transfer of micronutrients in and different waste product out of the biofilm construction[6]. Bacteria interconnect by means of synthesizing and reacting to warning sign molecules. This special communication mechanism is called Quorum sensing (QS). Quorum sensing allows bacteria to intellect the concentration of other bacteria present within a small microenvironment with different. Virulence factors i.e. enzymes and toxins[7].

The dweller bacterial population in a biofilm has many defenses and more mechanisms for survival such as defenses against microphage for phagocytosis, UV radiation, viral attack, cell stress and dehydration.

Biofilms made by various bacteria are resistant to antimicrobial , chemical disinfectant and other components of the innate and adaptive inflammatory defense system of the body[8].

It is clear that biofilms have the ability to resist in 1000 times the concentrations of different antibiotics and biocides that can inhibit and kill free living bacterial cells[9].

The main implants that have the biofilm infections are; teeth and dental implants, middle ear implants like hearing aids in the middle and internal ear , stents in GIT, urogenital tract stents, different airway and lung tissue, urinary tract artificial implants , eye, different catheters ofperitoneal membrane and peritoneal dialysis, indwelling catheters for haemodialysis and for chronic administration of chemotherapeutic agents(Hickman catheters). Biofilms are often responsible for nosocomial infections and chronic illnesses. *Pseudomonas aeruginosa*, a common biofilm-forming opportunistic pathogen, can lead to lung damage in both CF and immune compromised patients [8]. Bacterial biofilms may affect cutaneous wound healing and reduce

[1]Associate Prof. Pathology Sh Z Medical College Rahim Yar Khan
[2]Assistant Prof Pathology Aziz Fatimah Medical College Fasilabad.
[3]Assistant Prof Pathology Fatima memorial Medical college Lahore
Correspondence to Dr. Muhammad Shahbaz Email: drmshahbaz@szmc.edu.pk

topical antimicrobialefficacyin healing.[10] Continuous infections of biofilm-relatedartificial medical devices lead to inflammation and discomfort which requires removal or replacement of the infected devices. Removal of biofilm contaminated foreign devices endangers the patient's condition and creates additional costs.

The fact that conventional and routine antimicrobial therapy is frequently ineffective in removalof bacteria in the biofilm. In a current study biofilms were cured with citric acid/Zwitterionic surfactant (CAZS) [11]. Therefore due to pooreconomical conditions of developing countries like Pakistan, higher antibiotic cost which is not possible for individual and increasing antibiotic resistance give us an idea that biofilm infection can be treated with non-conventional modalities. Miraculous healing and repair qualities of honey are mentioned in almost all the Holy Scriptures e.g.; The Holy Quran, The Holy Bible and The Holy Torah [12]. Allah SubhanhuTaala says in The Holy Quran

وَأَوْحَىٰ رَبُّكَ إِلَى النَّحْلِ أَنِ اتَّخِذِي مِنَ الْجِبَالِ بُيُوتًا وَمِنَ الشَّجَرِ وَمِمَّا يَعْرِشُونَ ﴿٦٨﴾

68. *And thy Lord taught the Bee to build its cells in hills, on trees, and in (men's) habitations;*

ثُمَّ كُلِي مِن كُلِّ الثَّمَرَاتِ فَاسْلُكِي سُبُلَ رَبِّكِ ذُلُلًا يَخْرُجُ مِن بُطُونِهَا شَرَابٌ مُّخْتَلِفٌ أَلْوَانُهُ فِيهِ شِفَاءٌ لِّلنَّاسِ إِنَّ فِي ذَٰلِكَ لَآيَةً لِّقَوْمٍ يَتَفَكَّرُونَ ﴿٦٩﴾

69*. Then to eat of all the produce (of the earth), and find with skill the spacious paths of its Lord: there issues from within their bodies a drink of varying colours, wherein is healing for men: verily in this is a Sign for those who give thought.* (The Holy Qur'an, Al- Nahl 68-69)

The value of honey is recognized by medical experts. In the Australia, it has been accepted as a "Therapeutic Good" to be used as Antiseptic dressing to promote wound, burn ad skin ulcer healing and topical antibacterial agent for the treatment of acne spots and other dermatological infections[13]. Honey have also been used for the treatment of the gastrointestinal tractincluding periodontal and oral diseases[14].

Outstanding property of honey is to inhibit and disrupt the biofilm formed by different strains of *P. aeruginosa* and *S. aureus[15]*. Honey also inhibited the biofilm formed by meticillin resistant *Staphylococcus aureus* (MRSA), meticillin-sensitive S. aureus (MSSA) and vancomycinresistant *Enterococcusfaecalis* (VRE)[16]. In another study honey was used in treating bacterial biofilms embedded in chronic wound bacteria [17]. A study showed that Quorum sensing can be disrupted by honey[18].

To our knowledge, it appears that none has reported biofilm disruption property in the case of biofilm formation by *Pseudomonas aeruginosa and Staphylococcus aureus* . Therefore this study has main aim to evaluate the efficacy of indigenous Beri honey against these microorganism induced biofilm. Pakistani Beri honey was used in this study. Beri honey, collected from Karak district has more antibacterial activity is proved in the studyand also Beri honey has dark colour, so it has more antioxidant as well as antibacterial property than other honey. It also overcomes the emerging issues of Multi Drug Resistant induced biofilm.

The present study had three phases. First, we used a microtiter plate methods for the detection of biofilm formation. Then the ability of honey was assessed to inhibit the bacterial biofilm formation. In the next step we discovered the effect of honey on established biofilm.

MATERIALS & METHODS

This is an experimental study and it has been conducted in the Microbiology department, University of Health Sciences (UHS)Lahore, Pakistan**.** It took us six months to complete this study.

MATERIALS:

Honey Samples: Pakistani Beri honey was used in this study. This honey was collected from Karak district. The floral source of Pakistani Beri honey was identified by the apiarist supplying it. Identification was based on the geographical location, flowering plants, season, colour, flavour and aroma of Beri honey.

Manuka honeyUMF25 + (standardized honey / FDA approved) was used as standard honey. It was purchased from Comvita®, New Zealand.

Storage & sterility of Honey Samples: Honey samples were kept in the dark (closed under table drawers) at 18°C (64°F) to 23°C (73°F). The sterility of honey was checked by on Blood agar medium (Oxoid Ltd, UK). Honey samples which showed growth were treated with Gamma radiations at Pakistan Radiation Services (PARAS),

Lahore,Punjab, Pakistan. After gamma irradiation if the samples showed no growth then these samples were used in this study. This is best method of sterilization of honey[14].

Bacterial Strains: ATCC 25923 *Staphylococcus aureus* and ATCC 27853 *Pseudomonas aeruginosa* were used in this study. These were also stored in microbank at -70 ^{o}C in the Department of Microbiology, University of Health Sciences (UHS)Lahore,Punjab, Pakistan.Sterile,Flat-bollonia 96 wellmicrotiterELISA plates were used in this study to grow biofilm.Bacterial strains was identified by the morphological biochemical and cultural .

Bacterial suspension: ATCC strains (*Staphylococcus aureus*and *Pseudomonas aeruginosa*) were transferred from – 70^{o}C to Blood agar plate and incubated aerobically at 37 ^{o}C for 24 hrs. After identification of the strain, a few colonies with identical morphology were suspended in nutrient rich broth. The turbidity of bacterial suspension of this strain was adjusted to that of the 0.5 McFarland standards (10^{8} CFU/ml). It was performed by using a photometric device. At 600 nm the absorbance of 0.5 McFarland is 0.132. Then 1:100 dilution of this suspension in a fresh nutrient broth will result in the final testing inoculums of 10^{6} CFU.

Honey concentrations: The density of each honey is 1.37 g /ml. A 20 ml of stock solution was prepared by adding 13.7 grams of honey and 10 ml of deionized water. Thus 50 % (w/v) solution of each honey samples was obtained. From this stock solution, we used 40%, 30%, 20% and 10 %(w/v) concentration solutions of each honey samples. The procedure for biofilm formation was applied as described by theStepanovic[19].

RESULTS

Inhibition of Biofilm formation by Manuka and Beri Honey: Biofilms of ATCC strains of Pseudomonas and S.aureus were formed within 24 hours. However, the pattern of biofilm formation was not uniform. The biofilm formed by the ATCC strains were strongly adherent to microtiter plates .Manuka Honey inhibited the biofilm formation of ATCC strains at 20% (w/v) and above i.e. 30%, 40%, and 50 %(w/v) concentration of honey. Beri honey, on the other hand inhibited biofilm formation of *P aeruginosa* and *S. aureus* was at 30% (w/v) concentration of honey . The result showed that both honey at 10% (w/v) and lower concertationcould not inhibit biofilm formation.The extent of thebiofilm biomass and formation in each 96 well of microtiter plate was graphed against different concentration of Manuka and Beri honey. It is clear from the figure I and figureII that decreased and less concentration of honey leads to increased absorbance of light than the cutoff value. It means biofilm was formed that led to increased absorbance. When concentration of Manuka honey was more than 10% (w/v) and Beri honey concentration more than 20% (w/v) the absorbance was less than cutoff value, which means biofilm formation was inhibited by both honeys (Figure I,II).

Table I. Effect of Manuka honey on established biofilm

Concentration of Manuka honey (w/v)%	***P. aeruginosa* Biofilm absorbance at (570nm)**		***S. aureus* Biofilm absorbance at (570nm)**	
0	2.543	(+++)	2.599	(+++)
10	1.992	(+++)	2.228	(+++)
20	1.722	(+++)	2.208	(+++)
30	1.701	(++)	1.077	(++)
40	0.969	(+)	0.743	(+)
50	0.488	(-)	0.490	(-)

Non adherence- ; Weakly adherent+;
Moderately adherent ++; Strongly adherent +++

Table II. Effect of Beri honey on established biofilm

Concentration of Beri honey (w/v)%	***P. aeruginosa* Biofilm absorbance at (570nm)**		***S. aureus* Biofilm absorbance at (570nm)**	
0	2.571	(+++)	2.555	(+++)
10	2.499	(+++)	2.524	(+++)
20	2.446	(+++)	2.329	(+++)
30	1.895	(++)	1.708	(++)
40	0.963	(+)	0.790	(+)
50	0.565	(-)	0.492	(-)

Non adherence - ; Weakly adherent +; Moderately adherent ++; Strongly adherent+++

Disruption of the established biofilm by both honey (Manuka and Beri): Both Manuka and Beri Honey disrupted "established" i.e formed biofilm (Table I & II). *Pseudomonas aeruginosa* and *Staphylococcus aureus*, well known for strong biofilm formation, were found to be more resistant to be disrupted once their biofilm was formed. A higher concentration above 40 %(w/v) of both Manuka and Beri honey was required to disrupt these "established" biofilms (Table I& II). It indicates that when the concentration of both honeys is greater than 40 %(w/v), the absorbance is less than cut off value. It means that biofilm was disrupted.

Fig. I: Inhibition of *Pseudomonas aeruginosa*biofilm formation by Manuka&Beri honey.

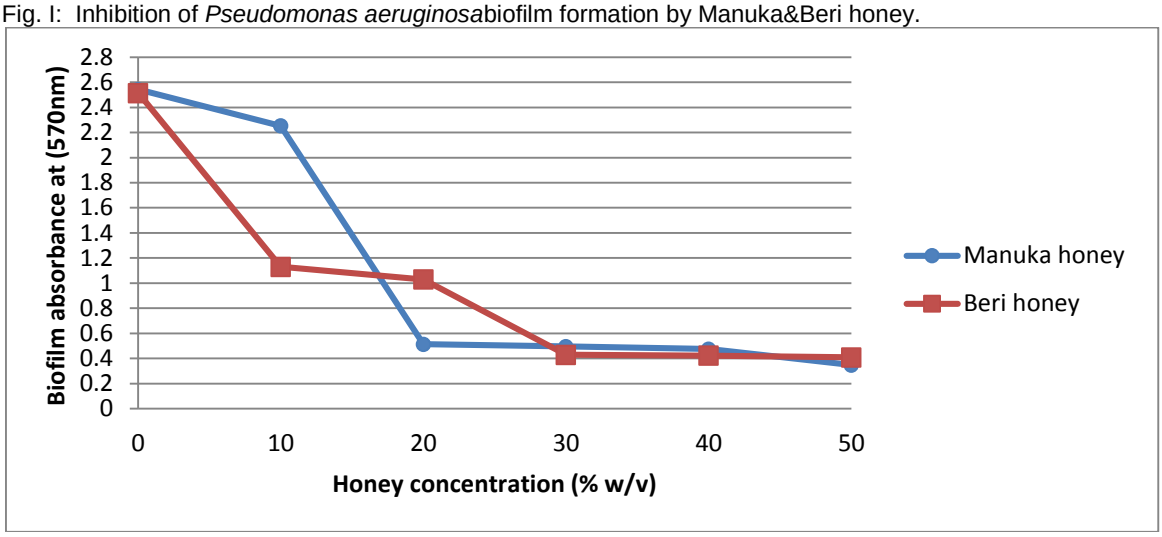
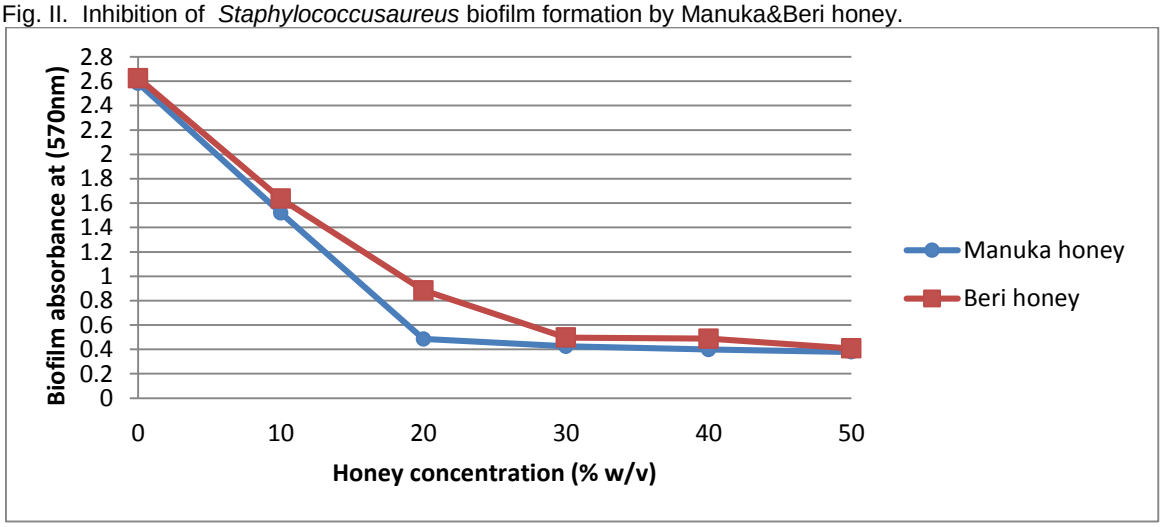


Fig. II. Inhibition of *Staphylococcusaureus* biofilm formation by Manuka&Beri honey.



DISCUSSION

The biofilm formed by the*Pseudomonasaeruginosa and Staphylococcus aureus*is resistant to many antibiotics. This contributes to its ability to resistance of the antibiotic therapy. The host thus becomes carrier to many diseases. These carriers are then capable of transmitting infection to the community through direct contact or indirectly by contaminating food or water. The resisistance to antibiotic is very common in biofilm producing organism. As a result, it had become verydifficult and expensive for physicians to treat biofilm, especially in underdeveloped countries like Pakistan, where thebioflim related diseases are usually in a large number. So it is necessary to find such methods that disrupt these biofilms by natural products having more antibacterial properties. Honey has been recognized a good antibacterial, healing and antioxidant natural product and said to possess the capability to inhibit the biofilm formation as well as disruption of the formed biofilm [20]. This study mainly evaluates the effect of Beri and Manuka honey in vitro on the biofilm formation and inhibition.This may be the first study in Pakistan regarding antibiofilm activity of Beri and Manuka honey to the best of our knowledge. Manuka honey is approved by FDA, and Beri Honey is Pakistan locah honey which is dark in colourused in this study. These both honeypossesses the best antibacterial and antibiofilm activity against MDR *organisms*[21]. It is clear in the present study that ATCC strains of *P. aeruginosa* and *S. aureus* can make a strongly adherent biofilms in vitro as well as in vivo. Talal*et al;* 2009 conducted a study in which Sidar and Manuka honey were evaluated for their efficacy in disruption of the established biofilm. They took Ten clinical isolates of *MRSA, MSSA* and *P. aeruginosa*with one reference strain for each. It was found that 1:2 dilution of both honeys were more

effective in inhibiting the planktonic bacteria than in the biofilm. It was, however, not clear in this study whether honey was used in (w/v) or (v/v) concentration[20].

Merckoll*et al*; 2009 used Medi honey and Norwegian honey in their study for disruption of biofilm formed by the *MRSA, MRSE, Klebsiella* and *P. aeruginosa.* According to their results both honeys have the antibiofilm activity against all strains of the tested bacteria. Medi honey disrupted the biofilm of MRSA and MRSE at 6% while ESBL and *P. aeruginosa* at 12%. Norwegian honey disrupted the biofilm of the same test strains at 12% and 25% respectively[17]. These two honeys also inhibited planktonic cultures of same test strain at 3% (w/v) concentration. This study differs however, from our study in two aspects. Firstly they worked only on established biofilm whereas we worked on inhibition of biofilm formation as well as the already formed biofilm. In another study (Okhiria*et al*; 2009) biofilm of six clinical isolates of *P. aeruginosa* were disrupted in time and concentration dependant way. In this study, *P. aeruginosa* biofilm when treated with 40% (w/v) Manuka honey showed significantly reduced biofilm biomass for all cultures. These results are consistent study[22]. The overall antibiofilm and antibacterial activity of both honey is synergistic effect of Methylglyoxal (MGO),H_2O_2,mainly fructose sugar components, phenolic compounds , acids and many minerals. MGO and fructose component are important factors which are found to be interfering with biofilm [17]. Further research and investigations of the effect of honey on the cell cycle, specific component in bacterial adhesion, biofilm and bacterial communication Quorum sensing may be useful in producing new classes of antibiotics.

CONCLUSIONS

The findings in this study help the fact that Manuk as well as Beri honey have the ability of inhibition and disruption the biofilm formed in vitro. This work also strongly support that honey whetherlocal or from outside, possesses good antibiofilm and antibacterial activity.

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