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Comparative Antibacterial and Antibiofilm Activities of Manuka Honey and Egyptian Clover Honey

Hisham A. Abbas
Department of Microbiology and Immunology
Faculty of PharmacyZagazig University
Zagazig- Egypt
Email: h_abdelmonem {at} yahoo.com

ABSTRACT--- ***The antibacterial and antibiofilm activities of Manuka and Egyptian clover honey against five clinical isolates of each of Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella spp. and Proteus mirabilis isolated from diabetic foot ulcers were compared. The antibacterial activity was estimated by determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Manuka honey showed higher antibacterial activities than clover honey as shown by lower MIC and MBC values. Pseudomonas aeruginosa and Proteus mirabilis showed higher sensitivity to manuka honey than Klebsiella spp. and Staphylococcus aureus. Both types of honey showed bactericidal activities against all tested strains.***

The antibiofilm activities were investigated against one strong biofilm forming isolate of each strain. The antibiofilm activities of Manuka honey were significantly more potent than clover honey. The biofilm inhibiting activity of manuka honey was greater against Klebsiella spp., Proteus mirabilis and Pseudomonas aeruginosa. Clover honey showed greater biofilm inhibiting activity against Klebsiella spp. and Proteus mirabilis. Biofilm disrupting activity of manuka honey was higher against Pseudomonas aeruginosa and Proteus mirabilis, while clover exerted higher biofilm eradication activity against Klebsiella spp. and Pseudomonas aeruginosa.

This study suggests the use of manuka and Egyptian clover honey for treatment of diabetic foot infections. Manuka honey was more effective but Egyptian clover honey is cheaper.

Keywords--- Manuka honey; Clover honey; antibacterial; antibiofilm; diabetic foot infection

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1. INTRODUCTION

Diabetic patients suffer from different complications including neuropathy and vascular problems that contribute to the occurrence of diabetic foot infections. These infections are hazardous and can end with diabetic gangrene and amputation of lower limbs [1,2].

Many bacteria can infect diabetic wounds and the most common ones are *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pnuemoniae*, *Escherichia coli* and *Proteus mirabilis* [3-5]. Infected wounds in patients with impaired immunity or circulation as in diabetic patients may be non-healing and chronic. Biofilms play an important role in diabetic foot infections. Biofilms are communities of sessile cells that are attached to a surface and enclosed within a matrix and they show high antimicrobial resistance [6,7]. The increase in emergence of antibiotic resistance among bacteria infecting wounds complicates the treatment. As a consequence, alternative therapeutic options are urgently needed to treat diabetic foot ulcers [8,9].

Honey has a broad spectrum of activity against both Gram-positive and Gram-negative bacteria. Moreover, it could inhibit biofilm formation and eradicate established biofilms [10,11]. Manuka honey is produced from *Leptospermum scoparium* (manuka) plant from New Zealand. It is a promising tool in the fight against bacteria. It has a broad antibacterial spectrum and could inhibit multi-drug resistant bacteria [12-14]. Moreover, it was found to inhibit biofilm formation and to remove pre-formed biofilms [15,16]. No resistance to manuka honey was reported [12-14]. More beneficial activities of manuka honey are its stimulating effect on immunity and wound healing [17]. Egyptian clover honey was reported to inhibit the growth of *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* and *Proteus mirabilis* [18,19].

The aim of the present study is to compare the antimicrobial and antibiofilm activities of manuka and Egyptian clover honeys against bacteria isolated from diabetic foot infections.

2. MATERIALS AND METHODS

2.1 Bacterial Strains

Five clinical isolates of each of *Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella spp.* and *Proteus mirabilis* isolated from diabetic foot ulcers were obtained from the stock culture collection of the Department of Microbiology and Immunology, Faculty of Pharmacy, Zagazig University.

2.2 Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The minimum inhibitory concentrations of manuka and clover honeys were determined by the broth microdilution method according to Clinical Laboratory and Standards Institute Guidelines (CLSI) [20]. Each well of a 96-well microtiter plate containing 50 µl of a series of dilutions of honeys (Manuka honey, Manuka health New Zealand Ltd., Te Awamutu, New Zealand and Clover honey, Isis Company, Egypt) in Muller-Hinton broth (Oxoid, Hampshire, England) was inoculated with 50 µl of bacterial suspensions to have a final inoculum of $5x10^5$ CFU/ml. The plates were incubated at 37 ºC for 20 h, and the lowest concentration of honey that showed no visible growth in the wells was considered as the MIC.

The minimum bactericidal concentrations of honeys were determined by adding 10µl of broth from the wells with no growth to plates of Mueller Hinton agar and incubating the plates for 24h at 37°C. The lowest concentration that caused 99.99% reduction in growth as shown by absence of growth or the appearance of less than five colonies was considered as the MBC. The bactericidal activities of honeys were determined by comparing MBC to MIC. Honey exerted bactericidal activity if the ratio of MBC to MIC is ≤ 4.

2.3 Assessment of Biofilm Production and Antibiofilm Activities of Manuka and Clover Honey

The method proposed by Stepanovic *et al*. [21] was used with some modifications. Bacterial strains were allowed to grow overnight in Tryptone soya broth (TSB) (Oxoid, Hampshire, England) and diluted to a cell density of 1×10^6 CFU/ml and 200 µl of bacterial suspensions were added to the wells of polystyrene 96-well microtiter plates. To form biofilms, the plates were incubated at at 37 ºC for 24 h. To evaluate the effect of honeys on biofilm synthesis, the same procedure was followed with the exception that sub-MICs of honeys were added to TSB to have a final concentration of 1/2 MICs of honeys. Planktonic bacteria were removed and the wells were washed three times with phosphate buffered saline (PBS). Adherent bacteria were fixed with 99% methanol and stained with 1% crystal violet solution. Excess dye was washed off with water and the plates were dried in air. The bound dye was solubilized with 95% ethanol and the adherent biofilms were quantified spectrophotometrically with a spectrofluorimeter (Biotek, USA) at 490 nm. The test was repeated three times and the mean optical densities were calculated and the percentages of biofilm inhibition were calculated.

To assess the biofilm eradicating activities of honeys, wells with established biofilms were aseptically washed three times with phosphate buffered saline (PBS) to remove planktonic bacteria , and 200 µl of 1/2 MICs of honeys in TSB were added. After 24 h incubation at 37°C, the honey solutions were removed and each well was washed three times with PBS. The adherent biofilms were quantified spectrophotometrically as in assessment of biofilm formation. The test was repeated twice. The percentages of biofilm eradication were estimated.

2.4 Statistical Analysis

The activities of manuka honey and clover honey on biofilm inhibition and biofilm eradication were compared by Two Way ANOVA followed by Benforroni post hoc test, Graph Pad Prism 5. P values <0.05 or <0.001 were considered statistically significant.

3. RESULTS

3.1 Antibacterial Activity of Manuka and Clover Honeys

Both types of honey showed antibacterial activity against all tested strains (Table 1). Manuka honey was more active than clover honey. The MICs of manuka honey ranged between 5% and 20% for Pseudomonas *aeruginosa* and *Proteus mirabilis*, 20% and 40% for *Staphylococcus aureus* and 5% and 40% for *Klebsiella spp.*

Table 1: Antibacterial activity of manuka and clover honeys

Isolate No.	Manuka honey			Clover honey		
	MIC (%)	MBC (%)	Bactericidal activity	MIC (%)	MBC (%)	Bactericidal activity
PA1	20	40	+	40	40	+
PA2	5	5	+	40	40	+
PA3	5	5	+	40	40	+
PA4	20	20	+	40	40	+
PA5	20	20	+	40	40	+
SA1	20	20	+	40	40	+
SA2	40	40	+	40	40	+
SA3	20	20	+	40	40	+
SA4	40	40	+	40	40	+
SA5	20	20	+	40	40	+
PM1	5	5	+	40	40	+
PM2	20	40	+	40	40	+
PM3	5	5	+	40	40	+
PM4	20	40	+	40	40	+
PM5	5	5	+	40	40	+
KL1	5	10	+	40	40	+
KL2	20	20	+	40	40	+
KL3	40	40	+	40	40	+
KL4	40	40	+	40	40	+
KL5	20	40	+	40	40	+

PA, *Pseudomonas aeruginosa*; SA, *Staphylococcus aureus*; PM, *Proteus mirabilis*; KL, *Klebsiella spp*.

The MBCs of manuka honey ranged between 5% and 40% for *Pseudomonas aeruginosa*, 20% and 40% for *Staphylococcus aureus*, 5% and 40% for *Proteus mirabilis* and 10% and 40% for *Klebsiella spp*. The MIC and MBC of clover honey were 40% for all strains. Both types of honey exerted bactericidal activities against all tested strains (MBC/MIC≤4).

3.2 Inhibition of Biofilm Formation

The effect of manuka and Egyptian clover honey was investigated against one strong biofilm isolate belonging to each of the bacteria tested. Both types of honey could inhibit biofilm formation by all tested strains (Table 2). Manuka honey was more potent in biofilm inhibition (90.89±5.77 to 97.7±0.44) as compared to clover honey (70.68±1.97 to 85.86±3.41).

Table 2: Biofilm inhibiting activities of manuka and clover honeys

Isolate No.	% inhibition of biofilm formation (Mean±SD)	
	Manuka honey	Clover honey
SA4	95.85±1.35	70.68±1.97
PA5	90.89±5.77	74.19±5.69
PM2	96.61±1.03	85.86±3.41
KL4	97.7±0.44	85.64±0.80

PA, *Pseudomonas aeruginosa*; SA, *Staphylococcus aureus*; PM, *Proteus mirabilis*; KL, *Klebsiella spp*.

3.3 Eradication of Established Biofilms

Both types of honey showed the ability to remove biofilms formed by all tested strains (Table 3). Manuka honey showed greater biofilm disrupting activity (72.20±1.90 to 77.62±4.22) than clover honey (40.33±0.24 to 63.67±3.31).

Table 3: Biofilm eradicating activities of manuka and clover honeys

Isolate No.	% eradication of biofilm formation (Mean±SD)	
	Manuka honey	Clover honey
SA4	72.20±1.90	40.33±0.24
PA5	77.62±4.22	55.60±5.81
PM2	74.64±1	50.67±3.08
KL4	72.98±2.56	63.67±3.31

PA, *Pseudomonas aeruginosa*; SA, *Staphylococcus aureus*; PM, *Proteus mirabilis*; KL, *Klebsiella spp*.

4. DISCUSSION

Honey was used as an efficient remedy for treating infected wounds since ancient civilizations [22]. Diabetic foot ulcers represent a therapeutic challenge because of antibiotic resistance and biofilm formation [23]. Honey was beneficial in management of wound infections that do not respond to antibiotics [24] and it can also remove pre-formed biofilms and inhibit biofilm formation [25].

In the present study both manuka and clover honeys showed good activity against planktonic and biofilm cells of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella spp*., and *Proteus mirabilis*. Manuka honey was more active as antibacterial agent than clover honey. *Pseudomonas aeruginosa* and *Proteus* mirabilis were more sensitive to manuka honey than *Klebsiella spp* and *Staphylococcus aureus*, while all tested strains showed the same susceptibility to clover honey. Both types of honey were found to be bactericidal.

Honey was reported to have good activity against bacteria that commonly infect diabetic foot ulcers such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pnuemoniae* and *Proteus mirabilis* [3-5]. The antibacterial activity of honey is attributed to several factors including mild acidity [23], high osmolarity [26], hydrogen peroxide [27], polyphenols [1], antioxidants [28], antibiotic peptides [14] and methylglyoxal [29]. The antibiofilm activity may originate from its quorum sensing inhibiting activity [30]. Quorum sensing controls biofilm formation [31].

The wound healing activity of honey may be a function of the infection barrier provided by its high viscosity, its low pH, hydrogen peroxide content and the moist wound environment maintained by honey that enhance wound healing [10]. Honey may be advantageous for treating wound infections because of its dual antimicrobial and wound healing activities.

In this study, both types of honey showed significant antibiofilm activities as compared to the control ($P<0.001$). Moreover, Manuka honey was significantly more potent than clover honey regarding biofilm inhibition ($P<0.001$). The biofilm inhibiting activity of manuka honey was greater against *Klebsiella spp*., *Proteus mirabilis* and *Pseudomonas aeruginosa* than against *Staphylococcus aureus*. On the other hand, clover honey showed greater biofilm inhibiting activity against *Klebsiella spp.* and *Proteus mirabilis* than against Pseudomonas *aeruginosa* and *Staphylococcus aureus*. Moreover, biofilm disrupting activity of manuka honey was more statistically significant than that of clover honey ($P<0.001$ for *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Proteus mirabilis* and $P<0.05$ for *Klebsiella*). Manuka honey showed a greater biofilm eradicating activity against *Pseudomonas aeruginosa* and *Proteus mirabilis* than against *Staphylococcus aureus* and *Klebsiella spp.* On the contrary, clover could remove biofilms of *Klebsiella spp* and *Pseudomonas aeruginosa* more efficiently than those of *Proteus mirabilis* and *Staphylococcus aureus*.

The antibacterial and antibiofilm activities of manuka honey and clover honey were reported in different studies. Clover honey showed good antibacterial activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus* [32]. Egyptian clover honey (20.3%) could inhibit the growth of *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* [19]. Moreover, *Proteus mirabilis* was inhibited by Egyptian clover honey at a concentration of 35% [18]. In a clinical trial on 30 patients with infected diabetic foot ulcers, the use of Egyptian clover honey dressings resulted in complete healing in 43.3% of ulcers, significant reduction in the size of ulcers in 43.3% of patients and lowering of the bacterial burden in all ulcers after the first week of therapy [33].

In agreement with this study, manuka honey exerted bactericidal activity against planktonic and biofilm cells of *Pseudomonas aeruginosa* and *Staphylococcus* honey *aureus* and its antibiofilm activity against *Pseudomonas aeruginosa* was higher than that against *Staphylococcus aureus* [34]. Manuka honey also was reported to inhibit enteric bacteria [35]. Methylglyoxal, a component of manuka honey was found to inhibit *Staphylococcus aureus* biofilms [36].

In summary, manuka and Egyptian clover honeys can be used for treatment of diabetic foot infections. Manuka honey is more active as antibacterial and antibiofilm agent than Egyptian clover honey, while clover has the advantage of low cost.

5. REFERENCES

[1] A.M. Aljadi, K.M. Yusoff, "Isolation and identification of phenolic acids in Malaysian honey with antibacterial properties", Turkish Journal of Medical Sciences, vol. 3, pp.229–236, 2003.
[2] M.P. Khanolkar, S.C. Bain, J.W. Stephens, "The diabetic foot", QJM, vol.101, No. 9, pp.685–695, 2008.
[3] R.A. Cooper, E. Halas, P.C. Molan, "The efficacy of honey in inhibiting strains of *Pseudomonas aeruginosa* from infected burns", The Journal of Burn Care& Rehabilitation, vol. 23, No. 6, pp.366–370, 2002.
[4] H. Estrada, M. Gamboa, M. Mdel, L. Arias, C. Chaves, "Evaluation of the antimicrobial action of honey against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella enteritidis*, *Listeria monocytogenes* and *Aspergillus niger*. Evaluation of its microbiological charge", Archivos latinoamericanos de nutrición, vol. 55, No. 2, pp.167–171, 2005.
[5] E. Bansal, A. Garg, S. Bhatia, A.K. Attri, J. Chander, "Spectrum of microbial flora in diabetic foot ulcers", Indian Journal of Pathology& Microbiology,vol. 51, No. 2, pp.204–208, 2008.

[6] S.E. Dowd, R.D. Wolcott, Y. Sun, T. McKeehan, E. Smith, D. Rhoads, "Polymicrobial nature of chronic diabetic foot ulcer biofilm infections determined using bacterial tag encoded FLX amplicon pyrosequencing (bTEFAP)", PLoS One, vol. 3, No. 10, pp.e3326, 2008.

[7] G. James, E. Swogger, R. Wolcott, E. Pulcini, P. Secor, J. Sestrich, "Biofilms in Chronic wounds", Wound Repair and Regeneration, vol. 16, No. 1, pp.37–44, 2008.

[8] S.J. Projan, P.J. Youngman, "Antimicrobials: new solutions badly needed", Current Opinion in Microbiology, vol. 5, No. 5, pp.463–465, 2002.

[9] J.J. Engemann, Y. Carmeli, S.E. Cosgrove, V.G. Fowler, M.Z. Bronstein, S.L. Trivette, J.P. Briggs, D.J. Sexton, K.S. Kaye, "Adverse clinical and economic outcomes attributable to methicillin resistance among patients with *Staphylococcus aureus* surgical site infection", Clinical Infectious Diseases, vol. 36, No. 5, pp.592–598, 2003.

[10] P.E.Lusby, A.L. Combes, J.M. Wilkinson, "Honey – a potential agent for wound healing?", Journal of Wound, Ostomy and Continence Nursing, vol. 29, No. 6, pp.296–300, 2002.

[11] J. Irish, S. Blair, D.A. Carter, "The antibacterial activity of honey derived from Australian flora", PLoS One, vol. 6, No. 3, pp.e18229, 2011.

[12] S.E. Blair, N.N. Cokcetin, E.J. Harry, D.A. Carter, "The unusual antibacterial activity of medical-grade Leptospermum honey: antibacterial spectrum, resistance and transcriptome analysis", European Journal of Clinical Microbiology& Infectious Diseases, vol. 28, No. 10, pp.1199–1208, 2009.

[13] A.F. Henriques, R.E. Jenkins, N.F. Burton, R.A. Cooper, "The intracellular effects of manuka honey on *Staphylococcus aureus*", European Journal of Clinical Microbiology& Infectious Diseases, vol. 29, No. 1, pp.45–50, 2010.

[14] P.H. Kwakman, L. de Boer, C.P. Ruyter-Spira, T. Creemers-Molenaar, J.P. Helsper, C.M. Vandenbroucke-Grauls , S.A. Zaat , A.A. te Velde , "Medical-grade honey enriched with antimicrobial peptides has enhanced activity against antibiotic-resistant pathogens", European Journal of Clinical Microbiology& Infectious Diseases, vol. 30, No. 2, pp.251–257, 2011.

[15] P. Merckoll, T.O. Jonassen, M.E. Vad, S.L. Jeansson, K.K. Melby, "Bacteria, biofilm and honey: a study of the effects of honey on 'planktonic' and biofilm embedded chronic wound bacteria", Scandinavian Journal of Infectious Diseases, vol. 41, No. 5, pp.341–347, 2009.

[16] S.E. Maddocks, M.S. Lopez, R.S. Rowlands, R.A. Cooper, "Manuka honey inhibits the development of *Streptococcus pyogenes* biofilms and causes reduced expression of two fibronectin binding proteins", Microbiology, vol. 158, No. 3, pp.781–790, 2012.

[17] S. Gannabathula, M.A. Skinner, D. Rosendale, J.M. Greenwood, A.N. Mutukumira, G. Steinhorn , J. Stephens, G.W. Krissansen , R.C. Schlothauer , "Arabinogalactan proteins contribute to the immunostimulatory properties of New Zealand honeys", Immunopharmacology and Immunotoxicology, vol. 34, No. 4, pp.598–607, 2012.

[18] H. El-Fadaly, F.S. Abdilla, E.E.Y. El-Badrawy, "Comparative study between Yemeni and Egyptian types of honey by means of antibacterial activity", Pakistan Journal of Biological Sciences, vol. 2, No. 1, pp.1–6, 1999.

[19] A.G. Hegazy, "Antimicrobial activity of different Egyptian honeys as comparison of Saudi Arabia honey", Research Journal of Microbiology, vol. 6, pp.488–495, 2011.

[20] Clinical and Laboratory Standards Institute, Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard, 7th edition, CLSI Document M7-A7. Wayne, PA, 2006.

[21] S. Stepanovic, D. Vukovic, I. Dakic, B. Savic, M. Svabic-Vlahovic, "A modified microtiter-plate test for quantification of staphylococcal biofilm formation", Journal of Microbiological Methods, vol. 40, No. 2, pp.175–179, 2000.

[22] V. Falanga, "Wound healing and its impairment in the diabetic foot", Lancet, vol. 366, No. 9498, pp.1736–1743, 2005.

[23] P.C. Molan, "The antibacterial activity of honey. 1. The nature of the antibacterial activity", Bee World, vol. 73, No. 1, pp.5–28, 1992.

[24] S. Efem, "Clinical observations on the wound healing properties of honey", The British Journal of Surgery, vol. 75, No. 7, pp. 679–681, 1988.

[25] R. Saraf, V. Bowry, D. Rao, P. Saraf, P.C. Molan, "The antimicrobial efficacy of Fijian honeys against clinical isolates from diabetic foot ulcers", Journal of ApiProduct and ApiMedical Science, vol. 1, No. 3, pp.64–71, 2009.

[26] H.A.L. Wahdan, "Causes of the antimicrobial activity of honey", Infection, vol. 26, No. 1, pp.26–31, 1998.

[27] K. Brudzynski, "Effect of hydrogen peroxide on antibacterial activities of Canadian honeys", Canadian Journal of Microbiology, vol. 52, No. 12, pp.1228–1237, 2006.

[28] K. Brudzynski, D. Miotto, "Honey melanoidins. Analysis of a composition of the high molecular weight melanoidin fractions exhibiting radical scavenging capacity", Food Chemistry, vol. 127, No. 3, pp.1023–1030, 2011.

[29] C.J. Adams, C.H. Boult, B.J. Deadman, J.M. Farr, M.N. Grainger, M. Manley-Harris, M.J. Snow, "Isolation by HPLC and characterisation of the bioactive fraction of New Zealand manuka (*leptospermum scoparium*) honey", Carbohydrate Research, vol. 343, No. 4, pp.651-659

[30] R. Wang , M. Starkey , R. Hazan , L.G. Rahme , "Honey's ability to counter bacterial infections arises from both bactericidal compounds and QS inhibition" Frontiers in Microbiology, vol. 3, pp.144, 2012.

[31] R. Daniels, J. Vanderleyden, J. Michiels, "Quorum sensing and swarming migration in bacteria", FEMS Microbiology Reviews, vol. 28, No. 3, pp.261–289, 2004.
[32] E.A. Khairy, R.H. Hedia, S.M. Dorgham, M. Effat, "Comparative studies on antimicrobial activities (AMA) of different types of honey using bacteria from animal origin", International Journal of Microbiological Research, vol. 4, No. 1, pp.50–55, 2013.
[33] A.M. Moghazy, M.E. Shams, O.A. Adly, A.H. Abbas, M.A. El-Badawy, D.M. Elsakka, S.A. Hassan, W.S. Abdelmohsen, O.S. Ali, B.A. Mohamed, "The clinical and cost effectiveness of bee honey dressing in the treatment of diabetic foot ulcers", Diabetes Research and Clinical Practice, vol. 89, No. 3, pp.276 – 781, 2010.
[34] T. Alandejani, J. Marsan, W. Ferris, R. Slinger, F. Chan, "Effectiveness of honey on *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilms" Otolaryngology and Head and Neck Surgery, vol. 141, No. 1, pp.114–118, 2009.
[35] S.M. Lin, P.C. Molan, R.T. Cursons, "The controlled *in vitro* susceptibility of gastrointestinal pathogens to the antibacterial effect of manuka honey", European Journal of Clinical Microbiology& Infectious Diseases, vol. 30, No. 4, pp.569–574, 2011.
[36] J. Jervis-Bardy, A. Foreman, S. Bray, L. Tan, P.J. Wormald, "Methylglyoxal-infused honey mimics the anti-*Staphylococcus aureus* biofilm activity of manuka honey: potential implication in chronic rhinosinusitis", Laryngoscope, vol. 121, No. 5, pp.1104–1107, 2011.