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Surface colonization when exposed to medical grade (Manuka) honey alone or in combination with other disinfectants

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Abstract

This study explored the use of medical grade (Manuka) honey as a surface disinfectant for *methicillin-resistant Staphylococcus aureus* (MRSA). The killing mechanism of this honey is almost entirely due to the presence and activity of methylglyoxal, H_2O_2, and other reactive oxygen species producing agents. To enhance the activity of Manuka, this study further added porphyrins, or light activated chemicals that produce reactive oxygen species. Results of this in vitro study showed that after 24 hours, a solution with 1mL of 1% Manuka honey reduced MRSA colonization by 1 log when seeded at a starting concentration of 10^8 colony forming units (CFU/mL) of MRSA. Similar results were found when this Manuka honey was paired with: 1) 1mL of a 1% v/v isopropyl alcohol solution, 2) 1mL of a 1% v/v isopropyl alcohol + 1mL of a porphyrin solution (0.01mg/mL Zn porphyrin solution), and 3) 1mL of 100mg of citric acid added to the solution that contained Manuka honey, isopropyl alcohol, and the porphyrin solution. All formulations with Manuka honey demonstrated antibacterial behavior. Moreover, it should be noted that all samples, between Manuka honey alone, Manuka in addition to isopropyl alcohol, Manuka in addition to isopropyl alcohol and porphyrins, and Manuka + isopropyl alcohol + porphyrins + citric acid were all deemed stable (after heat assays) at durations of one hour, one day, and one week. In this manner, the present study indicates that Manuka honey could be a suitable, safe, environmentally friendly, and effective MRSA disinfectant for everyday household use and should be further investigated either as a stand alone disinfectant or in combination with other disinfectants.

Keywords: Manuka, Honey, Porphyrins, MRSA, Disinfectant

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Introduction

For this study, *methicillin-resistant Staphylococcus aureus* was used as a bacterial sample to ascertain the antimicrobial efficacy of Manuka honey (1-3). *Methicillin-resistant Staphylococcus aureus* (MRSA) bacteria was, according to a 2017 poll, responsible for over 19,000 deaths in the United States alone (4). Extensive research has been conducted to mitigate the negative health impacts of MRSA, but due to its robust resistance to antibiotics (namely methicillin, which is in part where the bacterial species gets its name), this has been a daunting task, with recent evidence indicating that MRSA is evolving quickly enough to circumvent antibiotics that have recently been given commercial clearance (5). In fact, the U.S. Centers for Disease Control have indicated that by 2050, every 3 seconds someone will die from an antibiotic-resistant bacteria (6).

Clearly, MRSA and other antibiotic resistant bacteria present a clear and present danger to healthcare now and in the near future. While MRSA can be possibly treated with the antibiotics trimethoprim-sulfamethoxazole, clindamycin, minocycline, linezolid, or doxycycline there are significant extensive side effects of using such antibiotics such as blistering, peeling, or loosening of the skin; bloating; chills; clay-colored stools; constipation; cough; dark urine; decreased appetite; diarrhea (watery and severe, which may also be bloody); difficulty with swallowing; dizziness; fast heartbeat; feeling of discomfort; fever; headache; hives; itching; puffiness or swelling of the eyelids or around the eyes, face, lips, or tongue; hives or welts itching or rash; increased thirst; indigestion; inflammation of the joints; joint or muscle pain; large, hive-like swelling on the face, eyelids, lips, tongue, throat, hands, legs, feet, or sex organs; loss of appetite; nausea; numbness or tingling of the face, hands, or feet; pain in the stomach, side, or abdomen possibly radiating to the back; red skin lesions (often with a purple center); redness and soreness of the eyes; redness of the skin; sore throat; sores, ulcers or white spots in the mouth or on the lips; stomach cramps; stomach pain or tenderness; swelling of the feet or lower legs; swollen, painful, or tender lymph glands in the neck, armpit, or groin; tightness in the chest; unusual tiredness or weakness; unusual weight loss; vomiting; and/or yellow eyes or skin among others (7, 8).

Looking to the future, a future in which the sustainability of the active ingredients is deemed of equal paramount importance as the efficacy of the active ingredients themselves, Manuka honey, a monofloral honey derived from the manuka tree (*Leptospermum scoparium*), presents itself as a very strong candidate as a natural material to mitigate bacterial growth, both MRSA and otherwise (9). In fact, in recent studies of compounds similar in nature to Manuka honey, this medical grade honey was the most efficacious in reducing bacterial colonization. Moreover, this antimicrobial activity was evident at concentrations of both 10% and 20% v/v (10). Manuka honey is composed of dihydroxyacetone, leptosperin, and methylglyoxal which have all been studied individually for their antibacterial properties (9). Manuka honey has been studied for a wide range of applications from fighting bacteria to inhibiting cancer to even promoting tissue regeneration (11). However, Manuka honey may have some side effects such as allergic reaction, hyperglycemia, and contraindications with other food and drugs (11).

The use of Manuka to reduce bacterial spreading is nothing novel and this falls into a long line of naturally derived materials that have exhibited antibacterial activity and a reduction in bacterial proliferation. For example, ginseng and ginseng extract have both been long studied antimicrobial materials that are derived from naturally occurring, organic ingredients (12). Mechanistically, ginseng is able to alter the motility of certain bacteria and viruses, as well as disrupt biofilms that might be relevant to either of the microbe groups (12). Moreover, ginger has proven to be extremely efficacious at disrupting

bacterial replication under lab circumstances, and is often used in traditional homeopathic remedies to infection, in a similar vein as ginseng. Ginger has a myriad of medicinal uses, including but not limited to anti-tumor activity through cellular apoptosis, general antimicrobial activity, and inflammation reduction. Further research is required to holistically understand the full scope of antimicrobial mechanisms at play with respect to Manuka honey, but it is well understood that MGO and H_2O_2 play a significant role (11). In a comparison among different honeys, studies have shown that Revamil honey was the least effective medical-grade honey and comparing minimum inhibitory concentrations (MICs) to Manuka honey showed that, with regard to *S. aureus*, *E. coli* and *K. pneumoniae*, the Manuka honeys often had lower MICs (11).

Furthermore, another promising natural material for antibacterial applications includes porphyrins. Porphyrins are light activated plant-derived materials that can be assembled into nano-dimensional clusters to produce reactive oxygen species which can kill bacteria. Results of our prior in vitro study demonstrated that after 24 hours, both Zn and Fe nano porphyrins at 1 to 0.0001 mg/mL concentrations and when adsorbed from a solution to a solid surface, reduced MRSA colonization by about 1.5 logs in a light-exposed environment; in a dark environment, a little over a 1.0 log reduction in MRSA colonization was observed (13). Lastly, both of the novel Zn and Fe nano porphyrins were stable for up to one week on surfaces and passed ISO 10993 tests (meaning no toxicity to fibroblasts were observed after 24 hours).

Through the use of a standard triplicate in vitro bacterial proliferation assay, this study provided valuable information regarding how efficacious Manuka honey is with respect to reducing bacterial growth, in addition to combining Manuka with conventional natural ingredients commonly associated with surface disinfectants (such as isopropyl alcohol and citric acid) as well as synthetic antibacterial compounds that produce reactive oxygen species (porphyrins). A concentration of 1% v/v Manuka honey was both used independent of other ingredients (meaning the solution contained solely dilute honey, cell culture growth media, and MRSA), as well as in combination with 1% v/v isopropyl alcohol, 100mg of citric acid, and 1mL of a 0.01mg/mL Zn porphyrin solution, all in different combinations. A control group was also used exclusively with cell culture growth medium and MRSA bacteria only. The findings of this study indicated an approximate 1 log reduction across all samples, with the highest bacterial reduction being 1.05 log in a sample with all ingredients mentioned in this study: citric acid, isopropyl alcohol, Zn porphyrins, and Manuka honey. That said, the other log reduction values were approximately equal to the highest concentration, with the well containing Manuka honey and isopropyl alcohol yielding a log value of 0.93, the well containing just Manuka honey yielding a log of 0.944, and the well containing Manuka + Zn porphyrin + isopropyl alcohol yielding a log value of 0.911.

Materials and Methods

Materials

Medical grade Manuka honey was obtained from Manuka Doctor (Auckland, NZ). Isopropyl alcohol, citric acid, and nitrile gloves were obtained from Sigma (St. Louis, MO, USA). Nano-porphyrins were synthesized and characterized as previously described (13-15). Briefly, water soluble Zn porphyrins were obtained commercially from Frontier Scientific, Inc. (C40022 Zn(II) Coproporphyrin III Tetrasodium Salt; Newark, DE, USA). For a typical preparation, 0.45 mL of a fresh stock of the porphyrin solution (0.01 M porphyrins dissolved in a 0.05 M HCl solution (Sigma) stirred for 30 min before using) was

quickly added into 9.1 mL of a continuously stirred aqueous solution of cetyl trimethylammonium bromide (0.011M CTAB; Aldrich; Burlington, MA, USA) and NaOH (0.0027 M; Sigma) at room temperature (25 °C). Then, the mixture was stirred for 24 hours. The green solution was centrifuged at 12000 rpm (Sigma) and washed twice with Millipore water (Sigma) to remove the surfactants. The nano porphyrins were then lyophilized (Millrock Technologies, Kingston, NY, USA) until used later in experiments. Before their use, they were sterilized via UV light exposure for 1 hour.

With these materials, the following solutions were made and tested in this study (Table 1).

Table 1: Different Solutions Used in Bacterial Assays

Media (Positive Control)	**Nitrile Glove (Negative Control)**	**Manuka Honey**	**Manuka Honey + Isopropyl Alcohol**	**Media + Porphyrin + Isopropyl Alcohol**	**Porphyrin + Citric Acid+ Isopropyl Alcohol + Manuka Honey**
- 1 mL Media -3 mL Water	- 1 mL Media - 3 mL Water - Nitrile glove control	- 1 mL Media - 2 mL Water - 1 mL Dissolved Manuka Honey*	- 1 mL Media - 1 mL Water - 1 mL Isopropyl Alcohol (1% v/v) - 1 mL Dissolved Manuka Honey*	- 1 mL Media - 1 mL Water - 1 mL Isopropyl Alcohol (1% v/v) - 1 mL Dissolved Manuka Honey*	- 1 mL Media - 1 mL Water - 1 mL Isopropyl Alcohol (1% v/v) - 100 mg Citric Acid (anhydrous powder) - 1 mL Dissolved Manuka Honey*

*Dissolved Manuka Honey was at a concentration of 100mg/mL.

MRSA

MRSA were obtained from ATCC (43300; Manassas, VA, USA) and cultured in standard cell culture media consisting of Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum (Sigma). This cell culture solution was used instead of saline broth since it is more representative of solutions MRSA would be present in on contaminated surfaces.

MRSA Assays

Standard microbiology assays were followed [13]. Using MRSA that were incubated at 37°C, 10^8 cells/mL (a volume equivalent to this concentration was determined prior to bacteria addition to wells using a hemocytometer) were added to each well. The well volume change was assumed negligible in future calculations and the medium with MRSA that was added was typically about 10µL added to 3mL of disinfectant solution.

At the end of the time period, bacteria remaining in the well were removed and counted using a manual hemocytometer (Sigma) and this cell number was run through calculations to determine the bacteria concentration per mL. To ensure uniformity, the same hemocytometer that was used to add the initial 10^8 cells/mL to each well was used to determine the concentration of cells after the assay was completed.

Stability Assays Using Contact Angles

Contact angles are an easy, inexpensive, and effective way to determine the stability of solutions [16]. Here, using a home-made contact angle system consisting of a camera (BestBuy) and laptop computer (Dell), the contact angle of the various nano porphyrin solutions of interest to the present study were measured after one hour, one day, and one week. For this, 10 ml of the solutions were placed on tissue culture polystyrene (Sigma) and images were obtained and contact angles calculated after 10 seconds of applying the liquid onto the surface. The contact angles were compared to each other and if the contact angles were similar, the stability of the nano porphyrin containing solutions was confirmed.

Results and Discussion

Manuka Honey Assays

Results gathered in this study demonstrate that Manuka honey is a viable antibacterial disinfectant, both with and without isopropyl alcohol, the Zn porphyrins, and citric acid, all solutions previously shown to decrease MRSA colonization on surfaces (Figure 1). There was no direct correlation between the amount of isopropyl alcohol present and the degree of antimicrobial efficacy, indicating that Manuka honey was a salient component in reducing bacterial growth, regardless of the presence or absence of other active ingredients.

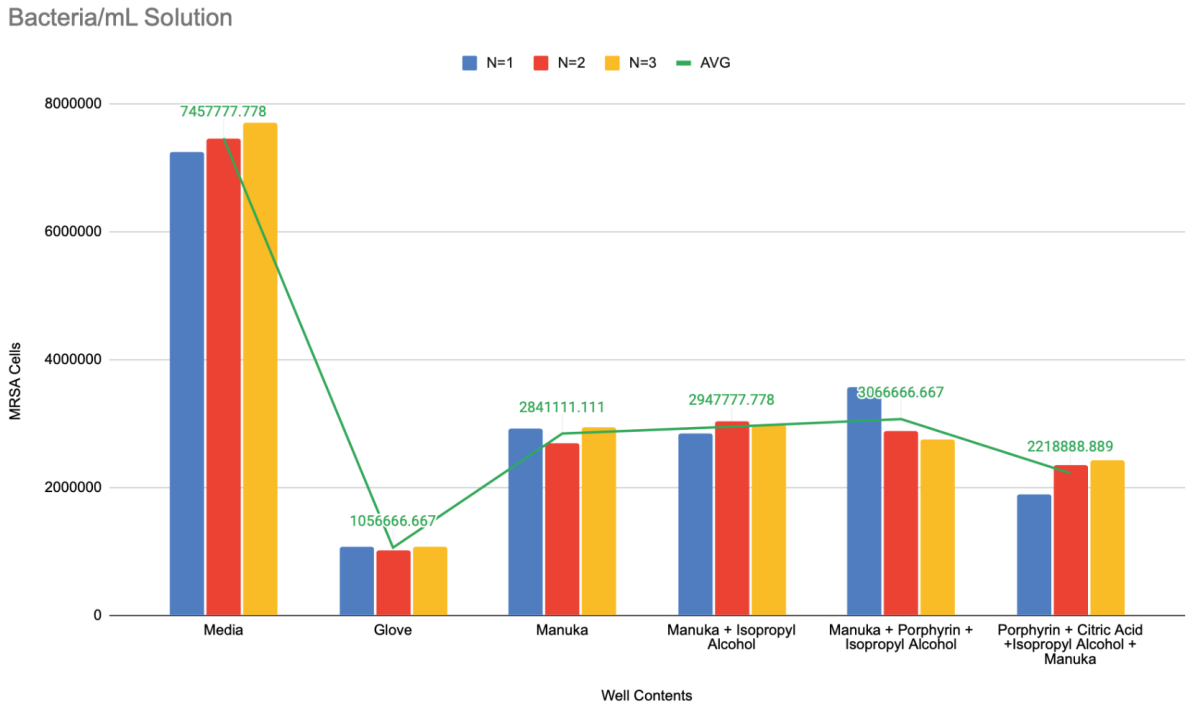

Figure 1: Manuka Honey (both with and without isopropyl alcohol, Zn porphyrins, and citric acid) Decreased MRSA Colonization After 24 Hours, with the sample containing citric acid surpassing log 1 colonization reduction. Data = mean +/- SEM; N = 3.

The most salient finding that should be noted in the above graphic is the degree of influence that Manuka honey had on the overall antimicrobial efficacy of the solution after a twenty four hour period in N=3 (triplicate). More specifically, even after the addition of other antimicrobial agents to the wells

in question, there was at most a fluctuation of a mere log 0.033 in the results between the non-control groups, likely indicating that the antibacterial activity is originating from the Medical Grade Manuka honey, and the assistance provided by the other means of antibacterial activity are marginal.

Relative to the MRSA growth media, which exhibited a log reduction value of only 0.52, the Manuka-based antimicrobial compounds exhibited log values that almost doubled the log value that was seen from Manuka alone, regardless of what other components were added in addition to the base ingredient of the honey. This further evidences the idea that Manuka honey, when applied to potential antimicrobial environments, could serve as an efficacious and environmentally friendly means of bacterial colonization reduction.

A supplemental assay was carried out to further examine the efficacy of Manuka honey with respect to antimicrobial activity; 100 mg of citric acid (Sigma) was added to a new mixture of porphyrins (0.01mg/mL Zn), 1%v/v isopropyl alcohol, and Manuka honey. This new mixture was again exposed to 10^8 MRSA cells in N=3 triplicate, with results indicating a notable increase in log reduction of bacterial colonization. With a log reduction value of 1.05, a noteworthy increase relative to the other antimicrobial solutions that were used in the prior assay, it can be deduced that there is a correlation between the diversity of antimicrobial compounds present in a given well during an assay and the degree of bacterial colony reduction after a 24 hour period, with Manuka honey being the driving force of this antibacterial activity.

Stability Assays Using Contact Angles

Results from the present study further confirmed that all Manuka honey samples were stable as the contact angles did not change between day one and a week later for the Honey-based solutions (both with and without the addition of isopropyl alcohol, citric acid, and porphyrins) on the tissue culture polystyrene.

Conclusion

This study explored the use of Medical Grade (Manuka) honey as an environmentally friendly effective surface disinfectant for *methicillin-resistant Staphylococcus aureus* (MRSA). Results of this in vitro study showed that after 24 hours, a solution with 1mL of 1% v/v Manuka honey reduced MRSA colonization by 1 log when seeded at a starting concentration of 10^8 colony forming units (CFU) of MRSA. Similar results were found when this Manuka honey was paired with: 1) 1mL of a 1% v/v isopropyl alcohol solution, 2) 1mL of a 1% v/v isopropyl alcohol + 1mL of a porphyrin solution (0.01mg/mL Zn porphyrin solution) and 3) 100mg of citric acid added to the solution that contained Manuka honey, isopropyl alcohol, and porphyrins. Moreover, it should be noted that all samples, between Manuka honey alone, Manuka in addition to isopropyl alcohol, Manuka in addition to isopropyl alcohol and porphyrins, and Manuka + isopropyl alcohol + porphyrins + citric acid were all deemed stable (after heat assays) at durations of one hour, one day, and one week. In this manner, the present study indicates that Manuka honey could be a suitable, environmentally friendly, safe and effective MRSA disinfectant for everyday household use and should be further investigated either as a stand alone disinfectant or in combination with other disinfectants.

Ethical Compliance

Not Applicable.

Conflicts of Interest

E.W. was an employee of Lunano, Inc. when these studies were completed and T.J.W. has equity in Lunano, Inc.

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