Original article

In vitro assessment of the antimicrobial potential of honey on common human pathogens

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Abstract

Background: Honey produced by honeybees (*Apis mellifera*) is one of the ancient traditional medicines used for treatment and prevention of various illnesses.

Objective: To assess the antimicrobial potential of honey on some common bacterial pathogen.

Methods: This experimental study was conducted in Jimma University, from February 10 – March 14, 2003. The Minimal Inhibitory Concentrations/ MIC and Minimal Bactericidal Concentrations/ MBC of two honey samples on *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Shigella shiga*, *Klebsiella aerogenes*, *Proteus vulgaris* and *Proteus mirabilis* was investigated by an agar dilution technique.

Results: The MIC of honey for 90% of test organism was 6.25% and 7.5% (V/V) for *P.aeruginosa*. The MBC of honey for 70% of the test organisms was again 6.25% (V/V). The MBC of honey for *S.shiga* (Standard test organism) and *P. aeruginosa* (both clinical isolates and control strain) was 7.5% (V/V).

Conclusions: Honey produced by honeybees (*Apis mellifera*) has both bacteriostatic and bactericidal activity when tested in vitro. However, Pharmacological standardization and clinical evaluation on the effect of honey are essential before using honey as a preventive and curative measure to common diseases related to the tested bacterial species.
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Introduction

In developing countries all over the world especially in Africa, large number of people die daily of preventable and curable diseases because of lack of even simple health care (1). Despite the enormous advance in health care made during the last half century, infectious diseases still account for 25% of mortality worldwide and 45% in low-income countries. Anti-infective drugs are critically important in reducing the global burden of infectious diseases. However, as resistant microbes develop and spread, the effectiveness of the drugs is diminished (2). This type of resistance to antimicrobial agent is an increasing problem in many areas of the world especially in developing countries (3,4).

The use of traditional medicine to treat infection has been practiced since the origin of man kind (1), and in past it was the only method available. Currently, due to the absence of sufficient modern health care system, particularly in rural areas, people prefer to visit traditional healers and herbal medicines (5-6). The integration traditional and modern medicine is gaining increased recognition globally (6-8).

Honey produced by honeybees (*Apis mellifera*) is one of the oldest traditional medicines considered to be important in the treatment of respiratory ailment, gastrointestinal infection and various other diseases. It is being used effectively as a dressing for wounds, (including surgical wounds), burns, and skin ulcers to reduce pain and odor quickly.

Recently, many researchers have reported the antibacterial activity of honey against *S.aureus, P. aeuruginosa, E.coli, P. mirabilis, S. pyogenus, S. flexneri* and *S .typhi* (9-11). It has been documented that honey has a bacteriostatic and bactericidal effect against various species of both gram positive and gram negative bacteria, as well as an anti-fungal effect (9, 12).

The ability of honey to kill microorganisms has been attributed to its high osmotic effect, high acidic nature (p^H being 3.2-4.5), hydrogen peroxide concentration and its phytochemical nature, i.e. its content of tetracycline derivatives, peroxides, amylase, fatty acids, phenols, ascorbic acid, flavonides, streptomycin, sulfathiazole, trepens, benzyl alcohol, and benzoic acids (9,13,14). However the production and type of honey produced by honeybees is dependent on the natural vegetative flowers blooming in different seasons. Thus the flowers from which bees gathered nectar to produce the honey may contribute to the difference in the antimicrobial activities of honey (15).

The purpose of the present study was therefore to evaluate scientifically the *in vitro* antimicrobial potential (bacteriostatic and bactericidal effect) of honey produced by honeybees (*Apis mellifera*) against eight bacterial species among those commonly involved in causing gastroenteritis, pneumonia, wound and urinary tract infections in humans.

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Materials and methods

This experimental study was conducted in Jimma University, School of Medical Laboratory Technology from February 10 - March 14, 2003.

Honey samples harvested during spring 2002 and winter 2003 were collected from Jimma University, College of Agriculture, Animal Science Department, Bee keeping Unit in sterile screwed cups. Each honey sample was first filtered with a sterile mesh to remove debris and then streaked on blood agar plate, and incubated overnight to check microbial purity and stored at 2-8 ^{o}C until used.

The following control bacterial strain, standard test organisms and clinical isolates most commonly involved in causing gastroenteritis, pneumonia, wound and urinary tract infections were used. Control [*E. coil* American Type Culture Collection /ATCC 25922, *S. aureus* ATCC 25923, *P.aeruginosa* ATCC 27813]; Standard [*S.typhi* 127, *S.shiga* 106, *K.aerogenes*, *P.vulgaris*]; Clinical isolates [*S.aureus* (ear discharge), *P.mirabilis* (ear discharge), *P.aeruginosa* (wound)].

Bacterial cultures, *S. aureus* ATCC 25923, and *S.shiga* 106 were obtained from Ethiopia Health and Nutrition Research Institute (EHNRI); *E. coil* ATCC 25922 *P.aeruginosa* ATCC 27813, and *S.typhi* 127 were obtained from Jimma University, School of Medical Laboratory Technology; *K.aerogenes*, *P.vulgaris* and all the clinical isolates were collected from Jimma University, Microbiology Department. The clinical isolates were identified based on the standard microbiological technique (16) and drug susceptibility test for each clinical isolate was done following the standard agar disc diffusion method (17). These organisms were maintained in the laboratory on nutrient agar slopes at 4^{o}C (18).

Morphologically identical colonies from overnight growth were picked with an inoculating loop and suspended in 3-4 ml of nutrient broth and incubated for 2-3 hours at 36-37^{o}C and diluted with sterile normal saline to a turbidity that matches 0.5 McFarland standard (10^6 Colony Forming Unit (CFU)/ml), and further diluted 1:100 in sterile nutrient broth to set an inoculum density of $1x10^4$ CFU/ml which was used for the test (18, 19).

Preliminary investigation had been carried out by using agar diffusion technique to test the activity of honey against control bacterial strains following the standard single disc diffusion method developed by Bauer *et al* (17). In brief, a loop full (4mm in diameter) of the prepared control bacterial suspensions ($1x10^4$CFU/ml) were separately applied to the center of a sterile Mueller Hinton plate and spread evenly using a sterile dry cotton wool, then 50 micro liter of different concentrations of honey were dispensed and incubated at 37^{o}C for 20 hours. Various inhibition zones, more than 5mm in diameter were observed at different concentrations of honey.

Following this screening test, further investigation of the antimicrobial effect of honey was carried out using the agar dilution technique, which was done by mixing molten Mueller Hinton agar [(Oxoid, UK) prepared by suspending 38 gram of the powder in 1 liter of distilled water and brought to boil to dissolve the medium completely and sterilized by autoclaving at 121^{o}C for 15 minutes], and held in water bath (45-50^{o}C) with honey (19). Hence a known volume (ml) of honey: 0.5, 0.75, 1, 1.25, 1.5, 2 per 20ml of media were used. These are equivalent to honey concentrations (percentage by volume) of 2.5, 3.75, 5, 6.25, 7.5 and 10 respectively. Similarly two selected antibiotics (penicillin G and chloramphenicol) and a supersaturated solution of sugar of the same proportion as honey (85%W/V) were diluted to get a similar concentration as honey and tested on separate plates and compared with the MIC & MBC of honey.

The test media were incubated at 36-37^{o}C overnight to check their microbial purity (18). Then, the test plates which showed no microbial contamination were inoculated with the prepared bacterial cultures (10^4 CFU/ ml) and incubated aerobically at 36-37^{o}C for 20 hours in inverted positions. Mueller Hinton plates with out honey were similarly inoculated to control the appropriate growth of the organisms.

The Partial Inhibitory effect /the lowest concentration that retarded growth/ and Complete Inhibitory effect of different concentration of honey were examined by placing plates on a dark background and observing macroscopically for the lowest concentration that retarded and completely inhibited growth (in comparison with the control plate) respectively. Thus the Partial Inhibitory Concentration /PIC was reported as the lowest concentration that retarded growth as compared to the control plate and the MIC was reported as the lowest concentration of honey that completely inhibited visible growth, and the MBC was determined by further sub culturing the last plate which showed visible growth and all the plates in which there was no growth in Mueller Hinton agar. The MBC was therefore the lowest concentration of honey required to produce sterile culture (19).

A stability test was also conducted as follows: Honey samples were first divided into two aliquots. The first aliquot was stored at -10^{o}C for one month and the second aliquot was autoclaved at 121^{o}C for 15 minutes and allowed to cool. Then each aliquot was tested for antimicrobial activity as before, and finally comparisons were made.

A single colony or a faint haze left by the initial inoculum was not regarded as growth. In plates with no

growth at lower concentration but growth at a higher concentration, test organisms were sub cultured to confirm purity, and the test was repeated.

The antimicrobial substances in honey were not assessed and determined. However, P^H was tested and the P^H of honey (undiluted) and media (with honey) were measured using a digital P^H meter. All tests were done in triplicate and with appropriate controls at each step.

Results

The results of the *in vitro* susceptibility of the test microorganisms to honey samples were similar. Of all the microorganisms tested, 90% were sensitive to honey at a concentration of 6.25% (V/V) of honey. *P. aeruginosa* (clinical isolate and control strain) was sensitive at a concentration of 7.5% (V/V) of honey. Both the control and clinical isolates of *P.aeruginosa* were the least sensitive of the test microorganism to honey (Table 1).

Partial Inhibition for 90% of the test microorganisms was observed starting from 2.5% (V/V) and Complete Inhibition was observed at 6.25% (V/V) of honey and Partial Inhibition and Complete Inhibition for clinical isolates of *P. aeruginosa* was observed at a concentration of 3.75% (V/V) and 7.5%(v/v) of honey respectively. Therefore, the Partial Inhibitory Concentration (PIC), the Minimum Inhibitory Concentration (MIC) value for 90% of the tested microorganisms was found to be 2.5 and 6.25% (V/V) and for *P.aeruginosa* which was found to be 3.5 and 7.5% (V/V) respectively. The Minimum Bactericidal Concentration (MBC) value for 70% of tested microorganisms was found to be similar to the MIC value of the 90% of tested organisms, i.e. 6.25% (V/V). But the MBC value for *S.shiga* (standard test organism) and *P. aeruginosa* (control strain and clinical isolates) was 7.5% (V/V).

This study also assessed the antibacterial activity of honey after autoclaving at 121^{o}C for 15 minutes and deep-freezing at -10^{o}C for one month on control bacterial strains and honey samples retained their antimicrobial activity. However, PIC and MIC of honey on all control strains after heat treatment increased by 1.2%, i.e. the PIC and MIC value for all control strains were 3.75 and 7.5% (V/V) respectively and the MBC value for *E.coli* and *S.aureus* was 7.5% (V/V) and that of *P.aeruginosa* 10% (V/V). On the other hand, the PIC, MIC and MBC values of honey on control bacterial strains after deep-freezing at -10^{o}C for one month were similar to untreated honey samples (Table 2).

Table 1: **The *in vitro* antimicrobial activity: PIC, MIC and MBC% (V/V) of honey produced by honeybees (*Apis millifera)* in Mueller Hinton agar by agar dilution method against various control strains, standard test organisms and clinical isolates**

Bacterial strains with inoculums density of 10^4 CFU/ml		Antimicrobial activity of honey % (V/V)		
		PIC	**MIC**	**MBC**
Control strains	*E.coli* ATCC 25922	2.5	6.25	6.25
	S.aureus ATCC 25923	2.5	6.25	6.25
	P.aeruginosa ATCC 27853	2.5	6.25	7.5
Standard test strains	*S.shiga* 127	2.5	6.25	7.5
	S.typhi 106	2.5	6.25	6.25
	P.vulgaris	2.5	6.25	6.25
	K.aerogenes NCTC 418	2.5	6.25	6.25
Clinical isolates	*P.mirabilis*(ear discharge)	2.5	6.25	6.25
	S.aureus (ear discharge)	2.5	6.25	6.25
	P.aeruginosa (wound)	3.75	7.5	7.5

Key: PIC-Partial Inhibitory Concentration **MIC-**Minimum Inhibitory Concentration **MBC-**Minimum Bactericidal Concentration

Table 2: **Comparisons of the *in vitro* antimicrobial activity: PIC, MIC, and MBC of honey produced by honeybees (*Apis mellifera*) in Mueller Hinton agar by agar dilution method before and after autoclaving at 121^{o}C for 15 minutes and deep freezing at -10^{o}C for one month on control bacterial strains**

Characteristics of honey	Antimicrobial activity of honey	Control bacterial strains with inoculums density of 10^4CFU/ml %(V/V)		
		E.coli ATCC 25922	*S.aureus* ATCC 25923	*P.aeruginosa* ATCC 27853
Untreated honey	PIC	2.5	2.5	2.5
	MIC	6.25	6.25	6.25
	MBC	6.25	6.25	7.5
Autoclaved honey	PIC	3.75	3.75	3.75
	MIC	7.5	7.5	7.5
	MBC	7.5	7.5	10
Deepfreeze honey	PIC	2.5	2.5	2.5
	MIC	6.25	6.25	6.25
	MBC	6.25	6.25	7.5

♦See Table 1 for key to abbreviations

The MIC and MBC of two selected common antibiotics, penicillin G and chloroamphenicol were assessed on control bacterial strains for control and comparison purposes and the result revealed that the MIC and MBC of penicillin G for *S. aureus* was less than 2.5% (V/V) or 0.5ml of stock penicillin G ($1x10^6$ IU per 2 ml of sterile water) per 20 ml of media. Again, all control bacterial strains were sensitive to chloroamphenicol, i.e. MIC was 6.25% (V/V) or 1.25 ml of stock chloroamphenicol (1gm/3ml of sterile water) per 20 ml of media. Control bacterial strains and clinical isolates were resistant to common antibiotics.

This study also compared the antibacterial activity of honey to a super saturated solution of sugar of the same sugar proportion as in honey (85% W/V) and the result showed that this supersaturated solution of sugar exhibited less degree of antibacterial activity as compared to honey (data not shown).

The P^H values of undiluted and different concentrations of honey were measured by digital P^H meter and these were found to be 6.92, 6.71, 6.5, 6.31, 6.11 and 3.8 for 2.5%, 3.75%, 5%, 6.25%, 7.5% (V/V) and undiluted honey respectively.

Discussion

In our study two honey samples were tested for their antimicrobial activity on selected bacterial species and the antimicrobial effect of these two honey samples on test microorganisms were similar. The honey samples were found to have both bacteriostatic and bactericidal properties on both gram-positive and gram-negative bacteria. Honey samples used in this study showed partial inhibitory (bacteriostatic) and bactericidal activities for all of the test organisms at concentrations 2.5 - 7.25% (V/V). Growth retardation and complete inhibition on 90% of the test organisms were observed at a concentration of 2.5 % [PIC] and 6.25 % [MIC] of honey respectively.

The highest PIC and MIC were recorded for clinical isolates (wound) of *P.aeruginosa*, i.e. 3.25 and 7.5 % (V/V) of honey respectively. The study showed that honey has less antimicrobial activity against *P. aeruginosa* and *S. shiga* as compared with other test microorganisms. The reason for this is not clear. Honey samples also exerted antimicrobial activities on *P. aeruginosa*, *P. mirabilis* and other bacteria, which were resistant to some common antibiotics discs such as penicillin, ampicillin, chloroamphenicol, cotrimoxazol, and gentamycin.

In Ethiopia, a study by Mogessie Ashenafi (1994) reported that '*tazma mar'* honey produced by sting- less bee (*Apis mellipodae*) was found to be effective against some food-borne pathogens of humans. Growth Retardation and inhibition on *S.typhimurim, S.enteritidis* and *E.coli* were noted at 15 and 20% concentration, while a more marked growth retardation and inhibition on *B. cereus* and *S. aureus* were observed at concentrations of 10% (20). In contrast to this report honey produced by honeybees (*Apis mellifera*), in the present study could inhibit most of the test organisms at a very low concentration (2.5-7.5%V/V). This might be due to the differences in the species of bees, which in turn results in difference in the production and type of honey (15) and the differences in the test methods and test organisms.

Studies on honey produced by honeybees (*Apis meliffera*) have shown that honey has antimicrobial activity against *S.aureus, P.aeruginosa, E.coli, P.mirabilis, Citrobacter ferundi, Streptococcus faecalis, S.flexinari,* and *S.typhi* (9,10). It completely inhibits major wound infection pathogens including *S.pyogenus* and *S.aureus* (11). The results of our study are consistent with the above study.

Molan demonstrated the activity of honey against *S.aureus*, Methicilin Resistance *S.aureus* and *Pseudomona* Spp. He also cited that Willix D found the percentage (by volume) of Manuka honey needed to completely prevent growth of each species of bacteria to be 1.8, 3.6, 3.7, 6.0, 6.3, 7.3, and 10.8 % (V/V) for *S.aureus, S. pyogeneus, E.coli, S. typhimurium, P.mirabilis and P.aeruginosa* respectively (12). But the percentage by volume of honey to completely prevent growth of *E.coli, S.aureus* and *P.mirabilis* in the present study was 6.5 and for *P.aeruginosa* it was 7.5; indicating that there is a variation in the antimicrobial potency of honey.

Another study by Molan reported the concentration of honey in nutrient agar (% V/V) against various strains of bacteria which cause gastroenteritis, and the PIC, MIC and MBC were found to be 6, 7, 10 for *E.coli*; 6, 7, 8 for *S.typhmurim*; 6, 7, 10 for *S.flexinari* and 6, 7, 10 for *S.sonnei* respectively (9). This is in contrast to our study. Here the variation in the antimicrobial potential of honey used in the present study as compared to the previous similar studies highlights that the source of the nectars may have contributed to the difference in the antimicrobial activities of honey; that is, the flowers from which bees gathered nectar to produce the honey, since flora source determines many of the attributes of honey, for example flavor, aroma, color and composition. And being a natural product, the composition of honey is highly variable (15). The variation in sensitivity is also attributable to differences in growth rate of pathogens, nutritional requirements, temperature, inoculum's size and the test method it self (19).

In the present study, the antimicrobial substances in honey were not estimated except for P^H. And the P^H of the media at which MIC and MBC observed were 6.3 and 6.11 respectively, which is low enough to be inhibitory to many pathogens; the P^H for growth of these pathogens normally falls between 7.2 and 7.4 (9).

The experiment also showed that antimicrobial substance in honey could withstand deep freezing at -10^{o}C for one month. However, the MBC of honey on all tested microorganisms decreased by 1.2% after autoclaving of honey at 121^{o}C for 15 minutes. This shows that its antimicrobial activity is not dependent alone on its phytochemical nature, i.e. tetracycline derivatives, ascorbic acid, peroxidase or amylases, streptomycin, sulfonamides which are claimed as heat labile (14). On the other hand, the antimicrobial effect of honey is attributed to its phenolic acid, flavonides, benzyl - alcohol, 2-hydroxy benzoic acid which are heat stable and may be active agents but their concentration in honey appears too low to sorely responsible (14).

Again, the experiment showed that, supersaturated solution of sugar of the same proportion as honey, i.e. 85% [W/V](10) did not have the same degree of antibacterial activity as honey, indicating that while the removal of water from bacteria is important; other factors are operating to provide the observed antibacterial effect.

In conclusions, honey produced by honeybees (*Apis mellifera*) has both a bactreiostatic and bactericidal activity when tested *in vitro*. However, pharmacological standardization and clinical evaluation on the effect of honey are essential before using honey as a preventive and curative measure to common diseases related to the tested bacterial species. The wider availability of honey in rural areas provides its utilization for certain diseases

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