



RESEARCH ARTICLE

ANTIMICROBIAL ACTIVITY OF DIFFERENT HONEY SAMPLES AND THEIR PROTEIN ESTIMATION

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ABSTRACT

**Background:** The ancient Medicine showed its versatility in applying various aspects of honey produced naturally. Honey has proved previously been antimicrobial agent as well as showing wound healing property by many authors. However, the properties of honey depend on its type, origin of location and flower which develops the final product. The current work was focused on the antimicrobial activity and protein content of honey originated from two commercial honey and two wild honey obtained from *Vachellia nilotica* and *Tamarindus indica*.

**Methods:** All the four honey were collected and an agar well diffusion assay was performed to determine antimicrobial activity against the selected microorganisms viz. *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Staphylococcus aureus*. Further study was continued with the best results obtained from *Vachellia nilotica* honey by using minimum inhibitory concentration (MIC) test through spectrophotometric analysis. The protein estimation from honey samples was performed through Lowry method.

**Results:** The agar diffusion assay showed that, *Vachellia nilotica* honey had greater antimicrobial activity against the bacterial strains compared to the other three honey types. The agar well diffusion method showed that *Vachellia nilotica* honey has the maximum zone of inhibition against *Escherichia coli* (24 mm), *Staphylococcus aureus* (20 mm), *Bacillus subtilis* (15 mm), and *Pseudomonas aeruginosa* (15 mm). The MIC test of the same showed that the honey sample has maximum inhibitory concentration at 6.3% for *Pseudomonas aeruginosa* (21 mm) and 50 % for *Staphylococcus aureus* (24 mm). The spectrophotometric analysis results for the minimum inhibitory concentration of *Vachellia nilotica* honey ranged from 0 (0.04%) to 0.19 (6.3%) for *Pseudomonas aeruginosa* and 0.02 (0.02%) to 0.24 (6.3%) for *Staphylococcus aureus*. The protein estimation showed that four honey samples have around 50% of protein compared to standard BSA.

**Conclusion:** *Vachellia nilotica* honey showed maximum antimicrobial activity compared to other three types of honey studied and also possess proteins. This honey can be used in alternative medicine.

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INTRODUCTION

The well known medicine of India "Ayurveda" widely uses honey in various purposes. The alternative medicine has included honey as its integral part to cure a wide range of disorders. The wound healing as well as easily digestive properties of honey made it as a wonderful medicine. Honey is known as a naturally synthesized sweet substance, collected by *Apis mellifera* from various secreted forms of different plants. These bees collect the nectar or secretions or excretions of

living parts of plants, and then, transform the honey by combining with their own substances. During the process, they deposit and dehydrate and allow the honey to mature (Codex Alimentarius, 2001). Hence, honey can be regarded as a complex of substances derived from nectar and pollen of plants along with the compounds added by honeybees. During the process of honey production, various biochemical reactions are resulted throughout the process of maturation to storage (Iglesias et al. 2006). The honey variety is mainly determined by its botanical origin of the nectar. Based on origin, honey is classified into blossom honey, honeydew honey and multifloral honey. Based on processing, honey is categorized as comb honey, strained honey, extracted honey, granulated honey and creamed honey. Following its utility as a sweetener for

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centuries, honey is also recognized for antioxidant and anti-inflammatory properties (Singh *et al.* 2009; Yaghoobi *et al.* 2013). The protein content of the honey in the form of enzymes also attracted several investigators and very few works showed protein presence in some honey (Ferrerres *et al.* 1993, Lee *et al.* 1998, Girolamo *et al.* 2012). *Vachellia nilotica*, taxonomically known as *Acacia nilotica* is commonly called Babul tree is known for its antimicrobial activity since ancient times in India. *Tamarindus indica* produces large numbers of yellow flowers which host copious nectar and collected by Indian bees (Ramanujam and Kalpana, 1992). Several investigators reported the antimicrobial activity of different types of honey against broad range of microorganisms, especially, *Staphylococcus aureus* (Cooper *et al.* 1999; Miorin *et al.* 2003; Demera and Angert, 2004; Stow *et al.* 2007; Temaru *et al.* 2007; Irish *et al.* 2008; Patel *et al.* 2009; Deshpande, 2013; Tissouras *et al.* 2013; Alkhyat *et al.* 2014), *Pseudomonas aeruginosa* (Demera and Angert, 2004), *Salmonella enteritidis* (Halawani and Shohayeb, 2011), *Klebsiella* (Riaz *et al.* 2011), *Escherichia coli* (Deshpande, 2013) and *Bacillus cereus* and *Bacillus subtilis* (Tissouras *et al.* 2013). Further antimicrobial studies have been extended to Vancomycin-resistant strains (Cooper *et al.* 2002; Jenkins *et al.* 2012), methicillin-resistant strains (Sherlock *et al.* 2010) and various clinical isolates including *Escherichia coli* (Doughari, 2006; Saraf *et al.* 2009), *Pseudomonas aeruginosa* (Doughari, 2006, Gethin and Cowman, 2008; Mullai and Menon, 2007; Saraf *et al.* 2009), *Helicobacter pylori* (Roland *et al.* 2007) and *Candida albicans* (Saraf *et al.* 2009). The practical approach of honey made ointments on burn wound infections (Tasleem *et al.* 2013) extended the applications of honey.

The main objective of the current work was to evaluate the antimicrobial activity of the honey originating from two commercial varieties (*Dabur* honey and *Meadows Wild Flower* (Minnesota Company, Denmark) and two local plant varieties (*Vachellia nilotica* and *Tamirindus indica*) along with the estimation of their protein contents by comparing with standard protein BSA.

## MATERIALS AND METHODS

**1. Sample collection-** Four honey samples were collected to test their antimicrobial activity. Sample 1 was a commercial honey sample purchased from the local market (*Dabur* honey). Sample 2 was a commercial honey sample obtained from Denmark (*Meadows Wild Flower*, Minnesota Company, Denmark) through personal communication. Sample 3 & 4 were collected from the plants *Tamirindus indica* (Tamirind) and *Vachellia nilotica* (babul). Following the chief honey flow season at Loni, Maharashtra during October to December, honey samples (100 g each) of *Apis florea* were collected. Honey was collected by squeezing the honey-storing portion of the combs only.

**2. Storage-** During the experimentation, the collected honey samples from different sources were stored at 4°C in the laboratory refrigerator. Before starting the experimentation, the cultures were grown over night at 37°C on nutrient agar and the bacterial suspensions were prepared separately with 0.85% saline. These suspensions were used as inoculants.

**3. Bacterial cultures-** The antimicrobial properties of four selected honeys were tested against four bacteria i.e. *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, collected from Pravara Medical Trust, Loni. These cultures were sub-cultured in nutrient agar slants and used for further antibacterial activity study.

**4. Antimicrobial activity-** The well diffusion assay method was used to determine the antimicrobial activity of four different honey samples against four bacteria. Nutrient agar plates were inoculated by spreading with specific microorganism and tested antimicrobial activity against the honey samples. After culture inoculation, 10 mm diameter wells were cut in the agar manually and 80 µl of four honey samples were loaded. The plates were incubated at 37°C for 24 hours. The zones of inhibitions were measured.

**5. MIC determination-**The MIC determination assay was performed for the better honey sample obtained from the plant *Vachellia nilotica* honey *achellia nilotica*. MIC assay was performed against two clinically important selected bacteria, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Spectrophotometric assay for MIC was determined by using sterile 12 well round bottomed commercially available microtitre plates. 100 µl of bacterial culture was inoculated to 1900 µl of test honey in each well at different concentrations (12 dilutions tested). Control wells were loaded with nutrient broth only by considering as negative control. The plates were incubated while shaking at 150 rpm for 24 h. The optical density was determined by the method given by Patton *et al.* (2006). In brief, the OD was obtained at T0 and after T24 at 600 nm. Following the procedure of Sherlock *et al.* (2011) the percent of inhibition of growth was determined.

**6. Protein precipitation and Estimation-**A homogenous mixture of 10 mL honey and 90 mL distilled water was prepared by keeping the mixture on magnetic stirrer for about one hour. This mixture was kept on ice and equal amount of 10% trichloroacetic acid was added. The mixture was incubated for 2 hours at 4°C. Then the mixture was centrifuged at 15,000 rpm for 10 minutes. The supernatant was decanted and the collected pellet was precipitated with 0.1M Phosphate buffer (pH-7.6). This precipitated protein was used for further analysis. The protein estimation was performed according to Lowry *et al.* (1951).

## RESULTS

Initial screening with agar diffusion assay method for antimicrobial activity of first three types of honey showed zone of inhibition against the four bacteria used (Table 1, Fig. 1a). Ampicillin was used as a standard antibiotic. Out of these three samples, *Meadows Wild flower* honey showed maximum zone of inhibition against *P. aeruginosa* (13 mm), *S. aureus* (10 mm) and *B. subtilis* (8 mm). The same sample has showed no zone of inhibition for *E. coli*. However, *Tamirindus indica* honey showed a zone of inhibition for *E. coli*. The honey sample from *Vachellia nilotica* showed the maximum zone of inhibition against all the bacteria, especially against *E. coli* (Table 1; Fig. 1b).

Table 1. Zone of inhibition showed by different honey samples against selected bacteria

S. No.	Honey	<i>E. coli</i>	<i>B. subtilis</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>
1	Dabur	-	3	9	-
2	Meadows Wild Flower	-	8	13	10
3	Tamirindus indica	7	6	1	8
4	<i>Vachellia nilotica</i>	24	20	15	20

Table 2. MIC assay for *Vachellia nilotica* honey & zone of inhibitions

S.No.	<i>Vachellia nilotica</i> honey Concentration (%)	O.D. taken at 660 nm				Zone of Inhibition	
		<i>P. aeruginosa</i>		<i>S. aureus</i>		<i>P. aeruginosa</i>	<i>S. aureus</i>
		T0	T24	T0	T24		
1	0.02	0.03	0.02	0.05	0.03	-	-
2	0.04	0.02	0.02	0.08	0.02	14	-
3	0.1	0.04	0.03	0.04	0.02	15	11
4	0.2	0.05	0.03	0.08	0.04	16	12
5	0.4	0.05	0.06	0.09	0.02	17	-
6	0.8	0.05	0.04	0.07	0.04	-	13
7	1.6	0.12	0.07	0.14	0.05	16	14
8	3.1	0.09	0.05	0.08	0.03	18	15
9	6.3	0.26	0.07	0.3	0.06	21	14
10	12.5	0.19	0.14	0.2	0.15	18	17
11	25	0.22	0.2	0.24	0.21	20	18
12	50	0.09	0.01	0.4	0.01	19	24

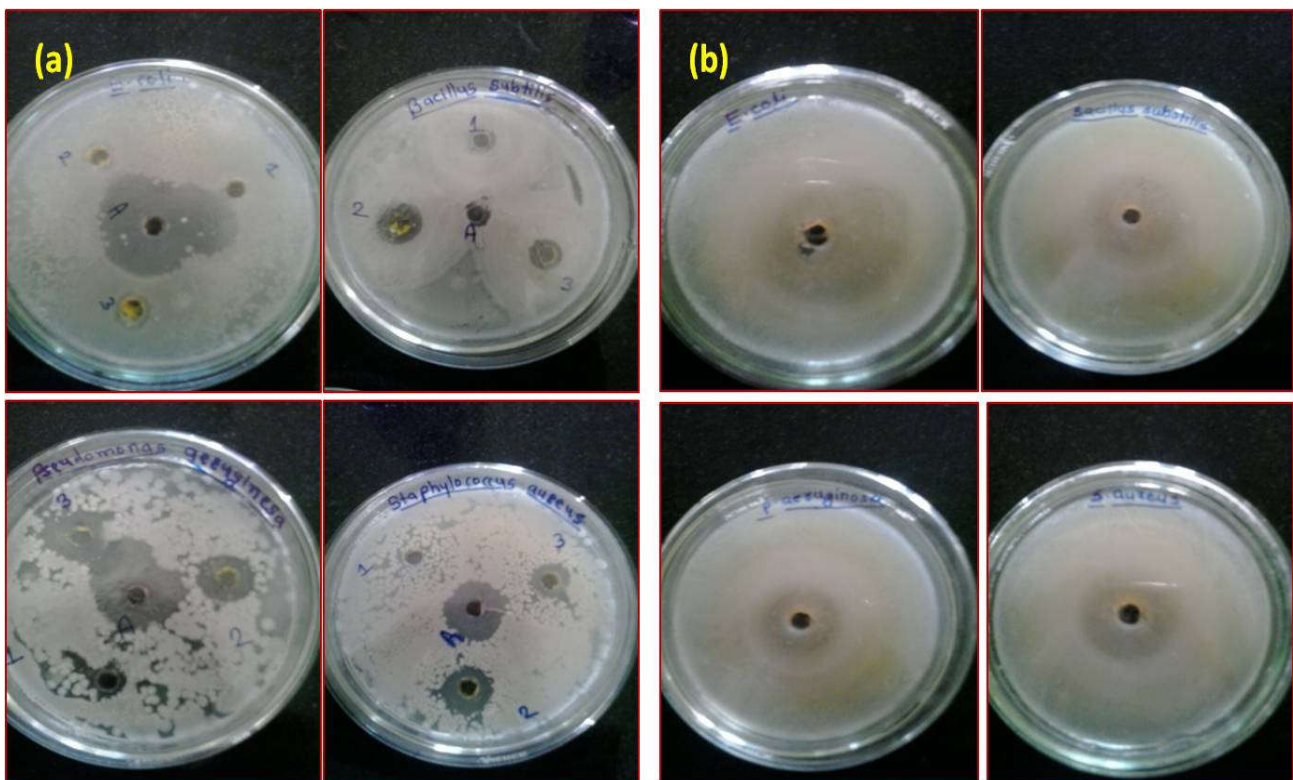


Figure 1 (a): Zone of inhibition showed by three honey and Ampicillin against selected bacteria.  
 (b): Zone of inhibition showed by *Vachellia nilotica* honey

*Vachellia nilotica* honey produces a large antimicrobial effect than any other three honeys for each dilution, when tested against different microorganisms. At a concentration of 6.3% *Vachellia nilotica* honey showed an antimicrobial effect over *Pseudomonas aeruginosa* and *Staphylococcus aureus*. From the OD obtained (Table 2), it is clearly observed that at a

concentration of 6.3% *Vachellia nilotica* honey is capable of inhibiting the microbial growth at a maximum rate. The agar well diffusion showed the antimicrobial activity of *Vachellia nilotica* honey against the clinically important strains of *P. aeruginosa* and *S. aureus* (Table 2; Fig. 2a-f).

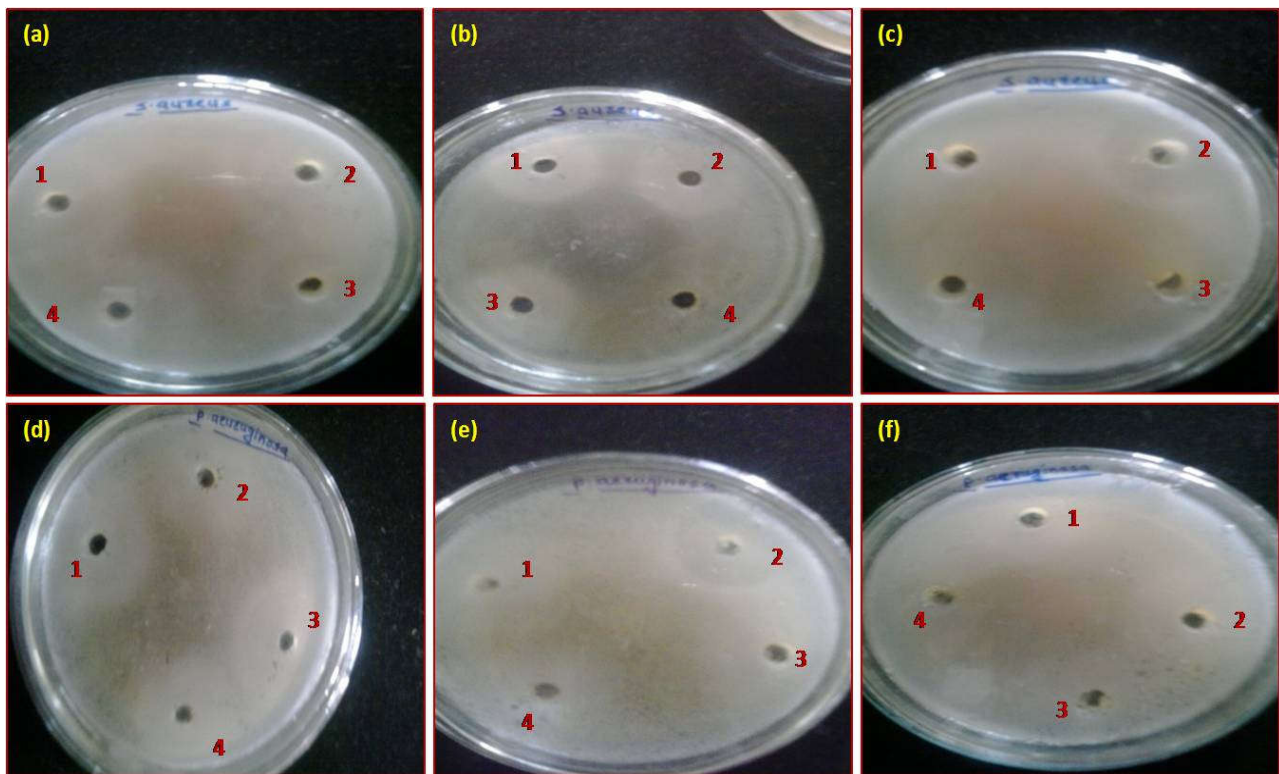


Figure 2. Minimum Inhibitory Concentrations of *Vachellia nilotica* honey against *S. aureus* (a, b, c) and *P. aeruginosa* (d, e, and f). MIC Dilutions (a, d)-well 1(50%), well 2 (25%), well 3 (12.5%), well 4 (6.3%); (b, e) well 1(3.1%), well 2 (1.6%), well 3 (0.8%), well 4 (0.4%); (c, f) well 1(0.2%), well 2 (0.1%), well 3 (0.04%), well 4 (0.02%)

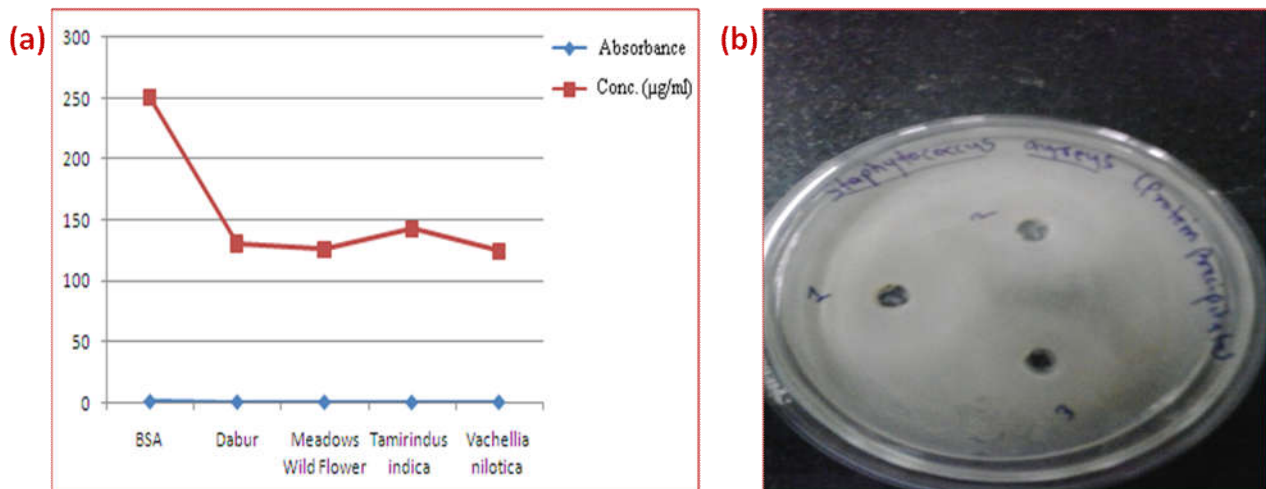


Figure 3. (a) Protein concentrations and absorbance from four honeys (b) Zone of inhibitions for protein precipitates of three honey samples

The total proteins of four selected honey samples were estimated by using Lowry method. The total concentrations of the protein precipitates from the four honey samples obtained were 131  $\mu\text{g/ml}$ , 0.75  $\mu\text{g/ml}$ , 143  $\mu\text{g/ml}$ , 125  $\mu\text{g/ml}$ , respectively (Fig. 3a). On comparison with standard protein Bovine Serum Albumin, these four samples contain only 50 % of protein concentration. Out of these four samples, only *S. aureus* was found to be sensitive for protein samples of *Dabur*, *Tamirindus indica* and *Vachellia nilotica*. These samples showed the zones of inhibitions as 25mm, 15 mm and 7 mm respectively (Fig. 3b).

## DISCUSSION

Honey is considered as an alternative medicine in Ayurveda and used in curing many diseases. The nutritional importance and calorific values of honey made it as a good medicine, which has no side effects in its usage. The current work was focused on the antimicrobial property of honey against different clinically important species of microorganisms. The presence of proteins in the honey and its antimicrobial activity were also considered for study. The honey samples and their antimicrobial action were studied by different investigators



globally. Sherlock *et al.* (2010) compared Ulmo, manuka and a laboratory synthesized honeys during their investigation. By using an agar well diffusion assay, they performed MIC Spectrophotometrically against Methicillin Resistant *S. aureus* (MRSA), *E. coli* and *P. aeruginosa*. The results showed by them indicated zones of inhibitions at 6.3% by Ulmo honey (The best honey claimed), only for MRSA strains. But, in the current study, *Vachellia nilotica* honey at 6.3% is inhibiting all the microorganisms clearly. *Meadows Wild flower* and *Tamarindus indica* honeys are also showed significant antimicrobial activity. Cooper *et al.* (1999) worked on *S. aureus* from infected wounds and showed antimicrobial activities of Manuka and mixed Pasture sources. They showed that the antibacterial activity of honey is due to hydrogen peroxide. In our study, *Vachellia nilotica* honey showed a reasonably good zone of inhibition against *S. aureus*, which may support the study of Cooper *et al.* (1999). By extending the studies, Cooper *et al.* (2002) isolated 18 strains of MRSA and 7 strains of Vancomycin-sensitive *Enterococci*. Miorin *et al.* (2003) showed antimicrobial activity of honey collected from *Apis mellifera* and *Tetragonisca angustula* and observed their activity against *S. aureus*. They showed that, both Propolis and honey samples will act as potential antimicrobial agents. Doughari (2006) demonstrated that *Tamarindus indica* honey has the highest antimicrobial activity against *S. aureus*. The current study results are similar to those obtained by Doughari (2006). Temaru *et al.* (2007) showed antimicrobial activity of stingless honey bees against *E. coli*, *P. aeruginosa* and *S. aureus*. Se-Ra Won *et al.* (2009) characterized a major honey protein derived from *A. cerana* and from *A. mellifera*. In the current study, the protein precipitate was prepared and estimated. A portion of protein was used to test its antimicrobial activity. However, this protein isolated from *Vachellia nilotica* honey showed 21 mm and 14 mm of zones in agar well diffusion assay. This is possibly due to the antibody property of a protein, which may need further purification to study its properties. This can enhance the pharmaceutical applications of honey in developing novel drugs against diverse microorganisms.

## Conclusion

Four types of honeys used in this study have proved their antimicrobial property. The origin of honey may differ, but its physiological functions were found to be the same. However, the range of antimicrobial activity differed drastically among the honey and the result was obtained with *Vachellia nilotica* honey. The strong activity of this honey demonstrated a clear outcome of antimicrobial nature of honey, especially against the pathogens *P. aeruginosa* and *S. aureus*. The protein content of the selected honey also proved to be an antimicrobial agent against *S. aureus*. Further studies on protein purification and structure determination, can enhance the research towards protein functioning and Proteomics.

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