

## THE ANTIMICROBIAL ACTIVITY OF HONEY OF THE STINGLESS BEE *Trigona* spp.

Assegid Garedew<sup>1</sup>, \*Erik Schmolz<sup>1</sup>,  
Ingolf Lamprecht<sup>2</sup>

<sup>1</sup>Free University of Berlin, Institute of Zoology, Königin-Luise-Strasse 1-3, D - 14195 Berlin

<sup>2</sup>Free University of Berlin, Institute of Animal Physiology, Ehrenbergstrasse 26 - 28, D-14195 Berlin

\*Corresponding author: e-mail: eschmolz@zedat.fu-berlin.de

### S u m m a r y

Stingless bees (*Trigona* spp.) produce a special honey which is less viscous and darker than honey of the honeybees (*Apis* spp.) and has a strong acid flavour. *Trigona* bee honey is highly prized in Ethiopia for medicinal use as a panacea for many ills. The antimicrobial activities of two samples of *Trigona* spp. honey obtained from Ethiopia and honeybee honey from Germany were tested *in vitro* against four fungal and six bacterial species.

Fungi were generally less sensitive than the bacteria or not sensitive at all to the treatments. Only *Aspergillus niger* and *Penicillium chrysogenum* responded slightly to one honey sample at a higher concentration. The bacteria reacted differently to the honey samples tested, with no relation to their Gram reaction. The minimal inhibitory concentrations of catalase treated honeys from stingless bees were higher than that of the non-treated samples for most bacterial species, indicating that hydrogen peroxide contributes to the antibacterial activity. *Apis* honey's hydrogen peroxide played no essential role in the antimicrobial activity against most bacteria tested. Concentrations below a certain value resulted in a microbial lawn of some bacteria around the inhibition zone indicating that the use of honey against infections at lower concentrations could be dangerous by encouraging the growth of bacteria.

**Keywords:** stingless bee, stingless bee honey, antimicrobial activity, non-peroxide activity, *Trigona* spp.

### INTRODUCTION

Therapy with bee products (apitherapy) is a worldwide old tradition, used for thousands of years but replaced by antibiotics in modern times by all but a few individuals preferring traditional or natural remedies or those with no access to antibiotics (Molan et al. 1988). It is, however, reviving currently, apart from others, due to the increasing report of resistance of bacteria against antibiotics (Greenwood 1995, Molan and Brett 1998). In addition to the drug resistance of bacteria, the side-effects of some pharmaceutical products may give rise to an aversion to synthetic drugs (Thompson 1976, Kauffman 1991) and to an

increasing interest in the use of alternative therapies. Although honey has been used as a medicine since ancient times, its effectiveness as a remedy has been revealed to be due to its antibacterial activity only a century ago (Molan 2001). The antibacterial activities of honey were considered to be due to: (I) osmotic effects - owing to the high osmolarity of honey whereby water is drawn away from microorganisms, reducing their ability to survive; (II) acidity - as honey is acidic with a pH value between 3.2 and 4.9 which inhibits growth of many pathogens whose optimal pH of growth is not acidic; (III) hydrogen peroxide - which is produced in the honey due to conversion

of glucose to gluconic acid by glucose oxidase, an enzyme produced by the hypopharyngeal gland of the bees; (IV) phytochemical factors - non peroxide antibacterial factors believed to be the various complex phenols and organic acids often referred to as flavonoids. These latter complex compounds do not breakdown easily under heat or light treatment and provide some types of honey with antibacterial activities even after these honeys were exposed to the mentioned factors, which usually destroy enzymes (Molan 2001).

The typical honey investigated by most researchers is produced by *Apis* spp. This honey is undoubtedly the most widely and massively collected and used by people for different purposes. But regionally, especially in the tropics and subtropics, there are other honeys made by different bee species, which are sometimes collected in substantial quantities (Krell 1996). One group of bees that produces a considerable amount of honey and hence can be used in beekeeping are the stingless bees.

Stingless bees are eusocial insects that belong to the family *Apidae* and the subfamily *Meliponinae* and lack a functional sting. Some species defend aggressively by biting the intruder - since they possess well developed mandibles, emitting a caustic liquid from the mouth, releasing unpleasant odours, and irritating by crawling in to the eyes, ears etc., of a predator (Ruttner 1992). Like the honeybees, stingless bees store honey and pollen (Michener 1974). The two important genera of *Meliponinae* that produce large amounts of honey and hence are used in stingless beekeeping are *Melipona* and *Trigona* (Crane 1992). The *Melipona* species are restricted to central and south America whereas *Trigona* species occur in all the tropical continental regions (Amano et al. 2000, Wille 1979).

*Trigona* spp. occur in Ethiopia at medium altitudes of up to 2300 m above sea

level and are about 10 mm long (Fichtl and Adi 1994). They construct their nests in underground cavities of naturally abandoned ant nests, termite mounds, and cavities under plant roots at a depth of about 1m. The nest consists of thimble-sized oval shaped honey/pollen pots placed around the one cell thick brood area of the combs that are arranged horizontally, unlike the vertical combs of honeybees.

Stingless bees produce a special honey known in Ethiopia as Tazma honey. The Tazma honey is less viscous and darker than *Apis* honey and has a stronger acid flavour. In addition to that it also possesses a stronger bacteriostatic effect (Krell 1996). Ripened honey of the stingless bees contains 30 to 35% water unlike *Apis* honey, with about 17.1% (Ruttner 1992). Though the water content of stingless bee honey is high, it does not ferment in the nest presumably due to the abundant resin chemicals, which impart dark colour to it, and hydrogen peroxide ([http://www.sibexlink.com.my/g15magazine/g15mag\\_vol3jan\\_science.html](http://www.sibexlink.com.my/g15magazine/g15mag_vol3jan_science.html)). This honey is often highly prized locally for medicinal use as a panacea for many ills, especially by the poor people of rural areas with less access to modern medicine. Due to its strong sour taste it is inedible and used only for medicinal purpose to treat cough, stomach disturbance, sore throat, tonsillitis, stomach and intestinal ulcers, cold, disease of the mouth and mucus membrane, and as wound dressing. Stingless bee honey is considered in folk medicine to be more powerful than honeybee honey for use as a "natural" cure for treating common diseases (Vit 2001).

The yield of honey from a colony rarely exceeds 1 to 2 litres. It is sold on market and is more expensive than *Apis* honey mainly due to its medicinal value and also the labour intensiveness of its harvesting process.

The purpose of our present *in vitro* investigation is to elucidate if the *Trigona*

honeys collected from Ethiopia have antimicrobial effects against different bacterial and fungal strains.

## MATERIALS AND METHODS

### Honey source

Two samples of *Trigona* spp. honey were obtained from two regions in Ethiopia, (I) Honey B from Bahir Dar (11° 35' N and 37° 28' E) Northwest Ethiopia, at an altitude of 1830 m above sea level; (II) Honey T from Temben (13° 53' N and 39° 53' E) Northern Ethiopia, at an altitude of 1500 m above sea level. The vegetation in both regions is categorized as Ethiopian undifferentiated woodland (White 1983). Honey samples were bought from Tazma experts and stored in a refrigerator, for one year, until needed for laboratory assay.

For comparison of the activity of *Trigona* honey with that of honeybee honey, a sample of honey was obtained from the research beehives of the Institute of Zoology, Free University of Berlin and named here as Honey D.

The pH of all the three honey samples was measured using a WTW Multi 340i pH meter (Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany) after diluting them 1:1 with distilled water.

### Biological material

Bioassays of the antimicrobial activities of the different honey samples were performed using (I) four fungal species: the yeast *Saccharomyces cerevisiae* (DSM 211) and three filamentous fungi, *Aspergillus niger* (DSM 737), *Penicillium chrysogenum* (DSM 844) and *Trichoderma viride* (DSM 63065); (II) four species of Gram positive bacteria: *Bacillus subtilis* (DSM 347), *Micrococcus luteus* (DSM 348), *Bacillus megaterium* (DSM 90), *Bacillus brevis* (DSM 5609); and (III) two species of Gram negative bacteria: *Escherichia coli* (DSM 31), *Pseudomonas syringae* (DSM 5176).

### Growth media

Cultivation of all bacterial cultures was done on Standard I nutrient broth (Merck Lot VL 630582) and/or Standard I nutrient agar (Merck, Lot VL 694681). The yeast was cultivated on a medium composed of 10 g Oxoid agar (Agar Bacteriological No. 1, Lot 817706-2), 1 g yeast extract (Sigma, CAS No. 8013-01-2), 2 g glucose (Merck), 0.5 g peptone (Sigma, Lot 128H0184), 10 ml Na-phosphate buffer (1M, pH 7) in 1 l distilled water. This same medium without agar was used as a nutrient broth for the yeast.

*Trichoderma viride* was cultivated on malt extract peptone agar (MEPA) composed of 30 g malt extract (Sigma, Lot 41k0181), 3 g soya peptone (Hy soy T, Sigma, Lot 128 H0184), 15 g Oxoid agar (Agar Bacteriological No. 1, Lot 817706-2) in 1 l distilled water. *Aspergillus niger* and *Penicillium chrysogenum* were cultivated on potato dextrose agar (PDA) composed of 20 g glucose (Merck), 15 g Oxoid agar (Agar Bacteriological No.1, Lot 817706-2), in 1 l potato infusion obtained by boiling 200 g scrubbed and sliced potato in 1 l water for 1h and passed through a fine sieve.

All types of media were sterilized by autoclaving at 121°C and 15 psi for 15 min.

### Bioassay

#### Preparation of the honey samples

The different honey samples mentioned were serially diluted to 1:1 (50%), 1:5 (20%), 1:10 (10%), 1:20 (5%), 1:50 (2%), and 1:100 (1%), with distilled water and used for the determination of total antimicrobial activity. The total antimicrobial activity of the honey samples is assigned the letter **T** followed by a subscript of the letter for the name of the corresponding honey samples **B**, **T**, **D** for honeys from Bahir Dar, Temben, and Germany, respectively. Therefore the total antimicrobial activity of these honey samples is hereafter referred to as **T<sub>B</sub>**, **T<sub>T</sub>**, **T<sub>D</sub>**. By analogy the

corresponding non-peroxide (NP) antimicrobial activities of these honey samples are hereafter referred to as NP<sub>B</sub>, NP<sub>T</sub>, NP<sub>D</sub>, respectively.

The honey samples were treated with catalase to remove H<sub>2</sub>O<sub>2</sub> and to elucidate their non-peroxide activity. Treatment was done by a 1:1 mixing of the honey sample with catalase solution of 20,000 units ml<sup>-1</sup> modified slightly after Molan and Russell (1988). The catalase solution was prepared by dissolving a 13,300 units/mg solid catalase from bovine liver (Sigma, Lot 32k 7031, E.C. 1. 11. 1. 6) in distilled water. The catalase treated honey, which is diluted 1:1 due to the treatment, was diluted further to 1:5, 1:10, 1:20, and 1:50 and used in the Petridish bioassay.

#### Petridish Bioassay

##### Bacteria and yeast

An isolated pure colony of an overnight grown strain was picked carefully using a sterile transfer loop, inoculated to a nutrient broth in an Erlenmeyer flask and grown overnight at 30°C. About 50 µl of the overnight culture was inoculated to a 20 ml solution of nutrient broth and grown further for about 3 to 5 h until an O.D. of 0.6 (546 nm) was achieved (method slightly modified after Faye and Wyatt 1980). The suspension was then diluted 1:50 with the corresponding nutrient broth in order to prepare the standard inoculum.

The sterilized nutrient agar was cooled to 48°C, 5 ml of the standard inoculum was mixed with the 1 l nutrient agar and poured in to plastic Petridish of  $\varnothing = 85$  mm, 10 ml in each. When the agar was solidified 3 holes were bored per Petridish using a cork borer of  $\varnothing = 9$  mm. Each hole was then filled with 50 µl honey of a certain dilution and the Petridishes were placed in a refrigerator for 24 h, giving the honey enough time to diffuse. Finally, the plates were removed from the refrigerator, incubated at 30°C for 24 to 48 h and the inhibition zones

were measured. Each concentration of all honey samples was investigated in triple Petridishes with three holes per Petridish, i.e., n = 9.

##### Filamentous fungi

An isolated pure colony of fungal culture which was grown for 72 h on solid medium and started to sporulate was scrubbed up using a sterile transfer loop and placed in 5 ml sterile distilled water in a test tube. The hyphae were disintegrated by adding sterile glass beads and shaking vigorously for 1 to 2 min, in order to get a uniform suspension. The 5 ml suspension was added to a 1 l agar solution at 48°C and the procedure above was followed further. The plates were taken out of the refrigerator and left at room temperature (*ca.* 25°C) for 72 h and the inhibition zones were measured.

The control experiments for both the bacterial and fungal cultures were done by inoculating the cultures with sugar syrup (Lyle's Golden syrup, UK) of dilutions similar to the honey samples. Sugar syrup has similar osmolarity to honey (Efem et al. 1992) and could reveal if the osmolarity of honey plays a significant role in the bacteriostatic/bactericidal activity of honey. The control experiments in the case of the non-peroxide activity tests were done using a catalase solution of 20,000 units ml<sup>-1</sup> in distilled water. Each concentration of all honey samples was investigated in triple Petridishes with three holes per Petridish, i.e., n = 9.

##### Turbidimetric bioassay

Though the agar well diffusion technique is convenient to use, it is less sensitive than the turbidimetric method in displaying the activity of an antimicrobial agent. The main reasons for its insensitivity is that the honey sample is further diluted by the agar medium surrounding the well and that the extent of the clear zone obtained is not directly proportional to the activity of the sample, because outlying

colonies could grow before an inhibitory concentration diffuses to them (James et al. 1972, Molan et al. 1988). In contrast to the agar well diffusion technique that displays cumulative result of incubation after 24 or 48 h, the turbidimetric method allows for kinetic investigations and displays the activity of the antimicrobial agents with incubation period "live". It demonstrates the activity of the antimicrobial agents that may not completely inhibit growth but prolong the cell cycle time and hence could not be observed by the Petridish bioassay method. For this reason the antimicrobial activities of the two stingless bee honeys were additionally investigated using this method. Since the 1:1 and 1:5 diluted honey samples were too turbid, it was impossible to investigate their antibacterial activities spectrophotometrically.

An actively growing bacterial culture was diluted with nutrient broth to an O.D. = 0.6 (546 nm) and 100  $\mu$ l of the suspension was added to an Erlenmeyer flask with side nose (for O.D. measurement) that contains 10 ml nutrient broth with 1:10, 1:20, 1:50, or 1:100 diluted honey. The culture was then incubated at 30°C by measuring the O.D. at 2 h intervals for 6 to 8 h. Nutrient broth with the corresponding dilution of honey was used for blanking the spectrophotometer during each measurement. The control experiments were bacterial cultures with no honey and also bacterial cultures inoculated with sugar syrup/golden syrup with concentrations corresponding to that of the honey samples. Each concentration was investigated six times (n = 6).

#### Statistical analysis

Results are presented as mean  $\pm$  S.D. values. Statistical tests were performed using the two-tailed student's test, Tukey's test, two-way ANOVA and  $\alpha = 0.05$  was considered as the critical value.

## RESULTS

The two honey samples from *Trigona* species were darker than the honey from *Apis mellifera* and also more acidic, with pH values of 3.2 and 3.45 for honeys B and T, respectively, compared to the higher pH of 4.9 for honey D.

The control experiments with sugar syrup did not show any inhibitory action on any bacterial or fungal species tested except for *B. brevis*, where a remarkable inhibition zone was observed at 100% and 50% concentration with inhibition zone diameters of  $12.3 \pm 0.3$  and  $10.7 \pm 3.5$  mm, respectively.

The responses of a specific microorganism to the different honey samples (Tukey's test) and to the two categories (total- and non-peroxide activities) of the same honey sample (two-tailed t-test) were significantly different in some cases and not in others, as seen in Table 1. The total activities of both honeys B and T ( $T_B$  and  $T_T$ ) were superior to the non-peroxide activities ( $NP_B$  and  $NP_T$ ) against the bacteria *B. brevis*, *M. luteus*, and *E. coli* at all concentrations tested, though the activities of only 50% concentrations of the different honey samples are displayed in Table 1. In addition to that 50%  $T_B$  exhibited stronger antibacterial activity against *B. megaterium* contrary to  $NP_B$  which encouraged lawn growth of this bacterium. There was no significant difference (two-tailed t-test) in the antibacterial activity of 50%  $T_T$  and  $NP_T$  against *B. megaterium*, both showing weak inhibitory activities. *Micrococcus luteus*, though inhibited with 50%  $T_B$  and  $T_T$ , showed a very dense microbial lawn due to incubation with the corresponding concentrations of hydrogen peroxide devoid samples, i.e., 50%  $NP_B$  and  $NP_T$ . *Escherichia coli* was inhibited strongly with 50% concentrations of hydrogen peroxide containing honeys B and T, but not with hydrogen peroxide free honey samples. Though  $T_B$  and  $NP_B$  showed slight differences in the strength of

Table 1

Antimicrobial activity of 50% dilution of different honey samples elucidated by the inhibition zone diameter (mm), mean  $\pm$ S.D. First letters denote the total activity (T) or non-peroxide activity (NP) and the subscripts indicate the origin of each sample; B: Bahir Dar, T: Temben (both from Ethiopia), D: Germany. One way ANOVA and Tukey's tests ( $\alpha = 0.05$ ,  $n = 9$ ) were performed across honey samples for each microbial species (along a row but not a column). Values within a row that possess the same letter do not have significant difference. l.g. denotes the promotion of lawn of growth

Organism	T <sub>B</sub>	T <sub>T</sub>	T <sub>D</sub>	NP <sub>B</sub>	NP <sub>T</sub>	NP <sub>D</sub>
<i>B. brevis</i>	21.1 $\pm$ 0.7 <sup>a</sup>	16.2 $\pm$ 1.7 <sup>ab</sup>	7.3 $\pm$ 2.3 <sup>c</sup>	13.3 $\pm$ 1.5 <sup>b</sup>	7.0 $\pm$ 1.0 <sup>c</sup>	10.3 $\pm$ 2.1 <sup>cb</sup>
<i>B. megaterium</i>	11.0 $\pm$ 0.8 <sup>a</sup>	3.0 $\pm$ 0.4 <sup>b</sup>	7.0 $\pm$ 1.0 <sup>b</sup>	l.g.	5.3 $\pm$ 1.2 <sup>b</sup>	6.0 $\pm$ 1.0 <sup>b</sup>
<i>B. subtilis</i>	14.8 $\pm$ 3.5 <sup>a</sup>	12.4 $\pm$ 0.9 <sup>a</sup>	42.3 $\pm$ 9.3 <sup>b</sup>	20.7 $\pm$ 5.0 <sup>a</sup>	29.0 $\pm$ 3.6 <sup>b</sup>	46.7 $\pm$ 4.2 <sup>b</sup>
<i>M. luteus</i>	10.8 $\pm$ 1.1 <sup>a</sup>	33.9 $\pm$ 3.0 <sup>b</sup>	42.0 $\pm$ 2.0 <sup>b</sup>	l.g.	l.g.	41.7 $\pm$ 2.1 <sup>b</sup>
<i>E. coli</i>	13.1 $\pm$ 0.8 <sup>a</sup>	27.6 $\pm$ 7.7 <sup>c</sup>	21.7 $\pm$ 2.9 <sup>c</sup>	0.0	0.0	20.3 $\pm$ 0.6 <sup>c</sup>
<i>P. syringae</i>	12.5 $\pm$ 0.5 <sup>a</sup>	4.1 $\pm$ 0.0 <sup>b</sup>	7.2 $\pm$ 0.8 <sup>c</sup>	15.7 $\pm$ 6.4 <sup>a</sup>	7.3 $\pm$ 2.3 <sup>c</sup>	10.0 $\pm$ 2.0 <sup>ac</sup>
<i>S. cerevisiae</i>	0.0	0.0	0.0	0.0	0.0	0.0
<i>A. niger</i>	5.0 $\pm$ 1.0 <sup>a</sup>	0.0	0.0	4.7 $\pm$ 1.2 <sup>a</sup>	0.0	0.0
<i>P. chrysogenum</i>	3.7 $\pm$ 0.6 <sup>a</sup>	0.0	0.0	4.0 $\pm$ 1.0 <sup>a</sup>	0.0	0.0
<i>T. viride</i>	0.0	0.0	0.0	0.0	0.0	0.0

Table 2

Comparison of responses of the different species of bacteria and fungi to treatment with 50% dilution of each honey sample represented by the mean diameter  $\pm$  S.D. of the inhibition zone in mm. Statistical significance test was done across species (along rows) using One way ANOVA and the Tukey's test ( $\alpha = 0.05$ ,  $n = 9$ ) for each honey sample. Values with in a row that possess the same letter do not have significant differences. Identical letters at the values along columns do not designate any statistical relation. l. g. denotes the promotion of lawn of growth

	T <sub>B</sub>	NP <sub>b</sub>	T <sub>T</sub>	NP <sub>T</sub>	T <sub>D</sub>	NP <sub>D</sub>
<i>B. brevis</i>	21.1 $\pm$ 0.7 <sup>a</sup>	13.3 $\pm$ 1.5 <sup>a</sup>	16.2 $\pm$ 1.7 <sup>a</sup>	7.0 $\pm$ 1.0 <sup>a</sup>	7.3 $\pm$ 2.3 <sup>a</sup>	10.3 $\pm$ 2.1 <sup>a</sup>
<i>B. megaterium</i>	11.0 $\pm$ 0.8 <sup>b</sup>	l.g.	3.0 $\pm$ 0.4 <sup>b</sup>	5.3 $\pm$ 1.2 <sup>a</sup>	7.0 $\pm$ 1.0 <sup>a</sup>	6.0 $\pm$ 1.0 <sup>a</sup>
<i>B. subtilis</i>	14.8 $\pm$ 3.5 <sup>b</sup>	20.7 $\pm$ 5.0 <sup>a</sup>	12.4 $\pm$ 0.9 <sup>a</sup>	29.0 $\pm$ 3.6 <sup>b</sup>	42.3 $\pm$ 9.3 <sup>b</sup>	46.7 $\pm$ 4.2 <sup>b</sup>
<i>M. luteus</i>	10.8 $\pm$ 1.1 <sup>b</sup>	l.g.	33.9 $\pm$ 3.0 <sup>c</sup>	l.g.	42.0 $\pm$ 2.0 <sup>b</sup>	41.7 $\pm$ 2.1 <sup>b</sup>
<i>E. coli</i>	13.1 $\pm$ 0.8 <sup>b</sup>	0.0	27.6 $\pm$ 7.7 <sup>c</sup>	0.0	21.7 $\pm$ 2.9 <sup>c</sup>	20.3 $\pm$ 0.6 <sup>c</sup>
<i>P. syringae</i>	12.5 $\pm$ 0.5 <sup>b</sup>	15.7 $\pm$ 6.4 <sup>a</sup>	4.1 $\pm$ 0.0 <sup>b</sup>	7.3 $\pm$ 2.3 <sup>a</sup>	7.2 $\pm$ 0.8 <sup>a</sup>	10.0 $\pm$ 2.0 <sup>a</sup>
<i>S. cerevisiae</i>	0.0	0.0	0.0	0.0	0.0	0.0
<i>A. niger</i>	5.0 $\pm$ 1.0 <sup>c</sup>	4.7 $\pm$ 1.2 <sup>b</sup>	0.0	0.0	0.0	0.0
<i>P. chrysogenum</i>	3.7 $\pm$ 0.6 <sup>c</sup>	4.0 $\pm$ 1.0 <sup>b</sup>	0.0	0.0	0.0	0.0
<i>T. viride</i>	0.0	0.0	0.0	0.0	0.0	0.0

Table 3

Minimal inhibitory concentrations (MIC) of the different honey samples (in %) against the tested bacterial and fungal species. \* = no inhibitory concentration, rather promotion of lawn of growth. □ = enhanced lawn of growth at the indicated and lower concentrations while inhibited by higher concentrations

	T <sub>B</sub>	NP <sub>B</sub>	T <sub>T</sub>	NP <sub>T</sub>	T <sub>D</sub>	NP <sub>D</sub>
<i>B. brevis</i>	>1	>10	>5	>20	>10	>5
<i>B. megaterium</i>	>5	*	>20	>20	>20	>20
<i>B. subtilis</i>	>5	>10	>20	>10	>10	>5
<i>M. luteus</i>	>20	*	>20	*	>20□	>20□
<i>E. coli</i>	>20	None	>20□	None	>20	>20
<i>P. syringae</i>	>20	>5	>20	>20	>20	>10
<i>S. cerevisiae</i>	None	None	None	None	None	None
<i>A. niger</i>	>20	>20	None	None	None	None
<i>P. chrysogenum</i>	>20	>20	None	None	None	None
<i>T. viride</i>	None	None	None	None	None	None

Table 4

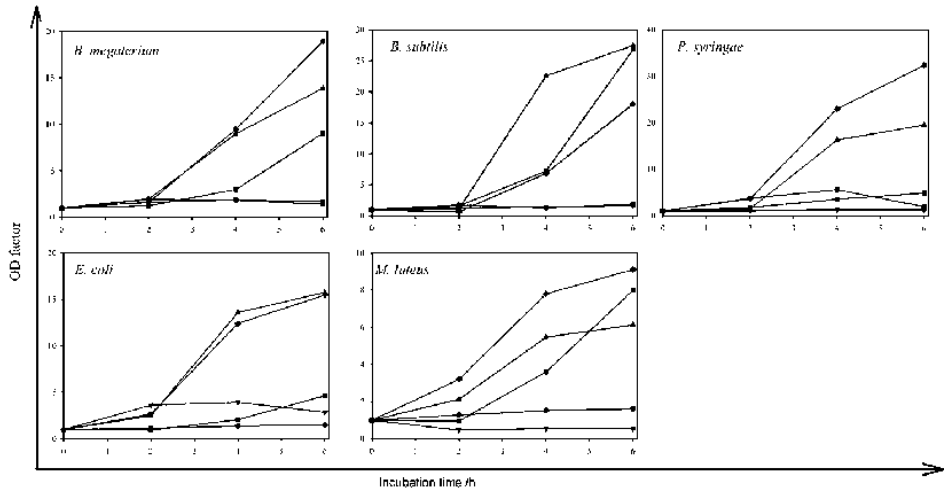
Effect of sugar syrup (control) on the growth rate of different bacterial species. Growth determined spectrophotometrically (546 nm) and given as O.D factor (O.D. at t = 6 h divided by that at t = 0 h). At the 6<sup>th</sup> h of incubation period all the bacterial species were growing logarithmically

	Control	50%	10%	5%
<i>B. megaterium</i>	13.9	2.3	36.3	13.5
<i>B. subtilis</i>	27.5	2.2	22.8	6.5
<i>E. coli</i>	15.7	2.0	30.9	16.4
<i>M. luteus</i>	6.1	2.5	50.9	12.6
<i>P. syringae</i>	19.5	3.5	50.9	31.6

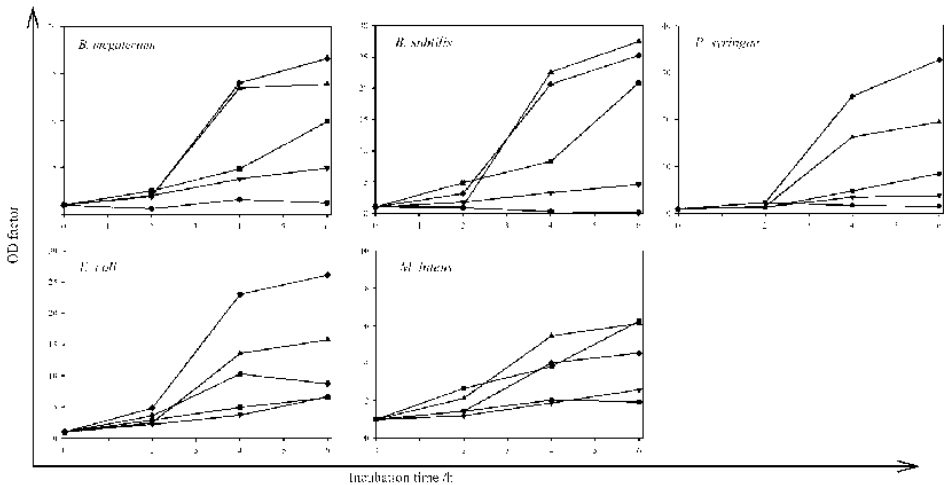
their inhibition on *B. subtilis*, this was not statistically significant (two-tailed t-test). However, NP<sub>T</sub> paradoxically exhibited a superior inhibitory activity against *B. subtilis* compared to that of T<sub>T</sub>. The total and non-peroxide preparations of the two stingless bee honeys did not show statistically significant difference in their inhibitory activities on *Pseudomonas syringae* (two-tailed t-test). Unlike in the case of the two honey samples from stingless bees whose strength of activity depended on the presence of hydrogen peroxide, the total-

(T<sub>D</sub>) and non-peroxide (NP<sub>D</sub>) activities of *Apis* honey showed no difference in the strength of bacterial inhibition for each bacterial species tested (two-tailed t-test) (Table 1).

Fungi were generally less sensitive, or even insensitive, to the treatments. Only T<sub>B</sub> and NP<sub>B</sub> showed slight inhibitory effects on the two fungal species: *A. niger* and *P. chrysogenum*, but no statistically significant difference exists (two-way ANOVA) either across these two species or across the two honey preparations (Table 2). *S. cerevisiae*



**Fig. 1** The effect of stingsless bee honey (Honey B) on the growth rate of different bacterial species determined spectrophotometrically. The OD factor is obtained by dividing the OD at  $t_t$  with the OD at  $t_0$ . The different symbols represent honey concentrations



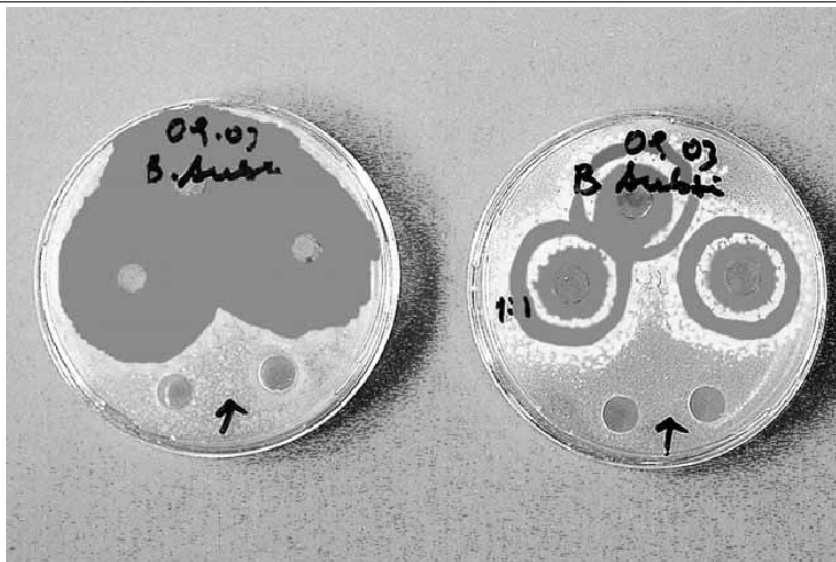
**Fig. 2** The effect of stingsless bee honey (Honey T) on the growth rate of different bacterial species determined spectrophotometrically. The OD factor is obtained by dividing the OD at  $t_t$  with the OD at  $t_0$ . The different symbols represent different concentrations of honey

and *T. viride* are insensitive to any of the honey samples. The response of the different bacterial species to the treatment with a certain honey sample is not dependent on the Gram reaction of the bacteria, both the Gram positive and Gram negative bacteria responding to the treatments. The responses of the bacterial species are rather dependent

on the type of honey sample and individual organisms within a category, Gram positive or Gram negative, rather than the Gram reaction (Tables 1 and 2).

In case of the three species of *Bacillus* and *M. luteus* there were dense bacterial growth zones next to the inhibition zones with the honey samples B and T even at a





**Fig. 3** Hormesis effect observed after treatment of *B. subtilis* with a 1:1 diluted Honey T. The arrows at the bottom side of the photo indicate the wells for the control treatment, i.e., sugar syrup of the corresponding concentrations

concentration of 100%. *M. luteus* and *B. subtilis* showed a unique response to the treatment with  $T_T$  where alternating rings of inhibition zones and dense growth zones were observed. The inner inhibition zones were clearer and larger, with complete inhibition of bacterial growth, and the outer inhibition zones were weaker and smaller, with the strength of inhibition decreasing outward. Up to three pairs of alternating rings were observed, with the number of rings declining with decreasing concentration of honey.

The minimal inhibitory concentration of catalase treated honey B was higher than that of the non-treated sample for most bacterial species, except for *P. syringae* where  $NP_B$  showed lower MIC than  $T_B$ . The MIC values of  $T_B$  and  $NP_B$  did not show much difference in the case of the two fungal species that were inhibited, *A. niger* and *P. chrysogenum*. The catalase treated honeys B and T failed to inhibit the growth of *E. coli* and also encouraged dense growth in case of *M. luteus*. Both  $T_D$  and  $NP_D$  resulted in microbial lawn of *M. luteus* at

concentrations 20%. A dense growth of *B. megaterium* was also encouraged by 50%  $NP_B$  (Table 3).

MIC values of  $T_B$  and  $T_T$  obtained using the Petridish bioassay method were higher than those from the turbidimetric method. Lower concentrations of both  $T_B$  and  $T_T$  encouraged fast growth of most bacteria with the growth rates surpassing that of the control, i.e. without honey (Fig. 1 and 2).

The effect of sugar syrup on the growth rate of bacteria, though not detectable with the Petridish bioassay method, was revealed with the spectrophotometric method (Table 4). The O.D. measurement showed that 50% sugar syrup retarded the growth rate of all bacterial species remarkably compared to the control. The 10% sugar syrup, however, encouraged growth of all bacteria tested. The 5% Sugar syrup displayed slight encouragement of growth in some bacteria.

## DISCUSSION

The insensitivity of the different bacterial and fungal species to the control, sugar

syrup, is an indication that the osmolarity of honey does not play an important role in the inhibition of growth of these microorganisms, but higher concentrations may prolong the cell cycle time. Hence, the other antibacterial factors of honey, i.e. hydrogen peroxide activity, non-peroxide activity mainly due to the flavonoids of honey, acidity, and lysozyme (Bogdanov and Blumer 2001) could be responsible for the inhibition of growth of these microorganisms. *Bacillus brevis*, however, is sensitive to and inhibited by the high osmolarity of sugar syrup indicating that, apart from others, the high osmolarity of honey could inhibit the growth of this bacterium.

The stronger total antibacterial activities of stingless bee honeys against some bacterial species compared to the non-peroxide activities are indications that hydrogen peroxide plays a significant role in the bactericidal/bacteriostatic action of these honey samples. This may be due to the fact that honey from stingless bees possesses considerable amount of hydrogen peroxide that could be, at least partially, responsible for preventing the fermentation of this honey which otherwise would have taken place due to its higher amount of water, 30 to 35%. Catalase treated honey B resulted in an enhancement of growth of *B. megaterium* and *M. luteus* though these bacteria were strongly inhibited by the non-treated honey samples. The most likely explanation for this could be that they are inhibited by hydrogen peroxide in honey in case of the total activity, and that the non-peroxide components are below inhibitory concentrations. Removal of hydrogen peroxide eliminates the inhibitory factor and hence bacterial growth could be encouraged either due to the higher sugar concentration, providing them with excess energy and nutritional sources, or the lower concentration of the phytochemical components below a certain critical level. The presence of certain bacteriostatic/bactericidal chemicals in the

growth medium at a concentration lower than a critical inhibitory level enhances the growth of the organism that otherwise would have been inhibited by higher concentrations, a phenomenon known as hormesis (Edward et al. 1998). This effect of hormesis was also clearly observed in the case of treatment of *M. luteus* with 50% NP<sub>T</sub>, though 50% T<sub>T</sub> showed a very strong inhibitory action with an inhibition zone of nearly 34 mm diameter. The activities of both types of stingless bee honeys against *E. coli* were only due to the presence of hydrogen peroxide, whose removal resulted in the normal growth of this bacterium without any detectable inhibition zone.

The formation of concentric rings of inhibition- and dense growth zones could also be explained based on the phenomenon of hormesis, with the different components of honey (mainly the phytochemical components with varying diffusion potentials) acting as hormesis factors in the different zones.

Both stingless bee honeys showed a higher non-peroxide activity against the bacterium *B. subtilis* than total activity, though it was statistically insignificant for honey B. This is in contrast to most literature and difficult to explain. It was, however, pointed out by Burdon (1995) that hydrogen peroxide, at a concentration below a certain critical level, could stimulate the division and proliferation of many cell types in mammalian tissue and plays a role in wound healing. This chemical acts at various points in the mechanisms that control cell growth and differentiation probably by oxidising proteins involved, and thus causing a change in the conformation of the protein molecules. Apart from this evidence on mammalian cells, a positive role of hydrogen peroxide supporting the growth of bacteria was not yet reported.

The comparable antimicrobial activity of catalase treated and non-treated samples of honeys T and B on some bacteria

demonstrates that the activities are not due to hydrogen peroxide. The lack of hydrogen peroxide based activity and the insensitivity to sugar syrup demonstrates that the activity of stingless bee honeys could be mainly due to the phytochemical (flavonoids), lysozyme, organic acid components (Bogdanov and Blumer 2001) and probably due to its strong acidity. Conclusive evidence has been demonstrated by Molan and Russell (1988) that the antibacterial activity of some New Zealand honeys was not only due to hydrogen peroxide but mainly to the antibacterial agents of plant origin. The authors also concluded that in honeys of higher antibacterial activities, non-peroxide activity is by far the largest part of the total activity.

The *Apis* honey tested did not show hydrogen peroxide based activity. One possible reason could be that the honey was ripe since it was stored for one year. Storage of the honey for one year was important since its activity had to be compared with *Trigona* honey that was also stored for one year. The enzyme glucose oxidase is practically inactive in ripe honey, no more replenishing the degraded hydrogen peroxide and hence lower concentration of the latter which hardly inhibits bacteria (Bogdanov and Blumer 2001, [http://privatewww.essex.ac.uk/~islamic/ilm/health-fit/honey\\_2.html](http://privatewww.essex.ac.uk/~islamic/ilm/health-fit/honey_2.html)). This is because the acidity produced in the action of the enzyme drops the pH to a value too low for the enzyme to work any more. In addition to that the activity of water ( $a_w$ ) of a ripe honey is very low inhibiting the process (Bogdanov and Blumer 2001). The lack hydrogen peroxide activity demonstrates that the antibacterial activity of this honey is mainly due to the phytochemical components, or lysozyme. The acidity of this honey (pH = 4.9) is not strong enough to inhibit the growth of most bacteria as it is further diluted and weakened in the growth medium. Osmolarity also does not play a role since the bacteria tested did not respond to the

osmolarity of sugar syrup, except *B. brevis*. The pH and osmolarity of different New Zealand honeys tested against various organisms did not inhibit bacterial growth (Molan and Russel 1988).

## CONCLUSIONS

The traditional use of *Trigona* honey as a panacea against different illnesses is rational if the infection to be treated is caused by bacteria, not by fungi, since only one of the honey samples showed a minor antifungal effect against two of the four fungal species tested, though reports exist that honey inhibits the growth of fungi.

The enhancement of growth of some bacterial species by lower concentrations of honey indicates that the use of honey at lower concentrations as a means of apitherapy could be dangerous. However, if used undiluted, *Trigona* honey offers many possibilities as a broad spectrum-healing agent against both Gram positive and Gram negative bacteria. Especially the use of this honey as a wound dressing agent may be effective since its dilution by body fluid is less intensive than for internal administration.

Though it is premature to conclude at this level that stingless bee honey can be used as a panacea, as claimed by the local people, it can be ascertained that it has a broad spectrum of antimicrobial action inhibiting the growth of both Gram positive and Gram negative bacteria. Further research in this field may help to recognize and use indigenous knowledge to help alleviate the ever-increasing report of resistance of pathogenic bacteria to current antibiotics.

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## AKTYWNOŚĆ PRZECIWDROBNOUSTROJOWA MIODU PSZCZÓŁ BEZZĄDŁYCH *Trigona* spp.

G a r e d e w A . , S c h m o l z E . , L a m p r e c h t I .

### S t r e s z c z e n i e

Pszczoły bezządle (*Trigona* spp.) wytwarzają charakterystyczny miód o niskiej lepkości, zdecydowanie bardziej ciemny i kwaśny w stosunku do miodu pszczoły miodnej. W Etiopii miód *Trigona* spp. jest droższy ze względu na jego zastosowanie jako panaceum na wiele schorzeń.

W pracy oznaczono aktywność antymikrobową dwóch próbek miodu pszczół bezządłych z Etiopii, i dla porównania, aktywność jednej próbki miodu pszczoły miodnej z Niemiec. Aktywność miodu określono testami *in vitro* w stosunku do czterech gatunków grzybów i sześciu gatunków bakterii.

Grzyby generalnie są dużo mniej wrażliwe na działanie miodu niż bakterie. Tylko *Aspergillus niger* i *Penicillium chrysogenum* w niewielkim stopniu reagowały na jedną z dwóch próbek miodu *Trigona* spp. i tylko przy jego wyższych stężeniach. Miód oddziaływał na bakterie bez związku z ich reakcją na test Grama. Minimalne stężenie miodu *Trigona* spp., które hamowało rozwój bakterii (MIC), było wyższe po zadziałaniu na miód katalazą, od tych próbek, które nie były traktowane katalazą (nie został rozłożony nadtlenek wodoru). Świadczy to o wpływie akumulacji nadtlenu wodoru na aktywność antymikrobową tego miodu. W przypadku miodu pszczoły miodnej (próbka porównawcza miodu niemieckiego) akumulacja nadtlenu wodoru nie odgrywała głównej roli w aktywności antibakteryjnej w reakcji przeciw większości użytych w doświadczeniu szczepów bakterii.

**Słowa kluczowe:** pszczoły bezządle, miód pszczół bezządłych, aktywność przeciwdrobnoustrojowa, aktywność nie-nadtlenkowa *Trigona* spp.