Journal of
Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Antimicrobial activity of honey from the stingless bee *Trigona carbonaria* determined by agar diffusion, agar dilution, broth microdilution and time-kill methodology

K.L. Boorn[1], Y.-Y. Khor[2], E. Sweetman[2], F. Tan[2], T.A. Heard[3] and K.A. Hammer[2]

1 School of Pathology and Laboratory Medicine, The University of Western Australia, Crawley, Western Australia, Australia
2 Microbiology and Immunology, School of Biomedical, Biomolecular and Chemical Sciences, The University of Western Australia, Crawley, Western Australia, Australia
3 CSIRO Entomology, Indooroopilly, Brisbane, Queensland, Australia

Keywords
antibacterial, minimum inhibitory concentration, MRSA, wounds.

Correspondence
Katherine A. Hammer, Microbiology & Immunology (M502), School of Biomedical, Biomolecular and Chemical Sciences, The University of Western Australia, 35 Stirling Hwy, Crawley, Western Australia 6009, Australia.
E-mail: khammer@cyllene.uwa.edu.au

2009/0970: received 2 June 2009, revised 28 July 2009 and accepted 17 August 2009

doi:10.1111/j.1365-2672.2009.04552.x

Abstract

Aims: The aim of this study was to determine the spectrum of antimicrobial activity of 11 samples of stingless bee honey compared to medicinal, table and artificial honeys.
Methods and Results: Activity was assessed by agar diffusion, agar dilution, broth microdilution and time-kill viability assays. By agar dilution, minimum inhibitory concentration (MIC) ranges were 4% to >10% (w/v) for Gram-positive bacteria, 6% to >16% (w/v) for Gram-negative bacteria and 6% to >10% (w/v) for *Candida* spp. By broth microdilution, all organisms with the exception of *Candida albicans* and *Candida glabrata* were inhibited at ≤32% (w/v). Geometric MIC (w/v) means for stingless bee honeys ranged from 7·1% to 16·0% and were 11·7% for medicinal honey and 26·5% for table honey. Treatment of organisms with 20% (w/v) stingless bee honey for 60 min resulted in decreases of 1–3 log for *Staphylococcus aureus*, >3 log for *Pseudomonas aeruginosa* and <1 log for *C. albicans*. Similar treatment with each control honey resulted in decreases of <1 log for all organisms.
Conclusions: Stingless bee honey has broad-spectrum antibacterial activity although activity against *Candida* was limited. Stingless bee honey samples varied in activity and the basis for this remains to be determined.
Significance and Impact of the Study: Stingless bee honey had similar activity to medicinal honey and may therefore have a role as a medicinal agent.

Introduction

The antimicrobial activity of honey has been both researched scientifically and exploited medicinally for many years. The majority of research has focused on honey produced by the European honeybee *Apis mellifera* and, until recently, relatively little attention has been paid to honey from stingless bees. Stingless bees (Apidae, Meliponini) are native to tropical and subtropical parts of the world such as Central and South America, Africa, Asia and northern Australia (Crane 1990). Like *Apis* honeybees, most species of eusocial stingless bees, such as *Tetragonisca angustula* and *Melipona quadrifasciata* from Brazil (Souza *et al.* 2006) and *Trigona carbonaria* from Australia, produce honey. Stingless bee honey is highly valued as a food source by the Aboriginal people of northern Australia and is of cultural significance, playing a role in the social traditions and rituals of the people (Akerman 1979; Isaacs 2000). Stingless bee honey has also been used in traditional medicine in Central and South America, and Africa (Cortopassi-Laurino *et al.* 2006), suggesting that stingless bee honey may have therapeutic properties that are similar to currently used medicinal honeys such as manuka honey from New Zealand (Cooper *et al.* 2000; George and Cutting 2007; Adams *et al.* 2008).

© 2009 The Authors

Two recent publications have described the antimicrobial activity of honey derived from the Australian native stingless bee *T. carbonaria* (Irish *et al.* 2008; Kimoto-Nira and Amano 2008). Activity was evaluated in both publications by an agar diffusion method but disparate results were found, with one study showing significant activity (Irish *et al.* 2008) and the other finding none (Kimoto-Nira and Amano 2008). Our aim was therefore to further examine the antimicrobial activity of several samples of *Trigona* stingless bee honey against a wide range of test organisms. Agar diffusion, agar and broth dilution methods, and a time-kill assay, were used to generate qualitative and quantitative data on the antimicrobial activity of stingless bee honey.

Materials and methods

Honey

Eleven stingless bee honey samples were collected from Brisbane (Queensland, Australia) and surrounding suburbs between October 2006 and February 2007. Ten samples (A–J) were from hives of *T. carbonaria* and the remaining sample (K) was from an unknown bee species (Table 1). The floral source for all honeys was a combination of native and exotic cultivated plant species. All stingless bee honeys were gamma-irradiated (minimum of 15 kGy) prior to testing. Irradiation has previously been demonstrated to have negligible effects on the antimicrobial activity of honey (Molan and Allen 1996). The following control honeys were tested in parallel for comparison; control honey (W: Wescobee 100% pure honey; Bayswater, Australia or C: Capilano Premium honey; Richlands, Australia); medical honey (Medihoney®; Antibacterial honey barrier, Richlands, Australia) and artificial honey. Artificial honey was prepared by dissolving 40·5 g fructose, 33·5 g glucose, 7·5 g maltose and 1·5 g sucrose in 17-ml sterile distilled water (Cooper *et al.* 2002). Stingless bee honeys were stored at 4°C and all other honeys were stored at room temperature. Table honey W was found to contain endemic micro-organisms which in some instances interfered with the interpretation of results. Table honey C was therefore used in subsequent tests. All honey solutions were prepared in sterile distilled water as weight for volume (w/v) solutions and used within 2 h of preparation.

Physicochemical honey properties

The pH of honey was determined by dissolving 10 g of honey in 75 ml of distilled water and measuring with a pH meter (Hach Company, Loveland, CO, USA) (Bogdanov *et al.* 1997). This was repeated on three separate occasions and the mean and standard deviation determined.

The water content of honey was estimated using an Abbé refractometer (Yagami International Trading Co. Ltd, Nagaya, Japan) according to the methods recommended by the International Honey Commission (Bogdanov *et al.* 1997). The refractive index of each sample was determined three times, corrected for temperature and the mean determined. The water content was then determined from the refractive index using a conversion table (Bogdanov *et al.* 1997).

The reducing sugar content of all honeys was determined using dinitrosalicylic (DNS) acid reagent. To make the reagent, 1 g of 3,5-DNS acid and 30 g of sodium potassium tartrate tetrahydrate were dissolved in *c.* 50 ml of distilled water. A 20 ml volume of 2 N NaOH was then added slowly and the total volume made up to 100 ml with distilled water (Bernfield 1955). Each honey

Table 1 Physical and chemical properties of honeys

Honey	Bee species	pH	Reducing sugars (g per 100 g)	Water content (g per 100 g)
A	*Trigona carbonaria*	3·66 ± 0·03	56·0 ± 1·0	23·9 ± 0·2
B	*T. carbonaria*	4·32 ± 0·09	57·3 ± 1·3	24·0 ± 0·0
C	*T. carbonaria*	3·75 ± 0·03	54·6 ± 1·1	25·7 ± 0·2
D	*T. carbonaria*	4·04 ± 0·04	58·7 ± 1·0	26·9 ± 0·0
E	*T. carbonaria*	3·86 ± 0·03	53·0 ± 2·2	25·2 ± 0·2
F	*T. carbonaria*	3·74 ± 0·02	50·5 ± 0·9	26·6 ± 0·3
G	*T. carbonaria*	3·78 ± 0·02	51·2 ± 1·4	26·4 ± 0·1
H	*T. carbonaria*	4·03 ± 0·02	55·7 ± 1·5	25·4 ± 0·1
I	*T. carbonaria*	3·83 ± 0·03	54·4 ± 1·3	25·7 ± 0·1
J	*T. carbonaria*	3·77 ± 0·02	52·3 ± 0·3	24·9 ± 0·2
K	Unknown	3·59 ± 0·03	51·9 ± 0·4	25·0 ± 0·1
Medical	*Apis mellifera*	3·86 ± 0·02	69·8 ± 0·8	17·9 ± 0·1
Table (C)	*A. mellifera*	3·83 ± 0·04	70·8 ± 0·5	16·2 ± 0·1
Artificial		4·82 ± 0·06	–	–

© 2009 The Authors

was diluted 1 in 1000 in distilled water to a reducing sugar concentration of 0·2–1 mg ml^{-1}. A 1·5 ml volume of DNS reagent was added to 0·5 ml of diluted honey and incubated in a heating block at 110°C for 5 min. Samples were cooled immediately in iced water and optical densities measured at 540 nm. The reducing sugar content of each sample was determined from a standard curve which used several concentrations of glucose (0·2–1 mg ml^{-1}). The assay was repeated in entirety three times and the mean and standard deviation determined for each sample.

Micro-organisms and culture conditions

Clinical isolates (n = 13) and reference strains (n = 21) were obtained from the Division of Microbiology and Infectious Diseases at PathWest Laboratory Medicine WA, Nedlands, Western Australia, and the Microbiology and Immunology Discipline of The University of Western Australia, Crawley, Western Australia. Reference isolates are shown in Tables 2, 3 and 4. Routine culture was performed on blood agar for bacteria and Sabouraud dextrose agar for *Candida* spp. All agar plates were incubated aerobically at 37°C for 24–36 h. Unless stated otherwise, inocula were prepared by subculturing each test organism onto the relevant growth medium and incubating overnight. Several colonies of each organism were then suspended in 0·85% saline, and cell suspensions were adjusted to *c.* 10^8 CFU ml^{-1} for bacteria and 10^7 CFU ml^{-1} for *Candida* spp.

Agar diffusion assay

The activity of four stingless bee honeys and three control honeys was assessed against six bacterial species using an agar well diffusion assay. Briefly, agar plates containing 20 ml of Mueller–Hinton agar were inoculated using a swab from a suspension of each organism containing *c.* 10^8 CFU ml^{-1}. An 8-mm diameter well was cut into the agar and 100 μl of 50% honey solution (w/v, prepared in sterile distilled water) was aliquoted into the well. Preliminary investigations with solutions of 25% honey showed no zones of inhibition. One honey was tested per agar plate. At the same time, a phenol standard curve was created for each test organism by aliquoting 100 μl of solutions containing 2%, 4%, 5%, 6%, 8% and 10% (w/v) phenol into single wells on additional plates. Each phenol solution was used for a maximum of 7 days after which time a fresh solution was prepared. After incubation at 37°C for 24 h, the diameter of the zone of inhibition was measured to the nearest millimetre. Each honey was tested a minimum of three times and means were calculated. The phenol equivalence (% w/v phenol) of honey was determined from a standard curve with the square of phenol zone size plotted against concentration.

Agar dilution assay

Bacteria and yeasts were both assessed for susceptibility to honeys using the agar dilution assay, which was performed according to method M7-A7 from the Clinical Laboratory Standards Institute (CLSI) (Clinical Laboratory Standards Institute 2006). Briefly, a stock solution of honey was prepared in sterile distilled water at 60% (w/v). Appropriate volumes of the honey stock solution, sterile distilled water and double-strength Mueller–Hinton agar were combined to result in final concentrations of 4%, 8%, 12% and 16% for medical honey and table honey W and 2%, 4%, 6% and 8% for stingless bee honey. Occasionally, additional plates containing 10% stingless bee honey were prepared. Inocula were prepared as described earlier, and agar plates were inoculated using a multipoint replicator that delivered a 1–3 μl inoculum spot, corresponding to *c.* 10^4 CFU per spot. Plates were incubated for 24 h at 35°C, and minimum inhibitory concentrations (MICs) were then determined as the lowest concentration inhibiting growth, disregarding 1–2 colonies (Clinical Laboratory Standards Institute 2006). The assay was repeated a minimum of three times and modal MIC values were selected.

Table 2 Mean zone of inhibition sizes (mm)* and phenol equivalence (% w/v phenol, shown in brackets) determined by agar diffusion

Honey	*Staphylococcus aureus* NCTC 6571	*Staphylococcus epidermidis* NCTC 11047	*Enterococcus faecalis* NCTC 775	*Escherichia coli* ATCC 10418	*Salmonella* Typhimurium ATCC 13311	*Pseudomonas aeruginosa* NCTC 10662
C	23·3 (8·9)	10·7 (3·5)	10·5 (5·2)	8·7 (2·8)	8·3 (1·5)	12·7 (2·1)
D	9·7 (2·3)	8·7 (1·3)	9·5 (4·0)	8·7 (2·8)	0	9·0 (1·2)
F	23·3 (8·9)	11·0 (3·8)	10·0 (4·6)	9·0 (2·9)	8·3 (1·5)	12·3 (2·0)
J	22·0 (8·3)	11·7 (4·6)	10·5 (5·2)	8·7 (2·8)	8·7 (1·6)	13·3 (2·2)
Medical	0	0	13·0 (8·0)	9·5 (3·0)	9·0 (1·6)	0

*One hundred microlitres of a 50% (w/v) honey solution was used per well (8 mm diameter).

© 2009 The Authors

Table 3 Minimum inhibitory concentrations of honey (% v/v) against Gram-positive bacteria and *Candida* determined by agar dilution

Honey	*Bacillus cereus* ATCC 13061	*Bacillus subtilis* ATCC 6633	*Enterococcus faecalis* NCTC 775	*Ent. faecalis* 24990	*Kocuria rhizophila* ATCC 9341	*Listeria monocytogenes* NCTC 7973	*Staphylococcus aureus* NCTC 6571	*Staph. aureus* 5455M	*Staph. aureus* 1571J	*Staph. aureus* (MRSA) 15046	*Staph. aureus* (MRSA) 15358	*Staph. aureus* (MRSA) 15913	*Staphylococcus epidermidis* NCTC 11047	*Staph. epidermidis* P3	*Staph. epidermidis* I1	*Staphylococcus xylosus* ATCC 29971	*Candida albicans* ATCC 10231	*Candida glabrata* ATCC 15545
Medical	8	8	8	12	8	8	8	8	8	8	8	8	8	8	8	8	8	>16
Table (W)	8	8	8	12	8	8	8	8	8	8	8	8	8	8	8	8	8	>16
A	4	6	8	6	6	8	>8	8	>8	>8	>8	8	8	>8	8	6	>8	>8
B	8	8	>10	>10	10	>10	>10	>10	>10	>10	>10	8	>10	>10	8	>10	>10	>10
C	6	6	8	8	4	6	8	8	6	8	8	6	6	8	6	6	6	>8
D	6	6	>10	>10	8	>10	>10	>10	>10	>10	>10	>10	>10	>10	8	>10	>10	>10
E	6	6	8	8	6	10	>10	>10	>10	>10	>10	>10	>10	>10	8	8	>10	>10
F	6	4	6	8	4	6	>8	8	8	>8	8	8	8	>8	6	6	8	>8
G	6	6	8	8	6	8	8	8	8	8	8	8	8	8	6	6	8	>8
H	6	>8	>8	>8	6	>8	>8	>8	>8	>8	>8	>8	>8	>8	>8	>8	>8	>8
I	4	6	10	8	6	8	>10	>10	>10	>10	>10	10	>10	>10	10	6	>10	>10
J	6	6	8	8	4	6	8	8	8	>8	8	8	8	>8	8	6	8	>8
K	6	8	8	8	6	8	>8	>8	>8	>8	>8	8	8	>8	8	6	>8	>8

Table 4 Minimum inhibitory concentrations of honey (% v/v) against Gram-negative bacteria determined by agar dilution

Honey	*Acinetobacter baumannii* NCTC 7844	*Citrobacter freundii* NCTC 9750	*Enterobacter cloacae* NCTC 10005	*Escherichia coli* NCTC 10538	*E. coli* 9489C	*Klebsiella pneumoniae* NCTC 8172	*Kl. pneumoniae* 5068	*Pseudomonas aeruginosa* NCTC 10662	*Ps. aeruginosa* 4938	*Salmonella* Typhimurium ATCC 13311	*Serratia marcescens* NCTC 1377	*Ser. marcescens* 8242	*Shigella sonnei* NCTC 8220	*Yersinia enterocolitica* 26608
Medical	8	12	12	8	12	12	12	12	>16	8	16	>16	12	8
Table (W)	8	12	12	8	12	12	12	12	>16	8	>16	>16	12	8
A	8	8	>8	>8	>8	>8	>8	>8	>8	>8	8	>8	8	6
B	8	>10	>10	>10	>10	>10	>10	>10	>10	8	>10	>10	>10	>10
C	6	6	6	6	6	8	8	6	6	4	6	6	6	6
D	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10
E	>10	>10	>10	>10	>10	>10	>10	8	8	10	>10	>10	8	8
F	6	6	8	6	8	8	8	6	6	6	6	6	6	6
G	8	8	8	8	8	8	8	6	6	6	8	8	6	6
H	>8	>8	>8	>8	>8	>8	>8	>8	>8	>8	>8	>8	>8	8
I	8	8	10	10	>10	>10	>10	8	8	8	10	10	8	6
J	6	6	8	8	8	8	8	6	6	6	8	8	6	6
K	8	8	>8	8	>8	>8	>8	6	6	8	8	8	8	6

© 2009 The Authors

Broth microdilution assay

The broth microdilution assay was performed according to CLSI reference methods M7-A7 for bacteria (Clinical Laboratory Standards Institute 2006) and M27-A3 for yeasts (Clinical Laboratory Standards Institute 2002). Briefly, twofold dilutions of each honey solution (64% w/v) were prepared in 96-well microtitre trays in Mueller–Hinton broth for bacteria or RPMI 1640 medium for yeasts. A final concentration of 5% lysed horse blood was included in tests with *Streptococcus* spp. to facilitate bacterial growth. Inocula prepared as described previously were diluted with 0·85% saline to result in final inocula concentrations of *c*. 5×10^5 CFU ml^{-1} for bacteria and 1×10^3–3×10^3 CFU ml^{-1} for *Candida*, confirmed by viable counts. After inoculation, final honey concentrations ranged from 1% to 32%. Incubation conditions and determination of MICs were performed according to the reference methods. In addition, minimum bactericidal concentrations (MBCs) and minimum fungicidal concentrations (MFCs) were determined by subculturing 10 μl volumes from nonturbid wells and spot inoculating onto the appropriate growth medium. After incubation, growth was recorded and MBCs/MFCs were determined as the lowest concentration resulting in the death of 99·9% on the inocula. The assay was repeated a minimum of three times and modal values selected. To analyse data, geometric means were calculated for each test organism and also for each honey sample. Data obtained for *Candida glabrata* were excluded from geometric mean calculations as this organism was not inhibited by any honey at ≤32%. For statistical analyses, MICs and/or MBCs were converted to log2 values and analysed using Student's 2- or 1-tailed *t*-test assuming unequal variance. Values of >32% were converted to the next highest value of 64% to enable analyses. *P*-values of <0·05 were considered significant.

Time-kill assay

Stingless bee honeys C, D, F and J were further evaluated for activity against *Staphylococcus aureus* NCTC 6571, *Pseudomonas aeruginosa* NCTC 10662 and *Candida albicans* ATCC 10231 using a time-kill assay. The three control honeys were also assessed. Bacterial inocula were prepared by inoculating 1–2 colonies into 10 ml of trypticase soy broth and incubating overnight at 37°C with shaking at 150 rev min^{-1}. The *C. albicans* inoculum was prepared by suspending colonies from a 24-h culture on Sabouraud dextrose agar in 0·85% saline. Cultures were adjusted with 0·85% saline to *c*. 10^8 CFU ml^{-1} for bacteria or *c*. 10^7 CFU ml^{-1} for *C. albicans*. Honey solutions were prepared in sterile distilled water so that after inoculation the final honey concentration was 20% (w/v). Time-kill assays were performed with sterile distilled water as the diluent to avoid the introduction of confounding factors such as ions, buffering or growth medium. Samples were taken immediately after inoculation (0 min) and again at 60 min for viable counts. Counts were performed by serially diluting samples tenfold in 0·85% saline and spreading 100 μl volumes from the relevant dilutions onto nutrient agar plates. The lower limit of detection was 3×10^2 CFU ml^{-1}. Plates were incubated for 24–48 h at 37°C, colonies counted and the numbers of surviving organisms calculated. The assay was repeated four times. The proportion of cells surviving at 60 min was calculated, as was the mean and standard deviation. Proportions of surviving cells were compared using Student's 2-tailed *t*-test, assuming unequal variance and differences were considered significant where $P < 0·05$.

Results

Examination of the physicochemical properties of stingless bee honey samples showed a mean pH of 3·85 (± 0·21 standard deviation) (Table 1), mean reducing sugar content of 54·2 (± 2·6) per 100 g of honey and mean water content of 25·4 (± 1·0) per 100 g honey.

By agar diffusion, all four stingless bee honeys produced zones of inhibition against all six test organisms, with the exception of honey D and *Salmonella enterica* ssp. *enterica* serovar Typhimurium (Table 2). The largest zones were seen for *Staph. aureus* for honeys C, F and J. For the remaining test organisms, zones were relatively small and ranged from 8·3 to 13·3 mm, with a well size of 8 mm. Phenol equivalence measurements ranged from 1·2% to 8·9% (w/v) phenol for all organisms. Table honey C and artificial honey did not produce zones for any organism at 50% and medical honey produced zones against *Enterococcus faecalis*, *Escherichia coli* and *Salmonella* Typhimurium only.

By agar dilution, the lowest MICs of stingless bee honey were 4% for honeys A, C, F, I and J against various organisms (Tables 3 and 4). Stingless bee honeys C and G showed the most overall activity, both inhibiting 31 of the 32 test organisms at ≤10%. Honeys H, D and B were the least active inhibiting only 3, 4 and 7 test organisms, respectively. Generally, the most susceptible organisms were *Bacillus cereus* and *Kocuria rhizophila*, which were both inhibited by all stingless bee honeys at ≤10%. The least susceptible organism was *C. glabrata*, which was not inhibited by any honey.

By broth microdilution, MIC geometric means ranged from 3·3% for *Staph. aureus* to 49·7% for *C. albicans* and MBC/MFC means ranged from 4·0% for *Staph. aureus* to 53·0% for *C. albicans* (Table 5). *Candida glabrata* was the least susceptible organism and was not inhibited at the

© 2009 The Authors

Table 5 Susceptibility of micro-organisms to honeys (% v/v) determined by broth microdilution

Honey	*Staphylococcus aureus* NCTC 6571		*Staph. aureus* (MRSA) 15913		*Staphylococcus epidermidis* NCTC 11047		*Streptococcus pyogenes* ATCC 10389		*Streptococcus pneumoniae* ATCC 12213		*Enterococcus faecalis* NCTC 775		*Escherichia coli* NCTC 10538		*Salmonella* Typhimurium ATCC 13311		*Pseudomonas aeruginosa* NCTC 10662		*Klebsiella pneumoniae* NCTC 8172		*Candida albicans* ATCC 10231		Geometric mean of the MIC*
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MFC	
Medical	4	8	4	8	8	16	32	32	16	16	16	>32	8	16	8	8	16	32	16	16	32	>32	11·7
Table (W)	8	32	32	>32	32	>32	>32	>32	4	4	32	>32	32	>32	32	>32	32	>32	32	>32	>32	>32	26·5
A	4	8	4	16	4	16	8	8	2	2	8	16	16	16	8	16	8	8	8	16	>32	>32	7·5
B	1	1	2	2	4	4	32	32	8	8	8	16	16	16	16	16	16	16	16	16	32	32	9·1
C	4	4	4	4	4	4	16	16	4	4	8	16	8	8	8	8	8	8	8	8	32	32	7·5
D	2	2	4	4	4	8	32	32	8	8	8	16	16	16	8	8	16	16	8	8	32	>32	9·1
E	2	2	2	2	4	4	16	16	4	4	8	16	16	16	8	16	8	16	16	16	32	32	7·5
F	4	4	4	8	4	16	16	16	4	4	8	16	8	8	8	8	4	8	8	8	>32	>32	7·5
G	2	2	4	4	4	8	8	8	4	4	8	16	8	8	8	8	8	8	8	8	>32	>32	7·1
H	4	4	8	8	8	8	16	16	8	8	16	32	16	16	16	16	16	16	16	16	>32	>32	13·2
I	16	16	16	32	16	32	16	16	8	8	16	32	16	32	16	32	8	16	16	16	>32	>32	16·0
J	2	2	4	4	4	4	16	16	4	4	8	16	8	8	8	8	8	8	16	16	>32	>32	8·0
K	8	32	16	32	16	32	16	16	4	4	16	32	8	32	16	32	8	16	16	16	>32	>32	13·2
Geometric mean (honeys A–K)	3·3	4·0	4·8	6·6	5·5	9·1	16	16	4·8	4·8	9·7	19·3	11·7	14·1	10·3	13·2	9·1	11·7	11·7	12·4	49·7	53·0	

MBC, minimum bactericidal concentration; MFC, minimum fungicidal concentration; MIC, minimum inhibitory concentration.

*Data for *C. albicans*, but not *Candida glabrata* are included in the calculation.

© 2009 The Authors

maximum test concentration of 32% (data not shown). Similarly, *C. albicans* showed little susceptibility to all honeys, with MICs of 32% for five honeys and >32% for the remaining eight. *Streptococcus pyogenes* (16% MIC geo. mean) was notably less susceptible to stingless bee honey compared to the other Gram-positive test organisms, including *Streptococcus pneumoniae* (4·8% MIC geo. mean). Whilst this may be because of interfering substances present in the lysed horse blood growth supplement, this appears to be unlikely given that *Strep. pneumoniae*, which was also tested in the presence of lysed horse blood, remained relatively susceptible. The remaining organisms were inhibited by stingless bee honey at concentrations ranging from 1% to 32%. Although *Staph. aureus* was one of the most susceptible organisms, MICs obtained for *Staph. aureus* did not differ significantly from those obtained for methicillin resistant *Staph. aureus* (MRSA), *Staphylococcus epidermidis* and *Strep. pneumoniae*. Similarly, MICs for the Gram-negative bacteria *E. coli*, *Salm.* Typhimurium, *Ps. aeruginosa* and *Klebsiella pneumoniae* did not differ significantly from each other, or from *Ent. faecalis*. Ranking of geometric means obtained for each stingless bee honey showed honey G to have the lowest geometric mean (7·1%) followed by honeys A, C, E and F, all with means of 7·5%. Honeys G and C both had significantly lower MICs than those obtained for honeys H ($P = 0{\cdot}037$), I ($P = 0{\cdot}009$) and K ($P = 0{\cdot}037$) only. Honeys G and C were not significantly different in activity ($P = 0{\cdot}849$). Honey I had the highest MIC geometric mean of 16% and MICs obtained for this honey were significantly higher than those obtained for honeys A, C, D, E, F, G and J, but not honeys B, H or K. No other significantly differences in activity between stingless bee honeys were seen. Comparison of MICs obtained for stingless bee honeys and medical honey showed no significant differences, whereas all stingless bee honeys with the exception of samples H, I and K were differed significantly from table honey. Medical honey was significantly more active than table honey ($P = 0{\cdot}035$). Finally, MICs and MBCs were generally either equivalent or differed by only one dilution for all honeys, indicating bactericidal activity.

Using the time-kill assay, treatment with all stingless bee honeys resulted in significant decreases in viability for *Staph. aureus* ($P < 0{\cdot}001$) and *Ps. aeruginosa* ($P < 0{\cdot}001$), with decreases in viability after 60 min in the order of 1–3 log and >3 log for each organism, respectively (Fig. 1). Significant, albeit slight, decreases were also seen after the treatment of *C. albicans* with stingless bee honeys C ($P = 0{\cdot}008$), D ($P = 0{\cdot}044$) and F ($P = 0{\cdot}016$). Results for honey J were not significant. Treatment with table honey C resulted in significant decreases in viability for both *Staph. aureus* and *Ps. aeruginosa* (<1 log), whereas treatment with medical honey resulted in significantly decreased viability for *Ps. aeruginosa* only. Treatment with artificial honey did not result in significantly decreased viability for any organism.

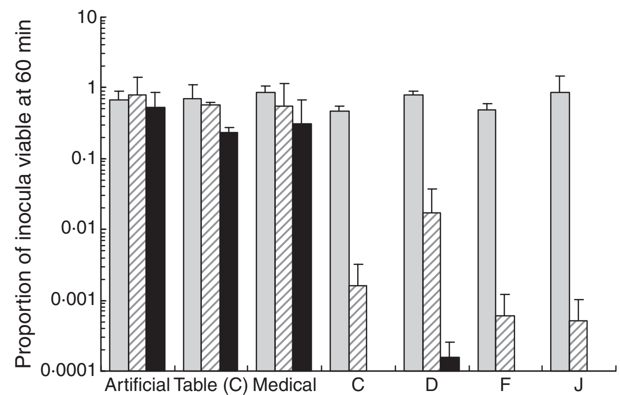

Figure 1 Proportion of viable cells (mean, standard deviation) of *Candida albicans* ATCC 10231 (light grey bars), *Staphylococcus aureus* NCTC 6571 (diagonal stripes) and *Pseudomonas aeruginosa* ATCC 10662 (black bars) after incubation with 20% (w/v) honey for 60 min.

Discussion

This study demonstrates that Australian *T. carbonaria* honey has broad-spectrum antibacterial activity but limited antifungal activity. Significant differences in antimicrobial activity were found amongst the 11 stingless bee honey samples, and individual test organisms were also found to differ in susceptibility. The physical and chemical properties of the stingless bee samples were similar to those found previously (Persano-Oddo *et al.* 2008).

Two recent publications have reported on the antimicrobial activity of stingless bee honey from *T. carbonaria* (Irish *et al.* 2008; Kimoto-Nira and Amano 2008). Both used the agar diffusion method with one reporting no antimicrobial activity (Kimoto-Nira and Amano 2008) whilst the other showed considerable activity (Irish *et al.* 2008). In the current study, little activity was seen by agar diffusion, which concurs with one of these previous studies. Although differences in results may be attributable to variation in the stingless bee honeys tested, the method by which activity was assessed and variations in protocols between different research groups are also likely to be factors. In particular, the method for inoculating the agar, whether it be surface inoculation such as was used in the current study or incorporation of the organisms into the agar plate itself (Irish *et al.* 2008) may influence results. The agar diffusion method has been widely used in the evaluation of the activity of honey, with many authors following the methods described by Allen *et al.* (1991). Several limitations of the agar diffusion method have

© 2009 The Authors

been noted previously by authors investigating honey or other natural antimicrobial substances. These include insensitivity, whereby low levels of antimicrobial activity are not necessarily detectable (Allen *et al.* 1991) and variability whereby minor variations in experimental conditions such as agar volume, inocula concentration and incubation conditions may cumulatively lead to significant variability in results. Furthermore, because nonpolar components may not readily diffuse through water-based agar (Griffin *et al.* 1999), agar diffusion may not be the most appropriate method for evaluating the activity of honey or other complex natural substances. Finally, the agar diffusion results obtained in the current study indicating little activity were not supported by the data obtained by the dilution methods and time-kill assay. The lack of a clear relationship between zone size and MIC was evident. This suggests that the agar diffusion method does not necessarily generate results that are representative of total antimicrobial activity.

Data from both the agar and broth dilution methods indicated that stingless bee honey has broad-spectrum antibacterial activity but limited activity against *Candida*. Stingless bee honey MICs were generally in the range of 6–8% by agar dilution and *c.* 4–16% by broth dilution. Many previous reports of the antimicrobial activity of stingless bee honey are difficult to compare to the present study because of methodological differences (Miorin *et al.* 2003; DeMera and Angert 2004; Garedew *et al.* 2004; Muli *et al.* 2008). Furthermore, most of these honeys were produced by bee species other than *Trigona* and from regions and floral sources distinct from those found in Australia, further limiting comparisons. However, one recent publication which used an agar dilution method has reported MICs of honey from several Guatemalan stingless bees including *Geotrigona acapulconis*, *Melipona beecheii*, *Scaptotrigona* spp. and *Tetra. angustula* ranging from 2·5% to >10% (v/v), with the majority of MICs being 5% (Dardón and Enríquez 2008). In terms of *Apis* honeys, published MICs for manuka honey or medical honey generally range between 2% and 8% (Cooper *et al.* 2002; Lusby *et al.* 2005; George and Cutting 2007), although MICs of >20% have been reported for *C. albicans* (Lusby *et al.* 2005; Irish *et al.* 2006) and *Serratia marcescens* (Lusby *et al.* 2005). MICs published for pasture honey with phenol equivalences similar to manuka honey were in the range of 3–10% (Cooper *et al.* 2000, 2002). Comparison of data from the current study with previously published results indicates that *Trigona* stingless bee honey has activity similar to other medical honeys or other antimicrobially active honeys. Furthermore, results generated using the time-kill method showed that stingless bee honey had a relatively rapid bactericidal action whereas the *Apis* honeys did not. A previous study found a similar trend where little effect was seen after treatment with 25% (w/v) *Apis* honeys, whereas significant cell death was evident after treatment with 25% *Trigona biroi* honey (Temaru *et al.* 2007). Although the majority of results indicated clear overall trends, there were a few instances where outcomes were atypical. In particular, it was expected that the medical honey would be significantly more antimicrobially active than the table honey. However, this was not always observed, and in some instances, the table honey even appeared to be more active than the medical honey. For example, it was unexpected that medical honey did not produce any zones of inhibition against *Staph. aureus*, which is typically the most sensitive organism. As stated earlier, methodological differences between the current and previous studies may account in part for this result. In addition, no major differences in activity were seen between the artificial, table and medical honeys in the time-kill assay. However, only one time point was assessed and it is therefore possible that differences in activity would be evident if the experiment was conducted over a longer time frame. Further studies are required to obtain a clearer understanding of these results.

Honey has several well-known characteristics that are generally accepted as contributing to total antimicrobial activity. These include low pH, an osmotic effect, hydrogen peroxide production and phytochemical factors (Cooper *et al.* 2002). The antimicrobial activity of many honeys can be attributed predominantly to hydrogen peroxide activity (Irish *et al.* 2008), evidenced by a decline in antimicrobial activity after treatment with the enzyme catalase. However, honeys such as manuka remain active after catalase treatment, indicating nonperoxide activity which is largely assumed to be because of the presence of unique phytochemicals (Allen *et al.* 1991; Cooper *et al.* 2002). The compound methylglyoxal has also been shown to contribute to the nonperoxide activity of manuka honey, although the origins of this compound are not yet known (Mavric *et al.* 2008). Nonperoxide activity has also been demonstrated previously for stingless bee honey (Temaru *et al.* 2007; Irish *et al.* 2008) and it has been suggested that both phytochemical and insect-derived factors are responsible for this. Although stingless bee honey differs from *Apis* honey in several important ways, it is of particular relevance that stingless bees store their honey in cerumen pots (Persano-Oddo *et al.* 2008) which are made of wax combined with propolis, which is largely composed of plant resins. In contrast, *Apis* honeybees store their honey in brood combs made only of wax (Crane 1990; Persano-Oddo *et al.* 2008). As noted previously (Temaru *et al.* 2007; Kimoto-Nira and Amano 2008), stingless bee honey therefore has a greater exposure to propolis and a greater opportunity to become

© 2009 The Authors
Journal compilation © 2009 The Society for Applied Microbiology,

infused with plant-derived antimicrobial compounds than *Apis* honey. The variation in activity seen amongst the 11 stingless bee samples tested in the current study may be because of differences in the levels of plant- and/or bee-derived components, because there was no obvious correlation between antimicrobial activity and any of the physical or chemical characteristics examined.

In conclusion, this study has demonstrated that honey from the Australian stingless bee *T. carbonaria* has antibacterial activity similar to that seen for other antimicrobial honeys. Currently, the main medicinal use of honey is in the treatment of wounds and burns and there are several commercially available products marketed specifically for these uses. It is likely that there could be a similar role for stingless bee honey in the treatment of wounds.

Acknowledgements

We thank Mr Ross Oxwell (Physiology, School of Biomedical, Biomolecular and Chemical Sciences, The University of Western Australia) for technical assistance with the refractometer. K.A.H. was funded by a priming grant from the Raine Medical Research Foundation, Western Australia.

References

Adams, C.J., Boult, C.H., Deadman, B.J., Farr, J.M., Grainger, M.N., Manley-Harris, M. and Snow, M.J. (2008) Isolation by HPLC and characterisation of the bioactive fraction of New Zealand manuka (*Leptospermum scoparium*) honey. *Carbohydr Res* **343**, 651–659.

Akerman, K. (1979) Honey in the life of the aboriginals of the Kimberleys. *Oceania* **49**, 169–178.

Allen, K.L., Molan, P.C. and Reid, G.M. (1991) A survey of the antibacterial activity of some New Zealand honeys. *J Pharm Pharmacol* **43**, 817–822.

Bernfield, M. (1955) Amylases, α and β. In *Methods in Enzymology* ed. Colowick, S.P. and Kaplan, N.O. pp. 149–158. New York: Academic Press.

Bogdanov, S., Martin, P., Lüllmann, C., Borneck, R., Flamini, C., Morlot, M., J., H., Vorwohl, G. *et al.* (1997) Harmonised methods of the European honey commission. *Apidologie* **28** (extra issue), 1–59.

Clinical and Laboratory Standards Institute (2002) *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standard – Second Edition*. Wayne, PA: CLCI.

Clinical and Laboratory Standards Institute (2006) *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard – Seventh Edition*. Wayne, PA: CLSI.

Cooper, R., Wigley, P. and Burton, N. (2000) Susceptibility of multiresistant strains of *Burkholderia cepacia* to honey. *Lett Appl Microbiol* **31**, 20–24.

Cooper, R.A., Molan, P.C. and Harding, K.G. (2002) The sensitivity to honey of Gram-positive cocci of clinical significance isolated from wounds. *J Appl Microbiol* **93**, 857–863.

Cortopassi-Laurino, M., Imperatriz-Fonseca, V.L., Roubik, D.W., Dollin, A., Heard, T., Aguilar, I., Venturieri, G.C., Eardley, C. *et al.* (2006) Global meliponiculture: challenges and opportunities. *Apidologie* **37**, 275–292.

Crane, E. (1990) *Bees and Beekeeping: Science, Practice and World Resources*. Oxford, UK: Heinemann Newnes.

Dardón, M. and Enríquez, E. (2008) Physicochemical and antimicrobial characterisation of nine stingless bees (Meliponini) honey from Guatemala. *Interciencia* **33**, 916–922.

DeMera, J. and Angert, E. (2004) Comparison of the antimicrobial activity of honey produced by *Tetragonisca angustula* (Meliponinae) and *Apis mellifera* from different phytogeographic regions of Costa Rica. *Apidologie* **35**, 411–417.

Garedew, A., Schmolz, E. and Lamprecht, I. (2004) Microcalorimetric investigation on the antimicrobial activity of honey of the stingless bee *Trigona* spp. and comparison of some parameters with those obtained with standard methods. *Thermochim Acta* **415**, 99–106.

George, N.M. and Cutting, K.F. (2007) Antibacterial honey (Medihoney™): in-vitro activity against clinical isolates of MRSA, VRE, and other multiresistant Gram-negative organisms including *Pseudomonas aeruginosa*. *Wounds* **19**, 231–236.

Griffin, S.G., Wyllie, S.G., Markham, J.L. and Leach, D. (1999) The role of structure and molecular properties of terpenoids in determining their antimicrobial activity. *Flav Fragr J* **14**, 322–332.

Irish, J., Carter, D.A., Shokohi, T. and Blair, S.E. (2006) Honey has an antifungal effect against *Candida* species. *Med Mycol* **44**, 289–291.

Irish, J., Carter, D.A., Blair, S.E. and Heard, T.A. (2008) Antibacterial activity of honey from the Australian stingless bee *Trigona carbonaria*. *Int J Antimicrob Agents* **32**, 89–90.

Isaacs, J. (2000) *Bush Food: Aboriginal Food and Herbal Medicine*. Sydney: Lansdowne Publishing Pty Ltd.

Kimoto-Nira, H. and Amano, K. (2008) Antimicrobial activity of honey produced by stingless honey bees. *J Apic Res* **47**, 325–327.

Lusby, P.E., Coombes, A.L. and Wilkinson, J.M. (2005) Bactericidal activity of different honeys against pathogenic bacteria. *Arch Med Res* **36**, 464–467.

Mavric, E., Wittmann, S., Barth, G. and Henle, T. (2008) Identification and quantification of methylglyoxal as the dominant antibacterial constituent of manuka (*Leptospermum scoparium*) honeys from New Zealand. *Mol Nutr Food Res* **52**, 483–489.

Miorin, P.L., Levy Junior, N.C., Custodio, A.R., Bretz, W.A. and Marcucci, M.C. (2003) Antibacterial activity of honey

© 2009 The Authors

and propolis from *Apis mellifera* and *Tetragonisca angustula* against *Staphylococcus aureus*. *J Appl Microbiol* **95**, 913–920.

Molan, P.C. and Allen, K.L. (1996) The effect of gamma-irradiation on the antibacterial activity of honey. *J Pharm Pharmacol* **48**, 1206–1209.

Muli, E., Maingi, J. and Macharia, J. (2008) Antimicrobial properties of propolis and honey from the Kenyan stingless bee, *Dactylurina schimidti*. *Apiacta* **43**, 49–61.

Persano-Oddo, L., Heard, T.A., Rodriguez-Malaver, A., Perez, R.A., Fernandez-Muino, M., Sancho, M.T., Sesta, G., Lusco, L. *et al.* (2008) Composition and antioxidant activity of *Trigona carbonaria* honey from Australia. *J Med Food* **11**, 789–794.

Souza, B.A., Roubik, D.W., Barth, O., Heard, T.A., Enriquez, E., Carvalho, C.A., Villas-Boas, J., Marchini, L. *et al.* (2006) Composition of stingless bee honey: setting quality standards. *Interciencia* **31**, 867–875.

Temaru, E., Shimura, S., Amano, K. and Karasawa, T. (2007) Antibacterial activity of honey from stingless honeybees (Hymenoptera; Apidae; Meliponinae). *Pol J Microbiol* **56**, 281–285.

© 2009 The Authors