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SHORT COMMUNICATION

Antibacterial Activity of Honey from Stingless Honeybees (Hymenoptera; Apidae; Meliponinae)

EMI TEMARU[1], SATOSHI SHIMURA[1], KAZUHIRO AMANO[2] and TADAHIRO KARASAWA[1*]

[1] Department of Clinical Laboratory Science, Division of Health Sciences
Kanazawa University Graduate School of Medical Science, Kanazawa, Japan
[2] Laboratory of Apiculture, National Institute of Livestock and Grassland Science
National Agricultural Research Organization, Tsukuba, Ibaraki, Japan

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Abstract

The aim of the study was to examine antibacterial activity of the honey of stingless honeybees (Meliponinae). An agar well diffusion assay demonstrated that many honey samples of stingless honeybees inhibited the growth of test strains of *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli* and *Pseudomonas aeruginosa*; moreover, they exhibited non-peroxide antibacterial activity against those strains. This is the first time that non-peroxide antimicrobial activity of honey from a number of species of stingless honeybees has been demonstrated. These antibacterial activities appear to be powerful, even when compared to those of "manuka honey" from Apinae honeybees.

Key words: honey, non-peroxide antibacterial activity, propolis, stingless honeybees

There are two types of beekeeping in the world. One is the geographically widespread practice of apiculture, utilizing honeybees, or Apinae. The Apinae family is poorly differentiated phylogenetically; fewer than 10 species have been recognized. *Apis mellifera* is widely present in the wild in Africa and Europe, and the subspecies domesticated by mankind are distributed worldwide and kept for beekeeping. Only *A. mellifera* and *Apis cerana* are used for apiculture. The other type of beekeeping utilizes stingless honeybees, Meliponinae, and is called meliponiculture (Crane, 1992; Amano *et al.*, 2000; Amano, 2002). Their stingers are vestigial and non functional. Meliponinae is well-differentiated phylogenetically, and more than 400 species have been recognized. They live in tropical and subtropical areas. Some species of Meliponinae had been kept by mankind in such areas before *A. mellifera* was distributed worldwide. As one of the most recognized examples of meliponiculture, Mayan people in Mesoamerica have been collecting honey for more than a thousand years. In addition to eating the honey, they use it as an antibiotic medicine.

Many studies of honey from Apinae honeybees have been reported, and the antimicrobial activity of the honey has been investigated (Molan, 1992a and 1992b; Willix *et al.*, 1992; al Somal *et al.*, 1994; Cooper *et al.*, 2002; French *et al.*, 2005; Waikato Honey Research Unit, 2007a). However, there have been few reports on the subject of stingless honeybee honey. In the present study, we investigated the antimicrobial activity of the honey of stingless honeybees.

Honey samples were kindly provided by: Professor M.R. Quinonex, Asuncion University, Paraguay; Ing Igor Fleisxher F., Paraguay; Ing M.C. Grajales, Chiapas University, Mexico; Professional beekeeper M.C.J. Espadas, Mexico; Professor M.C.O. Macias, Centro Universitario de la Costa Sur, Mexico; Dr. T. Heard, Australia; Professional beekeeper R.S. Zabel, Australia; Professor S. Boongird, DOK, Thailand; and Professor M.D.J. Mostoles, Camarines Sur State Agricultural College, Philippines. A total of 19 honey samples, 14 from stingless honeybees (A to N) and 5 from Apinae honeybees (O to S), were used. Samples were derived from *Trigona austra1is* (A, Qeensland, Australia), *Melipona beecheii* (B, L, M and N,

* Corresponding author: T. Karasawa, Department of Clinical Laboratory Science, Division of Health Sciences, Kanazawa University Graduate School of Medical Science, 5-11-80 Kodatsuno, Kanazawa 920-0942, Japan; phone: (81) 76 2652597; fax: (81) 76 2652611; **e-mail: karasawa@mhs.mp.kanazawa-u.ac.jp**

Yucatan, Mexico; H, Chiapas, Mexico), *Scaptotrigona pectora1is* (C and K, Yucatan), *Friesiomelita nigra* (D, Yucatan), *Melipona solani* (E, Chiapas), *Scaptotrigona bipunctata* (F, Asuncion, Paraguay), *Melipona quadrifasciata* (G, Asuncion), *Scaptotorigona mexicana* (I, Chiapas), *Trigona biroi* (J, Pili, Philippines), *A. mellifera* (O, R and S, Aichi, Japan), *A. cerana* (P, Bangkok, Thailand), and *A. dorsata* (Q, Kathmandu, Nepal). S*taphylococcus aureus* ATCC 25923, *S. aureus* ATCC 9144 *Escherichia coli* ATCC 25922, *E. coli* W13 (Karasawa *et al.*, 1999), *Enterococcus faecalis* JISSHU 020510, and *Pseudomonas aeruginosa* ATCC 27853 were used.

Agar well diffusion assay was based on a method used for measuring antibacterial activity defined as the "Unique Manuka Factor" (UMF) (Allen *et al.*, 1991a). Professor P.C. Molan, University of Waikato, Hamilton, New Zealand, kindly provided the current version of the method (personal communication), and we followed it closely. In brief, large square plates (Corning) inoculated with bacteria were prepared by adding 100 µl of the bacterial culture in tryptic soy broth (Merck) adjusted to 0.5 OD_{540} to 150 ml nutrient agar (Difco). The honey samples (25% w/v honey in water) were tested in quadruplicate by adding 100 µl to each of four wells. For testing non-peroxide antibacterial activity (*i.e.*, UMF activity), equal volumes of honey samples (50% w/v honey in water) and 2 mg/ml solution of catalase from bovine liver (2860 units/mg, Sigma) were mixed. After incubation for 18 h at 37°C, the diameter of the growth inhibitory zone was measured with a digimatic caliper. A quantitative curve was prepared with the square of the mean diameter of the growth inhibitory zone by phenol diluted with water (2, 3, 4, 5, 6, 7, and 10% w/v solution). A best-fit straight line was fitted and the equation of this line was used to calculate the activity. Considering the dilution and density of the honey, this figure was multiplied by a factor of 4.69, which is based on a mean honey density of 1.35 g/ml, and the activity was expressed with the equivalent percent phenol. For disc diffusion assay, bacteria were grown to 0.1 OD_{540} in tryptic soy broth and inoculated on nutrient agar (90 mm petri dish) with a cotton swab. Paper discs (diameter 8 mm and thickness 1 mm; Advantec, Tokyo, Japan) containing 50 µl of honey samples (50% w/v) were put on nutrient agar.

In the agar well diffusion assay, many honey samples from stingless honeybees exhibited total antibacterial activity (*i.e.*, activity without catalase) to various strains (Table I). Total antibacterial activity was also examined by a disc diffusion assay, and results consistent with those of the well assay were obtained. The well assay was likely to be more sensitive and precise in detecting the activity than the disc assay. *S. aureus* ATCC 9144 appeared to be more susceptible to honey than the other test strains. Data of five samples from Apinae honeybees are presented as references. Non-peroxide antibacterial activity of honey from stingless honeybees was detected when tested for the following strains: *S. aureus* ATCC 25923, 71% (no. of non-peroxide antibacterial activity-positive samples from stingless honeybees per a total of 14 samples from stingless honeybees); *S. aureus* ATCC 9144, 100%; *E. faecalis* JISSHU 020510, 50%; *E. coli* ATCC 25922, 57%; *E. coli* W13, 43%; and *P. aeruginosa* ATCC 27853, 43%. To confirm the antibacterial activity, the survival of *S. aureus* ATCC 9144 in the presence of honey samples was investigated. Two hundred microliters of each honey sample (50% w/v) was mixed with an equal volume of overnight culture of *S. aureus* in tryptic soy broth and incubated at 25°C. The mixed solution was withdrawn at 0, 1, 3, 9, and 24 h, diluted with phosphate-buffered saline immediately, and cultured on tryptic soy agar. From 0 to 24 h, honey samples from the Apinae honeybees (O, Q, and R), which showed no activity in the well and disc assays, did not affect the number of *S. aureus*, while samples from stingless honeybees markedly decreased the number of *S. aureus* (Fig. 1). These results demonstrate the bactericidal activity of honey samples from the stingless honeybees tested.

Honeybee honey from manuka (*Leptospermum scoparium*) in New Zealand has been found to have high antibacterial activity, with approximately half of this type of honey having an exceptionally high level of non-peroxide activity (Allen *et al.*, 1991b). However, almost all samples of honeybee honey from sources other than manuka showed no detectable non-peroxide activity (Allen *et al.*, 1991a). In the present study, we clearly showed that honey from stingless honeybees possesses strong total and non-peroxide antibacterial activities using the same method for measuring the UMF number, suggesting that the antibacterial activity of stingless honeybees is powerful, even when compared to those of manuka honey. Recently, DeMera and Angert (2005) have observed antimicrobial activity of honey from *Tetragonisca angustula*, Meliponinae. However, to the best of our knowledge, this is the first time that non-peroxide antimicrobial activity of honey from a number of species of stingless honeybees has been demonstrated.

The antimicrobial activity of the usual honey is attributed to four properties, including osmotic effect, acidity, hydrogen peroxide, and the intermingling of phytochemical factors (Molan, 1992a; Waikato Honey Research Unit, 2007a). The major antibacterial activity has been found to be due to hydrogen peroxide. However, when considering the use of honey as an antibacterial agent, non-peroxide activity is important, since the potency of the antibacterial activity is likely to be reduced by the action of catalase present in human body tissue and serum. This non-peroxide activity is believed to be due to the intermingling of

Table I
Antibacterial activity of honey from stingless honeybees measured by agar well diffusion assay and disc diffusion assay

Sample	*S. aureus* ATCC 25923		*S. aureus* ATCC 9144			*E. faecalis* JISSYU 020510			*E. coli* ATCC 25922			*E. coli* W13		*P. aeruginosa* ATCC 27853		
	Well		Well		Disc	Well		Disc	Well		Disc	Well		Well		Disc
	(–)	(+)	(–)	(+)	(–)	(–)	(+)	(–)	(–)	(+)	(–)	(–)	(+)	(–)	(+)	(–)
A	1.7±0.4	0.5±0.7	13.6±1.0	13.2±1.2	0	0	0	0	0	0	0	0	0	0	0	0
B	24.7±2.4	16.0±0.7	38.9±2.4	42.2±3.3	>16.4	29.4±0.6	18.8±0.5	>16.4	37.7±2.8	24.2±3.6	>16.4	19.8±1.9	25.3±2.5	13.8±1.2	12.6±1.4	7.5±0.1
C	28.1±2.3	15.0±1.2	42.1±4.2	34.5±2.8	>16.4	24.0±1.9	19.2±0.9	>16.4	32.2±2.0	33.8±2.9	>16.4	23.9±2.4	18.8±1.7	10.3±2.6	10.6±1.2	7.5±0.1
D	21.9±1.3	8.0±0.3	30.7±3.8	26.6±0.7	>16.4	17.5±1.2	6.7±0.8	>16.4	19.7±4.6	14.3±2.9	12.9±0.5	20.7±1.0	17.5±1.2	8.5±1.8	6.6±0.4	6.5±0.1
E	0	0	8.3±1.6	11.5±2.1	0	0	0	0	0	0	0	0	0	0	0	0
F	16.2±3.6	4.2±1.7	35.8±2.8	13.8±0.7	>16.4	7.0±1.3	0	0	21.0±0.7	1.9±2.9	0	4.0±1.2	0	7.9±1.4	0	0
G	0.3±0.4	0.2±0.4	17.8±2.2	18.8±4.4	10.5±0.5	0	0	0	0	2.1±4.6	0	0	0	0	0	0
H	10.6±1.4	2.5±1.3	28.3±5.0	25.5±1.0	>16.4	7.2±1.2	12.6±1.4	0	9.7±2.7	0	0	0	0	4.9±1.2	0	6.0±0.1
I	21.6±1.4	9.6±0.8	33.8±1.1	41.9±2.3	>16.4	17.2±1.2	11.4±1.7	>16.4	21.3±1.8	13.9±3.5	10.8±0.6	13.0±0.3	14.1±3.0	9.9±0.9	6.9±1.4	6.3±0.1
J	20.7±3.4	17.3±2.2	42.1±1.1	38.1±0.9	>16.4	30.6±5.3	23.7±1.1	>16.4	28.7±1.5	28.9±7.4	10.3±0.6	19.5±4.0	28.2±3.6	9.3±1.2	6.5±1.8	8.4±0.8
K	22.6±1.7	15.0±12.8	41.2±3.7	33.8±0.1	>16.4	25.0±4.1	15.4±0.5	>16.4	29.7±3.4	16.9±1.5	11.4±0.1	16.5±2.0	14.5±0.3	13.8±0.7	1.9±3.8	6.6±0.2
L	15.5±2.6	0	18.7±1.0	19.8±2.2	>16.4	8.7±1.1	0	>16.4	11.0±3.5	0	0	0	0	5.4±0.5	0	6.2±0.1
M	0	0	8.1±0.7	3.9±1.7	0	0	0	0	0	0	0	0	0	0	0	0
N	21.3±0.1	0	14.4±3.2	14.2±1.1	>16.4	0	0	0	2.8±5.5	0	0	0	0	0	0	0
S	8.4±1.6	1.7±0.2	22.1±3.7	18.3±1.1	13.7±0.0	16.1±2.3	0	0	5.1±1.2	4.6±1.0	0	0	0	0	0	0

The antimicrobial activity values are expressed as mean ± SD percents (w/v) phenol. The values of samples from O to R were zero for all tested bacterial strains. In the disc diffusion assay, the linearity of the standard curve was demonstrated among 4, 5, 6, and 7% (w/v) phenol solutions (see Text). (–), without catalase; (+), with catalase.

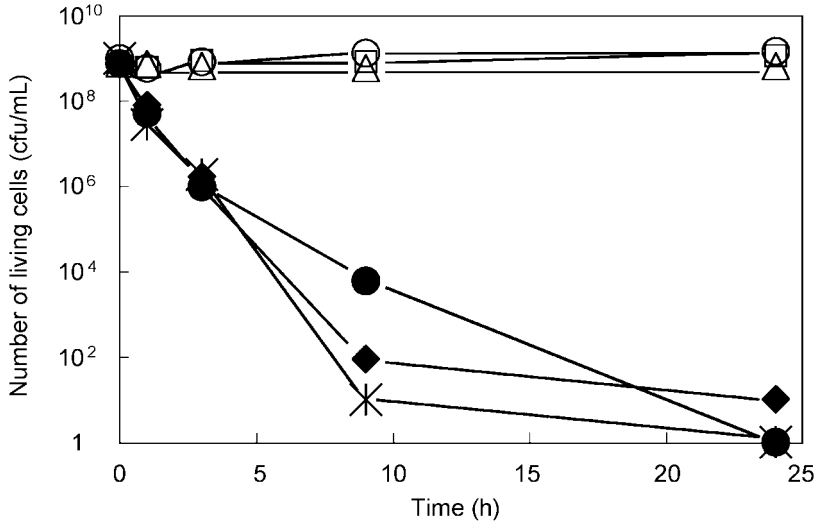

Fig. 1. Bactericidal activity of honey from stingless honeybees.
Honey samples were added as 50% solutions to an equal volume of culture of *S. aureus* ATCC 9144 and incubated at 25°C. Samples from Apinae honeybees: open circle, R (*A. mellifera*); open square, O (*A. mellifera*); and open triangle, Q (*A. dorsata*). Samples from stingless honeybees: filled circle, C (*S. pectoralis*); filled diamond, J (*T. biroi*); and asterisk, B (*M. beecheii*). In samples B and C, the bacterial number was less than the detection limit (10 cfu/ml) at 24 h incubation.

phytochemical factors, although there is not enough evidence for such definite conclusions to be justified (Molan, 1992b; Weston *et al.*, 1999; Waikato Honey Research Unit, 2007a and 2007b). Regarding the former three properties, there are fundamentally no differences between honeys of Apinae honeybees and stingless honeybees. Stingless honeybees keep honey in storage pots built of cerumen (Crane, 1992; Amano *et al.*, 2000). Cerumen is made of wax secreted from the glands on the abdomens of workers, combined with propolis, which is derived from resins collected from plants. Therefore, the honey is influenced by the infiltration of the propolis content. On the other hand, Apinae honeybees keep the collected honey in hexagon brood combs, which are built of pure wax alone. Propolis is only used to seal the extra space between the nest and the cavity; as a result, the honey of Apinae honeybees is not affected by propolis during storage. Propolis possesses antibacterial, antifungal and antiviral properties (Marcucci, 1995; Burdock, 1998; Bankova *et al.*, 2000). The resin content of the plants affects the antibacterial activity of the propolis (Marcucci, 1995; Burdock, 1998; Bankova *et al.*, 2000). In summary, differences in the antimicrobial activity of honeys from Apinae honeybees and stingless honeybees would be due to propolis. In addition, we consider that the non-peroxide activity of the honey from Apinae honeybees such as "active" manuka honey is due to antibacterial substances intermingled coincidentally when Apinae honeybees collect plant materials. In the present work, only honeybee honey sample S from buckwheat showed antibacterial activity. From our experience, this kind of honey is frequently found to possess antibacterial activity, the reason of which would be same with "active" manuka honey.

Attempts are now being made to use honey with UMF for medical treatments (*e.g.*, wound dressing, treatment of *Helicobacter pylori*, *etc*) (Cooper *et al.*, 2001; Molan, 2002 and 2006; Waikato Honey Research Unit, 2007a). Especially, there is a number of evidence to support the use of honey as a wound dressing. Not only antimicrobial activity but also anti-inflammatory activity, stimulation of cytokine production, and other biological activities of honey are found to be beneficial in wound care (Molan, 2006). Honey from stingless honeybees, as well as honeybee honey with UMF, would be suitable for dressing products.

In conclusion, the honey of stingless honeybees is an anciently known but newly rediscovered bioresource possessing antimicrobial activity, probably derived from phytochemical factors, and its availability for use in nutritional supplements and cosmetics as well as for pharmaceutical and medical use will be revealed by further research.

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