

ANTIMICROBIAL ACTIVITY BY *TRIGONA LAEVICEPS* (STINGLESS BEE) HONEY FROM THAILAND

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ABSTRACT

Objective: To determine the key properties of *Trigona laeviceps* honey from Thailand, including its antimicrobial activity.

Methodology: Proline content and the percentage of invert sugar were evaluated as described. Major protein bands were resolved and analyzed by SDS PAGE and MALDI TOF analyses. Antimicrobial activity was assayed by agar well diffusion.

Results: The honey was acidic (pH 3.37) but probably undoctored (proline content of 1,723mg/kg) with a normal sugar content (e.g. invert sugar 15.2% (w/w)) but a higher than expected total protein content (0.28g/100g). From some ten distinct protein bands, six major protein bands were revealed of which the 29 kDa band was likely to be pollen allergen Lol p VA precursor *Lolium perenne* (Perennial ryegrass). Neat honey is the most effective for use as a contact antimicrobial agent, and *Staphylococcus aureus* was the most susceptible tested pathogen to honey at all dilutions.

Conclusion: The studied honey could perhaps be used as an antimicrobial agent. Since pollen allergen protein was found, it may cause honey intoxication.

KEYWORDS: *Trigona laeviceps*, Honey, Proline, Invert sugar, Major protein, Inhibition zone.

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INTRODUCTION

Honey is one of the economic bee products. After bees forage for nectar from plants, enzymes in honeybees such as α -glucosidase will hydrolyze disaccharides in the nectar to monosaccharides, such as glucose and fructose.¹ In almost all honey types assayed,

fructose is the prevalent component (38%) whilst glucose is the second commonest component (31%).² Honey is, therefore, a hyperosmotic solution due to the high concentration of monosaccharide sugars, which alone will provide some antimicrobial activity in this neat state. It is widely consumed as a natural food in all countries with a very sweet taste. However, the presence of enzymes such as glucose oxidase produces acids like gluconic acid and hydrogen peroxide. Whilst the lowered pH and oxidizing agent (formerly known as inhibine) serve as antimicrobials to preserve the honey, the low pH tends to impart a sour taste which if too high a level (low pH) makes the honey unpalatable. In addition, taking advantage of its three antimicrobial components (hyperosmotic, low pH and presence of hydrogen peroxide), it has long been used in tradi-

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tional medicine since it is easily obtained in the countryside and is cheap. In the ancient times, the Egyptians and Greeks used natural unprocessed honey as a topical application to help prevent microbial infections and aid wound healing.³

Although many researches have reported the benefits of honey, some disadvantages have also been found. Honey is frequently (typically) contaminated by yeasts (*Saccharomyces*, *Schizosaccharomyces* and *Torula* strains), by fungi (*Penicillium* and *Mucor* strains) and by bacterial spores (*Bacillus* and *Clostridium* genus).⁴⁻⁶ Upon dilution of the honey to a less hyperosmotic condition typically > 19% water (v/v), such as occurs either after oral ingestion or topical application, these microbial contaminants can serve as opportunistic pathogens in susceptible people. Furthermore, altered mental status could be caused by grayanotoxin contaminated in honey.⁷

The physicochemical characteristics of honey, such as its composition, sweetness, color, odor and pH, are somewhat variable and diverse between bee species and between locations or seasons due to foraging from different plant sources. This is directly related to the fact that the properties of honey depend on various factors, including the plant sources, climate, environment and bee species.⁸ In Thailand, bee diversity is relatively high. Other than *Apis mellifera*, an imported species, there are four native *Apis* spp. and over seven species of stingless bees.⁹ In this research we investigated honey from the stingless bee, *Trigona laeviceps* Smith (Meliponini: Trigona), since it is the most common stingless bee species in Thailand. Although the taste and odor of honey from *T. laeviceps* is not favorable amongst many human consumers, it has long been used in traditional medicine in Thailand for both topical and oral applications. The properties of *T. laeviceps* honey from one supplier in Thailand, in terms of the pH, percentage of invert sugar, total protein content and the types of the major proteins, were obtained. In addition, antimicrobial activity on various pathogens was observed and analyzed.

METHODOLOGY

Sample collection: Wild honey of *Trigona laeviceps* was purchased from an apiary in Samut Songkram, Thailand, and was stored at RT until use.

Proline content: The proline content was determined as described previously,¹⁰ using a standard curve of proline derived from 0, 100, 200, 300, 400 and 500 µg/ml samples. Triple aliquots (250 µl) of each sample was mixed with 125 µl formic acid followed by 500 µl of 3% (w/v) Ninhydrin solution and then boiled for 15 minutes. Isopropanol (2.5 ml) was added, mixed and then the absorbance at 520 nm (1 cm light path) was measured. The proline content was calculated from the standard curve.

Percentage of invert sugar: Total invert sugar levels were evaluated as described previously.¹⁰ Ten ml of 2% (v/v) honey was mixed with 2.5 ml Fehling solution (3.47% (w/v) copper sulfate, 8.7% (w/v) potassium sodium tartrate and 2.5% (w/v) NaOH) and briefly boiled prior to the addition of 500 µl of 0.2% (w/v) methylene blue. Thereafter, the sample honey was titrated in until the color of methylene blue was totally gone (end point), and the volume of the sample honey was recorded (X ml). Then the sample honey (750 µl) was mixed with d-H₂O at the volume of 12.5 - X ml and 2.5 ml of Fehling solution added, boiled, 0.2% (w/v) methylene blue (500 µl) added and the amount of the sample honey required to titrated to the end point (loss of blue color) was recorded as Y. The percentage of invert sugar was then obtained from $(25 \times 100) / (\text{gram of honey} \times Y)$.

Protein assay: The total protein content of honey was evaluated as described previously,¹¹ using Bovine Serum Albumin (BSA) at a concentration of 0, 0.05, 0.10, 0.15, 0.20, 0.25 and 0.30 mg/ml for the standard curve. Sample honey was serially diluted and a sample (20 µl) was mixed with 200 µl of Bradford solution, incubated at RT for five minutes, and then the absorbance at 595 nm was measured. Assays were performed in triplicate and the protein content was calculated from the standard curve.

SDS PAGE of crude protein: Protein preparations were partially resolved by one dimensional discontinuous reducing gel, with a 12% and 4% (w/v) PA resolving and stacking gel, respectively. For 15 ml of loading sample, crude protein at a total amount of 10, 20, 30 and 40 mg was each mixed with 1 x SDS reducing loading dye, heated to 80 °C for one minute and chilled on ice prior to loading. The electrophoresis was performed with 1 x running buffer at 10 V/cm until the coomassie blue (CBB) dye front reached the bottom of the gel. The gel was incubated in 1.25% (w/v) CBB/ 10% (v/v) acetic acid/ 10% (v/v) methanol for 30 minutes. Then, it was destained (above solution but without the CBB) as required.

Amino acid sequencing: After being CBB stained and destained, major bands were cut, excised and tryptic digested. Partial amino acid sequences were analyzed by Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry (MOLDI TOF MS). The matching between obtained amino acids and recorded amino acid was searched by Mascot search program.

Agar well diffusion method: Antimicrobial activity, using the agar-well diffusion method was evaluated as described previously.¹² *Staphylococcus aureus* ATCC 25923 (Gram⁺ve bacteria) and *Escherichia coli* ATCC 35218 (Gram⁻ve bacteria) were grown in Luria Bertani (LB) media, whilst the yeasts *Candida albicans* ATCC 10231 and *Auriobasidium pullulans* ATCC 11942 and the fungi *Aspergillus niger* ATCC 16404 were grown in Potato Dextrose Broth (PDB), at 130 rpm and 37 °C (for bacteria) or 30°C (for yeast and fungi). Bacterial inoculum was adjusted to 0.5 McFarland turbidity standard (10⁸ CFU/ml) and then diluted 1: 10 while the concentration of fungi and yeast was adjusted to 3.4 x 10⁷ cells/ml.¹³ Honey was diluted in LB or PDB, as appropriate, to yield solutions of 0, 25 %, 50 %, 75 % and 100 % (v/v). After agar medium (LB agar for bacteria and Potato Dextrose Agar (PDA) for yeast and fungi) in a 11 cm diameter plate was spread with 100 ml of the test microbe culture, a hole in the center was made using a cork borer

(# 6) and 200 ml of the selected honey dilution was filled in the hole. Assays were performed in triplicate for each honey dilution-test microbe combination, with all five microbes being screened with all four honey concentrations. The bacterial samples were incubated at 37 °C for three days whilst the yeast and fungi samples were incubated at 30 °C for three days. The diameter of the inhibition zone on the plates was measured daily. The comparison of inhibition zones was analyzed by One Way ANOVA (SPSS program).

RESULTS

In order to characterize some of the key properties of *Trigona laeviceps* honey, the four parameters of pH, proline content, the percentage of invert sugar and the total protein content were evaluated (Table-I). The test sample of honey was found to be relatively acidic with a pH of 3.37. The proline content, assayed as an indicator of honey adulteration, was determined to be 1,723 mg/kg and as such indicated that the studied honey was genuine. The total invert sugar composition of the honey was 15.2% (w/w). However, since the taste of *T. laeviceps* honey was very sour, it is likely that the taste was more dependent on the acidity than the sweetness.

Other than sugar, which was the main component in honey, a slightly higher protein content than normally found in other honeys, such as *Apis mellifera*, was also observed (0.28 g/100g). Honey samples were resolved by discontinuous reducing SDS PAGE and then stained with Coomassie blue. Approximately ten distinct bands could be resolved with six major protein bands (Fig-1). From these the bands labeled A - E (Fig-1) were excised from the gel and, after tryptic digestion, were analyzed for

Table-I: Parameters used to determine the properties of *Trigona laeviceps* honey.

Parameter of measurement	Obtained value
pH	3.37
Proline content (mg/kg)	1,723
Percentage of invert sugar	15.2
Protein content (g/100 g)	0.28

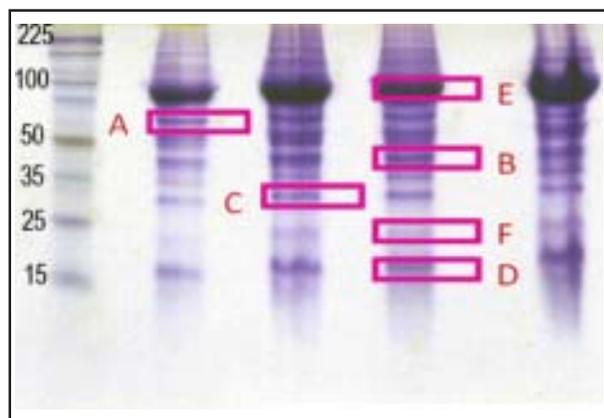


Fig-1: One dimensional reducing SDS PAGE resolution of the total proteins in honey with coomassie blue staining. Lane 1 contains broad range protein weight markers with sizes in kDa shown to the left, whilst lanes 2 - 5 contain crude protein at 10, 20, 30 and 40 mg, respectively. Bands labeled A - E were cut from the gel, excised and after tryptic digestion were analyzed for partial amino acids by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MOLDI TOF MS) and was searched for homology by Mascot search database. The matched peptides are shown in Table-II.

partial amino acids by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MOLDI TOF MS), deduced likely peptide fragment sequences were then used to search for likely candidate proteins (or homologs) by amino acid sequence similarity using Mascot search database. The matched peptides are shown in Table-II.

For antimicrobial activity, the diameter of inhibition zone of pathogens by honey at various dilutions was measured each day. That for the three days is summarized in Table-III, where both honey dose-dependent antimicrobial activity and microbe species-dependent susceptibility effects were both evident and statistically significant. Of the microbes, *S. aureus* was the most susceptible to honey-mediated inhibition of growth, but in all both the mechanism of action and whether it was microbiostatic or microbiocidal was not elucidated.

DISCUSSION

Since honey varies in its properties and bio-activities with different locations, seasons and bee species, the honey of *Trigona laeviceps* from Thailand was selected for this study because little is known about either the different honeys from Thailand or from this common bee species. Multifloral and natural unprocessed honey was used since this is the consumer preferred (conditioned to) format.

The quality criteria of honey used were those specified by the Codex Alimentarius standards, which include the selected parameters recorded in Table-I. Since the major components of honey are simple monosaccharide sugars (principally glucose and fructose), it is no surprise that we report that this honey was comprised of 15.2% invert sugar. However, we also found the honey to be composed of a

Table-II: Potential identification of the six major proteins resolved in *T. laeviceps* honey. They were obtained from searching for the sequence similarity / identity to recorded amino acid sequences in the database.

Major band	Score	Queries matched	Mass	Expected protein
A	60	17	67237	AK131402 NID <i>Homo sapiens</i>
B	58	12	40128	Polyprotein (Fragment) Turnip mosaic virus (strain Japanese) (TuMV)
C	78	17	29749	Pollen allergen Lol p VA Precursor <i>Lolium perenne</i> (Perennial ryegrass)
D	72	10	20809	Hypothetical protein <i>Tetrahymena thermophila</i> SB210
E	75	16	73035	Oligopeptide-binding protein aliA precursor (Exported protein 1) <i>Streptococcus pneumonia</i>
F	66	12	25490	GTP-binding protein RAB2 homolog (Rab_C66 protein) <i>Paramecium tetraurelia</i>

Table-III: The mean inhibition zone sizes of honey at various dilutions measured after 3 days of culture since the application of the honey. Significant differences between means were analyzed by One Way ANOVA.

Dilution of Honey	Diameter of inhibition zone in cm (mean + S.E.)				
	<i>S. aureus</i>	<i>E. coli</i>	<i>C. albicans</i>	<i>A. pullulans</i>	<i>A. niger</i>
0 % (Control)	0 ^{aa}	0 ^{aa}	0 ^{aa}	0 ^{aa}	0 ^{aa}
25 %	1.14 + 0.42 ^{aa}	0 ^{aa}	1.80 + 0.00 ^{bb}	1.74 + 0.03 ^{bb}	1.14 + 0.06 ^{aa}
50 %	2.54 + 0.14 ^{ab}	2.14 + 0.06 ^{bb}	1.80 + 0.06 ^{bb}	2.14 + 0.03 ^{cc}	1.66 + 0.03 ^{bb}
75 %	3.20 + 0.06 ^{bb}	2.54 + 0.06 ^{bc}	2.26 + 0.06 ^{cc}	2.06 + 0.06 ^{cc}	1.86 + 0.03 ^{bb}
100 % (Neat)	3.80 + 0.41 ^{bb}	2.94 + 0.10 ^{cc}	2.66 + 0.03 ^{dd}	2.74 + 0.03 ^{dd}	2.46 + 0.14 ^{cc}

Data are shown as the mean + S.E. and are derived from three independent repeats. Means within a column with different uppercase letters are significantly different at the $p < 0.05$ level.

higher than expected level of protein (0.28% (w/w)), being some 2.7- to 7-fold higher than that normally found in *A. mellifera* honey (40 - 100 mg/ 100g).

Following resolution of the honey proteins by reducing SDS PAGE and excision of the major bands and, following tryptic digestion, MALDI TOF MS analysis of the likely amino acid peptide sequences, the major protein band (band C) most closely matched with the pollen allergen Lol p VA Precursor from *Lolium perenne* (Perennial ryegrass) (Fig-1 and Table-II). This result is to some extent congruent with those of Gunduz *et al* and Finola *et al.*,^{7,14} and suggests that honey could be contaminated with pollen. This is almost to be expected given that bees forage for and return pollen to the hive and indeed the same individual bees may forage and return bit pollen and nectar to the hive at the same time. However, in contrast to *A. mellifera* where of the ten principal honey proteins four were derived from the bee, here none of the six major proteins appeared to be derived from *T. laeviceps*. The presence of pollen and pollen proteins (antigens) in honey raises the issue of the risk to sensitive (hypoallergenic) individuals already sensitized and allergic to pollen antigens from consuming honey, whilst the presence of an apparent human protein (polyprotein fragment) awaits confirmation and clarification.

Nevertheless, this research is the first report that *T. laeviceps* honey can perform any antimicrobial activity *in vitro*. The antimicrobial capacity of honey may be from a combination or synergistic effect of its low pH (pH 3.37),

high osmolarity and some certain molecules such as hydrogen peroxide, antimicrobial peptides, or other volatile substances. In general, the pH level of honey is in the range of 3.4 - 5.5. In addition, bacterial colonization or infection occurs at pH >7.3.¹⁵ Marcucci *et al.* stated that the antimicrobial activity of honey was basically against Gram^{+ve} bacteria,¹⁶ is in agreement with the limited taxonomic based data of this report (Table-III) where, of the tested isolates, *S. aureus* was the most susceptible. After honey was diluted, and thus the hyperosmolarity was reduced, it could still inhibit the growth of selected pathogens, perhaps suggesting the presence of an anti-microbial activity other than simply the sugar dependent hyperosmolarity. Given the low pH and sour taste of this honey, and thus the likely presence of high levels of gluconic acid, this may hence be due to hydrogen peroxide, a by product of glucose oxidation to gluconic acid by glucose oxidase. Regardless, since this *T. laeviceps* honey was not popular amongst consumers, being too acidic and thus sour tasting, it may be better to use this honey in traditional medicine, especially for the topical treatment of antibiotic-resistant pathogenic strains. In the light of this it is relevant that Cooper *et al.* reported that *leptospermum* honey (medical honey) from Australia could kill methicillin-resistant *Staphylococcus aureus* after a local antiseptic (octenidin) failed to kill the pathogen.¹⁷ From the data mentioned above, *T. laeviceps* honey contains both good and bad points. Some precautions may be required in order to avoid either allergy in hyperallergenic people

already sensitized to pollen allergens, or to sensitizing other susceptible members of the population as honey consumption increased to reduce suffering from bee or pollen related allergens.

CONCLUSION

Trigona laeviceps honey could inhibit the growth of selected pathogens in three aspects. The first one was the pH which at 3.37 may inhibit the growth of all but acidophiles. The second aspect was that, until diluted, honey is saturated with sugar (e.g. invert sugar was 15.2% (w/w)), providing a strong hyperosmotic effect. The last antimicrobial action was an apparent antimicrobial activity itself, although the component(s) responsible remain to be evaluated including the possible role of hydrogen peroxidase or antimicrobial peptides. Neat honey was the most suitable for growth inhibition, although this may simply represent the hyperosmotic effects rather than the concentration of relatively rare bioactive components. However, whilst the level of antimicrobial activity was dose-dependent, susceptibility was microbe dependent with *Staphylococcus aureus* being the most susceptible.

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