Integrative Biosciences 10: 163-167, 2006

Integrative
Biosciences
http://www.ibiosci.or.kr

Prevalence of Antibiotics in Nectar and Honey in South Tamilnadu, India

RD. Jebakumar Solomon*, V. Satheeja Santhi and Vimalan Jayaraj

Department of Molecular Microbiology, School of Biotechnology, Madurai Kamaraj University, Madurai 625 021, India

Abstract: Reverse-Phase High-performance Liquid Chromatography (RP-HPLC) based technique is one of the most sensitive techniques to detect the antibiotics present in honey. In the southern part of Tamilnadu, India, majority of the farmlands are occupied by plantations such as coconut, banana and rubber. A variety of antimicrobial compounds and antibiotics, which have been reported in pollen, nectar and other floral parts of the plant, gets accumulated in honey through honeybees (*Apis mellifera*). We have collected the nectar samples from banana (*Musa paridasiaca*) and rubber (*Ficus elastica*) flowers and the honey from honey hives of banana and rubber cultivated areas. The extracted nectar and honey samples are subjected to RP-HPLC analysis with authentic antibiotic standards. Nectar and honey samples showed 4-17, 11-29 μg/kg of streptomycin, 2-29, 3-44 μg/kg of ampicillin and 17-34, 26-48 μg/kg of kanamycin respectively.

Key words: Honey, nectar, pollen, streptomycin, ampicillin, kanamycin, RP-HPLC, MRSA, vancomycin-resistant enterococci

Honey is the combination of nectar secretions from the flowers of some plants and other sweet plant deposits that are gathered and modified by honeybees. Bees store honey in honeycombs and then use it for food in winter. It is a natural sweetener because of its high glucose and fructose content; it is widely used in candies, cereals, and baked goods. There are more than 300 types of honey varying in flavor and colors (from pale yellow to dark amber), depending on the type of blossoms visited by the honeybee.

It is accepted as an effective therapeutic agent by both practitioners of conventional medicine and the general public since the ancient time. The ancient Greeks, Romans, Chinese and Egyptians Medical personnel used honey to heal wounds and cure disease of the gut. Honey exerts bactericidal effect on all common wound-infecting species including multi-drug-resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE) and *Acinetobacter abumari*. The undiluted honey has been also observed to stop the growth of Candida species while *Pseudomonas aeruginosa, Clostridium oedematiens, Streptococcus pyogenes* remained resistant (Efem et al., 1992). Some species of Aspergillus did not produce aflotoxin in various dilutions of honey (Wellford et al., 1978) and it also had bacteriostatic effect on the growth of *Salmonella, Escherichia coli, Aspergillus niger and Penicillium chrysogenium* (Radwan, 1984). It has been postulated that the antibacterial activity of honey depends on the type of the flower that acts as the source of the nectar (Allen et al., 1991).

There are two types of antibacterial components in honey. The peroxide component is destructed when honey is heated or stored in the light and the other non-peroxide component is stable to heat and storage. Most of the non-peroxide antibacterial activity originates from the bee, but some of it comes from the honey source (nectar or honeydew).

Honey bee secretes enzymes into the nectar, which breaks them down into simple sugars to make them more digestible for the bees. The nectar is then spread throughout the honeycombs where it evaporates into thick syrup, aided by the bees fanning it with their wings. This fanning not only removes excess moisture, but keeps the hive at a constant temperature of 34°C. Thus antibiotics and other compounds concentration increase in honey than in nectar. One of those enzymes produces hydrogen peroxide and gluconic acid, which is involved in cleaning wounds and killing bacteria. Honey releases its hydrogen peroxide slowly, so it is less damaging to skin than other drugs (Al-Jabri, 2005). The antimicrobial activity of honey is mainly because of the differences in the amount of hydrogen peroxide generated, but sometimes is also because of additional antimicrobial components from specific plant sources

*To whom correspondence should be addressed.
Tel: 91-452-2459480; Fax: 91-452-2459105
E-mail: jsolomon@mrna.tn.nic.in

(Molan, 2000). However, any honey can be expected to suppress infection in wounds because of its high sugar content, but dressings of sugar on a wound have to be changed more frequently than honey dressings to maintain an osmolarity that is inhibitory to bacteria, as honey has additional antibacterial components (Molan, 1999).

Honey contains some natural antibiotics, which gives antimicrobial property and other therapeutic values. Antibiotics like sulfonamides (sulfathiazole, sulfamethazine, sulfamethaxazole, sulfanilamide), aminoglycosides (streptomycin), Tetracycline (oxytetracycline and chlortetracycline), and amphenicols (chloramphenicol) (Bogdanov, 2003) have been detected in honey that can resist the growth of multi-drug resistant microorganisms. Antibiotics in honey can be quantitatively determined by HPLC and LC-MS.

The epidemic disease that affects the beehives is foul brood caused by several bacteria; the most common instances of the disease are those caused by *Paenibaccilus* and *Mellisococcus*. To treat the disease, bee keepers either destroy the affected hives or, as they did in other countries, apply antibiotics to the hives. A portion of the applied antibiotics can then become incorporated into the bee products (Bogdanov and Fluri, 2000; Dharmananda, 2003).

Although some antibiotics have been used by beekeepers for the treatment of bacterial brood diseases world wide (Spivak, 2000), it is not widely practiced in southern parts of Tamilnadu.

MATERIALS AND METHODS

The nectar samples were collected from the flowers like banana and rubber in a biochemically sterilized condition from the study field. Similarly, the honey samples were collected from bee hives, setup in the plantations of banana and rubber of the same area. Both nectar and honey samples were collected during the peak flowering seasons of rubber (March to April) and banana (December to January) plantation crops. The study areas have been limited to eight different places as follows: Arumanai, Colachal, Gothamangalam, Neyyar Dam, Nedumangadu, Keeriparai, Kulasekaram, Panichamoodu from Cape Comorin District, southern part of Tamilnadu, India. Antibiotics (streptomycin sulfate, ampicilin, and kanamycin) were used as standards (Sigma Chemical Co., USA).

Protein quantification in nectar and honey

Honey and nectar samples of 1 g were made up to 1 ml with double distilled water and protein was estimated using Lowry's method. This was treated with 5 ml of alkaline mixture, and then the contents of the tubes were treated with 0.5 ml of Folin's- phenol immediately and kept at dark for 30 minutes. All the treated samples were read at 660 nm. Using standard graph the concentration of protein in the samples was determined (Lowry et al., 1951).

Analysis of glucose

Total glucose level was estimated by Nelson-Somogyi's method. Honey and nectar of 1 g were made up to 1 ml with double distilled water and treated with 2 ml of copper reagent to each tube. The tubes were then placed in a boiling water bath for 10 minutes and then cooled in a trough containing tap water. Then 2 ml of arsenomolybdate color reagent was added to each tube and vortexed (colour develops very rapidly with evolution of CO_2). The colour was read at 520 nm using the blank. Using standard graph the concentration of glucose in the samples was determined (Nelson, 1944).

Extraction and HPLC analysis of antibiotics from honey and nectar

35 g (about 20 ml) of honey sample was extracted with 50 ml of chloroform: methanol in the ratio of 9 : 1 and kept in a shaker for over night at room temperature. The organic layer was collected, passed through anhydrous sodium sulfate, evaporated to dryness and dissolved in methanol. Likewise the antibiotics from 3 g (2 ml) of nectar sample were extracted as mentioned above. The confirmation of ampicilin, streptomycin, and kanamycin were performed using high-performance liquid chromatography (HPLC) with ultra-violet detector. The following instrument conditions were used. (a) Shim pack CLC ODS (4.6 mm × 15 cm) column with guard column. Mobile phase-methanol : double distilled water (65 : 35), flow rate: 1.00 ml/min, the antibiotics were detected at different wavelengths as streptomycin sulfate-254 nm, ampicillin-254 nm and kanamycin-290 nm.

RESULTS

In the present study, the levels of protein, glucose and antibiotics have been determined from the honey and nectar. All the experiments were carried out in triplicates.

Proteins in honey and nectar

The result of the protein content in honey was represented in Fig. 1. In honey, the level of protein ranges from 0.62 to 3.70 mg/g. Among the eight places, the honey collected from Panichamoodu showed the lowest protein level (0.62 ± 0.02 mg/g) and the highest in Colachel area (3.70 ± 0.01 mg/g). The protein level in nectar ranges from 0.55 to 1.89 mg/g. The nectar from Neyyar dam has the lowest level of protein (0.55 ± 0.01 mg/g) and Kulasekeram place showed the highest level of protein (1.89 ± 0.04 mg/g). In general, the nectar showed lower protein levels than the honey samples.

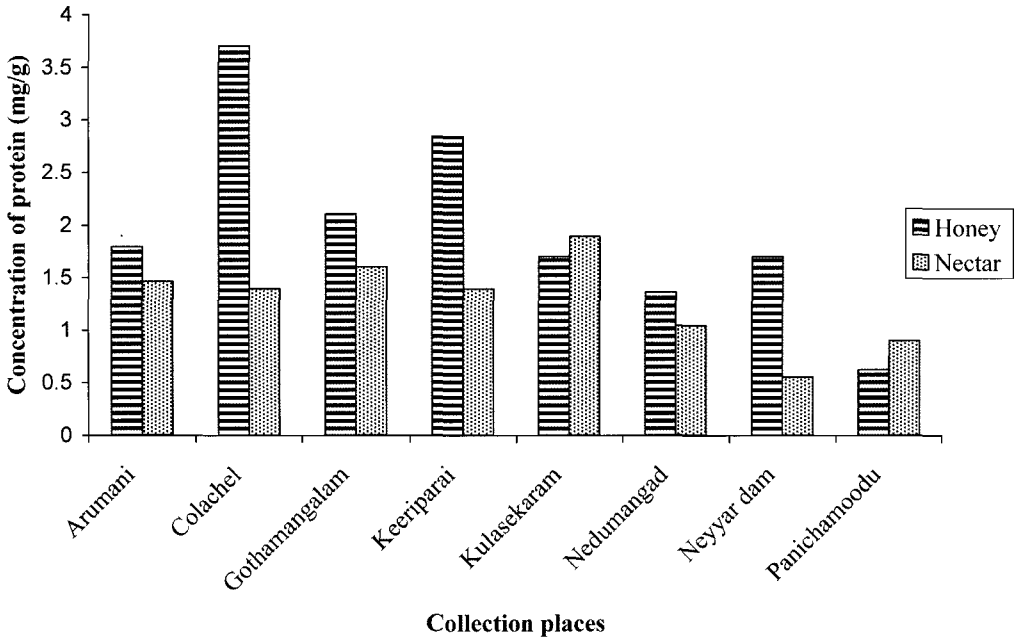

Fig. 1. Amount of protein in honey and nectar. All the samples from each collection places were analyzed in triplicates and the mean values with their S.D.

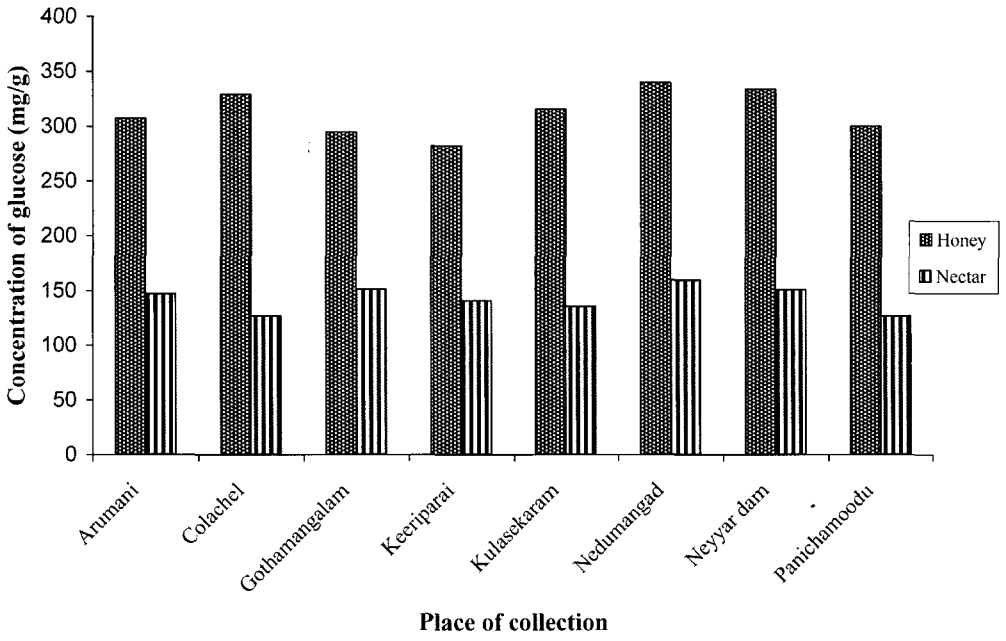

Fig. 2. Amount of glucose in honey and nectar.

Glucose in honey and nectar

The result of the levels of glucose in honey and nectar were summarized in Fig. 2. In honey, the glucose content ranges from 281.62 to 339.28 mg/g. Among the eight places, the honey collected from Keeriparai showed the lowest glucose content (281.62 ± 0.84 mg/g) and the highest in Nedumangad area (339.28 ± 0.49 mg/g). The glucose content in nectar varies from 126.52 ± 0.75 to 159.55 ± 0.46 mg/g. The nectar from Panichamoodu has the lowest level of glucose (126.52 ± 0.75 mg/g) and Nedumangad place contained the highest level of glucose (159.55 ± 0.46 mg/g). In general, the nectar showed the lowest level of glucose when compared to honey samples.

Antibiotic levels in honey and nectar

The results of the determination of ampicillin, streptomycin

Table 1. Level of antibiotics in honey and nectar

Place of Collection	Ampicilin (μg/kg)		Streptomycin (μg/kg)		Kanamycin (μg/kg)	
	Honey	Nectar	Honey	Nectar	Honey	Nectar
Arumanai	5.72 ± 0.25	3.95 ± 0.15	-	-	26.45 ± 0.35	17.54 ± 0.53
Colachel	3.95 ± 0.17	2.40 ± 0.93	29.45 ± 0.47	17.35 ± 0.68	39.54 ± 0.25	25.64 ± 0.12
Gothamangalam	22.04 ± 0.11	13.24 ± 0.53	11.47 ± 0.15	6.04 ± 0.16	46.01 ± 0.54	27.17 ± 0.11
Keeriparai	-	1.61 ± 0.12	-	5.65 ± 0.14	38.57 ± 0.22	22.27 ± 0.42
Kulasekaram	58.24 ± 0.15	31.16 ± 0.15	-	-	28.21 ± 0.23	19.12 ± 0.79
Nedumargad	44.17 ± 0.28	29.12 ± 0.45	-	4.35 ± 0.17	38.17 ± 0.34	24.48 ± 0.44
Neyyar Dam	23.39 ± 0.34	11.62 ± 0.19	12.46 ± 0.48	7.74 ± 0.37	48.45 ± 0.37	34.35 ± 0.57
Panichanmoodu	57.27 ± 0.31	36.56 ± 0.27	-	5.13 ± 0.15	35.21 ± 0.05	18.35 ± 0.53

All the samples were analyzed in triplicates. Values are the means of three replications ± S.D.

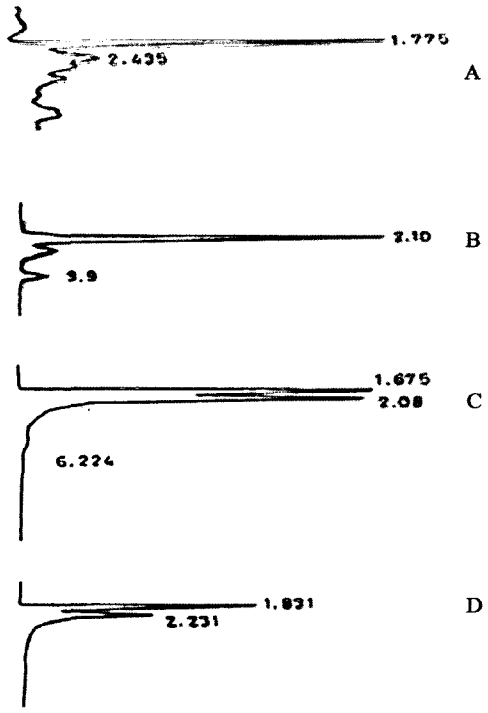

Fig. 3. HPLC chromatograms of A, Ampicillin (40 μl, mg/ml), B, Streptomycin sulfate (80 μl, mg/ml), C, Honey (50 μl), and D, Nectar (100 μl).

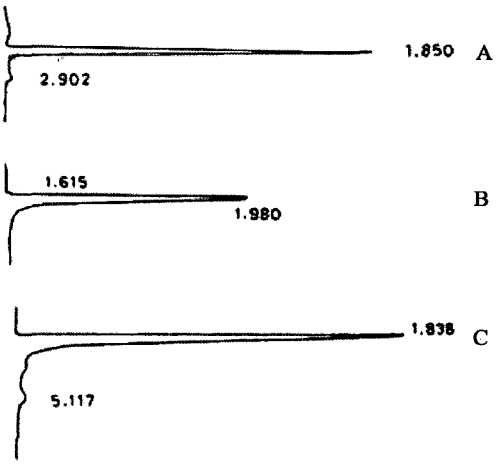

Fig. 4. HPLC chromatograms of A, Kanamycin (60 μl, mg/ml), B, Honey (50 μl), C, Nectar (100 μl).

and kanamycin in honey and nectar were summarized in Table 1. The highest ampicillin level was found in the honey collected from Nedumargad (44.17 ± 0.28 mg/kg), lowest in Colachel (3.95 ± 0.17 μg/kg) and not detected in honey collected from Keeriparai. The highest streptomycin level was found in the honey collected from Colachel (29.45 ± 0.47 μg/kg), lowest in Gothamangalam (11.47 ± 0.15 μg/kg) and not detected in the honey collected from Arumanai, Keeriparai, Kulasekaram, Nedumargad and Panichanmoodu. Likewise, the highest kanamycin level was found in the honey collected from Neyyar Dam (48.45 ± 0.37 μg/kg), lowest in Arumani (26.45 ± 0.35 μg/kg). So the amounts of all the antibiotics were higher in honey than in the nectar. HPLC results revealed that the respective antibiotics such as streptomycin sulfate, ampicillin and kanamycin were resolved at 2.10 ± 0.1, 1.77 ± 0.1 and 1.85 ± 0.0 (Fig. 3 and 4) and the detection limit was 100-200 ng respectively.

DISCUSSION

Bees obtained the protein from the natural food in the form of pollen and nectar. The ideal food source will contain pollen with more than 20% digestible crude protein and a surplus of nectar. The protein level in honey has been obtained in the range of 0.62 to 3.7 (mg/g) and it revealed the fact that the nutrient content varies from the source of nectar, collected by honeybees from different regions. In general, the protein content in honey is more when compared to nectar. It can provide the information that the processing of nectar by honeybee can improve the protein

content and it will be due to the consumption of honeybee's body protein to produce more amount of protein in honey.

Honey contains a number of enzymes including glucose oxidase, invertase, diastase (amylase), catalase and acid phosphatase. Invertase converts sucrose in the honey to fructose and glucose thus the glucose level in honey is more than that in nectar and also the bees can concentrate the nectar by beating wings to remove excess moisture.

Primary sources of microbial contamination are likely to include pollen, the digestive tracts of honeybees, dust, air, earth, and nectar. The contaminants in air, water and soil can reach plants, which on their part can reach the honey by nectar and honey dews through bees (Snowdon and Cliver, 1996). The presence of antibiotics in honey may be due to the presence of some beneficial microorganisms from the above said sources, which have the capacity to produce the antibiotics.

The streptomycin sulfate level is present on an average of 11.86 mg/g of honey, when it is compared with the previous study; the dihydro streptomycin was recorded as 2 mg/kg in honey from the Dutch market (Bruijnsvoort et al., 2004). Heering et al., 1998, purified the honey extract by liquid-liquid partition (tetracyclines), and by solid phase extraction-immunoaffinity chromatography (streptomycin, sulfathiazole). Detection limits were 20 μg/kg (tetracycline equivalents), 10 μg/kg (streptomycin), and 50 μg/kg (sulfathiazole equivalents). The present study has revealed that the level of streptomycin level in Indian honey is more when compared to the above said studies.

Honey contains around 100 antibacterial substances making it impossible for bacteria to become resistant to honey. If single bacteria become resistant to one of the antibacterial substances 99 others will kill it and the different antibiotics may be one among the antibacterial substances present in honey. Honey inhibited bacterial growth due to high sugar concentration (reduced water activity), hydrogen peroxide generation, proteinaceous compounds and other unidentified components present in the honey (Mundo et al., 2004) the antibiotics may be one of the components of antimicrobial compounds in honey.

The presence of small amounts of antibiotics in the honey although exhibits antimicrobial activity. It is still under debate that antibiotic such as chloramphenicol, which causes life-threatening side effect idiosyncratic aplastic anemia when used in large amounts.

ACKNOWLEDGMENT

The authors thank the International Atomic Energy Agency, Vienna, Austria for the support. We thank School of Biotechnology, Madurai Kamaraj University, Madurai, India for accessing its facilities.

REFERENCES

Al-Jabri AA (2005) Honey, milk and antibiotics. *Afr J Biotechnol* 4: 1580-1587.

Allen KL, Molan PC and Reid GM (1991) A survey of the antibacterial activity of some New Zealand honeys. *J Pharmacy Pharmacol* 43: 817-822.

Bogdanov S (2003) Current status of analytical methods for the detection of residues in bee products. *APIACTA* 38: 190-197.

Bogdanov S and Fluri P (2000) Honigqualitat und Antibiotikaruckstande, Schweiz. *Bienen-Zeitung* 123: 407-410.

Bruijnsvoort MV, Ottink SJ, Jonker KM and De Boer E (2004) Determination of streptomycin and dihydrostreptomycin in milk and honey by liquid chromatography with tandem mass spectrometry. *J Chroma A* 1058:137-142.

Dharmananda S (2003) Traces of chloramphenicol in Chinese Bee Products: Origin, Development, and Resolution. Institute for Traditional Medicine, Portland, Oregon, (http://www.itmonline.org/arts/bees.htm,).

Efem SE, Udoh KT and Iwara CI (1992) The antibacterial spectrum of honey and its clinical significance. *Infection* 20: 227-229.

Heering W, Usleber E, Dietrich R and Martlbauer E (1998) Immunochemical screening for antimicrobial drug residues in commercial honey. *Analyst* 123: 2759-2762.

Lowry OH, Rosebrough NJ, Farr AL and Randall RJ (1951) Protein measurement with the Folin-phenol reagents. *J Biol Chem* 193: 265-275.

Molan PC (1999) The role of honey in the management of wounds. *J Wound Care* 8:423-426.

Molan PC (1999) Why honey is effective as a medicine. Its use in modern medicine. *Bee World* 80: 80-92.

Molan PC (2000) Establishing honey as a recognized medicine. *J Amer Apitherapy Soc* 7: 1.

Mundo MA, Padilla-Zakour OI and Worobo RW (2004) Growth inhibition of food borne pathogens and food spoilage organisms by select raw honeys. *Int J Food Microbiol* 97: 1-8.

Nelson N (1944) A photometric adaptation of the Somogyi method for the determination of glucose. *J Biol Chem* 153: 375–380.

Radwan S, El-Essawy A and Sarhan MM (1984) Experimental evidence for the occurrence in honey of specific substances active against microorganisms. *Zentral Mikrobiol* 139: 249-255.

Snowdon JA and Cliver DO (1996) Microorganisms in honey. *Int J Food Microbiol* 31: 1-26.

Spivak M (2000) Preventative antibiotic treatments for honey bee colonies. *Amer Bee J* 140: 867-868.

Wellford TE, Eadie T and Llewellyn GC (1978) Evaluation of inhibitory action of honey on fungal growth, sporulation and aflotoxin production. *Zeitschrift Fur Lebensmittel Untersuchungund-forschung a-food Res* 166: 280-283.

[Received July 3, 2006; accepted August 25, 2006]