Chemical composition and antibacterial activity of honey collected from East Nusa Tenggara, Indonesia on pathogenic bacteria in aquaculture

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Abstract. The objectives of this research were to investigate the chemical composition and the antibacterial activity of honey from East Nusa Tenggara, produced by Trigona spp., on pathogenic bacteria in fish. The honey used in this research was collected specifically from Semau and Timor Islands while Aeromonas hydrophila and Vibrio alginoliticus were used as the testing bacteria. The chemical composition tests included moisture content (gravimetric), pH, total sugar (spectrophotometric). The honey was tested for the presence of the following active compounds: alkaloids, flavonoids, tannin, saponin, steroids and terpenoids. Antibacterial activity tests were conducted using “paper disk” method with undiluted honey samples. The moisture content of the Semau honey (33 %) was higher than that of the Timor honey (24.65 %), but the total sugar of the Semau honey (6.62 %) was lower than that of the Timor honey (74.22 %). All honey from both sources (Semau and Timor) in East Nusa Tenggara contained alkaloids, steroids and terpenoids but did not contain flavonoids, tannins and saponins. Antibacterial tests showed that the honey from both sources, Semau and Timor, showed antibacterial activity against V. alginoliticus and A. hydrophila; the V. alginoliticus inhibition zone diameters were 8.5 mm and 10.5 mm, respectively, while and the A. hydrophila inhibition zone diameter was 8 mm for both honeys.

1. Introduction
Bacterial infections on aquacultured fish are normally treated with drugs such as antibiotics and other chemicals [1]. However, continuous treatment with antibiotics can lead to severe side effects not only for the environment [2] but also for humans as consumers [3]. Continued treatment with excessive use of antibiotics can cause resistance in pathogenic microorganisms [1,2]. A further negative impact is that fish containing levels of antibiotics exceeding the standards for allowable concentrations will not be accepted for export.

Furthermore, many complaints regarding the supply of synthetic antibiotics on the market for preventing and treatment of bacterial infections have been reported by the Kupang Class I Fish Quarantine Station for the Quality Control and Security of Fish Products. Synthetic antibiotics are commonly expensive for fish farmers. Moreover, fish aquaculture is often located in remote areas making it hard to get the antibiotics in time [4]. Therefore, there is a need to develop new methods using natural products that are safer for the environment as well as for humans as consumers, which are cheaper, easily accessible and easily applied by fish farmers. Honey is one of the local trade commodities of East Nusa Tenggara Province (NTT) that could be developed as an antibacterial agent...
in fish aquaculture. One type of honey from NTT is produced by non-stinging bees *Trigona* spp. which nest in holes in the rocks.

Bees of the genus *Trigona* are honey bees found in tropical regions and some sub tropical regions which do not sting [5,6]. Honey produced by *Trigona* spp. bees has been found to possess antimicrobial, anticancer, antioxidant and anti inflammatory properties [7]. Antibacterial activity of honey against fish bacterial pathogens has been previously reported; for example Rapa honey and royal jelly from Bulgaria have been proven effective against *Aeromonas hydrophila* [8]. African honey is also known to possess antibacterial properties against *A. hydrophila* [9].

The study of honey from NTT, including honey produced by *Trigona* spp., is still very limited. Honey from NTT has commonly been used for medication without scientific study on its safety and efficacy for the treatment of humans and of fish. Therefore, this research studied the chemical compounds in rock honey produced by *Trigona* spp. and its antibacterial activity against pathogenic bacteria which commonly affect fish. The potential antibacterial properties of rock bee honey were evaluated in the context of its use as an antibacterial agent in fish aquaculture, in particular as an alternative to synthetic antibiotics.

2. Materials and Methods

2.1. Honey Collection

Rock bee honey collected from different localities was used in this study, including one type of rock bee honey from Semau and one from Soe, in Timor. A standard synthetic antibiotic was used as a positive control. Both of the honeys were obtained from traders in Semau and Soe.

2.2. TSA media Preparation

The media used for the antibacterial tests in this study was TSA (Tripticase Soy Agar). TSA was prepared in two layers, namely solid TSA and semi-solid TSA. Solid TSA was made earlier, one day before the antibacterial testing; while semi-solid TSA was made on the day of antibacterial testing by using 70% TSA. Two media types of both solid TSA and semi-solid TSA were prepared by adding NaCl 0,5% for the media used for *A. hydrophila* and by adding NaCl 2% to the media used for *V. alginolyticus*.

Aliquots of 10 mL of solid TSA were autoclaved and then poured onto petri dishes and allowed to cool until solid. The media were then kept in the freezer in an upside down position. After being autoclaved, semi-solid TSA was cooled down to 50°C. *A. hydrophila* bacteria were added to the TSA 0.5% media while *V. alginolyticus* bacteria were added to TSA 2% media, both at concentrations of 10^6 cells/mL. After that, 10 mL of each of the bacterial species was poured onto the solid TSA media which had been made one day earlier and then kept until frozen.

2.3. Antibacterial Test with Paper Disc Method

Sterile paper discs were soaked in each honey for 30 minutes without dilution. After 30 minutes, the paper discs were attached to the TSA media which had been inoculated with the bacteria *A. hydrophila* and *V. alginolyticus* and then incubated for 24 hours at 37°C. After 24 hours, the inhibition zone was observed and measured.

The strength of antimicrobial agent activity against specific tested bacteria can be determined by measuring diameter of the inhibition zone after incubation for 18-24 hours. If an antimicrobial agent inhibits the bacteria, the growth of bacteria will stop so that a clear zone will be observed around the paper disc [10]. The strength of antibacterial agents is classified based on the diameter of inhibition zone: 7-10 mm classified as weak; 10 mm – 15 mm as strong; and > 25 mm as very strong [11].

2.4. Honey Chemical Content Analysis

The chemical content analysis of each type of honey included testing for alkaloids with the Culvenor- Fiztgerald method, saponins with a foam test, terpenes and steroids with the Lieberman-Burchard
method, tannins with addition of FeCl$_3$, and flavonoids by adding HCl and powdered Mg. Total sugar content was measured with a spectrophotometer. Water content was analysed using a gravimetric method while pH was measured with a pH meter.

3. Results and Discussion

3.1. Total sugar, water content and pH of the rock bee honeys

Rock bee honey from Semau had a higher water content and pH compared to the honey from Timor. However sugar content was higher in the Timorese honey (Figure 1). Although both honeys were produced by the same kind of bee, _Trigona_ spp., they have different physico-chemical properties. It is well known that honey can have a different composition (e.g. water and sugar content) and contain different chemical compounds based on the source of the nectar the bees collect [12].

![Figure 1. Water content, total sugar content and pH of rock bee honey from NTT.](image)

Differences in the water content of honeys collected from different locations in Indonesia have been reported from several studies. For example, bitter black honey and sweet black honey from middle Kalimantan had 16.19% and 15.40% water content, respectively [13], while honey from Middle Bangka Regency contained 24.25% water [14] and honey from Trumon and Aceh contained 22.05% and 19.81% water, respectively [15]. The differences in honey water content can be affected by several factors such as climate, nectar type, the maturity of honey, production processes, post harvesting treatment, and storage [16].

The presence of water in honey comes from water absorption due to the hygroscopic properties of honey [14], which are related to the high sugar content. In addition, these sugars include glucose and fructose which are responsible for the osmotic properties and some antibacterial properties of honey [17]. Total sugar content in the rock honeys from NTT was lower than that of honey from Middle Bangka Regency (74.77%) [14]. Likewise, black bitter honey and sweet black honey from Middle Kalimantan both had a higher content of reducing sugar (average of 77.52% and 78.21%) compared to the two honeys in this study [13].

The pH value of both honeys from NTT was within the range of pH values reported for honey collected in other regions of Indonesia and other tropical countries. The pH values measured (Figure 1) were very similar to the pH of bitter and sweet black honey from Muara Teweh, Middle Kalimantan (pH 4 and pH 5, respectively) [13]. The pH value of rocky honey from NTT was slightly higher than that of rubber honey from Bangka (pH 3.92) [14], but similar to that of honey from India (pH 4.1) [18].
3.2. Chemical compounds in rock bee honeys from NTT
The qualitative tests for detecting active metabolites in the two rock bee honeys produced by *Trigona* spp. from NTT (Table 1) showed that both honeys from NTT contained alkaloids, steroids and terpenoids. However, no flavonoids, tannins or saponins were detected. The diversity of content of active chemical compounds in honey are thought to be related to the different nectars collected by the bees in a given area, season or hive [12,19].

| No | Active Metabolite Type | Rock Honey from Semau | Rock Honey from Timor |
|----|------------------------|-----------------------|----------------------|
| 1  | Alkaloid               | +                     | +                    |
| 2  | Flavonoid              | -                     | -                    |
| 3  | Tannin                 | -                     | -                    |
| 4  | Saponin                | -                     | -                    |
| 5  | Steroid                | +                     | +                    |
| 6  | terpenoid              | +                     | +                    |

Table 1. Active metabolite content of rock bee honey from NTT.

Differences in the chemical composition of honey are though to affect the strength of its antibacterial activity. The compounds found in the two honeys studied differ from those found in several other honeys. For example, a study on honey from Nigeria found tannins and flavonoids but no alkaloids [20]. Similarly, honey from Benin has also been reported to contain only flavonoids and tannins [21].

The chemical compounds identified in the two rock bee honeys (alkaloids, steroids and terpenoids) indicate that these honeys could be further developed as antibacterial agents, as these chemical compounds generally possess antibacterial properties. For example, triterpenoids has been reported to have antimicrobial activity against bacteria by disrupting cell membranes due to its hydrophobic functional group, while alkaloids are also known as antibacterial agents [22].

3.3. Antibacterial Activity of Rocky Honey from NTT
The strength of antibacterial activity of honey can be represented by the observed size of inhibition zones. The result of antibacterial testing of the two rock bee honeys from NTT against the pathogenic bacteria *V. alginoliticus* and *A. hydrophila* (Figure 2) showed that both honeys exhibited antibacterial activity against both bacteria. However the antibacterial activity of both honeys was lower than of the antibiotic used as a control. All substances tested (honeys and antibiotic) showed higher activity against *V. alginoliticus* than against *A. hydrophila*.
Figure 2. Antibacterial activity testing results for two rock bee honeys from NTT and a synthetic antibiotic (control) against the pathogenic bacteria *V. alginoliticus* and *A. hydrophil*.

Based on the results of the tests (Figure 1), the antibacterial activity of rock bee honey from Soe Timor in NTT against *V. alginoliticus* can be categorized as strong, as the inhibition zone diameter was over 10 mm (10.5 mm). However, activity against *A. hydrophila* was weak, with an inhibition zone diameter observed (8.5 mm) was in the weak class (7-10 mm). Rock bee honey from Semau showed weak antibacterial activity against both bacteria *A. hydrophila* and *V. alginoliticus*.

The differences in antibacterial activity by the two honeys may have been influenced by differences between honey collection sites, as [23] found that antibacterial activity of each honey is different depending on the geographic location. Furthermore the antibacterial activity of rock bee honey was different for *A. hydrophila* and *V. alginoliticus*, although both are gram negative bacteria. The factors that can affect the strength of antibacterial activity of a given antimicrobial agent include the functional group(s) of the active substance, the dose, resistance to the bioactive substance(s) and the inoculum concentration [24].

Antibacterial activity of honey can result from several mechanisms such as high sugar content which can inhibit the growth of bacteria [17,25], the presence of the radical hydrogen peroxide (H₂O₂) that can kill pathogenic microorganisms [25–27], the presence of organic antibacterial compounds [25] and the high degree of acidity (low pH) of honey that can reduce the growth rate of bacteria and lead to bacterial death [17,25–27].

4. Conclusion

Rock bee honey produced by *Trigona spp.* collected from Semau Island had a higher water content and pH but lower sugar content compared to honey collected from Soe Timor. Both of these honeys from East Nusa Tenggara Province (NTT) contained alkaloid, steroid and terpenoid compounds but no flavonoid, tannin or saponin compounds were observed. The antibacterial activity of both rock bee honeys against *A. hydrophila* bacteria was weak. Against *V. alginoliticus*, the activity of the honey from Soe Timor was strong, while that of the honey from Semau was weak.

References

[1] Maftuch, Setyawan F H and Suprastyani H 2018 UJI DAYA HAMBAT EKSTRAK Chaetoceros Calcitrans terhadap Bakteri Aeromonas salmonicida J. Fish. Mar. Res. 2 39–46

[2] Maisyaroh L F, Susilowati T, Haditomo A H C, Yuniarti T and Basuki F 2018 Penggunaan Ekstrak Kulit Buah Manggis (*Garcinia mangostana*) Sebagai Antibakteri Untuk Mengobati
Infeksi *Aeromonas hydrophila* Pada Ikan Nila (*Oreochromis niloticus*) *J. Sains Akuakultur Trop.* 236–43

[3] Nithikulworawong N 2012 Antibacterial Activity of Bauhinia sirindhorniae Extract Against *Aeromonas hydrophila* Isolated from Hybrid Catfish. *Walailak J. Sci. Technol.* 9 195-199

[4] Salosso Y 2012 Kandungan Senyawa Kimia Dan Aktivitas Antibakteri Tanaman Obat Tradisional Terhadap Bakteri *Aeromonas hydrophila* Prosiding Seminar Nasional Tahunan IX Hasil Penelitian Perikanan Dan Kelautan (Yogyakarta)

[5] Riendriasari S D and Krisnawati 2017 Produksi Propolis Mentah Lebah Madu *Trigona* spp. Di Pulau Lombok *J. Hutan Trop.* 171–5

[6] Saleng A, Syafirizal and Sari Y P 2016 Uji Aktivitas Antibakteri Ekstrak Propolis lebah *Trigono incise* terhadap Bakteri *Klebsiella pneumonia* dan *Staphylococcus aureus* *Bioprospek* 11 42–8

[7] Yaacob M, Rajab N F, Shahar S and Sharif R 2018 Stingless bee honey and its potential value: a systematic review *Food Res.* 2 124–33

[8] Stratev D, Vashin I, Balkanska R and Dinkov D H 2015 Antibacterial Activity Of Royal Jelly And Rape Honey Against *Aeromonas Hydrophila* (Atcc 7965) *J. Food Heal. Sci.* 1 67–74

[9] Ramalivhana J N, Obi C L, Samie A, Iweriebor B C, Uaboi-Egbenni P, Idiaghe J E and Momba M N B 2014 Antibacterial Activity of honey and medicinal plant extracts against Gram negative microorganisms *African J. Biotechnol.* 13 616–25

[10] Fadhmi, Syaukani E and Mudatsir 2015 Perbandingan daya Hambat Madu Seulawah dengan Madu Trumon terhadap Bakteri *Staphylococcus aureus* Secara In Vitro *J. Biot.* 3 9–14

[11] Rajasulochana P, Dhamotharan R, Krishnamoorthy P and Murugesan S 2009 Antibacterial Activity of The Extracts of Marine Red and Brown Algae *J. Am. Sci.* 5 20–5

[12] Parwata I M O A, Ratnayani K and Listya A 2010 Aktivitas Antiradikal Bebas Serta Kadar Beta Karoten Pada Madu Randu (*Ceiba pentandra*) Dan Madu Kelengkeng (*Nepheilium longata L.*) *J. Kim.* 4 54–62

[13] Fadjar M, Andayani S and Faizal M 2005 Purifikasi Fenolic Bahan Bioaktif Hydrozoa Bougainvillaea Sebagai Bakterisida terhadap Bakteri *Vibrio harveyi* *J. Aqua. Indones.* 5 79 – 83

[14] Fitrianingsih S P, Khairat A and Choestrina R 2014 Aktivitas antibakteri madu Pahit dan Madu Hitam manis terhadap *Escherichia coli* dan *Staphylococcus aureus* *J. Farm. Galen.* 1 32–7

[15] Evahelda, Pratama F and Santoso B 2017 Sifat Fisik dan Kimia Madu dari Nektar Pohon Karet di Kabupaten Bangka Tengah, Indonesia *Agritech* 37 363–8

[16] Fadhmni, Syaukani E and Mudatsir 2015 Perbandingan daya Hambat Madu Seulawah dengan Madu Trumon terhadap Bakteri *Staphylococcus aureus* Secara In Vitro *J. Biot.* 3 9–14

[17] Baroni M V, Arrua C, Nores M L, Fayé P, Diaz M D P, Chiabrando G A and Wunderlin D A 2009 Composition of honey from Córdoba (Argentina): Assessment of North/South Provenance by chemometrics *Food Chem.* 114 727–733

[18] Erguder B I, Kilicoglu S S, Namusu M, Kilicoglu B, Devrim E, Kismet K and Durak I 2008 Honey Prevent Hepatic Damage Induced by Obstruction of the Common Bile Duct. *World J. Gastroenterol* 12 3729–32

[19] Veeraputhiran V, Maribah T P E and Alfred A 2013 Physicochemical comparison and preservative of flora and non flora Honey *Agric. food Sci.* 3 128–30

[20] Ma’ruf M, Mawdahah G A, Eriana N N A, Swari F I, Aslamiyah S and Lutpiatina L 2018 Madu Lebah Kelulut (*Trigona* spp.) Dalam Aktifitas Terhadap Bakteri *Staphylococcus aureus* Resisten *J. Skala Kesehat. Politik. Kesehat. Banjarnasin* 9

[21] Adebeyo R, Torimiro N, Akinola S, Lawal S, Abolarinwa T and Adeoye W 2017 Study on antibacterial efficacy of Different Honey Types in South Western Nigeria Against Wound Associated Bacteria. *J. Aphitherapy* 2 15–9

[22] Francois A E, Armand P, Bertin G, Victoren D, Haziz S, Durand D N M, Esther D and Lamine B M 2016 Chemical Screening and Antibacterial Activity of Honey Produced in Benin *Indian J. Sci. Technol.* 10 1–11

[23] Cowan M M 1999 Plants Product as Antimicrobial agent *Clin. Microbiol. Rev.* 12 564–82
[23] Rio Y G P, Djamal A and Asterina A 2012 Perbandingan Efek antibakteri Madu Asli Sikabu dengan Madu Lubuk Minturun terhadap *Escherichia coli* dan *Staphylococcus aureus* secara in Vitro *J. Kesehat. Andalas* V 1 59–62

[24] Mallawa S and Halid I 2006 Aktivitas Bakteri Senyawa Bioaktif Spons laut terhadap *Stahylococcus aereus* dan *Vibrio cholerae* *J. Lutjanus* I

[25] Libonatti C, Varela S and Basualdo M 2014 Antibacterial activity of honey: A review of honey around the world *J. Microbiol. Antimicr. 6* 51–6

[26] Johnston M M, McBride D, Dahiya R, Owusu-Apenten and Nigam P S 2018 Antibacterial activity of Manuka honey and its components *AIMS Microbiol. 4* 655–664

[27] Nadhilla N F 2014 The Activity of antibacterial agent of Honey against *Staphylococcus aureus J. Major. 3* 94–101