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Isolation, Identification, and Antibacterial Assay of Indigenous Bacterial Isolates from *Apis cerana* Honeycomb

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Abstract

Apis cerana is a honey bee species with a cavity hive known as the eastern honey bee. Honey bee hives have Indigenous bacteria that have antimicrobial potential. Indigenous bacteria are free bacteria that can synthesize nitrogen compounds, sugars, and other bioactive substances. This study aims to isolate, identify and test the antibacterial of Indigenous bacteria isolate Apis cerana against gram-positive Staphylococus aureus and gram-negative bacteria Escherichia coli. This study used a descriptive research method to obtain the data from laboratory experiments. The study consisted of isolating Indigenous bacteria, identifying and testing the antibacterial agar diffusion method. The study results obtained seven isolates of AC 1, AC 2, AC 3, AC 4, AC 5, AC 6, AC 7 Indigenous bacteria from Apis cerana nests. AC 1 isolate has similarities with the genus Streptococcus, AC 2 isolates have similarities with the Klebsiella genus, while AC 3 isolates have similarities with the Bacillus genus, and AC 4, AC 5, AC 6 and AC 7 isolates have similarities with the Citrobacter genus. Indigenous bacterial isolates with potential antibacterial potential where the most significant inhibition against Staphylococus aureus bacteria was shown by isolate AC 1 (10.79 mm). At the same time, the smallest was found in isolate AC 7 (8.52 mm). The most significant inhibition against Escherichia coli bacteria was shown by isolate AC 1 (9.0 mm) while the smallest was found in isolate AC 3 (7.4 mm). Apis cerana nests have indigenous bacteria that have the potential to produce antibacterial substances.
Keywords: *Antibacterial, Honeycomb of Apis cerana, Indigenous Bacteria, Pathogenic Bacteria.*

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INTRODUCTION

The eastern honey bee or *Apis cerana* is one of about 20,000 species. It is commonly found in Southeast and South Asia at an altitude of 3,500 meters above sea level (Semuel, Kaunang, & Manoppo, 2019). *A. cerana* has a small physique. Its wingspan measures only 7-9 millimeters (Semuel & Rombot, 2023). In the wild *A. cerana* usually lives in tree holes

as a nest, including wood from fallen trees (Stanley et al., 2020). Beehives also contain secondary metabolite compounds in the form of flavonoids whose function is to protect and determine the quality of honey (Semuel, Kaunang, & Manopo, 2019). Flavonoid compounds can have the potential as antibacterials. Antibacterials are compounds used to control the growth of harmful bacteria. Controlling the growth of microorganisms aims to prevent the spread of disease and infection, eradicate microorganisms in infected hosts, and prevent the decay and destruction of materials by microorganisms (Becerril-sánchez et al., 2021) (Mokosuli, 2021).

Honey bees belong to the insect class, have various benefits for humans, and play an essential ecological role. Since prehistoric times humans have utilized their secondary metabolite products produced by honey bees, such as honey, propolis, and venom, as food and medicinal ingredients (Governa et al., 2019). As pollinator organisms, honey bee species pollinate more than 70% of flowering plants each year, and as much as 6.1 billion agricultural dollars produce products from honey bee pollination (Semuel, Kaunang, & Manopo, 2019). In nature, honeybees are essential in pollinating plants (Semuel & Rombot, 2023). The genus Apis honey bees are social insects known for their honey production. Indonesia has five successful honey bee species, namely A. andreniformis, A. dorsata, A. cerana, A. koschevnikovi, A. nigrocincta. A. nigrocincta is endemic to the island of Sulawesi and surrounding islands, while A. cerana is an introduced honey bee from other parts of Indonesia (Semuel, Kaunang, & Manoppo, 2019).

Antimicrobials are mechanisms for inhibiting bacterial growth by antibacterial compounds in the form of destroying cell walls by inhibiting their formation or changing them after they are formed, changes in the permeability of the cytoplasmic membrane causing food to escape from the cell, changes in protein and nucleic acid molecules, inhibition of enzyme action, and inhibition of nucleic acids and protein synthesis (Didaras et al., 2020); (Casillas-Vargas et al., 2021); (Bungenstock et al., 2020). Products produced by beehives include toxins, nest extracts, fungal isolates and honey which have antibacterial potential (Kaligis & Mokosuli, 2022); (Semuel & Rombot, 2023). However, there are still few research reports on the antibacterial potential of indigenous beehive bacteria. Research has been carried out with the aim of isolating, identifying, and testing the antibacterial activity of indigenous A. cerana nest bacteria against gram-positive *S. aureus* and gram-negative bacteria *E. coli*.

METHOD

The tools and materials used in this study are Knife, Dilution Bottle, Petri dish (Pyrex), Incubator (memmert), Erlenmeyer Flask (Pyrex), Measuring Glass (Pyrex), Drop Pipette (Brand), Ose needle, Spiritus lamp, Reaction Tubes (Pyrex), Tube Racks, Laminar Air Flow (B-One), Matches, Microscope, Autoclave (Gea), Bottles, Refrigerator, Micropipettes (Eppendorf), Object Glass, Analytical Scales, Scissors, Bunsen, and camera for documentation. The materials used in this study include NaCl 0.85%, Test Bacteria *S. aureus, E.coli,* Nutrient Agar (NA) Media, Nutrient Broth (NB), 70% Alcohol, 95% Alcohol, Fresh *A. cerana* Beehive Extract from Ratahan District, Southeast Minahasa

Regency, North Sulawesi. This research uses descriptive research methods to obtain the data from laboratory experiments.

Isolation of *Indigenous* Bacteria

Indigenous bacterial isolates were obtained from *A. cerana* beehives from Ratahan District, Southeast Minahasa, North Sulawesi. One gram of honeycomb was added to 9 mL of physiological saline solution (NaCl 0.85%). The isolate was incubated for 24 hours at 37 ˚C. Furthermore, a dilution series of 10-2 to 10-7 was made and then inoculated on Nutrient Agar (NA) media, Nutrient Broth (NB) media, and incubated at 37 ˚C for 24 hours.

Characterization of Cell Morphology of *Indigenous* Bacterial Isolates

Bacterial isolates are applied to a glass object and then fixed on a Bunsen flame; then drip crystal violet solution for 1 minute after washing using sterile water then drip iodine and let stand for 1 minute, then wash using 96% alcohol for 5 seconds, then wash using sterile water, then drip safranin and let stand for 1 minute, after that wash with sterile water and clean around the bacterial isolate using tissue, then observe the bacterial isolate under a microscope with a magnification of 100x to determine the shape of the cell, cell arrangement, bacterial color.

Bacterial Colony Morphology Test

Morphological Test of *indigenous* bacteria includes color, shape, edge, elevation, and appearance. Gram staining is done by standard method. The stages of biochemical testing carried out are the catalase, citrate, sulfate índole motility, catalase, and starch tests.

Antibacterial Test

Antibacterial Test using agar or well diffusion method. The test bacteria used were *S. Aureus* and *E. coli* bacteria. The antibacterials used are secondary metabolites produced by *indigenous* bacteria from *A. cerana* nests. A total of 3 ml (dilution) of test bacteria was added to NA media, poured into a sterile petri dish, and waited until it solidified. After solidifying, wells with a diameter of 6 mm were made. Secondary metabolites were inserted into the wells as much as 50 µl. Then incubated at 37 ^{0}C (Nurainy, 2012).

RESULTS AND DISCUSSION

Isolation Results of *Indigenous* Bacteria

A. cerana nests were obtained from Ratahan District, Southeast Minahasa Regency, North Sulawesi. *A. cerana* nests from the location were preserved in a sample box to avoid outside air. The *A. cerana* nest used for *indigenous* bacteria isolation was golden yellow; no eggs or bee larvae were found, and honey was still found in the nest chamber (Figure 1).

Figure 1. *Apis cerana* beehive obtained from Ratahan sub-district, Southeast Minahasa district - North Sulawesi

Figure 2. Dilution of 10-2 to 10-7 *indigenous* bacterial isolates in *Apis cerana* beehives using 9ml of distilled water.

The results at dilution 10-6 showed three *indigenous* bacterial isolates, and at dilution 10-7, 4 *indigenous* bacterial isolates. Figure 4 shows pure colonies of *indigenous* bacterial isolates in *A. cerana* beehives with 2x purification by the quadrant method.

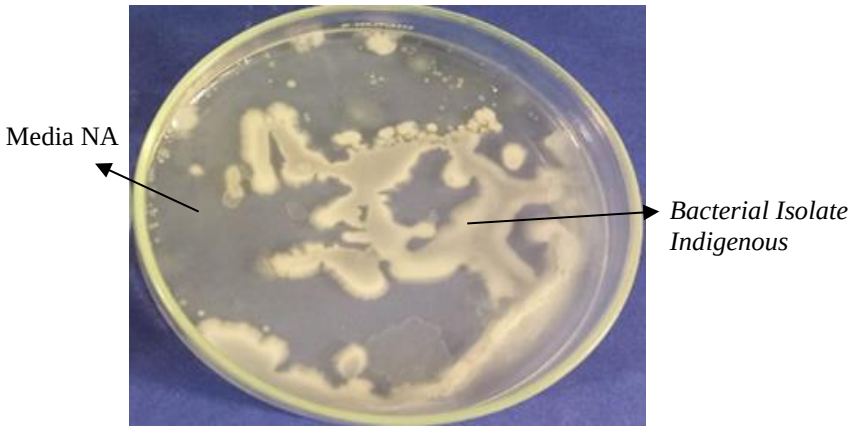


Figure 3. Colonies of *Indigenous* Bacterial Isolates in *Apis cerana* Beehive.

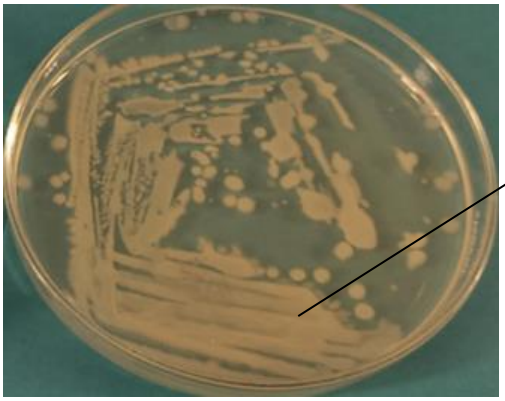


Figure 4. Pure Colonies of *Indigenous* Bacterial Isolates in *Apis cerana* Beehive.

Morphological Characterization of Indigenous Bacteria

In the isolation of indigenous bacteria, there are different morphologies of bacterial colonies; therefore, observations are made, including colony shape, colony surface, and colony color to colony edges after incubation for 24 hours on a Petri dish. The results of the morphology of *indigenous* bacterial isolates can be seen in table 1.

Table 1. Colony Morphology Results of *Indigenous* Bacterial Isolates in *Apis cerana* Honeycomb

Isolate Code	Morphology				
	Color	**Form**	**Edge**	**Elevation**	**Appearance**
AC 1	White	Punctiform	Raised	Entire	Glossy
AC 2	White	Punctiform	Umbonate	Entire	Glossy
AC 3	Dull	Punctiform	Raised	Embossed	Glossy
AC 4	Milk White	Circular	Raised	Lobate	Glossy
AC 5	Milk White	Circular	Convex	Lobate	Glossy
AC 6	Milk White	Punctiform	Convex	Undulate	Lobate
AC 7	Milk White	Punctiform	Raised	Embossed	Glossy

Characterization of Cell Morphology of *Indigenous* Bacterial Isolates

The gram staining results of 7 *indigenous* bacterial isolates of *A. cerana* nest are isolates AC_1 and AC_3 are gram-positive, and isolates AC_2, AC_4, AC_5, AC_6, and AC_7 are gram-negative.

Table 2. *Indigenous* bacterial isolates

Isolate code	Isolate Picture	Gram Staining

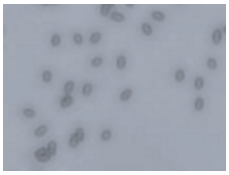
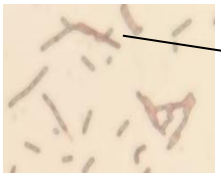
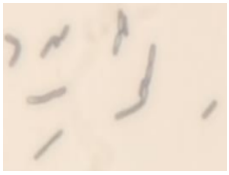
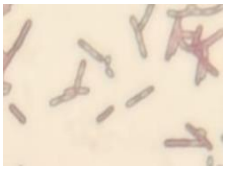
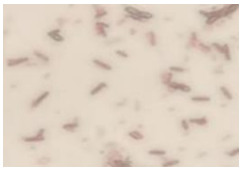
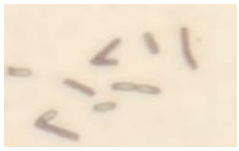
AC 1		**Gram Positive**
AC 2		**Gram Negative**
AC 3		**Gram Positive**
AC 4		**Gram Negative**
AC 5		**Gram Negative**
AC 6		**Gram Negative**
AC 7		**Gram Negative**

Table 3 shows that the characterization of cell morphology of *indigenous* bacterial isolates from *A. cerana* beehive shows that isolate AC_1 is coccus-shaped while isolates AC_2 , AC_3 , AC_4 , AC_5 , AC_6 , AC_7 are bacillus-shaped. The gram staining results of 7 isolates of *indigenous* bacteria of *A. cerana* hive are isolates AC 1 and AC 3 are gram positive and

isolates AC_2 , AC_4 , AC_5 , AC_6 , AC_7 are gram negative. In biochemical characterization, namely the catalase test isolate AC_1 showed negative results AC_2 , AC_3 , AC_4 , AC_5, AC_6 , AC_7 showed positive results. In the starch hydrolysis test isolates AC_1 , AC_2 , AC_3 , AC_4 , AC_5 , AC_6 , AC_7 showed positive results. In the SIM test AC_1 showed negative results, isolates AC_2 , AC_3 , AC_4 , AC_5 , AC_6 , AC_7 showed positive results. In the citrate test the seven isolates showed that AC_1 and AC_2 showed negative results, isolates AC_3 , AC_4 , AC_5 , AC_6 , AC_7 showed positive results. In the gelatinase hydrolysis test, the seven isolates showed negative results.

Table 4. Suspected Genus of *Indigenous* Bacteria

Alleged Genus	**Positive Results on Biochemical Tests**	**isolate code**	**Suspected Genus Showing**	**Literatur**
Streptococcus	Starch hydrolysis test and SIM test	AC_1	The presence of motile movement and capable of producing the enzyme amylase.	Isolation and Identification of Bacteria in the Waters of Koto Panjang Reservoir, Kampar Regency, Riau (Herni, 2016).
Klebsiella	Catalase test, Starch hydrolysis test starch hydrolysis and SIM test.	AC_2	Able to produce the enzyme catalase	Identification of Bacteria in Sotong Pangkong Traditional Food (Darna, 2018).
Bacillus	Catalase test, Test starch hydrolysis, SIM test and citrate test.	AC_3	Utilizing citrate as the sole source of energy and carbon.	Isolation and Morphological and Physiological Characterization of Bacteria. Endophytic Bacteria from Oil Palm Plants (Elaeiguineensis Jacq (Fifi, 2017).
Citrobacter	Catalase test, Test starch hydrolysis, SIM test and citrate.	AC_4 AC_5 AC_6 and AC_7	Utilizing citrate as the sole source of energy and carbon.	Isolation and Identification of Gram-Negative Bacteria from Soil Samples at the School of People's Animal Husbandry (SPR), Muara Enim Regency, South Sumatra (Ritonga, 2018).

Characterization of Cell Morphology of *Indigenous* Bacterial Isolates

Indigenous bacterial tests that have the ability of antibacterial content activity show seven *indigenous* bacterial isolates that have antibacterial against *S. Aureus* and *E. coli*, namely isolates AC_1 , AC_2 , AC_3 , AC_4 , AC_5 , AC_6 , AC_7 From the results of measuring the inhibition zone, the complete data can be seen in Table 5.

Table 5. Diameter of Zone of Inhibition of *Indigenous* Bacterial Isolates against Test Bacteria *E.coli* and *S.aureus* (mm)

Indigenous Bacterial Isolates	***Escherichia coli***	***Staphylococcus aureus***
AC_1	9,0	10,79
AC_2	8,2	9,29
AC_3	7,4	8,56
AC_4	8,7	9,12
AC_5	9,35	10,01
AC_6	8,65	9,07
AC_7	7,47	8,52

Description AC: *Apis Cerana*

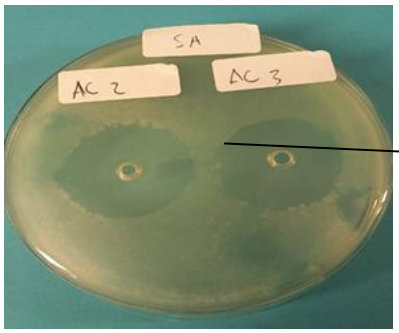


Figure 5 *Diameter of Zone of Inhibition of Indigenous Bacteria against Staphylococcus aureus*

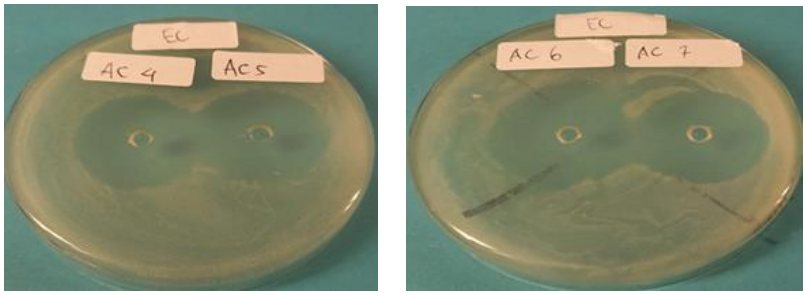


Figure 6. Diameter of Zone of Inhibition of *Indigenous* Bacteria against *Escherichia coli*

Discussion

Seven bacterial isolates were found from *Apis cerana* nests from Southeast Minahasa, North Sulawesi. The results of this study indicate that there are indigenous bacteria in Apis cerana nests with high diversity. The characterization of the isolate is based on the morphological identification of the isolate. Based on the identification of morphology, staining, cell shape showed different species. At the stage of isolation of indigenous bacteria that were successfully isolated from A.cerana beehives which were then incubated for one day and then diluted 10-2 to 10-7 bacteria which then put the results of dilutions 10-6 and 10-7 into Petri dishes with the pour plate method on Nutrient Agar media and then incubated for one day. From the results of the isolation process, the bacterial isolates were then identified microscopically. The microscopic morphological identification process results aimed to show the diversity of color, shape, edge, elevation, and appearance of bacterial isolates. Suppose for one day or 24 hours, there is no colony growth in the plant sample area. In that case, the surface sterilization is declared successful, the microbes are purified, and the purified microbial isolates are then identified morphologically based on the shape and colors of the colonies (Marzuki et al., 2021). Gram staining is a procedure to distinguish types of bacteria and yeast based on reactions that arise in the cell wall structure during the staining procedure. This stain uses gentian violet as a dye, iodine as a mordant, and ethanol for fading (Didaras et al., 2020). The next stage of the seven isolates of cell identification microscopically by performing the gram staining stage on the seven isolates of indigenous bacteria of A. cerana nest found AC_3 gram positive while AC_1,AC_2, AC_4, AC_5, AC_6, AC_7 gram-negative.

Observation of colony morphology and cell morphology is insufficient in identifying the seven indigenous bacterial isolates, therefore, further identification is needed to determine the metabolic activity caused by the enzyme workings of the seven indigenous isolates. Bacterial biochemical Test is a method or treatment carried out to identify and determine a pure culture of isolated bacteria through its physiological properties. A bacterium cannot be determined based only on its morphological properties, so it is necessary to see the biochemical properties and factors that affect its growth. Five biochemical tests were carried out: catalase, starch hydrolysis, citrate, Sulphate Indole Motility, and gelatin hydrolysis (Gao et al., 2020) (Rosalina et al., 2018).

After the identification process is arranged according to each biochemical Test's results, it is matched with the alleged genus according to Bergey's Manual Of seventh Edition. After carrying out the identification stage on the subsequent indigenous bacterial isolate, the antibacterial testing stage is carried out against the microbes E.coli and S.aureus, the selection of the two types of pathogenic microbes in this study has a base for antibacterial testing.

CONCLUSION

AC_1 isolate is similar to the genus Streptococcus, AC_2 isolate is similar to the genus Klebsiella, AC_3 isolate is similar to the genus Bacillus, and AC_4, AC_5, AC_6 and AC_7 isolates are similar to the genus Citrobacter. *Indigenous* bacterial isolates with

potential as antibacterials, namely inhibition against *Staphylococus aureus* bacteria, are the largest in isolate AC_1 (10.79 mm), while the smallest is found in isolate AC_7 (8.52 mm). The inhibition against *Escherichia coli* bacteria is largest in isolate AC_1 (9.0 mm) while the smallest is isolate AC_3 (7.4 mm).

REFERENCES

Becerril-sánchez, A. L., Quintero-salazar, B., Dublán-garcía, O., & Escalona-buendía, H. B. (2021). Phenolic compounds in honey and their relationship with antioxidant activity, botanical origin, and color. *Antioxidants*, *10*(11), 1–23. https://doi.org/10.3390/antiox10111700

Bungenstock, L., Abdulmawjood, A., & Reich, F. (2020). Evaluation of antibacterial properties of lactic acid bacteria from traditionally and industrially produced fermented sausages from Germany. *PLoS ONE*, *15*(3), 1–15. https://doi.org/10.1371/journal.pone.0230345

Casillas-Vargas, G., Ocasio-Malavé, C., Medina, S., Morales-Guzmán, C., Del Valle, R. G., Carballeira, N. M., & Sanabria-Ríos, D. J. (2021). Antibacterial fatty acids: An update of possible mechanisms of action and implications in the development of the next-generation of antibacterial agents. *Progress in Lipid Research*, *82*(November 2020). https://doi.org/10.1016/j.plipres.2021.101093

Didaras, N. A., Karatasou, K., Dimitriou, T. G., Amoutzias, G. D., & Mossialos, D. (2020). Antimicrobial activity of bee-collected pollen and beebread: State of the art and future perspectives. *Antibiotics*, *9*(11), 1–29. https://doi.org/10.3390/antibiotics9110811

Gao, Y., Stuhldreier, F., Schmitt, L., Wesselborg, S., Guo, Z., Zou, K., Mándi, A., Kurtán, T., Liu, Z., & Proksch, P. (2020). Induction of New Lactam Derivatives From the Endophytic Fungus Aplosporella javeedii Through an OSMAC Approach. *Frontiers in Microbiology*, *11*(November), 1–13. https://doi.org/10.3389/fmicb.2020.600983

Governa, P., Cusi, M. G., Borgonetti, V., Sforcin, J. M., Terrosi, C., Baini, G., Miraldi, E., & Biagi, M. (2019). Beyond the biological effect of a chemically characterized poplar propolis: Antibacterial and antiviral activity and comparison with flurbiprofen in cytokines release by LPS-stimulated human mononuclear cells. *Biomedicines*, *7*(4), 1–10. https://doi.org/10.3390/biomedicines7040073

Kaligis, M. I. G., & Mokosuli, Y. S. (2022). Characteristics and Flavonoid Content of Honey Apis dorsata Binghami from The Manembo Forest of South Minahasa. *Jurnal Biologi Tropis*, *22*(4). https://doi.org/10.29303/jbt.v22i4.4247

Marzuki, I., Kamaruddin, M., & Ahmad, R. (2021). Identification of marine sponges-symbiotic bacteria and their application in degrading polycyclic aromatic hydrocarbons. *Biodiversitas*, *22*(3), 1481–1488. https://doi.org/10.13057/BIODIV/D220352

Mokosuli, Y. S. (2021). Brine Shrimp Lethality Test (BSLT) Ekstrak Sarang Lebah Madu

(Apis dorsata Binghami. *Jurnal Pendidikan Biologi Undiksha*, *8*, 138–144. https://ejournal.undiksha.ac.id/index.php/JJPB/article/view/37506%0Ahttps://ejournal.undiksha.ac.id/index.php/JJPB/article/download/37506/19923

Rosalina, R., Ningrum, R. S., & Lukis, P. A. (2018). Aktifitas Antibakteri Ekstrak Jamur Endofit Mangga Podang (Mangifera indica L .) Asal Kabupaten Kediri Jawa Timur. *Majalah Ilmiah Biologi Biosfera: A Scientific Journal*, *35*(3), 139–144. https://doi.org/10.20884/1.mib.2018.35.3.757

Semuel, M. Y., Kaunang, E. S. N., & Manopo, J. S. (2019). The bioactive contents and antioxidant activity of honey bee nest extract of apis dorsata binghami from the North Sulawesi. *Molekul*, *14*(2), 92–102. https://doi.org/10.20884/1.jm.2019.14.2.502

Semuel, M. Y., Kaunang, E. S. N., & Manoppo, J. S. S. (2019). *Potensi Bioaktif dari Apis dorsata Binghami , Lebah Madu endemik Sulawesi*. 98.

Semuel, M. Y., & Rombot, D. O. (2023). Bioactivity of Apis dorsata Nest Extract from a Different Geographical Location in Minahasa, North Sulawesi, Indonesia. *International Journal of Agriculture and Biology*, *29*(1), 1–8. https://doi.org/10.17957/IJAB/15.1997

Stanley, D. A., Msweli, S. M., & Johnson, S. D. (2020). Native honeybees as flower visitors and pollinators in wild plant communities in a biodiversity hotspot. *Ecosphere*, *11*(2). https://doi.org/10.1002/ecs2.2957

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