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ANTAGONIST, ANTIBACTERIAL ACTIVITY OF *Stapylocossus aureus* AND ISOLATE OF ORAL BACTERIA FROM THE ENDOGENOUS FUNGUS *Apis dorsata* BINGHAMI NEST

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Abstract

Apis dorsata Binghami is a honey bee endemic to Indonesia, living naturally in the forests of Sulawesi. This study aimed to obtain isolates and characteristics of endogenous fungi antibiotic activity from *Apis dorsata* Binghami nest. The study consisted of isolation of fungi from beehives using potato dextro agar, pure culture of fungi, antagonist test and antibiotic test using disc diffusion method. Antibiotic test was performed on oral bacteria and *Staphylococcus aureus*. The results obtained six fungal isolates from *Apis dorsata* Binghami's nest, namely isolates FAB1, FAB2, FAB3, FAB4, FAB5 and FAB6. The results of the antagonist test showed that the isolates FAB1, FAB2, FAB3, FAB4 and FAB5 were antagonistic. The results of the antibacterial test showed that all isolates produced an average bacterial growth inhibition of 17-18 mm on *Staphylococcus aureus* and isolates of oral bacteria. The inhibition zone lasts up to 3 x 24 hours so that the activity of the bacteria is bactericidal. From the results of this study, it can be concluded that the endogenous fungus *Apis dorsata* Binghami is a potential source of antibacterial bioactives.

Key words: *Apis dorsata* Binghami Nest, Endogenous Fungi, Oral bacteria, *Staphylococcus aureus*, Antibacterial,

INTRODUCTION

Indonesia is a tropical country that has a lot of plant and animal diversity. More than seven endemic honey bee species are found in Indonesia, which is the largest in the world, of which there are two endemic species on the island of Sulawesi, namely *Apis dorsata* Binghami and *Apis nigrocincta*. Honey bees belong to the class of insects and have various benefits for humans and play an important role in ecology. Since prehistoric times, humans have used secondary metabolites, namely honey, propolis, and poisons as food and medicine. As pollinator organisms, for each year the honey bee species pollinates more than 70% of flowering plants and as much as 6.1 billion dollars in agriculture produces products from honey bee pollination. (Semuel *et al*., 2019a).

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A dorsata is the most prolific honeybee in producing honey, and has a nest with only one large comb, which usually hangs from tree branches and twigs, open ceilings and rock cliffs or ravines. Therefore, until now scientists have not been able to cultivate *A dorsata* (Semuel *et al*., 2019a). Honey bees are one of the main honey-producing factors that support the national economy and can contribute to the regeneration of forests and various plants (Nagir, 2016). Sulawesi Island is a biogeographical transition area for Asian and Australian flora and fauna. The giant forest honey bee (*Apis dorsata* Binghami) is one of Sulawesi's endemic species, currently *Apis dorsata* binghami has not been cultivated and still lives naturally in the forests of Sulawesi (Semuel *et al*., 2019b).

A honey bee nest is a complex arrangement used by bees as a place to live, as a space to store honey, to raise offspring, eggs, larvae, bee pollen and bee cocoons. Dorsata bees are the owner of the most species on earth compared to other honey bees, and have the largest body size and are known as giant bees (Semuel *et al*., 2019c). Honeycomb also contains secondary metabolite compounds in the form of flavonoids whose function is to protect and determine the quality of honey (Prestianti *et al*., 2017). Propolis is the main component used by honey bees in making hives. The general characteristics of propolis are gummy, sticky, and resinous substances from various plants collected by honey bees. The sap from plant shoots contained in bees is the main ingredient in the manufacture of propolis (Semuel *et al*., 2019c).

The content of compounds in honey bee hives serves as a protector and determinant of honey quality, including flavonoids which are natural phenolic compounds and bees wax. Based on the content of these compounds, the honeycomb of Trigona spp. has been studied and used as an antibacterial *Streptococcus mutans* (Price *et al*., 2017). The honey bee nest parts of Trigona sp that have potential as antimicrobials are not only found in the cover of the hive or propolis, but also in the pollen bag, honey bag, and egg bag. Parts of honey bee hives have different compound components as antimicrobial agents. The types of antimicrobials produced by honey bee nests include the group of antibiotics tetracycline, streptomycin, sulfonamides, tylosin, erythromycin, lincomycin, and chloramphenicol. This shows that honey bee hives have the potential to be used as antibiotics to suppress and kill various kinds of pathogenic bacteria so that the quality of honey is maintained. Honey bee nest antimicrobial agent compounds can be used as a source of natural antimicrobials derived from nature. Likewise, endogenous fungi in honey bee hives produce potential antibiotic compounds.

The prevalence of pathogenic bacterial infections has continued to increase in recent decades throughout the world. Climate change, animal-to-animal transmission, and antibiotic resistance have led to the emergence of many new species of pathogenic bacteria in humans that are resistant to antibiotics. Therefore, research to obtain new bioactive sources of antibiotics needs to be carried out continuously. This study aimed to obtain isolates of endogenous fungi in *Apis dorsata* Binghami beehives, isolate bioactives producing antibiotics/antibacterial and obtain bioactive antibacterial characteristics of endogenous fungi of *Apis dorsata* Binghami honeycombs.

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MATERIALS AND METHODS

Sample

This research was carried out from November to December 2021 at the Laboratory of Molecular Biology, Faculty of Mathematics and Natural Sciences, Manado State University.

Research methods

Fungi Isolation

The honeycomb used is fresh and cleaned of contamination. The nest used is a nest that has not been occupied by bee larvae, contains honey and is golden yellow in color. A total of 2 grams of nests were placed on PDA media in petri dishes. Incubation at 37ºC for 5-21 days depending on the growth rate of fungi (Rusliati *et al*., 2009). The fungal isolates that grew were then cultured purely on PDA media and incubated for 3-5 days at 37ºC.

Fungi Antagonist Test

The antagonist test refers to the dual culture method. In PDA media in one pertidish inoculation was carried out at two different places. Then incubated for seven days at room temperature. And observed every inhibition that occurs (Purwantisari & Hastuti, 2009).

Antibiotic Activity Test

The tools used were sterilized using a Mammert oven at 115ºC for ±60 minutes. PDA media was weighed as much as 7.25 grams and NA media was weighed as much as 7 grams then each was dissolved in 250 mL of sterile distilled water and stirred until the solution was complete. Each media solution was sterilized in an autoclave at 121ºC for 15 minutes. S. aureus bacteria and bacterial isolates from the mouth to be used were cultured using the agar slant method on nutrient agar media and incubated for 24 hours at room temperature (Luwu *et al*., 2017). The fungal isolates that grew were harvested and put in a centrifuge tube containing 100 l of sterile distilled water and centrifuged until H2O and the fungal isolates were separated and the fungal isolates settled at the bottom of the centrifuge tube.

The preparation of the test bacteria was carried out by preparing the NA medium to be poured into a sterile petri dish, then the bacteria from the mouth that had been cultured from agar slanted were transferred into a petri dish by scratching until the surface was covered with bacteria and a paper disc that had been made round. placed on the surface of the test bacteria and dripped with a solution of the fungal extract. The pellet from the extraction was placed in a well in the middle of the agar medium that had been inoculated with S. aureus. Incubation was carried out for seven days. Observation of inhibition was carried out after 24 hours until the seventh day. The research data were analyzed descriptively.

RESULTS AND DISCUSSION

Fungi Isolate from *Apis dorsata* Binghami's Nest

Pure cultures of fungal isolates grew optimally from the second day after planting. Identification of fungi using the determination book and online identification obtained the alleged genera, namely Syncephalastrum, Mortierella, Spacelia, Virgaria, Alternaria Sp, and Aureobasidium (Table 1).

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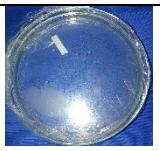
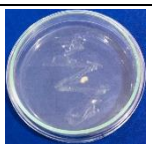
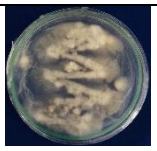
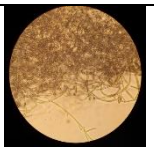
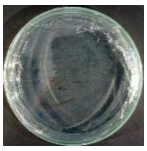
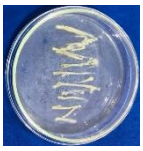
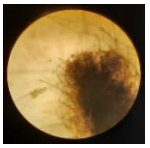
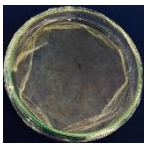
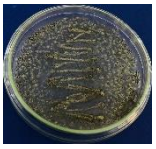
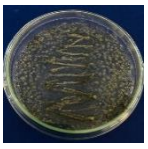
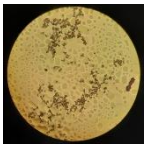
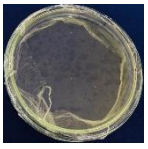
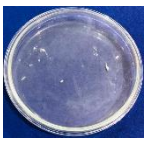
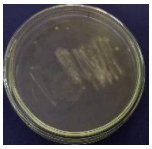
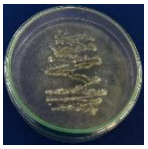
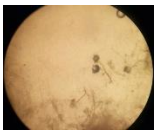
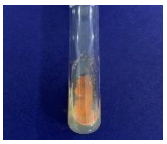
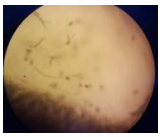
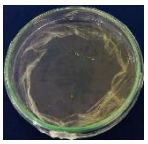
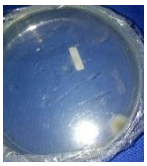
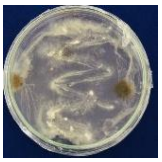
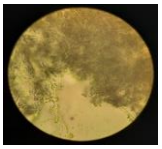
Table 1. Microscopic Fungi Isolates from *Apis dorsata* Binghami Nest

Name of Isolate and Genus Identified	First Day	Second Day	Third Day	Seventh	Observation Microscopic
FAB1 *Syncephalastrum*					
FAB2 *Mortierella*					
FAB3 *Spacelia*					
FAB4 *Virgaria*					
FAB5 *Alternaria Sp*					
FAB6 *Aureobasidium*					

Antagonist Activity Test

FAB1 and FAB2 isolates from the antagonist test of FAB1 and FAB2 isolates on the first day after inoculation, the two isolates grew normally. Antagonistic activity began to occur on the seventh day. On the third day, FAB2 began to spread but did not affect the growth of FAB1. The results of the antagonist test of the two isolates showed that FAB2 isolates were more dominant than FAB1 isolates (Figure 1).

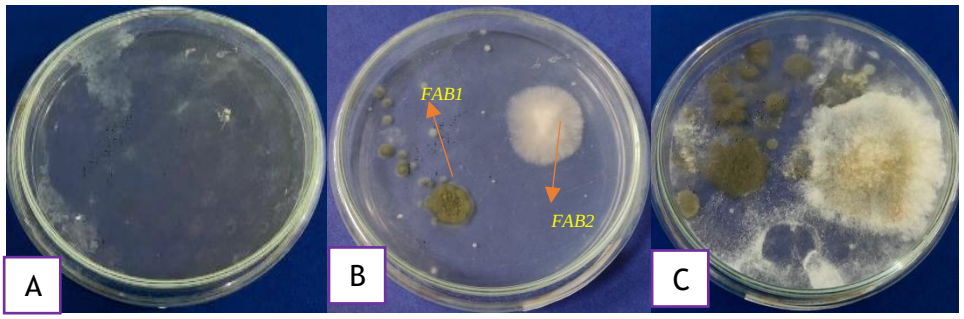


Figure 1. Test of FAB1 and FAB2 antagonists A. First day. B. the second day, and C. the seventh day

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FAB2 and FAB3 isolates

The antagonistic activity of FAB2 and FAB3 isolates began to appear on the third day. On the third day the growth pattern spread both FAB2 isolates and FAB3 isolates. However, on the seventh day, the growth dominance of FAB2 isolates over FAB3 isolates was very visible (Figure 2).

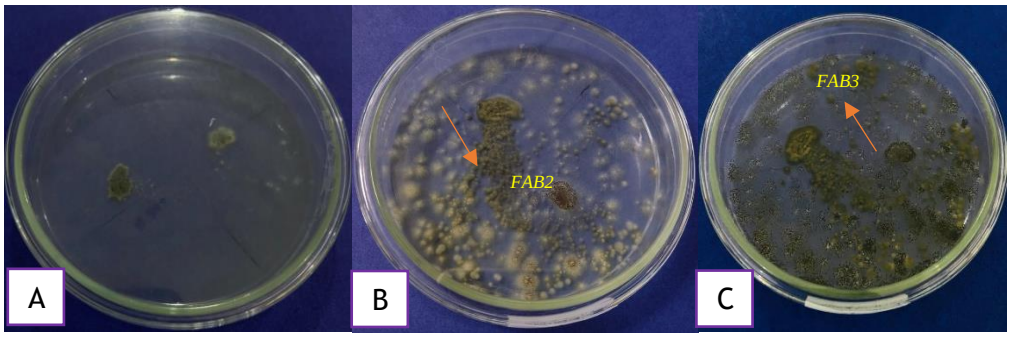

Figure 2. Antagonist test of fungi isolates FAB2 and FAB3. A. the first day. B. the second day, and C. the seventh day

FAB3 and FAB4 isolates

The first and second day antagonist tests have not shown mutually inhibiting growth. On the third day, hyphae from the two isolates were seen scattered throughout the growing media. On the seventh day, no inhibition zone was seen because the hyphae of the two isolates had covered the entire surface of the growing media (Figure 3).

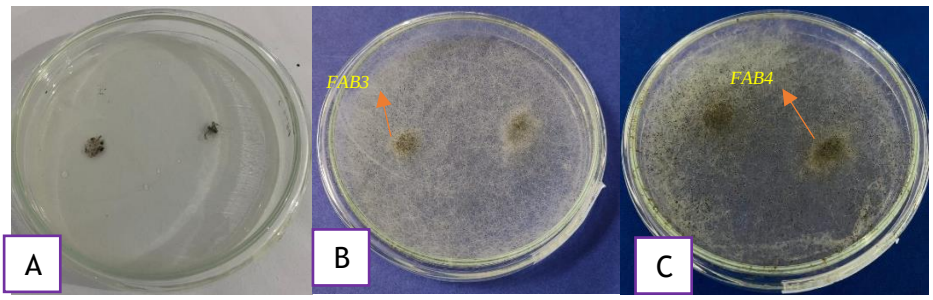

Figure 3. FAB2 and FAB3 isolate fungi antagonist test. A. the first day. B. the second day, and C. the seventh day

FAB4 and FAB5 isolates

On the first and second days of the antagonist test, there was no growth inhibition interaction. Antagonism began to appear on the third day. On the third day to the seventh day, it was seen that the FAB4 isolate had covered the entire petridish surface while the FAB5 isolate did not experience growth (Figure 4).

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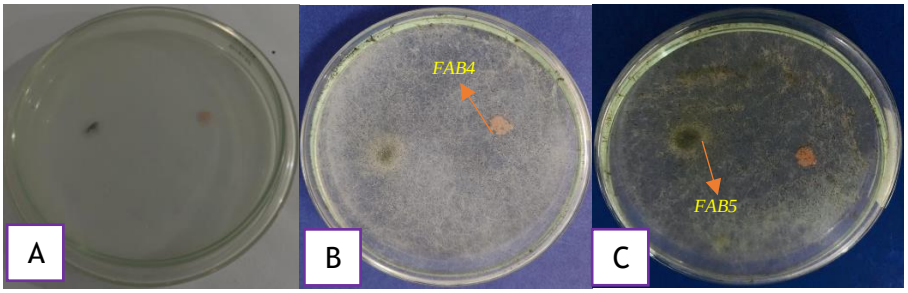

Figure 4. Antagonistic test of FAB4 and FAB5 fungi isolates. A. the first day. B. the second day, and C. the seventh day

FAB5 and FAB6 isolates

The antagonist test on the third day to the seventh day showed that the FAB5 isolate had covered the entire surface of the growing medium while the FAB6 isolate did not experience optimal growth. Thus the FAB5 isolate was able to inhibit the growth of the FAB6 isolate.

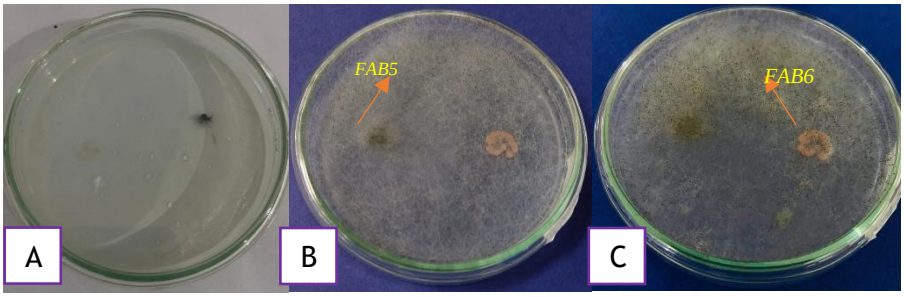

Figure 5. Antagonist test of Alternaria Sp and Aureobasidium; A. Observations on the first day, B. Observations on the third day, C. Seventh day observation

Fungi Extract Antibiotic Test

From table 2 below, it can be seen that the formation of the inhibitory zone on the first day to the third day experienced very good development and on the fourth day to the seventh day the inhibitory power only increased slightly or remained from this protection, it can be concluded that the results of the isolation of fungi from the beehive this is an antibiotic fungus Karen until the seventh day the inhibition zone formed did not disappear or even shrink.

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Table 2. Inhibitory Power of Fungi Isolate Against Oral Bacteria

Days	Average diameter of growth inhibition zone (mm)					
	FAB1	FAB2	FAB3	FAB4	FAB5	FAB6
1	12.53±0.15	14.23±0,06	8.07±0.28	10.30±0.10	9.93±0.15	12.60±0.20
2	13.60+0.10	15.40±0.10	13.57±0.32	11.77±0.15	10.43±0.15	13.70±0.10
3	14.43±0.12	15.63±0.06	14.43±0.12	12.37±0.32	11.17±0.15	14.27±0.06
4	15.20±0.10	16.07±0.12	14.43±0.21	13.30±0.26	11.37±0.15	14.27±0.31
5	15.40±0.10	16.27±0.12	14.43±0.32	13.27±0.12	11.67±0.15	15.33±0.15
6	15.63±0.21	16.27±0.15	14.63±0.06	13.40±0.20	11.53±0.31	15.43±0.15
7	15.40±0.26	16.20±0.10	14.70±0.17	13.33±0.15	11.67±0.23	15.27±0.06

From the results of the fungal antibiotic test against S. aureus bacteria and bacteria from the mouth, it can be seen that the inhibition formed from the first day to the fifth day experienced good growth and from the fifth day to the seventh day the growth was relatively small or constant.

Table 3. Inhibition of fungal isolates against S. aureus

Days	Average diameter of growth inhibition zone (mm)					
	FAB1	FAB2	FAB3	FAB4	FAB5	FAB6
1	17.43±0.21	15.40±0.20	8.30±0.10	16.40±0.17	0.00	9.57±0.12
2	18.3±0.10	19.27±0.25	9.67±0.15	18.27±0.12	0.00	10.23±0.06
3	19.30±0.10	23.50±0.20	10.37±0.15	12.97±0.35	0.00	10.33±0.15
4	20.10±0.10	17.33±0.14	11.23±0.25	20.80±0.10	0.00	11.30±0.17
5	20.33±0.21	26.30±0.10	11.30±0.10	21.37±0.21	0.00	11.40±0.17
6	20.20±0.10	26.63±0.32	11.33±0.12	21.60±0.10	0.00	11.40±0.26
7	20.67±0.50	26.73±0.25	11.50±0.17	21.57±0.06	0.00	11.67±0.15

This study proves that *Apis dorsata* Binghami beehive has endogenous fungi that are potential as a source of bioactive antibiotics. Six genera of honeycomb endogenous fungi that have been isolated have specific bioactive properties. This is evidenced by the results of the antagonist test showing different growth inhibition zones. Several isolates showed a predominance of growth on PDA media in antagonist assays. FAB2 isolate was able to inhibit the growth of FAB1 isolate. This indicates that the bioactive content contained in the FAB2 isolate inhibits the development of FAB1 hyphae. However, FAB2 growth was inhibited by FAB3 isolate in the antagonist test. FAB3 and FAB4 antagonist tests showed a balanced growth pattern. This balanced growth pattern indicated that the bioactives produced by the two isolates were not able to inhibit the growth of other isolates. The same results were also found in the isolates FAB4 and FAB5 as well as FAB5 and FAB6 isolates. From the results of this antagonist test, FAB2 and FAB3 isolates were potential to be further investigated as a source of bioactive antibiotics.

Fungal extract test on oral bacterial isolates showed an increasing trend of inhibition from the first

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day to the seventh day. The best bacterial growth inhibition was shown by isolate FAB2 where the average diameter of the inhibition zone for bacterial growth was the largest compared to all isolates (Table 2). The inhibition of bacterial growth is determined by the existing bioactive content. The high growth inhibition of FAB2 bacteria showed that the FAB2 isolate extract contained strong oral antibacterial secondary metabolites. However, all fungal isolates showed high inhibition when compared to standard clinical antibacterial activity tests. Oral bacterial isolates were used for reasons of future research, further investigation of the potential for oral bacterial antibiotics from the bioactive endogenous fungi of bee hives will be investigated further. Furthermore, the fungal extract test on pure cultured S. aureus bacteria actually showed a larger average diameter of the inhibition zone compared to the oral bacterial isolates. Thus the bioactive content in the fungal extract has a strong antibacterial activity of S. aureus. As with the oral antibacterial test, FAB2 isolate showed the best antibacterial activity. Thus the FAB2 isolate has the potential to be developed more as a source of antibiotics.

Fungal isolates of the genus Syncephalastrum were reported to have antagonistic activity with pathogenic bacteria (Rodrigues *et al*., 2009). Fungi of the genus Virgaria have also been reported to produce the antibiotic compounds cinatrine and virgaricin (Ishii *et al*., 2015). Virgaria extract was able to inhibit the growth of S. aureus 9.6 mm ± 0.3 (Tan et.al. 2018). Mycotoxins produced by Alternaria fungi are reported to be able to inhibit the growth of Pseudomonas (Muller *et al*., 2018). Liamocin produced by the fungus Aureobasidium is able to strongly inhibit the growth of antibiotic-resistant Streptococcus bacteria (Price *et al*., 2017). Aureabasidium fungi together with Aspergillus, Penicsillum and Saccharomyces are microscopic fungi that have the potential to be used in biotechnology and antibiotic exploration (Wang *et al*., 2022). Thus, this study succeeded in reporting fungal isolates that have prospects as a source of bioactive antibiotics in the future. In-depth research, especially the identification of antibiotic compounds and specific antibiotic tests in the future needs to be carried out.

CONCLUSION

From the results of honey bee nest isolation (Apis dorsaata Binghami) obtained six types of isolate fungi. Of the six types of isolate fungi produced by honey bee nests (*Apis dorsata* Binghami), FAB 1, FAB 2, FAB 3, FAB 4, and FAB 6 had antagonistic properties, while FAB 5 did not have antagonistic properties. From the results of the antibiotic test of *Apis dorsata* Binghami isolate against S. aureus bacteria and bacteria from the mouth, it was found that the fungi from the isolation of *Apis dorsata* Binghami's hive were antibiotic.

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