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Antifungal efficacy of stingless bee honey extracts against a black mould causing pathogen *Lasiodiplodia* spp.

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Abstract Fungal species causing the black sooty mould were isolated from fruit peels and subjected to molecular genetic analysis. The nucleotide sequences were found to be 100% identical to the fungus *Lasiodiplodia iranensis*. Results from testing the antifungal properties of stingless bee honey extracts showed that the extract from the *H. itama* species exhibited the highest inhibitory effect, followed by *T. pagdeni*, *G. thoracica*, and *T. fuscobalteata*. The average inhibition values were 21.43±1.27, 18.54±1.50, 17.51±1.76, and 17.18±0.91 mm., respectively.

Keywords: *Aglaia dookkoo, Lasiodiplodia* spp., Stingless bee honey

Introduction

The fruit plant, *Aglaia dookkoo* Griff., commonly known in Thailand as longkong, is an economically significant tropical, non-climacteric fruit in the country. The variety "Tan Yong Mus Longkong" is likely a regional or cultivar-specific name for this fruit. Due to its unique balance of sweet and slightly sour flavors and its aromatic, juicy flesh, demand for longkong has steadily increased, leading to a rise in its export value. Key international markets include the United States, Hong Kong, Vietnam, and Bangladesh (Paull, 2004). Morphologically, the fruit develops in clusters of 15 to 25 round balls. The skin, which is brittle, turns from pale green to bright yellow upon ripening, and often shows brown blemishes. The interior of the fruit is composed of five separate segments of white, translucent flesh, each containing one to five green seeds. As the fruit

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ripens, its sugar content increases approximately six folds, and the flesh becomes less astringent. However, its market viability is constrained by a very short postharvest shelf life, typically only 3-5 days, largely due to a variety of fungal diseases. The primary pathogens responsible for this decay include *Lasiodiplodia theobromae*, *Phomopsis* sp., *Fusarium* sp., *Leptoxyphium kurandae*, *Pestalotiopsis* sp., *Colletotrichum* sp., and *Cylindrocladium* sp. (Venkatachalam, 2016) Black mould disease is a significant problem affecting longkong fruit production for export (Figure 1). The disease manifests as black stains on the fruit's surface, which reduces its quality and market value, making it unacceptable for importing countries. While the fungus does not directly damage the fruit's flesh, instead feeding on the sugary nectar secreted from the fruit's extrafloral nectaries, it still poses a major obstacle for export because of strict quality standards that require blemish-free fruit (Sungsiri *et al*., 2011; Sungsiri and Sangchote, 2011). The objectives of this research were to isolate the fungus that causes black mold disease in Tan Yong Mus longkong and to test the efficacy of stingless bee honey extracts in inhibiting the causal pathogen.

Figure 1. Black mould disease on the peel of Tan Yong Mas longkong fruit (red arrow)

Materials and methods

Sample collection and isolation

A tissue transplanting method was used to isolate the fungus from Tan Yong Mas longkong fruit. Longkong peel infested with black mould were cut

into 0.5 mm pieces. These pieces were then washed with 1% Clorox for 3 minutes, followed by three a 3-minute rinse in sterilized distilled water. Then, they were blotted dry with sterilised tissue papers and then placed on a PDA (Potato Dextrose Agar) medium to study the morphology of the causal fungus. After obtaining a pure fungal colony, a spore suspension was prepared. A 50 µL aliquot of the spore suspension was added to 5 mL of PDB in a 50 mL tube and incubated on a rotary shaker at room temperature for 2 days. The mycelium was then collected by centrifuging the suspension at 5,000 rpm for 10 minutes. The liquid medium was discarded, and the mycelial pellet was washed twice with sterilised distilled water. This prepared mycelium was then used for DNA extraction.

Genomic DNA extraction

The protocol for fungal DNA extraction involves several key steps to ensure high purity and yield. Initially, isolated fungal mycelia were washed twice with 2 mL of TE buffer and centrifuged at 5,000 rpm for 5 minutes. The pellet was then resuspended in 1 mL of extraction buffer, ensuring the mycelia were completely dissolved. This is followed by the addition of 500 mL of 3 M sodium acetate, with the mixture incubated for 15 minutes twice. The solution was then centrifuged at 14,000 rpm for 10 minutes, and the supernatant was transferred to a new tube. Subsequently, 20 µL of proteinase K was added, and the sample was incubated at 60°C for 10 minutes. Then, a solution of 250/250 mL of phenol/chloroform was added and centrifugedat 14,000 rpm for 10 minutes. The DNA was precipitated by adding 500 µL of isopropanol with gentle inversion, with the sample chilled at -45°C to enhance precipitation. The precipitated DNA was then pelleted by centrifugation at 14,000 rpm for 15 minutes. The supernatant was discarded, and the pellet was washed with 500 µL of 70% ethanol, followed by centrifugation at 14,000 rpm for 10 minutes. The purified DNA pellet was air-dried before being rehydrated with 30-35 µL of nuclease-free water. The DNA was then stored at -20°C.

Polymerase chain reaction

The total reaction volume for DNA amplification using PCR was 50 µL. A master mix was prepared containing the following components: 5 µL of 10x PCR buffer; 2 µL of $MgCl_2$; 2.5 µL of dNTP; 0.5 µL of Primer ITS1 (5' TCCGTAGGTGAACCTGCGG 3'); 0.5 µL of Primer ITS4 (5' TCCTCCGCTTATTGATATGC 3'); 37.5 µL of sterile dH_2O; and 1 µL of Taq DNA polymerase. One microliter (100 ng) of the DNA template was then added

to the master mix in a PCR tube. The DNA was then amplified using a BIO-RAD T100™ Thermo Cycler preheated at a temperature of 95°C for 3 min and denatured at a temperature of 95°C for 45 s, annealed at a temperature of 60°C for 30 s and at an extended temperature of 72°C for 40 s for 41 cycles, then annealed at a final extension temperature of 72°C for 5 min. The obtained PCR products were then examined for size on 1% agarose gel under UV light. The amplicon was then extracted using the FavorPrep Plasmid Extraction Mini kit from Favorgen Biotech Corp. and sent for nucleotide sequencing analysis at Macrogen, Korea.

Nucleotide alignment and bioinformatic analysis

Nucleotide sequence analysis with bioinformatics software required several major tools and databases. The procedure usually employed ClustalW for multiple sequence alignment, CAP3, and the NCBI database for sequence comparison and annotation. A phylogenetic tree was constructed using the neighbour-joining method in MEGA version 11 (Tamura *et al*., 2021). The reliability of clusters within the tree was evaluated based upon 1000 bootstrap replications.

Stingless bee honey extraction

The stingless bee honey extraction method followed the method reported by Thongsaiklaing *et al.* (2024). One kilogram of each honey sample (*Heterotrigona itama*, *Tetragonula pagdeni*, *Geniotrigona thoracica*, and *Tetragonula fuscobalteata*) was diluted 1:1 (V/V) in water. The solution was aggressively mixed again before it was added with ethyl acetate in a 1:1 (V/V) ratio. To complete the extraction, the bottle containing the solution was extracted by ultrasound three times for 15 minutes at 40 ± 3 degrees Celsius in an ultrasound cleaning bath (BANDELIN, SONOREX DIGITEC) set to 37 kHz. After that, the separated ethyl acetate fraction was centrifuged for 10 minutes at 6,000 rpm. To extract the remaining honey, the same procedure was repeated twice. Following that, the ethyl acetate from all three extractions was mixed and evaporated using a vacuum evaporator. The extract was stored at -20°C.

Agar-well diffusion method

For the agar diffusion method, test plates were prepared with 20 mL of PDA. After solidifying the media, 100 µL of KT3 suspension (0.5-2.5 × 103) was evenly spread over several plates with an L-shaped rod. A sterile cork

borer with a diameter of 6 mm was then used to create a well in the centre of the plate. Subsequently, 50 µL of the four stingless bee honey extracts (*H. itama*, *T. pagdeni*, *G. thoracica*, and *T. fuscobalteata*) were dispensed into the well. The plates were then incubated under optimal growth conditions for 24-48 hours. Plates were examined after incubation for fungal growth inhibition zones, which were measured in millimeters around each well.

Statistical analysis

All data were analyzed by one-way analysis of variance (ANOVA). Duncan's Multiple Range Test (DMRT) was used for comparisons of means. Descriptive statistics were compared with the UNIVARIATE procedure of SAS software

Results

Nucleotide sequence analysis

To amplify ITS, DNA of KT3 was isolated from the peel of Tan Yong Mas longkong fruit infested with black mould disease. The PCR fragments for the ITS were approximately 540 bp in size (Figure 2). The *Lasiodiplodia sp.* was identified based on NCBI database. A phylogenetic analysis revealed a 100% sequence identity with the fungus *L. pseudotheobromae* and *L. theobromae* (Figure 3).

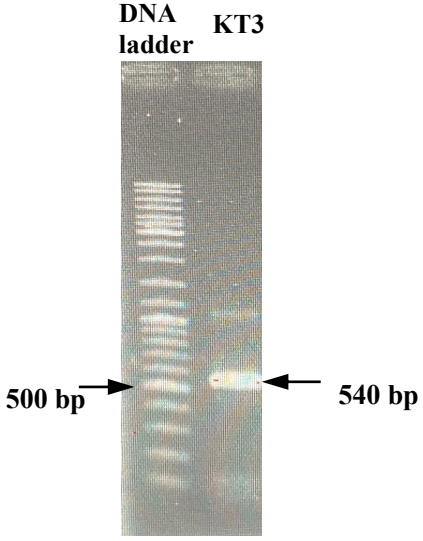

Figure 2. The PCR product 540 bp target size of ITS gene in the KT3 sample

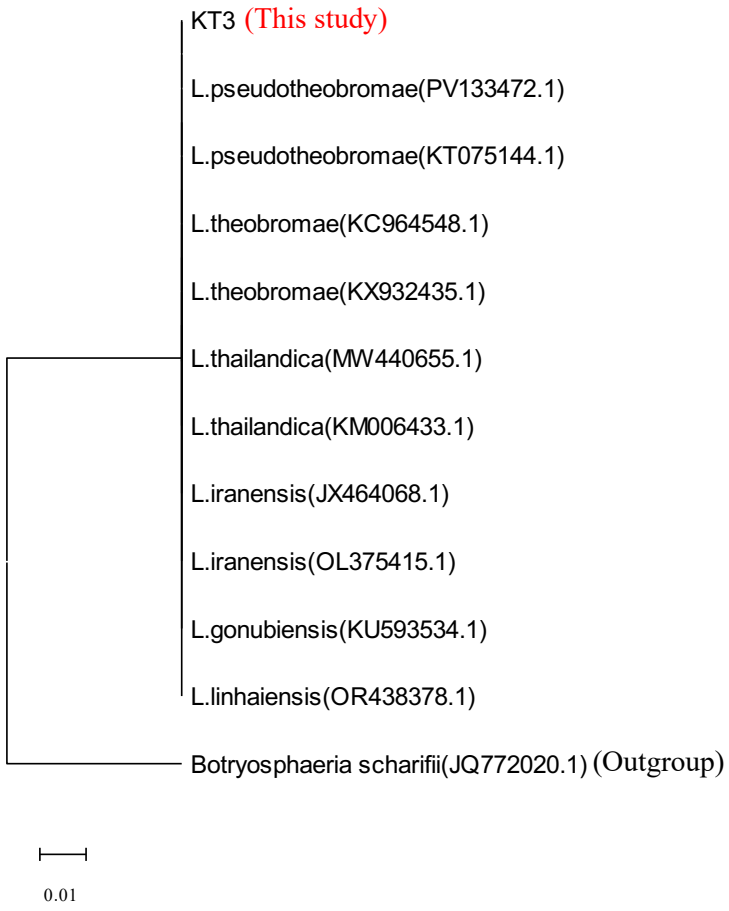

Figure 3. Neighbour-joining phylogenetic tree exhibiting the relationship between various *Lasiodiplodia* species based on ITS. The nucleotide sequences from this study are shown in red on the phylogenetic trees. An NJ technique was used to infer the evolutionary history using the Kimura 2-parameter model, and bootstrap support values from 1000 replicates are displayed at the nodes of the branches. The scale bar represents the number of substitutions per site.

Antifungal properties

By the agar-well diffusion method, Four stingless bee honey extracts were tested against the pathogen causing black mould. The values of inhibition zone diameters against KT3 of all four kinds of extracts are listed in Table 1, and their images are shown Figure 4.

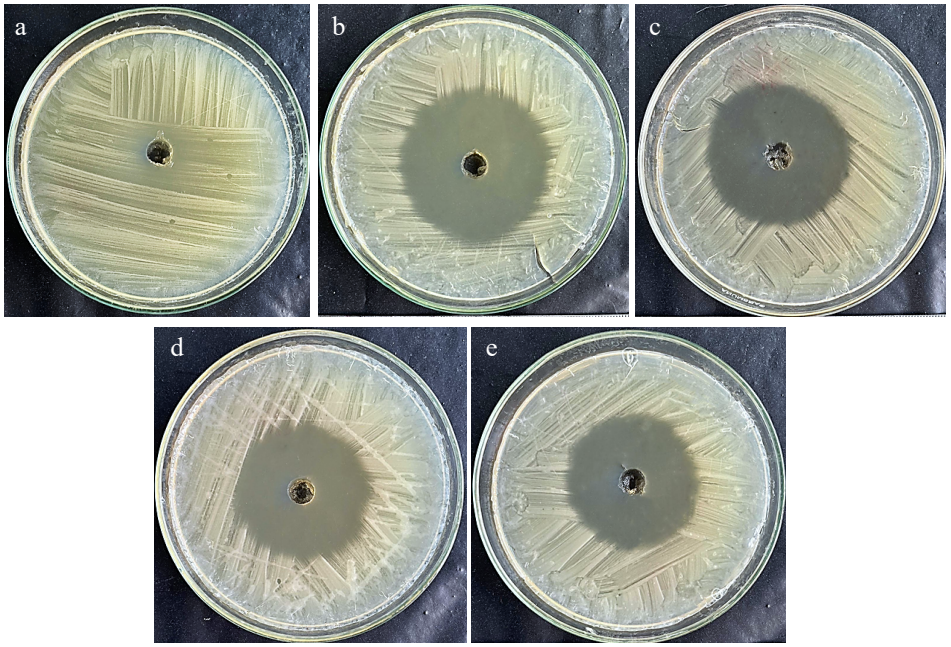

Figure 4. Agar well diffusion assay for the determination of antifungal KT3 activity of stingless bee honey extract: (a) ethyl acetate (control), (b) *H. itama* honey extract, (c) *G. thoracica* honey extract, (d) *T. pagdeni* honey extract, and (e) *T. fuscobalteata* honey extract

Table 1. Zone inhibition of four stingless bee honey extracts

Sample	Zone of inhibition (mm)
H. itama honey extract	21.43 ± 1.27^{a}
G. thoracica honey extract	18.54 ± 1.50^{b}
T. pagdeni honey extract	17.51 ± 1.76^{b}
T. fuscobalteata	17.18 ± 0.91^{b}
P-value	*P<0.05*

Discussion

The Tan Yong Mas longkong fruits were afflicted with black mould. The mould was isolated from a pathogenic fungus, identified as *L. pseudotheobromae* by molecular sequence analysis. *L. pseudotheobromae* is a significant plant

pathogen with a diverse host range that can infect the branches and fruits of a variety of plants, causing stem canker stem rot (Juliana *et al*., 2022), (Dou, 2017 and Wang *et al*., 2023), stem blight (Lv *et al*., 2022), leaf wilt (Fan *et al*.,2020), fruit rot (Munirah *et al*., 2017), and other symptoms that result in significant economic losses to agricultural production (Awan *et al*., 2016). Although the morphologies of *L. pseudotheobromae* and *L. theobromae* are so similar, *L. pseudotheobromae* has been called the "hidden species" in the *L. theobromae* complex. However, the two species have distinct conidial morphologies (He *et al.,* 2024). In 2008, *L. pseudotheobromae* was classified as a new species (Alves *et al*., 2008).

The activities of all analyzed pathogens were dose-dependent, and the results revealed that four stingless bee honey extracts had a good inhibitory efficacy against the *Lasiodiplodia* spp. tested. Among all four kinds of extracts, the honey extract from *H. itama* exhibited the strongest inhibitory action. In a study by Shehu *et al*. (2016) honey bees (*Apis dorsata*) and *Trigona thoracica* produced MTH and SBP, which have antifungal properties against *C. albicans* and *C. neoformans*. SBP outperformed MTH in terms of antifungal capabilities, possibly due to its high phenolic acid and flavonoid concentration. MTH and SBP may be effective treatment drugs against these two prevalent fungi. Pipattanapuckdee *et al.* (2023) Using a dual culture assay, the antagonistic bacterium *Bacillus siamensis* isolate RFCD306 shown strong antifungal effectiveness against *L. pseudotheobromae*, preventing fungal growth by 94.40%.

The fungus responsible for black mould disease on Tan Yong Mas longkong fruit in Thailand was identified as *L. pseudotheobromae* through molecular sequence analysis. This species is a serious plant pathogen that causes substantial economic losses in agriculture. This study demonstrated that honey extracts from four species of stingless bees– *H. itama*, *G. thoracica*, *T. pagdeni*, and *T. fuscobalteata*—showed strong inhibitory effects against the fungus, with the extract from *H. itama* exhibiting the strongest antifungal activity. These findings suggest that stingless bee honey extracts could serve as an effective treatment to control this prevalent fungal disease.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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