Jurnal Biodjati 11 (1): 1–20, May 2026

e-ISSN : 2541-4208
p-ISSN : 2548-1606

JURNAL BIODJATI

http://journal.uinsgd.ac.id/index.php/biodjati

Effectiveness of *Trigona laeviceps* Propolis Extract Against *Colletotrichum acutatum* and Chili Damping-Off Disease

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Received: March 1, 2026
Revise from: March 8, 2026
Accepted: April 4, 2026

DOI: 10.15575/biodjati.v11i1.54859

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Abstract. *Indonesia's biodiversity provides natural resources with potential for pest and plant disease control. Propolis from Trigona laeviceps has antimicrobial properties and can serve as an environmentally friendly alternative. The fungus Colletotrichum acutatum causes anthracnose disease in chilies, reducing plant production. This study aims to test the effectiveness of ethanol extract of T. laeviceps bee propolis in inhibiting the growth of C. acutatum in vitro and suppressing red chili damping-off. The experiment was carried out at the Phytopathology Laboratory, Department of Plant Pests and Diseases, Faculty of Agriculture, Universitas Padjadjaran. The research was conducted using a Completely Randomized Design (CRD) with two stages, and the data were analyzed using ANOVA followed by Duncan's multiple range test (DMRT). The first stage is a test to determine the Lethal Concentration 50% (LC_{50}) of propolis ethanol extract, yielding an LC_{50} of 12.8%. The second stage involved evaluating the ethanol extract of propolis using the poisoned food technique to assess its effects on colony growth and conidia germination of Colletotrichum acutatum, as well as its ability to suppress red chili damping-off. The experiment consisted of four treatments of propolis ethanol extract with concentrations of 12.8%, 19.2%, 25.6%, and 32%, a mancozeb fungicide treatment, and a control treatment. Repetition was carried out four times. T. laeviceps propolis ethanol extract concentrations of 12.8%, 19.2%, 25.6%, and 32% were effective in inhibiting the growth of C. acutatum fungal colonies with an inhibition range of 56.94%, 60.28%, 65.28%, and 81.39%, respectively. The ethanol extract of T. laeviceps propolis at concentrations of 25.6% and 32% reduced the incidence of red chili sprout blight by 37.5% and 35%, respectively.*

Keywords: *antimicrobial, anthracnose, bee, conidia germination, lethal concentration*

INTRODUCTION

Anthracnose is one of the diseases that can decrease both the quantity and quality of chili harvests. The yield loss caused by this disease can reach 10-50% and may increase up to 80% under severe conditions in chili plants (De Silva et al., 2019; Diao et al., 2017). During the rainy season, the incidence and severity of chili anthracnose tend to increase due to high humidity,

Citation
Hersanti, H., Suangga, A. A., & Bari, I. N. (2026). Effectiveness of *Trigona laeviceps* Propolis Extract Against *Colletotrichum acutatum* and Chili Damping-Off Disease. *Jurnal Biodjati*, *11*(1), 1-20.

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which favors the development and spread of *Colletotrichum* spp. Under severe infection and without proper control measures, yield losses can reach 100% in chili production (Mongkolporn & Taylor, 2018; Sharma et al., 2017).

Anthracnose disease in chili is caused by the pathogenic fungus *Colletotrichum acutatum*. This pathogen can attack all parts of the chili plant, including leaves, stems, and fruits (Semangun, 2007). According to Law et al. (2025), *Colletotrichum* spp. manifests as disease symptoms at multiple stages of chili growth, damping-off, leaf spotting, and stem dieback during vegetative growth, and fruit rot at the reproductive stage, contributing to severe tissue damage and yield loss under favorable environmental conditions. Attacks during the seedling phase can cause damping-off disease, reducing germination rates and seed vigor.

One common control technique used by farmers is synthetic fungicide application. This control method is often chosen for its rapid disease control (Sila & Sopialena, 2016). However, the continuous use of synthetic fungicides can lead to several negative impacts, including the development of pathogen resistance, risk to human health, and environmental pollution (Islam et al., 2024). Therefore, it is necessary to explore more effective, environmentally friendly control methods.

Natural fungicides have been widely used in environmentally friendly pest and disease management by exploiting bioactive compounds derived from natural sources (Hersanti et al., 2013). One potential source is propolis from *Trigona laeviceps*, which contains various antimicrobial compounds, including flavonoids, phenols, terpenoids, tannins, and alkaloids (Rismawati & Ismiyati, 2017). The effectiveness of these compounds depends on the extraction method; ethanol extraction has been reported to yield higher flavonoid content than water or non-polar solvents, indicating its efficiency in isolating bioactive substances (Bankova et al., 2018). Therefore, the ethanol extract of propolis has strong potential as an alternative for plant disease control.

Based on the background provided, this study investigates the effectiveness of a natural substance, namely ethanol extract of *T. laeviceps* honey bee propolis, in controlling the fungus *C. acutatum* in vitro and damping-off disease in red chili seedlings. This study is expected to provide scientific evidence regarding the antifungal potential of propolis extract as an environmentally friendly alternative to synthetic fungicides. Furthermore, the results may contribute to the development of sustainable disease management strategies for chili cultivation.

MATERIALS AND METHODS

Research site

The experiment was conducted at the Phytopathology, Biotechnology, Pesticides, and Environmental Toxicology Laboratory, Department of Plant Pests and Diseases, Faculty of Agriculture, Universitas Padjadjaran, as well as the Central Laboratory of Universitas Padjadjaran. The experiment was conducted from April to September 2021.

Procedures

Isolation of the fungus *Colletotrichum acutatum*

The isolation of *C. acutatum* was conducted following the method described by Nawaz et al. (2023). Chili fruits showing anthracnose symptoms were cut into 1 × 1 cm sections from both healthy and infected tissues, then surface-sterilized by immersion in 70% ethanol for 15 seconds, followed by 1% sodium hypochlorite for one minute. The samples were rinsed three times with

sterile distilled water, dried on sterile filter paper, and placed on PDA medium in Petri dishes, then incubated at room temperature (approximately 28°C). Emerging colonies were subcultured onto fresh PDA to obtain pure cultures, which were subsequently identified as *C. acutatum* based on macroscopic and microscopic characteristics, including colony morphology, spore shape, and hyphal structure.

Making propolis ethanol extract

A total of 200 g of propolis was macerated in 700 mL of 70% ethanol in a 1000 mL Erlenmeyer flask for 7 days with shaking at 130 rpm. The filtrate was then collected, and the residue was re-extracted with an additional 70% ethanol until a total volume of 1000 mL was reached, with each extraction followed by filtration until the extract became clearer (Hasan et al., 2013; Matienzo & Lamorena, 2004). The combined extract was subsequently evaporated using a rotary vacuum evaporator at approximately 40°C to remove the solvent, and the concentrated extract was weighed to determine its yield (Matienzo & Lamorena, 2004).

LC_{50} concentration determination test of propolis ethanol extract

The LC_{50} concentration was determined using the poisoned-food method described by Widiastuti et al. (2020), which involves pouring liquid PDA media mixed with ethanol propolis extract. The concentrated propolis extract was weighed according to the treatment concentrations and then mixed with liquid PDA at approximately 50°C. The PDA and extract were stirred using a stirring rod to ensure uniform mixing. Once evenly mixed, the media mixture was poured into Petri dishes, approximately 10 ml each, and left to solidify. The *C. acutatum* isolate was perforated using a cork borer with a diameter of 5 mm. Then, the isolate was transferred to the center of a Petri dish containing PDA media mixed with ethanol-propolis extract (Figure 1). The culture was then incubated at room temperature to observe colony growth.

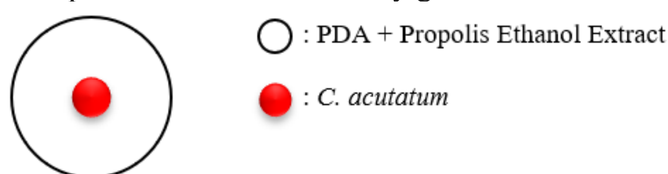

Figure 1. Layout for testing antimicrobial ability using the poisoned food technique method

Test of the inhibitory power of propolis ethanol extract on colony growth of *Colletotrichum acutatum*

The testing was conducted using the poisoned-food method (Widiastuti et al., 2020). The method used was consistent with that used to determine the LC_{50} concentration of ethanol propolis extract. The concentrations used were 1x, 1.5x, 2x, and 2.5x the LC_{50} concentration. The control treatment involved PDA media without the addition of extract. The cultures were then incubated at room temperature to observe colony growth.

Test of the inhibitory power of propolis ethanol extract on the germination of *Colletotrichum acutatum* Conidia

The conidial germination test of *C. acutatum* was conducted following the method used

by Uddin et al. (2023). Pure cultures of *C. acutatum* aged 10 days were taken in 5 pieces with a diameter of 5 mm each, then placed into test tubes containing 5 ml of ethanol propolis extract at each test concentration. The control treatment only received sterile distilled water. The suspension in the test tubes was shaken for 3 minutes using a shaker to release the fungal conidia. Subsequently, 1 ml of the suspension was taken using a micropipette and dropped onto a watch glass. The watch glass was then incubated in a sterile box with moist tissue.

Suppression test for the incidence of germ blight in chili seeds

The disease suppression test was conducted using a seed-soaking method based on Yulia et al. (2019), with modifications to the inoculation stage. Chili seeds were soaked in warm water for 2 hours, with non-viable seeds discarded, then air-dried on sterile filter paper. The selected seeds were treated by soaking in ethanol propolis extract at various concentrations, sterile distilled water (control), or a mancozeb-based fungicide (comparison) for 30 minutes, followed by drying. Subsequently, seeds were inoculated by immersion in a *C. acutatum* spore suspension (10^7 conidia/mL) for 30 minutes, drained, and placed on moist sterile filter paper in Petri dishes; non-inoculated seeds were placed directly without fungal exposure. Seed incubation lasted 14 days, with each treatment comprising 25 seeds.

Observation

Observation of *Colletotrichum acutatum* colony growth

The observed variable is the diameter of the fungal colonies. Observations were made until the *C. acutatum* colonies in the control treatment covered the surface of the Petri dish. The diameter of the fungal colonies was measured using a ruler. The inhibitory effect of ethanol propolis extract on the diameter of fungal colonies in each treatment was determined using the formula:

$$\% \text{ Inhibition} = (a-b)/a \times 100\%$$

where % inhibition is the percentage of fungal colony growth inhibition (%), a is the diameter of fungal colonies in the control treatment (cm) and b is the diameter of fungal colonies in each treatment (cm). Microscopic observations were also conducted to observe the effect of the application of ethanol extract of *T. laeviceps* propolis on the damage to the mycelium or hyphae of *C. acutatum* fungus. Observations were made by taking fungal culture samples from the Petri dish. The cultures were then placed on glass slides and observed using a microscope.

Observation of conidia germination of *Colletotrichum acutatum*

The observed variable was the number of germinated conidia, which were considered germinated when the germ tube length equaled or exceeded the length of the conidium (Ijadpanahsaravi et al., 2022). Observations were made 24 hours after incubation using a microscope at 400x magnification. The percentage of conidial germination is calculated using the following formula:

$$\text{Germination Rate } (\%) = \frac{\Sigma \text{ Number of germinate conidia}}{\text{Total number of observed conidia}} \times 100\%$$

The percentage inhibition of conidial germination is calculated using the following formula:

$$E= ((Gc-Gt))/Gc \times 100\%$$

Where *E* is percentage Inhibition of Germination (%), *Gc* is germination of conidia without treatment (control), and *Gt* is germination of conidia with treatment.

Observation of conidia germination of *Colletotrichum acutatum*

The data collected in the disease suppression test include the damping-off incubation period, the number of seedlings infected by *Colletotrichum* sp., germination rate, seedling height, root length, and the number of seedlings producing cotyledon leaves. Observations were conducted daily until the seeds reached 14 days after planting (DAP). Subsequently, the calculation was performed to determine the damping-off disease incidence rate in chili seedlings. The disease incidence (DI) is calculated using the formula according to Yulia & Widiantini (2018):

$$DI = n/N \times 100\%$$

Where *DI* is disease incidence (%), *n* is the number of chili seedlings affected by damping-off refers to the count of chili seedlings showing symptoms of damping-off disease, and *N* is the total number of chili seedlings observed represents the overall count of chili seedlings examined for damping-off symptoms

The formula determines the percentage inhibition of extract on damping-off disease incidence:

$$PI= (DIc- DIt)/DIt \times 100\%$$

Where *PI* is inhibition percentage (%), *DIc* is disease incidence of control, *DIt* is disease incidence of treatment.

Experimental design and analysis

This study employed an experimental method consisting of two stages. The first stage was a preliminary test to determine the Lethal Concentration 50% (LC_{50}) of ethanol extract against *C. acutatum* using probit analysis (Kumar et al., 2020). The LC_{50} assay was arranged in a Completely Randomized Design (CRD) with 6 concentration treatments (0.1%, 0.2%, 0.4%, 0.8%, 1.6%, and 3.2%) and one control, with 4 replications per treatment and conducted in duplicate. The LC_{50} values obtained were subsequently used in the second stage to evaluate the antifungal activity of ethanol propolis extract on colony growth, conidia germination, and damping-off suppression in chili.

The second stage included antifungal and disease suppression assays using CRD with four replications and duplicate experiments. Antifungal activity was tested using the poisoned food technique (Madhura et al., 2024), with treatments including control, ethanol propolis extract at concentrations of $1\times LC_{50}$, $1.5\times LC_{50}$, $2\times LC_{50}$, and $2.5\times LC_{50}$ (expressed as % v/v), and a mancozeb-based fungicide. The damping-off suppression test was conducted using the blotter method (Vishunavat et al., 2023) with 25 seeds per replication, including uninoculated control, inoculated control, ethanol propolis extract at $1\times LC_{50}$, $1.5\times LC_{50}$, $2\times LC_{50}$, and $2.5\times LC_{50}$ (expressed as % v/v), and a fungicide treatment.

Data Analysis

The data obtained were subsequently analyzed using SPSS version 21. The data from the experiment to determine the LC_{50} concentration were analyzed using Probit Analysis. The data from the experiments on inhibitory power and disease suppression were analyzed using Analysis of Variance (ANOVA). If the testing shows significant differences, Duncan's test will be used as a post hoc test at the 5% significance level.

RESULT AND DISCUSSION

Determination of the LC_{50} Concentration of Ethanol Extract of Propolis

The chili pathogen isolate grown on Potato Dextrose Agar (PDA) media forms colonies that are whitish-gray to orange, symmetrical, and fill the PDA plate in a petri dish (Figure 2A & 2B). The colonies reach maximum growth, completely covering the petri dish, at 12 Days After Inoculation (DAI). Microscopic observation reveals cylindrical, elongated conidia resembling tubes (Figure 2C). Based on these macroscopic and microscopic characteristics, the fungal isolate can be identified as *C. acutatum*. This aligns with the description provided by Syahputri (2022), indicating that *C. acutatum* exhibits cylindrical conidia with ellipsoidal ends, and its fungal colonies appear whitish-gray with red to yellowish hues. *C. acutatum* is identified as one of the species causing anthracnose disease in large red chili peppers in Indonesia.

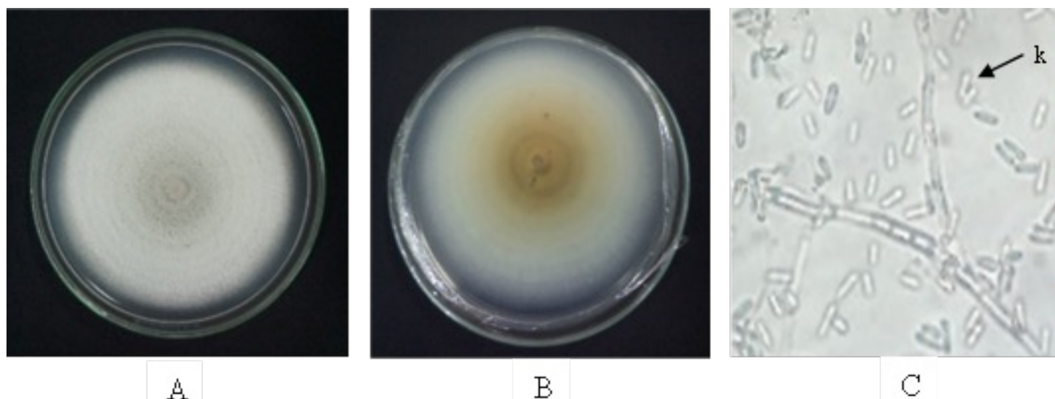

Figure 2. The isolate of *C. acutatum* fungus on red chili exhibits a colony with a front view (A), a rear view (B), and fungal conidia (C)

The results of ethanol extract of propolis testing using the poisoned food method indicate that the propolis extract is capable of inhibiting fungal colony growth by 50% at a specific concentration. This was determined based on the LC_{50} value obtained after probit analysis. The LC_{50} value of the ethanol extract of propolis against the growth of *C. acutatum* fungal colonies can be seen in Table 1. In Table 1, it is observed that the LC_{50} value of ethanol propolis extract on the growth of *C. acutatum* fungal colonies is 12.8%. This value represents the concentration required to inhibit 50% of fungal growth, indicating that the bioactive compounds in propolis exhibit antifungal activity against *C. acutatum*. Propolis contains antifungal constituents, such as flavonoids, phenolic acids, and terpenoids, that inhibit fungal growth by disrupting cell membranes and interfering with metabolic processes (Dudoit et al., 2021). Based on the obtained

LC_{50} value, concentrations of 12.8%, 19.2%, 25.6%, and 32% are used for testing the inhibitory effects of ethanol propolis extract on the growth of *C. acutatum* colonies and conidial germination, as well as for testing the suppression of damping-off disease incidence in chili seeds.

Table 1. LC_{50} Values of Ethanol Extract of Propolis on the Growth of *C. acutatum* Fungal Colonies

Extract Material	LC-50 (%)	Lower Bound	Upper Bound
Ethanol Propolis Extract	12.80	6.20	49.12

Testing the inhibitory effect of ethanol propolis extract on the growth of *C. acutatum* colonies

The test results indicate that the application of ethanol propolis extract significantly inhibits the growth of *C. acutatum* colonies. The colony diameters in each extract concentration treatment differ significantly from those in the control treatment. Observations were conducted until the fungal colonies in the control treatment completely covered the Petri dish, which occurred at 12 Days After Treatment (DAT). The average colony diameter of *C. acutatum* in each treatment is shown in Table 2. The ethanol propolis extract treatment with the highest inhibition percentage is treatment E (32% concentration), with 81.39% inhibition, followed by treatment D (25.6% concentration), with 65.28% inhibition, and treatment C (19.2% concentration), with 60.28% inhibition. The lowest inhibition is observed with treatment B (12.8% concentration), with 56.94% inhibition. These results indicate that increasing the concentration of ethanol-propolis extract enhanced inhibition of *C. acutatum* colony growth. This effect is attributed to higher concentration of bioactive antifungal compounds in propolis, particulary flavonoids and phenolic acids, which are known to disrupt fungal cell membranes, increase membrane permeability, and interfere with enzymatic activity and cellular metabolism, ultimately inhibiting fungal growth (Zulhendri et al., 2021; Ożarowski et al., 2022). In addition, these compounds may induce structural damage to hyphae and inhibit spore germination, thereby further contributing to propolis's antifungal activity.

Based on Table 2, the inhibition percentage of *C. acutatum* increases with increasing ethanol propolis extract concentrations. The lowest concentration (12.8%) showed an inhibition of 56.94%, while the highest concentration (32.0%) reached 81.39%. This indicates a dose-dependent response, where higher concentrations of propolis extract resulted in greater suppression of fungal growth. Statistical analysis also showed significant differences among treatments, indicating that each concentration had a distinct effect on colony growth inhibition.

Table 2. The colony diameter and percentage inhibition of *C. acutatum* fungal growth at 12 Days After Treatment (DAT)

Code	Treatment	The diameter of *C. acutatum* colonies (cm)	Inhibition Percentage (%)
A	Control	9.000^{e}	-
B	12.8% ethanol propolis extract	3.875^{d}	56.94
C	19.2% ethanol propolis extract	3.575^{cd}	60.28
D	25.6% ethanol propolis extract	3.125^{c}	65.28
E	32.0% ethanol propolis extract	1.675^{b}	81.39
F	Mancozeb fungicide	0.500^{a}	94.44

Notes: The same letters within a column in the table indicate that the data are not significantly different based on the Duncan's Multiple Range Test at 5% significance level.

The antifungal activity of propolis is primarily due to its rich composition of bioactive compounds, including flavonoids, phenolic acids, and terpenoids. These compounds are known to inhibit fungal growth through multiple mechanisms, such as disrupting cell membrane integrity, increasing membrane permeability, inhibiting enzymatic activity, and interfering with cellular respiration and energy metabolism. Flavonoids, for instance, can form complexes with extracellular and soluble proteins, leading to protein denaturation. At the same time phenolic compounds may cause structural damage to cell walls and membranes, ultimately resulting in cell lysis. In addition, terpenoids have been reported to promote fungal growth (Dudoit et al., 2021; Sforcin, 2016).

Macroscopic observations (Figure 3) further support these findings, showing that untreated colonies (control) exhibited rapid, uniform growth, completely covering the Petri dish surface. In contrast, colonies treated with lower concentrations of propolis extract (12.8% and 19.2%) showed reduced radial expansion, although growth was still evident. At higher concentrations (25.6% and 32%), colony growth was markedly restricted, with colonies appearing smaller, denser, and in some cases showing signs of growth cessation. This gradual inhibition pattern indicates that the antifungal compounds in propolis act in a concentration-dependent manner, progressively limiting fungal development as their concentration increases.

Propolis contains various antifungal bioactive compounds, including flavonoids such as pinocembrin, galangin, and pinobanksin, which contribute to its antimicrobial activity (Elangovan, 2024). These compounds can inhibit fungal growth by disrupting cell membranes, increasing membrane permeability, and interfering with metabolic processes. For instance, pinocembrin has been reported to inhibit *Penicillium italicum* by disrupting respiratory activity and energy homeostasis, leading to membrane damage and metabolic dysfunction (Sforcin, 2016). Other constituents such as 3-acetylpinobanksin, pinobanksin-3-acetate, p-coumaric acid, and caffeic acid also contribute to the antifungal properties of propolis (Ozcan, 1999; Jenny et al., 2024).

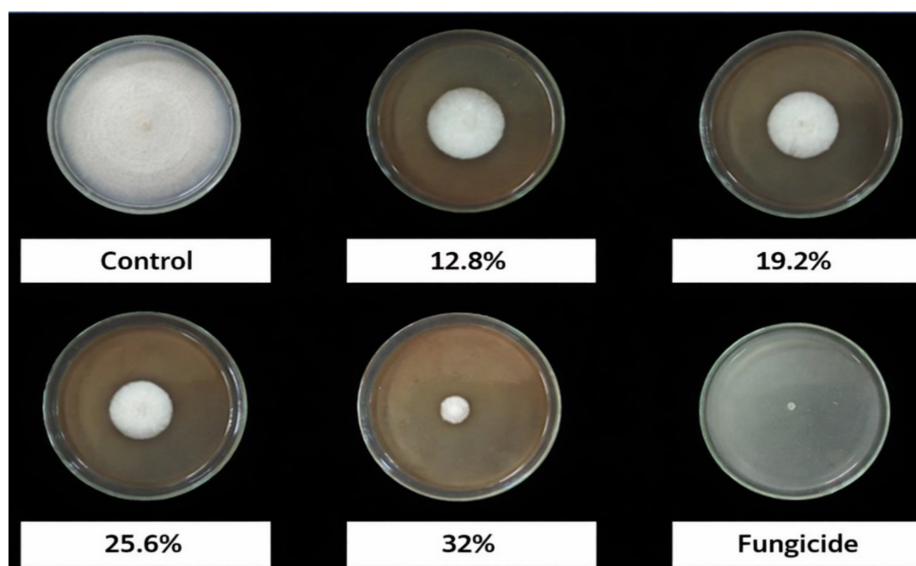

Figure 3. The growth of *C. acutatum* fungal colonies at 12 Days After Treatment (DAT) at various levels of ethanol propolis extract concentrations

The fungicide treatment with the active ingredient mancozeb showed the highest inhibition value, at 94.4%, compared to other treatments. In macroscopic observations, *C. acutatum* fungal

colonies were unable to grow on media treated with the fungicide. This suggests that the *C. acutatum* isolate's response to the active ingredient mancozeb remains effective. The concentration of the active ingredient is based on the manufacturer's recommended concentration in the formulation, 1 g/L. Mancozeb fungicide has a multi-site mode of action, meaning it generally works on contact and can inhibit more than one type of biochemistry in the pathogen fungus's organelles (Fungicide Resistance Action Committee, 2018). Due to its multi-site activity mode of action, this fungicide has a low risk of resistance development. This is because mancozeb simultaneously disrupts multiple metabolic processes in the fungal cell, including enzyme activities involved in respiration and amino acid synthesis, making it difficult for the pathogen to develop resistance through single genetic mutations (Andriani et al., 2017).

Testing the inhibitory effect of ethanol propolis extract on the germination of *C. acutatum* conidia

After 24 hours of incubation in sterile boxes, the *C. acutatum* suspension exhibited conidial germination, as observed under a microscope at 400x. Germinated conidia are characterized by the formation of a germ tube, and a conidium is considered germinated when the length of the germ tube becomes equal to or exceeds the diameter of the conidium (Ijadpanahsaravi et al., 2022). Conidial germination is shown in Figure 4. The observation results indicate that germinated conidia in the control treatment grow faster, thicker, and more branched, whereas in the ethanol propolis extract treatment, conidia are shorter and thinner. This suggests interference by compounds present in the ethanol propolis extract during conidial germination, leading to suboptimal growth. It is known that flavonoid and pinobanksin compounds in propolis can damage cell membranes, increase permeability, and disrupt fungal metabolism, resulting in damage to fungal cells (Sforcin, 2016). In line with the study by Maringgal et al. (2019), it was reported that there was damage to the hyphae of *Colletotrichum brevisporum* and spores undergoing cytolysis after treatment with Trigona honey extract.

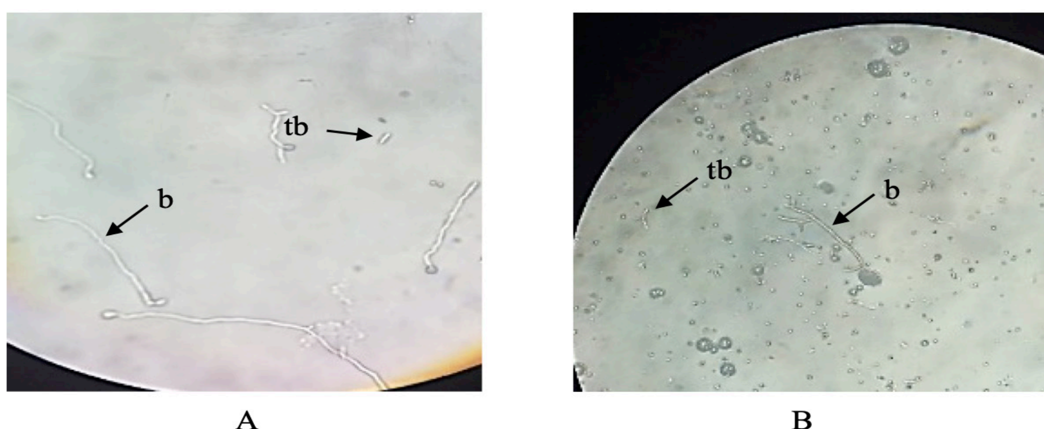

Figure 4. Conidial germination of *C. acutatum* fungus at 400x magnification: (A) Control Treatment, (B) Ethanol Propolis Extract Treatment. Note: b = germinated conidia, tb = non-germinated conidia

The test results for ethanol propolis extract inhibition of *C. acutatum* conidia germination are shown in Table 3. The germination values of conidia in almost all ethanol propolis extract

treatments show a significant difference compared to the control treatment. Only in the 12.8% extract concentration treatment, the germination value did not show a significant difference compared to the control treatment. This may be attributed to the saturation effect of bioactive compounds, in which the antifungal activity reaches a threshold beyond which further increases in concentration do not result in a proportional increase in inhibitory effect.

Table 3. The percentage of germinated conidia of *C. acutatum* fungus and the percentage inhibition of germination of *C. acutatum* fungus conidia

Code	Treatment	The diameter of *C. acutatum* colonies (%)	Inhibition percentage (%)
A	Control	76.33[c]	-
B	12.8% ethanol propolis extract	69.13[bc]	9.44
C	19.2% ethanol propolis extract	58.94[b]	22.78
D	25.6% ethanol propolis extract	59.43[b]	22.15
E	32.0% ethanol propolis extract	60.27[b]	21.05
F	Mancozeb fungicide	3.17[a]	95.84

Notes: The same letters within a column in the table indicate that the data are not significantly different based on the Duncan's Multiple Range Test at 5% significance level

Propolis contains various bioactive compounds such as flavonoids and phenolic acids that act on fungal cells by disrupting membrane integrity and inhibiting enzymatic processes (Bankova et al., 2021). At certain concentrations, these compounds may already have exerted their maximum inhibitory effect on conidial germination, resulting in similar levels of inhibition at higher concentrations. In addition, limited solubility and diffusion of active compounds in the growth medium may also reduce the effectiveness of higher concentrations, leading to significant differences among treatments (Przybyłek & Karpiński, 2019).

The results indicate that conidial germination of *C. acutatum* was inhibited in all treatments. Successively from the smallest value, treatment B (concentration 12.8%) showed inhibition of 9.44%, treatment E (concentration 32%) inhibition of 21.05%, treatment D (concentration 25.6%) inhibition of 22.15%, treatment C (concentration 19.2%) inhibition of 22.78%, and treatment F (fungicide) resulted in significant inhibition of 95.84% compared to other treatments. Among the treatments, the ethanol propolis extract did not show significantly different results, indicating that increasing its concentration did not affect inhibitory activity against conidial germination.

Propolis is rich in flavonoids and phenolic compounds that exert antifungal activity by disrupting cell membrane integrity and inhibiting enzymatic processes. These effects can reach an optimal level at certain concentrations (Wagh, 2023). Furthermore, the limited solubility and diffusion of propolis compounds in the growth medium may reduce their bioavailability at higher concentrations, leading to similar levels of inhibition across treatments (Grecka et al., 2021). In addition, interactions among the complex mixture of compounds in propolis may result in antagonistic or non-synergistic effects at higher concentrations, thereby preventing further increases in antifungal activity (Anjum et al., 2019).

Test of suppression on damping-off disease incidence in chili seeds

The incubation period is measured from seed inoculation until the appearance of disease symptoms (Figure 5). Infection by *C. acutatum* causes early symptoms of brown spots on the hypocotyl area of the seedlings. The pathogen also causes seed failure to germinate, leading to the growth of hyphae that form aservuli on the surface of chili seeds that fail to germinate. This is consistent with the statement by Naznin et al. (2016), which notes that infection by *C. acutatum* leads to necrotic symptoms, beginning with the stem becoming soft and brown, then turning white-grayish, and, in some symptomatic areas, aservuli developing.

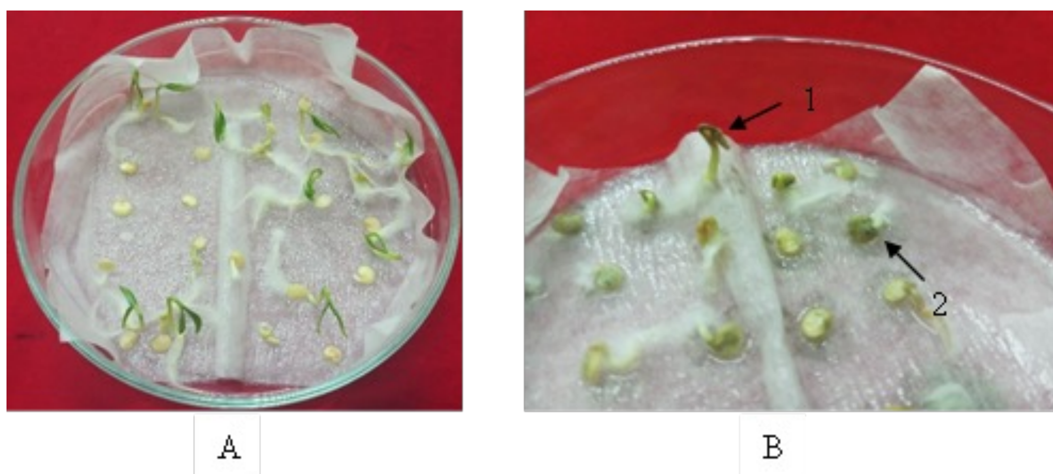

Figure 5. Infection of *C. acutatum* on chili seedlings. (A) Chili seedling without *C. acutatum* infection in the treatment without inoculation. (B) Chili seedling infected with *C. acutatum*. Legend: 1 = brown spot symptoms on the hypocotyl of the seedling, 2 = symptoms of fungal hyphae growth on chili seeds.

The results of the incubation period for damping-off disease in chili seedlings indicate that the first symptoms of the disease appeared at 4 days after seed inoculation (DSI) in the control treatment (Code A). Meanwhile, all treatments with ethanol propolis extract (Codes B-E) and fungicide (Code F) showed the first disease symptoms appearing at 5 DSI. This indicates that treatments with ethanol-propolis extract and the fungicide tended to delay the appearance of disease symptoms by 1 day compared to the control. However, the effect was not statistically significant (Table 4).

This delay may also be related to propolis compounds' ability to inhibit fungal enzymes involved in host tissue penetration, such as cell wall-degrading enzymes. By suppressing these enzymes, the pathogen's ability to invade plant tissues is reduced, thereby slowing the infection process and delaying symptom development. In addition, propolis may induce physiological changes in plant tissues that enhance their defense responses, thereby delaying disease onset (Sforcin & Bankova, 2011; Silva et al., 2022).

The disease incidence at the onset of first symptoms indicates that the ethanol propolis extract treatment did not differ significantly from the control treatment inoculated with *C. acutatum*. This means that the application of ethanol-propolis extract did not inhibit disease occurrence at the initial stage of infection. It was noted that the ethanol propolis extract treatment at 12.8% concentration had the highest disease incidence (4%) compared to other treatments.

Table 4. The Incubation Period of Red Chili Damping-off Disease

Code	Treatment	Day after inoculation	Disease incidence (%)
A	Control	4	2[a]
B	12.8% ethanol propolis extract	5	4[a]
C	19.2% ethanol propolis extract	5	2[a]
D	25.6% ethanol propolis extract	5	1[a]
E	32.0% ethanol propolis extract	5	3[a]
F	Mancozeb fungicide	5	3[a]

Note: The same letter in one column in the table indicates no statistically significant difference based on the Duncan Multiple Range Test at 5% significance level.

Incidence and suppression of red chili damping-off disease

Observations of damping-off disease incidence in red chili seedlings were conducted from the moment of seed inoculation until the plants reached 14 days after seed inoculation (DAI). Symptoms of damping-off disease in red chili seedlings were first detected at 4 DAI in the control treatment inoculated with *C. acutatum* (Figure 6, Table 5).

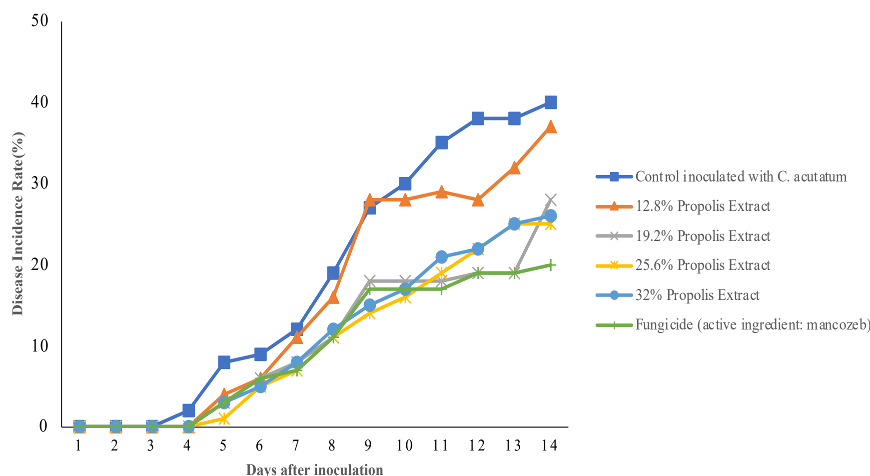

Figure 6. The development of damping-off disease in red chili seedlings in each treatment

The average disease incidence in propolis extract treatments C, D, and E did not show statistically significant differences, indicating that increasing the concentration did not enhance the suppression of damping-off disease. Therefore, 19.2% can be considered the minimum effective concentration, as higher concentrations did not result in a significant increase in disease suppression. This finding is consistent with previous studies indicating that the antifungal activity of propolis is concentration-dependent but may not increase proportionally at higher concentrations due to the complexity of its bioactive compounds (Zulhendri et al., 2021; Ożarowski et al., 2022). Furthermore, no significant difference was observed between propolis extract treatments and the fungicide treatment, suggesting that propolis extract has comparable effectiveness under the conditions of this study and thus has potential as an alternative control agent. This is supported by previous reports showing that seed coating using natural materials can enhance seed germination, improve seedling growth, and protect against seed- and soil-borne diseases (Haskett et al., 2021),

while Castro et al. (2018) demonstrated that ethanol propolis extract improved the resistance of *Pinus sylvestris* seeds to *Fusarium circinatum* infection.

Table 5. Incidence and Suppression of Red Chili Damping-off Disease

Code	Treatment	Disease incidence (%)	Inhibition percentage (%)
A	Control with inoculation	40[b]	-
B	12.8% ethanol propolis extract	37[b]	7.5
C	19.2% ethanol propolis extract	28[a]	30.0
D	25.6% ethanol propolis extract	25[a]	37.5
E	32.0% ethanol propolis extract	26[a]	35.0
F	Mancozeb fungicide	20[a]	50.0

Note: The same letter in one column in the table indicates no statistically significant difference based on the Duncan Multiple Range Test at 5% significance level.

Components of red chili seed growth

The growth components observed consist of germination rate, seedling height, root length, and the number of seedlings producing cotyledon leaves. The chili seeds used are the Tanjung 2 variety obtained from BALITSA. The seed package description indicates a germination rate of 79%, purity of 100%, and moisture content of 6.7%. The observations show that the germination rate in the ethanol propolis extract treatment is lower compared to the germination rate described for the variety (Table 6). However, the statistical analysis indicates that the germination rate in the ethanol propolis extract treatment is not significantly different from that in the control treatment.

Table 6. Germination capacity of chili seeds at 14 days after seed inoculation (DAI)

Code	Treatment	Germination percentage (%)
A	Control without inoculation	89[b]
B	Control with inoculation	85[ab]
C	12.8% ethanol propolis extract	74[ab]
D	19.2% ethanol propolis extract	78[ab]
E	25.6% ethanol propolis extract	73[ab]
F	32.0% ethanol propolis extract	69[a]
G	Mancozeb fungicide	86[ab]

Note: The same letter in one column in the table indicates no statistically significant difference based on the Duncan Multiple Range Test at 5% significance level.

The germination capacity in both the control treatments, whether inoculated or not with *C. acutatum*, and in the fungicide treatment was higher than in the described variety. This is suspected to be due to the effect of pre-treatment with hot water before testing. Hot water treatment can accelerate seed dormancy breaking, leading to increased seed germination percentage (Zanzibar, 2017). Water uptake during seed imbibition initiates the reactivation of metabolic processes in the embryo, which ultimately leads to seed germination and embryo growth (El-Maarouf-Bouteau, 2022).

The observation results for seedling height, root length, and the number of seedlings producing cotyledon leaves were measured based on germinated seeds. Observations were conducted on 5

germinated seeds from each replication. The growth components of chili seeds are shown in Table 7. The observation results for seedling height indicate that the ethanol propolis extract treatment increased plant height growth. However, unlike in other growth components, the ethanol propolis extract treatment did not increase root length or the number of seedlings producing cotyledon leaves.

The differences in seedling growth responses among treatments indicate that ethanol propolis extract selectively influences plant physiological processes. The increase in seedling height may be associated with bioactive compounds such as flavonoids that can stimulate cell elongation and enhance metabolic activity, thereby promoting shoot growth (Silva et al., 2022). However, the lack of improvement in root length may be due to the presence of phenolic compounds that can inhibit root meristem activity and limit root elongation at certain concentrations (Xie et al., 2021). In addition, under pathogen stress, plants tend to prioritize shoot growth over root development due to trade-offs in resource allocation between growth and defense responses (Huot et al., 2014).

Table 7. Components of Red Chili Seed Growth at 14 DAI

Code	Treatment	GL (cm)	RL (cm)	SCL
A	Control without inoculation	1.775[d]	4.525[b]	15[b]
B	Control with inoculation	0.775[a]	1.525[a]	0[a]
C	12.8% ethanol propolis extract	1.375[c]	1.225[a]	0[a]
D	19.2% ethanol propolis extract	1.225[bc]	1.200[a]	0[a]
E	25.6% ethanol propolis extract	0.950[ab]	1.000[a]	0[a]
F	32.0% ethanol propolis extract	1.075[abc]	1.025[a]	0[a]
G	Mancozeb fungicide	1.375[c]	0.800[a]	0[a]

Note: The same letter in one column in the table indicates no statistically significant difference based on the Duncan Multiple Range Test at 5% significance level. GL: germination length, RL: Root Length, SCL: Number of Seedlings with Cotyledon Leaves

The observation results for seedling height indicate that ethanol propolis treatments at lower concentrations, namely 12.8% and 19.2%, significantly increased plant height compared to the control treatment inoculated with *C. acutatum*. Meanwhile, treatments with higher concentrations did not show a significantly different effect on height increase. This suggests the influence of compounds contained in propolis on plant height growth.The effect of propolis on plant height growth may be associated with its bioactive compounds, which can act as natural biostimulants, enhancing physiological processes such as cell division and hormonal regulation. Flavonoids present in propolis have been reported to interact with plant hormone pathways, particularly auxin, which plays a key role in stem elongation and shoot development (Zhang et al., 2022). In addition, propolis contains antioxidant compounds that can reduce oxidative stress in plants, thereby maintaining cellular function and promoting better growth under biotic stress conditions (Almuhayawi, 2020). However, at higher concentrations, certain compounds in propolis may exert inhibitory effects due to phytotoxicity or hormonal imbalance, limiting further growth enhancement and resulting in no significant differences compared to lower concentrations (Hassan et al., 2023).

In the growth component of root length, the application of ethanol propolis extract resulted in a decrease in root length growth. Moreover, increasing the concentration of ethanol propolis extract further decreased the length of seedling roots produced. However, statistical analysis indicates that this decrease in root length did not differ significantly (Figure 7).

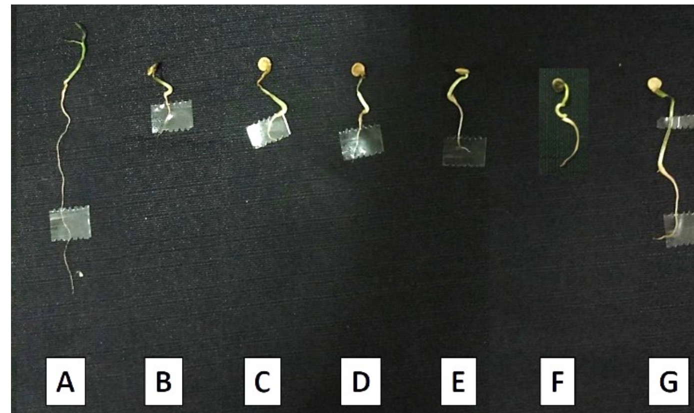

Figure 7. Seedling height and root length of red chili at 14 DAI. (A) Control without *C. acutatum* inoculation; (B) Control inoculated with *C. acutatum*; (C) 12.8% ethanol propolis extract; (D) 19.2% ethanol propolis extract; E) 25.6% ethanol propolis extract; (F) 32% ethanol propolis extract; (G) Mancozeb fungicide

Based on seedling growth parameters, the ethanol extract of propolis may contain bioactive compounds that can negatively affect seedling vigor at certain concentrations. This is consistent with Almeida et al. (2021), who reported that plant extracts used as seed treatments can suppress pathogens but may also reduce germination and vigor due to phytotoxic effects, similar to fungicides that interfere with cellular metabolism and physiological processes (Song et al., 2025). However, propolis extract has also been reported to enhance plant growth, including increased leaf weight, plant height, and leaf area (El-Hefny et al., 2022), as well as improved nutrient content and yield in pea (*Pisum sativum* L.) (Pereira et al., 2018). These contrasting effects may be attributed to variations in propolis composition, which depend on environmental conditions and the botanical origin of Trigona bees (Sforcin, 2016). Observations of the number of seedlings that exhibited cotyledon leaves indicated that in all treatments inoculated with *C. acutatum*, no seedlings exhibited cotyledon leaves at 14 days after inoculation (DAI). Conversely, under normal conditions (control without *C. acutatum* inoculation), an average of 15 out of 25 seedlings were able to exhibit cotyledon leaves. Visual observations of seedlings treated with a 19.2% ethanol extract of propolis revealed that *C. acutatum* mycelium covered the seed surface (testa), as depicted in Figure 8. A similar situation was reported by Hersanti et al. (2021), where *Fusarium oxysporum* f. sp. *lycopersici* mycelium adhered to the outer skin (testa) of tomato seeds, resulting in the inhibition of cotyledon leaf emergence.

Colletotrichum species, including *C. acutatum*, are capable of infecting plant tissues through specialized infection structures that penetrate the host surface and colonize plant cells. During infection, the pathogen secretes various cell wall-degrading enzymes, such as cellulases, polygalacturonases, and pectinases, which break down the structural components of plant cell walls, facilitating tissue maceration and leading to host cell damage and death (Zhang et al., 2024). The degradation of plant cell walls enables the pathogen to invade deeper tissues and disrupt normal cellular functions, ultimately reducing seed viability and inhibiting seedling growth. In addition, *Colletotrichum* spp. produce various secondary metabolites and effectors that interfere with host cellular processes and induce oxidative stress, thereby contributing to cellular leakage, tissue necrosis, and disease development during the necrotrophic phase of infection (Han et al., 2024).

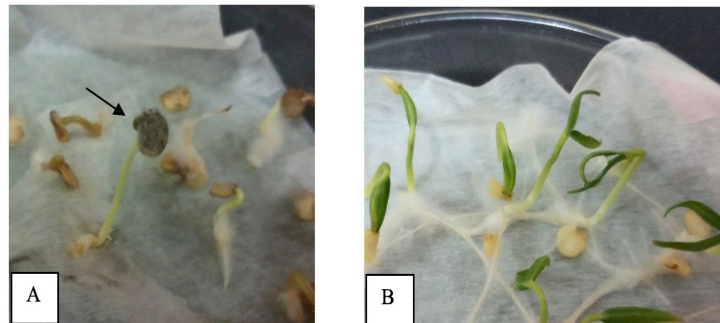

Figure 8. The mycelium of the fungus *C. acutatum* inhibits the emergence of cotyledon leaves. (A) The outer skin of the seed covered with *C. acutatum* mycelium in the treatment with 19.2% ethanol extract of propolis; (B) The outer skin of the seed free from *C. acutatum* mycelium in the control treatment without inoculation

CONCLUSION

The ethanol extract of propolis inhibits the growth of *C. acutatum* colonies (56.94%-81.39%) and conidial germination (9.44%-22.78%). Additionally, it can suppress damping-off disease in red chili seedlings, with an inhibition percentage ranging from 7.5% to 37.5%. The ethanol extract of propolis at a concentration of 19.2% showed relatively high inhibition of colony growth (60.28%) and conidial germination of *C. acutatum* (22.78%), as well as suppressing the incidence of damping-off disease in chili seedlings (30%). However, these effects were not significantly different from other propolis treatments.

AUTHOR CONTRIBUTION

H. conceptualized and designed the research, supervised all stages of the study, provided research funding, and guided the overall research process. **A.A.S**. carried out the research activities, including initial preparation, laboratory experiments, observations, data collection, and preparation of the research findings. **I.N.B**. contributed to supervising the research, revising the manuscript and the research process as needed, and providing valuable ideas and suggestions to improve the quality of the study.

ACKNOWLEDGMENTS

The authors sincerely appreciate the institutional laboratory facilities and technical support that contributed to the completion of this research.

CONFLICT OF INTEREST

All co-authors have reviewed and agreed to the manuscript's contents, and there is no financial interest to report.

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