Bioactive Antifungal Metabolites Produced by *Streptomyces albus* CINv1 Against Strawberry Anthracnose Pathogen

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Keywords: Antifungal compound; *Streptomyces* sp.; Strawberry anthracnose pathogen; LC-MS analysis

Abstract

The endophytic *Streptomyces albus* CINv1, isolated from *Cinnamomum verum*, has previously demonstrated the ability to promote plant growth, colonize effectively, and exhibit antifungal activity against *Colletotrichum* sp. isolate CA0110, the causal agent of strawberry anthracnose. This study aims to assess the in vitro antifungal potential of ethyl acetate from *S. albus* CINv1. The investigated including culture broth medium, optimal incubation conditions as well as determining their antifungal activities, comparing with their standard phenolic compounds through LC-MS analysis. The culture broth medium and optimal incubation conditions for antifungal production by *S. albus* CINv1 were investigated. The results revealed that the ISP-2 culture broth, at a pH range of 6–9 with incubation periods of 7–10 days, achieved the highest percentage inhibition of radial growth (PIRG) of *Colletotrichum* sp. CA0110. As the concentration of the culture filtrate increased from 20% to 100%, the percentage of conidial germination inhibition (%PCGI) also increased, ranging from 22.77% to 91.80%. The ethyl acetate crude extract of *S. albus* CINv1 exhibited the highest antifungal activity against *Colletotrichum* sp. CA0110, with a percentage inhibition of radial growth (%PIRG) of 92.25%. The crude extract was subsequently fractionated using column chromatography and evaluated for its %PCGI against *Colletotrichum* sp. CA0110. Among the 15 fractions, five (F2, F9, F10, F11, and F12) showed the highest %PCGI, ranging from 92.67% to 100%. LC-MS analysis of the five fractions, using 16 standard phenolic compounds, revealed varying quantities of detected phenolics. The results exhibited the predominant antifungal compounds, such Naringenin, Hydroxybenzoic acid, O-coumaric acid, Quercetin, Kaempferol, and Chlorogenic acid. Notably, ferulic acid was identified as a well-known fungicide and antifungal compound. The findings suggest that the metabolites of endophytic *S. albus* CINv1, particularly phenolics, hold significant promise for developing potent biocontrol agents or bio-fungicide to control anthracnose disease in various crops in Thailand.

Keywords: antifungal compound; *Streptomyces* sp.; Strawberry anthracnose pathogen; LC-MS analysis

1. Introduction

Strawberry anthracnose is a serious crop disease in the highlands of Chiang Mai, Thailand. It is primarily caused by *Colletotrichum* spp., including *Colletotrichum fragariae, C. gloeosporioides,* and *C. acutatum* (Denoyes-Rothan et al., 2003; Han et al., 2016). The disease can damage strawberry crowns, petioles, leaves, fruit trusses, flowers, buds, and fruits (Howard et al., 1992). Plant growth and yield are significantly reduced by crown and fruit rot (Ward and Hartman, 2021). Biological control is a sustainable and effective alternative to conventional pesticides for managing fungal phytopathogens (Rios-Muniz and Evangellista-Martinez, 2022). In recent years, a diverse range of microbial biocontrol agents (BCAs) has been developed to address these issues. Among the most extensively studied are bacteria from the genera *Pseudomonas, Bacillus,* and *Streptomyces*, which have been registered and commercialized as biocontrol products (Bonaterra et al., 2022).

The *Streptomyces* genus is responsible for producing over 70% of all known natural products derived from actinomycetes. In addition to antibiotics, which form the largest category, *Streptomyces* also generates secondary metabolites with various biological applications. (Rashad et al., 2015; Awla et al., 2017). These include antagonistic agents such as antibacterials, antifungals, antiprotozoals, and antivirals; agro-biological agents like insecticides, pesticides, and herbicides; and compounds with regulatory activities (Awla and Rashid, 2020). The *Streptomyces* sp. can successfully control fungal plant pathogen by hydrolyzing their cell walls, for example the streptomycin was first successful control blight disease; antimycin (Bentley et al., 2002). Also, compounds that are synthesized by *Streptomyces* exhibit extreme chemical diversity such as *S. hygroscopicus* is a notable example, producing over 180 secondary metabolites that exhibit multiple simultaneous bioactivities (Harir et al., 2018). Furthermore, the extract from *Streptomyces* sp. H4 exhibited strong antifungal activity against *C. fragariae* on strawberry fruit. It inhibited mycelial growth and conidial germination, suppressed disease development, and reduced disease incidence in strawberries, demonstrating broad-spectrum antifungal properties (Li et al., 2021).

Antibiotic biosynthesis is a unique characteristic of microorganisms, heavily influenced by culture conditions. Similarly, the ability of *Streptomyces* cultures to produce bioactive compounds depends on factors such as pH, incubation time, temperature, and the types of carbon and nitrogen sources utilized. For example, *S. spectabilis* achieved the highest production of bioactive metabolites when grown with cellobiose and peptone as the carbon and nitrogen sources under conditions of pH 5, 30°C, for 5 days. Similarly, the optimal conditions for *S. purpurascens* were pH 7, 30°C, for 8 days in a medium supplemented with starch and casein, while *S. lavendofoliae* showed maximum production at pH 7, 30°C, for ten days in a medium containing starch and peptone (Bundale et al., 2015). In addition, seven *Streptomyces* species (*S. rimosus, S. albus, S. riseofavus, S. fumosus, S. spiralis, S. aureofaciens*, and *S. favogriseus*) demonstrated their ability to produce chitinase, which is associated with antifungal activity to control plant pathogens. Furthermore, the culture filtrate of *S. albus*, grown at 30°C and pH 6.0 for 6 days, favored maximum chitinase production and effectively inhibited pathogenic fungi such as *M. oryzae, F. graminearum, R. solani, Puccinia* sp., and *B. cinerea* (Ekundayo et al., 2022). Likewise,

ethyl acetate extract of *S. albus* RC2 was demonstrated strong growth inhibition of *Scopulariopsis gossypii* Co1 and *F. fujikuroi* L3. The crude extract also suppressed the spore germination (Quach et al., 2023).

Bioassay-directed screening of compounds and extracts is a crucial step in the discovery of new pesticides and agrochemicals. The separation of specific components presents unique challenges, requiring a fast and efficient technique to isolate significant compounds. However, the size and scale of the isolation method directly influence the final requirements for obtaining pure compounds. Today, high-performance liquid chromatography (HPLC), as well as LC-MS and GC-MS techniques, are commonly used for compound screening. For example, the *Streptomyces* sp. isolate TS9 was extracted using ethyl acetate, and the active compound that inhibited the pathogen was analyzed via HPLC and compared with 16 standards, such as quercetin, gallic acid, caffeic acid, and rutin, among others (Awla and Rashid, 2020). In addition, following the extraction of bioactive compounds of *Streptomyces amritsarensis* V31 using four solvents; ethyl acetate, dimethyl sulfoxide, n-Hexane, and methanol using 1:3 (w/v) pellet: solvent ratio, and the DMSO extract was recorded the highest antifungal activity against six fungal infections. The LC-MS analyses clearly showed that *S. amritsarensis* V31 produced a mixture of potential antifungal compounds that inhibited the growth of various phytopathogenic fungi. The findings of this study suggest that the metabolite extracts of this strain could be utilized as a bio-fungicide for controlling phytopathogenic fungi (Shahid et al., 2021).

2. Materials and Methods

2.1 Fungal Pathogen and Endophytic Streptomyces

The *Colletotrichum* spp. strain CA0110 and *Streptomyces albus* CINv1 were obtained from the collection in the Virology Laboratory, Department of Entomology and Plant Pathology, Faculty of Agriculture, Chiang Mai University, Thailand. These strains were previously isolated from strawberry anthracnose in Chiang Mai province, Thailand. The fungal pathogen was cultured on potato dextrose agar (PDA) at 25°C for 14 days. The antagonistic *S. albus* CINv1, originally isolated from *Cinnamomum verum*, has demonstrated fungal inhibition against *Phytophthora cactorum*, which causes Phytophthora crown rot disease in strawberries (Potiyot & Kunasakdakul, 2014), and *Colletotrichum* spp. strain CA0110, which causes anthracnose of strawberry (Pupakdeepan et al., 2023). *S. albus* CINv1 was cultured on IMA-2 medium at 30°C for seven days prior to use.

2.2 Culture Broth Selection

Five different media broths were evaluated to determine the most suitable medium for the antifungal activity of the *S. albus* CINv1 strain. Ten culture discs (5 mm diameter) of *S. albus* CINv1 were transferred into 100 ml of each medium broth and incubated at 30°C for seven days on a digital orbital shaker (SCILOGEX SCI-O330-Pro) at 150 rpm. After incubation, the culture filtrates were centrifuged at 10,000 rpm for 15 min at 4°C, and the supernatants were filtered through a 0.2 μm membrane to obtain the culture filtrates. The antifungal activity of each culture filtrate was evaluated using the agar well diffusion technique (Kavitha et al., 2010). Eighty microliters

of each culture filtrate were added to wells in PDA plates. A fungal mycelium disc (5 mm in diameter) was placed one cm away from the wells. Sterile distilled water served as a control treatment. Four replicates were performed per treatment, and the experiment was conducted twice. After three days of incubation at room temperature, the radius of the fungal growth on each plate was measured. The percent inhibition of radial growth (PIRG) was calculated using the following equation (1):

$$PIRG = (R1-R2) / R1 \times 100 \quad (1)$$

where R1 is the radial growth of the control treatment and R2 is the radial growth of the treatments (Khamna et al., 2009).

2.3 Optimal Incubation Conditions

The selected broth medium from the previous experiment was used to determine the optimal incubation conditions, including pH and incubation period. The optimal pH was determined by varying the pH levels (4, 5, 6, 7, 8, 9, and 10) of 100 ml of the selected broth medium in 250 ml flasks. Ten discs of *S. albus* CINv1, cultured on IMA-2 medium for seven days, were transferred to each flask and incubated at 30°C for seven days on a digital orbital shaker at 150 rpm. The antifungal activity of the culture filtrate obtained from each pH trial was determined using the agar well diffusion technique (Kavitha et al., 2010). The optimal pH was then used to prepare the culture broth medium for the incubation period trial. *S. albus* CINv1 was cultured using the selected broth medium with the optimal pH.

The optimal incubation period was determined by testing at 3, 5, 7, and 10 days. The antifungal activity was evaluated using the agar well diffusion method (Kavitha et al., 2010). The optimal conditions for antifungal metabolite production by the *S. albus* CINv1 were determined by the percent inhibition of radial growth (PIRG) on the fungal pathogen as previously described. Two experiments were conducted twice with four replications per treatment, based on a completely randomized design (CRD). The PIRG was calculated using the aforementioned method. Experimental data were analyzed using IBM SPSS Statistics version 26 and subjected to analysis of variance (ANOVA). Separation of the means was performed using the LSD test at $p \leq 0.05$.

2.4 Preparation of the Crude Extract of *Streptomyces albus* CINv1

The *S. albus* CINv1 strain was cultured using the optimal broth medium and incubation conditions, with a digital orbital shaker set at 150 rpm and 30°C. The culture filtrate was prepared by transferring ten culture discs (5 mm in diameter) into 100 ml of ISP-2 broth (pH 7) and incubating on a digital orbital shaker at 150 rpm and 30°C for seven days. After incubation, the culture filtrate was centrifuged at 12,000 rpm for 15 min at 4°C and filtered through a 0.2 µm Millipore membrane. Five concentrations of the culture filtrate were prepared using sterile distilled water at 20%, 40%, 60%, 80%, and 100% (v/v), respectively. Sterile distilled water served as a control treatment (El-Abyad et al., 1993).

2.5 Inhibition of Fungal Conidial Germination

The culture filtrate obtained from the previous experiment (section 2.4) was used to determine antifungal activity. The inhibition of fungal conidial germination was assessed using the slide culture technique. A 10 µl spore suspension (10^4 conidia/ml) from the fungal isolate was inoculated into a 1.5 ml microcentrifuge tube containing 1 ml of each concentration of *S. albus* CINv1 strain culture filtrate. After gentle mixing, the trial microcentrifuge tubes were incubated at 25°C for 12 hours. Subsequently, 10 µl from each trial was observed under a compound microscope (Gemeda et al., 2014). Sterile distilled water served as a control. Three replications were performed, and 100 conidia per replication were recorded. Germ tubes longer than their conidia lengths were counted as germinating conidia. The percentage of conidial germination inhibition (PCGI) in each treatment was calculated relative to the control treatment using the following equation (2):

$$PCGI = (Cc-Ct) / Cc \times 100 \quad (2)$$

where Cc represents the average number of germinated conidia in the control treatment and Ct represents the average number of germinated conidia in the treatment.

2.6 Effects of Crude Extract on Fungal Mycelium Growth

The *S. albus* CINv1 strain was cultured on IMA-2 medium at 30°C for seven days. The culture filtrate was prepared by transferring 100 culture discs (5 mm diameter) into 1,000 ml of ISP-2 broth (pH 7) and incubated on a digital orbital shaker at 150 rpm and 30°C for seven days. The culture filtrate was passed through filter paper No. 5 (2.5 µm) twice using a Buchner funnel and suction flask with a vacuum pump. Subsequently, 2 liters of concentrate was prepared from 15 liters of culture filtrate using a rotary evaporator set at 40°C in a water bath. To extract the crude compounds, three solvents (hexane, dichloromethane, and ethyl acetate) were used separately. The extraction process for each solvent was performed three times using *S. albus* CINv1 strain culture filtrate and solvent solution at a 1:5 (v/v) ratio. Following the fraction separation method, a separating funnel was used to separate the crude extract compounds based on polarity differences with the solvents (Phuakjaiphaeo, 2017). After 30 minutes of partitioning, the solvent layer was collected and dried under vacuum conditions using a rotary evaporator set at 40°C in a water bath. The obtained crude extract was used for further evaluation of its inhibitory effects on fungal mycelial growth. To evaluate the inhibitory effects of the crude extract on fungal mycelial growth, the agar well diffusion method was employed. The stock solution of crude extract was prepared at a concentration of 1 mg/ml in 10% DMSO and filtered through a 0.22 µm membrane filter. A cork borer (5 mm diameter) was used to create wells in the PDA medium. Then, 30 µl of the crude extract obtained from each solvent was added to each well. An equal volume of 10% DMSO served as the control. The fungal mycelium disc (5 mm in diameter) of the severe isolate was placed 1 cm away from the wells and incubated at 30°C until the control mycelial growth reached the edge of the wells. Four replicates per treatment were performed. The inhibition zones of the treatments were

measured (Qi et al., 2019). The effective crude extract treatment was selected based on the percent inhibition of radial growth (PIRG). The experiments were conducted twice using a completely randomized design (CRD). Experimental data were analyzed using IBM SPSS Statistics version 26 and subjected to analysis of variance (ANOVA). Separation of means was performed using the LSD test at $p \leq 0.05$.

2.7 Antifungal Compound Detection

2.7.1 Column Chromatography

The effective crude extract treatment obtained from the previous experiment was used to isolate contained antifungal compounds using preparative column chromatography. A total of 5 g of crude extract was dissolved in 20 ml of a 1:1 (v/v) mixture of hexane and ethyl acetate and loaded into a chromatography column (2 x 50 cm) packed with silica gel (230-400 mesh ASTM, Merck, Darmstadt, Germany), which was completely saturated with hexane. The crude extract at the top of the column was washed with fresh solvent, and the eluent was collected at the bottom as the solvent flowed through. Fresh solvent was continuously added to the top of the column to prevent it from drying out. The solvent mixture was prepared from hexane and ethyl acetate at ratios of 200:0 (v/v), 180:20 (v/v), 160:40 (v/v), and so on, up to 20:180 (v/v) and 0:200 (v/v), with increments of ±20 ml each time. Finally, n-butanol was used four times. During the column run, 50 ml of the eluent was collected in Erlenmeyer flasks. Thin-layer chromatography (TLC) was used to take a drop from each solution and combine similar band patterns. The combined fractions were dried using a rotary evaporator set at 40°C in a water bath. The dried fractions were kept in sterile scintillation vials for further antifungal compound detection.

2.7.2 Inhibition of fungal conidia germination

The antifungal activity of the combined fractions was evaluated for their effects on the conidial germination of the severe fungal isolate using the slide technique (Gemeda et al., 2014). Each fraction was prepared at a concentration of 1,000 ppm (1 mg/ml) in 2% DMSO. A 2% DMSO solution served as a control treatment. Three replications per treatment were used. After incubating at 25°C for 12 h, conidial germination was recorded for 100 conidia per replication. Only germ tubes longer than their conidia length were counted using a compound microscope. The percentage of conidial germination inhibition (PCGI) in each treatment compared to the control treatment was calculated as described in method 2.5.

2.7.3 Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) of the antifungal activity was determined using the conidial germination as previously described. MIC90 and MIC50 values were utilized to define and compare the inhibitory efficiency of the chromatography fractions at concentrations of 90% and 50% (Patrick & Eric, 2020). The fraction was dissolved in 0.5% DMSO, and then serially double diluted with water to obtain final concentrations of 1,000, 500, 250, 125, 62.5, 31.25, and 15.62 ppm. A 0.5% DMSO solution served as a control treatment.

These assays were conducted using a completely randomized design (CRD) with 100 conidia, and three replications per treatment. The number of germinated conidia was counted and calculated by the percentage of conidial

germination inhibition (PCGI). The experiment was conducted twice. Experimental data were analyzed using IBM SPSS Statistics version 26 and subjected to analysis of variance (ANOVA). Separation of means was performed using the LSD test at $p \leq 0.05$.

2.8 Secondary Metabolite Analysis

To evaluate the potential of the *S. albus* CINv1 strain to produce secondary metabolites, Liquid Chromatography-Mass Spectrometry (LC-MS) was performed. The samples were diluted with 50% ethanol and filtered through a 0.45 µm nylon filter into 1.5 ml vials prior to injection. Analytical liquid chromatography was performed using an Agilent 1260 Infinity II series chromatograph, coupled with an Agilent 6130 electrospray ionization quadrupole mass spectrometer (Agilent Tech., Santa Clara, CA, USA). Separation was executed using an Ultra C18 column (5 µm, 4.6 × 250 mm, Restek, Bellefonte, PA, USA). The mobile phase consisted of two solutions: A (0.2% acetic acid in 5% MeOH) and B (0.2% acetic acid in 50% acetonitrile). A linear elution gradient was applied as follows: 0–45 min, 10–20% B, 45–85 min, 20–55% B, 85–97 min, 55–100% B, 97–110 min, 100% B. The initial conditions were held for 10 min as a re-equilibration step. The flow rate of the mobile phase was maintained at 0.5 mL/min, the column temperature was kept at 40°C, and the injection volume was 20 µL. Spectra were obtained in the negative selected ion monitoring mode and processed using OpenLab software. High-purity nitrogen was used as both a nebulizer and auxiliary gas. The mass spectrometer was operated in negative ion mode with the following parameters:

- Capillary voltage: -3.5 V
- Nebulizing gas flow: 1.5 L/min
- Dry gas flow rate: 12 L/min
- Dissolving line temperature: 250°C
- Voltage detector: 1.35 V
- Full scan spectra range: 100 to 1,200 m/z
- Scan time: 250 ms/spectrum

This LC-MS analysis allowed for the identification and characterization of secondary metabolites produced by *S. albus* CINv1, providing insights into its potential antifungal properties.

3. Empirical Results

3.1 Culture Broth Selection

The culture filtrates obtained from the cultures of *S. albus* CINv1 strain in five different media broths were evaluated to determine the suitable medium for activating antifungal production. After seven days of incubation, the results showed differences in the color of the medium-broth culture filtrates (Figure 3.1). The color of the culture filtrates ranged from red-orange to dark brown, with varying intensities in the order: IMB-2, BN, GYM, ISP-2, and YMD. Furthermore, all media induced the antifungal production of the *S. albus* CINv1 strain, as shown by

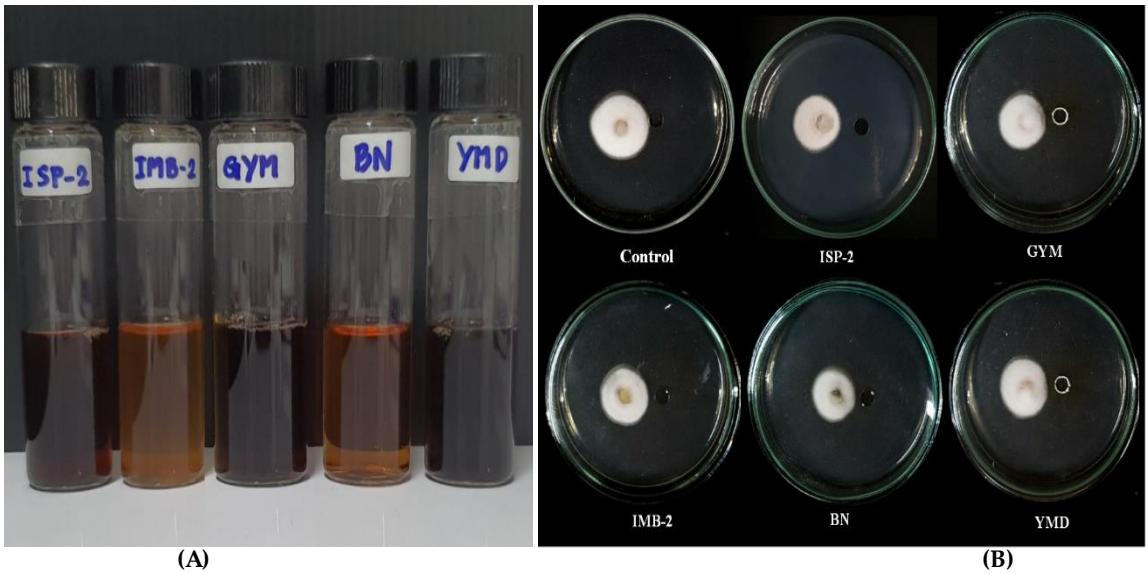
the percentage of inhibition of radial growth (PIRG) values against the *Colletotrichum* spp. CA0110. The culture filtrate of the ISP-2 broth medium showed the highest significant inhibition of the *Colletotrichum* spp. CA0110, with 69.75% inhibition. This was followed by the culture filtrates of GYM and YMD broths, which showed 58.00% and 53.75% inhibition, respectively. Meanwhile, the culture filtrates of BN and IMB-2 media exhibited lower inhibition of the *Colletotrichum* spp. CA0110, with 48.75% and 40.25% inhibition, respectively (Table 3.1, Figures 3.1).

Table 3.1 Percent Inhibition of Radial Growth of *Colletotrichum* spp. CA0110 by Culture Filtrates of *Streptomyces albus* CINv1 in Different Broth Media Tested with Agar Well Diffusion Technique for Three Days

Name of media	Codes name	%PIRG[1]
Inhibitory Mold Broth	IMB-2	40.25±1.25c[2]
Bennett's Medium	BN	48.75±2.50bc
International Streptomyces Project-2	ISP-2	69.75±11.89a
Yeast-Extract-Glucose Broth	GYM	58.00±2.16b
Yeast-Extract-Malt Extract-Dextrose Broth	YMD	53.75±6.23b
%CV		11.482
LSD0.05		9.067

Note : [1] Numbers in the columns indicate PIRG values as the mean of four replicates.

[2] The means within the column followed by the same letter(s) are not significantly different at p≤0.05, using the least significant difference test



(A) **(B)**

Figure 3.1 : (A) Color of culture filtrates obtained from *Streptomyces albus* CINv1 cultured in five different broth media. (B) Antifungal activities exhibited by various culture filtrates of *Streptomyces albus* CINv1 on the mycelium growth of 3-day-old *Colletotrichum* spp. CA0110 on PDA using the agar well diffusion method.

3.2 Optimal Incubation Conditions

The optimal pH for antifungal metabolite production by the *S. albus* CINv1 strain was determined by evaluating the percentage of inhibition of radial growth (PIRG) on the fungal mycelium growth. The results showed the highest inhibition of the fungal pathogen at pH 7 (75.5%), which was not significantly different from pH 6 (73.0%) and pH 8 (68.0%). However, the inhibition was significantly lower at pH 9 (55.5%), pH 10 (48.0%), pH 5 (35.5%), and pH 4 (30.5%) (Table 3.2).

The optimal incubation period for antifungal metabolite production by the *S. albus* CINv1 strain was also evaluated by assessing the PIRG on the mycelium growth of *Colletotrichum* spp. CA0110. The results showed the highest inhibition of 60.75% after seven days of incubation, which was not significantly different from ten days (52.50%). The inhibition decreased to 47.25% and 35.75% after 5 days and 3 days of incubation, respectively (Table 3.3).

Table 3.2 Effects of Culture Filtrates of *Streptomyces albus* CINv1 Cultured in ISP-2 Medium at Various pH Levels on Inhibition of Radial Growth of *Colletotrichum* spp. CA0110 After Tested by Agar Well Diffusion Method for Three Days

Treatment	pH Level	Percent Inhibition of Radial Growth (%PIRG)[1]
1	4	30.5±9.57d[2]
2	5	35.5±9.57cd
3	6	73.0±5.77a
4	7	75.5±12.58a
5	8	68.0±18.25ab
6	9	55.5±17.07abc
7	10	48.0±21.60bcd
	%CV	21.125
	LSD0.05	26.249

Note : [1] Numbers in columns indicated PIRG values by mean of four replicates.

[2] Means within the column followed by the same letter(s) are not significantly different at p≤0.05 using the least significant difference test.

Table 3.3 Effects of Culture Filtrates of *Streptomyces albus* CINv1 Cultured in ISP-2 Medium in Different Incubation Periods on Inhibition of Radial Growth of *Colletotrichum* spp. CA0110 After Tested by Agar Well Diffusion Method for 3 Days

Treatment	Incubation Periods (Days)	Percent Inhibition of Radial Growth (%PIRG)[1]
1	3	35.75±2.986c[2]
2	5	47.25±8.958b
3	7	60.75±2.986a
4	10	52.50±5.000ab
	%CV	11.306
	LSD0.05	8.092

Note : [1] Numbers in columns indicated PIRG values by mean of four replicates.

[2] Means within the column followed by the same letter(s) are not significantly different at p≤0.05 using the least significant difference test.

3.3 Inhibition of Fungal Conidial Germination

Five concentrations of the culture filtrate were prepared using sterile distilled water at 20%, 40%, 60%, 80%, and 100% (v/v). Each concentration was used to determine the inhibition of fungal conidial germination using the slide culture technique. The results are shown in Table 3.4. All concentrations of the *S. albus* CINv1 culture filtrates inhibited conidial germination. The inhibition ranged from 22.77% to 91.80%, and the percentage of conidial germination inhibition (PCGI) increased significantly with increasing concentration.

Table 3.4 Percent of Inhibition of Conidia Germination of *Colletotrichum* spp. CA0110 After Cultured in Five Concentrations of the Filtrates of *Streptomyces albus* CINv1 Culture in ISP-2 Broth After Incubation at 25°C for 12 h

Treatment	Percent (%) Concentration of Culture Filtrate	Percentage of (%) Conidia Germination Inhibition[1]
1	Control	00.00±1.445f[2]
2	20	22.77±6.048e
3	40	59.92±4.586d
4	60	70.67±1.258c
5	80	82.15±1.669b
6	100	91.80±1.971a
	%CV	6.168
	LSD0.05	5.671

Note : [1] Numbers in columns indicated PIRG values by mean of three replicates.

[2] Means within the column followed by the same letter(s) are not significantly different at p≤0.05 using the least significant difference test.

3.4 Effects of Crude Extract on Fungal Mycelium Growth

A separatory funnel was used to extract two-liter concentrations from 15 liters of evaporated culture filtrate. The yields of hexane, dichloromethane, and ethyl acetate extractions were 1.214 g, 2.705 g, and 5.004 g, respectively. The colors of each crude extract were white, dark yellow, and dark purple, respectively (Figure 3.2). At a concentration of 30 µl of 1,000 ppm, each solvent crude extract was tested for inhibition of radial growth of the *Colletotrichum* spp. CA0110 on PDA medium using the agar well technique after 5 days of incubation. The results showed the ethyl acetate crude extract was the best to inhibit the fungal growth at 92.25%, followed by dichloromethane and hexane at 78.50% and 67.00%, respectively (Tables 3.5, Figure 3.2).

Table 3.5 Percent Inhibition of Radial Growth of 5-Day-Old *Colletotrichum* spp. CA0110 by Crude Extracts of *Streptomyces albus* CINv1 which were Extracted with Three Different Solvents

Treatment	Solvents	Percent Inhibition of Radial Growth (PIRG)[1]
1	10% DMSO (Control)	00.00±0.00d[2]
2	Hexane	67.00±2.44c
3	Dichloromethane	78.50±3.00b
4	Ethyl acetate	92.25±2.02a
	%CV	3.674
	LSD0.05	3.186

Note : [1] Numbers in columns indicate PIRG values as the mean of four replicates.

[2] Means within the column followed by the same letter(s) are not significantly different at $p \leq 0.05$ using the least significant difference test.

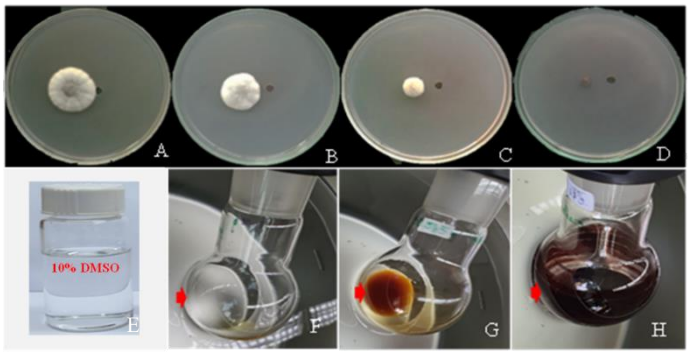


Figure 3.2 : Agar Well Diffusion Testing on PDA Medium to Inhibit Mycelium Growth of *Colletotrichum* spp. CA0110 with 10% DMSO (A, E) by *Streptomyces albus* CINv1 Crude Extract After Incubation for 3 Days Derived from Hexane (B, F), Dichloromethane (C, G), and Ethyl Acetate Extracting Solvents (D, H).

3.5 Antifungal Compound Detection

3.5.1 Inhibition of Fungal Conidia Germination

The antifungal activities of fifteen chromatography fractions (F1-F15) at a concentration of 1,000 ppm were evaluated for their effects on conidia germination of the *Colletotrichum* spp. CA0110 using the slide culture technique. The result exhibited the PCGI of the *Colletotrichum* spp. CA0110, with only 5 fractions including F2, F9, F10, F11, and F12 showing the best antifungal activity at 92.67-100% PCGI. This was followed by a moderate group of PCGI including F3, F15, and F8 showing 74.33%, 63.67%, and 52.00%, respectively. Less than 50% of PCGI was observed for F1, F5, F7, F6, F13, F14, and F4, showing 36.33%, 34.33%, 24.67%, 21.67%, 16.33%, 14.33%, and 13.67.00%, respectively. In contrast, the control treatment (2% DMSO) showed no antifungal activity (0%) (Table 3.6).

Table 3.6 Percentages of Conidial Germination Inhibition of *Colletotrichum* spp. CA0110 Treated with the 15 Fractions from Silica Gel Column Chromatography at a Concentration of 1,000 ppm

Treatment	Percentage of (%)Conidia Germination Inhibition[1]	Treatment	Percentage of (%)Conidia Germination Inhibition[1]
2%DMSO	0.00[2]	F8	52.00±4.00d
F1	36.33±4.72e	F9	100.00±0.00a
F2	100.00±0.00a	F10	100.00±0.00a
F3	74.33±4.50b	F11	100.00±0.00a
F4	13.67±4.50h	F12	92.67±3.05a
F5	34.33±3.51ef	F13	16.33±4.50gh
F6	21.67±2.51gh	F14	14.33±5.13gh
F7	24.67±5.03fg	F15	63.67±4.04c
	%CV		5.903
	LSD0.05		6.638

Note : [1] Numbers in columns indicated PIRG values by mean ±SD of three replicates.

[2] Means within the column followed by the same letter(s) are not significantly different at p≤0.05 using the least significant difference test.

3.5.2 Minimum Inhibitory Concentration

The minimum inhibitory concentrations at 50% (MIC50) and 90% (MIC90) of five fractions were determined based on the percentage of conidia germination inhibition presented in Table 3.7. The results exhibited the antifungal activity of the fraction F2, which showed the MIC50 at 31.25 ppm and the MIC90 at 125 ppm. This was followed by fraction F9, which showed the MIC50 at 62.5 ppm and the MIC90 at 250 ppm. Fraction F10 showed the MIC50 at 62.5 ppm and the MIC90 at 500 ppm, while fraction F11 had the MIC50 at 125 ppm and the MIC90 at 500 ppm. The last fraction, F12, showed the MIC50 at 250 ppm and the MIC90 at 1,000 ppm. Overall,

the best fraction was F2, which exhibited the lowest MIC50 and MIC90 values, followed by fractions F9, F10, F11, and F12, respectively.

Table 3.7 Inhibition Percentage of *Colletotrichum* spp. CA0110 Conidia Germination Testing After Treated with Different Concentrations of Five Fractions from Column Chromatography

Concentration (ppm)	Percentage of Conidia Germination Inhibition				
	F2[1]	F9	F10	F11	F12
0.5%DMSO	0.00±0.00f	0.00±0.00g	0.00±0.00g	0.00±0.00h	0.00±0.00g
15.62	22.00±1.00e	16.00±1.00f	15.0±1.00f	12.33±1.52g	2.33±1.52g
31.25	50.33±2.08d[3]	32.33±1.52e	29.00±2.00e	23.00±2.64f	16.33±1.52f
62.5	63.33±1.52c	51.33±2.08d[3]	50.67±1.52d[3]	35.33±2.51e	24.33±1.52e
125	89.67±2.30b[4]	66.67±2.51c	60.33±1.52c	49.67±1.15d[3]	30.67±3.21d
250	100.0±0.00a	89.67±1.15b[4]	84.67±1.52b	79.33±5.13c	50.33±2.08c[3]
500	100.0±0.00a	100.00±0.00a	100.00±0.00a[4]	92.33±1.52b[4]	71.33±2.08b
1,000	100.0±0.00a	100.00±0.00a	100.00±0.00a	100.00±0.00a	89.67±3.05a[4]
%CV	1.940	2.428	2.228	4.875	5.898
LSD0.05	2.146	2.332	2.064	4.049	3.539

Note : [1] F = The best fractions from fifteen fraction were tested for their effects on conidia germination inhibition of the severe CA0110 isolate using the slide culture technique

[2] Numbers in the columns indicate PIRG values as mean ± SD of three replicates. The means within the column followed by the same letter(s) are not significantly different at $p \leq 0.05$ using the least significant difference test.

[3] The values defined as MIC50

[4] The values defined as MIC90

3.6 Secondary Metabolite Analysis

To evaluate the potential of the *S. albus* CINv1 strain to produce secondary metabolites, five fractions were further analyzed using liquid chromatography-mass spectrometry (LC-MS). The results showed the presence of various phenolic compounds, with 16 compound groups detected (Table 3.8). The F2 fraction revealed the presence of 11 compounds, including hydroxybenzoic acid, catechin, epicatechin, caffeic acid, rutin, o-coumaric acid, ferulic acid, rosmarinic acid, quercetin, naringenin, and chlorogenic acid. Notably, naringenin was detected at a concentration of 3,648.23 µg/g, significantly higher than the control, which showed 828.94 µg/g. The F9 fraction contained 10 compounds, including hydroxybenzoic acid, epicatechin, caffeic acid, o-coumaric acid, ferulic acid, naringin, quercetin, naringenin, kaempferol, and chlorogenic acid. Hydroxybenzoic acid showed the highest concentration at 4,027.44 µg/g, compared to 1,527.94 µg/g in the control. Additionally, caffeic acid was detected at 1,127.37 µg/g, significantly higher than the control (342.81 µg/g), and chlorogenic acid was found at 1,012.26 µg/g, compared to 445.50 µg/g in the control. The F10 fraction revealed the presence of 13 compounds, including gallic acid, hydroxybenzoic acid, catechin, EGCG, epicatechin, caffeic acid, o-coumaric acid, naringin, rosmarinic acid,

quercetin, naringenin, kaempferol, and chlorogenic acid. Hydroxybenzoic acid showed the highest concentration at 4,576.69 µg/g, while the control showed 1,527.94 µg/g. This was followed by kaempferol, chlorogenic acid, and o-coumaric acid, respectively. The F11 fraction contained 12 compounds, including hydroxybenzoic acid, catechin, epicatechin, caffeic acid, rutin, o-coumaric acid, naringin, ferulic acid, rosmarinic acid, quercetin, naringenin, and kaempferol. Notably, o-coumaric acid was detected at 2,300.64 µg/g, while the control showed 327.96 µg/g. Significant amounts of hydroxybenzoic acid, caffeic acid, naringenin, and chlorogenic acid were also detected. The F12 fraction revealed 11 compounds, including gallic acid, hydroxybenzoic acid, caffeic acid, rutin, o-coumaric acid, ferulic acid, rosmarinic acid, quercetin, naringenin, kaempferol, and chlorogenic acid. Ferulic acid was detected at a high concentration of 10,996.26 µg/g, while the control showed 901.76 µg/g. Both naringenin and hydroxybenzoic acid were found in substantial quantities. It is worth noting that this analytical technique did not detect the presence of phytic acid, p-coumaric acid, or the compounds found in fractions 10 and 12 (gallic acid and naringin) and fraction 9 (naringin), as indicated in Table 3.8.

Table 3.8 Phenolic compounds substrate containing five fraction of column chromatograph detected by LC-MS technique.

Compounds	LOD (ug/ml)	Control	F2[1]	F9	F10	F11	F12
Gallic acid	0.12	217.20±4.62	ND2	ND	179.69±3.03	ND	240.80±3.69
Hydroxybenzoic acid	0.95	1527.94±21.02	532.54±11.08	4027.44±0.64	4576.69±59.84	187.08±1.63	624.84±14.10
Catechin	0.30	42.73±1.58	89.50±23.26	ND	55.37±0.43	109.59±0.11	ND
EGCG	3.01	93.23±3.32	ND	ND	182.70±4.55	ND	ND
Epicatechin	0.32	99.83±1.00	180.77±5.86	328.73±0.89	83.42±1.65	53.98	ND
Caffeic acid	0.06	342.81±1.11	138.44±0.51	1127.37±5.55	150.48±2.49	124.02±1.44	105.95±0.77
Rutin	1.07	241.62±4.43	58.55±0.88	ND	ND	318.25±7.80	200.81±1.30
p-coumaric acid	0.07	75.88±3.30	ND	ND	ND	ND	ND
o-coumaric acid	0.26	327.96±8.49	982.19±36.42	143.40±0.28	195.77±0.01	2300.64±41.41	168.08±2.80
Ferulic acid	0.19	901.76±17.19	348.52±5.44	362.53±0.21	ND	606.87±12.28	10996.26±23.49
Naringin	0.14	ND	ND	9.86±0.18	17.10±0.54	ND	ND
Rosmarinic acid	0.88	285.02±2.23	50.35±1.48	ND	56.81±0.50	37.74±0.00	117.05±2.51
Quercetin	3.78	749.89±35.86	137.04±4.20	203.89±1.28	268.96±6.14	106.67±1.85	237.72±6.29
Naringenin	0.56	828.94±17.51	3648.23±30.47	391.90±7.41	360.22±1.56	665.25±6.21	1877.48±37.74
Kaempferol	0.92	423.95±13.83	ND	42.47±0.08	543.47±17.36	627.03±7.71	200.76±2.16
Chlorogenic acid	3.26	445.50±10.01	109.34±1.99	1012.26±20.06	456.92±4.50	130.08±1.85	339.18±5.49

Note : [1] Mean ± SD of three replicates.

[2] ND = Not detected, LOD= limit of detection, Control = crude extract.

4. Discussion

Strawberry anthracnose is prevalent in Chiang Mai Province, Thailand. In this study, an endophytic strain of *Streptomyces albus* CINv1 was isolated from the medicinal plant *Cinnamomum verum*. Its antagonistic ability was evaluated against several pathogens affecting strawberries, including *Phytophthora cactorum*, which causes crown rot disease, and *Colletotrichum* spp. strain CA0110, responsible for anthracnose. Previous studies have reported that endophytic *S. albus* CINv1 colonizes the roots, petioles, and leaves of strawberry plants, improving growth parameters while reducing disease incidence and severity [Potiyot & Kunasakdakul, 2014, Pupakdeepan et al., 2023]. To our knowledge, there are no studies on the secondary metabolites or compounds of this strain for controlling strawberry anthracnose. This research represents the first instance of selecting culture media and optimal conditions to isolate compounds effective against *Colletotrichum* spp. strain CA0110. The *S. albus* CINv1 strain exhibited the highest antifungal activity in ISP-2 medium at 150 rpm over a period of seven days. Our findings align with those of Rajan and Kannabiran (2014), who optimized culturing conditions for the *Streptomyces* sp. VITBRK2 strain according to the International Streptomyces Project's recommendations for carbon and nitrogen source assimilation. Additionally, Marzoug et al. (2023) demonstrated that *Streptomyces ayarius* S115 cultured in ISP-2 and modified ISP-2 exhibited effective cell growth and antimicrobial activity against pathogenic bacteria, as evidenced by substantial inhibition zones. Similarly, Phongsopitanun et al. (2014) reported that two actinomycete strains, *S. iranensis* D2-1 and *S. sundarbansensis* D2-2, cultured on ISP-2 medium displayed antimicrobial activity against fungi, yeasts, and bacteria. The optimal conditions for antifungal metabolite production by the *S. albus* CINv1 strain were determined to be a pH range of 6-9 and an incubation period of 7-10 days. These results are consistent with findings from Rammali et al. (2022), who noted that *Streptomyces* sp. strains cultured in ISP-2 broth at 150 rpm and 28°C for ten days exhibited significant antimicrobial activity. Furthermore, our results corroborate those of Singh et al. (2017), who optimized the culture conditions for Actinomycetes isolate ACITM-1 to produce bioactive metabolites, identifying an optimal pH of 7.5 and a temperature of 30°C, however, their study differed in culture medium and incubation duration, utilizing starch casein broth for a 4-day period. Five concentrations of the culture filtrate were prepared at 20%, 40%, 60%, 80%, and 100% (v/v). The results indicated that all concentrations of the *S. albus* CINv1 strain culture filtrates effectively inhibited conidia germination of the fungal pathogen. Our findings are consistent with those reported by Li et al. (2011), who found that *Streptomyces globisporus* JK-1 suppressed mycelial growth in three plant pathogenic fungi: *Magnaporthe oryzae, Bipolaris maydis,* and *Cryphonectria parasitica*. Additionally, increasing concentrations of the culture filtrate resulted in decreased conidia germination and mycelial growth among these fungal pathogens.

In the crude extract assay of *S. albus* CINv1, the highest yield was 5.004 g of ethyl acetate, characterized by a dark purple color. Testing the extract's ability to inhibit fungal growth revealed a maximum inhibition rate of 92.25%. This result is consistent with numerous studies on the selection of solvents for culture filtrate extraction. For instance, Ahsan et al. (2017) demonstrated the antifungal activity of *Streptomyces* strain KX852460 against

Rhizoctonia solani AG-3 strain KX852461, the pathogen responsible for tobacco leaf spot disease. They utilized various solvents for culture filtrate extraction, finding ethyl acetate to be particularly effective against *R. solani* AG-3 strain KX852461. Similarly, Abdel-Aziz et al. (2021) reported that ethyl acetate extracts from *Streptomyces carpaticus* EGY-S7 were more effective than fungicides in controlling *Fusarium oxysporum* f. sp. *lycopersici*, which causes tomato wilt. Additionally, Piyaboon (2017) investigated crude extracts from ethyl acetate of *Streptomyces* spp. isolate CH15, showing effectiveness against *Colletotrichum gloeosporioides*, which causes chili anthracnose. Furthermore, these results align with findings from Awla and Rashid (2020), who used ethyl acetate to extract antifungal compounds from *Streptomyces zaomyceticus*. Their study revealed that the compound effectively controls *C. acutatum* and shows potential as a future fungicide.

Many types of antifungal substances are derived from microorganisms in the *Streptomyces* spp. group. This group of bacteria is recognized as a source of bioactive secondary metabolites, contributing approximately 7,600 out of 10,000 compounds produced within the Actinomycetes group and about 23,000 compounds reported from all microorganisms (Berdy, 2005). The bioactive metabolites produced by Streptomyces have vital applications in medicine and agriculture (Demain & Sanchez, 2009, Awla et al., 2017, Nandhini et al., 2018). In this study, five fractions were obtained from a total of fifteen using silica gel column chromatography. These fractions were analyzed using LC/MS, revealing sixteen groups of phenolic compounds that showed potential as promising fungicides for food preservation and antifungal treatment of plant pathogens (Yan et al., 2023). Notably, ferulic acid was identified as a compound exhibiting antibacterial, antioxidant, and anti-inflammatory properties. Other compounds detected, including hydroxybenzoic acid, caffeic acid, o-coumaric acid, naringenin, kaempferol, and chlorogenic acid, have also demonstrated antibacterial, antifungal, anti-inflammatory, and antioxidant activities. In Fraction 2 (F2), naringenin was detected and demonstrated antifungal activity against phytopathogenic fungi. For instance, da Silva Santos et al. (2022) identified antifungal flavanones, including naringenin, which were effective against *Sclerotinia sclerotiorum, Rhizopus stolonifer,* and *Colletotrichum gloeosporioides*. Our results are consistent with those of Duda-Madej et al. (2022), Soberon et al. (2020), and Son et al. (2017), who reported the antifungal and antimicrobial activity of naringenin against human pathogens, including *Candida albicans, Saccharomyces cerevisiae, Aspergillus flavus,* and *Fusarium oxysporum* M42, as well as *Fusarium linii* KB-F1 (Kozlowska et al., 2017) and *Magnaporthe oryzae* (Kozlowska et al., 2017, Dean et al., 2012). Additionally, o-coumaric acid was detected in Fraction 2 (F2) and was reported to exhibit antifungal activity against *F. oxysporum f.* sp. *capsici*, a fungal pathogen (Vega-Rodriguez et al., 2021). In Fraction 9 (F9), hydroxybenzoic acid demonstrated both antibacterial and antifungal activities (Kalinowska et al., 2021). Caffeic acid was also identified, exhibiting antibacterial, antifungal, and antioxidant properties (Ma & Ma, 2015). Chlorogenic acid showed antifungal activity and was effective in controlling the growth of various plant pathogenic fungi (Martinez et al., 2017). In Fraction 10 (F10), kaempferol, chlorogenic acid, and o-coumaric acid were detected, with kaempferol showing antibacterial, antifungal, anti-inflammatory, and antioxidant activities (Ma & Ma, 2015, Kalinowska et al., 2021, da Silva Santos et al., 2022). In Fraction 11 (F11),

o-coumaric acid, hydroxybenzoic acid, caffeic acid, naringenin, and chlorogenic acid were all detected, demonstrating antibacterial, antifungal, anti-inflammatory, and antioxidant activities. Importantly, both kaempferol and naringenin were found in substantial quantities, reinforcing their bioactive properties (da Silva Santos et al., 2022). Predominantly, ferulic acid was identified in Fraction 12 (F12) and is recognized as a promising fungicide for the preservation of food and the antifungal treatment of plant pathogens (Yan et al., 2023). Additionally, ferulic acid has antibacterial, antioxidant, and anti-inflammatory properties. Notably, Fraction 2 (F2) exhibited higher concentrations of naringenin and o-coumaric acid than the other fractions. These compounds have been reported as antifungal agents against various phytopathogenic fungi affecting strawberry plants, including *Colletotrichum gloeosporioides, Sclerotinia sclerotiorum, Rhizopus stolonifer, Aspergillus flavus,* and *Fusarium oxysporum* (da Silva Santos et al., 2022, Duda-Madej et al., 2022, Soberon et al., 2020, Son et al., 2017, Kozlowska et al., 2017, Dean et al., 2012, Vega-Rodriguez et al., 2021). Of particular interest, *Colletotrichum* spp. are responsible for causing strawberry anthracnose disease, which is a primary focus of these studies.

5. Conclusions

The *Streptomyces albus* CINv1 strain was evaluated in five different culture broths, with varying pH levels and incubation periods, to identify the most suitable medium for antifungal activity. The results indicated that the culture filtrate of ISP-2 broth at a pH of 6-9, incubated for 7 to 10 days, significantly inhibited the fungal growth of *Colletotrichum* spp. isolate CA0110. Furthermore, the crude extract obtained from ethyl acetate exhibited high efficacy in inhibiting the growth of fungal pathogens. Fifteen fractions derived from column chromatography were assessed for their antifungal activity against conidia germination (PCGI). The results revealed that five fractions (F2, F9, F10, F11, and F12) demonstrated PCGI ranging from 92.67% to 100%. Specifically, F2 exhibited MIC50 and MIC90 values of 31.25 ppm and 125 ppm, respectively, F9 showed values of 62.5 ppm and 250 ppm, F10 had 62.5 ppm and 500 ppm, F11 presented 125 ppm and 500 ppm, and F12 recorded values of 250 ppm and 1,000 ppm, respectively. All five fractions were analyzed for secondary metabolite secretion using LC/MS, resulting in the detection of sixteen distinct groups of compounds. Notably, F2 contained higher concentrations of naringenin and o-coumaric acid compared to the other fractions. These compounds are recognized as antifungal agents against various phytopathogenic fungi affecting strawberry plants, particularly *Colletotrichum* spp., which are responsible for causing strawberry anthracnose disease, the primary focus of this study.

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