Dibutyl succinate, produced by an insect-pathogenic fungus, *Isaria javanica* pf185, is a metabolite that controls of aphids and a fungal disease, anthracnose

Running title: Dibutyl succinate plays a key role in biocontrol

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Abstract

BACKGROUND

An entomopathogenic fungus, *Isaria javanica* pf185, causes mortality in nymphs of the green peach aphid and inhibits the growth of fungal plant pathogens. However, the metabolites of pf185 involved in these antifungal and aphicidal activities are

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unknown. This study was performed to identify the metabolites with these activities.

RESULTS

An antifungal metabolite was purified by repetitive column chromatography and preparative high performance liquid chromatography. Based on data from mass spectrometry and nuclear magnetic resonance, the active metabolite was identified as dibutyl succinate. The minimum concentration of dibutyl succinate to inhibit germination of conidia of the cause of anthracnose, *Colletotrichum acutatum*, was 4 mg/mL. Dibutyl succinate at 2 μg/mL inhibited mycelial growth. It also had strong aphicidal activity against the nymphs of the green peach aphid, *Myzus persicae,* median lethal concentration (LC50) of 306 mg/L at 24 h exposure, and median lethal time (LT50) of 14.5 h at 388 mg/L exposure.

CONCLUSION

This is the first report indicating that a single metabolite, dibutyl succinate, from the beneficial fungus *I. javanica* has potential for use in integrated pest management to inhibit both insect and fungal plant pathogens.

Keywords: aphid; antifungal activity; dibutyl succinate; dual biocontrol; *Isaria javanica*

1 INTRODUCTION

Interest in biological control agents to control plant diseases and insects has been growing steadily because of concerns over environmental pollution and health issues of chemical pesticides and the development of pest resistance to such treatments [1]. Biopesticides are derived from viable fungi, bacteria, and viruses, and/or their active metabolites [2, 3]. Dual

biocontrol of microbial plant pathogens and insects by entomopathogenic fungi could be cost-effective in sustainable agriculture [3, 4]. For example, pre-treatment with the insect pathogen, *Beauveria bassiana,* suppresses damping-off disease and downy mildew in different crops [5, 6], bacterial blight disease [6, 7], and *Zucchini yellow mosaic virus* [8]. Similarly, *Isaria fumosorosea* (formerly *Paecilomyces fumosoroseus*), an entomopathogen for both whitefly and aphid [9-13] also controls cucumber powdery mildew [14]. In addition, emerging research has revealed additional roles for fungal entomopathogens in providing crop growth promotion [15, 16]. Consequently, such fungi are attractive candidates to study their use in the formulation of products for integrated pest management.

Beauveria bassiana, *Metarhizium anisopliae*, and *I. fumosorosea* are well characterized with respect to their pathogenicity on economically important insect pests, such as whitefly and aphids. They are formulated as biocontrol agents worldwide [17]. *Isaria fumosorosea* is commercialized to control sweet potato whitefly [18]. Recent reviews indicate that parasitism on susceptible insects requires an infection process and the production of toxins [7, 17]. Thus, the modes of action by which entomopathogenic fungi combat plant diseases may include direct inhibition of plant pathogens by antibiosis [7].

Characterizing the active metabolites of entomopathogens would be important in the development of new tools for management of pests in agriculture. The production of several uncharacterized secondary metabolites with antimicrobial activities has been detected in cell-free culture filtrates of entomopathogenic fungi [19, 20]. Characterized bioactive compounds from entomopathogenic fungi include polyketides (PK) and nonribosomal peptides (NRP). The NRP beauvericins, produced by *Beauveria* and *Isaria* spp., are both antifungal and insecticidal [21, 22]. However, other PK toxins are weak insecticides but have eukaryotic cytotoxicity through the inhibition of enzyme activity [22]. For instance, fumosorinone from *I. fumosorosea*, a pyridone alkaloid compound, inhibits protein tyrosine

phosphatase 1B and is being explored for potential use in treatments for type II diabetes [11, 22].

This study focuses on *I. javanica* pf185, isolated from mountain soil in Korea, because it has both antimicrobial activity, against the cause of damping-off, *Pythium ultimum,* and aphicidal activity, against green peach and cotton aphids (*Myzus persicae* and *Aphis gossypii*, respectively) [23, 24]. In this study, we examined the isolate pf185 for its ability to inhibit a fungal plant pathogen which causes anthracnose disease in pepper. We purified and identified a metabolite from the cell-free culture supernatant of *I. javanica*, strain pf185, because of its antifungal activity and then determined whether it was also aphicidal. This study is the first to report microbial production of dibutyl succinate and the antifungal as well as aphicidal activity of this metabolite.

2 MATERIALS AND METHODS

2.1 Fungal strains and growth conditions

Isaria javanica pf185 (KACC93241P) was obtained from the Korean Agricultural Culture Collection (KACC), National Agrobiodiversity Center, Wanju, South Korea. The phytopathogenic fungus, *C. acutatum* (KACC 40689), a causal agent of anthracnose disease in red pepper, was also obtained from KACC. The fungi were grown and maintained on potato dextrose agar (PDA; Difco Inc., Detroit, MI, USA). For preparation of *I. javanica* pf185 conidia, the fungus was cultured at 25 °C on PDA for 7 d and the fungal mass was suspended in sterile water. The suspension was filtered through two layers of sterile cheese clothes to remove hyphal debris.

2.2 Chemicals

The chemicals used in this study were analytical grade purchased from Sigma-Aldrich Co. (St. Louis, MO, USA), unless otherwise indicated. Solvents used in this study were of high-performance liquid chromatography (HPLC) grade and purchased from Fisher Scientific (Pittsburgh, PA).

2.3 *In vitro* antifungal assays

Antifungal activity of *I. javanica* pf185 was tested by co-culturing on PDA with *C. acutatum* by placing inocula of 7 mm^2 mycelial plugs 5 cm apart. These inocula were from 5 d-old PDA cultures. Growth inhibition of the fungal pathogens was determined 7 d after co-incubation.

2.4 Purification of the antifungal compound produced by *I. javanica* pf185

A mycelial plug of *I. javanica* pf185 was transferred into sterile potato dextrose broth (PDB) (Difco) and growth cultivated in a shaking incubator with agitation of 150 rpm at 25 °C for 7 d. The culture (8 L) was centrifuged at 6,000 *g* for 20 min at 4 °C using a Supra 21K centrifuge (Hanil Science Industrial, Korea) to pellet the fungal mass. The supernatant was filtered through Whatman No. 2 filter paper. The cell-free supernatant was acidified with concentrated HCl to pH 2.0, and extracted twice with an equal volume of n-butanol. The soluble organic fraction was concentrated using a rotary evaporator (Büchi, Rheinstetten, Germany) and dried. The mass was 10.0 g. This crude extract was dissolved in methanol and applied to a silica gel column (Kieselgel 60, 70–230 mesh; Merk, Darmstadt, Germany). Materials were eluted with a stepwise gradient of increasing concentration of mobile phases (100:0, 90:10, 80:20, 70:30, 60:40, 0:100, $CHCl_3$/MeOH; ml/ml). The eluted fractions were collected, concentrated by rotary evaporation, and applied to paper disk to test for antifungal activity against the pepper pathogenic fungus *C.*

acutatum on PDA. The fraction (546 mg) that showed antifungal activity was further purified on preparative TLC plates (20 cm × 20 cm, silica gel 60 F_{254}**,** EMD/Millipore, Temecula, CA, USA) with a mobile phase (ethyl acetate:chloroform:formic acid; 7:3:0.5; v/v/v). The TLC plate was cut into equal 2 cm fractions and was scraped off with a sharp knife and collected separately. Each fraction was extracted with methanol and concentrated with a vacuum concentrator. The recovered fractions were dissolved in methanol and tested for antifungal activity against *C. acutatum* on PDA. The extract showing antifungal activity (8 mg) was detected from the TLC band with Rf 0.85 and was further purified by HPLC using a Symmetry C18 column (4.6 × 250 mm, 5 µm, Waters Co., Milford, MI, USA). The mobile phase was H_2O:acetonitrile (60:40; v:v) with a flow rate of 1.5 ml min^{-1}, and peaks were detected at 210 nm by a SPD-10UV-VIS detector (Shimadzu, Japan). All solvents used were of HPLC grade and were purchased from Fisher Scientific (Pittsburgh, PA, USA). The collected HPLC fraction that showed antifungal activity was used to determine the chemical structure as described in section 2.5.

2.5 Identification of the antifungal compound produced by *I. javanica* pf185

The nuclear magnetic resonance (NMR) analysis was performed by dissolving 6 mg of the purified compound in 600 µL of methanol-d_4 in an NMR tube. The NMR spectrum was generated using a 500 MHz NMR spectrometer (VNMRS, Agilent Tech., Santa Clara, CA, USA) equipped with a PFG triple-resonance cold probe. Chemical shifts were calculated using tetramethylsilane. The assignments of ^{1}H and ^{13}C NMR spectra were proven by the analysis of $^{1}H-^{1}H$ correlation spectroscopy (COSY), heteronuclear single quantum correlation (HSQC), and heteronuclear multiple bond correlation (HMBC) experiments by the Gwangju branch of the Korean Basic Science Institute.

2.6 Assessment of inhibition of *C. acutatum*

The effect of the purified compounds from *I. javanica* pf185 on germination of *C. acutatum* spores was tested. Spores were cultured on PDA at 25 °C for 14 d. Sterile water was added using 10 ml/plate and a suspension was generated by rubbing with a sterilized glass rod. The spore suspension was filtered through 4 layers of sterile cheese cloth. Spores were counted on a hemocytometer (Marienfeld Superior, Lauda-Königshofen, Germany) under a light microscope (Leica Microsystems, Wetzlar, Germany) and diluted with sterile PDB to a concentration of 5×10^6 conidia/ml. Fifty microliters of this suspension was added to 2 ml of sterilized PDB containing 0.05% Tween 80. Authentic dibutyl succinate (Sigma-Aldrich) was added at defined final concentrations. Spore germination of *C. acutatum* was determined under a light microscope (Leica Microsystems, Wetzlar, Germany) after 6 h of incubation at 25 °C. At least 100 spores for each treatment were examined. Spores that produced germ tubes longer than their diameter were considered to have germinated. For mycelial growth inhibition, 20 µl of dibutyl succinate diluted to known extents was applied on one side of the PDA plate as described in section 2.3. After 7 d, the extent of inhibition for the growth of *C. acutatum* mycelia was measured. Each experiment was repeated twice in triplicates for each treatment.

2.7 Aphicidal activity.

The aphicidal activity of dibutyl succinate was tested by using defined concentrations diluted in 0.05% Tween 80: 0 mg/L, 194 mg/L (0.84 mM), 388 mg/L (1.68 mM), 970 mg/L (4.21 mM), 1,940 mg/L (8.42 mM), and 3,880 mg/L (16.85 mM). The 0.05% Tween 80 solution was used as the control. The green peach aphid (*M. persicae*) was obtained from Dr. Han of the Agricultural Microbiology Division, National Academy of Agricultural Science, and maintained on tobacco. Tobacco plants (*Nicotiana tabacum*

'Xanthi') were planted in potting soil and grown at 25 °C under a 16 h:8 h light/darkness cycle with a relative humidity of 60%. Leaves of tobacco plants infested with aphids were used in this investigation.

Two days before performing the assay, healthy adult apterous aphids were selected and transferred onto young tobacco leaves, using approximately 15–20 adults per leaf. Tobacco plants were then caged in the plant growth chamber and the insects allowed to reproduce for 2 d. Consequently, one to two day-old second instar nymphs were used for the bioassays.

For the aphid insecticidal experiment, tobacco leaves (3.5 × 3.5 cm) were placed in an insect breeding dish containing sterile cotton wool soaked in distilled water (10 cm × 40 cm, SPL Science, Daejeon, Korea). Twenty second instar nymphs were placed onto each tobacco leaf using a soft brush. Solutions of dibutyl succinate at defined dilutions were directly applied to each nymph using a micropipette. After treatment, the insect breeding dishes were incubated in the plant growth chamber, and mortality was measured at 24, 48, 72, and 96 h after incubation. The total mortality for each treatment was corrected according to Abbott's formula based on the mortality observed in the control treatment [25]. The median lethal time (LT50) and the median lethal concentration (LC50) of dibutyl succinate were calculated using a complementary log–log model [26]. All experiments were performed in 3 replicates with 20 second instar nymphs per treatment.

2.8 Data Analysis

Data were analyzed by ANOVA ($P < 0.05$), using SPSS (version 23, SPSS Inc., Chicago, IL, USA). If the F test showed significant differences, the differences between measurements were further elucidated through Duncan's multiple range test ($P < 0.05$). The LC50 data was analyzed using the Tukey post-test if the variance analysis results were

significant at 95% confidence level, using SPSS. The LT50 was also assessed by using Probit analysis [27] using SPSS.

3 RESULTS

3.1 Dibutyl succinate is a key antifungal metabolite

In dual *in vitro* growth antagonism assays on PDA, *I. javanica* pf185 showed strong growth inhibition against *C. acutatum* (Fig. 1). This pathogenic fungus was used as the target pathogen to purify the antifungal compound from pf185. The antifungal metabolite from *I. javanica* pf185 was extracted from culture filtrates with acidified butanol (Fig. 1A). High-performance liquid chromatography (HPLC) analyses of these extracts from cultures of *I. javanica* pf185 indicated that a product with an elution time of 21.6 min had high antifungal activity (Fig. 1B). Based on ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra (Table 1, Fig. 2, and Fig. 1S) and mass spectrometry (LC-MS) of the LC purified fractions, the antifungal metabolite was identified as dibutyl succinate. This product showed main ion peaks at $[M+H]^{+}$ at *m/z* 231.1 corresponding to a chemical formula $C_{12}H_{22}O_4$ (Fig. 3). The chemical structure and the ^{1}H-^{1}H COSY and HMBC spectra of the antifungal compound are shown in Fig. 3. Additional data for structural analysis for the assignments of ^{1}H and ^{13}C NMR spectra were proven by the analyses of $^{1}H-^{1}H$ correlation spectroscopy (COSY), heteronuclear single quantum correlation (HSQC), and heteronuclear multiple bond correlation (HMBC) (Fig. 1S). Identical results with these data from the fungal cultures were obtained when authentic dibutyl succinate was used in these approaches to deduce chemical structure (Fig. 2S).

3.2 Antifungal activity of dibutyl succinate

Minimal inhibitory concentration (MIC) of authentic dibutyl succinate on conidia of *C. acutatum* was 4 mg/mL, whereas the minimum concentration required for mycelial growth inhibition was 2 μg/mL (Fig. 4). Application of 0.05% Tween 80 did not show any effects on spore germination or mycelial growth of *C. acutatum*.

3.3 Effect of dibutyl succinate on green peach aphid

Dibutyl succinate rapidly killed green peach aphid nymphs in concentration- and time-dependent manners (Table 2). A higher aphicide effect was observed in aphids treated with dibutyl succinate at >970 mg/L within 24 h. The aphicidal activity of dibutyl succinate increased with time after treatment at 194 and 388 mg/L (Table 2). The LC50 was 306 mg/L (95% CL; 54–624 mg/L; $R^2 = 0.99$) in the first 24 h of treatment, and 231 mg/L (95% CL; 8–661 mg/L; $R^2 = 0.98$) by day 2 (Table 3). The LT50 values of dibutyl succinate were 14.5 h ($R^2 = 0.72$) at 388 mg/L, 10.5 h ($R^2 = 0.83$) at 970 mg/L, and 7.5 h ($R^2 = 0.98$) at 1,940 mg/L (Table 3).

4 DISCUSSION

This study indicated that the insect pathogen *I. javanica* pf185 produced dibutyl succinate as a potent inhibitory metabolite for both fungal pathogens and aphids. This finding is similar to results obtained with some of the metabolites produced by biocontrol pseudomonads. These bacteria release metabolites such as hydrogen cyanide and cycliclipopeptides that are active against plant microbial pathogens, insects and nematodes [28-30].

This is the first report of the production of dibutyl succinate by microbes and its role in biocontrol. The biosynthetic pathway of dibutyl succinate and its mode of action in biocontrol are unknown. In industry, dibutyl succinate is produced by direct esterification of

succinic acid with butyl alcohol in the presence of ion-exchange resins or sulfonated coal as a catalyst [31]. However, the biological effects of dibutyl succinate have not been examined extensively. Dibutyl succinate (synonyms; Butanedioic acid, dibutyl ester, and Tabatrex) is reported to act as a repellant for flies, cockroaches, and ants [31, 32]. In contrast, dibutyl succinate is an attractant for Coleoptera (beetles) and Psychodidae (sand flies) [33].

The growth conditions that maximize dibutyl succinate production require investigation. We found that the antifungal activity of *I. javanica* pf185 was influenced by different carbon and nitrogen sources and growth temperature (unpublished data). Our previous study showed that pathogenicity on a whitefly of another *I. javanica* isolate, pf05, was affected by growth medium and temperature [34]. The spectrum of insects targeted by dibutyl succinate is being expanded by current investigations with whitefly and mites. The *in vitro* dual growth assay showed pf185 to have strong inhibitory effect against mycelial growth of *C. acutatum,* but not against *Rhizoctonia solani* (manuscript in preparation). The findings from this paper also show dibutyl succinate to be more effective for mycelial growth inhibition than limiting spore germination. In other studies, we found that volatiles of pf185 promoted tobacco growth. The degree to which dibutyl succinate interacts with other pathogenicity factors, such as the PK and NPK toxins during challenge of the *Isaria* fungus, of its different hosts also awaits investigation. Unlike bacteria and viruses, insect pathogenic fungi directly infect their hosts by penetrating the cuticle [7, 17]. The relationships between the timing and location of dibutyl succinate and toxin production during pathogenesis need to be investigated.

In summary, this study has identified a novel antifungal and aphicidal metabolite, dibutyl succinate, from the entomopathogenic fungi *I. javanica* pf185. This chemical, with its cross kingdom biocidal effects, offers potential in sustainable agriculture for biocontrol of more than one type of plant pests.

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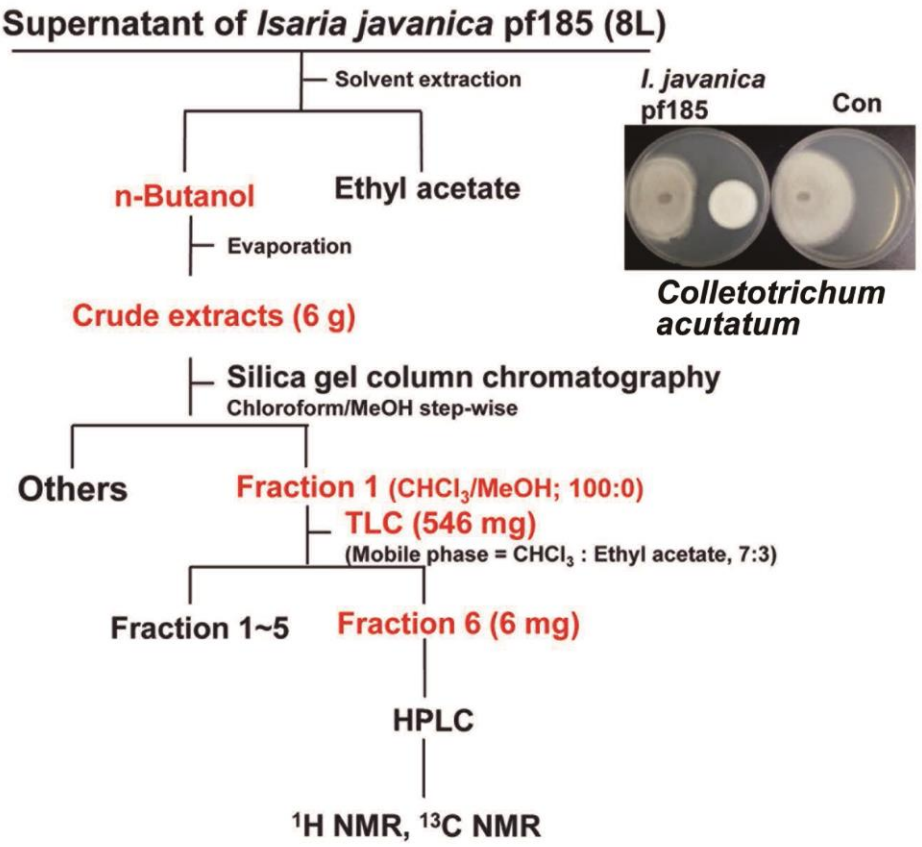
Figure legends

Figure 1. (A) Fractionation procedure for the purification of the antifungal compound from the culture filtrate of *I. javanica* pf185. (B) The spectrum detected at 210 nm from high performance liquid chromatography (HPLC) of the extract from TLC that had antifungal activity.

A.



B.

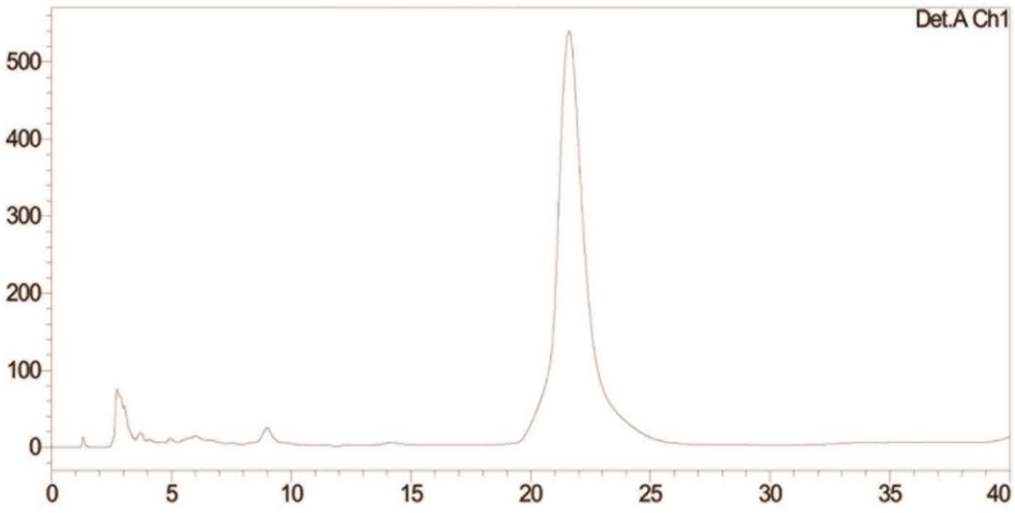


Figure 2. The ^{1}H (A) and ^{13}C (B) nuclear magnetic resonance (NMR) spectra of the purified metabolite, dibutyl succinate, produced by *I. javanica* pf185 and dissolved in $CD_3OD/CDCl_3$ (1:1; v/v) at 500 MHz for ^{1}H and 125 MHz for ^{13}C at room temperature.

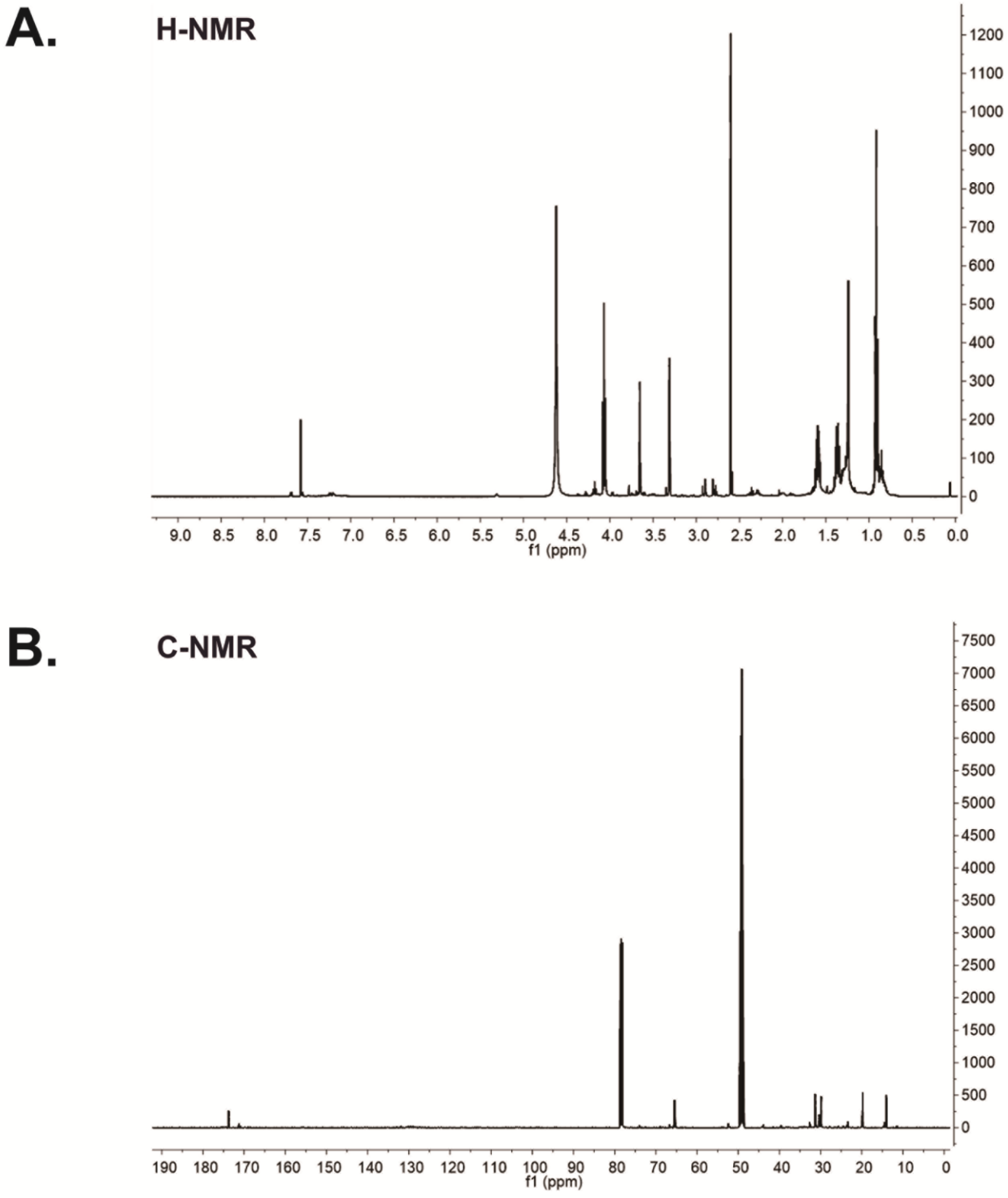


Figure 3. Liquid chromatography mass spectrometry (LC-MS) spectrum of the compound purified through high liquid chromatographic purification showing main ion peaks at $[M+H]^+$ at *m/z* 231.1 in the positive ESI scan. Chemical structure and significant correlation in ^1H-^1H COSY (solid lines), and HMBC (arrows) spectra of the purified metabolite, dibutyl succinate, produced by *I . javanica* pf185.

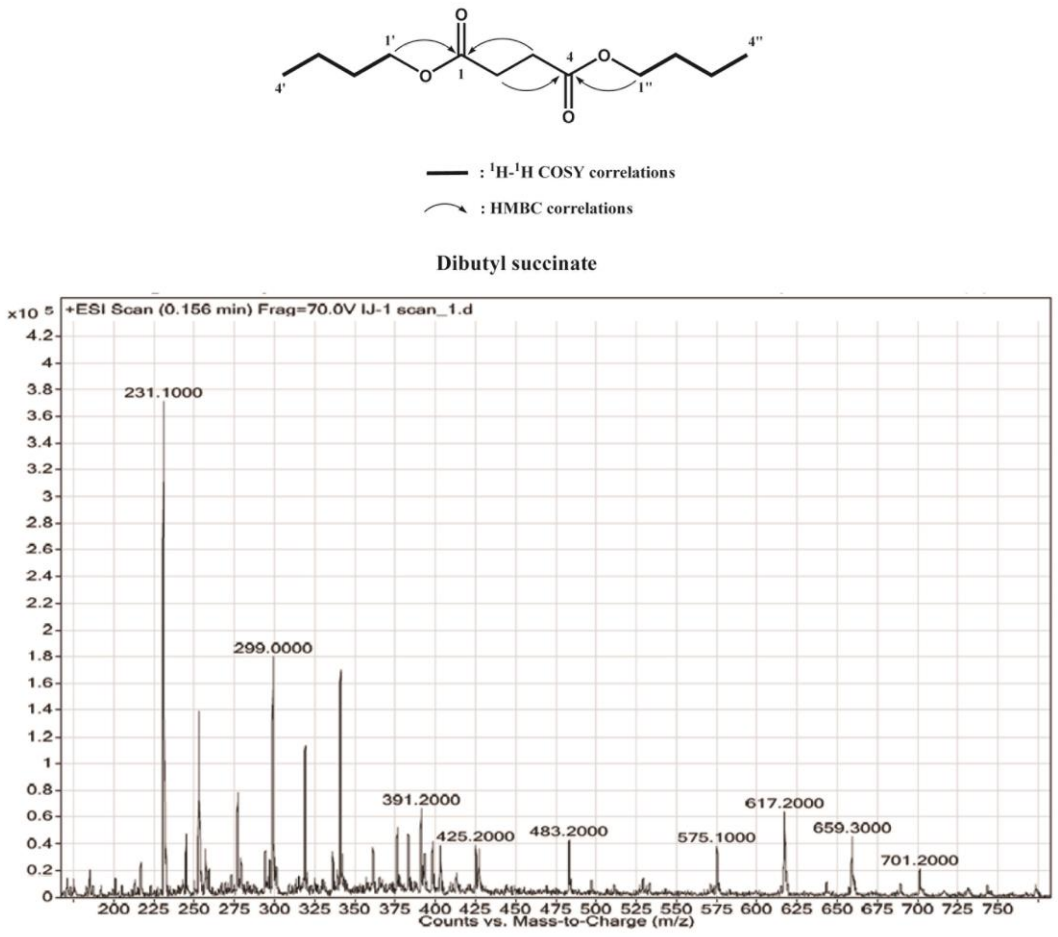


Figure 4. The antifungal activity of dibutyl succinate against *C. acutatum*. (A) Inhibition of mycelial growth of *C. acutatum* by defined concentrations of authentic dibutyl succinate. Images were taken 7 d after treatment. (B) Inhibition of conidial germination after 6 h of incubation. At least 100 spores for each treatment were examined. Spores that produced germ tubes longer than their diameter were considered to have germinated. Each experiment was repeated twice in triplicates per treatment. Means and standard errors for two independent experiments are shown. Different letters indicate statistically significant differences ($P < 0.05$) by Duncan's multiple test.

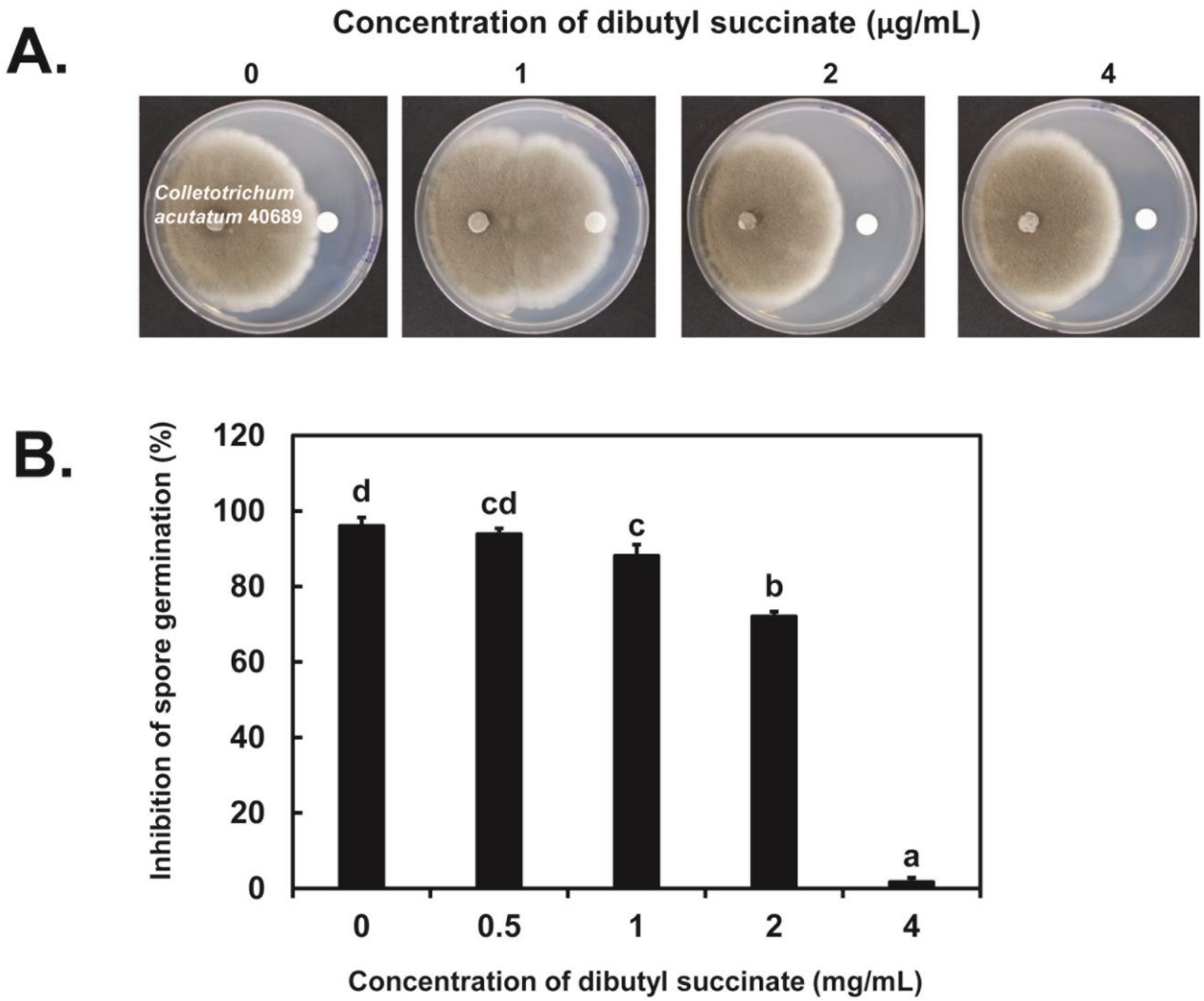


Table 1. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectral data of dibutyl succinate in CD_3OD.

Position	δ_H (*Int.*, *Multi.*, *J* in Hz)	δ_C
1, 4	-	173.8
2, 3	2.61 (4H, s)	29.8
1', 1''	4.07 (4H, d, 6.8)	65.5
2', 2''	1.36 (4H, m)	19.8
3', 3''	1.59 (4H, m)	29.9
4', 4''	0.92 (6H, t, 7.5)	14.1

Table 2. Mortality of nymphs of the aphid *M. persicae* caused by dibutyl succinate, the active metabolite from *I. javanica* pf185.

Concentrations (mg/L)	Aphicidal activity (%) at days (d) after treatments			
	1 d	2 d	3 d	4 d
3,880	100.0±0.0 [a]	-	-	-
1,940	94.4±0.0 [a]	96.2±3.8 [a]	100.0±0.0 [a]	100.0±0.0 [a]
970	85.5±9.1 [a]	86.7±10.5 [a]	87.3±9.6 [ab]	91.5±5.6 [ab]
388	61.9±3.2 [b]	71.6±0.1 [a]	76.7±4.1 [ab]	89.3±2.1 [ab]
194	12.9±3.7 [c]	41.9±5.0 [b]	56.3±9.6 [b]	66.5±11.6 [b]
0 mg/L	0	0	0	5.0+2.1 [c]

Twenty *M. persicae* aphids and three replicates were used for each concentration of authentic dibutyl succinate. Data are means ± standard deviation from three replications/treatment; means with different lowercase letters within a column represent significantly differences between different concentrations at $P < 0.05$ when compared by least significance differences (LSD).

Table 3. Probit analysis of detached-leaf bioassays to estimate median lethal concentrations (LC50) and times (LT50) of the authentic dibutyl succinate.

Concentrations (mg/L) Hours after treatment (HAT)	LC50 (95% confidence limits)	LT50 (95% confidence limits)	Regression line and coefficient	
388 mg/L	-	14.5 h (3.813 – 18.385)	y = 1.43 + 1.62*x	R^2=0.72
970 mg/L	-	10.5 h (6.037 - 14.404)	y = 0.38 + 1.13*x	R^2=0.83
1,940 mg/L	-	7.5	y = 0.81 + 0.32*x	R^2=0.88
24 HAT	306 mg/L (54 – 624)	-	y = 0.25 + 2.04*X	R^2=0.99
48 HAT	231 mg/L (8 – 661)	-	y = 9.68E-3 + 1.89*x	R^2=0.98

Regression line: the equation reflects the relationship between the aphicidal activity and the concentration of dibutyl succinate; LT50, median lethal time value; LC50, median lethal concentration value, 95% fiducial limit (FL), the overall parameter is 95% in this range.