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Pathogenicity of *Beauveria bassiana* (Balsamo) Vuillemin (Hypocreales: Cordycipitaceae) and crude secondary metabolites against *Dendroctonus micans* (Kugelann,1974) (Coleoptera: Curculionidae)

Beauveria bassiana (Balsamo) Vuillemin (Hypocreales: Cordycipitaceae) ve ham sekonder metabolitin *Dendroctonus micans* (Kugelann,1974) (Coleoptera: Curculionidae)'a karşı patojenitesi

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Abstract

Beauveria bassiana (Balsamo-Vuillemin, 1912) (Hypocreales: Clavicipitaceae) is a valuable source of natural bioactive compounds, particularly secondary metabolites. This study, conducted in 2024 at Trabzon Forest Pests and Biological Control Laboratory, aimed to evaluate the insecticidal efficacy of a crude ethyl acetate extract of its secondary metabolites against *Dendroctonus micans* (Kugelann, 1794) (Coleoptera: Curculionidae) larvae and its antimicrobial activities. Pa-4 strain caused 100% mortality of *D. micans* larvae within ten days of 1×10^9 spores/mL, and the LC$_{50}$ value was determined to be 1.63×10^5 spores/mL. The crude secondary metabolites extract concentration was 0.065 g/mL in the biomass and insoluble residues (mycelial extract) and 0.68 g/mL in the supernatant. The LC$_{50}$ value for micelle-extract was 1019 ppm, while for the supernatant extract it was 1382 ppm ($p<0.05$). The antimicrobial activity of the crude secondary metabolites mycelial extract exhibited the largest zone of inhibition against *Enterococcus faecalis* ATCC 51299, with a diameter of 9.73 mm, followed by *Bacillus subtilis* ATCC 6633 (9.28 mm), and *Candida albicans* ATCC 10351 (6.94 mm) ($p<0.001$). This study suggests that the Pa-4 strain and crude secondary metabolites extract could be potential agents for the biological control of *D. micans* and antimicrobial properties.

Keywords: Biological control, crude extract, entomopathogenic fungus, ethyl acetate, forest pest management

Öz

Beauveria bassiana (Balsamo-Vuillemin, 1912) (Hypocreales: Clavicipitaceae), özellikle sekonder metabolitleri ile doğal biyolojik aktif bileşiklerin önemli bir kaynağıdır. Bu çalışma, 2024 yılında Trabzon Orman Zararlıları ile Biyolojik Mücadele Laboratuvarı'nda yapılmış olup, *B. bassiana*'nın Pa-4 suşunun *Dendroctonus micans* (Kugelann, 1794) (Coleoptera: Curculionidae) larvalarına karşı etil asetat ekstraktının insektisidal etkinliği ve antimikrobiyal aktivitelerini değerlendirmeyi amaçlamıştır. Pa-4 suşu, 1×10^9 spor/mL'lik konsantrasyonda, *D. micans* larvalarında on gün içinde %100 mortaliteye yol açtı ve LC$_{50}$ değeri 1.63×10^5 spor/mL olarak belirlendi. Ham sekonder metabolitler ekstraktının konsantrasyonu biyokütle ve çözünmeyen kalıntılarda (miseliyal ekstrat) 0.065 g/mL, süpernatantta ise 0.68 g/mL olarak ölçüldü. Misel ekstraktının LC$_{50}$ değeri 1019 ppm, süpernatant ekstraktının ise 1382 ppm olarak belirlendi ($p<0.05$). Ham sekonder metabolitler miseliyal ekstraktının antimikrobiyal aktivitesi, *Enterococcus faecalis* ATCC 51299'a karşı 9.73 mm çapında en büyük inhibisyon zonunu gösterirken, *Bacillus subtilis* ATCC 6633 (9.28 mm) ve *Candida albicans* ATCC 10351 (6.94 mm) içinde etkili oldu ($p<0.001$). Bu çalışma, Pa-4 suşu ve ham sekonder metabolitler ekstraktının *D. micans*'ın biyolojik mücadelesi ve antimikrobiyal özellikler için potansiyel ajanlar olabileceğini önermektedir.

Anahtar sözcükler: Biyolojik mücadele, entomopatojenik fungus, etil asetat, ham ekstrakt, orman zararlıları ile mücadele

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Introduction

Insects represent over one million species and generally provide significant benefits to humanity. However, particular species can negatively impact food production, health, and overall well-being. Herbivorous insects, in particular, are responsible for 18% of the damage to global agricultural output (Jankielsohn, 2018). Massive quantities of pesticides are used annually to prevent crop damage, and these pesticides accumulate in water resources and soil. The overuse or indiscriminate application of these pesticides poses a significant threat to beneficial insects. It threatens humanity in the long term if pests resist them (Sandhu et al., 2012; Sinha et al., 2016; Baron et al., 2019).

Dendroctonus micans (Kugelann, 1974) (Coleoptera: Curculionidae) is the primary pest of spruce in Eurasia, causing severe tree mortality, especially in the marginal regions of its distribution areas. The main host in Türkiye is eastern spruce, *Picea orientalis* (L.) Peterm. (Pinales: Pinaceae). This species comprises 3.4% (approximately 234,000 hectares) of the common forests and has a natural distribution on the northern slopes of the Eastern Black Sea Mountains (Forestry Statistics, 2021). The insect, which is common in Artvin and Giresun, also progresses to the forests in Trabzon and causes damage of high economic importance (Alkan-Akıncı et al., 2014; Büyükterzi et al., 2022). However, the silvicultural and chemical methods applied have not been sufficient in the control of the pest, so research on biological control factors has increased in recent years (Langor, 1991; Yılmaz et al., 2006; Yaman & Radek, 2008; Sevim et al., 2010; Yaman et al., 2010; Kocacevik et al., 2015; Alkan Akıncı & Grégoire, 2025).

The most effective biological control strategy for pest management is the use of parasitoids or pathogens that target the pest. Insect pathogens, commonly referred to as entomopathogens, encompass a diverse range of organisms, including viruses, bacteria, fungi, protists, and nematodes (Silva et al., 2020; Usta et al., 2025). Entomopathogenic fungi have been widely used to reduce insect populations and decrease crop losses. Over 750 species of fungi have been isolated from insect bodies and classified into 100 different genera (Sinha et al., 2016; Shin et al., 2020). In particular, *Beauveria*, *Cordyceps*, *Isaria*, *Lecanicillium*, *Metarhizium*, and *Nomuraea* are genera of entomopathogenic fungi that have been developed into products for controlling economically crucial agricultural and forest pests effectively (Faria & Wraight, 2007; Islam et al., 2021; Tu et al., 2023).

The mitosporic genera *Metarhizium* and *Beauveria* are two notable genera of entomopathogenic fungi (EPF) recognized for their effectiveness in managing insect pests. These fungi have gained considerable attention due to their potential as eco-friendly alternatives to chemical pesticides (Shahid et al., 2023). *Beauveria* and *Metarhizium* species are primarily soil-dwelling fungi that successfully adapt to a wide range of soil types and ecological conditions. They have been reported from agricultural systems, forested areas, grasslands, and various other terrestrial habitats (Vidhate et al., 2023).

Entomopathogenic fungi generate a range of secondary metabolites (SMs) that exhibit potential biological activities, including antimicrobial and insecticidal properties (Ibrahim et al., 2012). The effects of fungal SMs on insect physiology and biological behaviors have been the subject of considerable research. Volatile compounds may act as either repellents or attractants, whereas nonvolatile compounds often function as toxins or modulators of insect immune responses. In addition, the nonvolatile compounds contribute to the defense against the host immune system by functioning as toxins or demonstrating both deterrent and stimulant effects (Holighaus & Rohlfs, 2016). Furthermore, these components play essential roles in the ability of hosts to compete with opportunistic pathogens for nutritional resources (Gillespie & Claydon, 1989). Several studies have demonstrated that SMs comprise nonribosomal peptides, alkaloids, terpenes, and polyketides, acting as virulence factors and playing essential roles in the successful invasion and colonization of insect hosts (Namasivayam et al., 2014; Mannino et al., 2021; Elhamouly et al., 2022). The production and release of these toxins are closely controlled, with recent progress in genetic manipulation shedding light on the genes responsible for their synthesis and regulation (Staats et al., 2014).

Beauveria species produce a wide range of toxic bioactive compounds, including low-molecular-weight beauvericin, bassianolide, bassianin, tenellin, beauverolide L, and oosporein (Logrieco et al., 2002; Keswani et al., 2019; Litwin et al., 2020). The association of EPF with the pest is complex because *Beauveria* species have evolved a range of strategies, including the production of toxins, to overcome host defenses and establish infection, effectively colonize the midgut, and ultimately precipitate disease and mortality in the insect host (Mwamburi, 2020; Dannon et al., 2020).

Secondary metabolites obtained from *Beauveria bassiana* (Bb) (Balsamo-Vuillemin, 1912) *(*Hypocreales: Clavicipitaceae) have demonstrated significant antimicrobial properties. Research indicates that these metabolites exhibit activities that are antibiotic, antifungal, antiviral, larvicidal, and insecticidal (Daniel et al., 2017; Vivekanandhan et al., 2018; Soesanto et al., 2021; Soth et al., 2022; Camele et al., 2023). The antimicrobial properties of *B. bassiana* SMs have been attributed to various compounds, including oosporein, beauvericin, bassianolide, bassianin, beauveriolide, bassiacridin, and cyclosporine (Camele et al., 2023). Certain SMs exhibit inhibitory effects on a range of pathogens and pests, such as *Fusarium oxysporum (*Schltdl.) (Hypocreales: Nectriaceae), *Aedes aegypti* (L., 1762) (Diptera: Culicidae) larvae, and diamondback moth larvae (Daniel et al., 2017; Soesanto et al., 2021; Soth et al., 2022). Additionally, SMs have been found to impact the immune defenses of insects such as *Eurygaster integriceps* (Puton, 1881) (Hemiptera: Scutelleridae) (Zibaee et al., 2011; Gustianingtyas et al., 2020). Furthermore, research has highlighted the role of Bb in producing bioactive molecules that possess insecticidal properties, making them effective alternatives for pest control (Vivekanandhan et al., 2018; Sinno et al., 2021). The metabolites from Bb have also been investigated for their larvicidal activity against mosquitoes and their potential as biopesticides (Daniel et al., 2017; Vivekanandhan et al., 2018). Moreover, these metabolites have been found to weaken the immune system of host insects, contributing to their antimicrobial effects (Gustianingtyas et al., 2020).

Considering all these studies, the damage caused by *D. micans* is gradually increasing, and the control methods applied are insufficient to stop the effect of the pest. Therefore, the development of various control methods has become necessary. It is noteworthy that secondary metabolites obtained from entomopathogenic fungi have the potential to be utilized in pest control. These metabolites not only exert insecticidal action but also affect the pathophysiology of the host tree and plant (McKinnon et al., 2017). Additionally, some fungal secondary metabolites have potential antimicrobial effects (Glare et al., 2020). As a matter of fact, Nicoletti et al. (2023) emphasize that increasing toxin production through in vitro synthesis may be an effective strategy in reducing the development of insecticide resistance and increasing insecticidal efficacy, especially with a comprehensive understanding of the structural properties of secondary metabolites, their in vitro insecticidal mechanisms, their insecticidal efficacy, and their effects on non-target organisms.

In this direction, this study aims to (i) evaluate the insecticidal activity of the Pa-4 strain of *B. bassiana* and its crude SMs extract using ethyl acetate (EtOAc) against *D. micans* larvae, and (ii) assess the antimicrobial potential of these extracts against selected human pathogenic bacteria and fungi. This study is one of the rare studies in Türkiye that examines both the insecticidal and antimicrobial aspects of *B. bassiana* holistically.

Materials and Methods

Fungal cultures

The Pa-4 strain selected for this study was chosen because it causes significant mortality against *Pristiphora abietina (*Christ, 1791) (Hymenoptera: Tenthredinidae) and different pests, as shown in the research by Biryol et al. (2021a, b, 2022, 2025).

Preparation of conidiospore suspensions

The stock culture was cultured on Sabouraud dextrose agar (SDA+yeast extract, 15 g/L agar) in sterile cryovials containing 10% glycerol for 10 days at 25±2ºC. Aerial conidia were scraped from the surface of the adequately sporulated Petri dishes and collected by adding 10 mL of 0.01% Tween 80 to the cultures on agar plates. The hyphae were removed from the conidial suspensions, and the concentration of spores was determined with a Neubauer hemocytometer. The viability of the conidia was determined by spraying 0.1 mL of 1 × 10^7 spores/mL onto Petri dishes containing 1.5% SDA. After 24 hours, the percentage of germinated spores in each plate was evaluated through microscopic observation at 400 × magnification. Spore germination was considered successful when the germ tube length equaled or exceeded half the length of the original conidium. The viability of isolates was assessed according to Hywell-Jones & Gillespie (1990), and only those with a viability above 90% were used in the experiments.

In vitro dose-response bioassay of Pa-4 strain against *Dendroctonus micans*

The larval beetles were sourced from the Trabzon Forest Pests and Biological Control Laboratory in 2024. The bioassay study was conducted according to Biryol et al. (2024) and was performed on randomly selected healthy last-instar larvae. The bioassay dose range was established as 1 × 10^5 to 10^9 spores/mL and was administered by spraying using a hand-held spray atomizer. A sterilized 0.01% Tween 80 solution was supplied to the control groups. The larvae were placed within square-shaped enclosures between the shells of the test plants in plastic boxes (20 × 20 cm, *n*= 3 × 30). All larval test groups were kept in plastic boxes and reared in a climate-controlled incubator at 20±2ºC and 65±5 relative humidity (RH), following a light:dark photoperiod (L12:D12). The mortality of the beetles in each treatment was observed daily for seven days. Each treatment was replicated three times on separate days.

Extraction of crude secondary metabolites of Pa-4 strain with ethyl acetate

This study utilized a potato dextrose broth (PDB) medium, preparing 150 mL in a 500 mL conical flask for the Pa-4 strain, which was distributed under sterile conditions. The spore mixture of the Pa-4 strain was adjusted to 1 × 10^7 spores/mL, and 1 mL of inoculum was applied to the liquid media (eight replicate flasks) and incubated in a dry shaker at 150 rpm for 14 days at 28ºC (Vizcaíno et al., 2005). After the incubation period, the biomass and insoluble residues were separated from the supernatants by filtration using sterile Whatman No. 1 qualitative filter paper and the micelles were removed. The supernatants were transferred into Falcon tubes and centrifuged at 9000 rpm at +4ºC for 15 minutes, after which the spores were removed. The filtrate underwent equal parts of liquid-liquid extractions with ethyl acetate (EtOAc). The biomass and insoluble residues underwent solid-liquid extractions first with EtOAc and then with methanol (MeOH) (Hamill et al., 1969). Equal volumes of extract and EtOAc (1:1 v/v) were combined in a 250 mL balloon using a magnetic stirrer at room temperature for one hour. The mixture was then transferred into a separation funnel and left for 15 minutes to separate into two phases. The lower phase was discarded, and the upper filtrate was transferred to balloons and evaporated using an evaporator at a water temperature of 42ºC. The remaining crude SM extracts in the balloon were dissolved in 1 mL of dimethyl sulfoxide (DMSO, 10% w/v), transferred into an Eppendorf tube, and stored at -20ºC until needed (Vizcaíno et al., 2005). The weight and yield of the dry extracts were determined for the supernatants and mycelial extract.

Bioassay of crude secondary metabolites of Pa-4 strain against *Dendroctonus micans*

The efficiency of the crude SMs extracts of the tested fungal isolate, Pa-4 strain, was evaluated against *D. micans* larvae. The metabolite derived from the biomass and insoluble residues extract was designated the "mycelial extract" (M). In contrast, the metabolite derived from the culture supernatant was designated the "Supernatant" (SE). The bioassay experimental setup was conducted with the specified of beetles and experimental conditions outlined in the dose-response bioassay experiment (*n*=3 × 30). Various concentrations of the EPF crude SMs extract from the Pa-4 strain, i.e., 500 ppm, 1000 ppm, 2000 ppm, and 5000 ppm, were prepared and sprayed on the rectangular frame opened on the food via spraying. The mortality of the beetles in each treatment was observed daily for ten days. The experiments were performed in three iterations and three sub-repetitions. The solvent DMSO (0.1%) of the metabolites produced was used in the control group.

Disc diffusion methods

The agar diffusion method was utilized to evaluate the antibacterial and antifungal activities of the crude SMs extracts. The strains were examined for their antimicrobial activity against *Enterobacter cloacae* ATCC 2468, *Enterococcus faecalis* ATCC 51299, *Escherichia coli* ATCC 2471, *Klebsiella pneumoniae* ATCC 700603, *Salmonella typhimurium* ATCC 13311, *Serratia marcescens* ATCC 13880, *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633, and *Candida albicans* ATCC 10351. The Mueller–Hinton agar (MHA) and Mueller–Hinton broth (MHB) media were prepared according to the instructions provided by the manufacturer for the cultivation of bacterial strains. Potato dextrose agar (PDA) and potato dextrose broth (PDB) were used for the fungal strains. After autoclaving the media, it was transferred into sterile 90 mm diameter Petri dishes with a thickness of 4±0.5 mm, while the liquid preparation was immediately refrigerated at 4ºC upon cooling. The specified human pathogens were smeared on suitable media, sterile paper discs (6 mm diameter) were impregnated with 50 µl of the crude SMs extract (150 µg/mL) and allowed to dry under sterile conditions before being placed onto the inoculated agar plates. Petri was incubated at 37ºC for 24-48 hours. Kanamycin (for bacteria, 1 mg/mL) an antibiotic and fluconazole (10 µg/disc, for fungi) were used as positive controls, and the pure solvent DMSO was used as a negative control. The antimicrobial activity was recorded as the diameter of the inhibition zone formed around the disc (Bauer et al., 1966; Barry et al., 1970; Sharma et al., 2014).

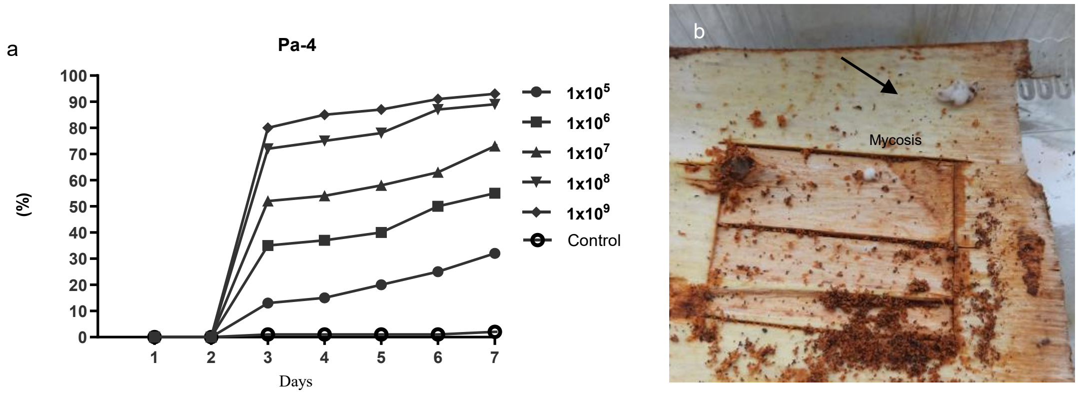
Statistical analysis

The mortality data obtained from the trials were corrected using Abbott's formula (Abbott, 1925), and the analyses were performed on the adjusted values (Abbott, 1925). However, natural mortality observed in the control group is presented in the Figure1a as recorded, without correction. The mortality data obtained from the laboratory experiments were subjected to probit analysis to determine the lethal concentration (LC_{50}) of the Pa-4 strain. The Kaplan-Meier method was used to construct cumulative survival curves. The log-rank (Mantel-Cox) test was employed to evaluate the discrepancy in survival rates between each concentration. Antimicrobial activity was evaluated using a disk diffusion assay, and data were analyzed with One-way ANOVA for each microorganism, followed by Duncan's multiple range test to distinguish means at a significance level of $p<0.05$. The data were analyzed via SPSS 22.0 software (SPSS Inc., Chicago, IL), and the graphs were plotted via GraphPad Prism software (version 6.0, GraphPad Software, USA).

Results

The efficacy of different doses was evaluated on the local isolate Pa-4 strain against *D. micans*. These *B. bassiana* have been demonstrated to be effective against harmful beetles. In the dose-response bioassay, the Pa-4 strain caused 100% mortality of *D. micans* larvae within 10 days after applying the conidial concentration of 1×10^9 spores/mL (Figure 1a). For the other concentrations used (1×10^5, 1×10^6, 1×10^7, and 1×10^8 spores/mL), beetle pathogenicity ranged between 45%, 68.7%, 86.8% and 93.7%, respectively ($p<0.05$) (Figure 1a). During and after the experiment, mortality in the insects was attributed to the Pa-4 strain as confirmed by the observed mycosis in the deceased individuals (Figure 1b).

The Pa-4 strain caused a significant increase in mortality rates in *D. micans* larvae compared with those in the control group and the increase was due to fungal concentration ($p<0.05$). The LC_{50} value was determined to be 1.63×10^5 spores/mL (Table 1).



Beauveria bassiana (Pa-4)

Figure 1. Dose-response effects of Pa-4 strain (*Beauveria bassiana*) on *Dendroctonus micans* larvae. (a) Mortality rates (%) at different concentrations (mean±SD of three replicates). (b) Cadaver mycosis observed in infected larvae.

Table 1. LC_{50} value of Pa-4 strain against *Dendroctonus micans* larvae based on probit analysis

Beetle	LC_{50} (spores/mL) (*FL* 95%)	Intercept	(Slope±*SE*)	X^2	*df*	*N*
Dendroctonus micans	$1.63×10^5$ ($7.08×10^4$-$3.07×10^5$)	-3.203	0.62±0.65	1.954	3	270

*Abbreviations: *df*, degrees of freedom; *FL*, fiducial limit; *SE*, standard error; X^2, chi-square. N: Total number of larvae.

The yield of crude SMs extract was 0.065 g/mL for the mycelial extract (M) and 0.68 g/mL for the culture supernatant extract (SE), calculated as dry weight per mL of DMSO. Different concentrations from 500 ppm to 5000 ppm were prepared from the crude SMs extract and tested on the larvae of *D. micans* (Figure 2). The mortality rate of larvae exposed to the lowest concentration (500 ppm) of M-extract or SE-extract was 35%. In contrast, larvae exposed to the highest concentration of M-extract (5000 ppm) were 90%. However, SE-extract had a mortality rate of 75%. In addition, at a concentration of 2000 ppm, M-extract and SE-extract mortality rates were observed as 65% and 55%, respectively (Figure 2).

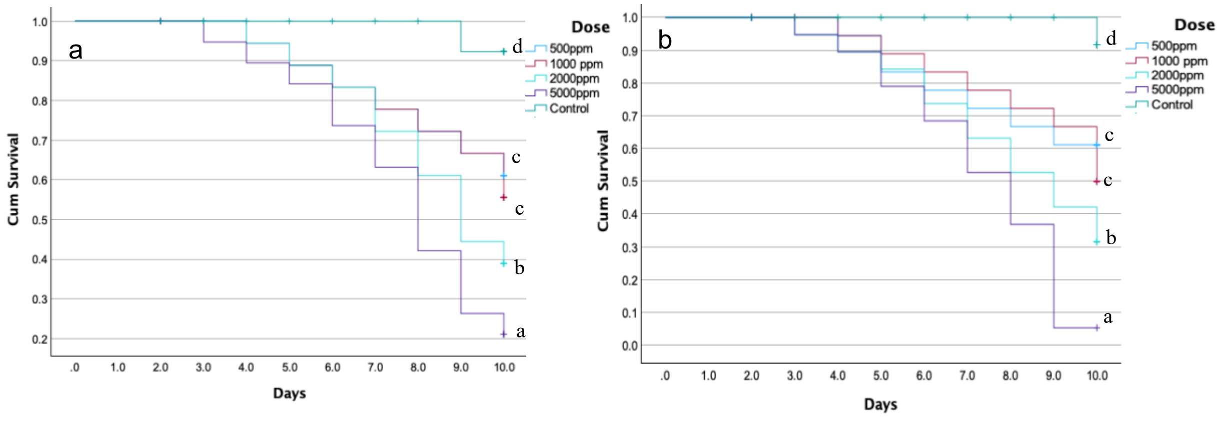


Figure 2. Kaplan-Meier survival diagram for supernatant extract (A) and mycelial extract (B) of *Dendroctonus micans* exposed to crude SMs extract at four different concentrations. The lowercase letters indicated the important differences among concentrations.

Figure 2a illustrates the Kaplan–Meier survival curves for the control group compared to the SE-extract infected group. The LC_{50} value of the SE-extract for the larval stage was determined to be 1382 ppm (Table 3). Figure 2b presents the survival curves for the larvae in both the control group and the group infected with M-extract. The LC_{50} value of M-extract for the larval stage was determined to be 1019 ppm (Table 3). As shown in Figures 2a and 2b, both M- and SE-extracts reduced survival in a concentration-dependent manner, with the highest concentration (5000 ppm) showing the lowest survival rates in larvae. This result shows that intracellular compounds have a more substantial toxic effect on the larvae.

Table 2. Results of log-rank (Mantel-Cox) analysis of *Dendroctonus micans* larvae exposed to different concentrations of crude secondary metabolite extracts

Crude secondary metabolite	Concentrations (ppm)	500		1000		2000		5000		Control	
		X^2	*p*	X^2	*p*	X^2	*p*	X^2	*p*	X^2	*p*
M	500			0.165	0.685	2.348	0.125	8.843	0.003	4.826	0.028
	1000	0.165	0.685			1.665	0.197	10.500	0.001	6.752	0.009
	2000	2.348	0.125	1.665	0.197			3.235	0.072	12.945	0.000
	5000	8.843	0.003	10.500	0.000	3.235	0.072			26.734	0.000
	Control	4.826	0.028	6.752	0.009	12.945	0.000	26.734	0.000		
SE	500			0.070	0.792	1.410	0.235	5.427	0.020	4.545	0.033
	1000	0.070	0.792			0.940	0.332	4.673	0.031	5.547	0.019
	2000	1.410	0.235	0.940	0.332			1.525	0.217	9.988	0.002
	5000	5.427	0.020	4.673	0.031	1.525	0.217			17.208	0.000
	Control	4.545	0.033	5.547	0.019	9.988	0.002	17.208	0.000		

*X^2: Chi-square, *p*: significance, M: Micelle extracts, ES: Supernatant extracts.

According to the log-rank test results, all groups of *D. micans* larvae exposed to fungal SMs significantly differed from the control group (Table 2). Table 2 demonstrated that crude SM extracts reduced larval survival in a dose-dependent manner. For the M-extract, larvae treated with 2000 ppm (X^2=12.945) and 5000 ppm (X^2=26.734) showed the most substantial differences compared with the control, while 500 ppm (X^2=4.826) and 1000 ppm (X^2=6.752) produced moderate effects. In contrast, the SE-extract was less effective; only the highest concentration (5000 ppm, X^2=17.208) had a substantial impact, whereas 2000 ppm (X^2=9.988), 1000 ppm (X^2=5.547), and 500 ppm (X^2=4.545) showed weaker effects. Overall, the M-extract was more effective than the SE-extract (X^2=30.21 vs. X^2=19.07, df=4, p<0.01).

Table 3. Dose-response mortality estimated by probit analysis of crude secondary metabolite extracts against *Dendroctonus micans* larvae

Crude SMs	LC$_{50}$ (ppm)	95%*CI*		Intercept	(Slope±SE)	LC$_{90}$ (ppm)	X^2	*df*	*N*
		Lower Bound	Upper Bound						
SE	1382	1033	1824	-3.403	1.08±0.178	20.970	1.567	2	1350
M	1019	820	1228	-4.919	1.58±0.196	6195	2.984	2	1350

* Abbreviations: *df*: degrees of freedom; *CI*: confidence interval; *SE*: standard error; X^2: chi-square test, M: mycelial extract, SE: supernatant extracts, N: Total number of larvae.

The results of the disk diffusion experiment indicate that the SE-extract created the most effective inhibition zone against *E. faecalis* ATCC 51299 (Table 4) (F (3,8) = 632.13, *p*<0.001). However, the crude SMs extracts showed no statistically significant inhibitory effect against *B. subtilis* ATCC 6633, *S. marcescens* ATCC 13880 and *C. albicans* ATCC 10351 (*p*> 0.05), as indicated by the absence of inhibition zones.

Similarly, limited effects were observed for *E. coli* ATCC 2471 and *S. typhimurium* ATCC 13311 (*p*>0.001). Overall, according to the disk diffusion results, the secondary metabolite extracts produced clear inhibition zones, particularly against the Gram-positive bacteria *E. faecalis* (9.73 mm) and *B. subtilis* (9.28 mm), whereas the inhibition zones observed in Gram-negative species were smaller, indicating more limited activity. These findings suggest that the metabolites of the Pa-4 strain exhibit stronger target selectivity towards Gram-positive bacteria. In addition, the M-extract resulted in a zone of inhibition in all species except for *S. marcescens* ATCC 13880 (F (3,8) = 1500.16, *p*<0.001). The M-extract had the most significant zone of inhibition against *E. faecalis* ATCC 51299, with a diameter of 9.73 mm, followed by *B. subtilis* ATCC 6633, with a diameter of 9.28 mm (Table 4). The findings of this study demonstrate that the antimicrobial effect of M-extract is particularly remarkable. Furthermore, while no inhibition zone was observed for *C. albicans* ATCC 10351 in the SE-extract, the M-extract fungal metabolite formed a zone of 6.94 mm (Table 4) (F (3,8) =399.42, *p*<0.001).

Table 4. Antimicrobial activity of crude secondary metabolite extracts against human pathogenic bacteria and fungi (inhibition zone mm±SD)

Bacteria/Fungi	ES	M	DMSO	Control
Escherichia coli ATCC 2471	7.86±2.09^{c}	8.17±0.68^{b}	1.47±0.82^{d}	20.70±3.88^{a}
Klebsiella pneumoniae ATCC 700603	7.05±0.91^{c}	7.75±0.76^{b}	1.44±0.51^{d}	25.93±1.10^{a}
Enterobacter cloacae ATCC 2468	6.98±0.34^{b}	6.95±0.30^{c}	NZ	23.69±0.38^{a}
Bacillus subtilis ATCC 6633	NZ	9.28±0.78^{b}	NZ	30.62±0.61^{a}
Serretia marcescens ATCC 13880	NZ	NZ	NZ	18.74±0.83^{a}
Staphylococcus aureus ATCC 25923	6.14±0.18^{c}	6.52±0.42^{b}	NZ	19.64±1.02^{a}
Salmonella typhimurium ATCC 13311	7.88±2.07^{c}	8.62±0.94^{b}	1.07±0.11^{d}	23.33±0.95^{a}
Enterococcus faecalis ATCC 51299	8.31±0.18^{c}	9.73±0.53^{b}	NZ	21.54±1.08^{a}
*Candida albicans*ATCC10351	NZ	6.94±0.87^{b}	NZ	13.82±0.74^{a}

*NZ: Non-Zone, M: Micelle extracts, ES: Supernatant extracts. Different superscript letters within the same row indicate statistically significant differences ($p<0.05$, one-way ANOVA followed by Duncan's multiple range test).

Discussion

This investigation provided evidence that not only the Pa-4 strain itself but also its crude secondary metabolite extracts possess pronounced insecticidal efficacy and display antimicrobial potential. The findings demonstrate that, in addition to using *B. bassiana* as a biological control agent, secondary metabolites may be viewed as potential agents for biotechnological and pharmaceutical applications due to their antimicrobial properties. Such dual functionality highlights the importance of further characterizing these metabolites and their potential modes of action. Additionally, this study may serve as a foundation for future research to identify various SMs. In this context, the broad range of actions provided by the Pa-4 strain indicates that it has potential for biological control and diverse applications in biotechnology and pharmaceutical development.

Beauveria bassiana, a naturally occurring soil-borne fungus, is widely recognized for its significant entomopathogenic properties, attracting considerable attention in the scientific community. It has a remarkable capacity to infect a wide range of insect species, thereby reducing their damage in agricultural and forest areas. Consequently, it is highly effective for pest control (Wahengbam et al., 2021). The Pa-4 strain employed in this study is notable for its high virulence and efficacy against a broad spectrum of forest and agricultural pests (Biryol et al., 2021a, b, 2022, 2025). Furthermore, the Bb strains have been demonstrated to influence forest pests on a global scale (Lutczyk & Swieczynska, 1984; Hallet et al., 1994; Kreutz et al., 2004; Sevim et al., 2009; Kocacevik et al., 2015; Davis et al., 2018; Dembilio et al., 2018). In this study, the impact on *D. micans* was also investigated. The results demonstrated that the Pa-4 strain caused 100% mortality in *D. micans* within ten days of administration of a conidial concentration of 1 × 10^9 spores/mL. Sevim et al. (2010) reported that KTU-53 (*B. bassiana*) was lethal to larvae and adults of *D. micans*, which are fungi isolated from various pests and soils. Therefore, the impact of the Pa-4 strain derived from a different spruce pest (*P. abietina*) on *D. micans*, a bark beetle inhabiting the same environment, was again confirmed.

These findings strongly show that the Pa-4 strain is not only effective in controlled laboratory conditions but also has excellent potential to be applied in the management programs of forest pests, as revealed in the study conducted by Biryol et al. (2025). The high mortality observed on *D. micans* suggests that Pa-4 may be a reliable alternative to conventional chemical insecticides. Previous studies (Kreutz et al., 2004; Sevim et al., 2010) have also reported similar levels of efficacy with different *B. bassiana* isolates and supported the ability of entomopathogenic fungi to offer a consistent and sustainable control strategy in various geographical regions. These findings suggest that the dose-dependent pathogenicity of the Pa-4 strain is consistent with other *B. bassiana* isolates reported in literature (Kocacevik et al., 2015; Fora et al., 2020; Kim et al., 2022; Yanar et al., 2023). In addition, integrating Pa-4 applications with other biological control factors, such as predator *R. grandis*, can improve the effectiveness of integrated pest management

(IPM) strategies. In fact, a study conducted by Biryol et al. (2025) determined that Pa4-OD (prototype bioinsecticides) exhibits low pathogenicity on the predator even at high doses. Overall, the development of Pa-4 as a commercial biopesticide offers an environmentally friendly, sustainable and effective alternative to the long-term fight against forest pests. In addition, not only the fungus itself, but also the secondary metabolites produced by the Pa-4 strain are essential in terms of biological control potential. The effects, mechanisms and ecological roles of the metabolites produced by strains with high virulence have not yet been fully elucidated, and this continues to exist as an important topic that continues to be researched in the scientific world.

Building on this, recent studies have increasingly focused on natural products derived from plants or microorganisms, including secondary metabolites, as effective alternatives to chemical insecticides in integrated IPM programs (Schrader et al., 2010; Singh & Gaikwad, 2020; Silva et al., 2021). In particular, SMs derived from plants, bacteria and fungi have been widely reported as effective agents for pest management (Balumahendhiran et al., 2019; Logeswaran et al., 2019; Vivekanandhan et al., 2022a, b; Kannan et al., 2023; Perumal et al., 2023a, b, c). Notably, secondary metabolites produced by Bb exhibit significant antifungal and antibacterial activities against diverse pathogenic microorganisms, highlighting their potential role in biological control (Parine et al., 2010; Sahab, 2012; Lozano-Tovar et al., 2013). Thus, Bb represents a promising source of bioactive compounds with proven efficacy against both plant pathogens and insect pests. In this regard, the present study specifically examined the insecticidal and antimicrobial properties of the secondary metabolite derived from the Pa-4 strain.

According to Abdullah (2019), the toxic effects of two species of leafhopper, *Spodoptera littoralis* (Boisduval,1833) (Lepidoptera: Noctuidae) and *Aphis gossypii* Glover, 1877 (Homoptera: Aphididae), were studied by extracting SMs from the extracellular culture media of two fungal species, *B. bassiana* and *Trichoderma harzianum* (Rifai) (Hypocreales: Hypocreaceae). The study revealed that the SMs derived from *B. bassiana* exhibited greater efficacy than those from *T. harzianum*. In this study, the LC_{50} value for *S. littoralis*, applied with a metabolite of *B. bassiana* was reported to be 575 ppm while the LC_{50} value was 226 ppm for *A. gossypii*. In our study, the LC_{50} value of the SE-extract for the larval stage was determined to be 1382 ppm. These differences in LC_{50} values likely reflect interspecific variations in susceptibility, feeding behavior, and cuticle permeability among different insect orders. According to Gurulingappa et al. (2011), metabolites derived from the culture filtrates and mycelial extracts of *B. bassiana* exhibited a significant insecticidal effect, reducing *A. gossypii* survival by 25-97.5% and limiting aphid colonization on treated leaf discs. In our study, the mortality rates of the Bb mycelial extracts were determined to be between 35% and 90%. It supports that SMs have a broad-spectrum activity on different groups of insects. In line with these findings, McGee (2002) reported that methanol extracts of four fungal isolates reduced larval growth rates of *Helicoverpa armigera* (Hübner, 1808) (Lepidoptera: Noctuidae) and *Helicoverpa punctigera* (Wallengren, 1860) (Lepidoptera: Noctuidae) families, and that crude extracts of Bb had a significant mortality effect when introduced to insects through the food of *S. littoralis* larvae (Quesada-Moraga et al., 2006).The impact of mortality and larval instar alterations on nutritional intake according to our intershell feeding methodology is consistent with the abovementioned research. As the dosage administered has increased, mortality has increased, particularly among crude M-extract SMs. Notably, the dose-dependent increase in mortality, particularly in crude M-extracts, clearly highlights the potential of secondary metabolites as promising bioinsecticidal agents. Similarly, Bakr et al. (2025) demonstrated that ethyl-acetate-derived fractions from Bb including the first reported detection of the highly toxic compound 2,4-di-tert-butylphenol in the mycelia caused significant mortality in *Spodoptera littoralis* larvae, thereby reinforcing the pivotal role of crude secondary metabolites as promising agents in integrated pest management.

Antimicrobial resistance (AMR) has emerged as one of the most formidable and multifaceted threats to global public health, particularly in relation to the effective management of infectious diseases. As evidenced by numerous recent studies (Dadgostar, 2019; Tsoupras et al., 2022; Wang et al., 2022), the escalating resistance of pathogenic microorganisms to widely employed antimicrobial agents is undermining the effectiveness of current therapeutic options, thereby exacerbating the global burden of infectious diseases. This growing challenge not only increases the morbidity and mortality associated with infections but also leads to a significant rise in healthcare expenditures, necessitating urgent and coordinated global efforts to address the crisis. For this reason, fungal SMs are currently being investigated as potential new drug candidates. Numerous studies have confirmed the bioactivity of entomopathogenic fungi (EPFs) (Schmidt et al., 2003; Madla et al., 2005). In particular, Shin et al. (2013a, b, 2014) isolated and identified 342 EPF isolates, comprising 28 species across 20 genera, from diverse habitats throughout Korea, and highlighted that several of these isolates possess antibacterial, antioxidant, and anticancer properties. In a further study by Singh et al. (2017), secondary metabolites of fungi were found to have multifunctional lower molecular weight properties, which could be beneficial in several areas, including medicine, biopharmaceuticals, biopesticides, plant growth-promoting agents, and other molecules/drugs that are important for animal health. Lee et al. (2005) reported anti-*Bacillus* or anti-*Staphylococcus* activity in 47 fungal isolates. This study revealed that secondary metabolites of Bb have antimicrobial effects on some gram-positive and gram-negative bacteria and *C. albicans*, except for *S. marcescens* ATCC 13880.

The study conducted by Aksoy et al. (2023) demonstrated the antimicrobial efficacy of *B. bassiana* extract against *K. pneumoniae*, *C. albicans*, *S. aureus* and *E. coli*, with inhibition zones of 22, 23, 25, and 27 mm, respectively, at a concentration of 150 µg/mL. In our study, the inhibition zones of *K. pneumoniae*, *C. albicans*, *S. aureus* and *E. coli* were 7.75, 6.94, 6.52 and 8.17 mm in diameter, respectively. This discrepancy between the inhibition zone diameters may be attributed to factors such as strain variability, the degree of extract purification, or differences in culture and experimental conditions. Nevertheless, despite these differences in magnitude, the observed zones of inhibition in our study confirm that Pa-4 derived metabolites retain broad-spectrum antimicrobial potential, consistent with previous reports. According to Parine et al. (2010), the antibacterial activity of the crude *B. bassiana* extract can be attributed to the presence of several bioactive compounds in its composition. Among these compounds, bassianolide, oosporein, oxalic acid, beauvericin, bassianin, and tenellin are particularly significant. Notably, the compound beauvericin is widely recognized as an insecticidal virulence factor, demonstrating potent antimicrobial effects against a broad spectrum of pathogenic bacteria (Patocka, 2016; Yin et al., 2022).

Recent studies further confirm this potential. For example, Ávila-Hernández et al. (2020) reported that oosporein inhibited fungal growth, while Camele et al. (2023) showed that both diffusible and volatile metabolites of Bb display potent antimicrobial activity. Similarly, Deb et al. (2023) highlighted significant inhibition of *Rhizoctonia solani*, supporting its use against plant pathogens. Pedrini (2022) provided molecular insights into NRPS/PKS gene clusters responsible for metabolite biosynthesis, explaining the diversity and bioactivity of *B. bassiana* compounds. Finally, Bakr et al. (2025) showed that different metabolite fractions (mycelia and filtrate) displayed significant insecticidal activity, with GC/MS confirming the presence of active compounds. Collectively, these findings support the growing recognition of entomopathogenic fungi as an important source of bioactive secondary metabolites with potential applications extending beyond their entomopathogenic role. By positioning *B. bassiana* within this broader scientific context, our study contributes to the growing body of evidence that fungal metabolites may serve as valuable leads in the search for novel antimicrobial and pest management solutions.

Conclusion

The literature indicates that such natural substances have the potential to provide both alternatives to pesticides and sustainable solutions in agricultural applications. Consequently, the role of compounds derived from microorganisms, such as the Pa-4 strain, represents a significant advancement in developing novel pest management strategies. Moreover, this study experimentally confirmed that crude culture filtrates of *B. bassiana* Pa-4 exhibit measurable antimicrobial activity against selected human pathogens. These findings highlight the dual potential of Pa-4 as both a biocontrol agent and a candidate source for antimicrobial drug development. In line with the growing global interest in alternative pest control methods, including those for agricultural, forestry, and human health-related pathogens, the development of entomopathogenic fungal-based products has gained significant momentum. Although the crude extracts showed promising bioactivity, this study did not identify specific active compounds. Future studies should focus on purification, structure elucidation, and mode-of-action analysis of the bioactive metabolites. Additionally, in vivo efficacy and non-target effects should be evaluated to support field applicability. To our knowledge, this is the first report demonstrating both insecticidal and antimicrobial activities of the Pa-4 strain against *D. micans* and a panel of human pathogens.

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