

## Antimicrobial Activity of Fungal Endophytes from *Vaccinium dunalianum* var. *urophyllum* (Aktiviti Antimikrob Kulat Endofit daripada *Vaccinium dunalianum* var. *urophyllum*)

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### ABSTRACT

Fungi associated with *Vaccinium* species play important roles in plant growth and disease control, especially in the final blueberry production. *Vaccinium dunalianum* var. *urophyllum* (Ericaceae) is a well-known medicinal plant in Southern China used to treat inflammation and microbial infections. The endophytic fungi from these plants are therefore anticipated as potential new sources of antimicrobials. In this report, the inhibitory effects of endophytes against clinical bacteria and yeast were comprehensively screened and 11 isolates indicated high bioactivity by the agar diffusion method. The corresponding crude extracts of these fungi under submerged fermentation also demonstrated distinct differences and *n*-butyl alcohol displayed the lowest extraction efficiency among the extracts. The ethyl acetate and dichloromethane extracts of filtrates from the *Colletotrichum* sp. VD001, *Epicoccum nigrum* VD021 and *E. nigrum* VD022 strains displayed good properties against pathogenic microorganisms according to disc diffusion assays and minimal inhibitory concentration (MIC). This study is the first indicating that cultivable endophytic fungi associated with blueberry plants produce potential compounds against clinical pathogens.

**Keywords:** Antimicrobial activity; fungal endophytes; minimum inhibitory concentration; *Vaccinium* species

### ABSTRAK

Kulat yang dikaitkan dengan spesies *Vaccinium* memainkan peranan penting dalam pertumbuhan tumbuhan dan kawalan penyakit, terutamanya dalam pengeluaran beri biru akhir. *Vaccinium dunalianum* var. *urophyllum* (Ericaceae) ialah tumbuhan ubatan yang terkenal di Selatan China yang digunakan untuk merawat jangkitan keradangan dan mikrob. Oleh itu, kulat endofit daripada tumbuhan ini dijangka sebagai sumber baru antimikrob yang berpotensi. Dalam laporan ini, kesan perencatan endofit terhadap bakteria klinikal dan suatu ragi telah ditayangkan secara komprehensif dan 11 pencilan menunjukkan bioaktiviti tinggi melalui kaedah penyebaran agar. Ekstrak mentah yang sepadan dengan kulat ini di bawah penaipaan terendam juga menunjukkan perbezaan yang ketara serta *n*-butil alkohol menunjukkan kecekapan pengekstrakan terendah dalam kalangan ekstrak. Ekstrak etil asetat dan diklorometana filtrat daripada *Colletotrichum* sp. VD001, *Epicoccum nigrum* VD021 dan *E. nigrum* VD022 menunjukkan sifat-sifat yang baik terhadap mikroorganisma patogen berdasarkan ujian penyebaran cakera dan kepekatan perencatan minimum (MIC). Kajian ini adalah kajian yang pertama menunjukkan bahawa kulat endofit yang boleh dikaitkan dengan tumbuhan beri biru menghasilkan sebatian yang berpotensi terhadap patogen klinikal.

**Kata kunci:** Aktiviti antimikrob; kepekatan perencatan minimum; kulat endofit; spesies *Vaccinium*

### INTRODUCTION

Many bioactive metabolites from fungal endophytes isolated from healthy plants have displayed important pharmaceutical properties (Ali & Olivo 2002; Faeth & Fagan 2002; Ma et al. 2004; Pusztahelyi et al. 2015). Analysis of antimicrobial activity against pathogens can be employed as a simple and efficient screening tool and could be used to isolate potentially useful strains and to further identify their bioactive agents (Dos Santos et al. 2015; Kusari et al. 2013; Qadri et al. 2014; Zhang et al. 2012). Currently, countless fungal endophytes with antimicrobial activities have been isolated from various plants and many bioactive compounds have been discovered from these strains, including terpenoids, alkaloids, phenylpropanoids, aliphatic compounds, polyketides and peptides (Mousa &

Raizada 2013). Paclitaxel (Taxol®) was directly extracted from *Taxomyces andreanae*, an endophytic fungus (Stierle et al. 1993). In our laboratory, 350 fungal strains have been isolated from moso bamboo (*Phyllostachys edulis*) seeds and one of these strains have been identified to have high potential of hypocrellin production (Shen et al. 2014).

Extracts directly isolated from blueberry plants (*Vaccinium* species) indicated good antioxidant (Elfar et al. 2013; Miao et al. 2013; Stewart et al. 2015; Tadych et al. 2012) as well as antimicrobial activities (Ermis et al. 2015; Girardot et al. 2014; Kalt et al. 2007; Puišo et al. 2014; Silva et al. 2013). Many endophytic fungi have been isolated and characterized from *Vaccinium* spp., some of them belonging to pathogenic fungi whereas others are beneficial fungi (Farr et al. 2002; Jeffers 1991; Miao et al.

2013; Reeh & Cutler 2013; Sauer et al. 2002; Tadych et al. 2012; Vohnik et al. 2012). Furthermore, several natural products from these fungi have been identified, such as griseofulvin, flavonoids and bioactive polyketides (Li et al. 2009; Richardson et al. 2014; Sauer et al. 2002). However, fungal resources isolated from *Vaccinium* spp., especially endophytes with potential antimicrobial activities, remain to be explored.

The South China blueberry, *Vaccinium dunalianum* Wight var. *urophyllum* Rehd. & Wils., mainly grows in south China and unlike other *Vaccinium* species, this plant also has important roles in ethnomedicine (Huang & Xie 1988). In our laboratory, a total of 374 fungal endophyte strains from *V. dunalianum* var. *urophyllum* have been obtained covering 15 genera and 25 species (Li et al. 2016). In this study, the antimicrobial activity of these fungi was screened and three different crude extracts (dichloromethane, ethyl acetate and n-butyl alcohol) were analysed for the relevant bioactivity.

## MATERIALS AND METHODS

### STRAINS

Endophytic fungi in this study were isolated by Li et al. (2016) and deposited in the China Forestry Culture Collection Center (CFCC).

For the antimicrobial activity assay, the tested strains were *Staphylococcus aureus* (CGMCC1.2386), *Bacillus subtilis* (CGMCC1.769), *Listeria monocytogenes* (ATCC27708), *Salmonella bacteria* (ATCC14208), *Proteus vulgaris* (ATCC33420) and a yeast *Candida albicans* (ATCC10231). *S. aureus* and *B. subtilis* were obtained from the China General Microbiological Culture Collection Center and *L. monocytogenes*, *S. bacteria*, *P. vulgaris* and *C. albicans* from the American Type Culture Collection.

### ANTIMICROBIAL ACTIVITY OF ENDOPHYTES AND CRUDE EXTRACTS

The fresh mycelia of different endophytic fungi were grown on plates at 25°C for 1-3 weeks and five plugs (5 mm in diameter) of mycelia with culture medium were subsequently added to 250 mL Erlenmeyer flasks containing 100 mL of Potato Dextrose (PD) broth medium (in g/L: fresh potato 200 and dextrose 20; pH6.0). All liquid cultures were maintained at 25°C for 7-20 days with shaking (150 rpm), depending on the growth rates of these fungal strains (the dry weight of the mycelia was calculated as about 10 g/L). The fermentation broths were filtered to segregate the filtrates from the mycelia, which were extracted separately with three organic solvents (dichloromethane, ethyl acetate and n-butyl alcohol), to obtain mycelium and filtrate extracts. These extracts were redissolved in dimethyl sulfoxide (DMSO) at a concentration of 50 mg/ $\mu$ L.

The antimicrobial activity of cultural fungi was screened by the agar diffusion method, which is generally

used to rapidly and qualitatively select bioactive microorganisms. Agar plugs (5 mm in diameter) of growing culture with different mycelial strains were subsequently added to Luria-Bertani Agar (LBA) medium (in g/L: tryptone 10, yeast extract 5, NaCl 10 and agar 20; pH6.0) and PDA medium that was supplemented with 0.5% olive oil previously spread with bacteria (*S. aureus*, *B. subtilis*, *L. monocytogenes*, *S. bacteria* and *P. vulgaris*) and a yeast (*C. albicans*), which were limited to  $1\sim 2 \times 10^8$  colony-forming units (CFU)/mL. The mixed plates were incubated at 37°C for 24 h for the bacteria and 25°C for 2 days for the yeast. The inhibition zones around the agar plugs were calculated to investigate the antimicrobial activities of the fungal isolates (Shen et al. 2014).

The extracts prepared from the endophytes were evaluated for antimicrobial activity against *S. aureus*, *B. subtilis*, *L. monocytogenes*, *S. bacteria*, *P. vulgaris* in LB medium and *C. albicans* in PD medium (the same conditions as in the inoculation tests). They were assessed by the disc diffusion method at a concentration of 100  $\mu$ g/disc and the antimicrobial bioactivity against pathogens was estimated by the size (diameter in mm) of growth inhibition zones, where DMSO was used as the negative control.

The minimal inhibitory concentration (MIC) values, which represent the lowest extract concentration that completely inhibit the growth of test bacteria and yeast, were determined by the micro-well dilution method on 96-well microtitre plates (Nunc Nunclon, Denmark), as described by Eloff et al. (1998) with some modifications. Every well contained 100  $\mu$ L LB broth medium (for *S. aureus*, *B. subtilis*, *L. monocytogenes*, *S. bacteria* and *P. vulgaris*) or PD broth medium (for *C. albicans*). Different extracts from endophytes were separately distributed 100  $\mu$ L in the first row at a concentration of 50 mg/mL and gradually diluted to 25, 12.5, 6.25, 3.125, 1.5625, 0.78125, 0.390625, 0.1953125 and 0.09765625 mg/mL. The clinical pathogens ( $1\sim 2 \times 10^8$  CFU/mL) 100  $\mu$ L were added to the corresponding wells and cultured at 37°C for the bacteria or 25°C for the yeast for 24 h. The reaction was measured by an MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 0.5 mg/mL) assay (Eloff et al. 1998). For comparison, ciprofloxacin and nystatin were used as positive controls.

Tryptone, yeast extract, dextrose, NaCl and agar were obtained from Oxoid (Basingstoke, Hampshire, England), while ciprofloxacin, nystatin and DMSO were obtained from Sigma-Aldrich (Saint Louis, Missouri, USA). In addition other reagents were all obtained from Beijing Chemical Works (Daxing, Beijing, China).

### HPLC ANALYSIS

The crude extracts were analysed using the Agilent 1200 HPLC-DAD system, equipped with an Angilent Eclipse XDB-C18 column (4.6 mm  $\times$  150 mm, 5  $\mu$ m). The operating condition involve a flow rate of 1.0 mL/min and a mobile phase of methanol/water (70/30, v/v). For each sample, the

TABLE 1. Antimicrobial activity of fungal isolates from *Vaccinium dunalianum* var. *urophyllum* against pathogens

Isolate No.	<i>Candida albicans</i>	<i>Bacillus subtilis</i>	<i>Proteus vulgaris</i>	<i>Listeria monocytogenes</i>	<i>Salmonella</i> <i>bacteria</i>	<i>Staphylococcus aureus</i>
<i>Colletotrichum</i> sp. VD001	+	+++	+++	++	++	-
<i>Epicoccum nigrum</i> VD003	+	++	+	+	+	-
<i>Pestalotiopsis vicala</i> VD006	+	++	++	+	+++	+
<i>Monochaetia kansensis</i> VD008	-	+	+	+	++	-
<i>Colletotrichum siamense</i> VD011	++	+	+++	++	++	+
<i>Sordariomyces</i> sp. VD014	-	+	-	+	+	-
<i>Colletotrichum acutatum</i> VD015	+	++	++	++	++	++
<i>Ceuthospora pinastri</i> VD020	+	++	++	+	++	-
<i>Epicoccum nigrum</i> VD021	++++	++++	++++	++++	++++	+++
<i>Epicoccum nigrum</i> VD022	++	+++	+++	+++	+++	++
<i>Nemania diffusa</i> VD027	+	+	+	++	++	-

Footnotes: - : no activity (<6 mm); +: weak activity (6-10 mm), ++: activity (10-15 mm), +++: good activity (15-20 mm), ++++: very good activity (>20 mm)

injection volume was 10 µL and the detecting wavelength was set at 254 nm. HPLC grade methanol used was obtained from Fisher Scientific (Geel, Belgium, UK).

## RESULTS AND DISCUSSION

### DETECTING ANTIMICROBIAL ACTIVITIES FROM THE CULTURABLE STRAINS

Using the agar diffusion assay, all of the culturable fungi associated with *V. dunalianum* var. *urophyllum* (Li et al. 2016) were analyzed to screen for antimicrobial bioactivity and several promising strains with potential bioactive applications were selected for further study. The clinical pathogens tested included typical bacteria (*S. aureus*, *B. subtilis*, *L. monocytogenes*, *S. bacteria* and *P. vulgaris*) and a yeast (*C. albicans*) (Table 1). In particular, the two isolates *E. nigrum* VD021 and *E. nigrum* VD022 significantly inhibited the growth of all tested bacteria and displayed activity against *C. albicans*. The size (diameter) of growth inhibition zones are all above 15 mm. Strains *Pestalotiopsis vicola* VD006, *Colletotrichum* sp. VD011 and *Colletotrichum acutatum* VD015 had broad antimicrobial activities but displayed less bioactivity against clinical microorganisms compared to *E. nigrum* VD021 and *E. nigrum* VD022. In addition, six mycelial isolates also had strong inhibitory effects. In total, 11 strains were singled out for further study.

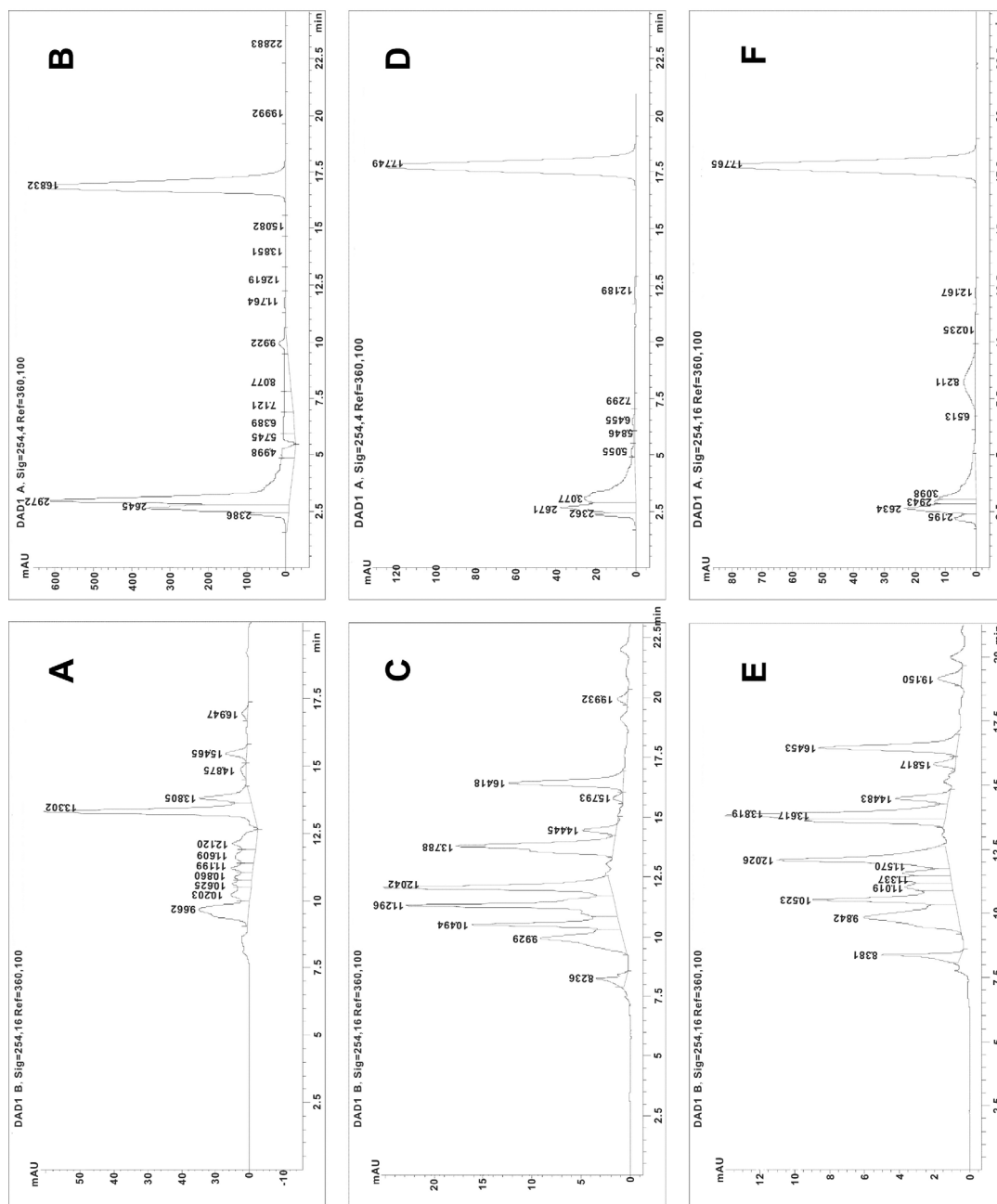
### ANALYSING EXTRACT BIOACTIVITIES

In order to characterize further the 11 fungal isolates, the antimicrobial properties of different solvent extracts from each fungal candidate were evaluated by the size (diameter in mm) of growth inhibition zones (Figure 1). Among the crude extracts of these endophytes, the ethyl acetate and dichloromethane extracts from the filtrates demonstrated increased inhibitory effects and broader scopes. In the disc diffusion assay, none of the crude extracts had significant bioactivity against *S. aureus* and the dichloromethane extract of the filtrates from *E. nigrum* VD021 significantly inhibited the growth of all clinical strains. Based on growth inhibition zone determinations, two extracts (ethyl acetate and dichloromethane extracts) from three strains (*Colletotrichum* sp. VD001, *E. nigrum* VD021 and *E. nigrum* VD022) had marked bioactivity over a broad spectrum of bacteria. The activities of these extracts were also determined from MIC values, which ranged from 1.5625 to 12.5 mg/mL. Using ethylene acetate as the extraction solvent, the fermentation broth from strains *Colletotrichum* sp. VD001 and *E. nigrum* VD022 exhibited high activities against most microbial species (Figure 1) and the lowest MIC value, 1.5625 mg/mL, was obtained against *C. albicans* (Table 2). The dichloromethane extract from the VD022 fermentation broth also indicated broad-spectrum inhibitory effects and optimal properties against *S. bacteria* with the smallest MIC value (1.5625 mg/mL). According to both the disc diffusion assay results and

the MIC value calculations (Figure 1 and Table 2), the dichloromethane and ethylene acetate extracts from the fermentation broth for strains *Colletotrichum* sp. VD001, *E. nigrum* VD021 and *E. nigrum* VD022 displayed high bioactivities. HPLC analysis was also applied to preliminarily screen the various derivatives (Figure 2) and the complex multi-peak chromatograms indicated the presence of many natural products in the crude extracts, especially in the dichloromethane extracts from the fermentation broth of strains of *E. nigrum* VD021 and VD022.

The endophytic fungus *Clonostachys rosea* inhibits the growth of *Botrytis cinerea* on lowbush blueberry and displays tolerance to fungicides *in vitro* (Reeh & Cutler 2013). Therefore, the main purpose of this study was to screen for bioactive strains and to investigate the effective activities of the relevant extracts. After further exploration, several culturable fungi, such as strains *Colletotrichum* sp. VD001, as well as *E. nigrum* VD021 and VD022, displayed prominent antimicrobial activities which could be utilized for fungal biocontrol and combat diseases. In contrast to other natural compound producers, such as plants, fungal species are highly diverse but poorly explored and endophytes are generally regarded as potential sources of biocontrol preparations and drug-like molecules. Further investigation of the effective agents from these endophytes is required and we plan to study the dichloromethane and ethylene acetate extracts from the fermentation broth of strains *Colletotrichum* sp. VD001, *E. nigrum* VD021 and *E. nigrum* VD022 in a future study. The species from *Colletotrichum* play important roles in plant diseases, which are generally regarded as anthracnoses and appear worldwide (Cannon et al. 2012; Hyde et al. 2009). Some metabolites from them (Garcíaapajón et al. 2003), such as colletotric acid, mycosporine alanine, antiauxin, 2-pyruvoylaminobenzamide and WF14861 displayed different bioactivities. *E. nigrum* has been used as a tool of biological control for plant pathogens and produces a variety of bioactive compounds such as flavipin (Bamford et al. 1961), epicorazins A-B (Baute et al. 1978), epirodin (Ikawa et al. 1978), orevactaene (Shu et al. 1997), epicocconone (Bell et al. 2003), epicoccins A–D (Zhang et al. 2007) and epicolactone (Silva et al. 2012). Recently, Perveen et al. (2017) extracted three new agents (2-methyl-3-nonyl prodiginine, Bis (2-ethylhexyl) phthalate and a meroterpenoid, Preaustinoid A), which displayed anticancer and antimicrobial activities, from *E. nigrum*. Future studies will be directed toward structure determination of effective compounds from *Colletotrichum* sp. VD001, *E. nigrum* VD021 and VD022. In addition, morphological and phylogenetic analyses will be carried out to identify the taxa of the *Colletotrichum* species.

In conclusion, three fungal endophytes (*Colletotrichum* sp. VD001, *E. nigrum* VD021 and *E. nigrum* VD022) screened from the South China blueberry for antimicrobial activity, displayed high potential in medicinal and biocontrol industries.



\*Diameter of growth inhibition in mm. mm±SD: millimeter ± standard deviation. FD: Dichloromethane extracts of the filtrates; FE: Ethyl acetate extracts of the filtrates; FB: n-butyl alcohol extracts of the filtrates; ME: Ethyl acetate extracts of the mycelia. Statistical analysis of the data was performed with SPSS 18.0 using LSD test for determining significant difference ( $\alpha = 0.05$ ). The same superscript letter ( $^{(a-b)}$ ) indicates no significant difference ( $p > 0.05$ ) between inhibition zone values of the extracts from different strains against the same pathogen

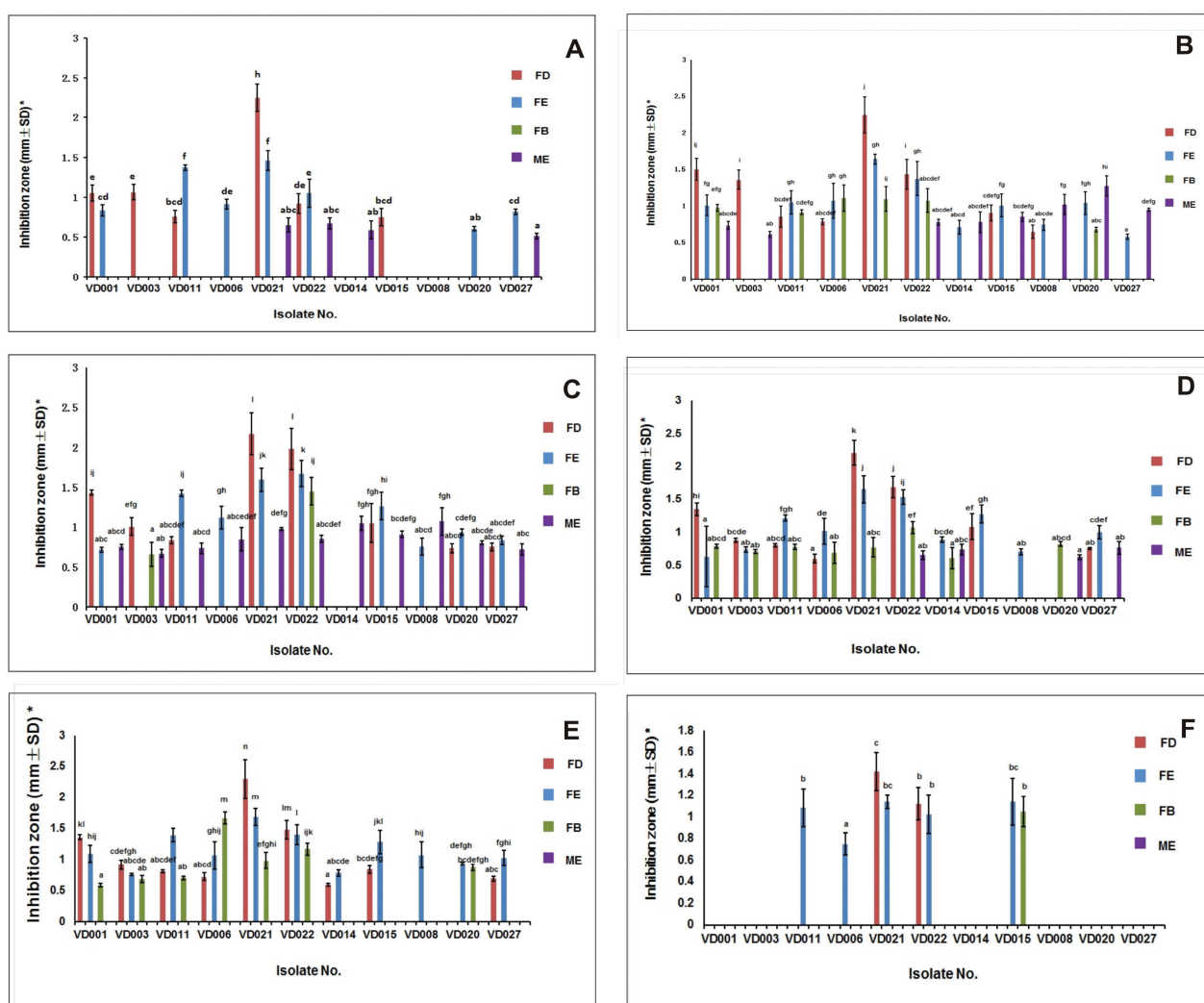
FIGURE 1. Bioactivity of crude extracts of the mycelia and filtrates of endophytic fungi from *Vaccinium dunalianum* var. *urophyllum* tested by disk diffusion assay. A: *C. albicans*; B: *B. subtilis*; C: *P. vulgare*; D: *L.monocytogenes*; E: *S. bacteria*; F: *S. aureus*



TABLE 2. MIC values of crude extracts of the filtrates of endophytic fungi from *Vaccinium dunalianum* var. *urophyllum*

Test	MIC value (mg/mL)					
	<i>C. albicans</i>	<i>B. subtilis</i>	<i>P. vulgaris</i>	<i>L. monocytogenes</i>	<i>S. bacteria</i>	<i>S. aureus</i>
ciprofloxacin	-	0.00625	0.00098	0.003125	0.003125	0.0125
nystatin	0.05	-	-	-	-	-
<i>Colletotrichum</i> sp. VD001 <sup>FD</sup>	6.25	12.5	12.5	6.25	6.25	12.5
<i>E. nigrum</i> VD021 <sup>FD</sup>	3.125	3.125	3.125	3.125	1.5625	6.25
<i>E. nigrum</i> VD022 <sup>FD</sup>	6.25	6.25	6.25	3.125	3.125	6.25
<i>Colletotrichum</i> sp. VD001 <sup>FE</sup>	1.5625	6.25	6.25	6.25	6.25	6.25
<i>E. nigrum</i> VD021 <sup>FE</sup>	3.125	12.5	6.25	12.5	6.25	12.5
<i>E. nigrum</i> VD022 <sup>FE</sup>	1.5625	3.125	3.124	6.25	6.25	6.25

<sup>FD</sup>Dichloromethane extracts of the filtrates. <sup>FE</sup>Ethyl acetate extracts of the filtrates



\*Diameter of growth inhibition in mm. mm±SD: millimeter ± standard deviation. FD: Dichloromethane extracts of the filtrates; FE: Ethyl acetate extracts of the filtrates; FB: n-butyl alcohol extracts of the filtrates; ME: Ethyl acetate extracts of the mycelia. Statistical analysis of the data was performed with SPSS 18.0 using LSD test for determining significant difference ( $\alpha = 0.05$ ). The same superscript letter (<sup>a-n</sup>) indicates no significant difference ( $p > 0.05$ ) between Inhibition zone values of the extracts from different strains against the same pathogen

FIGURE 2. HPLC profiles of the extracts from endophytic fungi associated with *Vaccinium dunalianum* var. *urophyllum*. A: The dichloromethane fraction of filtered extracts from *Colletotrichum* sp. VD001; B: the ethyl acetate fraction of filtered extracts from *Colletotrichum* sp. VD001; C: the dichloromethane fraction of filtered extracts from *E. nigrum* VD021; D: the ethyl acetate fraction of filtered extracts from *E. nigrum* VD021; E: the dichloromethane fraction of filtered extracts from *E. nigrum* VD022; and F: the ethyl acetate fraction of filtered extracts from *E. nigrum* VD022

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