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Antibacterial and antioxidant aromatic butenolide derivatives from the endophytic fungus *Aspergillus flavipes* XC5-7

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ABSTRACT: Five bioactive aromatic butenolide derivatives were isolated from ethyl acetate (EtOAc) extracts of the endophytic fungus *Aspergillus flavipes* XC5-7, which was isolated from tobacco in China. Their structures were identified by ^{1}H nuclear magnetic resonance (NMR), ^{13}C NMR, and mass spectrometry (MS) spectroscopic analyses as aspernolide C (**1**), aspernolide H (**2**), butyrolactone II (**3**), aspulvinone P (**4**), and aspulvinone Q (**5**). Compound **1** exhibited significant antimicrobial activity against ten strains of bacteria with minimal inhibitory concentrations (MICs) in the range of 6.6 to 27.9 µg/ml, especially against *Enterococcus faecalis* (MIC = 7.7 µg/ml) and *Escherichia coli* (MIC = 6.6 µg/ml). Aspulvinone P (**4**) and aspulvinone Q (**5**) were found to exhibit prominent antioxidant effects for the first time, with potencies [EC_{50} (half effective) = 13.3 and 16.0 µM] that were two-fold greater than those of the positive control Trolox (EC_{50} = 32.7 µM) in 2,2-di(4-tert-octylphenyl)-1-picrylhydrazyl free radical (DPPH) assays. In addition, the antibacterial and antioxidant structure-function relationships of these compounds were also studied and discussed.

KEYWORDS: fungal metabolites, *Aspergillus flavipes*, antibacterial activity, antioxidant activity

INTRODUCTION

For a long period of time, drug resistant bacteria have posed increasing threats to human health and sanitary safety [1]. Numerous bacterial species, such as drug-resistant *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Escherichia coli*, have been reported to cause severe respiratory, gastrointestinal, bloodstream, and skin infections [2, 3]. Since the discovery of famous antibiotics such as penicillin and cephalosporin, fungal secondary metabolites have been regarded as potential and promising resources for the development of new antimicrobial agents. Many fungal secondary metabolites with powerful antibacterial activities, such as dimeric tetrahydroxanthones [4], prenylated xanthones [5], alkaloids [6], diphenyl ethers [7], and terpenes [8], have been reported. On the other hand, the extracts from a large number of fungi species such as *Pleurotus ostreatus* [9], *Aspergillus fumigatus* [6], and *Talaromyces islandicus* [10] have exhibited potential antioxidant value, which could be used for treating oxidative damage diseases. Their metabolites with free radical scavenging effects have also been reported including dimeric naphtho-γ-pyrones [11], phenolics [6], and hydroanthraquinones [10].

In our continuous search for antimicrobial and antioxidant fungal metabolites [12], *Aspergillus flavipes* XC5-7 showed the potential value since its ethyl acetate (EtOAC) extract could inhibit the growth of *S. aureus* ATCC 25923, with a minimal inhibitory concentration (MIC) value of 146.3 µg/ml. Therefore, a systematic chemical study of its EtOAC extract was carried out, which afforded five aromatic butenolide derivatives (Fig. 1). Using a combination of ^{1}H nuclear magnetic resonance (NMR), ^{13}C NMR, and mass spectrometry (MS) spectroscopic analyses, the structures of butenolide compounds were identified as compared to previous studies from other *Aspergillus* species or strain [13–16]. Herein, their structural elucidation, antimicrobial and antioxidant activities, compound isolation, and fungal fermentation are described.

MATERIALS AND METHODS

General experimental procedures

The primary purification of secondary metabolites was carried out by column chromatographies (CCs) and the packing material was selected as Sephadex LH-20 (Pharmacia, Uppsala, Sweden), middle chromatogram isolated Gel (MCI) (Mitsubishi, Tokyo, Japan), and silica gel (Qingdao Haiyang Chemical Co., Ltd., Qingdao, China). The accurate purification was performed using 1525-2998 semi-preparative high performance liquid chromatography (HPLC, Waters, Milford, USA) that possessed with YMC-ODS-A HPLC column (250 × 10 mm, 5 µm, YMC, Kyoto, Japan), An Infinite-F50 microplate reader was chosen for optical density (OD) measurements (Tecan, Hombrechtikon, Switzerland). NMR data were determined by Bruker AVIII-600 and AVIII-500 NMR instruments with internal standard of tetramethyl silane (Bruker, Billerica, USA). A LTQ XL equipment was used for ESIMS determination (Thermofisher Technologies, Waltham, USA).

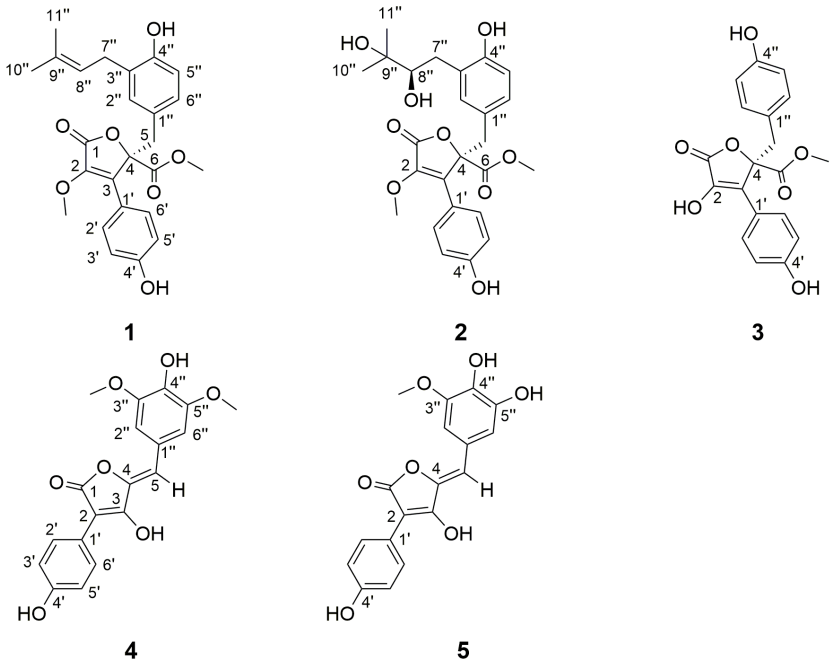

Fig. 1 Structures of compounds **1**–**5**.

Fungal isolation and cultivation

Fresh leaf stalks of tobacco (*Nicotiana tabacum*) were obtained at the end of July 2021. The plant tissues were surface-sterilized immediately with 0.8% NaClO and 70.0% ethanol solution, and further placed on potato dextrose agar (PDA, Shanghai Yuanye Bio-Technology Co., Ltd., China) [12]. After cultivation for one week, the fungus strain XC5-7 was obtained. This strain was purified, subcultured, and used in this study.

The fungus was activated at 28 °C for one week. Then several small species of PDA with fungus were cut and put in potato dextrose broth (PDB), which was incubated at 25 °C and 130 rpm to obtain seed cultures. Solid fermentation was carried out on the rice cultures, and a total of 30 Erlenmeyer flasks (500 ml) were used and prepared with 80 g of sterilized rice and 120 g of sterile water. Subsequently, 20 ml of the rice seed culture mixture was added to the Erlenmeyer flasks containing the rice cultures, which were maintained at 28 °C in the dark for four weeks.

Extraction and isolation

After being combined, the rice cultures of the strain XC5-7 were extracted twice using EtOAc as the solvent. The EtOAc was evaporated under reduced pressure, which yielded a crude extract (41.2 g). Using 40:1 to 1:3 petroleum ether – EtOAc as the eluent, the extract was fractionated by silica gel CC to afford fractions A–I. Fractions E–H (10.5 g) were combined and subsequently fractionated by MCI CC to obtain six subfractions (M1–M6) by different concentration of methanol (MeOH) in water. Subfraction M3 (5.2 g, 70% MeOH part) was purified by a combination of semi-preparative HPLC [60% MeOH + 0.1% trifluoroacetic acid (CF_3COOH)] and Sephadex LH-20 CC to give **2** (6.5 mg, t_R 18.0 min) and **1** (67.5 mg, t_R 28.2 min). Subsequently, compounds **4** (28.5 mg, t_R 18.9 min), **5** (130.5 mg, t_R 20.6 min), and **3** (17.2 mg, t_R 43.6 min) were isolated from fraction M2 by semi-preparative HPLC [25% acetonitrile + 0.1% formic acid (HCOOH)].

Antimicrobial assays

Antimicrobial assays were carried out on the basis of the previous broth microdilution method [6]. Briefly, the bacteria were inoculated in Mueller–Hinton (MH) liquid broth and incubated in a shaker (130 rpm, 35 °C) for 6–9 h. The bacterial suspension was diluted to 1.0×10^4–1.0×10^5 CFU/ml. The test compounds were dissolved in dimethyl sulfoxide (DMSO) to a concentration of 10 mg/ml as the stock solution and further diluted with MH broth to concentrations ranging from 0.5 to 250 µg/ml. Subsequently, 100 µl of each of the bacterial suspensions and compound solutions were transferred into 96-well plates, and the plates were kept at 37 °C for 24 h. The OD values at 530 nm were determined, and the MIC values were defined as the concentrations at which the compounds reduced the growth of the bacteria by 50%. In this study, a total of eight strains of human pathogenic bacteria and two strains of agricultural pathogenic bacteria were selected, including Gram-positive (*S. au-*

reus ATCC 25923, *S. aureus* clinical isolate, *E. faecalis* ATCC 29212, *Bacillus subtilis* ATCC 6633, and *Bacillus altitudinis*) and Gram-negative (*K. pneumoniae* clinical isolate, *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Pseudomonas syringae* pv. *actinidiae*, and *Erwinia carotovora* var. *carotovora*).

Antioxidant assays

The 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS) and DPPH scavenging assays of compounds **1**–**5** were performed with Trolox as the positive control [17]. The tested solutions of **1**–**5** were diluted to 0.005–2.00 mM. Equal volumes of oxidant solution and ABTS were added into a brown bottle, which was maintained in the dark at 28 °C for 24 h. After the addition of 80% ethanol, the dark blue mixture was diluted, and 180 µl of diluted $ABTS^+$ solution was transferred to 96-well plates. Afterward, the compound solutions (20 µl) were quickly added and reacted for 6 min in the dark to obtain OD values at 734 nm. For the DPPH assay, DPPH solutions (0.15 mM) were prepared, and 100 µl of solution was pipetted into 96-well plates. Then, 100 µl of sample solution was quickly transferred to the well of plates to react in the dark for 30 min, after which the absorbance was determined at 517 nm. The EC_{50} values were defined as the concentrations at which 50% of the free radicals were scavenged, and were calculated as follows: radical scavenging rates (%) = $[(A_{blank} - A_{test})/A_{blank} \times 100]$. A_{blank} represents the absorbance of the blank group, and A_{test} represents the absorbance of the test group.

Statistical analysis

All the tests were performed in triplicate. ± Standard deviation (SD) values were assigned according to three individual measurements. Antibacterial data of aspernolide C (**1**) were analyzed via one-way analysis of variance (ANOVA) by GraphPad Prism software using Turkey's Test. The EC_{50} data in the antioxidant assays were analyzed with two-way analysis of variance through Turkey's Test.

RESULTS

Fungal identification

After being cultivated on PDA for one week, the fungus strain *A. flavipes* XC5-7 generated a brown-orange paniform limited colony with a large number of powdery spores on the front. No obvious pigmentation could be observed on the back of the colony (Fig. 2). Under the microscope (10 × 40), several inflated stipes could be observed with hair-like sporidesm on the top of the conidiophores (about 250–800 µm length × 6–10 µm width), and the scattered conidia were round (2–3 µm diameter) (Fig. 2) [12]. The lengths of phialides were about 5–8 µm and the widths were 2–3 µm.

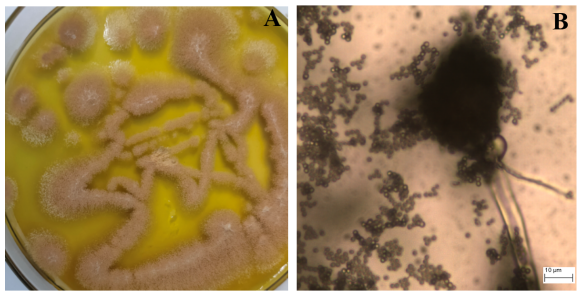

Fig. 2 Morphological (A) and microscopic (B, 10 × 40) characteristics of *A. flavipes* XC5-7.

Morphological and microscopic observations were initially performed for the fungus XC5-7 in the genus *Aspergillus*, and the internal transcribed spacer (ITS) and 18S rDNA sequence analyses (GeneBank accession No. MW522866) showed 100% identity to the reported *Aspergillus flavipes*. Therefore, the fungus strain XC5-7 was identified to be *Aspergillus flavipes*.

Structure elucidation

Aspernolide C (**1**): yellow oil; electrospray ionization mass spectrometry (ESIMS) m/z 456.4 $[M+NH_4]^+$, $C_{25}H_{26}O_7$; ^{1}H-NMR (CD_3OD, 600 MHz), δ_H 7.52 (2H, d, J = 8.9 Hz, H-2′ and H-6′), 6.89 (2H, d, J = 8.8 Hz, H-3′ and H-5′), 6.53 (1H, brs, H-6″), 6.53 (1H, brs, H-5″), 6.43 (1H, brs, H-2″), 5.08 (1H, tt, J = 7.4, 1.3 Hz, H-8″), 3.78 (3H, s, 6-OCH_3), 3.63 (3H, s, 2-OCH_3), 3.42 (2H, brs, H-5), 3.09 (2H, d, J = 9.9 Hz, H-7″), 1.66 (3H, s, H-10″), 1.58 (3H, s, H-11″); ^{13}C-NMR (CD_3OD, 150 MHz), δ_C 170.9 (C-6), 169.3 (C-1), 160.7 (C-4′), 155.4 (C-4″), 142.0 (C-2), 139.1 (C-3), 133.1 (C-2″), 132.5 (C-2″), 131.1 (C-2′, C-6′), 129.9 (C-6″), 128.7 (C-3″), 124.9 (C-1″), 123.7 (C-8″), 122.0 (C-1′), 116.9 (C-3′, C-5′), 115.2 (C-5″), 86.6 (C-4), 59.0 (2-OCH_3), 54.1 (6-OCH_3), 40.0 (C-5), 28.9 (C-7″), 26.1 (C-10″), 17.9 (C-11″). The NMR data were in accordance with those in the literature and compound **1** was determined as aspernolide C [13]. The structure of **1** was further verified by its heteronuclear single quantum coherence (HSQC) and ^{1}H detected heteronuclear multiple bond correlations (HMBCs). And the locations of 2-OCH_3 and 6-OCH_3 were unambiguously confirmed by the key HMBC from δ_H 3.78 (6-OCH_3) to δ_C 170.9 (C-6) and from δ_H 3.63 (2-OCH_3) to δ_C 142.0 (C-2).

Aspernolide H (**2**): colorless oil; ESIMS m/z: 495.0 $[M+Na]^+$, $C_{25}H_{28}O_9$; ^{1}H-NMR (CD_3OD, 500 MHz), δ_H 7.51 (2H, d, J = 8.7 Hz, H-2′ and H-6′), 6.89 (2H, d, J = 8.7 Hz, H-3′ and H-5′), 6.62 (1H, d, J = 1.3 Hz, H-2″), 6.59 (1H, d, J = 8.2 Hz, H-5″), 6.51 (1H, dd, J = 8.2, 1.3 Hz, H-6″), 3.81 (3H, s, 6-OCH_3), 3.69 (3H, s, 2-OCH_3), 3.48 (1H, m, H-8″), 3.45 (1H, m, H-5a), 3.42 (1H, m, H-5b), 2.67 (1H, d, J = 13.9 Hz, H-7″a), 2.47 (1H, dd, J = 10.2, 13.9 Hz, H-7″b), 1.29

(3H, s, H-10″), 1.18 (3H, s, H-11″); ^{13}C-NMR ($CDCl_3$, 125 MHz), δ_C 170.7 (C-6), 169.1 (C-1), 160.5 (C-4′), 156.1 (C-4″), 141.9 (C-2), 138.9 (C-3), 134.4 (C-2″), 130.9 (C-2′, C-6′), 130.5 (C-6″), 127.4 (C-3″), 125.2 (C-1″), 121.8 (C-1), 116.8 (C-3′, C-5′), 116.0 (C-5″), 86.5 (C-4), 80.7 (C-8″), 73.7 (C-9″), 59.0 (2-OCH_3), 54.0 (6-OCH_3), 39.7 (C-5), 34.2 (C-7″), 25.5 (C-11″), 25.2 (C-10″). The NMR data were in accordance with those in the literature and compound **2** was determined as aspernolide H [14].

Butyrolactone II (**3**): yellow oil; ESIMS m/z: 357.5 $[M+H]^+$, $C_{19}H_{16}O_7$; ^{1}H-NMR (CD_3OD, 600 MHz), δ_H 7.59 (2H, d, J = 8.9, H-2′ and H-6′), 6.87 (2H, d, J = 8.8, H-3′ and H-5′), 6.64 (2H, d, J = 8.5, H-2″ and H-6″), 6.52 (2H, d, J = 8.5, H-3″ and H-5″), 3.78 (3H, s, 6-OCH_3), 3.47 (2H, brd, H-5); ^{13}C-NMR (CD_3OD, 150 MHz), δ_C 171.7 (C-6), 170.5 (C-1), 159.5 (C-4′), 157.6 (C-4″), 139.9 (C-2), 132.7 (C-2″, C-6″), 130.5 (C-2′, C-6′), 129.3 (C-3), 125.4 (C-1″), 123.2 (C-1′), 116.8 (C-3′, C-5′), 115.7 (C-3″, C-5″), 86.9 (C-4), 54.0 (6-OCH_3), 39.6 (C-5). The NMR data were in accordance with those in the literature and compound **3** was determined as butyrolactone II [15].

Aspulvinone P (**4**): yellow powder; ESIMS m/z 357.3 $[M+H]^+$, $C_{19}H_{16}O_7$; ^{1}H-NMR (CD_3OD, 600 MHz), δ_H 7.76 (2H, d, J = 8.7 Hz, H-2′ and H-6′), 7.09 (2H, s, H-2″ and H-6″), 6.82 (2H, d, J = 8.7 Hz, H-3′ and H-5′), 6.34 (1H, s, H-5), 3.88 (6H, s, 3″-OCH_3 and 5″-OCH_3); ^{13}C-NMR (CD_3OD, 150 MHz), δ_C 171.9 (C-1), 165.0 (C-3), 157.9 (C-4′), 149.4 (C-3″, C-5″), 142.9 (C-4), 138.3 (C-1″), 130.3 (C-2′, C-6′), 125.6 (C-4″), 122.8 (C-1′), 116.2 (C-3′, C-5′), 109.2 (C-2″, C-6″), 109.0 (C-5), 101.8 (C-2), 56.9 (3″-OCH_3, 5″-OCH_3). The NMR data were in accordance with those in the literature and compound **4** was determined as aspulvinone P [16].

Aspulvinone Q (**5**): yellow powder; ESIMS m/z 349.2 $[M+Na]^+$, $C_{18}H_{14}O_7$; ^{1}H-NMR (CD_3OD, 600 MHz), δ_H 7.80 (2H, d, J = 8.6 Hz, H-2′ and H-6′), 7.47 (1H, s, H-2″), 7.18 (1H, dd, J = 8.3, 1.7 Hz, H-6″), 6.81 (3H, d, J = 8.5 Hz, H-3′, H-5′, and H-5″), 6.36 (1H, s, H-5), 3.90 (3H, s, 3″-OCH_3); ^{13}C-NMR (CD_3OD, 150 MHz), δ_C 172.7 (C-1), 167.4 (C-3), 157.4 (C-4′), 149.2 (C-3″), 148.8 (C-4″), 143.5 (C-4), 129.9 (C-13, C-17), 126.9 (C-6), 125.9 (C-11), 123.6 (C-12), 116.5 (C-10), 116.1 (C–2′, C-6′), 114.4 (C-2″), 108.1 (C-5), 100.5 (C-2), 56.5 (3″-OCH_3). The NMR data were in accordance with those in the literature and compound **5** was determined as aspulvinone Q [16].

Antibacterial activities

As summarized in Fig. 3 and Table 1, compound **1** showed potent antimicrobial activity against ten strains of bacteria, with MIC values ranging from 6.6 to 27.9 µg/ml. It could significantly inhibit the growth of *E. faecalis*, *P. aeruginosa*, and *E. coli* (MIC

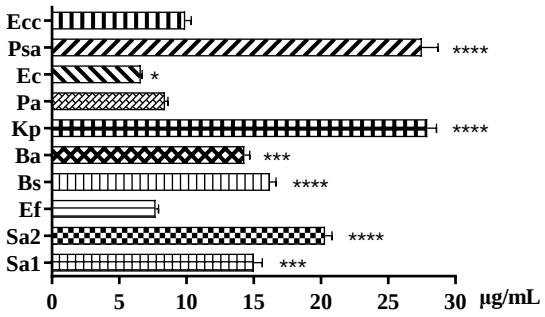

Fig. 3 Antibacterial effects of aspernolide C (**1**). ± SD values were calculated based on three individual experiments. Sa1 (*S. aureus* ATCC 25923), Sa2 (*S. aureus* clinical isolate), Ef (*E. faecalis* ATCC 29212), Bs (*B. subtilis* ATCC 6633), Ba (*B. altitudinis*), Kp (*K. pneumoniae* clinical isolate), Pa (*P. aeruginosa* ATCC 27853), Ec (*E. coli* ATCC 25922), Psa (*P. syringae* pv. *actinidiae*), Ecc (*E. carotovora* var. *carotovora*). * $p < 0.05$, compared with Ecc; *** $p < 0.001$, compared with Ecc; **** $p < 0.0001$, compared with Ecc.

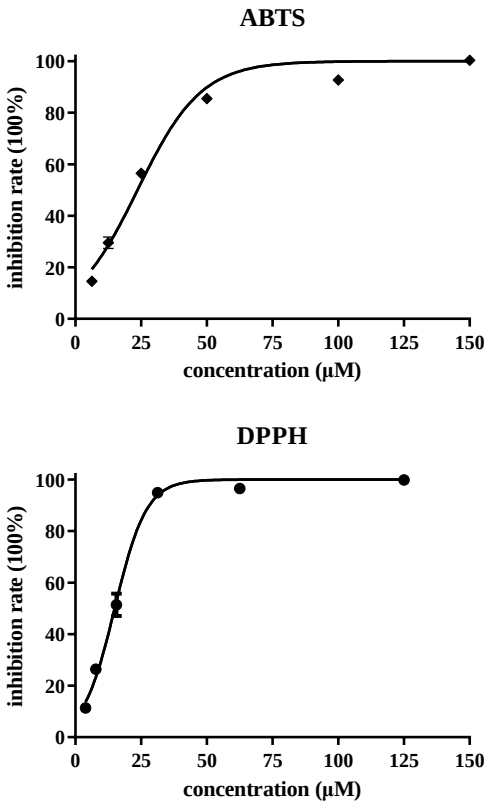

Fig. 4 Antioxidant effects of aspulvinone P (**4**) in the ABTS and DPPH assays.

Table 1 The MIC values of compounds **1**–**5** (μg/ml)[a].

Compound	Sa1	Sa2	Ef	Bs	Ba	Kp	Pa	Ec	Psa	Ecc
1	15.0 ± 1.1	20.3 ± 0.9	7.7 ± 0.4	16.2 ± 0.8	14.3 ± 0.7	27.9 ± 1.2	8.4 ± 0.4	6.6 ± 0.2	27.5 ± 2.1	9.9 ± 0.8
2	23.4 ± 1.9	42.8 ± 3.1	20.5 ± 0.9	38.2 ± 1.5	28.5 ± 1.9	> 100.0	19.5 ± 1.2	26.8 ± 1.9	68.3 ± 3.8	32.1 ± 1.6
3	78.5 ± 4.2	> 100.0	64.8 ± 3.7	45.8 ± 2.6	> 100.0	> 100.0	> 100.0	> 100.0	> 100.0	> 100.0
4	> 100.0	> 100.0	> 100.0	> 100.0	> 100.0	> 100.0	> 100.0	> 100.0	> 100.0	> 100.0
5	> 100.0	> 100.0	> 100.0	> 100.0	> 100.0	> 100.0	> 100.0	> 100.0	> 100.0	> 100.0
Penicillin G[b]	0.5 ± 0.1	1.2 ± 0.2	0.4 ± 0.1	0.6 ± 0.1	0.8 ± 0.1	NT	NT	NT	NT	NT
Streptomycin[b]	NT	NT	NT	NT	NT	1.2 ± 0.1	1.0 ± 0.2	0.8 ± 0.1	0.8 ± 0.1	0.7 ± 0.1

[a] Compounds were considered as inactive when MICs > 100.0 μg/ml, ± SD values were calculated based on three individual experiments, Sa1 (*S. aureus* ATCC 25923), Sa2 (*S. aureus* clinical isolate), Ef (*E. faecalis* ATCC 29212), Bs (*B. subtilis* ATCC 6633), Ba (*B. altitudinis*), Kp (*K. pneumoniae* clinical isolate), Pa (*P. aeruginosa* ATCC 27853), Ec (*E. coli* ATCC 25922), Psa (*P. syringae* pv. *actinidiae*), Ecc (*E. carotovora* var. *carotovora*).
[b] Positive control; NT = not tested.

Table 2 EC_{50} values of **1**–**5** in the antioxidant assays (μM)[a].

Compound	ABTS	DPPH
1	147.6 ± 22.1****	324.9 ± 28.2****
2	108.3 ± 17.9****	254.3 ± 21.9****
3	31.7 ± 2.8*	46.3 ± 4.5
4	20.7 ± 1.3	13.3 ± 1.3*
5	17.6 ± 1.8	16.0 ± 1.5*
Trolox[b]	18.4 ± 2.1	32.7 ± 3.0

[a] Compounds were considered as inactive when EC_{50} > 500.0 μM, ± SD values were calculated based on three individual experiments.
[b] Positive control; * $p < 0.05$, compared with control; **** $p < 0.0001$, compared with control.

=7.7, 8.4, and 6.6 μg/ml, respectively). The broad-spectrum antibacterial effects of **1** were revealed since it could inhibit the growth of all five strains of Gram-positive bacteria and five strains of Gram-negative bacteria. Aspernolide C (**1**) also showed the potential for agricultural antibiotic development due to its potent antibacterial effects against two strains of the crop pathogenic bacteria *P. syringae* and *E. carotovora* (MIC = 27.5 and 9.9 μg/ml). As an oxygenated derivative of **1**, aspernolide H (**2**) showed weaker antimicrobial activity than that of **1**, with MICs in the range of 19.5 to 68.3 μg/ml. The isopentene side chain at C-3″ of **1** could be an important functional group for its antibacterial activities [5, 18]. Compound **3** only exhibited weak activity against three strains of Gram-positive bacteria. However, compounds **4** and **5** were inactive against all the bacteria.

Antioxidant activities

Interestingly, in the ABTS and DPPH assays, aspulvinones P and Q (**4** and **5**) showed prominent radical scavenging effects (Table 2 and Fig. 4). In the DPPH assays, the potencies of **4** and **5** (EC_{50} = 13.3 and 16.0 μM) were twofold greater than those of the positive control Trolox (EC_{50} = 32.7 μM). Additionally, in the ABTS assays, the potencies of **4** and **5** were similar to those of the positive control. Previous studies have shown the potent α-glucosidase inhibitory and antiviral effects of aspulvinones P and Q, but their antioxidant effects have not been reported. In this study, their significant antioxidant activities were revealed for the first time [16].

Moreover, butyrolactone II (**3**) also exhibited potent radical scavenging effects (EC_{50} = 31.7 and 46.3 μM) respectively, while compounds **1** and **2** exhibited weak activities (Table 2). Considering the structure-function relationships of **1**–**5**, the presence of a bareness enolic hydroxy group at the butenolide ring could be one of the key functional groups for their antioxidant activities [6, 19]. Additionally, the enolic hydroxy group at C-3 seemed more active than that at C-2.

DISCUSSION

Fungi are known as promising potential resources for antimicrobial drug development worldwide, since fungal secondary metabolites such as polyketides, alkaloids, and terpenes have been reported to exhibit potent antibacterial effects [2, 5]. In this study, the strain *A. flavipes* XC5-7 showed potential antibacterial value because antibacterial activity against *S. aureus* was observed for its EtOAc extract. The antibacterial secondary metabolites were systematacially studied, and five polyketides (**1**–**5**) were isolated and identified as aromatic butenolide derivatives. Notably, aspernolide C (**1**) has been isolated from the fungi *A. versicolor* [13], *A. iizukae* [14], *A. flavipes* [20], gorgonian-derived *Aspergillus* sp. [21], and *Penicillium chrysogenum* SCSIO 41001 [22]. Aspernolide H (**2**) was regarded as the metabolite of *A. iizukae* [14], and butyrolactone II (**3**) was isolated from *A. terreus* and *A. flavipes* [15, 16, 23]. Moreover, aspulvinones P and Q (**4** and **5**) could be metabolized by *A. flavipes* PJ03-11 [16], *A. flavipes* HN4-13 [24], and *Aspergillus* sp. CPCC 400735 [25].

Moreover, a previous study has revealed the strong antibacterial effects of aspernolide C (**1**) against *S. aureus* with MIC value of 1.56 μm [21]. And in the present study, aspernolide C (**1**) exhibited potent antibacterial effects on *S. aureus* ATCC and clinical strains

(MIC = 15.0 and 20.3 μg/ml), which was in accordance with those in the literature [21]. Additionally, the antibacterial spectrum of **1** was preliminarily established, and it was shown to have pronounced antibacterial effects on *E. faecalis*, *P. aeruginosa*, and *E. coli* (MIC =7.7, 8.4, and 6.6 μg/ml, respectively) [12]. The antibacterial effects of **1** suggested that it was a broad-spectrum and nonselective agent against Gram-positive and Gram-negative bacteria.

However, the antibacterial data about aspernolide H (**2**) have not been reported. In the present study, compound **2** showed overall weaker antimicrobial activity than those of **1**. Analysis of their structures revealed the oxidation of the isopentene side chain, which indicated that such a low polarity isopentene side chain could be an important functional group for antibacterial activity [5, 18]. This deduction was further proven by the antimicrobial structure-function relationship of prenylated diphenyl ethers and prenylated xanthones, and the low polarity isopentene side chain could target on the membrane of multidrug-resistant gram-positive bacteria [5, 18, 26]. In addition, it has been reported that butyrolactone II (**3**) and its derivative were moderately active against *P. syringae* and *Botrytis cinerea* with MIC of 15.6 μg/ml, which was also in accordance with our study [23]. Thus, additional studies on the structure-function relationships and targets of these compounds are needed both *in vitro* and *in vivo*.

On the other hand, although aspulvinones P and Q (**4** and **5**) could not inhibit the growth of bacteria, they showed prominent antioxidant effects. It is known that aspulvinones P and Q exhibit α-glucosidase inhibition, anti-HIV, and anti-SARS-CoV-2 activities [13, 27], but their antioxidant effects have not been reported. Thus, the significant of these compounds in terms of antioxidant activity was also revealed for the first time in our study. Notably, the potencies of **4** and **5** were twofold greater than that of the positive control Trolox in DPPH assays and similar to that of the positive control in ABTS assays. It has been reported that butyrolactone II (**3**) exhibited potent antioxidant effects with an EC_{50} of 17.64 ± 6.41 μM, which was also in accordance with our study (EC_{50} = 31.7 and 46.3 μM, respectively) [24]. Rapid clearance of the excessive reactive oxygen species (ROS) in the human body is beneficial for the treatment of the illnesses such as atherosclerosis, diabetes, arthritis, and cancer [9, 14]. Thus, their radical scavenging effects need additional research in the future.

Furthermore, it has been reported that the antioxidant activities of these compounds could be associated with the phenolic and alcoholic hydroxy groups in their structures. In our study, butyrolactone II (**3**) also exhibited potent effects, but **1** and **2** had weak effects. Thus, the bulky enolic hydroxy group at the butenolide ring might be one of the key active groups for the radical scavenging effects seen in the structure-function relationship of **1**–**5** [6, 19]. Additionally, the enolic hydroxy group at C-3 could enhance this activity.

In conclusion, five bioactive aromatic butenolide derivatives were isolated from the endophytic fungus *A. flavipes* XC5-7. Aspernolides C (**1**) and H (**2**) exhibited potent and broad-spectrum antibacterial activities. Aspulvinones P (**4**) and Q (**5**) showed prominent antioxidant effects. Additional studies exploring the targets, structure-function relationships, and clinical use of these compounds are necessary.

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