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Antimicrobial Activity of Secondary Metabolites from Endophytic Fungus *Fusarium* sp. Isolated from *Eichhornia crassipes* Linn.

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Abstract: The active metabolites producing endophytic fungus, *Fusarium* sp., was isolated from *Eichhornia crassipes* Linn. yielded four compounds: "altersolanol (1)", "4-hydroxydihydronorjavanicin (2)", "5-hydroxy-7-methoxy-2-isopropylchromone (3)" and "fusaraichromenone (4)". Structures of 1-4 were elucidated by analysis of their spectroscopic data. The antimicrobial activity was tested by using four bacteria; methicillin-resistant *Staphylococcus aureus* (MRSA SK1), *Staphylococcus aureus* (SA), *Escherichia coli* (EC) and *Pseudomonas aeruginosa* ATCC27853 (PA) and two yeast; *Cryptococcus neoformans* ATCC90112 flucytosine – resistant (CN90112) and *Candida albicans* ATCC 90028 (CA90028) to adverse effect showed that compound **1** and **3** had moderate antibacterial activity against MRSA, *SA*, *PA* and *SK1* with same MIC value 16 μg/mL. However, all pure compounds showed relatively low activities to inhibit the growth of CA90028 and CN91112 yeast.

Keywords: Antimicrobial Activity, Endophytic Fungi, *Fusarium* sp., *Eichhornia crassipes* Linn

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1. Introduction

Fungi were a great source that could produce varieties of structural and bioactive compounds. They are rich sources of secondary metabolites; terpenoids, flavonoids, steroids, alkaloids, etc. Endophytic fungi live in the fruits, leaves, flowers and stems of plants. Fungal endophytes were attractive sources of bioactive secondary metabolites, such as antiviral [1], antioxidant [2], antimicrobial [3], anticancer [4] and anti-inflammatory [5] activities. Bioactive compounds that endophytic fungus *Fusarium* produce showed various activities, such as antibacterial [6], [7], antifungal [7], cytotoxic activity [8]. Research aimed to study active compounds from fungal endophytes. An unidentified endophytic fungus *Fusarium* sp. was isolated from *Eichhornia crassipes* Linn. This

research reported four known compounds' isolation, structural characterization, and antimicrobial activity. *E. crassipes* Linn. is herbal rich in antimicrobial phytochemicals, such as vitamins, terpenoids, lignin, alkaloids, phenolic acid and other metabolites [9]. The antimicrobial activity of crude extracts from endophytic fungi isolated from *E. crassipes* Linn was evaluated. The result showed that *Fusarium* sp. had the strongest antimicrobial activity. So isolated *Fusarium* sp. was interested in further studying to isolate structures that determine and test their antimicrobial activity.

2. Materials and Methods

2.1 Isolation of endophytic fungi

Isolation of endophytic fungi was proceeded by a modified method of Roopa [10]. The plant *Eichhornia crassipes* Linn. was collected from Thale Noi, Phatthalung. Leaves of *Eichhornia crassipes* Linn. were washed with soap and tap water, followed by immersion in 95% ethanol for 30 seconds and in 5% sodium hypochlorite for 4 min. After washing with distilled water three times, the samples were cut and transferred to Petri dishes containing potato dextrose agar (PDA) until fungi growth was observed. Purified and moved to another Petri dish. This step was produced until the Fusarium fungus was isolated.

2.2 Fermentation and extraction

Fusarium sp. fungus was inoculated in an Erlenmeyer flask containing 300 mL potato dextrose broth (PDB). These flasks were incubated at room temperature for 21 days. After that, the mycelium of endophyte was extracted with methanol and concentrated using a rotary evaporator to obtain methanol extract (CM)

2.3 Isolation of pure compounds

The methanol extract (CM) was separated by Sephadex column chromatography to give 7 fractions based on TLC characteristics (CM1-CM7). Fraction CM1 was first separated by reverse-phase carbon 18 column chromatography with 4% methanol in dichloromethane, yielding 3 sub-fractions (CM1.1-CM1.3); sub-fraction CM1.3 was further purified by column chromatography, producing compound **1** (39.4 mg) and compound **2** (31.7 mg). Fraction CM2 was separated by reverse-phase carbon 18 column chromatography with 2% methanol in dichloromethane yielding 4 subfractions (CM2.1 – CM2.4); subfraction CM 2.2 was purified by column chromatography followed by TLC to give compound **3** (5.4 mg). Subfraction CM 2.3 was purified by column chromatography yielding compound 4 (19.4 mg).

2.4 Antimicrobial activity

The antimicrobial activity was assessed by colourimetric broth micro-dilution assay using 6 human pathogenic microorganisms, namely, methicillin-resistant *S. aureus* (MRSA SK1), *Staphylococcus aureus* ATCC25923 (SA), *Escherichia coli* ATCC25922 (EC), *Cryptococcus neoformans* ATCC90112 (CN90112), *Pseudomonas aeruginosa* ATCC27853 (PA) and *Candida albicans* ATCC90028 (CA90028). These tested microorganisms were obtained from Mycology Laboratory, Microbiology Department, Prince of Songkhla University, Thailand. All experiments were carried out in triplicate. A stock concentration of 10 mg/mL in dimethylsulfoxide (DMSO) was prepared for each extract and stored at -20 °C. The samples were dissolved in dimethylsulfoxide (DMSO) to a 1 mg/mL final concentration for the antimicrobial test. This study used commercial antibacterial drugs (vancomycin, gentamicin) and an antifungal drug (amphotericin B) as positive control and DMSO as a negative control. Vancomycin and gentamicin were dissolved completely in sterile distilled water at a prepared concentration of 16 mg/mL, while amphotericin B was dissolved in sterile distilled water at 10 mg/mL. Tested pathogenic microorganisms were inoculated in nutrient broth (NB) for bacteria and Sabouraud's dextrose broth (SDB) for yeasts and incubated in a shaker incubator for 3 h at room temperature. The suspension of each strain of bacteria was adjusted to 1.5×10^8 CFU/mL (0.5 MacFarland turbidity standard),

while each yeast strain was adjusted to 6×10^8 CFU/mL (2 McFarland turbidity standard. Then, bacteria and yeast suspensions were diluted with MHB and SDB to 1:200 and 1:20, respectively. All extracts were investigated for their antimicrobial activity at the final concentration of 200 μg/mL. The serial two-flow dilution was carried out with the colourimetric broth micro-dilution test in 96 well plates, according to Supaphon *et al.* (2014) [11]. Each sample was conducted in triplicate. Resazurin was used to detect microbial growth for the antimicrobial test. The blue colour indicated an absence of microbial growth and the pink colour indicated microbial growth. The active sample that showed the antimicrobial activity against tested microorganisms were further tested for MIC and MBC or MFC, respectively. The minimum inhibitory concentrations (MIC) of the active sample from the preliminary test were evaluated using the same method. Samples with final concentrations of 128 - 0.025 μg/mL were tested for MIC. The lowest concentration of extract that inhibited growth (blue colour) was recorded as the MIC. The sub-culturing method determined the minimum bactericidal concentration (MBC) or minimum fungicidal concentration (MFC). Each well of 96-well plate that showed positive results (blue colour) were streaked onto culture agar and the plates were incubated at optimal conditions. The lowest concentration of sample that showed no microbial growth on culture agar after incubation was recorded as the MBC or MFC.

2.5 Spectroscopic data of Compounds 1, 2, 3 and 4

Compound 1; colorless solid ; UV (MeOH) λ_{max} : 223, 270 and 332 nm; FT-IR (ATR) λ_{max} : 3207, 1715, 1680, 1602, 1578, 1423 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ: 6.96 (1H, *d*, *J* = 2.3 Hz, H-8), 6.66 (1H, *d*, *J* = 2.3 Hz, H-6), 4.91 (1H, *d*, *J* = 2.5 Hz, H-10), 3.45 (1H, *dd*, *J*= 11.5,4.5 Hz, H-3), 3.78, (3H, *s*, OCH_3-12), 3.05 (*td*, *J* = 12.0, 4.1 Hz, H-9a), 2.37 (*dd*, *J* = 14.1, 4.1 Hz, H-1_{eq}), 2.20 (*td*, *J* = 12.2, 11.6, H-4_{ax}), 1.91 (*tdd*, *J* = 12.2, 3.4, 2.5 Hz, H-4a), 1.70 (*ddd*, *J* = 12.2, 4.5, 3.4 Hz, H-4_{eq}), 1.30 (*dd*, *J* = 14.4, 12.0 Hz, H-1_{ax}), 1.29 (*s*, H-11); ^{13}C NMR (75 MHz, $CDCl_3$): δ 203.7 (C-9), 162.4 (C-7), 157.5 (C-5), 134.8 (C-8a), 125.3 (C-10a), 107.6 (C-6), 100.5 (C-8), 75.8 (C-3), 73.4 (C-2), 63.8 (C-10), 54.8 (C-12), 42.9 (C-4a), 41.5 (C-9a), 37.1 (C-1), 31.1 (C-4), 26.8 (C-11)

Compound 2; red solid; UV (MeOH) λ_{max} : 218, 250, 292 and 351 nm ; FT-IR (ATR) λ_{max} 3408, 1716, 1622, 1454, cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) : δ 12.68 (1H, *s*, 8-OH), 7.86 (1H, *s*, 5-OH), 6.48 (1H, *s*, H-7), 5.12 (1H, *dd*, *J* = 4.9, 4.5 Hz, H-4), 4.81 (1H, *d*, *J* = 4.5Hz, 4-OH), 3.96 (3H, *s*, 12-OCH_3), 3.13 (1H, *dd*, *J* = 4.9, 17.6 Hz, H_b-2), 2.75 (1H, *m*, H-3); 2.61 (1H, *dd*, *J* = 17.6, 5.3 Hz, H_b-9), 2.49 (1H, *dd*, *J* = 17.6, 5.3 Hz, H_a-9), 2.31 (1H, *dd*, *J* = 17.6, 5.3 Hz, H_a-2), 2.12 (3H, *s*, 11-CH_3); ^{13}C NMR (75 MHz, $CDCl_3$) : δ 205.5 (C-10), 203.4 (C-1), 157.5 (C-8), 154.2 (C-6), 134.9 (C-5), 124.1 (C-4a), 105.6 (C-8a), 95.8 (C-7), 67.5 (C-4), 54.8 (C-12), 42.6 (C-9), 39.9 (C-2), 37.5 (C-3), 28.1 (C-11)

Compound 3; yellow solid ; UV (MeOH) λ_{max} : 229 and 321 nm ; FT-IR (ATR) λ_{max} 3004, 1653, 1612, 1584 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) : δ 12.72 (1H, s, 5-OH), 6.33 (1H, *d*, *J* = 2.0 Hz, H-8), 6.25 (1H, *d*, *J* = 2.0 Hz, H-6), 6.04 (1H, *s*, H-3), 3.88 (3H, *s*, OMe-7), 2.85 (1H, *sept*, *J* = 6.5 Hz, H-11), 1.32 (6H, *d*, *J* = 6.5 Hz, H-12,13); ^{13}C NMR (75 MHz, $CDCl_3$): δ 181.6 (C-4), 172.5 (C-2), 164.2 (C-7), 163.4 (C-5), 156.7 (C-9), 104.1 (C-3), 102.4 (C-13), 102.1 (C-10), 96.8 (C-6), 92.7 (C-8), 56.3 (OMe-6), 32.8 (C-11), 19.6 (C12,13)

Compound 4; colorless gum; UV (MeOH) λ_{max} : 265 nm ; FT-IR (ATR) λ_{max} 3400, 1713, 1682, 1154 cm^{-1} ; ^{1}H NMR (300 MHz, $CDCl_3$) δ : 11.12 (1H, *s*, 8-OH), 7.85 (1H, *s*, 4-OH), 6.48 (1H, *s*, H-7), 4.18 (1H, *brm*, H-3), 3.85 (3H, *s*, H-12), 2.15 (3H, *s*, H-10), 1.78 (3H, *s*, H-5), 0.91(3H, *d*, *J* = 6.4 Hz, H-9); ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.6 (C-1), 162.5 (C-2), 156.2 (C-8), 148.4 (C-4a), 114.7 (C-8a), 102.4 (C-4), 92.8 (C-5), 98.7 (C-7), 70.3 (C-3), 56.8 (C-OMe), 19.7 (C-10), 18.9 (C-9), 12.3 (C-11)

3. Results and Discussion

3.1 Identification of pure compounds

The methanol extract of the endophytic *Fusarium* sp. mycelia was purified by column chromatography and TLC to give four known compounds, altersolanol Q (**1**), 4-hydroxydihydronorjavanicin (**2**), 5-hydroxy-7-methoxy-2-isopropylchromone (**3**) and fusaraichromenone (4). The separated were identified by comparison of their spectroscopic data with past reported.

Compound 1 was obtained as a colorless solid. Compounds 1 showed UV absorbances at λ_{max} 223, 270 and 332 nm, similar to those of known altersolanol Q [12]. The IR spectrum showed absorption bands at 3207 (OH stretching), 1715 (C=O stretching) cm^{-1}. The ^1H NMR spectrum of **1** showed two aromatic protons at δ_H 6.96 (1H, *d*, *J* = 2.3 Hz, H-8) and 6.66 (1H, d, J = 2.3 Hz, H-6), two oxymethine protons at δ_H 4.91 (1H, *d*, *J* = 2.5 Hz, H-10) and 3.45 (1H, *dd*, *J* = 11.5, 4.5 Hz, H-3), one methoxy proton at δ_H 3.78 (3H, *s*, OCH_3-12) and two methine protons at δ_H 3.05 (1H, *td*, *J* = 12.0, 4.1 Hz, H-9_a) and 1.91 (1H, *tdd*, *J* = 12.2, 3.4, 2.5 Hz, H-4_a). Moreover showed two methylene protons at δ_H2.20 (1H, *td*, *J* = 12.2, 11.6 Hz, H-4_{ax}), 1.70 (1H, *ddd*, *J* = 12.2, 4.5, 3.4 Hz, H-4_{eq}) and 2.37 (1H, *dd*, *J* = 14.1, 4.1 Hz, H-1_{eq}), 1.30 (1H, *dd*, *J* = 14.4, 12.0 Hz, H-1_{ax}). Finally showed one methyl proton at δ_H 1.29 (3H, s, H-11). ^{13}C NMR data indicated that compound **1** features 16 carbon atoms. One carbonyl carbons at δ 203.7 (C-9), three oxymethine carbons at δ 75.8 (C-3), 73.4 (C-2) and 63.8 (C-10), two methane carbons at δ 42.9 (C-4a) and 41.5 (C-9a), two methylene carbons at δ 37.1 (C-1) and 31.1 (C-4), one methoxy carbon at δ 54.8 (C-12), one methyl carbon at 26.8 (C-11) and six tertiary carbons at δ 162.4 (C-7), 157.5 (C-5), 134.8 (C-8a), 125.3 (C-10a), 107.6 (C-6) and 100.5 (C-8). Based on the spectroscopic data and comparison with the literature reported compound **1** was elucidated to be **altersolanol Q** [12].

Compound 2 was obtained as a red solid. Compounds 2 showed UV absorbances at λ_{max} 218, 250, 292 and 351 nm, similar to those of known 4-hydroxydihydronorjavanicin [13]. The IR spectrum showed absorption for carbonyl groups at 1716 and 1622 cm^{-1} and the absorption band of O-H stretching at 3408 cm^{-1}. The ^1H NMR spectrum of 2 showed one chelated hydroxyl group at δ_H 12.68 (1H, *s*, 8-OH), one hydroxyl proton at δ_H 7.86 (1H, *s*, 5-OH), one aromatic, oxymethine, methoxy, methine, protons at δ_H 6.48 (1H, *s*, H-7), 3.96 (3H, *s*, 12-OCH_3), 2.75 (1H, *m*, H-3), 5.12 (1H, *dd*, *J* = 4.9, 4.5 Hz, H-4), respectively. Moreover showed two methylene protons at δ_H 3.13 (1H, *dd*, *J* = 17.6, 4.9 Hz, H_b-2), 2.31 (1H, *dd*, *J* = 17.6, 5.3 Hz, H_a-2) and 2.61 (1H, *dd*, *J* = 17.6, 4.9 Hz, H_b-9), 2.49 (1H, *dd*, *J* = 17.6, 5.3 Hz, H_a-9). Lastly, showed one methyl proton at δ_H 2.12 (3H, s, 11-CH_3). ^{13}C NMR data indicated that compound **2** features 12 carbon atoms. Two carbonyl carbons at δ 205.5 (C-10) and 203.4 (C-1), four oxymethine carbons at δ 157.5 (C-8), 154.2 (C-6), 134.9 (C-5) and 67.5 (C-4), two methylene carbons at 42.6 (C-9) and 39.6 (C-2), one methyl, methine, methoxy and aromatic carbons at δ 28.1 (C-11), 37.5 (C-3), 54.8 (C-12) and 95.8 (C-7), respectively. Moreover showed tertiary carbons at δ 124.1 (C-4a) and 105.6 (C-8a). From the spectroscopy spectrum and comparison with the other reported, compound **2** was **4-hydroxydihydronorjavanicin** [13].

Compound 3 was obtained as a yellow solid. The IR spectrum showed absorption band at 3004 cm^{-1} (OH stretching), 1653 cm^{-1} (C=O stretching) ^1H NMR spectrum of compound **3** showed one chelating hydroxyl proton at δ_H 12.72 (1H, *s*, 5-OH), two aromatic protons at
δ_H 6.33 (1H, *d*, *J* = 2.0 Hz, H-8) and 6.25 (1H, *d*, *J* = 2.0 Hz, H-6), each one olephenic, methoxy and methine proton at δ_H 6.04 (1H, *s*, H-3), 3.88 (3H, *s*, OCH_3-7) and 2.85 (1H, *sept*, *J* = 6.5 Hz, H-11), respectively. Finally, showed two methyl protons at δ_H 1.32 (6H, *d*, *J* = 6.5 Hz, H-12,13). ^{13}C NMR data indicated that compound **3** showed 13 carbon atoms. One carbonyl carbon at δ 181.6 (C-4), three oxyaromatic carbons at δ 164.2 (C-7), 163.4 (C-5) and 156.7 (C-9), two aromaticmethine carbons at δ 96.8 (C-6) and 92.7 (C-8), each one oxyolephenic, olephenic, methoxy and methine carbon at δ 172.0 (C-2), 104.1 (C-3), 56.3 (OCH_3-6) and 32.8 (C-11), respectively. Moreover showed two methyl carbons at 19.6 (C12,13) and one tertiary carbon at 102.1 (C-10). Based on the spectroscopic data and comparison with the reported literature, compound 3 was identified as **5-hydroxy-7-methoxy-2-isopropylchromone** [14].

Compound 4 was obtained as a colorless solid. The IR spectrum showed absorption bands at 3400 (OH stretching), 1713 (C=O stretching) cm^{-1}. The ^1H NMR spectrum of **4** showed two hydroxy proton at δ_H 11.12 (1H, S, 8-OH) and 7.85 (1H, S, 4-OH); one aromatic proton at δ_H 6.48 (1H, *s*, H-8), one methine protons at δ_H 4.18 (1H, *brm*, H-3). Moreover showed one methoxy proton at δ_H 3.85 (3H, *s*, H-12). Finally showed three methyl protons at δ_H 2.15 (3H, s, H-10); 1.78 (3H, s, H-11); 0.91 (3H, *d*, J = 6.4 Hz H-9). ^{13}C NMR data indicated that compound 4 feature 13 carbon atoms. One carbonyl carbons at δ 171.6 (C-1), two oxyaromatic carbon at δ 162.5 (C-6) and 156.2 (C-8), Four aromatic carbon at δ 148.4 (C-4a), 114.7 (C-8a), 92.8 (C-5) and 98.7 (C-7), one oxymethine carbon at δ 102.4 (C-4), one methine carbon at δ 70.3 (C-3), Moreover had one methoxycarbon and three methyl carbons at δ 56.8 (C-OCH_3), 19.7 (C-10), 18.9 (C-9) and 12.3 (C-11), respectively. Based on the spectroscopic data and compared to the past research, compound 4 was elucidated to be **fusaraichromenone** [15].

3.2 Antimicrobial activity

Antibacterial activity of isolated compounds exhibited against four bacteria, SA, MRSA SK1, PA and EC. In contrast, SA and MRSA SK1 compared with vancomycin as a positive control, while PA and EC compared with gentamicin as a positive control. The result showed that vancomycin inhibited bacteria SA and MRSA SK1 with the same MIC value 1 μg/mL (similar to the literature review that vancomycin inhibited SA with MIC value 1.5 μg/mL [16] and inhibited MRSA SK with MIC 2 μg/mL [17]. The result antimicrobial activities of isolated compounds showed in table 1. Compound 1 showed moderate activity towards SA, MRSA SK1 and PA with the same MIC value 16 µg/mL, but low activity towards EC with a MIC value 128 μg/mL. Compound 2 was not active against bacteria SA and MRSA SK1 with MIC value of more than 200 μg/mL, and weak activity to inhibit PA and EC with MIC value 200 μg/mL. Compound 3 showed moderate activity towards SA, MRSA SK and PA with the same MIC value 16 μg/mL, and inhibited EC with a MIC value 32 μg/mL. Compound 4 showed relatively low against SA with MIC value 32 μg/mL, but low activity against MRSA SK1, PA and EC with 128, 200 and 200 μg/mL, respectively. The anti-yeast activity of isolated compounds against CA90028 and CN90112 compared with amphotericin B as a positive control. The result showed that compound 3 showed moderate activity towards CA90028 and CN91112 with the same MIC value 32 μg/mL. But compounds 1, 2 and 4 showed weak activity to inhibit the growth of CA90028 and CN90112 with the same MIC value 128 μg/mL. While amphotericin B that as positive control, showed activity towards CA90028 and CN91112 with the same MIC value 0.5 μg/mL, that similar to the literature review that amphotericin B inhibited CA90028 with MIC value 1 μg/mL [18] and inhibited CN91112 with MIC 1 μg/mL [19].

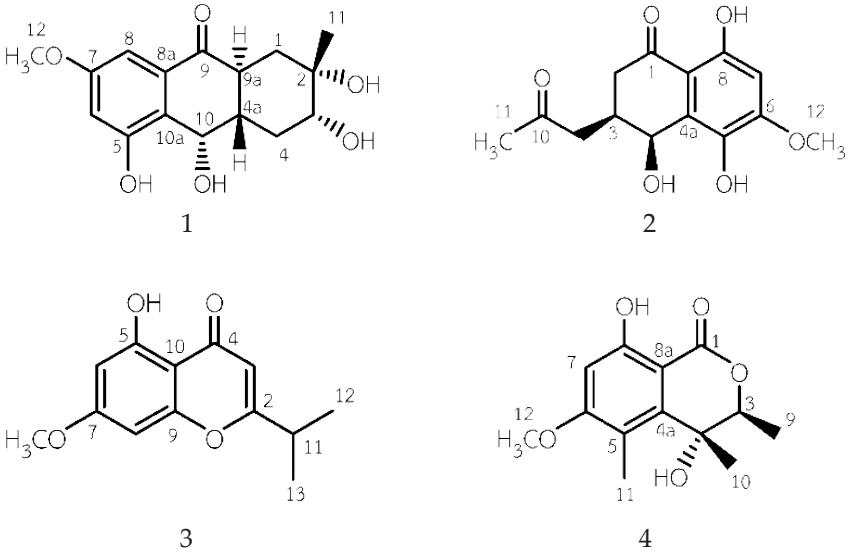

Figure 1. compounds 1-4

Table 1 Antimicrobial activity of compounds 1-4

compound	Bacteria								Yeasts			
	SA		MRSA SK1		PA		EC		CA 90028		CN 91112	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
1	16	200	16	>200	16	>200	128	>200	128	128	128	128
2	>200	>200	>200	>200	200	>200	200	>200	128	128	128	128
3	16	200	16	128	16	>200	32	>200	32	32	32	128
4	32	200	128	>200	200	>200	200	>200	128	128	128	>200
Vancomycin	1	2	1	1								
Gentamicin					2	4	2	4				
Amphotericin B									0.5	1	0.5	1

MIC = Minimal Inhibitory Concentration (μg/mL) MBC = Minimum Bactericidal Concentration (μg/mL)

4. Conclusions

Four known compounds were purified from the methanol extract of the fungus *Fusarium* sp Compounds 1, 2, 3, and assigned as altersolanol Q, 4-hydroxydihydronorjavanicin, 5-hydroxy-7-methoxy-2-isopropylchromone and fusaraichromenone, respectively. The antimicrobial activity of 1, 2, 3, and 4 was observed against SA, MRSA SK1, PA, EC, CN90112 and CA90028. The activity of compounds against SA and MRSA SK1 found similar results that compounds 1 and 3 showed moderate activity, while compounds 2 and 4 showed relatively low activity and were not active, respectively. Inhibition to PA found that compounds 1 and 3 showed moderate activity, while compounds 2 and 4 showed low activity. EC was best against compound 3, followed by 1, 2 and 4. Anti-yeast CN90112 and CA90028 showed less sensitivity against all isolated compounds.

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Conflicts of Interest: The authors declare no conflicts of interest.

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