New Polyketides from Endophytic Diaporthe sp. XZ-07

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Four new polyketides, namely *rel*-(2*R*,3*S*,4*R*,5*R*)-4-ethyltetrahydro-3-methyl-5-propylfuran-2,3-diol (1), methyl 5-[(1*R*)-1-hydroxyethyl]- γ -oxofuran-2-butanoate (2), butyl 5-[(1*R*)-1-hydroxyethyl]- γ -oxofuran-2-butanoate (3), and 3,4-dihydro-5'-[(1*R*)-1-hydroxyethyl][2,2'-bifuran]-5(2*H*)-one (4), together with three known ones, 5-[(1*R*)-1-hydroxethyl)- γ -oxofuran-2-butanoic acid (5), phomopsolide B (6), and (2*S*,3*S*)-3,6-dihydro-6-oxo-2-[(1*E*)-2-{(4*S*,5*S*)-2,2,5-trimethyl-1,3-dioxolan-4-yl]ethenyl}-2*H*-pyran-3-yl (2*E*)-2-methylbut-2-enoate (7), were isolated from the submerged cultures of the endophytic fungal strain *Diaporthe* sp. XZ-07 of *Camptotheca acuminate*. The structures of the new compounds were established by 1D- and 2D-NMR and MS data and by *Mosher*'s method. Compound 6 significantly inhibited the growth of human-tumor HeLa cells with an *IC*₅₀ of 5.7 µg/ml by the MTT assay (MTT=3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide).

Introduction. - Plant endophytes are a group of microorganisms, including fungi and bacteria, which not only live within plants' internal tissues or organs without causing any apparent symptoms or diseases in the host plants but also serve as important sources of bioactive compounds, presumably due to the symbiotic relationship with their hosts [1-3]. In the course of our study for chemical constituents from the endophytic microorganisms of medicinal plants, a series of new compounds were previously isolated [4-8]. Continuous research on the secondary metabolites from Diaporthe sp. XZ-07 - an endophytic fungal strain isolated from the surface-sterilized twig tissues of *Camptotheca acuminate* whose extract showed potent cytotoxic activity against human tumor cells [9] - led to the discovery of four new polyketides, rel-(2R,3S,4R,5R)-4-ethyltetrahydro-3-methyl-5-propylfuran-2,3-diol (1), methyl 5-[(1R)-1-hydroxyethyl]- γ -oxofuran-2-butanoate (2), butyl 5-[(1R)-1-hydroxyethyl]- γ -oxofuran-2-butanoate (3), 3,4-dihydro-5'-[(1R)-1-hydroxyethyl][2,2'-bifuran]-5(2H)-one (4), and three known ones, 5-[(1R)-1-hydroxyethyl]- γ -oxofuran-2-butanoic acid (5) [10], phomopsolide B (6) [11], and (2S,3S)-3,6-dihydro-6-oxo-2-{(1E)-2-[(4S,5S)-2,2,5trimethyl-1,3-dioxolan-4-yl]ethenyl-2H-pyran-3-yl (2E)-2-methylbut-2-enoate (7) [12][13]. In this article, we report the isolation and structure characterization of compounds 1-7 and antitumor and antimicrobial activities of 5-7.

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Results and Discussion. – Compound **1** was obtained as white powder. Its molecular formula was determined as $C_{10}H_{20}O_3$ by HR-ESI-MS (m/z 211.1309 ([M+Na]⁺), corresponding to one unsaturation degree. The IR absorptions at 3418 and 3313 cm⁻¹ indicated the presence of OH groups. The ¹³C-NMR spectra (*Table 1*) of **1** displayed ten C-atoms comprising one oxygenated quaternary C-atom (δ (C) 79.3), three CH (including two oxymethines at δ (C) 100.6 and 83.3), three CH₂ (δ (C) 39.2, 19.9, and 19.2), and three Me groups (δ (C) 18.8, 14.0, and 12.6). The ¹H-NMR spectra (*Table 1*) of **1** displayed two OH resonances at δ (H) 2.98 and 2.47. The ¹H,¹H-COSY cross-peaks δ (H) 2.98 (OH)/ δ (H) 4.81 (H–C(2)) indicated that this OH was connected with C(2) (δ (C) 100.6)²). The HMBC spectra showed correlations from both HO–C(2) and HO–C(3) to C(2) and C(3) (δ (C) 79.3), indicating the vicinal position of the two OH groups. In addition, H–C(2) showed an HMBC to the C-atom at δ (C) 83.3 (d), and Me(9) showed HMBCs to C-atoms at δ (C) 100.6, 79.3, and 51.8 (d), revealing the

Table 1.	^{1}H - and	¹³ C-NMR	Data (CDCh)	of 1^{2}).	δin	ppm, J	in Hz.
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	$\delta(\mathrm{H})$	$\delta(C)$	HMBC
H-C(2)	4.81 (s, H_{β})	100.6(d)	C(3), C(4), C(5), C(9)
C(3)		79.3 (s)	
H-C(4)	$1.72 - 1.77 \ (m, H_a)$	51.8(d)	C(3), C(5), C(6), C(10), C(11)
H-C(5)	$3.52 - 3.56 (m, H_{\beta})$	83.3 (<i>d</i>)	C(7), C(10)
$CH_{2}(6)$	1.55 - 1.60 (m)	39.2 (t)	C(4), C(5), C(7)
$CH_{2}(7)$	1.49-1.54, 1.32-1.38 (2m, overlapped)	19.2 (t)	
Me(8)	0.93 (t, J = 7.0)	14.0(q)	C(6), C(7)
Me(9)	1.19 (s, β -pos.)	18.8(q)	C(2), C(3), C(4)
$CH_{2}(10)$	1.49-1.54, 1.32-1.38 (2m, overlapped)	19.9 (t)	
Me(11)	1.03 (t, J = 7.4)	12.6(q)	C(4), C(10)
HO-C(2)	2.98 (br. s)		C(2), C(3)
HO-C(3)	2.47 (br. <i>s</i>)		C(2), C(3), C(9)

2) Arbitrary atom numbering; for systematic names, see Exper. Part.

presence of a tetrahydrofuran moiety. Moreover, with the molecular formula $C_{10}H_{20}O_3$ and two Me groups appearing as *t* in the ¹H-NMR spectrum, two side chains were recognized as an ethyl and propyl group. The CH₂(7) and CH₂(10) groups heavily overlapped at $\delta(H)$ 1.49–1.54 and 1.32–1.38 (2*m*); they were distinguished and assigned by the HMBCs Me(11)/C(4) ($\delta(C)$ 51.8) and C(10) ($\delta(C)$ 19.9), and Me(8)/ C(6) ($\delta(C)$ 39.2) and C(7) ($\delta(C)$ 19.2), and therefore, the ethyl substituent was positioned at C(4) and the propyl group at C(5).

The relative configuration of **1** was determined by the ROESY experiments. The ROESY correlations HO-C(3) (δ (H) 2.47)/H-C(4) and Me(9)/H-C(2) and H-C(5) suggested that HO-C(2), HO-C(3), and H-C(4) were α -oriented, and H-C(5) was β -oriented. Thus, compound **1** was determined to be *rel*-(2*R*,3*S*,4*R*,5*R*)-4-ethyltetrahydro-3-methyl-5-propylfuran-2,3-diol. The absolute configuration of **1** could be determined by chemical transformations, *e.g.*, by the *Mosher* method [14][15]. However, due to the limited amount of material available, we were unable to carry out those reactions.

Compound **2**, a colorless oil, had the molecular formula $C_{11}H_{14}O_5$, as determined by the HR-ESI-MS (m/z 249.0743 ([M + Na]⁺)). The IR spectra indicated the presence of OH groups (3430 cm⁻¹) and a C=O group (1736 cm⁻¹). The ¹H-NMR spectra of **2** (*Table* 2) displayed two Me groups, including one MeO group (δ (H) 3.65 (s)), two olefinic H-atoms (δ (H) 6.48 and 7.30 (t, J = 3.4)), and one H-atom at an oxygenated Catom. The ¹³C-NMR and DEPT spectra (*Table* 2) showed signals for four quaternary C-atoms, three CH, two CH₂, and two Me groups. Comparison of the ¹H- and ¹³C-NMR data of **2** with those of compound **5** revealed that **2** and **5** had very similar structures. The structure of **5** was elucidated from 2D-NMR data and showed NMR data and an optical rotation ($[a]_{D}^{22} = -16.0$ (c = 1.0, MeOH)) similar to those of the known 5-(1hydroxyethyl)- γ -oxofuran-2-butanoic acid ($[a]_{D}^{25} = -3.8$ (c = 0.3, CH₂Cl₂)) with unreported configuration [10]. The only difference between **2** and **5** was the replacement

	2		3		4	
	$\delta(H)$	$\delta(C)$	$\delta(H)$	$\delta(C)$	$\delta(H)$	$\delta(C)$
C(1)		167.9 (s)		174.4 (s)		179.3 (s)
$CH_{2}(2)$	2.70(t, J = 6.4)	28.4(t)	2.69(t, J = 6.6)	28.8(t)	2.67–2.71 (<i>m</i>)	29.6 (t)
$CH_{2}(3)$	3.17(t, J = 6.4)	33.7 (t)	3.14(t, J = 6.4)	33.7 (t)	2.48 - 2.53 (m)	27.3 (t)
C(4) or $H-C(4)$		189.1 (s)		189.2 (s)	5.53 (t, J = 5.9)	76.4(d)
C(5)		152.5 (s)		152.6 (s)		151.7 (s)
H-C(6)	7.30 (d, J = 3.4)	120.0(d)	7.31 $(d, J = 3.4)$	120.0(d)	6.46 (d, J = 3.2)	111.1(d)
H-C(7)	6.48 (d, J = 3.4)	108.6(d)	6.48 (d, J = 3.5)	108.6(d)	6.25 (d, J = 3.2)	106.8(d)
C(8)		164.9 (s)		164.9 (s)		160.6 (s)
H-C(9)	4.87 (q, J = 6.4)	64.3(d)	4.84 (q, J = 6.4)	64.3(d)	4.80(q, J = 6.5)	64.0(d)
Me(10)	1.50 (d, J = 6.6)	21.8(q)	1.51 (d, J = 6.7)	21.8(q)	1.47 (d, J = 6.2)	21.7(q)
$Me(1')$ or $CH_2(1')$	3.65(s)	52.2(q)	4.07(t, J = 6.5)	65.5(t)		
$CH_{2}(2')$			1.54 - 1.60 (m)	31.7(t)		
CH ₂ (3')			1.31 - 1.39(m)	20.1(t)		
Me(4')			0.91 (t, J = 7.4)	13.9 (q)		

Table 2. ¹*H*- and ¹³*C*-*NMR* Data (CD₃OD) of $2-4^2$). δ in ppm, J in Hz.

of the OH of carboxyl group by a MeO group, which was indicated by the upfield shift of C(1) from δ (C) 176.4 in 5 to δ (C) 167.9 in 2, and confirmed by the HMBC MeO/C(1).

The absolute configuration of **2** was determined by the modified *Mosher* method [14][15]. Due to the limited amounts of **2**, and the fact that **5** was obtained in relatively large amounts, we transformed **5** into **2** by the reaction with (diazomethyl)trimethylsilane (Me₃SiCHN₂) [16]. As the natural and semisynthetic **2** had identical optical rotations, the (*S*)-MTPA (MTPA-Cl = α -methoxy- α -(trifluoromethyl)benzeneacetyl chloride) and (*R*)-MTPA esters were prepared by the procedure described previously [17]. The absolute configuration of C(9) was determined to be (*R*) based on the values of $\Delta\delta$ ($\delta(S) - \delta(R)$) = +0.067 (Me(10)), -0.098 (H-C(7)), and -0.038 (H-C(6)). Therefore, compound **2** was established to be methyl 5-[(1*R*)-1-hydroxyethyl]- γ -oxofuran-2-butanoate. Accordingly, compound **5** was assigned as 5-[(1*R*)-1-hydroxyethyl]- γ -oxofuran-2-butanoic acid.

Compound **3** was obtained as colorless oil. The molecular formula $C_{14}H_{20}O_5$ was established by HR-ESI-MS (m/z 291.1209 ($[M + Na]^+$)). The ¹H- and ¹³C-NMR spectra of **3** (*Table 2*) closely resembled those of **2**, except for the disappearance of the MeO group and appearance of three CH₂ groups including a CH₂O, and one Me group. The correlations CH₂(1')/CH₂(2') and CH₂(3')/Me(4') were observed in the ¹H,¹H-COSY plots. The HMBCs Me(4') (δ (H) 0.91)/C(2') (δ (C) 31.7) and C(3') (δ (C) 20.1), and CH₂(1') (δ (H) 4.07)/C(1) (δ (C) 174.4), C(2'), and C(3') established the structure of **3** as shown. Additionally, the similarity of the optical rotation to that of **2** helped to identify **3** as butyl 5-[(1*R*)-1-hydroxyethyl]- γ -oxofuran-2-butanoate because the influence of the Bu *vs.* Me group on the optical activity might be negligible as they are far away from the chiral center.

Compound **4** was obtained as colorless oil. The HR-ESI-MS data indicated the molecular formula $C_{10}H_{12}O_4$ based on the $[M + Na]^+$ ion at m/z 219.0590. Compound **4** showed NMR data (*Table 2*) similar to those of **2**, except that the C(4)=O group (δ (C) 189.1) of **2** was replaced by an OCH group (δ (C) 76.4) in **4**. The HMBCs from H–C(4) (δ (H) 5.53)/C(1) (δ (C) 179.3) and C(3) (δ (C) 27.3), and CH₂(3) (δ (H) 2.48–2.53)/C(1), C(2), C(4) (δ (C) 76.4), and C(5) (δ (C) 151.7), along with the ¹H,¹H-COSY cross-peaks CH₂(2)/CH₂(3)/H–C(4), established the structure of a γ -lactone moiety, and the linkage between C(4) and C(5)²).

The ROESY data of **4** showed correlations $CH_2(3)/CH_2(4)$, and H-C(9)/Me(10). These data did not yet allow to assign the configuration of **4**. The absolute configuration at C(9) was assumed to be (*R*) from biosynthetic considerations, as **4** was possibly derived from **2** or *vice versa*. However, the configuration at C(4) could not be determined due to the minute amount of material available. Therefore, compound **4** was tentatively determined as 3,4-dihydro-5'-[(1*R*)-1-hydroxyethyl][2,2'-bifuran]-5(2*H*)-one.

The structures of compounds **6** and **7** were identified as phomopsolide B and (2S,3S)-3,6-dihydro-6-oxo-2-{(1E)-2-[(4S,5S)-2,2,5-trimethyl-1,3-dioxolan-4-yl]ethen-yl}-2H-pyran-3-yl (2E)-2-methylbut-2-enoate by comparing their NMR data and optical rotations **6**: $[\alpha]_D^{22} = +219.6$ (c = 0.38, MeOH) ([11]: $[\alpha]_D^{20} = +250$ (c = 0.202, MeOH), see also [12][13]); $[\alpha]_D^{18} = +116.1$ (c = 0.29, MeOH) ([13]: $[\alpha]_D^{22} = +120$ (c = 0.35, EtOH)). Compound **7** was reported as an intermediate of the total synthesis of **6**,

and it is considered to be an artifact in this study because acetone was used in experiments.

Biological Activities. – Compounds **5** and **6**, which were isolated in this study in relatively large amounts, were evaluated for their inhibitory activities against the growth of human tumor HeLa cells. Compound **5** showed no activities, but **6** exhibited strong cytotoxicity with an IC_{50} of 5.7 µg/ml (0.019 µm/ml) (IC_{50} of the positive control (*cis*-platin), 3.5 µg/ml (0.012 µm/ml)).

Compounds **5**–**7** were tested for their antibacterial and antifungal activities against *Shigella dysenteriae*, *Candida albicans*, *Saccharomyces cerevisiae*, and *Penicillium avellaneum* UC-4376 by the disk diffusion method with rifampicin and nystatin as positive controls, respectively. At the concentration of 50 μ g/disk, compound **6** showed an inhibitory zone with a diameter of 1.4 cm against *S. dysenteriae* after incubation for 6 h at 37°, but **6** had no antifungal activities. Compounds **5** and **7** were inactive against all organisms, and the positive control, rifampicin, showed antibacterial activities against *S. dysenteriae* with an inhibitory zone of 2.1 cm, and nystatin showed antifungal activities against *C. albicans*, *S. cerevisiae*, and *P. avellaneum* UC-4376 with inhibitory zones of 2.5, 2.1, and 3.1 cm, respectively.

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Experimental Part

General. Liquid fermentation: GUJT series semi-prep. stainless-steel fermentor with mechanical agitator (501; Zhenjiang East Biotech Equipment and Technology Co., Ltd., China). (Diazomethyl)-trimethylsilane (=trimethylsilyl)diazomethane) was purchased from Aldrich. (+)-(R)- and (-)-(S)-a-methoxy-a-(trifluoromethyl)benzeneacetyl chloride (MTPA-Cl) were bought from Alfa Aesar. The Cell Proliferation Kit I was from Roche Diagnostics. TLC: silica gel GF_{254} (SiO₂; 10–40 µm; Qingdao). Prep. TLC (1.0–1.5 mm): precoated SiO₂ GF_{254} (10–40 µm; Qingdao) glass plates (10 × 20 or 20 × 20 cm); visualization under UV light. Column chromatography (CC): SiO₂ H (200–300 mesh or 10–40 µm; Qingdao), Sephadex LH-20 (Amersham Pharmacia, Sweden), and RP-18 (reversed-phase C_{18}) SiO₂ (40–63 µm; Merck, Germany). Optical rotations: Jasco-DIP-370 digital polarimeter. UV Spectra: Shimadzu-2401PC spectrophotometer; λ_{max} (log ε) in nm. IR Spectra: Bio-Rad-FTS-135 spectrophotometer; KBr discs; in cm⁻¹. 1D- and 2D-NMR Spectra: Bruker AM-400 and -DRX-500 instruments, resp.; chemical shifts δ in ppm rel. to Me₄Si, coupling constants J in Hz. ESI- and HR-ESI-MS: Finnigan-LCQ-Advantage and VG-Auto-Spec-3000 mass spectrometers, resp.; in m/z.

Microbial Material. The fungal strain XZ-07 was isolated from the surface-sterilized twig of *C. acuminata* collected from the Shaowujiangshi National Nature Conservation District, Fujian Province, P. R. China, by methods described previously [9]. It was identified as a nonsporulating fungus by traditional morphology. Internal transcribed spaces (ITS) of strain XZ-07 were cloned and sequenced (GenBank accession number: DQ272498). The blast search result showed that the ITS sequence of strain XZ-07 was highly homologous to species of the genus *Diaporthe*. The strain XZ-07 was inoculated in a 5 1 *Erlenmeyer* flask containing 1.5 1 of GPY medium (consisting of glucose (10 g/l), peptone (2 g/l), and yeast extract (0.5 g/l); pH 7.0) for 1-2 d at 28° to afford seed cultures. Then, the seed was inoculated in the fermentor with 30 l of GPY medium for 7 d at 28° and 200 rpm.

Extraction and Isolation. The culture was filtered through gauze. The filtrate was extracted five times with an equal volume of AcOEt. The org. solvent was evaporated at 45° to afford a residue (4.5 g) which was subjected to medium-pressure liquid chromatography (*RP-18* SiO₂ (145 g), H₂O, then 30, 50, and 70% MeOH/H₂O, then MeOH (21 for each gradient): *Fractions 1–6. Fr. 2* (809 mg) was divided into

eight subfractions, *Fr.* 2.1 – *Fr.* 2.8, by CC (SiO₂ *H* (200–300 mesh), CHCl₃/MeOH gradient). Further separations by repeated CC (SiO₂ *H* (10–40 μ m), prep. TLC (CHCl₃/MeOH), and CC (*Sephadex LH*-20, MeOH) yielded **2** (2 mg), **4** (2 mg), and **5** (10 mg). *Fr.* 3 (800 mg) was subjected to CC (SiO₂, gradient CHCl₃/AcOEt): *Fr.* 3.1 – *Fr.* 3.7. After purification of *Fr.* 3.3 by CC (SiO₂, CHCl₃/MeOH 50:1), **6** (12 mg) was obtained. Compounds **1** (1 mg) and **3** (3 mg) were isolated from *Fr.* 4 (191 mg) by repeated CC (SiO₂ *H* (200–300 mesh or 10–40 μ m) and prep. TLC (CHCl₃/MeOH) and purified by CC (*Sephadex LH*-20, acetone). *Fr.* 5 (554 mg) was subjected to CC (SiO₂, gradient petroleum ether/AcOEt, twice): **7** (4 mg).

Mosher *Esters of* **2**. To a soln. **2** (5 mg) in anh. pyridine (0.25 ml) was added either (*S*)- or (*R*)-MTPA-Cl (7 μ). The mixture was stirred at 30° for 12 h, evaporated, and purified by CC (*Sephadex LH-20*, acetone).

rel-(2R,3S,4R,5R)-4-Ethyltetrahydro-3-methyl-5-propylfuran-2,3-diol (1). White powder. $[\alpha]_{\rm D}^{18} = -81.0 \ (c = 0.08, \ {\rm CHCl}_3). \ {\rm UV} \ ({\rm CHCl}_3): 222 \ (2.56), 235 \ (2.47), 256 \ (2.57). \ {\rm IR}: 3418, 3313, 2960, 2931, 2873, 1465. \ 1377, 1028, 1007. \ {\rm 'H-} \ {\rm and} \ {\rm ^{13}C-NMR}: \ Table 1. \ {\rm HR-ESI-MS} \ ({\rm pos.}): 211.1309 \ ([M+{\rm Na}]^+, {\rm C}_{10}{\rm H}_{20}{\rm NaO}_3^+; \ {\rm calc.} \ 211.1310).$

Methyl 5-[(1R)-1-Hydroxyethyl]- γ -oxofuran-2-butanoate (**2**). Colorless oil. $[\alpha]_{18}^{18} = -54.0 \ (c = 0.1, MeOH)$. UV (MeOH): 202 (3.69), 280 (3.65). IR: 3430, 2926, 2853, 1736, 1674, 1439, 1369, 1031. ¹H- and ¹³C-NMR: *Table* 2. HR-ESI-MS (pos.): 249.0743 ($[M + Na]^+$, C₁₁H₁₄NaO⁺₅; calc. 249.0738).

Butyl 5-[(1R)-1-Hydroxyethyl]-γ-oxofuran-2-butanoate (**3**). Colorless oil. $[a]_{15}^{18} = -45.1$ (c = 0.31, MeOH). UV (MeOH): 200 (3.98), 279 (4.07). IR: 3434, 2961, 2933, 1734, 1676, 1519, 1383, 1173, 1079, 1026. ¹H- and ¹³C-NMR: *Table 2.* HR-ESI-MS (pos.): 291.1209 ([M + Na]⁺, C₁₄H₂₀NaO₅⁺; calc. 291.1208).

3,4-Dihydro-5'-[(1R)-1-Hydroxyethyl][2,2'-bifuran-5(2H)-one (**4**). Colorless oil. $[a]_{\rm B}^{\rm 18} = -54.5$ (c = 0.11, MeOH). UV (MeOH): 209 (3.51), 262 (2.90). IR: 3435, 2926, 2872, 1777, 1451, 1384, 1188, 1058. ¹H- and ¹³C-NMR: *Table 2*. HR-ESI-MS (pos.): 219.0590 ($[M + Na]^+$, C₁₀H₁₂NaO₄⁺; calc. 219.0633).

Bioassays. Cytotoxic activities were measured by the MTT method with *cis*-platin as positive control [18] [19] (MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). HeLa cells were plated in the 96-well plate at the density of 5000 cells per well and incubated for 24 h at 37° before treatment. After treatment with varying concentrations of tested compounds for 72 h, cell proliferation was analyzed by the *Cell Proliferation Kit I* (MTT) according to the manufacturer's instructions. The optical densities were measured at 570 nm with a microplate reader. All assays were done in triplicate. Growth-inhibition rate was calculated by the following equation: inhibition rate = $(OD_{control well} - OD_{treated well})/OD_{controwell} \times 100\%$. *IC*₅₀ is defined as the concentration of compounds that resulted in 50% inhibition of growth rate.

The antibacterial and antifungal activities were assessed against *S. dysenteriae*, *C. albicans*, *S. cerevisiae*, and *P. avellaneum* UC-4376 at the concentration of 50 μ g/disk, by means of the disk diffusion assay on agar plates, as described previously [20][21]. The experiments were performed in triplicate, and the results are presented as mean values of the three measurements.

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