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A new phthalazinone derivative and a new isoflavonoid glycoside from lichen-associated *Amycolatopsis* sp.

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ABSTRACT

A new phthalazinone derivative, named amycophthalazinone A (1), and a new isoflavonoid glycoside, 7-*O*-methyl-5-*O*-α-L-rhamnopyranosylgenestein (2), along with an isoflavonoid glycoside, 7-*O*-α-D-arabinofuranosyl daidzein (3) firstly found from natural sources, and eight known compounds (4–11), were isolated from the culture broth of the lichen-associated *Amycolatopsis* sp. YIM 130642. The structures of new compounds were elucidated on the basis of spectroscopic analysis. Compound 1 was the first example of naturally occurring phthalazinone derivative. The antimicrobial activities of all compounds towards five pathogenic strains were evaluated by a broth microdilution assay. Compound 1 exhibited the most potent inhibitory activity against *Staphylococcus aureus*, *Salmonella typhi*, and *Candida albicans* with MIC values of 32, 32, and 64 μg/mL, respectively.

1. Introduction

Actinobacteria has long been regarded as one of the important resources of bioactive metabolites in discovery of natural drugs [1]. Among them, the genus of *Streptomyces* is the main contributor of producing antibiotics in the past several decades. Over time, the ratio of bioactive substances from “rare actinomycetes” had been gradually increased [1]. The genus of *Amycolatopsis* is characterized as a rare actinomycete group. Some *Amycolatopsis* species had been reported to produce various structural types of antibiotics, such as glycopeptides [2], cyclic thiazolyl peptides [3], vancoresmycin [4], pargamicin A [5], and rifamorpholines [6]. Most notably, vancomycin and rifamycin with strong antibacterial activity produced by *Amycolatopsis* sp. had been used as the common clinical antibiotic drugs.

Lichen is a symbiotic community between fungi and a photosynthetic alga and/or cyanobacteria [7]. Most lichens are promising sources of specific secondary metabolites possessing antioxidant, cytotoxic, and antimicrobial activities [7,8]. Up to now, very little study on the chemical components of the lichen *Squamarina* had been reported. A naphthoquinone named squamarone was isolated from the lichen *Squamarina cartilaginea* [9]. Psoromic acid derived from *Squamarina cartilaginea* showed potent antibacterial activity against the oral pathogens *Streptococcus gordonii* and *Porphyromonas gingivalis* [10]. So far, a number of publications have been reported on the cultivable bacterial associated with lichens. However, only a few structurally identified bioactive molecules are reported from several strains, especially most of which are *Streptomyces* species [11]. In addition, several 21-membered macrocyclic benzenoid ansamycins with antiproliferative and anti-neuroinflammatory activity were isolated recently from moss-soil-derived actinomycete *Streptomyces cacaoi* subsp. *asoensis* H2S5 [12]. Recent studies revealed that lichen-associated bacteria represent a potential but under explored resource for discovery of new bioactive natural products [11,13].

Our current research work is focused on investigating bioactive secondary metabolite produced by lichen-associated actinobacteria. During the course of our bioactive screening for the culture extract of actinomycete strains isolated from lichens growing in Yunnan dry-hot valley region, the strain *Amycolatopsis* sp. YIM 130642 showed potent antimicrobial activity against several pathogens. Further phytochemical studies on the culture broth of this strain led to the isolation of one new phthalazinone derivative, amycophthalazinone A (**1**), and a new isoflavonoid glycoside, 7-*O*-methyl-5-*O*-α-L-rhamnopyranosylgenestein (**2**), along with an isoflavonoid glycoside 7-*O*-α-D-arabinofuranosyldaidzein (**3**) firstly found from natural sources, and eight known compounds, prunetin (**4**) [14], kakkatin (**5**) [15], isoformononetin (**6**) [16], genistein (**7**) [17], formononetin (**8**) [18], turnagainolide B (**9**)

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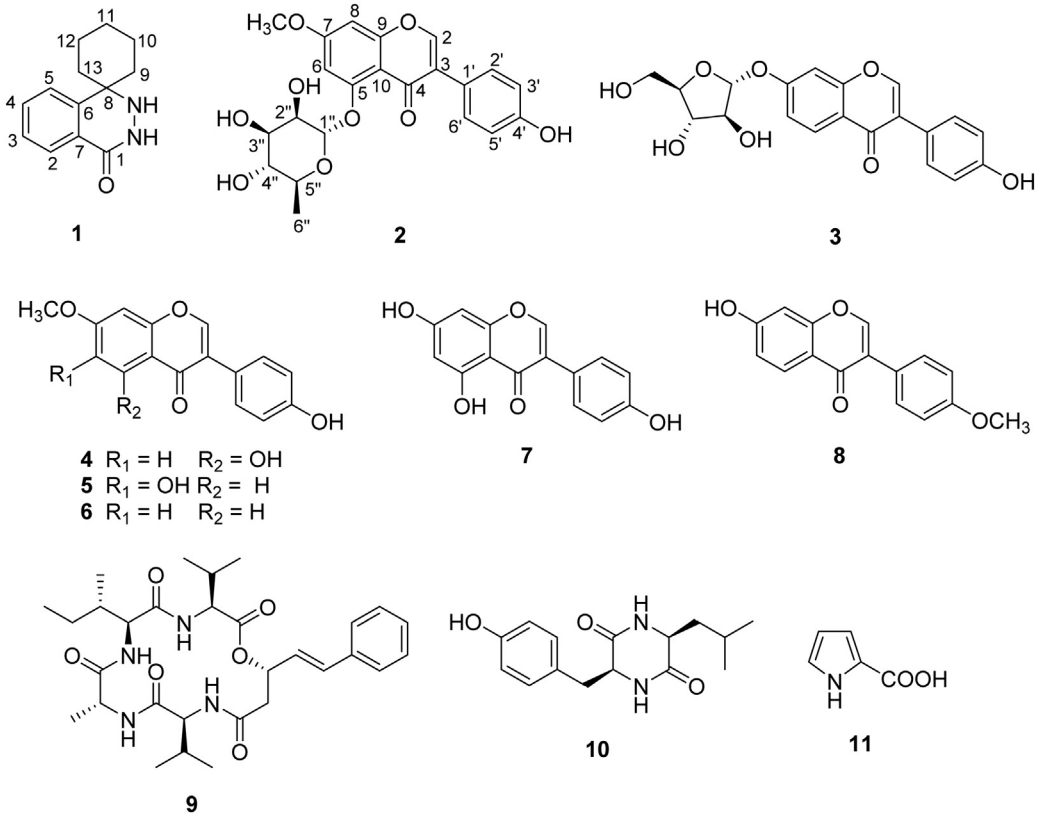

Fig. 1. Chemical structures of compounds **1–11**.

[19], cyclo (L-Leu-L-Tyr) (**10**) [20], minaline (**11**) [21] (Fig. 1). Recent research revealed that genistein could inhibit α-glucosidase *in vitro*, more potent than the positive control, acarbose, and minaline could inhibit the growth of radish (*Raphanus sativus*) seedlings, which was comparable to the positive control glyphosate [22]. So far, a number of bioactive substances had been isolated from *Amycolatopsis* strains derived from various habitats, whereas there is no any report of secondary metabolites from lichen-associated *Amycolatopsis* species till now. In this paper, we describe the isolation, structural elucidation, and antimicrobial activities of the isolated compounds from the strain *Amycolatopsis* sp. YIM 130642.

2. Experimental

2.1. General experimental procedures

Optical rotations were recorded on a Jasco P-2000 polarimeter. UV spectra were detected on a U-4100 spectrophotometer. IR spectra were collected on a Bio-Rad FTS spectrometer, with KBr pellets. NMR spectra were recorded on a Bruker DRX-500 and Bruker *Avance III-HD 600* NMR spectrometers, using TMS as an internal standard. HRESIMS were acquired by an Agilent 3250AA LC-MSD TOF mass spectrometer. Column chromatography (CC) was employed on silica gel (200–300 mesh, Qingdao Marine Chemical Inc., China), Sephadex LH-20 (Pharmacia), and RP-18 silica gel (40–63 μm, Merck).

2.2. Cultivation of actinomycete strain

The strain YIM 130642 was isolated from the lichen *Squamarina* sp. collected in dry and hot valley region of Jinsha River, Yunnan Province. The specimen of the lichen was identified by Prof. Lisong Wang, Kunming Institute of Botany, The Chinese Academy of Sciences, Kunming, Yunnan, China. The strain was determined as *Amycolatopsis* sp. by the partial 16S rRNA gene sequence and deposited in Yunnan Institute of Microbiology, Yunnan University, China. The fresh mycelium of the strain was inoculated into 100 × 250 mL Erlenmeyer flasks, each containing 40 mL of seed medium (yeast extract, 4 g; glucose, 4 g; malt extract, 3 g; trace salt 1 g; multi-vitamins 3.5 mg; H_2O, 1 L; pH 7.2). After 3 d of the incubation at 28 ± 1 °C on a rotary shaker at 200 r.p.m., each 40-mL of the culture liquid was transfered as seed into 2 L Erlenmeyer flasks (× 100) containing 400 mL of fermentation medium (soybean extract, 20 g; peptone, 2 g; glucose, 20 g; starch, 5 g; yeast extact, 2 g; NaCl, 4 g; K_2HPO_4, 0.5 g; $MgSO_4{\cdot}7H_2O$, 0.5 g; $CaCO_3$, 2 g; H_2O, 1 L; pH 7.8). The cultivation was kept for 7 days at 28 ± 1 °C on a rotary shaker at 200 r.p.m.

2.3. Extraction and isolation

The cultures were extracted with an equal volume of EtOAc for four times. The combined organic phase was then concentrated *in vacuo* to obtain a brown gum of crude extract (34 g). The extract was submitted to silica gel CC, eluting with a gradient of $CHCl_3$/MeOH from 1:0 to 0:1 (*v*/v) to obtain eight fractions (Frs. 1–7). Fr. 2 was purified by crystallization to get compound **4** (440 mg). Fr. 3 was subjected to silica gel CC (petroleum ether/EtOAc 4:1 → 1:1) to afford two subfractions (Frs. 3.1–3.2). Fr. 3.1 was further purified by silica gel CC (petroleum ether/acetone 6:4, 1:1) to afford compound **5** (15 mg). Compound **11** (17 mg) was yielded by crystallization from Fr. 3.2. Fr. 4 was subjected to silica gel CC ($CHCl_3$/MeOH 9:1) to afford compound **9** (15 mg). Fr. 5 was purified by silica gel CC ($CHCl_3$/MeOH 9:1, 85:15) and RP-18 CC (MeOH/H_2O, 3:7, 4:6, 1:1) to afford compounds **1** (7 mg) and **10** (10 mg). Fr. 6 was seperated by RP-18 CC, eluting with a gradient of MeOH/H_2O from 2:8 to 1:0 to afford three subfractions (Frs. 6.1–6.3). Fr. 6.1 was then purified by crystallization to obtain compound **3** (15 mg). Fr. 6.2 was subjected to RP-18 CC (MeOH/H_2O 3:7, 4:6, 1:1) to afford compounds **6** (5 mg) and **8** (5 mg). Fr. 6.3 was purified by Sephadex LH-20 (MeOH) to provide compound **2** (8 mg). Fr. 7 was subjected to silica gel CC ($CHCl_3$/MeOH 9:1, 8:2) to yield compound **7**

Table 1
^{1}H and ^{13}C NMR data of compounds **1** and **2**.

Position	1[a]		2[b]	
	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C
1		164.3, C		
2	8.35, d (7.5)	128.3, CH	7.90, s	151.4, CH
3	6.81, t (7.5)	117.4, CH		126.0, C
4	7.28, t (7.5)	133.5, CH		176.0, C
5	7.04, d (8.0)	114.9, CH		157.3, C
6		147.5, C	6.64, d (2.4)	100.9, CH
7		116.0, C		164.2, C
8		68.6, C	6.63, d (2.4)	94.4, CH
9	1.94–2.01, m	38.1, CH_2		160.0, C
10	1.60–1.65, m	21.7, CH_2		110.0, C
11	1.27–1.31, m	25.1, CH_2		
12	1.60–1.65, m	21.7, CH_2		
13	1.94–2.01, m	38.1, CH_2		
1′				122.9, C
2′, 6′			7.22, dd (7.2, 2.4)	130.3, CH
3′, 5′			6.72, dd (7.2, 2.4)	114.8, CH
4′				157.2, C
1″			5.42, d (1.2)	99.3, CH
2″			4.08, dd (3.6, 1.8)	70.4, CH
3″			3.99, dd (9.6, 3.6)	70.6, CH
4″			3.36, t (9.6)	72,5, CH
5″			3.58, m	69.9, CH
6″			1.12, d (6.0)	16.6, CH
7-OCH_3			3.81, s	55.1, CH_3
1-NH	7.13, br s			
8-NH	8.73, br s			

[a] Measured in pyridine-d_5.
[b] Measured in CD_3OD.

(4 mg).

*2.3.1. Amycophthalazinone A (**1**)*

Yellow powder; UV (MeOH) λ_{max} (log ε) 272 (1.60), 350 (2.47) nm; IR (KBr) ν_{max} 3432, 2923, 2851, 1644, 1612, 1507, 1485, 1383, 1269, 1148, 1041, 754 cm^{-1}; ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data, see Table 1; HRESIMS *m/z* 217.1344 $[M + H]^+$, 239.1159 $[M + Na]^+$ (calcd for $C_{13}H_{16}N_2ONa$, 239.1160).

*2.3.2. 7-O-Methyl-5-O-α-L-rhamnopyranosylgenestein (**2**)*

Yellow gum; $[\alpha]_D^{20}$ − 21.7 (*c* 0.13, MeOH); UV (MeOH) λ_{max} (log ε) 202 (2.51), 258 (3.68) nm; IR (KBr) ν_{max} 3443, 1628, 1516, 1426, 1255, 1119, 1053, 1023 cm^{-1}; ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR data, see Table 1; HRESIMS *m/z* 453.1168 $[M + Na]^+$ (calcd for $C_{22}H_{22}O_9Na$, 453.1162).

2.4. Antimicrobial assays

Antimicrobial assays were performed by the broth microdilution method in 96-well sterilized microplates as described previously [23]. The yeast *Candida albicans* (YM 2005) was grown in Sabouraud dextrose broth medium for 48 h at 28 °C with the test concentration of 1.0×10^5 spores/mL. The bacterial strains *Staphylococcus aureus* (YM 3105), methicillin-resistant *Staphylococcus aureus* (MRSA, YM 3106), *Salmonella typhi* (YM 3115), and *Escherichia coli* (YM 3130) were grown in LB medium (1.0% tryptone, 1.0% NaCl, 0.5% yeast extract, pH 7.0) for 18 h at 37 °C, and the final suspensions were adjusted to 1.0×10^5 colony-forming units/mL. Each of 50 μL inoculum suspension was added into the wells of 96-well plates. The test compounds were dissolved in a small volume of sterile DMSO and diluted in the appropriate medium to afford the final concentrations ranging from 512.0 to 1 μg/mL using 2-fold serial dilution method. The plates were incubated at 28 °C (48 h) for yeast and 37 °C (24 h) for bacteria. Nystatin and chloramphenicol **were** used as positive control for yeast and bacteria, respectively. The minimal inhibitory concentrations (MICs) were determined as the lowest concentrations at which the microorganism did not demonstrate visible growth. The experiments were repeated three times.

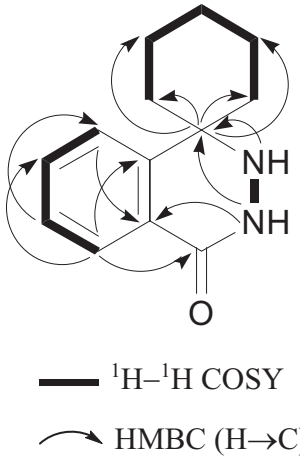

Fig. 2. Selected ^{1}H–^{1}H COSY and HMBC correlations of compound **1**.

3. Results and discussion

Compound **1** was obtained as a yellow powder. Its molecular formula was determined to be $C_{13}H_{16}N_2O$ by the positive HRESIMS ion at *m/z* 239.1159 $[M + Na]^+$ (calcd 239.1160), indicating seven degrees of unsaturation. The ^{1}H NMR spectrum (Table 1) showed four aromatic protons, including two doublet signals at δ_H 7.04 (*J* = 8.0 Hz) and 8.35 (*J* = 7.5 Hz), two triplet signals at δ_H 6.81 (*J* = 7.5 Hz) and 7.28 (*J* = 7.5 Hz). Interpretation of the ^{1}H-^{1}H COSY spectrum (Fig. 2) revealed that these four protons were linked together, indicating an *ortho-disubstituted* aromatic ring. The ^{13}C NMR and HSQC spectra displayed 13 signals corresponding to one carbonyl carbon (δ_C 164.3), six olefinic carbons (δ_C 147.5, 133.5, 128.3, 117.4, 116.0, 114.9), one aliphatic quaternary carbon (δ_C 68.6), five aliphatic methylenes (δ_C 25.1, each two signals at δ_C 38.1 and 21.7). The two exchangeable protons at δ_H 8.73 and 7.13 observed in the original ^{1}H NMR spectrum were shifted to δ_H 8.71 and 7.21, respectively, which were overlapped by the solvent residual signals of pyridine-d_5 during the 2D-NMR experiment (see Supplementary materials). These two exchangeable protons were coupled with each other in the ^{1}H-^{1}H COSY spectrum, indicating the existence of acylhydrazine unit (O = C-NH-NH-) due to its atomic composition. This was further supported by the strong absorption bands for NH group (3432 cm^{-1}) and conjugated carbonyl group (1644 cm^{-1}). Since seven degrees of unsaturation were accounted for, it was implied that compound **1** should contain two additional rings except for a carbonyl group and a benzene ring. The coupling sequence of the aliphatic protons from H-9 to H-13 could be distinguished by tracking correlations in the ^{1}H-^{1}H COSY spectrum. In the HMBC spectrum (Fig. 2), the quaternary carbon at δ_C 68.6 (C-8) showed strong long-range correlations with two pairs of methylene protons at δ_H 1.94–2.01 (H-9, 13) and 1.60–1.65 (H-10, 12), indicating the cyclohexane ring consisted of C-8 and five methylene groups. The weak HMBC correlations from two extrangable protons at δ_H 8.71 and 7.21 to C-8 implied the linkage of -NH-NH- group with C-8. Furthermore, the key HMBC correlations could be observed from H-2 (δ_H 8.35) to C-1 (δ_C 164.3), C-4 (δ_C 133.5), and C-6 (δ_C 147.5), and from H-5 (δ_H 7.04) to C-1, C-3 (δ_C 117.4), and C-7 (δ_C 116.0). These above data readily confirmed that compound **1** had a phthalazinone skeleton bearing a spiro cyclohexane at C-8. Thus, its structure was formulated as in Fig. 1 and given its trivial name of amycophthalazinone A.

Compound **2** was obtained as a yellow gum with the molecular formula $C_{22}H_{22}O_9$, deduced from HRESIMS ($[M + Na]^+$ *m/z* 453.1168,

calcd 453.1162), indicating twelve degrees of unsaturation. The IR spectrum exhibited absorption bands for hydroxy group at 3443 cm^{-1} and carbonyl group at 1628 cm^{-1}. The ^1H NMR spectrum showed seven aromatic proton signals at δ_H 7.90 (1H, s), 7.22 (2H, dd, J = 7.2, 2.4 Hz), 6.72 (2H, dd, J = 7.2, 2.4 Hz), 6.64 (1H, d, J = 2.4 Hz), 6.63 (1H, d, J = 2.4 Hz); eight proton signals attributable to a sugar moiety at δ_H 5.42 (1H, d, J = 1.2 Hz), 4.08 (1H, dd, J = 3.6, 1.8 Hz), 3.99 (1H, dd, J = 9.6, 3.6 Hz), 3.58 (1H, m), 3.36 (1H, t, J = 9.6 Hz), 1.12 (1H, d, J = 6.0 Hz), and one methoxy group at δ_H 3.81 (3H, s). It is notable that the aromatic proton signal at δ_H 7.90 (1H, s) is characteristics of H-2 in isoflavonoid skeleton. The ^{13}C NMR and DEPT spectra showed 22 carbon signals including 15 carbons for the isoflavonoid aglycone and 6 carbons for a sugar moiety, along with a methoxy carbon (δ_C 55.1). Its ^1H and ^{13}C NMR data showed strong resemblance with those of reported isoflavonoid glycosides [24]. The main difference is owing to the substitution position of the sugar moiety and the methoxy group. The quaternary carbon at δ_C 160.0 was assigned to be C-9 based on its HMBC correlation with H-2. The two aromatic protons at δ_H 6.64 and 6.63 were coupled with a small constant of J = 2.4 Hz, indicating that they are in meta position of aromatic ring. These two protons were determined as H-6 (δ_H 6.64) and H-8 (δ_H 6.63), respectively, due to both of their long-range correlations with C-10 and the only correlation from H-8 to C-9. The two quaternary carbons at δ_C 157.3 and 164.2 were positioned in C-5 and C-7, respectively, on the basis of the HMBC correlations from H-6 and H-8 to C-7, and from H-6 to C-5. Since the unambiguous assignment of H-6, H-8, C-5 and C-7, the location of glycosylation and the methoxy group could be easily determined by analysis of the HMBC spectrum. The HMBC cross peaks were observed from the anomeric proton H-1″ (δ_H 5.42) to C-5 and from the methoxy protons at δ_H 3.81 to C-7. Thus, the glycosylation and the methoxy group were sited at C-5 and C-7, respectively. The chemical shifts of the sugar signals and the small coupling constant of J = 1.2 Hz for the anomeric proton clearly suggested the *α*-rhamnopyranoside moiety. Acid hydrolysis of compound **2** refluxed with 2 N HCl for 2 h afforded L-rhamnose ($[\alpha]_D^{20}$ + 9.8, H_2O). The L-conformation of the rhamnose was confirmed by its positive specific rotation. Therefore, the structure of compound **2** was determined to be 7-*O*-methyl-5-*O*-α-L-rhamnopyranosylgenestein.

To the best of our knowledge, the phthalazinone skeleton in **1** has not been encountered in natural products previously, although a few synthetic compounds with similar substructures have been reported [25,26]. The related structures of phthalazine and tetrahydrophthalazine, containing a hydrazine moiety in the ring system, are incorporated into important bioactive compounds, including examples of pseudopeptides and C-nucleosides [25]. Many synthetic derivatives containing benzoylhydrazine skeleton had been reported. Some of them possess a wide range of biological activities, such as leishmanicidal activity [27], antiangiogenic activity [28], antichagasic activity [29], insecticidal activity [30], and antifungal activity [31]. Compound **1** was the first example of naturally occurring phthalazinone derivative.

Isoflavonoids were commonly occurred in various microorganism sources, mainly obtained from *Streptomyces*. Recent reported examples included isoflavone rhamnopyranosides from soil-derived *Streptomyces* [24,32], chlorinated isoflavonoids from soil-derived *Streptomyces* [33–35], and isoflavonoid glycosides from termite-associated *Streptomyces* [36,37]. In this paper, we firstly report the phthalazinone derivative and isoflavonoids from the actinomycete *Amycolatopsis*.

The naturally occurring hydrazine compounds are rarely observed, most of which are derived by synthetic approach. A number of structurally related cyclic depsipeptides bearing such group had been reported, *e.g.* polyoxypeptins [38], aurantimycins [39], citropeptin [40], kettapeptin [41], azinothricin [42], diperamycin [43], pipalamycin [44], variapeptin [45], and pargamicins [46,47]. As described in literature [48], the hydrazine moiety in these peptides was formed possibly by the conversion of ornithine into piperazic acid *via* oxidation to a nitroso species, followed by cyclization and reduction. Nevertheless, the detailed biosynthetic mechanisms for the formation of piperazic acid are still not well understood currently [49]. Similarly, in our study, some peptides and amino acid, such as compounds **9–11**, were also isolated. Therefore, a similar conversion from amino group to hydrazine moiety was also possibly existed in this microbial strain. However, more extensive investigations are necessarily required to clarify this biogenetic proposal.

Table 2
Antimicrobial activities of compounds **1–5** against different pathogens.

Compounds	MIC (μg/mL)				
	C. albicans	*S. aureus*	MRSA	*S. typhi*	*E. coli*
1	64	32	256	32	128
2	128	64	256	512	> 512
3	256	256	512	64	128
4	256	128	256	128	32
5	256	128	128	256	128
6	> 512	> 512	> 512	256	256
7	128	64	128	256	32
8	> 512	> 512	> 512	256	512
9	128	> 512	> 512	128	64
10	> 512	> 512	> 512	> 512	> 512
11	> 512	> 512	> 512	> 512	> 512
Nystatin	8	–	–	–	–
Chloramphenicol	–	8	16	8	8

All isolated compounds were tested for their antimicrobial activities towards *S. aureus*, methicillin-resistant *S. aureus*, *S. typhi*, and *E. coli* by using broth microdilution method. The results were shown in Table 2. Among them, compound **1** exhibited the most potent inhibitory activity against *S. aureus*, *S. typhi*, and *C. albicans* with MIC values of 32, 32, and 64 μg/mL, respectively. Compound **2** showed moderate inhibitory activity towards *S. aureus* and *E. coli* with MIC value of 64 μg/mL. Compound **3** displayed moderate activity against *S. typhi* with MIC value of 64 μg/mL. Besides, compounds **4** and **9** inhibited the growth of *E. coli* with MICs of 32 and 64 μg/mL, respectively. Compound **7** showed moderate inhibitory activity against *S. aureus* and *E. coli* with MICs of 64 and 32 μg/mL, respectively. The remaining compounds **5**, **6**, **8**, **10**, and **11** showed weak or no antimicrobial activities towards the five tested pathogenic strains.

Acknowledgments

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Appendix A. Supplementary data

1D and 2D NMR spectra of compounds **1** and **2** are available as Supporting information.

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