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Cytotoxicity and antibacterial activities of crude extract of *Streptomyces* sp. W08, an endophyte of *Amomum krervanh* Pierre

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ABSTRACT

Strain W08 was isolated from the pseudostem tissue of *Amomum krervanh* Pierre (Zingiberaceae) and identified as *Streptomyces* sp. by analyzing its morphology, chemotaxonomy, and 16SrDNA sequence. It was shown to exert bactericidal effects against *Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus*, and methicillin-resistant *S. aureus*. Crude extract of its culture inhibited the tested bacteria, with minimum inhibitory concentrations of 32–128 µg/ml. The cytotoxicity of the crude extract was also assessed against cell lines using [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] colorimetric assay. The crude extract showed weak cytotoxic activity on L929 and Vero cells with IC_{50} values of 453.22 and >512.00 µg/ml, respectively. The most powerful cytotoxicity of the crude extract was observed in human cervical carcinoma cells and human breast carcinoma cells with IC_{50} values of 78.45 and 106.50 µg/ml, respectively, while the IC_{50} value of human hepatocellular carcinoma cells was 425.86 µg/ml. The bioactive compounds in the extract were isolated by column chromatography and thin-layer chromatography, which were revealed by IR and NMR analyses to be 1-hydroxy-2-methyl-6-methoxyanthraquinone (**1**) and 6-methoxy-2-methylquinizarin (**2**). The findings showed that the crude extract of *Streptomyces* sp. W08 containing the bioactive compounds exhibited antibacterial activity and selective cytotoxicity toward some cancer cells. The studies on these compounds could thus lead to useful approaches for managing some bacterial infections and cancers in the future.

INTRODUCTION

Amomum krervanh Pierre (Zingiberaceae) is a tropical plant that is widely distributed in Southeast Asia. It is used globally for spices and is commonly applied as folk medicine to treat stomach disorders (Yin *et al*., 2013a). The chemical composition of this plant has been reported (Wu *et al*., 2006; Yin *et al*., 2013a, 2013b; Zeng *et al*., 2012) and it has also been indicated that plants of the genus *Amomum* exhibit antioxidant (Teresita *et al*., 2000), antimicrobial (Diao *et al*., 2014; Kwon *et al*., 2003; Malti *et al*., 2007; Moon *et al*., 2004), anti-inflammatory (Choi *et al*., 2018; Lee *et al*., 2008; Mathew *et al*., 2003), and antimalarial activities (Kamchonwongpaisan *et al*., 1995).

Recently, in studies on the endophytic actinomycetes, we isolated strain W08 from the pseudostem tissue of *Amomum krervanh* Pierre. It has antibacterial activity against mostly Gram-positive bacteria. Herein, we report the cytotoxicity of its crude extract against two normal cell lines [murine epithelial cells (L929) and African green monkey kidney *cells (Vero)] and* three cancer cell lines [human breast carcinoma cells (MCF-7), human cervical carcinoma cells (HeLa), and human hepatocellular carcinoma cells (HepG2)] using a (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (MTT) colorimetric assay. We also identified this strain, purified the major compounds, and also elucidated their structures.

MATERIALS AND METHODS

Isolation, cultivation, and antibacterial screening of endophytic actinomycetes

Various tissues of *Amomum krervanh* Pierre were used to isolate the endophytic actinomycetes as described in a previous study

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(Taechowisan *et al*., 2017). Ten actinomycete isolates were obtained and tested for their ability to produce antibacterial substances against *Bacillus cereus* ATCC 7064, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, methicillin-resistant *Staphylococcus aureus* Sp6, *Pseudomonas aeruginosa* ATCC 28753, and *S. aureus* ATCC 25923 using an overlay assay on ISP-2 agar plates, as previously described (Wu *et al*., 2015) with some modifications. Antibacterial screening of actinomycetes was carried out as described in a previous study (Taechowisan *et al*., 2017). Among the 10 isolates of actinomycetes, the results showed that the best production of antibacterial substances was achieved by isolate W08. This isolate was identified according to the methods of Taechowisan *et al*. (2003) and Taechowisan *et al*. (2017). The isolate W08 was grown on ISP-2 agar at 30°C for 14 days, and the culture was extracted with ethyl acetate as described in a previous study (Taechowisan *et al*., 2017).

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MICs of the crude extract were carried out by the National Committee for Clinical Laboratory Standards microbroth dilution methods (National Committee for Clinical Laboratory Standards, 2000). The crude extract was initially dissolved in dimethyl sulfoxide (DMSO). The MIC and MBC were carried out as described in a previous study (Taechowisan *et al*., 2017).

MTT assay for cytotoxicity activity (IC_{50})

The normal cell lines [murine epithelial cells (L929) and African green monkey kidney cells (Vero)] and three cancer cell lines [HeLa, human hepatocellular carcinoma cells (HepG2), and MCF-7] were used to assess the IC_{50} of the crude extract using MTT assay as described in a previous study (Taechowisan *et al*., 2017).

Purification and structural elucidation of major components

Ethyl acetate extract (10.75 g) was fractionated by silica gel 60 column chromatography and eluted with a mixture of petroleum ether–ethyl acetate with increasing polarity. Fractions eluted with 3.5% and then 8% ethyl acetate in petroleum ether were purified by thin-layer chromatography (TLC) (solvent: petroleum ether–ethyl acetate, 8.5:1.5 and 4:1) to give compounds **1** and **2**, respectively. The purified compounds were then subjected to NMR spectroscopy.

RESULTS AND DISCUSSION

A total of 100 samples each of leaf, pseudostem, rhizome, and root tissues of *Amomum krervanh* Pierre were examined. The root tissue was the site where actinomycetes were most commonly isolated, where a total of 20% of actinomycetes were found (Table 1), which matches our previous report (Taechowisan *et al*., 2003). The roots thus present a good habitat for these endophytic actinomycetes. This may be related to the abundance of actinomycetes within the rhizosphere microbial flora (Sardi *et al*., 1992), enabling easier infection of host plants. The results showed that, against the tested microorganisms, isolate W08 showed promising activities (Table 2). A large number of endophytic actinomycete isolates have no potential antibacterial

Table 1. Isolated actinomycetes from different parts of *Amomum krervanh* Pierre.

Plant	Actinomycete isolates from each plant parts			
	Leaf	Pseudostem	Rhizome	Root
Amomum krervanh Pierre	W02	W08	W09	W15
			W32	W24
			W33	W29
				W31
				W34
Number of isolates	1(4%)	1(4%)	3(12%)	5(20%)

Table 2. Screening for antibacterial activity of actinomycetes using an overlay assay.

Actinomycete isolates	Inhibition index on tested microorganisms					
	B.c.[a]	B.s.	S.a.	MRSA	E.c.	P.a.
W02	–	–	–	–	–	–
W08	5.89 ± 0.20	4.21 ± 0.23	6.30 ± 0.39	5.42 ± 0.33	–	–
W09	–	–	–	–	–	–
W15	–	–	–	–	–	–
W24	–	–	–	–	–	–
W29	–	–	–	–	–	–
W31	–	1.43 ± 0.52	1.43 ± 0.57	–	–	–
W32	–	3.02 ± 1.00	3.63 ± 0.05	–	–	–
W33	–	4.13 ± 0.30	3.88 ± 0.52	–	–	–
W34	–	2.16 ± 0.51	–	–	–	–

[a]B.c.; *B. cereus* ATCC 7064, B.s.; *B. subtilis* ATCC 1248, S.a.; *S. aureus.* ATCC 25923, MRSA; methicillin-resistant *S. aureus* Sp6 (the clinical isolate), E.c.; *E. coli* ATCC 25922 and P.a.; *P. aeruginosa* ATCC 28753.

activity against the tested bacteria. As stated in several reports, endophytic actinomycete activity influences the metabolic products of actinomycetes acting on plant growth and physiology (Hasegawa *et al*., 2006; Igarashi *et al*., 2002; Manulis *et al*., 1994; Meguro *et al*., 2006; Mishra *et al*., 1987). Thus, most of them did not produce antimicrobial metabolic products.

Based on the results of morphological observation, isolate W08 formed extensive substrate mycelia and abundant aerial mycelia on agar medium. It produced aerial mycelia that differentiated into long spore chains. These spore chains were straight to flexuous (rectiflexibiles) with oval and smooth surface spores (Figure. 1). Its substrate mycelia were grayish-green to dark olive-brown and the aerial mycelia were greenish-white to light greenish-grey. Owing to features including the presence of LL-type diaminopimelic acid in the whole-cell extracts, isolate W08 was identified as *Streptomyces*. The 16S rDNA sequence was determined for isolate W08 (1,473 bp). The BLAST search results and the phylogenetic tree are presented in Table 3 and Figure 2, respectively. These results showed that isolate W08 has high levels of sequence similarity to Streptomyces *coelicolor* NBRC 12854 (accession number: AB184196) and Streptomyces *felleus* NBRC 12766 (accession number: AB184129). The 16S rDNA analysis showed that isolate W08 is phylogenetically also closely related to these strains. The 16S rDNA sequence data reported in this paper are available in GenBank under accession number AB845558.

The antibacterial activity of the crude extract from *Streptomyces* sp. W08 culture is shown in Table 4. The crude extract inhibited the tested bacteria, with MICs and MBCs of 32–128 µg/ml and 256 to >512 µg/ml, respectively. However, no activity against Gram-negative bacteria was exhibited by this extract, which was due to the different composition of bacterial cell walls. The mechanisms of action of the crude extract may involve antimicrobial effects, having different activity against Gram-negative and Gram-positive bacteria.

The crude extract showed weak cytotoxic activity against L929 and Vero cells, with IC_{50} values of 453.22 and >512.00 µg/ml, respectively (Table 5). The most powerful cytotoxicity of the crude extract was observed in HeLa and MCF-7 cells, with IC_{50} values of 78.45 and 106.50 µg/ml, respectively, while the IC_{50} value against HepG2 cells was 425.86 µg/ml. These data are interesting as they suggest that this crude extract is more toxic to some cancer cells than to normal cells, implying its potential as an anticancer agent. Although this preliminary anticancer study demonstrated that the crude extract exhibited an anticancer effect, more detailed investigation is required to isolate the bioactive compounds from the crude extract.

Among our observations of the cytotoxicity toward cancer cells, it was particularly interesting that the crude extract exhibited greater toxicity against HeLa and MCF-7 cells than against L929 and Vero cells. In this context, the therapeutic index (TI) is an important parameter to select samples for developing drugs. This value is the ratio of the concentration of the crude extract at which 50% of the normal cell death to that of the crude extract at which 50% cancer cell death occurred. Further investigation is considered to be warranted if a TI value of ≥4 is identified. The present study found TI values for this crude extract of ≥4 for HeLa and MCF-7 cells. This extract is thus promising for further investigation as an anticancer agent.

Silica gel column chromatography and TLC with petroleum ether–ethyl acetate as the mobile phase resulted in the isolation of two major compounds, 1-hydroxy-2-methyl-6-methoxyanthraquinone (**1**) and 6-methoxy-2-methylquinizarin (**2**), TOFMS *m/z*: 291.2533 (calcd for $C_{16}H_{12}O_4Na$: 291.2532) and 307.2527 (calcd for $C_{16}H_{12}O_5Na$: 307.2525), respectively (Figure. 3).

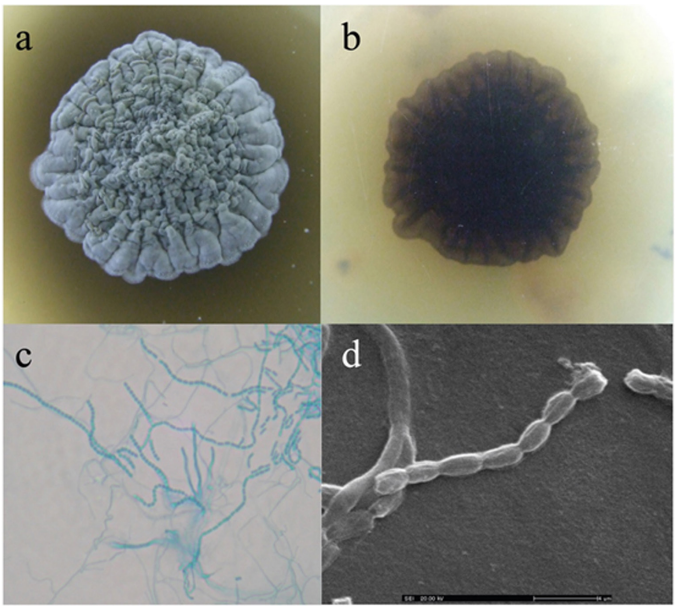


Figure 1. The morphological characteristics of *Streptomyces* sp. W08. The colony appearance [surface (a) and reverse (b)] of *Streptomyces* sp. W08 after 21 days of growth on ISP-2 agar at 30°C incubation. (c) a light micrograph and (d) a scanning electron micrograph; bar = 2 µm.

Table 3. 16S rDNA similarity values between *Streptomyces* sp. W08 and representatives of the genus *Streptomyces*.

***Streptomyces* species**	**Similarity to strain W08**	**No. of nucleotide differences/total no. of nucleotides compared**
S. coelicolor NBRC 12854	100.00	0/1452
S. felleus NBRC 12766	100.00	0/1452
Streptomyces albidoflavus NBRC 13010	99.93	1/1452
Streptomyces canescens NBRC 12751	99.93	1/1452
Streptomyces coriofaciens NBRC 13403	99.93	1/1452
Streptomyces champavatii NRRL B-5682	99.86	2/1472
Streptomyces fungicidicus YH04	99.66	5/1473
Streptomyces koyangensis VK-A60	99.52	7/1473
Streptomyces flavofungini NBRC 13371	98.34	24/1453
Streptomyces exfoliatus NBRC 13191	96.06	57/1450
Streptomyces albus subsp. *albus* DSM 40313T	96.01	58/1455
Streptomyces griseus KACC 20084	95.45	66/1451
Streptomyces globisporus subsp. *caucasicus* NRRL B-2593	94.58	77/1423

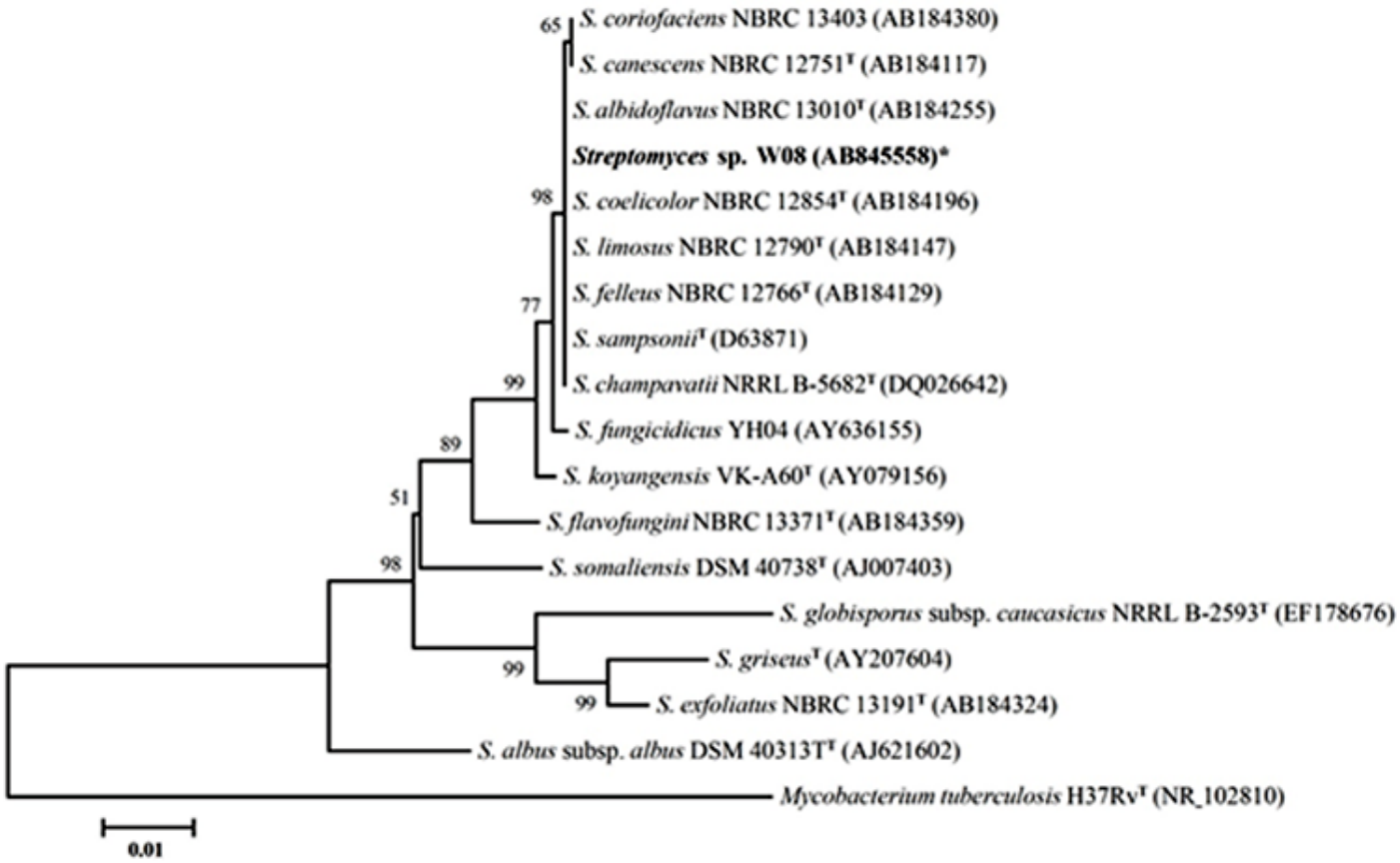


Figure 2. Neighbor-joining phylogenetic tree of isolate W08, including the closely related type strain base on 16SrDNA gene sequences which were retrieved from GenBank. Accession numbers appear in parentheses. Bootstrap (1,000 replicates) values are given in percentage. Bar, 0.01 substitutions per site.

Table 4. MIC and MBC of the crude extract from *Streptomyces* sp. W08 culture.

Tested microorganisms	MIC (µg/ml)	MBC (µg/ml)
B. cereus ATCC 7064	64	512
B. subtilis ATCC 6633	128	>512
S. aureus ATCC 25932	32	256
Methicillin-resistant *S. aureus* Sp6	64	256
E. coli ATCC 25922	>512	ND
P. aeruginosa ATCC 28753	>512	ND

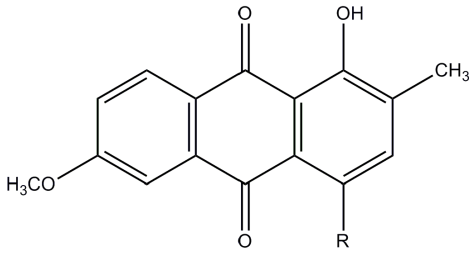
Table 5. IC_{50} and TI of the crude extract on various cell lines.

IC_{50a} (µg/ml)					TI[b]		
L929	Vero	MCF-7	HeLa	HepG2	MCF-7	HeLa	HepG2
453.22	>512.00	106.50	78.45	425.86	4.25	5.77	1.06

[a]IC_{50} values represent the concentration causing 50% growth inhibition.

[b]TI is defined as the ratio of 50% toxic dose on normal cells to 50% effective dose on cancer cells (L929 cells were selected to determine the TI, while the IC50 value of Vero cells was >512.00 µg/ml).

Their ^{1}H-NMR and ^{13}C-NMR spectral data were identical with those of 1-hydroxy-2-methyl-6-methoxyanthraquinone and 6-methoxy-2-methylquinizarin previously reported (El-Lakany et al., 2004). According to data from the literature, anthraquinone



1 R = H
2 R = OH

Figure 3. Chemical structures of 1-hydroxy-2-methyl-6-methoxyanthraquinone (**1**) and 6-methoxy-2-methylquinizarin (**2**).

compounds have been reported on antimicrobial activity (El-Lakany *et al*., 2004; Mbaveng *et al*., 2008).

CONCLUSION

In summary, crude extract from the culture of *Streptomyces* sp. W08 showed antibacterial activity against Gram-positive bacteria. It also exhibited potent cytotoxic effects on HeLa and MCF-7 cells, while exhibiting low cytotoxicity against normal cells (L929 and Vero cells). These results indicate that this

crude extract may be a potential therapeutic candidate for treating some bacterial infections and cancers.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

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