Trop J Nat Prod Res, May 2021; 5(5):814-818 **ISSN 2616-0684 (Print)**
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

Characterization and Anti-*Candida* Activity of the Endophytic *Streptomyces* Isolated from *Asystasia gangetica*

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ARTICLE INFO

Article history:
Received 26 October 2020
Revised 01 March 2021
Accepted 12 May 2021
Published online 03 June 2021

ABSTRACT

Oral candidiasis, or oral thrush, is a common fungal infection of the oral cavity caused by the *Candida* species. This infection can cause oral discomfort, pain, and loss of appetite. Because of the increase in antifungal resistance among *Candida* species, alternative antifungal compounds are required to be developed. Actinobacteria, specifically the *Streptomyces* species, are well known as producers of bioactive compounds and play an important role in drug discovery. The endophytic actinobacteria strain 5R010, isolated from the root of *Asystasia gangetica*, was identified using phenotypic properties and 16S rRNA gene analysis. The spiral spore chains were observed on the aerial mycelia of strain 5R010. *LL*-diaminopimelic acid was detected in the whole-cell hydrolysate. The strain showed the closest 16S rRNA gene similarity of 99.72% with *Streptomyces sioyaensis* as well as sharing the same node with *S. sioyaensis* in the phylogenetic tree. On the basis of the agar disc diffusion method, the crude ethyl acetate extract of strain 5R010 showed inhibitory activity against seven tested *Candida* species including *Candida albicans* TISTR 5554, *Candida glabrata* TISTR 5006, *Candida guilliermondii* TISTR 5206, *Candida krusei* TISTR 5351, *Candida parapsilosis* TISTR 5007, *Candida pseudotropicalis* TISTR 5336, and *Candida tropicalis* TISTR 5268. Based on the results obtained from this study, *S. sioyaensis* 5R010 can be employed in further research involving the isolation of antifungal agents.

Keywords: Actinobacteria, Endophyte, Candidiasis, *Streptomyces*.

Introduction

The members of the genus *Candida*, an opportunistic fungus, are 'yeast-like' fungi, comprising more than 150 species. Among the *Candida* species, *C. albicans* is found to be the most persistent commensal microorganism within the human oral cavity, occurring in approximately 50% of the cases of candidiasis.[1,2] The malady of thrush or oral candidiasis, caused by an excess of *Candida* species, is a common opportunistic infection of the oral cavity affecting the oral mucosa. This disease is underdiagnosed among the elderly who wear dentures, when in fact, it has an inextricable connection with other health conditions such as immunodeficiency and diabetes mellitus.[3] Some other *Candida* species, known as non-albicans, that causes oral candidiasis are *C. guilliermondii, C. glabrata, C. tropicalis, C. tropicalis, C. lusitaniae, C. parapsilosis, C. krusei, and C. stellatoidea*.[4]

Two main classes of antifungal agent which are most widely used for the treatment of candidiasis are the polyenes (amphotericin B and nystatin) and the azoles (fluconazole, miconazole, ketoconazole, clotrimazole, itraconazole, voriconazole, and posaconazole).[5]

However, a limited number of antifungal agents from a few drug classes and widespread incidents of drug-resistance among *Candida* species required research and development of new antifungal compounds.[6,7] Actinobacteria, the phylum of Gram-positive bacteria having high G+C content, is well known as an antibiotic producer, and includes antifungal agents such as amphotericin B nystatin. This group of bacteria can be found globally in various environments, including soil and marine environments, and symbioses with plants and some social insects.

Plants harbour several novel endophytic actinobacteria such as *Streptomyces mimosae*, *Microbispora catharanthi*, and *Nonomuraea phyllanthi*.[8, 9, 10] In contrast to phytopathogens, endophytes are microbes which live in the plant tissue without causing plant diseases.[11] Recently, endophytic actinobacteria are considered a promising source of novel bioactive compounds.[12] Here, we evaluated the *in vitro* activity of the crude extract obtained from endophytic actinobacteria strain 5R010 against seven *Candida* species. In addition, strain 5R010 was characterized and identified in this study.

Materials and Methods

Microorganisms

Actinobacteria strains were isolated from the root of *Asystasia gangetica* grown in the Botanical Garden of the Faculty of Science, Ramkhamhaeng University. The plant sample was freshly prepared for the actinobacterial isolation. Briefly, the sample was washed using tap water to remove the soil prior to surface sterilization. The surface sterilization was accomplished by washing the sample in 3% NaOCl for 5 minutes and then for 1 minute in ethanol (95% v/v) and sterile distilled water. The sample was ground by means of a mortar, followed by the standard serial dilution (10^{-1} – 10^{-4}). The resultant of each solution (0.1 mL) was spread on the surface of humic acid vitamin agar and incubated for 14 days. The colony of strain 5R010

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Citation: Phongsopitanun D, Sripreechasak P, Piewpong K, Phongsopitanun W, Prateeptongkum E. Characterization and Anti-*Candida* Activity of the Endophytic *Streptomyces* Isolated from *Asystasia gangetica*. Trop J Nat Prod Res. 2021; 5(5):814-818. doi.org/10.26538/tjnpr/v5i5.4

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria.

was selected and purified using the international *Streptomyces* medium number 2 agar (ISP2). The strain was maintained on ISP2 as the working culture.[13]

Seven *Candida* strains including *Candida albicans* TISTR 5554, *Candida glabrata* TISTR 5006, *Candida guilliermondii* TISTR 5206, *Candida krusei* TISTR 5351, *Candida parapsilosis* TISTR 5007, *Candida pseudotropicalis* TISTR 5336, and *Candida tropicalis* TISTR 5268 were obtained from the TISTR culture collection, Bangkok, Thailand. All *Candida* species were cultured on Sabouraud dextrose agar (SDA).

Characterization of actinobacteria

Cultural characteristics of the strain were observed using the culture grown on ISP media at 30°C for 14 days. The colour of the colony and the colour of the pigment were determined using the NBS/ISCC colour chart.[14] The morphology of the spore was observed using a light microscope (Olympus, CH2). The biochemical test was determined using API20E (*bioMérieux*). Growth pH, NaCl, and growth temperature were determined using ISP2 agar media. The freeze-dried cells were used for the chemotaxonomic study. The diaminopimelic acid isomers and the sugar in whole-cell hydrolysates were hydrolyzed from the freeze-dried cells and determined using thin-layer chromatography (TLC) methods.[15]

The strain was identified using 16S rRNA gene analysis. Briefly, the genomic DNA was extracted from the mycelia cultured in ISP2 broth at 30°C for four days. Then, the 16S rRNA gene was amplified using the universal primers, including 20F (5´-GAGTTTGATCCTGGCTCAG-3´) and 1530R (5´-GTTACCTTGTTACGACTT-3´). The nucleotide sequence of the PCR product was sequenced using the services of Macrogen (Korea). The nucleotide sequence was manually aligned and analyzed using BioEdit (Ibis Biosciences). The BLASTN search was analyzed using the EzbioCloud.[16] The phylogenetic tree, based on the 16S rRNA gene, was constructed using MEGA X.[17]

Preparation of the crude extract

The strain 5R010 was cultured in ISP2 broth (total volume 1200 mL) in shaking conditions at 180 rpm, 30°C for 14 days. After the end of the incubation period, the culture broth was partitioned with ethyl acetate three times. Then, the ethyl acetate layer was evaporated to obtained the crude ethyl acetate extract.

Anti-Candida activity

The determination of anti-*Candida* activity was accomplished by the agar disc diffusion method. Briefly, the crude extract was dissolved in dimethyl sulfoxide (DMSO) to obtain a 100 mg/ml final concentration. Then, 20 µL of the extract was applied to the paper disc (6 mm in diameter). The paper discs containing amphotericin B (20 µg, Himedia) and DMSO were used as the positive and the negative control, respectively. To prepare the tested microorganisms, each of the *Candida* strains grown on SDA agar at 30°C for 48 hours was transferred to normal saline solution (NSS) prior to the adjustment of the turbidity of the solution equivalent to 0.5 McFarland solution. Then, the NSS solution containing *Candida* cells was swabbed on to the surface of the SDA agar. Finally, the prepared paper discs were transferred on to the surface of the SDA agar and incubated at 30°C for 48 hours. The inhibition zones around the paper disc were measured. The broth microdilution technique was used to evaluate the minimum inhibition concentration (MIC) of the crude extract.[18]

Statistical analysis

The topology of the phylogenetic tree was calculated using the bootstrap test.[19]

Results and Discussion

Characterization of strain 5R010

Strain 5R010 was found to grow well on the different kinds of ISP media used in this study. The strain produced the white aerial mycelia and yellow tone of the substrate mycelia on various ISP agars. A soluble pigment was not observed (Table 1). The microscopic observation revealed that the strain produced a spiral spore chain on the aerial mycelia (Figure 1). Based on API20E, the strain showed activity of the enzymes *β*-galactosidase, arginine dihydrolase, and weak activity of lysine and ornithine decarboxylase. However, the activities of enzymes including urease, citrate utilization, tryptophan deaminase, and gelatinase, were not observed. The nitrate reduction was negative. Meanwhile, strain 5R010 grew at pH 5-9 and up to 7% (w/v) for NaCl. Growth was not observed at 45°C.

The results of the chemotaxonomic study revealed that strain 5R010 contained *LL*-diaminopimelic acid alongside glucose and ribose in the whole-cell hydrolysate. According to Lechevalier and Lechevalier,[20] this characteristic resembles cell-wall type I of the *Streptomyces* species. Based on 16S rRNA gene analysis, the strain 5R010 clearly displayed the highest 16S rRNA gene with 99.72% similarity to *Streptomyces sioyaensis* NRRL-B5408^T. The phylogenetic tree analysis revealed that the strain shared the node with *S. sioyaensis* NRRL-B5408^T with a bootstrap value of 70. Therefore, the strain 5R010 was identified as *Streptomyces sioyaensis*.

Anti-Candida activity of the crude extract

A total of 96.1 mg of the crude extract was obtained from the culture media of 5R010. Based on the agar disc diffusion assay, the crude extract showed anti-*Candida* activity against all tested *Candida* species. However, the potency of the crude extract against each of the *Candida* species was different. The crude extract of strain 5R010 showed the strongest activity against *C. albicans* TISTR 5554, *C. guilliermondii* TISTR 5206, and *C. parapsilosis* TISTR 5007, with the assay indicating an inhibition zone for the crude extract (20 mg/disc) with a diameter exceeding 37.5 mm. Moderate activity was observed against *Candida glabrata* TISTR 5006 and *Candida pseudotropicalis* TISTR 5336. However, the crude extract showed weak activity against *C. krusei* TISTR 5351 and *C. tropicalis* TISTR 5268 (Figure 3, Table 2). The MIC of the crude extract against *Candida* species ranged between 2.5 mg/ml to more than 2.5 mg/ml, depending on the *Candida* species. In addition, the crude extract exhibited antibacterial activity against Gram-positive bacteria (*K. rhizophila* ATCC 9341, *B. subtilis* ATCC 6633, and *S. aureus* ATCC 25923). Activity against Gram-negative bacteria was not observed.

Medicinal plants are the source of endophytic actinobacteria, an important source of antifungal activity. For example, the endophytic *Streptomyces* sp. TP-A0456 isolated from *Aucuba japonica* produced anti-*Candida* compounds called cedarmycins A and B.[21] Based on this study, the strain 5R010 was identified as *Streptomyces sioyaensis* 5R010. *S. sioyaensis* was first described by Nishimura *et al*.[22] Since the discovery, several new compounds have been isolated from this *Streptomyces* species. In 1980, Tokura *et al*.[23] isolated a new peptide antibiotic, siomycin D, together with two analogues, siomycins A-C, from the culture broth of *S. sioyaensis*. Siomycin D showed antimicrobial activity against Gram-positive bacteria. In 1985, Ikeda *et al*.[24] reported that *S. sioyaensis* strain MD753-C2 produced a new streptomycin group antibiotic (6´´´-O-α-D-mannopyranosyl mannosidostreptomycin) together with streptomycin and mannosidostreptomycin. This new compound showed weak antibacterial activity.

In 1989, Takahashi *et al*.[25] isolated *S. sioyaensis* SA-1758 from the marine environment. This strain produced a new insecticide called altemicidin, which was related to acaricidal and miticidal activity, antitumour activity, and antimicrobial activity against the *Xanthomonas* strain. Furthermore, *S. sioyaensis* was reported to produce the new lipophilic antibiotic, FR-900336. This new compound was active against Gram-positive bacteria and fungi including *C. albicans*, *Shizosaccharomyces pombe*, *Trichophyton mentagrophytes*, *Aureobasidium pullulans*, *Mucor hiemalis*, *Rhizopus acetorinus*, *Fusarium* sp. R2, and *Helminthosporium* sp. 2-1.[26] In addition to antibiotics, the enzyme endo-1,3-*β*-glucanase produced by *S. sioyaensis* isolated from peat moss was reported. This enzyme showed antifungal activity by degrading fungal cell walls.[27] Recently, the new strain of *S. sioyaensis* TM32 isolated from the rhizosphere soil of *Curcuma longa* showed potent antimicrobial activities against human pathogens including antibiotic-resistant *Staphylococcus haemolyticus* MR-CoNS.[28]

The antifungal agents commonly used for candidiasis treatment are the polyenes amphotericin B and nystatin. These two compounds were produced from *Streptomyces nodosus* and *Streptomyces noursei* for amphotericin B and nystatin B, respectively. Therefore, *Streptomyces* species have been shown to be potential bioactive compound producers.

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Table 1: Cultural characteristics of the strain 5R010

Culture media	Growth	Colour		
		Aerial mycelia	Substrate mycelia	Soluble pigment
ISP2	Good	White	Pale greenish yellow	-
ISP3	Very good	White	Brilliant yellow	-
ISP4	Good	White greenish gray	Strong orange	-
ISP5	Good	White	Light yellow green	-
ISP6	Good	White	Grayish yellow	-
ISP7	Very good	White	Light greenish yellow	-

Table 2: Anti-*Candida* activity and the MIC values of the crude extract of strain 5R010

Candida species	Inhibition zone (mm)		MIC (mg/ml)	
	Crude extract (2 mg/disc)	Amphotericin B (20 µg/disc)	Crude extract	Amphotericin B
C. albicans TISTR 5554	46	17.5	2.5	0.0024
C. glabrata TISTR 5006	24.8	18	> 2.5	0.0012
C. guilliermondii TISTR 5206	45.5	11	2.5	0.0097
C. krusei TISTR 5351	11.6 (w) *	12.1	> 2.5	0.0097
C. parapsilosis TISTR 5007	37.5	15	2.5	0.0024
C. pseudotropicalis TISTR 5336	16.5	14.8	> 2.5	0.0048
C. tropicalis TISTR 5268	38 (w) *	17	> 2.5	0.0024

*W = weak activity

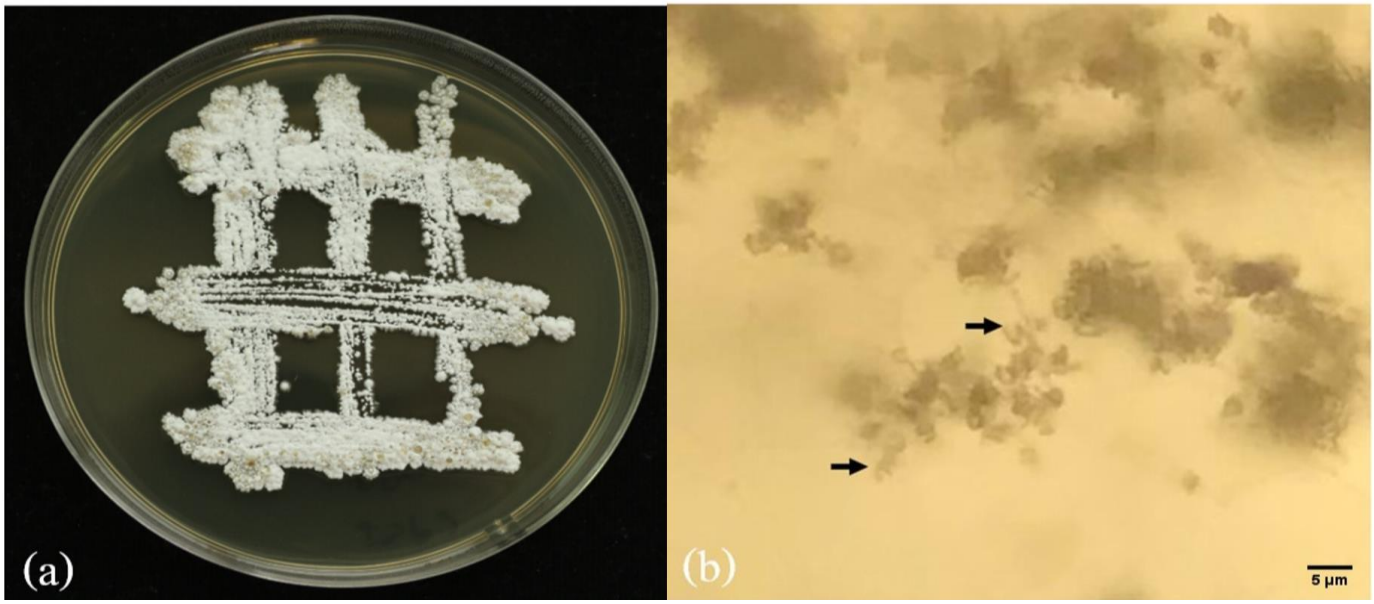

Figure 1: Cultural characteristics (a) and morphology of spore chains (b) of the strain 5R010 after growth on the ISP2 media at 30°C for 14 days.

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

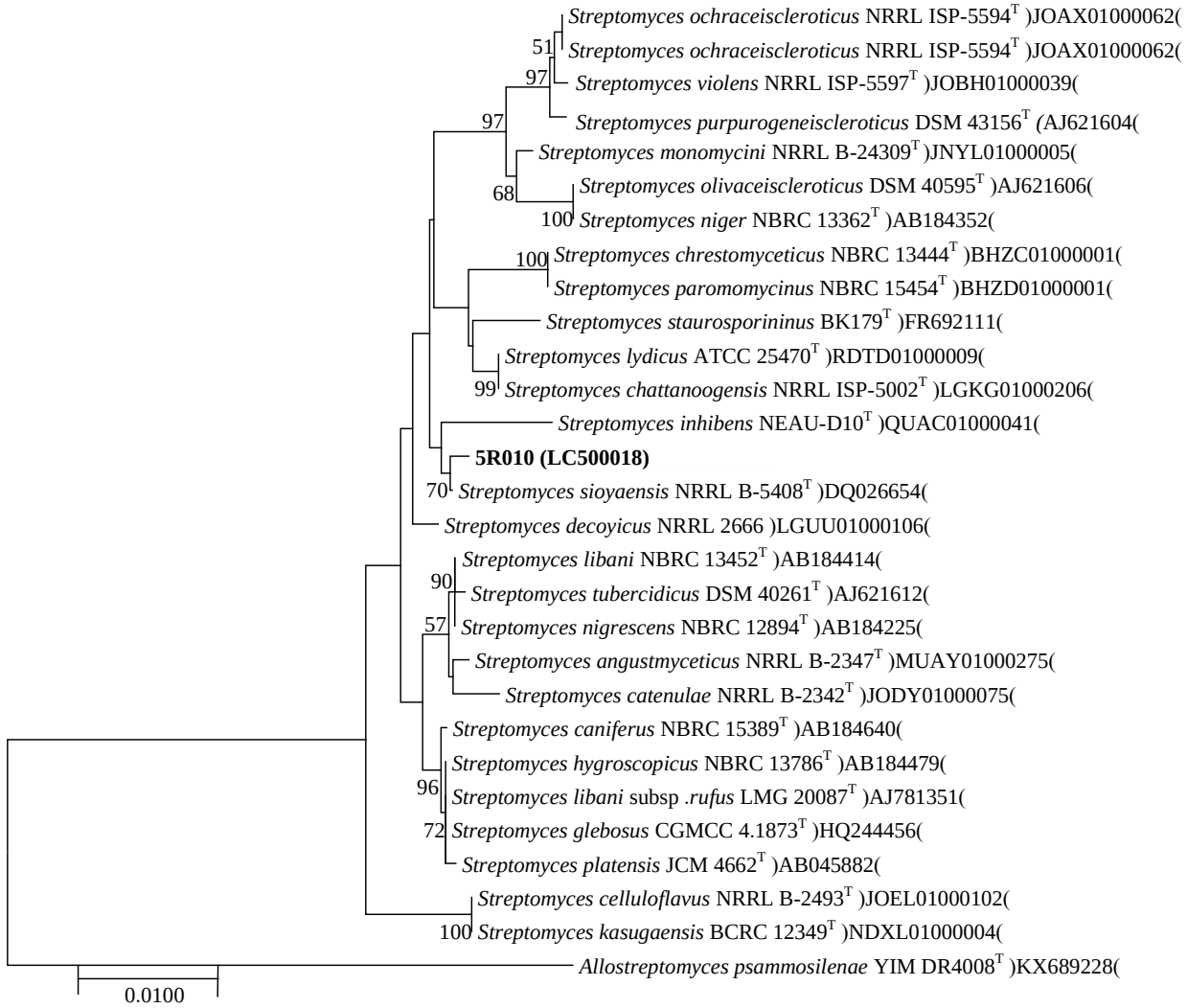

Figure 2: Phylogenetic relationship based on 16S rRNA gene sequences of the strain 5R010 and closely related *Streptomyces* species obtained from BLAST results. The numbers at the branch nodes indicate bootstrap percentages derived from 1,000 replications (only values >50% are shown). Bar, 0.01 substitutions per nucleotide position. *Allostreptomyces psammosilenae* YIM DR4008^T was used as the out group.

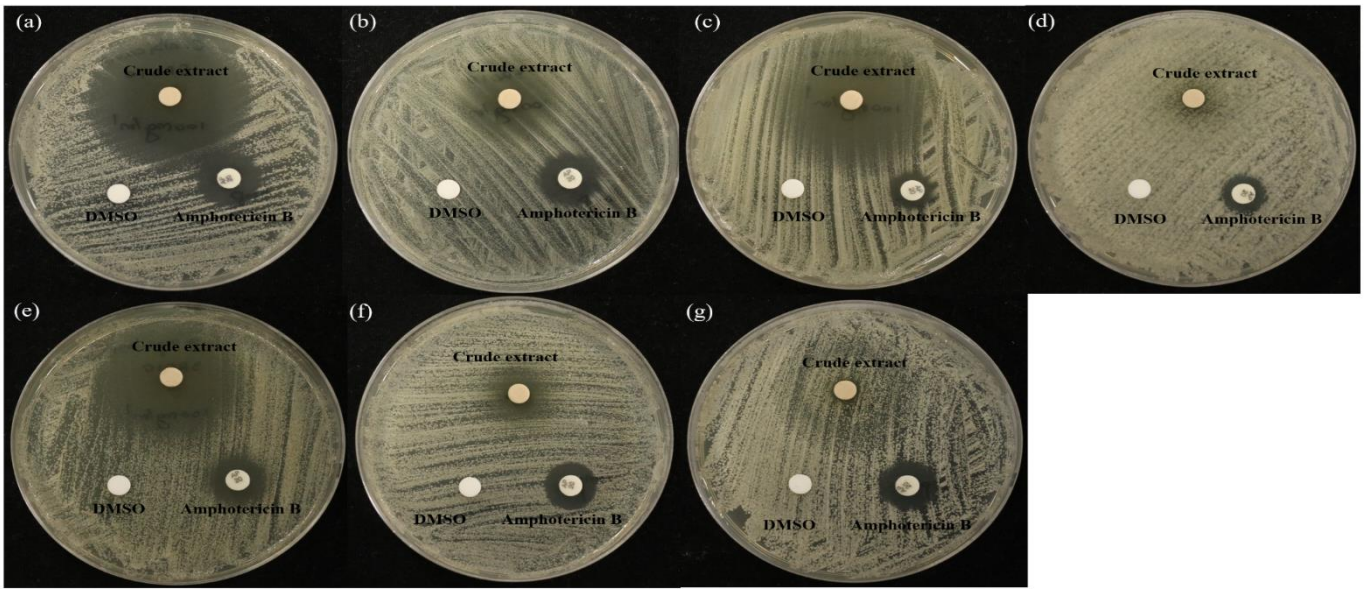

Figure 3: Anti-*Candida* activity testing based on agar disc diffusion methods. (a) *Candida albicans* TISTR 5554, (b) *Candida glabrata* TISTR 5006, (c) *Candida guilliermondii* TISTR 5206, (d) *Candida krusei* TISTR 5351, (e) *Candida parapsilosis* TISTR 5007, (f) *Candida pseudotropicalis* TISTR 5336, and (g) *Candida tropicalis* TISTR 5268

Conclusion

In this study, *S. sioyaensis* 5R010 isolated from *Asystasia gangetica* showed good anti-*Candida* activity and can be used for further pharmaceutical investigations of anti-*Candida* compounds in the future. The crude extract of strain 5R010 exhibited antifungal activity against various *Candida* species, especially *C. albicans*. However, the active compounds that exhibited antifungal activity in the crude extract were not determined in this study. For further studies, pure antifungal compounds should be isolated.

Conflict of Interest

The authors declare no conflicts of interest.

Author's Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Acknowledgments

This study was supported by the Resident Training Program in General Dentistry, Institute of Dentistry, Ministry of Public Health, Thailand, and the Thailand Research Fund MRG6180011**.**

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