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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

OPEN ACCESS Pakistan Journal of Biological Sciences

ISSN 1028-8880
DOI: 10.3923/pjbs.2024.469.478

Research Article
Antibacterial and Anticancer Properties of Endophenazines from *Streptomyces prasinus* ZO16, an Endophyte in *Zingiber officinale* Rosc.

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Abstract

Background and Objective: This study investigated a bacterial strain, ZO16, isolated from ginger (*Zingiber officinale*) roots. Analysis of its 16S ribosomal DNA (rDNA), along with chemical and physical properties, revealed it to be *Streptomyces prasinus*. This study aimed to isolate and characterize the main bioactive compounds from ZO16, evaluating their antibacterial and anticancer properties. **Materials and Methods:** Techniques like column chromatography and thin-layer chromatography (TLC) were used to purify the key compounds from ZO16's culture extract. Nuclear Magnetic Resonance (NMR) spectroscopy and mass spectrometry were utilized to confirm the identities of the purified compounds as endophenazine A (compound **1**) and endophenazine B (compound **2**). The antibacterial and anticancer properties of these compounds were then evaluated. **Results:** The isolated compounds displayed antibacterial activity against *Staphylococcus aureus* ATCC 25923 and Methicillin-Resistant *Staphylococcus aureus* (MRSA). The minimum inhibitory concentration (MIC) of the isolated compounds against bacteria ranged from 8 to 32 µg/mL, while the minimum bactericidal concentration (MBC) was between 32 and 128 µg/mL. These compounds exhibited effectiveness against tested cancer cells with IC_{50} values ranging from 30.40 to 32.51 µg/mL for cervical cancer (HeLa), 78.32 to 86.45 µg/mL for liver cancer (HepG2) and 23.41 to 28.26 µg/mL for breast cancer (MDA-MB-231) cells. However, these compounds also showed moderate toxicity towards non-cancerous Vero cells (IC_{50} = 317.44-328.63 µg/mL). **Conclusion:** The findings of this study suggest that *Streptomyces prasinus* strain ZO16 produces compounds with antibacterial and anticancer properties. Further investigation of these compounds has the potential to contribute to the development of improved methods for controlling and treating bacterial infections and some cancers.

Key words: Antibacterial activity, anticancer activity, endophenazines, endophyte, *Streptomyces prasinus* ZO16, *Zingiber officinale* Rosc.

Citation: Taechowisan, T., T. Chuen-Im and W.S. Phutdhawong, 2024. Antibacterial and anticancer properties of endophenazines from *Streptomyces prasinus* ZO16, an endophyte in *Zingiber officinale* Rosc. Pak. J. Biol. Sci., 27: 469-478.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The escalating threat of antibiotic resistance necessitates the exploration of novel therapeutic agents. Nature, in its remarkable diversity, harbors a wealth of potential solutions waiting to be discovered. One fascinating niche lies within the symbiotic relationships between plants and their resident endophytic microorganisms. Plants harbor a diverse community of microorganisms within their tissues, with some residing intercellularly and others intracellularly. Endophytes, the latter group, are a fascinating collection of bacteria and fungi that establish a unique relationship with their hosts. Unlike plant pathogens, endophytes do not cause any visible disease symptoms and instead, participate in a symbiotic association where both parties benefit.

Endophytic bacteria represent a particularly well-studied group within the endophyte community and offer a multitude of benefits to their plant hosts. Some endophytes possess the ability to fix atmospheric nitrogen or solubilize mineral nutrients, making them readily available for plant uptake and growth. Endophytic bacteria are especially skilled at fixing nitrogen in plant roots, potentially leading to a reduced reliance on synthetic fertilizers[1]. They also contribute to enhanced plant growth by promoting efficient nutrient acquisition[1]. Beyond aiding nutrient uptake, endophytic bacteria act as guardians against pathogens. They achieve this in several ways: By competing for space and resources, producing compounds that kill harmful microbes or stimulating the plant's defense mechanisms[2,3]. Endophytic bacteria further assist plants in weathering harsh environmental conditions by producing compounds and enzymes that help the plant detoxify[4]. Some even contribute to bioremediation by breaking down pollutants within the plant itself[4]. Particularly endophytic actinomycetes have garnered significant scientific interest due to their remarkable capacity to produce a structurally diverse array of bioactive compounds, including antibiotics[5-9].

The diverse nature of endophytes extends to their associations with various plant species[10-13]. Research on ginger's (*Zingiber officinale* Rosc.) endophytic bacteria has shown promise in promoting plant growth and fighting fungal infections, demonstrating how these microbes provide specific benefits to their ginger hosts[14-16]. Notably, the abundance of endophytic bacterial communities varies across the distinct growth stages of ginger. This suggests that both the plant itself and the surrounding environment contribute to the endophytic bacterial community and these microbes could produce valuable medicinal compounds[17]. These findings underscore the complex and dynamic nature of endophyte-plant interactions.

Considering the alarming rise of antibiotic resistance bacteria, there is a critical need to discover antimicrobial compounds from alternative sources. This study aims to explore the potential of endophytic actinomycetes residing within ginger tissues as a source of such compounds. Endophytic actinomycetes will be isolated from ginger and screened for antibacterial activity against human pathogens. The most potent isolate will be selected for further investigation, including the identification and characterization of the active compounds responsible for its antibacterial activity. Ultimately, the study aimed to assess the potential of these compounds to combat infectious diseases and explore their possible applications against cancer.

MATERIALS AND METHODS

Study area: The study was conducted at Departments of Microbiology and Chemistry, Silpakorn University, Nakhon Pathom, Thailand between April, 2023 and May, 2024.

Isolation and antibacterial screening of actinomycetes: Eighteen ginger (*Zingiber officinale* Rosc.) root samples were collected near Nakhon Pathom, Thailand (coordinates: 13.8189417'N, 100.0413870'E). To isolate actinomycetes, the roots were thoroughly washed, severed into small segments and then treated with a multi-step sterilization process. This process involved rinsing with Tween 20 solution, sodium hypochlorite and ethanol to remove surface contaminants. Finally, the sterilized root pieces were dried aseptically in a laminar flow cabinet (Esco Scientific, Pennsylvania, USA). The surface-sterilized root segments were plated onto a special medium called humic acid-vitamins (HV) agar[18]. To prevent fungal and yeast growth, 100 µg/mL of cycloheximide and nystatin were added to the agar. These plates were incubated at 32°C for 3 weeks. The colonies with characteristic actinomycete morphologies were picked and transferred to fresh plates containing ISP-2 medium for further analysis[19]. A total of 37 actinomycete isolates were evaluated for their ability to inhibit the growth of bacteria. This screening included *Staphylococcus aureus* ATCC 25923 and clinical isolates of Methicillin-Resistant *Staphylococcus aureus* (MRSA) strains RI, Sp2, Sp3 and T2. A modified soft-agar overlay method was used[20] and the size of the inhibition zones was measured. This experiment was performed in triplicate to ensure accuracy. Among the 37 isolates, ZO16 displayed the strongest antibacterial activity. This isolate was then identified using a combination of morphological, physiological and chemotaxonomic techniques following the methods established by

Cassarini *et al.*[21]. Strain ZO16 was grown on a large scale (600 Petri dishes) using ISP-2 agar for 21 days at 32°C. The culture was then extracted with Ethyl Acetate (EtOAc) to recover potential bioactive compounds[22]. The combined organic extracts were concentrated using a rotary evaporator R-300 (BUCHI Labortechnik AG, Switzerland), resulting in a dark brown solid (12.50 g). This crude extract was then divided into two parts: One part was dissolved in DMSO for antibacterial and anticancer testing, while the other part was prepared for further purification and characterization of individual compounds using Dichloromethane (CH_2Cl_2).

Scanning electron microscopy: To investigate the morphology of the ZO16 strain, Scanning Electron Microscopy (SEM) was employed following the methods outlined by Castillo *et al.*[23]. Briefly, the samples were prepared, dried using a critical point dryer with liquid CO_2 (Quorum K850, UK), sputter-coated with gold (Safematic CCU-010 HV, Switzerland) and examined under a scanning electron microscope (TESCAN Mira3, Czech Republic). This process allowed us to observe the morphology of the ZO16 isolate's spore chains and any ornamentation on their surfaces.

Identification of ZO16 strain using 16S rDNA sequencing and phylogenetic analysis: To identify the ZO16 strain, 16S ribosomal RNA gene (rDNA) sequencing and phylogenetic analysis were performed. First, the ZO16 strain, exhibiting the strongest antibacterial activity, was cultured in ISP-2 broth for seven days at 32°C with shaking (150 rpm). The bacterial cells were then harvested by centrifugation and washed with phosphate-buffered saline (PBS). Genomic DNA was extracted from the purified cells using a commercially available DNA extraction kit (Geneaid, Taiwan). The 16S rDNA gene was amplified using Polymerase Chain Reaction (PCR) with specific primers (A7-26f and B1523-1504r). The PCR cycling conditions involved initial denaturation, followed by repeated cycles of denaturation, annealing (primer binding) and extension (amplification). A final extension step ensured complete product formation, followed by cooling. The amplified 16S rDNA fragment (around 15,000 base pairs) was separated using gel electrophoresis and purified using a commercial gel extraction kit (Qiagen, Germany). This purified fragment was then directly sequenced using the Sanger dideoxy chain termination method. A sequencing kit (Big Dye Terminator cycle sequencing kit, Applied Biosystems, USA) and an automated sequencer (ABI PRISM 310 Genetic Analyzer, Applied Biosystems, USA) were employed for this purpose. Sequencing utilized both the original PCR primers (A7-26f and B1523-1504r) and additional internal primers (C704-685r and D1115-1100r) to ensure complete sequence coverage. The obtained DNA sequences were assembled into a complete 16S rDNA sequence for the ZO16 strain. This assembled sequence was compared to known 16S rDNA sequences deposited in the National Center for Biotechnology Information (NCBI) GenBank database using the Basic Local Alignment Search Tool (BLAST) program. This comparison identified reference strains (organisms with known 16S rDNA sequences) that were most similar to the ZO16 strain. The 16S rDNA sequences from the ZO16 strain, along with the retrieved reference sequences, were then aligned using software called CLUSTAL W (version 1.74). This alignment process arranges the sequences to identify regions of similarity and difference. The alignment was manually checked and adjusted for accuracy before constructing a phylogenetic tree. Finally, a phylogenetic tree was constructed using the neighbor-joining method implemented in MEGA 11 software[24]. This tree visually depicts the evolutionary relationships between the ZO16 strain and its closest relatives based on the 16S rDNA sequence similarities.

Determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentrations (MBC): The antibacterial efficacy evaluated of both the crude extract and the purified compounds by determining their minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against relevant bacterial strains. Established protocols by Pfaller *et al.*[25] were followed to ensure consistent and reliable testing procedures. Chloramphenicol (Thermo Fisher Scientific, USA) served as a positive control in this experiment.

Determination of the cytotoxicity of the crude extract and purified compound: The potential anticancer properties of the crude extract and purified compounds were investigated using the MTT assay[26]. The substances were tested against a panel of three cancer cell lines: Cervical cancer (HeLa), liver cancer (HepG2) and breast cancer (MDA-MB-231) cells. A range of concentrations (1-512 µg/mL) was used to assess their effect. To determine the extract or compounds' specificity for cancer cells, a non-cancerous cell line (Vero) was also included in the experiment. Selectivity index (SI) was calculated to measure this preference. The SI is the ratio of the concentration needed to inhibit 50% growth in the non-cancerous cells (Vero) compared to the concentration needed for the same effect in cancer cells. A higher SI indicates the compound is more selective for targeting cancer

cells with minimal harm to healthy cells. Doxorubicin hydrochloride (Thermo Fisher Scientific, USA) served as a positive control for cytotoxicity testing.

Compound purification and characterization: The crude extract (12.0 g) was separated into its components using column chromatography. This involved packing a column with silica gel and passing the extract through it with a gradually increasing solvent mixture (CH_2Cl_2: MeOH). Fractions containing potentially active compounds were eluted (extracted) using 7-10% methanol in Dichloromethane (CH_2Cl_2). These fractions were further purified using thin-layer chromatography (TLC) with a different solvent mixture (CH_2Cl_2: EtOAc, 3:5). This yielded 10.75 mg of a purified compound, named compound **1**. Similarly, fractions obtained using 10-12% methanol in CH_2Cl_2 were further purified using TLC, resulting in 12.48 mg of another purified compound (compound **2**). The structures of the purified compounds (compounds **1** and **2**) were determined using various spectroscopic techniques. Melting points were measured using a melting point apparatus. Ultraviolet (UV) absorption spectra were recorded to gain insights into the compounds' electronic structures. Additionally, Nuclear Magnetic Resonance (NMR) spectroscopy provided detailed information about the compounds' atomic arrangements. Both ^{1}H-NMR and ^{13}C-NMR spectra were obtained using a high-field NMR spectrometer. Finally, mass spectrometry was used to determine the molecular weights of the purified compounds.

Statistical analyses: This study employed descriptive statistics to analyze the results. This approach helps to describe and summarize the key characteristics of data. The findings are presented as averages (means) with an indication of variability (standard deviations, SD). Statistical analyses were performed using SPSS for Windows version 11.01 (SPSS Inc., Chicago, Illinois, USA). Treatment effects were analyzed using one-way ANOVA, followed by Tukey's multiple comparisons. A $p<0.05$ was used to indicate significance.

RESULTS

The 37 different strains of actinomycetes were isolated from the roots of ginger (*Zingiber officinale*). All strains were subsequently tested for their ability to inhibit the growth of tested bacteria. This was done by observing the inhibition zone around each bacterial colony on a plate. Among all the strains, ZO16 showed the most promising antibacterial activity, with inhibition zones reaching 30 to 35 mm against a specific panel of pathogenic bacteria (Fig. 1a).

The aerial mycelia initially appeared greyish green, turning dark grey after seven days of incubation (Fig. 1b). Notably, ZO16 produced light brown soluble pigment. Examining ZO16 under a microscope revealed structures called sporophores that branch out at single points (monopodially). These sporophores bore flexible, oval-shaped spores with a hairy surface. Both the aerial and substrate mycelia (thread-like fungal structures) were well-developed

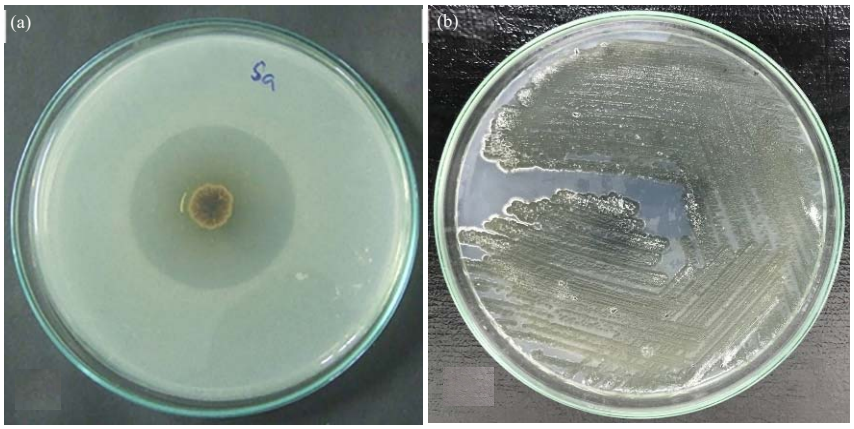

Fig. 1(a-b): Screening of antibacterial activity and subculture of *Streptomyces prasinus* ZO16. After 24 hrs of incubation at 37°C, the actinomycetes colony's clear zone were examined to check for antibacterial activity using the soft-agar overlay technique, (a) *Staphylococcus aureus* ATCC 25923 was added to the 7-day-old preculture of *Streptomyces prasinus* ZO16 on ISP-2 medium and (b) The aerial mycelia initially appeared greyish green, turning dark grey after 7-days of incubation

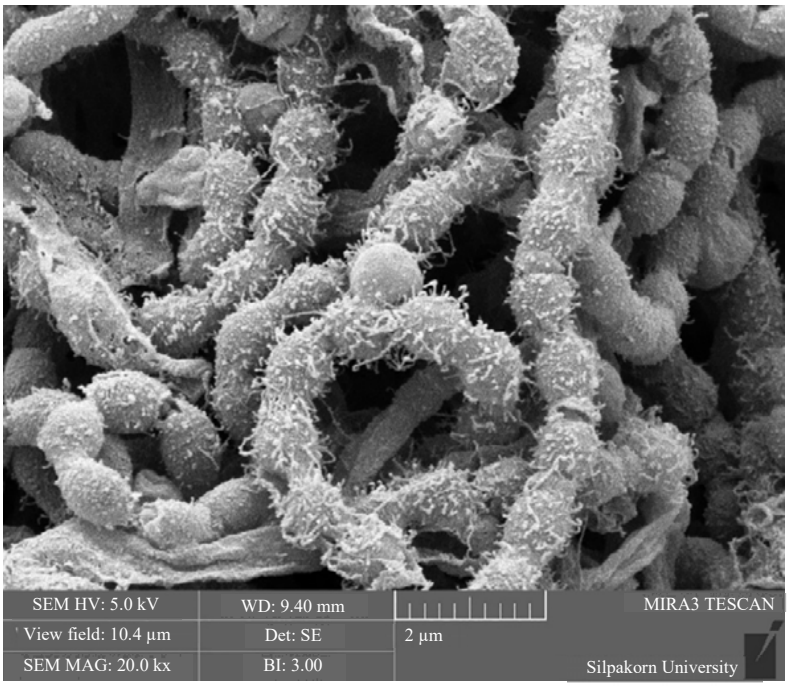

Fig. 2: Scanning electron micrograph of *Streptomyces prasinus* ZO16 grown on the ISP-2 agar after 15 days at 32°C incubation

and showed no fragmentation (Fig. 2). Based on the microscopic observations and the detection of a specific molecule (LL-diaminopimelic acid) in the cell extract, ZO16 was identified as belonging to the *Streptomyces* genus. Further analysis of the 16S ribosomal RNA gene (rDNA) using a technique called BLAST indicated that ZO16 is closely related (99.19% sequence similarity) to *Streptomyces prasinus* strain NBRC 12810. A phylogenetic tree (Fig. 3) confirmed this close relationship, showing ZO16 clustering with *S. prasinus* strains NBRC 12810, NRRL B-2712 and IHBA 9329. Finally, the 16S rDNA sequence of ZO16 was deposited in GenBank, a public database for genetic sequences, under the accession number PP800765.

This study successfully isolated two purified compounds from the crude extract obtained from *Streptomyces prasinus* strain ZO16. Information about the chemical structure of these compounds, determined using various spectroscopic techniques, is presented in the following sections.

Compound 1: Was a pale yellow amorphous powder, MP 173-175°C, UV (MeOH)λ_{max} nm (logε): 255 (4.82), 365 (4.05); IRν_{max} (KBr) cm^{-1}: 3440, 1736, 1531, 1461; ESI-MS m/z (rel. int.): 293 $[M+H]^+$, 315 $[M+Na]^+$; molecular formula: $C_{18}H_{16}N_2O_2$; ^{1}H-NMR ($CDCl_3$, δ, ppm, J/Hz): 8.96 (1H, d, J = 7.3, H-2), 8.53 (1H, d, J = 8.5, H-4), 8.18 (1H, d, J = 8.6, H-6), 8.05 (1H, t, J = 7.4, H-3), 7.91 (1H, t, J = 7.1, H-7), 7.88 (1H, d, J = 8.4, H-8), 5.45 (1H, t, J = 7.1, H-2'), 4.06 (2H, d, J = 6.9, H-1') and 1.82 (6H, s, H-4', 5'). ^{13}C-NMR ($CDCl_3$, δ, ppm, J/Hz): 166.6 (s, 1-COOH), 145.8 (s, C-5a), 144.6 (s, C-4a), 140.8 (s, C-9), 140.6 (s, C-9a, 10a), 138.6 (d, C-2), 136.8 (s, C-3'), 136.2 (d, C-4), 133.2 (d, C-7), 132.8 (d, C-8), 131.6 (d, C-3), 129.5 (d, C-6), 126.5 (s, C-1), 121.7 (d, C-2'), 31.4 (t, C-1'), 27.3 (q, C-4') and 18.5 (C-5').

Compound 2: Was a pale purple amorphous powder, MP 175-177°C, UV (MeOH)λ_{max} nm (logε): 236 (4.32), 283 (4.43), 374 (3.85), 516 (3.87), 545(3.89); IRν_{max} (KBr) cm^{-1}: 3438, 1736, 1628, 1594, 1546; ESI-MS m/z (rel. int.): 323 $[M+H]^+$, 345 $[M+Na]^+$; molecular formula: $C_{19}H_{18}N_2O_3$; ^{1}H-NMR ($CDCl_3$, δ, ppm, J/Hz): 8.42 (1H, d, J = 7.5, H-2), 7.83 (1H, t, J = 8.5, H-3), 7.74 (1H, d, J = 8.4, H-4), 6.97 (1H, s, H-8), 6.16 (1H, s, H-6), 5.33 (1H, t, J = 6.9, H-2'), 3.81 (3H, s, CH_3-5), 3.61 (2H, d, J = 6.9, H-1'), 1.81 (3H, s, H-4') and 1.64 (3H, s, H-5'). ^{13}C-NMR ($CDCl_3$, δ, ppm, J/Hz): 184.5 (s, C-7), 166.8 (s, 1-COOH), 148.8 (s, C-9a), 144.2 (s, C-9), 140.3 (s, C-5a), 138.4 (s, C-3'), 137.2 (d, C-8), 134.2 (s, C-4a), 133.8 (d, C-3), 132.8 (s, C-10a), 129.7 (d, C-2), 127.9 (s, C-1), 119.7 (d, C-4), 119.5 (d, C-2'), 102.2 (d, C-6), 36.1 (s, CH_3-5), 30.6 (t, C-1'), 27.3 (q, C-4') and 19.5 (q, C-5').

Structural elucidation of compound **1** revealed its identity as endophenazine A [9-(3-methyl-2-buten-1-yl)-1-phenazinecarboxylic acid] (Fig. 4a). Similarly, compound **2** was

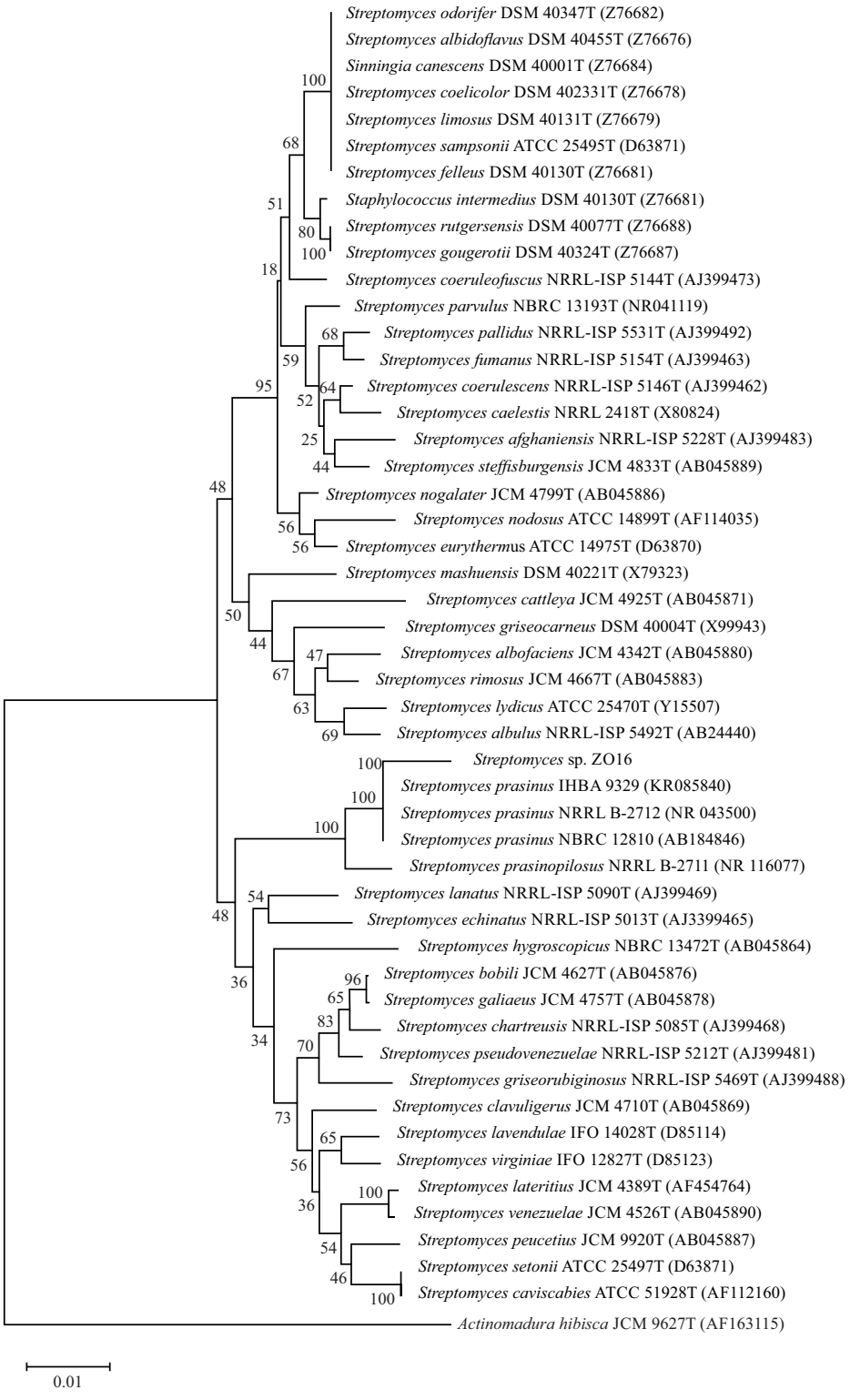

Fig. 3: Neighbor-joining tree based on 16SrDNA gene sequences showing the position of *Streptomyces prasinus* ZO16 and related taxa

Numbers at the nodes indicate levels of bootstrap support (%) based on a neighbor-joining analysis of 1000 resampled datasets. Bar, 0.01 substitutions per sites and *Actinomadura hibisca* JCM9627T (AF163115) was used as the outgroup

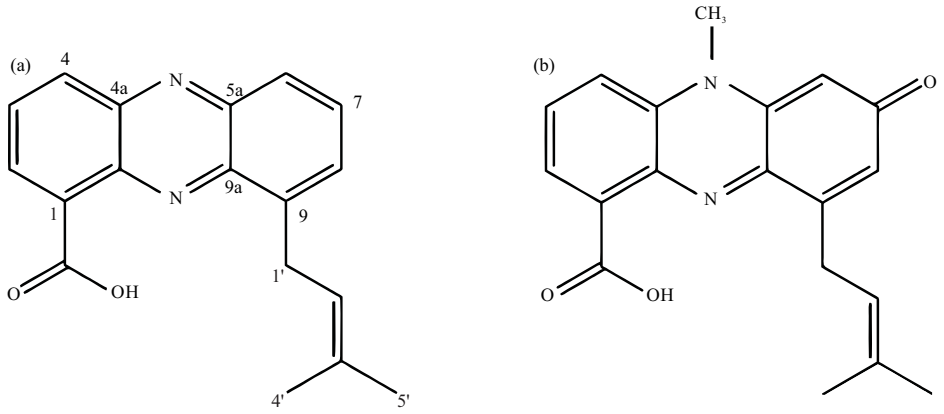

Fig. 4(a-b): Structures of the compounds, (a) Endophenazine A [9-(3-methyl-2-buten-1-yl)-1-phenazinecarboxylic acid] and (b) Endophenazine B [5,7-dihydro-5-methyl-9-(3-methyl-2-buten-1-yl)-7-oxo-1-phenazinecarboxylic acid]

Table 1: MIC and MBC of the purified compounds and crude extract against tested bacteria

	MIC[a] (µg/mL)					MBC (µg/mL)				
Test substances	[b]S.a.	MRSA Sp2	MRSA Sp3	MRSA RI	MRSA T2	S.a.	MRSA Sp2	MRSA Sp3	MRSA RI	MRSA T2
Crude extract	16	32	32	32	64	64	64	128	128	256
Compound **1**	8	8	8	16	32	32	32	32	64	128
Compound **2**	8	8	8	16	32	32	32	32	64	128
Chloramphenicol	2	2	1	2	8	4	4	2	4	16

[a]MIC: Minimum inhibitory concentration, MBC: Minimum bactericidal concentration, [b]S.a.: *Staphylococcus aureus* ATCC 25923, MRSA: Methicillin-Resistant *Staphylococcus aureus* RI, Sp2, Sp3 and T2

Table 2: IC_{50} values and selectivity indices (SI) of crude extract and purified compounds against cancer cell lines

	Vero cells*	MDA-MB-231 cells		HeLa cells		HepG2 cells	
Test substances	IC_{50} (µg/mL)**	IC_{50} (µg/mL)	SI***	IC_{50} (µg/mL)	SI	IC_{50} (µg/mL)	SI
Crude extract	465.52±32.45[a]	62.20±6.24[a]	7.48	82.72±12.34[a]	5.63	126.54±25.34[a]	3.68
Compound **1**	328.63±24.86[b]	23.41±5.87[b]	14.04	30.40±7.85[b]	10.81	86.45±16.71[b]	3.80
Compound **2**	317.44±28.25[b]	28.26±7.13[b]	11.23	32.51±0.37[b]	9.76	78.32±12.65[b]	4.05
Doxorubicin hydrochloride	99.48±10.46[c]	6.25±1.14[c]	15.92	1.95±0.20[c]	51.02	92.16±12.23[b]	1.08

*Vero cells: African green monkey kidney cell line, MDA-MB-231 cells: Human breast cancer cell line, HeLa cells: Human cervical carcinoma cell line, HepG2: Human hepatocellular carcinoma cell line and **IC_{50} values represent the concentration causing 50% growth inhibition. The values are expressed as Mean±Standard Deviation of the three replicates, ***SI: Selectivity indices (SI) were calculated as the ratio of the IC_{50} in the Vero cell line to the IC_{50} in the cancer cell lines and [a,b,c]Different letters indicated statistically significant differences ($p<0.05$)

identified as endophenazine B [5,7-dihydro-5-methyl-9-(3-methyl-2-buten-1-yl)-7-oxo-1-phenazinecarboxylic acid] (Fig. 4b). In the experiment conducted for isolation of endophenazines A and B, it was found that the isolate ZO16 could produce endophenazines A and B on 0.90 and 1.04 mg/g of crude extract or 0.94 and 1.08 mg/L of culture medium.

Table 1 summarizes the antibacterial activity of the crude extract and purified compounds against various bacterial strains, determined by their MIC and MBC values. Notably, the purified compounds displayed a broad spectrum of activity, inhibiting the growth of *S. aureus* ATCC 25923 and clinical isolates of MRSA (RI, Sp2, Sp3 and T2) with MICs and MBCs ranging from 8-32 and 32-128 µg/mL, respectively. The cytotoxicity of the crude extract and purified compounds was evaluated against Vero cells and three human cancer cell lines (HeLa, HepG2 and MDA-MB-231). They exhibited high cytotoxicity, with IC_{50} values ranging from 23.41-126.54 µg/mL to the cancer cell lines. The crude extract and purified compounds showed moderate cytotoxicity against non-cancerous cell line (Vero), with IC_{50} values ranging from 317.44-465.52 µg/mL. The selectivity indices (SI) observed for the crude extract and purified compounds against MDA-MB-231 and HeLa cell lines were lower compared to doxorubicin hydrochloride, indicating a less selective cytotoxic effect on these cancer cell lines. Conversely, the SI for the extract and purified compounds against HepG2 cells was higher compared to doxorubicin hydrochloride, suggesting a more targeted cytotoxicity towards this specific cancer cell line (Table 2).

The current study findings demonstrate that endophenazines A and B, purified from *Streptomyces prasinus* ZO16 isolated from *Zingiber officinale* root tissues, exhibit antibacterial activity and significant anticancer properties. These compounds also display moderate cytotoxicity in normal cells. These results suggest that *Zingiber officinale* root tissues represent a promising source for isolating actinomycetes capable of producing valuable bioactive compounds.

DISCUSSION

This study successfully isolated and identified two known bioactive compounds, endophenazine A (compound **1**) and endophenazine B (compound **2**), from *Streptomyces prasinus* ZO16. This identification was achieved by comparing the spectral data of the purified compounds with data from previous research[27-29].

Endophenazines are a diverse group of nitrogen-containing heterocyclic compounds produced by various microorganisms for example; *Streptomyces* and *Pseudomonas* genera[30], exhibiting a broad range of biological activities. The potential of endophenazines for various applications, such as the development of novel antibiotics in medicine, biocontrol agents in agriculture and antifouling coatings in industry, has garnered significant recent attention[31-34]. The current study investigated the isolation, taxonomic identification of the producing organism (*Streptomyces prasinus* ZO16), antibacterial activities and purification and evaluation of the anticancer endophenazines produced by this strain. The yield of endophenazines can vary significantly between different *Streptomyces* species. For example, *Streptomyces kunmingensis* YIM 121234 and *Streptomyces anulatus* 9663 have been reported to produce 0.08 and 5.4 mg/L of endophenazine A, respectively[28,35]. Notably, the highest reported yield of endophenazine A was obtained from a genetically engineered strain, *Streptomyces coelicolor* M512, which produced 20 mg/L via the heterologous expression of the entire endophenazine gene cluster[36]. This study reported the isolation of *Streptomyces prasinus* ZO16 from the root tissues of *Zingiber officinale* (ginger). This strain exhibited a moderate yield of endophenazine A (0.94 mg/L) under non-optimized cultivation conditions. Future studies could explore optimization strategies to potentially enhance the production of these valuable compounds. Based on the phylogenetic tree (Fig. 2), the ZO16 isolate shares a close evolutionary relationship with *Streptomyces prasinus* strains NBRC 12810, NRRL B-2712 and IHBA 9329. This close relationship is supported by the high 16S rDNA gene sequence similarity of 99.19% between ZO16 and these strains. Organisms with a more recent common ancestor tend to be classified within the same genus because their genetic makeup is more similar. Previously, *Streptomyces prasinus* has never been reported to produce endophenazine. On the contrary, *Streptomyces prasinus* was found to produce prasinomycins which were active against Gram-positive bacteria[37]. *Streptomyces prasinus* ZO16 was investigated for its endophenazine production in this study. The isolated endophenazines were primarily endophenazine A and B, exhibiting antimicrobial activities against the clinical isolates of MRSA and *Staphylococcus aureus* ATCC 25923, consistent with previously published data on these compounds[35,38-40]. While this study focused on these 2 major endophenazines, the literature reports a wider diversity of endophenazine forms, including endophenazines A-D[35], endophenazine A1[38], glycosylated A-E[39] and N-prenylated F1[40]. These various endophenazine forms have been shown to possess antibacterial activity against a range of pathogenic bacteria, including *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*. Furthermore, endophenazines have demonstrated cytotoxic activity against the human breast cancer cell line (MCF-7), with an IC_{50} value of 20.23±1.37 µg/mL[28]. The results demonstrate promising cytotoxic activity of endophenazines against various cancer cell lines (MDA-MB-231, HeLa and HepG2). The IC_{50} values ranging from 23.41 to 126.54 µg/mL indicate a potent effect on cancer cell viability. This suggests potential for further development as anticancer agents. However, the cytotoxicity towards the non-cancerous Vero cell line (IC_{50}: 317.44-465.52 µg/mL) highlights the need for improved selectivity. While these values show moderate cytotoxicity compared to cancer cells, minimizing damage to healthy cells is crucial for therapeutic applications. The results also indicate a more targeted effect on HepG2 cells compared to doxorubicin hydrochloride, as evidenced by higher selectivity indices (SI). This suggests the potential for endophenazines to be more effective against HepG2 liver cancer specifically.

CONCLUSION

Endophenazine A and B were isolated from *Streptomyces prasinus* ZO16. These compounds exhibited antibacterial properties against the clinical isolates of MRSA

and *Staphylococcus aureus* ATCC 25923. It also exhibited anticancer properties against various cancer cell lines. These compounds exhibited potent effects on cancer cell viability, particularly HepG2 liver cancer cells, with higher selectivity compared to doxorubicin hydrochloride. However, further research is necessary to improve selectivity towards cancer cells and elucidate the mechanism of action. Additionally, *in vivo* studies and exploration of strategies to enhance targeting will be crucial for the development of endophenazines as potential therapeutic agents for cancer. Further investigation of these compounds has the potential to contribute to the development of improved methods for controlling and treating bacterial infections and some cancers.

SIGNIFICANCE STATEMENT

This study identified a promising new source of bioactive compounds. The isolated *Streptomyces prasinus* ZO16 from the root tissues of ginger (*Zingiber officinale*). This strain produces endophenazine A and B, which exhibit antibacterial and anticancer properties with minimal toxicity towards healthy cells. These findings suggest the potential of endophenazines from *Streptomyces prasinus* ZO16 as alternative therapeutic agents. These compounds exhibit antibacterial and anticancer properties, making them attractive candidates for the development of novel treatments for bacterial infections and cancers.

ACKNOWLEDGMENT

This work was supported by a research grant (No. SRIF-JRG-2567-04) from the Faculty of Science, Silpakorn University, Nakhon Pathom, Thailand.

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