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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.2025.27.37

Research Article
Antibacterial and Anticancer Properties of Diketopiperazines from *Streptomyces antimicrobicus* BN122, an Endophyte in *Oryza sativa* var. glutinosa

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Abstract

Background and Objectives: This study characterized a bacterial strain, BN122, isolated from the root tissues of purple sticky rice (*Oryza sativa* L. var. glutinosa). Identified as *Streptomyces antimicrobicus* based on 16S rDNA analysis and physical-chemical properties, the aim was to isolate and evaluate the antibacterial and anticancer activities of its bioactive compounds. **Materials and Methods:** The major compounds were purified from BN122's culture extract using column chromatography and TLC. The NMR spectroscopy and mass spectrometry confirmed their identities as Cyclo-(L-Pro-L-Val), Cyclo-(L-Pro-L-Leu), Cyclo-(L-Pro-L-Trp) and Cyclo-(L-Pro-L-Phe). The antibacterial and anticancer activities of these compounds were subsequently assessed. Statistical significance was determined using SPSS software. **Results:** Isolated compounds exhibited potent antibacterial activity against Gram-positive bacteria. Minimum inhibitory concentrations (MICs) ranged from 32 to 256 µg/mL, while minimum bactericidal concentrations (MBCs) were between 128 and 512 µg/mL. Compounds demonstrated potent cytotoxic activity against cancer cells, with IC_{50} values ranging from 32.00 to 57.08 µg/mL for MDA-MB-231 cells, 85.73 to 158.93 µg/mL for HeLa cells and 276.89 to 323.48 µg/mL for HepG2 cells. Notably, these compounds exhibited moderate toxicity towards non-cancerous Vero cells (IC_{50} = 482.73 to 680.87 µg/mL). **Conclusion:** The findings suggested that *Streptomyces antimicrobicus* BN122 produces compounds with promising antibacterial and anticancer properties. Further research on these compounds could contribute to developing novel therapeutic strategies for bacterial infections and certain cancers.

Key words: Antibacterial activity, anticancer activity, diketopiperazines, endophyte, *Streptomyces antimicrobicus* BN122, *Oryza sativa* L. var. glutinosa

Citation: Taechowisan, T., T. Chuen-Im and W.S. Phutdhawong, 2025. Antibacterial and anticancer properties of diketopiperazines from *Streptomyces antimicrobicus* BN122, an endophyte in *Oryza sativa* var. glutinosa. Pak. J. Biol. Sci., 28: 27-37.

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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

Endophytic actinomycetes are unicellular, Gram-positive, aerobic bacteria classified within the phylum Actinomycetota. These microorganisms exhibit a branched morphology and actively contribute to the breakdown of organic matter, thereby participating in nutrient cycling within their plant hosts[1]. In recent years, endophytic actinomycetes have gained significant interest as a promising source for novel drug discovery efforts[2]. Their exploration has yielded the identification, isolation and exploitation of several strains with biomedical potential. These endophytic actinomycetes serve as valuable resources for the development of new and essential drugs, addressing the growing challenge of discovering novel bioactive compounds with therapeutic applications[3]. Endophytic actinomycetes have been successfully isolated from the rice plant (*Oryza sativa* L.). Notably, *Streptomyces* species are prevalent endophytes within rice and represent promising candidates for the extraction of biologically active compounds[4]. Rice is the dominant crop in Thai agriculture, exhibiting a high diversity of varieties due to the country's rich and varied ecological landscape[5]. Purple sticky rice (*Oryza sativa* L. var. glutinosa) is a glutinous rice variety native to Thailand. This rice is characterized by its distinctive purple pigmentation in the bran and pericarp. In recent years, purple rice consumption has gained popularity due to its reported health benefits associated with bioactive compounds, including anthocyanins and γ-oryzanol[6-8]. While numerous studies have investigated the actinobacterial diversity associated with different rice cultivars[9-14], endophytic actinobacteria specifically from *Oryza sativa* L. var. glutinosa remain unexplored. This study presents the first report of endophytic actinobacterial isolation from this glutinous rice variety in Yasothon Province, Thailand. This study aimed to investigate the endophytic actinomycetes from purple rice (*Oryza sativa* L. var. glutinosa) in Yasothon Province and screen 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. This study aimed to evaluate 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 Silpakorn University, Nakhon Pathom, Thailand (Departments of Microbiology and Chemistry), between May, 2023 and April, 2024.

Isolation and antibacterial screening of actinomycetes: Five *Oryza sativa* L. var. glutinosa samples were collected from Pho Sai (subdistrict), Patiu District, Yasothon Province, Thailand (coordinates: 15.84823″N, 104.39223″E). To isolate actinomycetes, the leaves, stems and roots were cut into 50 small pieces (5×5 mm^2) each and thoroughly washed 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 tissue pieces were dried aseptically in a laminar flow cabinet (Esco Scientific, Pennsylvania, USA). The surface-sterilized plant tissues were plated onto a special medium called humic acid-vitamins (HV) agar[15]. 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[16]. A total of 23 actinomycete isolates were evaluated for their ability to inhibit the growth of bacteria. This screening included *Staphylococcus aureus* TISTR885, *Staphylococcus epidermidis* TISTR518, *Bacillus cereus* TISTR687, *Bacillus subtilis* TISTR008, *Escherichia coli* TISTR887, *Salmonella typhimurium* TISTR292, *Pseudomonas aeruginosa* TISTR1287 and a clinical isolate of Methicillin-Resistant *Staphylococcus aureus* (MRSA) Sp3. A modified soft-agar overlay method was used[17] and the size of the inhibition zones was measured. This experiment was performed in triplicate to ensure accuracy. Among the 23 isolates, BN122 (the isolate from the root tissue of *Oryza sativa* L. var. glutinosa) 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.*[18]. Strain BN122 was grown on a large scale (600 Petri dishes (about 12 L)) using ISP-2 agar for 21 days at 32°C. The culture was then extracted with ethyl acetate (EtOAc) to recover potential bioactive compounds[19]. The combined organic extracts were concentrated using a rotary evaporator R-300 (BUCHI Labortechnik AG, Switzerland), resulting in a dark brown solid (15.25 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: The Scanning Electron Microscopy (SEM) was used to observe the morphology of the selected strain (BN122). The sample was prepared according to the methods described by Castillo *et al.*[20]. The resulting

preparations were dried at the critical point of liquid CO_2 (Quorum K850, United Kingdom), sputter coated with gold (Safematic CCU-010 HV, Switzerland) and examined with a scanning electron microscope (TESCAN Mira3, Czech Republic). Spore chain morphology and spore surface ornamentation of the selected isolate were recorded.

Identification of BN122 strain using 16S rDNA sequencing and phylogenetic analysis: The 16S rDNA sequencing and phylogenetic analysis were conducted to identify the BN122 strain. First, the BN122 strain, exhibiting the strongest antibacterial activity, was cultured in ISP-2 broth for 7 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 and the 16S rDNA gene was amplified using the methods described by Taechowisa *et al.*[21]. The primers used for amplification were A7-26f (5′-CCGTCGACGAGCTCAGAGTTTG ATCCTGGCTCAG-3′) and B1523-1504r (5′-CCCGGGTACCAAGCTTAAGGAGGTGATCC AGCCGCA-3′). The PCR products were purified using a QIAquick gel extraction kit (Qiagen, Germany) following the manufacturer's protocol. Sanger sequencing of purified PCR products was performed at 1st BASE, Singapore. The NCBI BLAST was employed to confirm the similarity of actinomycetes to reference species. Sequences of closely related type strains, obtained from the GenBank database, were subjected to multiple alignments using Clustal W. Subsequently, a phylogenetic tree was constructed using the neighbor-joining method in MEGA 11 software[22]. The 16S rDNA sequence of *Microbispora corallina* DF-32 (JCM 10267) served as an outgroup.

Determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentrations (MBC): The antibacterial efficacy of both the crude extract and the purified compounds was evaluated by determining their minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against relevant bacterial strains. Established protocols by Pfaller *et al.*[23] 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[24]. 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. Concentrations ranging from 10 to 160 µg/mL were used to evaluate their effect. To evaluate the specificity of the extract or compounds for cancer cells, a non-cancerous cell line (Vero) was added to the experiment. The 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 (15.25 g) was separated into its components by column chromatography using silica gel 60 (Merck, 0.040-0.063 mm). 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 5-7% 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 13.14 and 13.23 mg of purified compounds, named compounds **1** and **2**, respectively. Similarly, fractions obtained using 7-10% methanol in CH_2Cl_2 were further purified using TLC, resulting in 12.48 and 12.65 mg of other purified compounds (compounds **3** and **4**). The structures of the purified compounds (compounds **1-4**) were determined using various spectroscopic techniques. Melting points were measured using a Stuart SMP20 apparatus (Cole-Parmer, Staffordshire, UK). Ultraviolet (UV) spectra were recorded using a Perkin Elmer Lambda 35 spectrophotometer (PerkinElmer Life and Analytical Sciences, USA) 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 (400 MHz) and ^{13}C-NMR (100 MHz) spectra were obtained using a Bruker Avance III NMR Spectrometer (Bruker, Germany). Finally, mass spectrometry analysis was also performed on a POLARIS Q mass spectrometer (Thermo Fisher Scientific, USA) to determine the molecular weights of the purified compounds.

Statistical analysis: Each experiment was performed in triplicate. Data were expressed as Mean ± 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. Values of $p<0.05$ were considered to indicate statistical significance.

RESULTS

A total of 750 leaf, stem and root tissues of *O. sativa* L. var. glutinosa were used to isolate endophytic actinomycetes, of the 42 isolates recovered, 24 were from roots, 13 from stems and 5 isolates from leaves with a prevalence of 9.6, 5.2 and 2%, respectively. Based on screening for antimicrobial activity using the soft-agar overlay method, one actinomycete isolate (BN122) showed potent activity (4.5 to 6.7 cm diameter inhibition zones) against the tested bacteria; Fig. 1a: *Staphylococcus aureus* TISTR885, Fig. 1b: *Staphylococcus epidermidis* TISTR518, Fig. 1c: *Bacillus cereus* TISTR687 and Fig. 1d: Methicillin-resistant *Staphylococcus aureus* Sp3.

Examining BN122 under a microscope revealed structures called sporophores that branch out at single points (monopodially). These sporophores bore flexible, rod-shaped spores with a smooth surface. Both the aerial and substrate mycelia (thread-like fungal structures) were well-developed and showed no fragmentation (Fig. 2). The aerial mycelia initially appeared creamy white, turning creamy yellow after seven days of incubation. Notably, BN122 produced light yellow soluble pigment. Based on the microscopic observations and the detection of a specific molecule (LL-diaminopimelic acid) in the cell extract, we identified BN122 as belonging to the *Streptomyces* genus. Further analysis of the 16S ribosomal RNA gene (rDNA) using a technique called BLAST indicated that BN122 is closely related (99.19% sequence similarity) to *Streptomyces antimicrobicus* strain SMC 277. A phylogenetic tree (Fig. 3) confirmed this close relationship, showing BN122 clustering with *Streptomyces antimicrobicus* strain SMC 277. Finally, the 16S rDNA sequence of BN122 was deposited in GenBank, a public database for genetic sequences, under the accession number PQ164395.

This study successfully isolated four purified compounds from the crude extract obtained from *Streptomyces antimicrobicus* BN122. Information about the chemical structure of these compounds, determined using various spectroscopic techniques, is presented in the following sections.

Compound 1: It was a white amorphous powder, MP 190-192°C, ESI-MS m/z (rel. int.): 196.9 $[M+H]^+$; molecular formula: $C_{10}H_{16}N_2O_2$; ^{1}H-NMR ($CDCl_3$, δ, ppm, *J*/Hz): 5.98 (1H, brs, N-H), 4.07 (1H, t, *J* = 7.6, H-6), 3.93 (1H, s, H-9), 3.50-3.67 (2H, m, H-3), 2.58-2.66 (1H, m, H-10), 2.34-2.40 (2H, m, H-5), 2.01-2.10 (1H, m, H-4b), 1.86-1.94 (1H, m, H-4a), 1.06 (3H, d, *J* = 7.3, CH_3) and 0.93 (3H, d, *J* = 7.6, CH_3). ^{13}C-NMR ($CDCl_3$, δ, ppm, *J*/Hz): 169.72 (s, C-1), 164.61 (s, C-7), 60.26 (d, C-6), 58.71 (d, C-9),

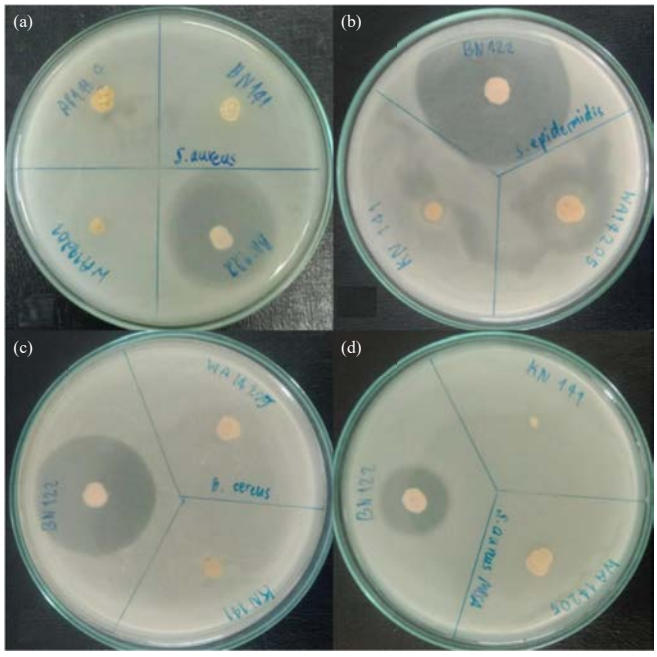

Fig. 1(a-d): Screening of antibacterial activity using the soft-agar overlay technique, (a) *Staphylococcus aureus* TISTR885, (b) *Staphylococcus epidermidis* TISTR518, (c) *Bacillus cereus* TISTR687 and (d) Methicillin-resistant *Staphylococcus aureus* Sp3

The tested bacteria in soft-agar were overlaid onto the 7-day-old preculture of *Streptomyces antimicrobicus* BN122 on ISP-2 medium. After 24 hrs of incubation at 37°C, the actinomycetes colony's clear zone was measured

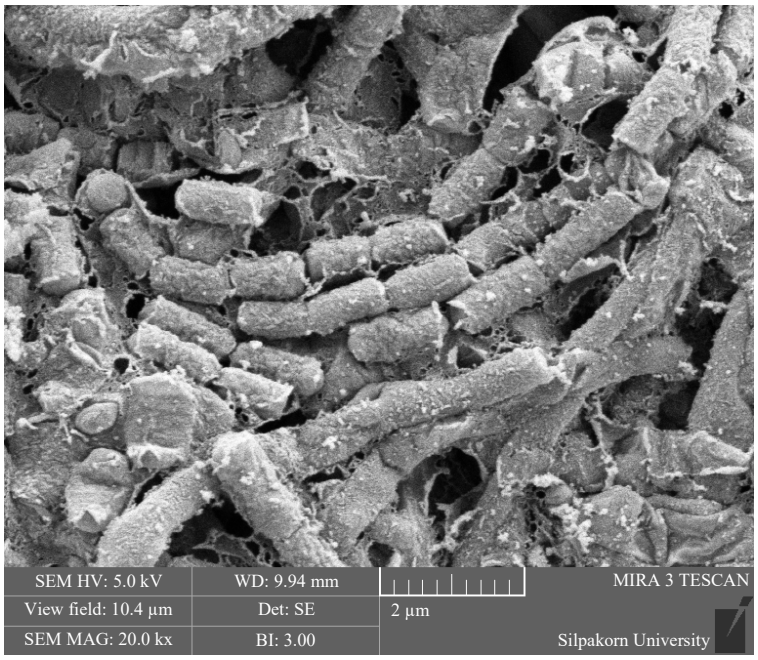

Fig. 2: Scanning electron micrograph of *Streptomyces antimicrobicus* BN122 grown on the ISP-2 agar after 15 days at 32°C incubation

45.06 (t, C-3), 28.47 (t, C-5), 28.33 (d, C-10), 22.32 (t, C-4), 19.21 (q, C-11) and 16.04 (q, C-11′).

Compound 2: It was a white amorphous powder, MP 163-165°C, ESI-MS m/z (rel. int.): 210.8 $[M+H]^+$; molecular formula: $C_{11}H_{18}N_2O_2$; ^{1}H-NMR ($CDCl_3$, δ, ppm, *J*/Hz): 6.11 (1H, brs, N-H), 4.13 (1H, t, *J* = 8.3, H-6), 4.03 (1H, d, *J* = 9.3, d, *J* = 3.9, H-9), 3.47-3.65 (2H, m, H-3), 2.31-2.37 (2H, m, H-5), 2.01-2.19 (2H, m, H-4), 1.87-1.95 (1H, m, H-11), 1.76 (1H, m, H-10b), 1.54 (1H, m, H-10a), 0.98 (3H, d, *J* = 6.6, CH_3), 0.94 (3H, d, *J* = 6.6, CH_3). ^{13}C-NMR ($CDCl_3$, δ, ppm, *J*/Hz): 170.26 (s, C-1), 166.24 (s, C-7), 59.23 (d, C-6), 53.65 (d, C-9), 45.78 (t, C-3), 38.89 (t, C-10), 28.41 (t, C-5), 24.98 (d, C-11), 23.61 (t, C-4), 23.07 (q, C-12), 21.54 (q, C-12′).

Compound 3: It was a white amorphous powder, MP 165-167°C, ESI-MS m/z (rel. int.): 283.9 $[M+H]^+$; molecular formula: $C_{16}H_{17}N_3O_2$; ^{1}H-NMR ($CDCl_3$, d, ppm, *J*/Hz): 8.25 (1H, brs, N-H (Indole)), 7.13-7.61 (5H, m, Ar-H), 5.75 (1H, brs, N-H), 4.39 (1H, d, *J* = 10.6, d, *J* = 2.7, H-9), 4.07 (1H, t, *J* = 8.0, H-6), 3.77 (1H, d, *J* = 15.0, d, *J* = 3.9, Hb-10), 3.61 (2H, m, H-3), 2.96 (1H, d, *J* = 15.0, d, *J* = 10.7, Ha-10), 2.31-2.38 (2H, m, H-5), 1.88-2.04 (2H, m, H-4). ^{13}C-NMR ($CDCl_3$, d, ppm, *J*/Hz): 168.98 (s, C-1), 165.23 (s, C-7), 136.37 (s, C-9′), 126.45 (s, C-8′), 123.06 (d, C-2′), 122.57 (d, C-6′), 119.78 (d, C-5′), 118.26 (d, C-4′), 111.33 (d, C-7′), 109.75 (s, C-3′), 59.13 (d, C-6), 54.45 (d, C-9), 45.35 (t, C-3), 28.26 (t, C-5), 26.78 (t, C-10) and 22.57 (t, C-4).

Compound 4: It was a white amorphous powder, MP 146-148°C, ESI-MS m/z (rel. int.): 244.9 $[M+H]^+$; molecular formula: $C_{14}H_{16}N_2O_2$; ^{1}H-NMR ($CDCl_3$, δ, ppm, *J*/Hz): 7.23-7.39 (5H, m, Ar-H), 5.64 (1H, brs, N-H), 4.28 (1H, d, *J* = 10.6, d, *J* = 2.7, H-9), 4.09 (1H, t, *J* = 7.6, H-6), 3.56-3.66 (2H, m, H-3), 2.77 (2H, d, *J* = 14.4, d, *J* = 10.5, H-10), 2.32-2.38 (2H, m, H-5), 1.98-2.06 (1H, m, H-4b), 1.87-1.92 (1H, m, H-4a). ^{13}C-NMR ($CDCl_3$, δ, ppm, *J*/Hz): 169.05 (s, C-1), 164.75 (s, C-7), 135.66 (s, C-1′), 129.07 (d, C-2′), 128.85 (d, C-3′), 127.33 (d, C-4′), 59.04 (d, C-6), 56.08 (d, C-9), 45.39 (t, C-3), 36.72 (t, C-10), 28.32 (t, C-5) and 22.53 (t, C-4).

Structural elucidation of compounds **1**, **2**, **3** and **4** revealed their identity to diketopiperazines as Cyclo-(L-Pro-L-Val) (Fig. 4a), Cyclo-(L-Pro-L-Leu) (Fig. 4b), Cyclo-(L-Pro-L-Trp) (Fig. 4c) and Cyclo-(L-Pro-L-Phe) (Fig. 4d), respectively. In the experiment conducted for isolation of these diketopiperazines, it was found that the isolate BN122 could produce diketopiperazines; Cyclo-(L-Pro-L-Val), Cyclo-(L-Pro-L-Leu), Cyclo-(L-Pro-L-Trp) and Cyclo-(L-Pro-L-Phe) on 0.86, 0.87, 0.82 and 0.83 mg/g of crude extract or 1.09, 1.10, 1.04 and 1.05 mg/L of culture medium.

Table 1-2 summarize 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 Gram-positive bacteria; *Staphylococcus aureus* TISTR885, *Staphylococcus epidermidis*

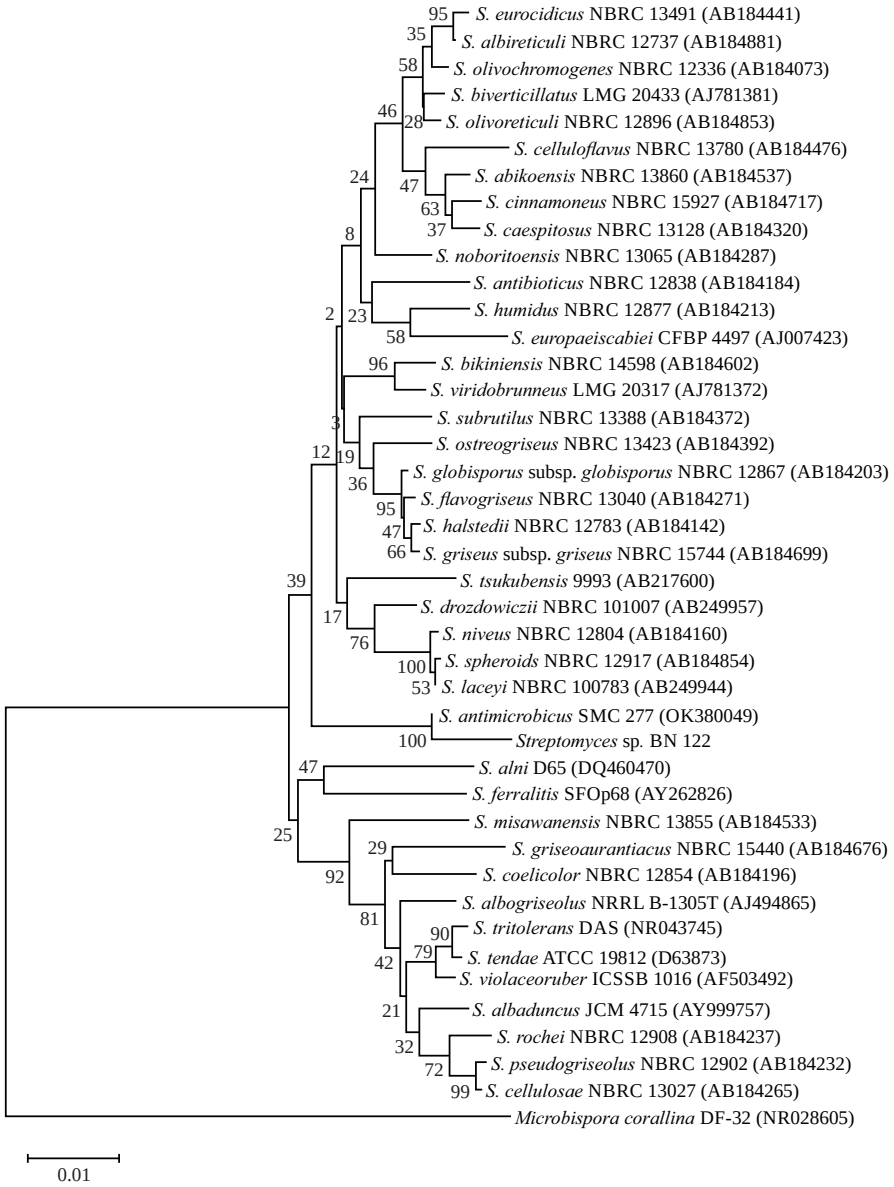

Fig. 3: Phylogenetic analysis of 16S rDNA gene sequences of *Streptomyces antimicrobicus* strain BN122 and related strains were retrieved from GenBank (accession numbers in parentheses)

A phylogenetic tree was constructed using the neighbor-joining method implemented in MEGA11 software. Bootstrap analysis (1000 replicates) was performed to assess the robustness of the tree topology, with bootstrap percentages displayed for each node (branch length representing 0.01 substitutions per site)

TISTR518, *Bacillus cereus* TISTR687, *Bacillus subtilis* TISTR008 and methicillin-resistant *Staphylococcus aureus* Sp3 with MICs and MBCs ranging from 32-256 and 128-512 µg/mL, respectively. The cytotoxic effects of the crude extract and purified compounds were assessed against Vero cells and three human cancer cell lines (HeLa, HepG2 and MDA-MB-231). The crude extract demonstrated lower cytotoxicity compared to the purified compounds (Fig. 5a). Notably, the purified compounds induced a progressive decline in cell viability with increasing concentrations (Fig. 5b-e). However, doxorubicin hydrochloride exhibited superior cytotoxicity to the purified compounds at higher concentrations (Fig. 5f). These effects were not observed in untreated controls. The purified compounds exhibited high cytotoxicity towards MDA-MB-231 and Hela cell lines with IC_{50} values ranged from 32.00-57.08 and 85.73-158.93 µg/mL, respectively. They showed weak cytotoxicity against HepG2 cells with IC_{50} values ranged from 276.89-323.48 µg/mL. The

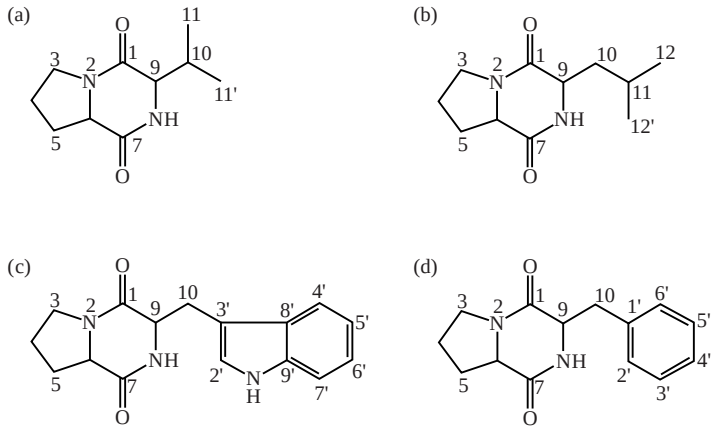

Fig. 4(a-d): Structures of the compounds, (a) Cyclo-(L-Pro-L-Val), (b) Cyclo-(L-Pro-L-Leu), (c) Cyclo-(L-Pro-L-Trp) and (d) Cyclo-(L-Pro-L-Phe)

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

	MIC (µg/mL)							
Test substances	[a]S.a.	S.e.	B.c.	B.s.	MRSA Sp3	E.c.	S.t.	P.a.
Crude extract	256	256	256	256	512	>512	>512	>512
Compound 1	32	32	32	64	256	>512	>512	>512
Compound 2	32	32	32	64	256	>512	>512	>512
Compound 3	32	32	32	64	128	>512	>512	>512
Compound 4	32	32	32	32	128	>512	>512	>512
Chloramphenicol	2	2	2	2	8	16	16	32

[a]S.a.: *Staphylococcus aureus* TISTR885, S.e.: *Staphylococcus epidermidis* TISTR518, B.c.: *Bacillus cereus* TISTR687, B.s.: *Bacillus subtilis* TISTR008, MRSA Sp3: Methicillin-Resistant *Staphylococcus aureus* Sp3, E.c.: *Escherichia coli* TISTR887, S.t.: *Salmonella typhimurium* TISTR292 and P.a.: *Pseudomonas aeruginosa* TISTR1287

Table 2: MBC of the purified compounds and crude extract against tested bacteria

	MBC (µg/mL)							
Test substances	[a]S.a.	S.e.	B.c.	B.s.	MRSA Sp3	E.c.	S.t.	P.a.
Crude extract	512	512	512	512	>512	>512	>512	>512
Compound 1	128	128	256	256	512	>512	>512	>512
Compound 2	128	128	256	256	512	>512	>512	>512
Compound 3	128	128	256	256	512	>512	>512	>512
Compound 4	128	128	256	256	512	>512	>512	>512
Chloramphenicol	16	16	16	16	32	32	32	64

[a]S.a.: *Staphylococcus aureus* TISTR885, S.e.: *Staphylococcus epidermidis* TISTR518, B.c.: *Bacillus cereus* TISTR687, B.s.: *Bacillus subtilis* TISTR008, MRSA Sp3: Methicillin-Resistant *Staphylococcus aureus* Sp3, E.c.: *Escherichia coli* TISTR887, S.t.: *Salmonella typhimurium* TISTR292 and P.a.: *Pseudomonas aeruginosa* TISTR1287

crude extract and purified compounds showed no cytotoxicity against non-cancerous cell line (Vero), with IC_{50}>400 µg/mL. The MDA-MB-231 is mostly affected by the purified compounds similar to doxorubicin hydrochloride, while HeLa and HepG2 are affected less than MDA-MB-231. The most effective compound in these cell lines after 24 hrs of treatment was compound **4** (IC_{50} values in MDA-MB-231 and HeLa cells were 32.00±8.54 and 85.73±15.31 µg/mL), respectively. The obtained IC_{50} values of the purified compounds were much lower than those against Vero cells. The selectivity indices (SI) observed for the crude extract and purified compounds against cancer cell lines were lower compared to doxorubicin hydrochloride, indicating a less selective cytotoxic effect on these cancer cell lines. However, the SI for compound **4** against MDA-MB-231 cells was higher compared to doxorubicin hydrochloride, suggesting a more targeted cytotoxicity towards this specific cancer cell line (Table 3).

The findings demonstrate that diketopiperazines, purified from *Streptomyces antimicrobicus* BN122 isolated from the root tissue of *O. sativa* L. var. glutinosa, exhibit antibacterial activity and significant anticancer properties. These compounds exhibit no cytotoxicity in normal cells, but some compounds demonstrate more selective cytotoxicity against specific cancer cell lines. These results suggested that

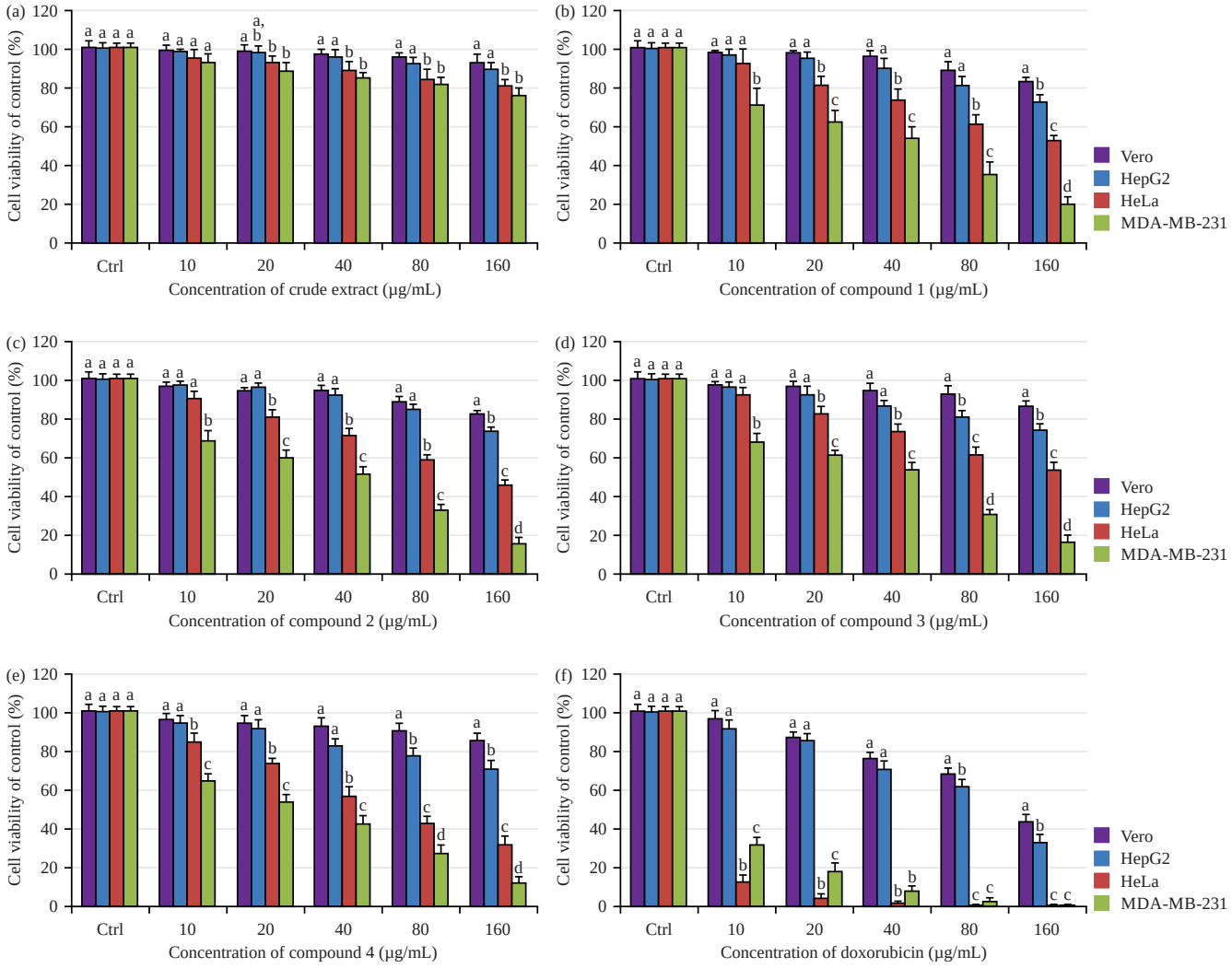

Fig. 5(a-f): Effects of (a) Crude extract, (b-e) Purified compounds **1-4** and (f) Doxorubicin hydrochloride with different concentrations on cancer cell lines (HeLa, HepG2 and MDA-MB-231) and non-cancerous cell line (Vero) after 24 hrs of treatment using MTT assay

Results were expressed as percentages over controls without compound treatments in triplicate experiments. Statistical analysis was performed using one-way ANOVA with Tukey's test for multiple comparisons using SPSS 11.01. The different alphabet labels within cell types are significantly different at the same concentration of the compounds ($p<0.05$)

Table 3: 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	1186.83±100.75^{a}	411.63±43.91^{a}	2.88	478.09±67.73^{a}	2.48	801.22±90.38^{a}	1.48
Compound **1**	482.73±61.06^{b}	57.08±12.17^{b}	8.46	155.59±22.10^{b}	3.10	287.81±55.80^{b}	1.68
Compound **2**	484.11±63.61^{b}	49.73±14.04^{b}	9.73	132.01±10.54^{b}	3.67	298.69±46.92^{b}	1.62
Compound **3**	676.38±46.62^{c}	49.52±14.51^{b}	13.66	158.93±25.95^{b}	4.26	323.48±55.34^{b}	2.09
Compound **4**	680.87±55.59^{c}	32.00±8.54^{b}	21.28	85.73±15.31^{b,c}	7.94	276.89±41.08^{b}	2.46
Doxorubicin hydrochloride	137.44±21.15^{d}	9.99±5.85^{b}	13.76	7.72±6.22^{c}	17.80	111.08±26.96^{c}	1.24

*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, **IC_{50} values represent the concentration causing 50% growth inhibition, 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 ^{abcd}Different letters indicated statistically significant differences within the same category ($p<0.05$)

Oryza sativa L. var. glutinosa root tissues represent a promising source for isolating actinomycetes capable of producing valuable bioactive compounds.

DISCUSSION

This study successfully isolated and identified four known bioactive compounds, diketopiperazines, from *Streptomyces antimicrobicus* BN122. This identification was achieved by comparing the spectral data of the purified compounds with data from previous research[25-28].

Diketopiperazines constitute a diverse class of secondary metabolites ubiquitously produced by a broad spectrum of organisms, including bacteria, fungi, plants, animals[29] and marine organisms[30]. Numerous natural products featuring the diketopiperazines scaffold have demonstrated a wide range of pharmacological properties, such as antiviral, antifungal, antibacterial and antitumor activities[31]. Present study investigated the isolation and taxonomic identification of the producing organism (*Streptomyces antimicrobicus* BN122), antibacterial activities and purification and evaluation of the anticancer diketopiperazines produced by this strain. The yield of diketopiperazines can vary significantly between different *Streptomyces* species. For example, *Streptomyces fungicidicus*, *Streptomyces* sp. RK44 and *Streptomyces* sp. Call-36 has been reported to produce 0.23-0.33, 0.20-0.38 and 0.23-0.36 mg/L of diketopiperazines, respectively[26,28,29]. This study reported the isolation of *Streptomyces antimicrobicus* BN122 from the root tissue of *Oryza sativa* L. var. glutinosa. This strain exhibited a high yield of diketopiperazines (1.04-1.10 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. 3), the BN122 isolate shares a close evolutionary relationship with *Streptomyces antimicrobicus* strain SMC 277. This close relationship is supported by the high 16S rDNA gene sequence similarity of 99.19% between BN122 and SMC 277. Strain SMC 277 has been isolated from the clay soil in a paddy field in Chonburi Province, Thailand. This strain could inhibit the growth of several bacterial pathogens[30]. However, the purified compounds from *Streptomyces antimicrobicus* have never been reported. Since *Streptomyces antimicrobicus* BN122 was investigated for diketopiperazines production in this study. The isolated diketopiperazines were primarily Cyclo-(L-Pro-L-Val), Cyclo-(L-Pro-L-Leu), Cyclo-(L-Pro-L-Trp) and Cyclo-(L-Pro-L-Phe), exhibiting antimicrobial activities against Gram-positive bacteria and the clinical isolates of MRSA. However, there are no antimicrobial activities against Gram-negative bacteria, consistent with previously published data on these compounds[27,28]. The Cyclo-(L-Pro-L-Leu), a cyclic dipeptide isolated from *Streptomyces* sp., KH-614, a taxon closely related to *Streptomyces lydicus*, demonstrated antibacterial activity against vancomycin-resistant *Enterococcus faecalis* strains. The minimum inhibitory concentration (MIC) values of Cyclo-(L-Pro-L-Leu) against *Enterococcus faecalis* K-99-34, K-00-184 and K-00-221 were determined to be 12.5 μg/mL[31]. A synergistic effect was observed when cyclo-(L-Pro-L-Leu) was combined with cyclo-(L-Pro-L-Phe) against a variety of pathogens, including *Escherichia coli*, *Staphylococcus aureus*, *Micrococcus luteus*, *Candida albicans* and *Cryptococcus neoformans*. The MIC values for these combinations ranged from 0.25 to 0.5 μg/mL[32]. Seven diketopiperazines, including cyclo-(L-Pro-L-Leu), cyclo-(L-Pro-L-Phe), cyclo-(L-Pro-L-Gly), cyclo-(L-Pro-L-Pro), cyclo-(L-Pro-L-Val), cyclo-(L-trans4-OH-Pro-L-Leu) and cyclo-(L-trans-4-OH-Pro-L-Phe), were isolated from a fermentation broth of *Aspergillus fumigatus*. These fungal diketopiperazines exhibited weak antibacterial activity against *Staphylococcus aureus* and *Micrococcus luteus* at a concentration of 2.9 mmol/L[33]. The Cyclo-(L-Pro-L-Leu), also known as gancidin W, isolated from two *Streptomyces* species, inhibited the growth of vancomycin-resistant *Enterococcus faecalis* strains with MIC values of 12.5 μg/mL[34].

Diketopiperazines have demonstrated promising anticancer properties in preclinical studies[35,36]. These cyclic dipeptides have been shown to inhibit cancer cell growth and proliferation and to induce apoptosis in cancer cells, suggesting their potential as promising candidates for further drug development[37]. The findings demonstrate the potent cytotoxic effects of diketopiperazines against various cancer cell lines, including MDA-MB-231, HeLa and HepG2. The calculated IC_{50} values, ranging from 32.00 to 323.48 μg/mL, underscore their significant impact on cancer cell viability, suggesting their potential as promising anticancer agents. However, the observed cytotoxicity against the non-cancerous Vero cell line (IC_{50}: 482.73 to 680.87 μg/mL) highlights the need for further optimization to enhance selectivity. While these values indicate moderate cytotoxicity compared to cancer cells, minimizing damage to healthy tissues remains a critical consideration for therapeutic applications. Notably, compound **4** (cyclo-(Pro-Phe)) exhibited a more targeted effect on MDA-MB-231 cells compared to doxorubicin hydrochloride, as evidenced by its higher selectivity index (SI). This suggests the potential of diketopiperazines to be particularly effective against MDA-MB-231 breast cancer.

CONCLUSION

In summary, diketopiperazines were isolated from *Streptomyces antimicrobicus* BN122. These compounds exhibited antibacterial properties against Gram-positive bacteria. They also exhibited anticancer properties against various cancer cell lines. These compounds exhibited potent effects on cancer cell viability, particularly MDA-MB-231 breast 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 diketopiperazines as potential therapeutic agents for cancer.

SIGNIFICANCE STATEMENT

This study identified a promising new source of bioactive compounds. *Streptomyces antimicrobicus* BN122 isolated from the root tissues of purple sticky rice (*Oryza sativa* L. var. glutinosa). This strain produces diketopiperazines, which exhibit antibacterial and anticancer properties with minimal toxicity toward healthy cells. These findings suggest the potential of diketopiperazines from *S. antimicrobicus* BN122 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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