# A Crystal Compound "Cyclo-D-Prolyl-D-Leucyl" From Endophytic *Bacillus* Sp.

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## Abstract

An endophytic bacterial strain was isolated from the leaves of *Agapanthus* sp. in Sydney, Australia. Taxonomic identification of isolated strain based on 16S rDNA sequences was conducted at Department of Biological Science, The University of New South Wales, Sydney. This endophytic strain was assigned to the genus *Bacillus* based on phylogenetic inference. Fermentation studies (size of inoculum and various media) of endophytic *Bacillus* sp. were conducted by using four test organisms. Size of inoculum (2.0%) and nutrient broth medium or glucose/yeast extract medium are suitable for the cultivation of this strain. Fermentation (7 L) of isolated *Bacillus* sp. was undertaken for three days on shaker culture, and extraction of active metabolites from 7 L fermentation was utilized with ethyl acetate at pH 5. Isolation of the active compounds from the fermented broth of this strain was carried out by chromatographic technique with the various solvent systems. The molecular structure of the crystal compound was untaken by crystallographic analysis at X-ray Crystallography Laboratory, UNSW Analytical Centre, The New South Wales University, Sydney. The two compounds including a crystal compound "Cyclo-D-Prolyl-D-leucyl" were isolated from this strain, and the crystal compound was mentioned in this paper. Antimicrobial activity of the active crystal compound was undertaken at the Department of Botany, University of Yangon. The isolated crystal compound possessed highly antibacterial activity on *Agrobacterium* sp., *Escherichia coli*, *Micrococcus luteus*, *Staphylococcus aureus* and *Xanthomonas oryzae* while moderately antifungal activity on *Candida albicans* and *Malassezia furfur* in 20 μg *in vitro*.

**Keywords**: *Agapanthus* sp., Cyclo-D-Prolyl-D-Leucyl, Endophytic *Bacillus* sp.

## Introduction

Endophytes are "Microbes which colonize inside the internal tissues of the healthy plants without any negative effects" (Bacon and White, 2000). Endophytes include both commensal microorganisms that have no direct effect on the host plant, and mutualistic symbionts which could be used in the biological control of plant pathogens or for plant growth promotion (Petrini, 1991).

A fungus *Penicillium notatum* contamination showed a potent inhibition of *Staphylococcus* sp. growing on the agar plates, and then this famous accident led to the discovery of antibiotic penicillin by Alexander Fleming (Fleming, 1929). Since the discovery of penicillin in 1929, intensive studies of bacteria and fungi have been shown that microorganisms are a rich source of pharmaceutically important bioactive substances (Fenical, 1993). Common endophytes include bacteria, fungi and actinomycetes, and they can be isolated from the healthy and wild plants (Brooks *et al*., 1994) or the cultivated crops of monocotyledonous (Fisher *et al*., 1992).

Antibiotics such as cephalosporin, daptomycin, streptomycin, vancomycin, antifungal amphotericin B, griseofulvin, antiviral aciclovir, doxorubicin, and many others play a vital role in therapeutics (Terlau *et al*., 2000 and Zeeck, *et al*., 2001). The objectives of this research work are to isolate an endophytic bacterial strain, to investigate fermentation studies of isolated endophyitc strain, to isolate the active compound from the fermented broth, and to evaluate its antimicrobial activity on seven pathogenic microorganisms.

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## Materials and Methods

### Isolation of an endophytic strain from *Agapanthus* sp.

An endophytic bacterial strain was isolated from the leaves of *Agapanthus* sp. (Family: Agapanthaceae) in Sydney, Australia for screening of antibiotic producing strain. The isolation of endophytic strain was followed by the method of Lee *et al*. (1996). Plants parts were washed in running tap water for 15 min. Plants parts were cut into about 1 $cm^2$ pieces. The surfaces of cut-plant pieces were sterilized by soaking in 75% ethanol for 2 min. Next, sterile surfaces were socked in 5.3% sodium hypocloride for 5 min. Cut-plant pieces were washed out sodium hypocloride by socking in 75% ethanol for 0.5 min. They were dried and cut into smaller pieces, and placed on the nutrient broth agar plates and then incubated for 3 days to 1 week.

### Identification of endophytic strain by DNA sequencing

The microbial taxonomy of endophytic strain was studied by DNA sequencing, sequence databases, analysis of sequences and genomic sequencing projects (Lane, 1991; Tang *et al.* 1998).

A. Isolation procedure of bacterial genomics

In order to identify the microbial taxonomy of endophytic bacterial strain, its genomic was isolated by the method of Rapley (1998). Inoculate bacteria in 15 mL LB broth under shaker condition for 12-14 hours. Then, harvest the cell pellet by centrifuging at 10,000 rpm for 3 minutes. Discard the supernatant and re-suspend the pellet in 567 µL TE buffer. Add 30 µL of 10% SDS and 3 µL of (20 mg/ml) proteinase K. Incubate at 37°C for 1 hour in a water bath. Add 100 µL of 5M NaCl and mix thoroughly. Incubate at 65°C for 10 minutes in a water bath.

After incubation, add about 750 µL of Chloroform/Isoamyl alcohol ratio 24:1 ($CHCl_3$ 720 µL: IA 30 µL). Mix thoroughly (maximum 10 sec./tube) and centrifuge at high speed for 10 min. Collect the viscous aqueous supernatant into a new microcentrifuge tube. Add equal volume (400-600 µL) of Phenol-Chloroform-Isoamyl alcohol, mix thoroughly (maximum 10 seconds/tube), centrifuge at high speed for 5 or 10 min. Collect the supernatant into a new microcentrifuge tube. Add 0.6 volume of Iso-propanol, mix from top to bottom until a stringy whitish DNA precipitate unclearly visible. Centrifuge at high speed for 10 min, and pour out the supernatant. Wash the DNA pellet with 500 µL of 70% ethanol, mix thoroughly and spin for 10 min. Remove the supernatant and re-dissolve the DNA pellet in 20-100 µL TE buffer and then store the DNA at -20°C.

B. Sequencing of 16S rDNA by Polymerase Chain Reaction (PCR)

The 16S rDNA of isolated bacterial strain was enzymacally amplified by PCR. The PCR amplification was done with 29 cycles of initial denaturation at 94ºC for 3 min, template denaturation at 94ºC for 30 sec, annealing at 50ºC for 1 min, and extension at 72°C for 3 min, using primers that were designed based on a conserved region between 16S rDNA sequences of bacterial species. The forward primer was 27F: AGAGTTTGATCTGGCTCAG and the reverse primer was 1492R: ACGGTTACCTTGTTACGACTT (Lane, 1991).

**Antimicrobial activity of isolated endophytic strain**

Isolated bacterial strain grown on the nutrient broth agar medium plate was transferred into 50 mL falcom tube containing 10 mL of nutrient broth medium, and the falcom tube was incubated for 2 days as the seed culture. After two-days incubation, the seed culture 1.0% of isolated strain was transferred into 250 mL conical flask containing 100 mL of fermentation medium and then fermentation was carried out for 2-5 days (Stoble and Sullivum, 1999).

At the end of fermentation, the fermentation broth was used for the paper disc diffusion assay. In inhibitory test of bioactive bacterial strain, test organisms such as *Candida albicans*, *Malassezia furfur*, *Escherichia coli* and *Staphylococcus aureus* were used. The malt extract agar medium (25g/L) was used for fungal test organisms while nutrient agar medium 13 g/L was for bacterial test organisms. The test organisms were inoculated in 10 mL assay broth in the 50 mL falcon tubes at 30ºC for bacteria and at 37ºC for fungi respectively and incubated overnight.

After overnight, 100 µL of test organisms was added in each assay plate and spread on the agar plates. Then, the paper discs impregnated with the fermented broth were applied on the test plates, and the plates were incubated for 24-36 hours at 30°C for bacteria and 37°C for fungi. Clear zone (inhibitory zone) surrounding the test disc indicates the presence of the bioactive compound that inhibits the growth of the test organism selectively. Disc size is 6.0 mm, and the volume the fermented broth is 10 µL/disc (Davis and Stout, 1971).

**Fermentation studies of active strain**

A. Size of inoculum

In order to isolate antimicrobial metabolites from active strain, fermentation study for maximal production of the bioactive compounds was investigated by optimizing the proper growth kinetic (cultivation and transfer) of inoculum. The strain grown on the nutrient agar medium plates was transferred into a 50 mL falcom tube containing 10 mL of nutrient broth medium and incubated for 2 days as the seed culture. After incubation, the seed culture (size of inoculum: 0.5%, 1.0%, 1.5%, 2.0%, 2.5%) was transferred into the five of 50 mL falcom tubes containing 10 mL fermentation medium in each. Fermentation was carried out for 5 days, and the fermentation broth was used for paper disc diffusion assay (Monaghan, *et al*., 1999).

B. Fermentation in various media

Active bacterial strain grown on the nutrient agar medium plates was transferred into the 50 mL falcom tube containing 10 mL of nutrient broth medium in each tube, and the falcom tube was incubated for 2 days as the seed culture. After two-days incubation, the seed culture 2.0% was transferred into the 50 ml conical flasks containing 10 mL of various fermentation media such as nutrient broth medium (M1), glucose/yeast extract medium (M2) and LB medium (M3). The fermentation was carried out for 2-5 days and the fermentation broth was used for the paper disc diffusion assay. Test organisms were *Candida albicans*, *Malassezia furfur*, *Escherichia coli* and *Staphylococcus aureus* (Stoble and Sullium, 1999).

**Extraction, separation and isolation of the bioactive compounds from fermented broth**

Two days old of the seed culture (140 mL) was transferred into the 7 flasks of 2.5 L conical flask containing 1 L of the fermentation medium. Then, the flasks were incubated at 30°C on the shaker at 180 rpm. Fermentation was carried out for three days. After three days of fermentation, the fermentation broth (7 L) was centrifuged at 4500 rpm for 20 minutes. Then, the cultural filtrate (the supernatant) was extracted with ethyl acetate (pH 5) in three times. The extracted samples were concentrated by a rotary evaporator, and applied on TLC plates and allowed to dry (Grabley *et al.*, 1999). Separation and isolation of metabolites from the ethyl acetate extract were carried out by chromatographic methods with the various solvent systems. Identification of the crystal compound was undertaken by crystallographic analysis at X-ray Crystallography Laboratory, UNSW Analytical Centre, The New South Wales University, Sydney, Australia.

**Antimicrobial activity of the isolated crystal compound**

Antimicrobial activity of the isolated crystal compound was evaluated with seven test organisms (*Agrobacterium* sp., *Candida albicans*, *Escherichia coli*, *Malassezia furfur*, *Micrococcus luteus*, *Staphylococcus aureus* and *Xanthomonas oryzae*). The paper discs (φ 6 mm) were soaked with 20 μg of the compound (Davis and Stout, 1971).

## Results

**Isolation of endophytic bacterial strain**

In the course of screening for the antimicrobial compounds producing microorganisms, an endophytic bacterial strain was isolated from the leaves of *Agapanthus* sp. The surface and reverse color of isolated strain was cream, and its cultural character was shown in Fig. 1. The bacterial cells are rod–shaped and gram-positive.

Cultural character Rod-shaped bacterium

Fig. 1. Isolated bacterial strain

**Identification of isolated endophytic strain**

Analysis of the 16S rDNA of isolated strain showed that it was a member of the genus *Bacillus*. High similarities were observed between isolated strain and *Bacillus* sp., and isolated strain was also closed related to *Bacillus* sp. Based on phylogenetic inference, this endophytic strain was assigned to the genus *Bacillus*. Direct sequencing of the PCR-amplified 16S rDNA of isolated strain was shown in Fig. 2.

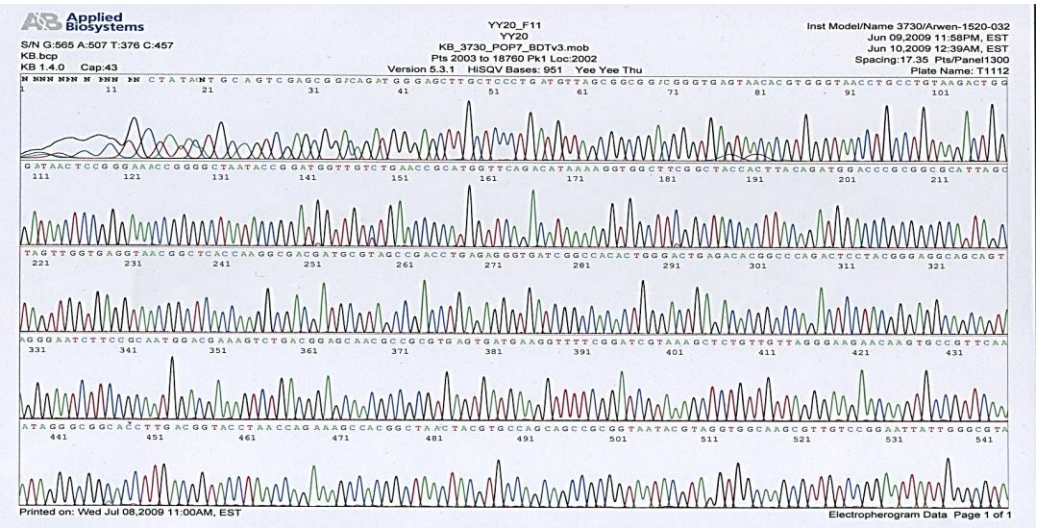


Fig. 2. Direct sequencing of the PCR-amplified 16S rDNA of isolated strain

**Antimicrobial activity of endophytic *Bacillus* sp.**

According to the result of inhibitory zones, the fermented broth of isolated strain *Bacillus* sp. indicated antimicrobial activities on *C. albicans* (16 mm), *M. furfur* (16 mm), *E. coli* (18 mm) and *S. aureus* (20 mm).

**Fermentation studies of endophytic *Bacillus* sp.**

A. Size of inoculum

According to the result of seed culture, 2.0% size of inoculum showed the largest inhibitory zone against four test organisms at third day fermentation of *Bacillus* sp. (Tables 1 to 4).

Table 1. Inhibitory zones (mm) against *C. albicans*

| Fermentation | 0.5% | 1.0% | 1.5% | 2.0% | 2.5% |
|---|---|---|---|---|---|
| Fer. 2nd day | 11 | 15 | 15 | 16 | 16 |
| Fer. 3rd day | 12 | 19 | 20 | **20** | 18 |
| Fer. 4th day | 14 | 16 | 17 | 17 | 16 |
| Fer. 5th day | 10 | 12 | 13 | 13 | 12 |

Table 2. Inhibitory zones (mm) against *M. furfur*

| Fermentation | 0.5% | 1.0% | 1.5% | 2.0% | 2.5% |
|---|---|---|---|---|---|
| Fer. 2nd day | 8 | 15 | 12 | 13 | 13 |
| Fer. 3rd day | 11 | 18 | 18 | **21** | 18 |
| Fer. 4th day | 13 | 15 | 15 | 16 | 15 |
| Fer. 5th day | 10 | 11 | 11 | 12 | 11 |

Table 3. Inhibitory zones (mm) against *E. coli*

| Fermentation | 0.5% | 1.0% | 1.5% | 2.0% | 2.5% |
|---|---|---|---|---|---|
| Fer. 2nd day | 11 | 16 | 17 | 17 | 17 |
| Fer. 3rd day | 15 | 20 | 20 | **21** | 19 |
| Fer. 4th day | 12 | 15 | 15 | 16 | 16 |
| Fer. 5th day | 10 | 12 | 12 | 12 | 12 |

Table 4. Inhibitory zones (mm) against *S. aureus*

| Fermentation | 0.5% | 1.0% | 1.5% | 2.0% | 2.5% |
|---|---|---|---|---|---|
| Fer. 2nd day | 12 | 16 | 16 | 17 | 17 |
| Fer. 3rd day | 13 | 18 | 18 | **20** | 18 |
| Fer. 4th day | 10 | 12 | 14 | 16 | 15 |
| Fer. 5th day | 8 | 8 | 9 | 10 | 10 |

B. Antimicrobial activity of *Bacillus* sp. in various media

According to the result of inhibitory zones, the fermented broth of *Bacillus* sp. in three different media showed antimicrobial activity on *C. albicans*, *M. furfur*, *E. coli* and *S. aureus*. Media M1 and M2 were better than medium M3 as shown in Table 5.

Table 5. Inhibitory zones (mm) of *Bacillus* sp. in different media

| Strain | *C. albicans* | *M. furfur* | *E. coli* | *S. aureus* |
|---|---|---|---|---|
| | 1d 2d 3d 4d | 1d 2d 3d 4d | 1d 2d 3d 4d | 1d 2d 3d 4d |
| Medium M1 | 11, 15, **20**, 16 | 12, 15, **18,** 14 | 10, 16, **20,** 11 | 11, 17, **20,** 9 |
| Medium M2 | 8, 12, **18**, 15 | 13, 17, **20**, 16 | 8, 12, **17**, 8 | 12, 16, **18**, 8 |
| Medium M3 | 8, 11, 17, 14 | 10, 14, 17, 13 | 9, 14, 20, 12 | 8, 10, 16, 8 |

**Isolation and purification of the active compound**

Ethyl acetate extract (5.5 g) were obtained for the isolation of antimicrobial metabolites from 7 L fermentation of strain *Bacillus* sp. There were seven fractions that were separated from the ethyl acetate crude extract by silica gel column (5 cm × 20 cm) with the various solvent systems (dichloromethane: methanol, 95:5, 9:1, 4:1, 2:1 and 1:1). After the crude extract column, the seven large fractions were collected, then they were tested antimicrobial activity to choose the active fractions. Among these fractions, the three fractions did not show any antimicrobial activity whereas the other fractions indicated antimicrobial activity on *C. albicans*, *M. furfur* and *S. aureus*.

After that, the most active fraction F4 (415 mg) was run by silica gel column (dichloromethane: methanol, 9:1) and preparative thin layer chromatography (PTLC) with dichloromethane: methanol (95:5) in order to get more purified compounds. The isolation procedure of active metabolites was shown in Fig. 3.

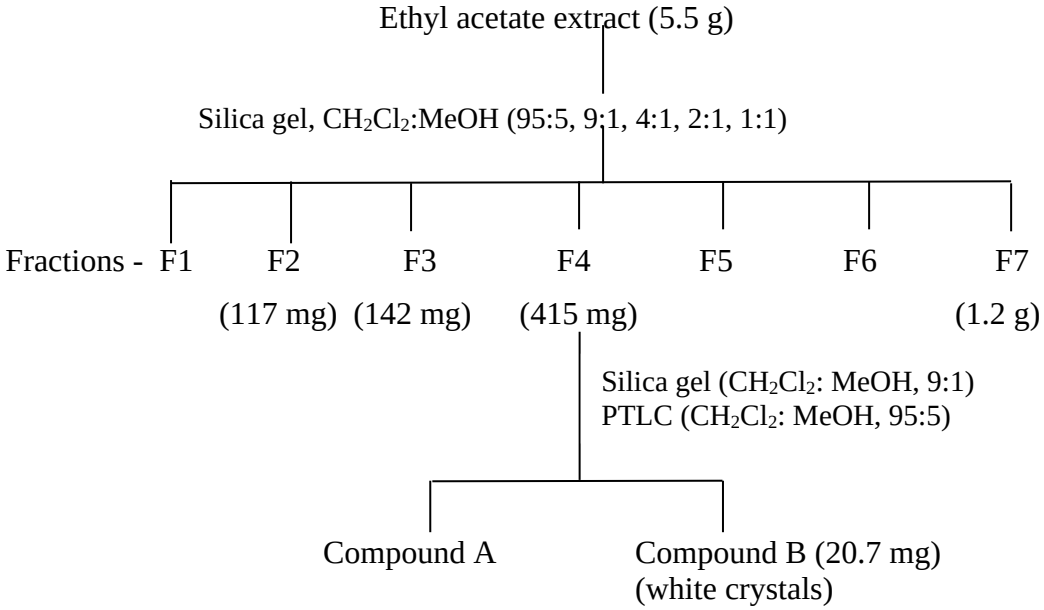


Fig. 3. Isolation procedure of the active compounds

**Physico-chemical properties of the crystal compound**

The isolated compound B in crystal form was isolated from the fraction F4 an UV absorbing band at 254 nm. It has $R_f$ 0.35 (chloroform/methanol, 9:1) and 0.72 (chloroform/methanol, 8:2). It gave white spot with anisaldehyde/sulphuric acid reagent. This substance is good soluble in acetone and methanol. It was identified as "Cyclo-D-Prolyl-D-Leucyl" according to crystallographic X-ray. It is a cyclic dipeptide and its molecular formula is $C_{11}H_{18}N_2O_3$. Its molecular structure and X-ray structure were shown in Fig. 4a and 4b.

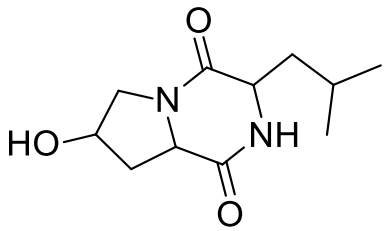


Fig. 4a. Cyclo-D-Prolyl-D-leucyl

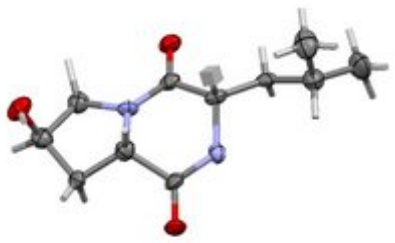

Fig. 4b. X-ray structure

**Antimicrobial activity of the active compound**

The isolated crystal compound showed highly antibacterial activity on *Agrobacterium* sp., *Escherichia coli*, *Micrococcus luteus*, *Staphylococcus aureus* and

*Xanthomonus oryzae* while it also indicated moderately antifungal activity on *Candida albicans* and *Malassezia furfur* (Table 6).

Table 6. Antimicrobial activity (mm) of the crystal compound

| Test organism | *Agrobac-. terium* sp. | *Escheri-. chia coli* | *Microc. luteus* | *Staphyloc.. aureus* | *Xantho. oryzae* | *Candida albicans* | *Malas. furfur* |
|---|---|---|---|---|---|---|---|
| Crystal compound | 18 | 18 | 19 | 18 | 20 | 15 | 16 |

10-13 mm (weak activity); 14-17 mm (moderate activity); >18 mm (high activity)

## Discussion

In the present research, an endophytic bacterial strain *Bacillus* sp. was isolated from the leaves of *Agapanthus* sp. in Sydney, Australia. Isolated endophytic strain was a member of the genus *Bacillus* because high similarities were observed between isolated strain and *Bacillus* spp. according to 16S rDNA sequencing techniques. Duijff *et al.* (1997) and Sturz *et al*. (1998) isolated endophytic *Bacillus* sp. strain from disinfected plant tissues, and it exhibited potential as biocontrol agents against microbial pathogens. Pleban *et al*. (1995) also isolated many endophytic *Bacillus* spp., and they investigated biocontrol on some crops by using these species.

Fermentation parameters are very important for large-scale cultivation of microbial strains. The fermented broth of strain *Bacillus* sp. showed highly antimicrobial activity against *C. albicans*, *M. furfur*, *E. coli* and *S. aureus*. In this research work, 2.0% size of inoculum showed the high inhibitory zone against various test organisms at third day fermentation for growth kinetic of inoculum for isolated strain. Monaghan *et al*. (1999) and Stoble & Sullium (1999) investigated culture preservation, inoculum development and fermentation parameters for large-scale cultivation of microbial strains.

The crude extracts are evaluated by using chemical, biological and pharmaceutical screening to discover the new bioactive compounds. Zähner (1982) and many other researchers started systematically chemical screening of the crude extracts in the 1980s (Grabley *et al*., 1999). In isolation and purification of the bioactive compounds, the two compounds including a crystal compound "Cyclo-D-Prolyl-D-leucyl" were isolated from 7 L fermentation of *Bacillus* sp. This paper mentioned only the crystal compound B which showed antimicrobial activity on seven pathogenic test-organisms. This finding was agreed with the statements of Rosa *et al*., (2003); Gräfe, (1992) and Lopes *et al*., (2018). This compound was previously isolated from marine bacterium by Rosa *et al*. (2003) but it was not in the crystal form. They have reported that it has antimicrobial activity on *E. coli* and *M. furfur*.

Gräfe (1992) has reported that active metabolites from natural sources showed inhibition of the growth of higher organisms (e.g. tumour cells) or pathogens (e.g. bacteria, fungi, viruses) at low concentration, and subsequently can be used to cure

infectious diseases. Lopes *et al*. (2018) stated that endophytic bacteria species protected the plants against plant diseases and promoted plant growth.

## Conclusion

The microbial agents continue to play a major role in drug discovery and in the pharmaceutical industry. They also are essential for pharmaceutical and natural products research. The need of the effective bioactive compounds is important because life-threatening microbial infections are increasing recently. The discovery of biological agents that possess selective toxicity against human and plant pathogens is an important scientific challenge. It was found that the active crystal compound in this research possessed better antibacterial activity on *Escherichia coli*, *Micrococcus luteus*, *Staphylococcus aureus*, *Agrobacterium* sp. and *Xanthomonas oryzae* than antifungal activity on *C. albicans* and *M. furfur*. Therefore, this compound could be applied to inhibit cholera, diarrhea and skin infections and diseases on animals including man as well as crown gall and leaf blight diseases on crops and plants.

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