# The evaluation of Antibacterial Activity of Fungal Endophyte *Ceratobasidium ramicola* IBRLCM127 Colonizing in Rhizomes of Medicinal Plant, *Curcuma mangga* Valeton & Zijp

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# The evaluation of Antibacterial Activity of Fungal Endophyte *Ceratobasidium ramicola* IBRLCM127 Colonizing in Rhizomes of Medicinal Plant, *Curcuma mangga* Valeton & Zijp

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**Abstract**. The current study was conducted to evaluate the antibacterial activity of fungal endophyte isolate, *Ceratobasidium ramicola* IBRLCM127 colonizing in rhizomes of a medicinal plant, *Curcuma mangga* Valeton & Zijp. Primary screening of its antibacterial potential was performed by employing agar plug assay, and the results revealed that the fungal isolate was capable to inhibit all the 11 test bacteria used in the study. All four Gram-positive bacteria (*Staphylococcus aureus, Bacillus cereus, Bacillus subtilis* and Methicillin-resistant *Staphylococcus aureus* ATCC 33591) showed the highest susceptibility degree to the fungal isolate with the size of inhibition zones of ≥21 mm. As for Gram-negative bacteria, 6 out of 7 tested bacteria (*Proteus mirabilis, Yersinia enterocolitica, Escherichia coli* IBRL 0157, *Salmonella typhimurium, Klebsiella pneumoniae* ATCC 13883 and *Acinetobacter antratus*) were the most susceptible species with inhibition zone size of ≥21 mm, whilst only *Pseudomonas aeruginosa* ATCC 27844 less susceptible to fungal isolate with the size of inhibition zone of 11 to ≤ 20 mm. Secondary screening using disc diffusion assay revealed that fungal ethyl acetate extract derived from fermentative broth (extracellular) demonstrated better potential of antibacterial activity compared to the methanol extract derived from fungal biomass (intracellular). The results showed that 8 out of 11 test bacteria were susceptible to fungal ethyl acetate extract with the diameter of inhibition zone ranging from 8.0±0.0 mm to 13.0±0.0 mm and 8.3±0.6 mm to 11.7±0.6 mm in Gram-positive and Gram-negative bacteria, respectively. Contradictorily, methanol extract only capable to inhibit *S. aureus* with inhibition zone of 8.3±0.6 mm in diameter. The minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of ethyl acetate extract towards Gram-positive bacteria were both in the range of 125.00 to 500.00 µg/mL, whilst for Gram-negative bacteria were in the range of 125.00 to 250.00 and 250.00 to 500.00 µg/mL, respectively. On the other hand, the MIC and MBC values for methanol extract towards Gram-positive bacteria were 250.00 and 500.00 µg/mL, respectively. Both ethyl acetate and methanol extracts exerted bactericidal effects on test bacteria with the ratio of MBC/MIC ≤4. The detail of the ethyl acetate extract effects on the bacterial cells was observed using scanning electron microscopy (SEM), in which the micrographs obtained from SEM revealed that the severity of the cell damages caused by the extract were beyond repair, and the mode of action could possibly due to the disruption in the cell wall biosynthesis and cell membrane permeability.

## 1. Introduction

Since the first discovery of antibiotic in the 1940s, it has been used for decades to treat various infections which in turn resulted in the antimicrobial resistance (AMR) due to the overuse and misuse of antibiotics, along with incapability of pharmaceutical companies in developing a novel drug. The emergence of new AMR strains such as *Enterococcus faecium, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Candida albicans* for the past few years have been widely reported as major classes of pathogens that developed resistance towards commercialized antibiotics and drugs [1]. Hence, there is a dire need in seeking the novel antibiotics to replace the older antibiotics that already loss their effectiveness.

Endophytic microorganisms are believed to be a potential source of novel bioactive compounds that possess a potential in treating new emerging disease in human, animal and plant due to its amazing species diversity [2]. Among of those endophytic microorganisms, fungi have been identified as the most common endophytes owing to the fact that over a million of fungal species existed in nature with only 5% of the species have been recognized so far [3]. Endophytic fungi that are harbouring asymptomatically in plant tissues play numerous roles from the adaptation and morphogenesis to defending mechanism against predators, pathogens and growth promoters and even producing a plethora of bioactive secondary metabolites [4].

*Ceratobasidium ramicola* is a common fungal pathogen that can causes a range of diseases to commercial crops such as black root rot in strawberry [5], diseases of peas, soya beans and pak choy [6], vascular streak dieback in cacao [7] and dieback disease in sengon and rice seedlings [8]. Interestingly, to the best of our knowledge, this is the first report of *C. ramicola* that has been isolated as endophytic fungus from *C. mangga*. Most of the previous literatures only reported *C. ramicola* and its corresponding genus as mycorrhizal fungi isolated from terrestrial orchid [9, 10].

## 2. Materials and Methods

### *2.1. Fungal Endophyte Isolate, Culture and Maintenance*

The isolate of fungal endophyte was priorly isolated from the rhizome of *C. mangga* Valeton & Zijp and provided by the culture collection of Industrial Biotechnology Research Laboratory (IBRL), Universiti Sains Malaysia, Penang, Malaysia.

### *2.2. Cultivation and Extraction of Secondary Metabolites of Fungal Endophyte*

The preparation of inoculum was conducted by introducing two mycelial agar plugs which were excised from the periphery of 7-day-old fungal endophyte culture into 250 mL Erlenmeyer flask consisting of 100 mL of YES broth culture medium. The culture was cultivated in a dark condition for 20 days at 30°C with agitation speed of 120 rpm. After that, the fermentative broth and fungal biomass were separated out by using Whatman No. 1 filter paper. The filtered fermentative broth was extracted thrice using equal volume of ethyl acetate (1:1, v/v). The upper organic phase was collected and concentrated to dryness using rotary evaporator under reduced pressure to obtain a crude paste of ethyl acetate extract. Meanwhile, the separated fungal biomass was washed using sterile distilled water twice followed by freeze-drying and macerating in methanol (1:50, w/v) overnight. The macerated mixture was then filtered using muslin cloth, followed by Whatman No. 1 filter paper for the complete separation of biomass residue from biomass filtrate. The biomass filtrate was then further concentrated to dryness using rotary evaporator under reduced pressure to acquire a crude paste of methanolic extract.

### *2.3. Test Microorganism and Culture Maintenance*

A total of 11 test bacteria supplied by IBRL, School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia, were employed in the current study, of which consisting of four Gram-positive bacteria (*S. aureus, B. cereus* ATCC 10876, *B. subtilis* IBRL A3 and MRSA ATCC 33591) and seven Gram-negative bacteria (*P. mirabilis, Y. enterocolitica, E. coli* IBRL 0157, *S. typhimurium*, *K.*

*pneumoniae* ATCC 13883, *A. antratus* and *P. aeruginosa* ATCC 27844). The bacterial inoculum was prepared according to the protocol assigned by Clinical and Laboratory Standard Institute [11].

*2.4. Agar Plug Diffusion Assay*

The agar plug diffusion assay was conducted according to Radakrishnan *et al.* [12] with some modifications. Positive control was included in this experiment consisting of chloramphenicol (30 µg/mL) and the formation of inhibition zone surrounded the agar plug was measured. The experiments were performed in three replicates in separate occasions and the results obtained were presented as mean value ± standard deviation.

*2.5. Disc Diffusion Susceptibilty Assay*

Disc diffusion susceptibility assay was conducted according to the protocol recommended by CLSI standard M2-A9 [13]. The test bacterial inoculum was then seeded on MHA plate using a sterile cotton swab using a spread plate technique. The preparation of fungal crude extract was done by dissolving 10.0 mg of extract in 0.2 mL of 5% dimethyl sulfoxide (DMSO) and followed by addition of 0.8 mL of sterile distilled water to obtain 1 mg/mL of extract concentration. The sterile antibiotic discs with 6.0 mm diameter were impregnated with 20 µL of the prepared extract and left to air-dry prior to placing them on the MHA plate seeded with test bacteria. Positive and negative controls used in this experiment were chloramphenicol (30 µg/mL) and 1.0% DMSO, respectively.

*2.6. Determination of MIC and MBC*

Broth microdilution assay was opted to determine the MIC value of fungal ethyl acetate crude extract against test bacteria [14]. Mueller-Hinton broth (MHB) was used to prepare a single-fold dilution of the fungal extract. Next, 100 µL of fungal extract was dispensed into each well of microtiter plate followed by the addition of 100 µL of test bacterial inocula with approximately 1 x $10^7$ CFU/mL to make up a final volume and concentration of microbial inocula in each well of 200 µL and 1 x $10^7$ CFU/mL, respectively. Chloramphenicol was included as reference drug while 5% methanol and bacterial inoculum was served as control. The plate was then incubated at 37°C for 24 hours prior to the addition of growth indicator, 40 µL of 0.2 mg/mL p-iodonitrotetrazolium violet (INT) dissolved in 99.5% ethanol into each well. The value of MIC was determined and recorded as the lowest concentration of fungal ethyl acetate crude extract that can inhibit the visible growth of test bacteria [15]. The determination of MBC value of fungal ethyl acetate extract was done afterwards based on the reading of the MIC values. The viable cells from the wells with no visible microbial growth were enumerated using a standard viable plate count on MHA plates and these plates were incubated at 37°C for overnight. The MBC was observed and recorded as the lowest concentration of fungal extract that caused the reduction of 99.9% in bacterial growth as compared to the growth control [16].

*2.7. Scanning Electron Microscopy (SEM) Observation*

The sample preparation was done by transferring 50 µL of bacterial inoculum (approximately 1 x $10^8$ CFU/mL) into 25 mL Erlenmeyer flask consisting of 945 µL of MHB and subjected to incubation in an orbital shaker at 150 rpm, 37°C for 18 to 20 hours. Next, 0.5 mL of fungal ethyl acetate crude extract (5 mg/mL) was added into the culture to make up a total volume of mixture of 10 mL with final extract concentration was 0.5 mg/mL. A control was also included in this experiment by adding 0.5 mL of 20% DMSO (v/v) into the bacterial culture to replace the fungal extract. The mixture was incubated subsequently at 150 rpm, 37°C for 36 hours in an orbital shaker. The cultures were then harvested at 0 and 36 hours of incubation time and subjected to SEM observation.

*2.8. Statistical Analysis*

Student t-test was used to analyze the data in the current study using SPSS Version 24.0. All tests involved were performed independently in three replicates. The results obtained were then analyzed by using a one-way ANOVA test and presented as the mean ± standard deviation.

## 3. Results

*3.1. Qualitative Screening of Antibacterial Activity*

The antibacterial activity of endophytic fungus, *C. ramicola* IBRLCM127 was shown in Table 1, of which the fungal extract was able to inhibit all the 11 test bacteria. Interestingly, all the 4 Gram-positive bacteria (*S. aureus, B. cereus* ATCC 10876, *B. subtilis* IBRL A3, MRSA ATCC 33591) showed the highest susceptibility rate towards fungal extract with diameter of inhibition zone ≥ 21mm. As for Gram-negative bacteria, only 1 bacterial species (*P. aeruginosa* ATCC 27844) showed moderate susceptibility rate with the size of inhibition zone of 11 to ≤ 20 mm, while the remaining of 6 bacterial species (*P. mirabilis, Y. enterocolitica, E. coli* IBRL 0157, *S. typhimurium, K. pneumoniae* ATCC 13883 and *A. antratus*) showed the highest susceptibility rate towards fungal extract with the inhibition zone of ≥ 21mm. The results obtained in this study revealed that *C. ramicola* IBRLCM127 exhibited prominent antibacterial activity against pathogenic bacteria from both classes of Gram-positive and Gram-negative bacteria, of which some of them also caused significant infectious disease to human.

**Table 1.** Primary screening of antibacterial activity of *Ceratobasidium ramicola* IBRLCM127 against selected test bacteria using agar plug diffusion assay

| Test bacteria | Inhibition zone |
|---|---|
| **Gram positive bacteria** | |
| *S. aureus* | +++ |
| *B. cereus* ATCC 10876 | +++ |
| *B. subtilis* IBRL A3 | +++ |
| Methicillin-resistant *S. aureus* ATCC 33591 | +++ |
| **Gram negative bacteria** | |
| *P. mirabilis* | +++ |
| *Y. enterocolitica* | +++ |
| *E. coli IBRL 0157* | +++ |
| *S. typhimurium* | +++ |
| *K. pneumoniae ATCC 13883* | +++ |
| *A. antratus* | +++ |
| *P. aeruginosa ATCC 27844* | ++ |

+=Inhibition zone ≤10 mm, ++=Inhibition zone 11 to ≤20 mm, +++=Inhibition zone ≥21 mm

*3.2 Disc Diffusion Assay*

The antibacterial activity of ethyl acetate extract of *C. ramicola* IBRLCM127 was shown in Table 2. The results revealed that the ethyl acetate extract displayed antibacterial activity against 8 out of 11 test bacteria, of which all 4 Gram-positive bacteria were inhibited successfully with the diameter of inhibition zone ranging from 8.0±0.0 to 13.0±0.0 mm. Meanwhile, for Gram-negative bacteria, 4 out of 7 bacteria (*Y. enterocolitica, K. pneumoniae* ATCC 13883, *A. antratus* and *P. aeruginosa* ATCC 27844) were inhibited by the ethyl acetate extract with the inhibition zone sizes of 8.3±0.6 to 11.7±0.6 mm. On the other hand, only 1 test bacteria, viz., Gram-positive bacteria (*S. aureus*) was inhibited by methanol extract with diameter inhibition zone of 8.3±0.6 mm.

Based on the current finding, the antibacterial compounds were believed to be secreted extracellularly by endophytic fungus into the fermentative broth as it showed remarkable antibacterial activity compared to its biomass (intracellular). The main advantage of using microorganisms to produce biologically active compounds was the relative simplicity to obtain high yield as well as the possibility of genetic and culture condition manipulation [17].

**Table 2.** Antibacterial activity of *C. ramicola* IBRLCM127 ethyl acetate (extracellular) and methanol (intracellular) extracts against selected test bacteria using disc diffusion assay

| Test bacteria | Ethyl acetate extract (fermentative broth; mm) | Methanol extract (fungal biomass; mm) | Chloramphenicol (30 µg/mL; mm) |
|---|---|---|---|
| **Gram positive bacteria** | | | |
| *S. aureus* | 13.0±0.0 | 8.3±0.6 | 19.7±0.6 |
| *B. cereus* | 10.0±0.0 | - | 21.3±0.6 |
| *B. subtilis* | 10.0±0.0 | - | 21.7±0.7 |
| Methicillin-resistant *S. aureus* ATCC 33591 | 8.0±0.0 | - | 22.7±0.6 |
| **Gram negative bacteria** | | | |
| *P. mirabilis* | - | - | 21.0±1.0 |
| *Y. enterocolitica* | 11.7±0.6 | - | 30.7±0.6 |
| *E. coli* IBRL 0157 | - | - | 20.7±0.6 |
| *S. typhimurium* | - | - | 20.7±0.6 |
| *K. pneumoniae* ATCC 13883 | 8.3±0.6 | - | 20.0±0.0 |
| *A. antratus* | 11.7±0.6 | - | 22.0±0.0 |
| *P. aeruginosa* ATCC 27844 | 9.0±0.0 | - | 15.3±0.6 |

*3.3 Determination of MIC and MBC*

MIC value obtained can be defined as the lowest concentration of the assayed antibacterial or antifungal agents that inhibit the visible growth of the tested microorganisms**.**The MIC values of Gram-positive bacteria for ethyl acetate extract were ranging from 125.00 to 500 µg/mL and methanol extract was 250.00 µg/mL (Table 3). On the other hand, Gram-negative bacteria were slightly resisted to ethyl acetate extract with the MIC values ranged from 125 to 250 µg/mL, while all Gram-negative bacteria were not susceptible to methanol extract. In a meantime, the MBC values of ethyl acetate extract were also determined in this experiment, of which the values for Gram-positive and Gram-negative bacteria were ranging from 125.00 to 500.00 µg/mL and 250.00 to 500.00 µg/mL, respectively. Meanwhile, the MBC value of methanol extract for Gram-positive bacteria was 500 µg/mL. In short, both ethyl acetate and methanol extracts were demonstrated bactericidal effects on test microorganisms since the MBC/MIC ratio ≤ 4.

**Table 3.** Antibacterial activity of *Ceratobasidium ramicola* IBRLCM127 ethyl acetate (extracellular) and methanolic (intracellular) extracts against test microorganisms

| Test bacteria | Ethyl acetate extract (fermentative broth) | | | Methanol extract (fungal biomass) | | |
|---|---|---|---|---|---|---|
| | MIC (µg/mL) | MBC ((µg/mL) | MBC/MIC | MIC (µg/mL) | MBC ((µg/mL) | MBC/MIC |
| Gram positive bacteria | | | | | | |
| *S. aureus* | 125.0 | 125.0 | 1 | 250.0 | 500.0 | 2 |
| *B. cereus* | 250.0 | 500.0 | 2 | - | - | - |
| *Bacillus subtilis* | 125.0 | 250.0 | 2 | - | - | - |
| Methicillin-resistant *S. aureus ATCC 33591* | 500.0 | 500.0 | 1 | - | - | - |
| Gram negative bacteria | | | | | | |
| *P. mirabilis* | - | | | - | - | - |
| *Y. enterocolitica* | 250.0 | 500.0 | 2 | - | - | - |
| *E. coli IBRL 0157* | - | - | - | - | - | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| *S. typhimurium* | - | - | - | - | - | - |
| *K. pneumoniae ATCC 13883* | 250.0 | 500.0 | 2 | - | - | - |
| *A. antratus* | 125.0 | 250.0 | 2 | - | - | - |
| *P. aeruginosa ATCC 27844* | 125.0 | 500.0 | 4 | - | - | - |

*3.4. Structural Degeneration and Morphological Changes of the Bacterial Cells after Extract Treatment*
The effects of ethyl acetate extract on bacterial cells was evaluated using SEM and the result is shown in Figure 1. Figure 1a is the control (untreated) cells of *S. aureus* which displays the intact coccal shaped with smooth cell surface and still maintain its rigidity. However, after 36 hours of exposure to the extract (Figure 1b), the bacterial cells start to lose their intact cocci shape, lysed and shrunk abruptly (Figure 1b).

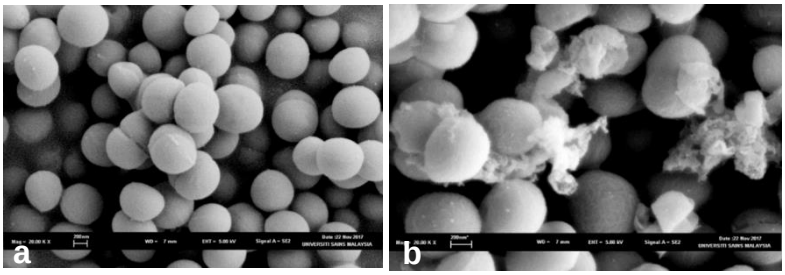


**Figure 1.** SEM micrographs showing the effects of *C. ramicola* IBRLCM127 ethyl acetate extract at the concentration of 1.0 mg/mL on *S. aureus* cells, (a) untreated cell (control) and (b) extract-treated cell (×2000)

Figure 2 shows the micrographs of a control and treated of Gram-negative bacterial cells, *K. pneumoniae* ATCC 13883 before and after treatment with extract. Figure 2a is the control or untreated cells that exist in normal cell condition with rugose surface, rod shape rigid and smooth cell surface. However, after the cells were treated with the extract for 36 hours, significant morphological changes of the cells could be observed including the invagination and formation of cavities on the cell surfaces. The SEM observations showed that the extract-treated cells had undergone severe structural damages which finally caused the cell damages beyond repair.

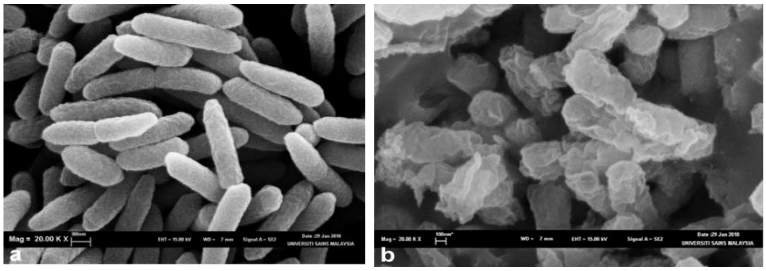


**Figure 2.** SEM micrographs showing the effects of *C. ramicola* IBRLCM127 ethyl ethyl acetate extract at the concentration of 1.0 mg/mL on *K. pneumoniae* ATCC 13883 cells, (a) untreated cell (control) and (b) extract-treated cell (×2000)

## 4. Discussion

The antibacterial activity of endophytic fungus *C. ramicola* IBRLCM127 extract was preliminary screened quantitatively by opting agar plug diffusion assay method as this method was only used to detect the presence of antibacterial compounds secreted by the fungal agar plugs. Preliminary screening results revealed a wide-spectrum of antibacterial activity of *C. ramicola* IBRLCM127 wherein it can inhibit all the 11 test bacteria consisting of 4 Gram-positive bacteria and 7 Gram-negative bacteria. In a meantime, the disc diffusion assay method was selected to precisely quantify the antibacterial activity of fermentative broth and fungal biomass as this method had been acknowledged as a wise choice for the secondary screening stage [13]. Naturally, endophytic fungi tend to secrete their bioactive metabolites extracellularly into the fermentation medium as the function of these metabolites is to keep them save from predators or pathogens.

The bacterial response to ethyl acetate extract was greater in Gram-positive bacteria compared to Gram-negative bacteria, in line with the previous studies that reported Gram-positive bacteria were more susceptible to fungal extract than Gram-negative bacteria [18]. This condition was presumably caused by the difference in cell wall structure of these two classes of bacteria that serves as a permeability barrier to impede the toxic substances penetration into the cells [19]. Theoretically, the cell wall of Gram-positive bacteria consists of predominantly thick and porous peptidoglycan layer without the existing of outer membrane which causes it to become permeable to most antibiotics [18]. Contradictorily, the cell wall of Gram-negative bacteria contains more complex substances with outer membrane layer that caused them to be more resistant to the antibacterial agent

The structural damages at subcellular resolution of the bacterial cells could be observed clearly using SEM and TEM, of which significant cell damages were shown by the cell lysis and ruptured possibly due to the disintegration of the cell wall and cell membrane which finally lead to the leakage of cytoplasmic content. In this case, the bioactive molecules contained in ethyl acetate extract of *C. ramicola* IBRLCM127 can bind strongly to the bacterial outer membrane causing the permeation of antibacterial agent across the membrane and caused the cells to become unstable and collapsed structurally that resulted to the cell death. This prediction was congruent with the finding of Chatterjee and his colleagues that reported the ethyl acetate extract of *Alternaria alternata* AE1 caused the destruction of the bacterial cell wall which led to the total damage of the cell [20]. The current result showed that *C. ramicola* IBRLCM127 demonstrated remarkable antibacterial activity and to the best of our knowledge, this is the first report concerning endophytic *C. ramicola* as a potential antibacterial agent.

## 5. Conclusion

Endophytic fungus *C. ramicola* IBRLCM127 demonstrated significant antibacterial activity against a wide-array of Gram-positive and Gram-negative bacteria with bactericidal effects that causing severe morphological damages beyond repair. The possible mode of action of ethyl acetate extract of *C. ramicola* IBRLCM127 against bacterial cells is through their cell wall or cell membrane.

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