Screening of antimicrobial and toxicity activity of endophytic bacteria associated with Curcuma Zedoaria

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Abstract. Indonesian has been used Curcuma zedoaria as a herbal in cancer treatment. The bioactive compounds contained in the medicinal plants are products by the plant itself or by endophytes living inside the plant. Zebrafish has been used for the study in several areas of cancer research, including angiogenesis, metastasis, antitumor drug screening, and toxicity. This study was to investigate the antimicrobial and toxicity activity of secondary metabolite from endophytic bacteria isolated from C. zedoaria. Endophytic isolates were screened for antimicrobial activities against six microbial pathogens Bacillus subtilis, Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli, Candida albicans, and Sacharomyces cerevisiae. Four of endophytic bacteria showed antimicrobial activity were tested in toxic compound producing through Fish Embryo Toxicity (FET) test using zebrafish. The results indicated that from 73 selected isolates, 16 isolates (21.92%) showed antimicrobial activities against at least one of the test organisms. Four of endophytic bacteria that showed high antimicrobial activities, were identified as Citrobacter freundii, Bacillus subtilis, Pseudomonas otitidis, and Burkholderia cenocepacia. FET test of ethyl acetate extracts from the four selected isolates revealed that they had LC₅₀ values 63.5; 24.7; 13.4; and 12.6 ppm, respectively. These result showed that the highest toxicity was obtained from B. cenocepacia extract.

1. Introduction
Endophytes are a potential source of bioactive compounds that may produce the same bioactive compounds as those of the host plants. Chemical profile and biological activity of bioactive compound produced by endophytes might be related to the bioactive produced by the host plants. Endophytes release secondary metabolite compounds that might be responsible for the medicinal value of plants. Some of the bioactive compound produced by endophytes have been proven to be useful in the pharmaceutical industry for drug discovery, such as anticancer, antibiotic, antymycotic and antiviral [1]. Therefore, many studies concerning the diversity of endophytes and the chemistry and bioactivity of endophyte metabolites have been conducted.

Curcuma zedoaria known as ‘kunyit putih’ has been used traditionally in many countries as a valuable medicinal plant. In Indonesia this plant has been used for disease treatment related to cancer. Extract of C. zedoaria rhizome showed antimicrobial activity [2], neuroprotective activity and
cytotoxic effects against human ovarian cancer cells [3]. According to Sri et al. [4] and Lakshmi [5], essential oil from rhizome of *C. zedoaria* has inhibition activity in the proliferation of cancer cells.

Bioactive compound contained in *C. zedoaria* was estimated to be closely related to the presence of microbial inside the plant. Endophytic diversity associated with *C. zedoaria* may indicate diversity in bioactive compound and therefore has a great potential as the source to find a new chemical compounds as a drugs candidate. Therefore, in this study we investigate the antimicrobial activity and their toxicity activity of endophytic bacteria associated with *C. zedoaria*.

2. Materials and Methods
Endophytic bacteria were recovered from a previous study [6]. A total of 73 isolates were isolated from *C. zedoaria* planted in 3 areas in Bogor, i.e. Bojong Gede, Cibinong and Dramaga, Indonesia during 2013. All bacteria were previously identified molecularly based on 16S rRNA gene sequence and MALDI-TOF based on protein profile [7].

The screening of antimicrobial activity was carried out using six different microbial test strains; *Bacillus subtilis* InaCC B1, *Pseudomonas aeruginosa* InaCC B3, *Staphylococcus aureus* InaCC B4, *Escherichia coli* InaCC B5, *Candida albicans* InaCC Y116, and *Saccharomyces cerevisiae* InaCC Y728. Bacteria test strains were grown on Nutrient Broth (NB) at 37 °C for 20 hours and yeast test strains were grown on Potato Dextrose Broth (PDB) at 30 °C for 20 hours. Bacteria cultures was diluted to 10⁶ cells/mL and yeast was diluted to 10⁶ cells/mL on semisolid agar, then overlayed on NA or PDA as basal media.

The endophytic bacteria were cultured on NA plates for five days. Each of the endophytic bacteria were plated on NA size of 6 mm in diameter and then placed on bioassay media. The plates were incubated at 37 °C for 24 hours for bacteria and yeast at 30 °C for 48 hours. The width of inhibition zones was measured and evaluated as follows: +++, ≥20 mm; ++, 11-19 mm; +, 2-10 mm; +, <1 mm; -, 0 mm. Each of antagonistic assay was done in three replicates.

Each of endophytic bacteria was cultured on NB liquid medium. Five mL of actively growing cells were poured into 100 mL of NB liquid medium. The cultures were incubated at 28 °C for five days with periodical shaking at 120 rpm. After the incubation period, the cultures were taken out and centrifuged at 4 °C, 10000 rpm for 15 minutes. The metabolites were extracted using ethyl acetate (1:1) [8] and extraction was repeated three times. The collected solvent was evaporated and dried in vacuum evaporator using MgSO₄ to yield the crude extract.

Anticancer properties of the metabolites of the endophytic were tested using toxicity test. Toxicity test of crude extracts was done using zebrafish embryo. Fish Embryo Toxicity (FET) test was conducted through in vitro assays to determine the LC50 values. Lethal Concentration 50 (LC50) is the concentration that causes 50% mortality in the experimental animals. Determination of LC50 values using zebrafish help us to understand the toxic effect of the drug compound to mammals [14]. Zebrafish fertilized eggs were selected using stereo microscope (Olympus) and placed in 300 µL of test solution in 96-well plates individually. The crude extracts were dissolved in several concentration e.g 0 (control), 50, 100, 150, 200, and 250 ppm, respectively. The plates were covered and were observed at 24, 48, and 72 hours post fertilization (hpf). Each concentration was done in triplicates and each replicate was made in five well. The selected eggs were exposed with endophytic bacteria extract for 72 hours and observed every 24 hours incubation using stereo microscope. The morphological development of the zebrafish were studied by observing some morphological characters such as axis, somite, trunk, pigmentation, yolk sac, heart, eye, ear, and blood circulation [9].

3. Results and Discussion
3.1 Endophytic bacteria as antimicrobial producer
The results showed that several endophytic bacteria associated with *C. zedoaria* showed antimicrobial activities. Among 73 selected isolates, 16 isolates showed antimicrobial activities against Gram-positive, Gram-negative bacteria, and yeast. The antimicrobial activities showed the range of inhibition activity from 1 to 22 mm (Figure 1). The strength of inhibition was categorized according to Davis and Stout (1971): a very strong (clear zone > 20 mm), robust (clear zone of 10 - 20 mm), medium (5-10 mm clear zone), weak (< 5 mm). Based on the inhibition strength, most of the
antimicrobial activities of bacterial isolates were weak. *B. subtilis* (RB.P.1) and *B. cenocepacia* (RI.P.1) could be categorized as a robust inhibitor, however, they only inhibited one type of pathogenic microbe (Table 1).

![Figure 1. Antimicrobial activities of endophytic bacteria against microbial test strains: A. C. albicans, B. S. cerevisiae, C. B. subtilis](image)

The data were displayed in Table 1 showed that antimicrobial of the endophytic bacteria were more active against bacteria than to yeast. The bacteria are more sensitive to antimicrobe compounds. Bacteria are prokaryotic cells with a simple structure of the cell wall which is made of peptidoglycan, whereas yeast is a eukaryotic cell which cell wall components consisted of beta-glucan and mannan-oligo-saccharides [10]. The results also showed that the antimicrobial activities of isolates tested were stronger against Gram-positive than Gram-negative bacteria. It might due to the differences of cell wall structure between Gram-positive and negative bacteria. Gram-positive bacteria are more susceptible caused the peptidoglycan is not effective as a barrier, whereas Gram-negative bacteria have an outer membrane which is cell wall polysaccharides and cannot be penetrated by antibiotics, dyes and detergents [11].

| Endophytic bacteria | Microbial test strains | Gram-positive | Gram-negative | Yeast |
|---------------------|------------------------|---------------|---------------|-------|
|                     |                        | *B. subtilis* | *S. aureus*   | *E. coli* | *P. aeruginosa* | *C. albicans* | *S. cerevisiae* |
| *B. subtilis*       |                        | -             | -             | -       | +++            | -             | +++            |
| *(RB.P.1)*          |                        | -             | -             | -       |                | -             | -              |
| *P. denitrificans*  |                        | +             | -             | -       | -              | -             | -              |
| *(RB.P.3)*          |                        | +             | ++            | -       | +              | -             | -              |
| *P. otitidis*       |                        | +             | ++            | -       | +              | -             | -              |
| *(BL.S.1)*          |                        | +             | -             | -       | -              | -             | -              |
| *M. cosmeticum*     |                        | +             | -             | -       | -              | -             | -              |
| *(BL.S.2)*          |                        | ++            | -             | -       | -              | -             | -              |
| *P. denitrificans*  |                        | ++            | -             | -       | -              | -             | -              |
| *(DL.P.3)*          |                        | +             | -             | -       | -              | -             | -              |
| *E. cancerogenus*   |                        | +             | -             | -       | -              | -             | -              |
| *(DL.P.1)*          |                        | +             | -             | -       | -              | -             | -              |
| *B. cenocepacia*    |                        | +++           | -             | -       | -              | -             | -              |
| *(RI.P.1)*          |                        | +             | -             | -       | -              | -             | -              |
| *B. cereus*         |                        | +             | -             | -       | -              | -             | -              |
| *(RI.P.5)*          |                        | +             | -             | -       | -              | -             | -              |
| *P. geniculata*     |                        | +             | -             | -       | -              | -             | -              |
| *(RI.P.6)*          |                        | +             | -             | -       | -              | -             | -              |
| *E. ludwigii*       |                        | +             | -             | -       | -              | -             | -              |
| *(RI.P.8)*          |                        | +             | -             | -       | -              | -             | -              |
| *P. nitroreducens*  |                        | +             | -             | -       | -              | -             | -              |

Table 1. Antimicrobial activites of endophytic bacteria from *C. zedoaria*
Medicinal plants with wide ethnobotany history have a potential to carry endophytic bacterial species that carry the characteristics of the host plant. According to Tan and Zou [12], several bioactive compounds produced by endophytic bacteria were normally associated with the characteristics of the host plant. This result also confirmed the study of Banisalam et al. [13], that extract of *C. zedoaria* rhizome has antibacterial pharmacological effects against pathogenic bacteria *E. coli*, *P. aeruginosa*, *B. cereus* and *S. aureus*.

### 3.2 Acute toxicity using zebra fish embryos

The death rate of embryos as well as growth abnormalities where observed after exposed with ethyl acetate extract. Ethyl acetate extract was more toxic than water phase. According to Figure 2, LC50 values differed between ethyl acetate and water phases. It was due to the active compound largely been carried away by ethyl acetate. LC50 of ethyl acetate extract were much lower than the water phase for all four endophytic bacteria tested (Figure 2). Those values indicating that the ethyl acetate extract were more toxic than the water phase. The ethyl acetate extract may carry much more bioactive compound than the water phase. The toxicity of the ethyl acetate extract ranged from around twice for *C. freundii* to ten times for the best of the endophytic bacteria tested (*B. subtilis*, *P. otitidis*, and *B. cenocepaica*).

| Organism               | LC50 (ppm) | 63.5 | 109.9 | 24.7 | 252.3 | 13.4 | 132.4 | 12.6 | 120.8 |
|------------------------|------------|------|-------|------|-------|------|-------|------|-------|
| *C. freundii* (BLS.3) |            |      |       |      |       |      |       |      |       |
| *B. subtilis* (RB.P.1) |            |      |       |      |       |      |       |      |       |
| *P. otitidis* (BLS.1)  |            |      |       |      |       |      |       |      |       |
| *B. cenocepaica* (RL.P.1) |            |      |       |      |       |      |       |      |       |

**Figure 2.** LC50 value of endophytic bacteria

The addition of ethyl acetate extract showed a significant morphological differences compared to control treatment (0 ppm of crude extract). Some eggs coagulated at 24 hours and there is no specific reason why the egg is coagulated. Coagulation usually occurs naturally, which is around 4-5% of eggs produced, and this probably is caused by nutrients deficiency obtained by zebrafish mothers (10).
Untreated zebrafish embryos as a control (0 ppm), 48 hours after fertilization hatched and developed into larvae with perfect organs; straight body axis, eyes, tail, head, spine, and normal pigmentation (Figure 3a). Normal zebrafish larvae have a straight body axis [15] and in the case of abnormalities, the pigment colour is different from the control [16].

Zebrafish embryos that have been exposed to ethyl acetate extract of *C. freundii*, *B. subtilis*, *P. otitidis*, and *B. cenocepacia* at 50, 100, 150, 200, 250 ppm after 72 hours of incubation resulted in embryo coagulation and some organs abnormalities (Figure 3). Most of the zebrafish embryos had major abnormalities in the body axis, egg yolk sac, heart, blood coagulation, and body pigmentation. The size of yolk sac swelled in all embryos exposed to the ethyl acetate extract. The yolk sac is a membrane that wraps the yolk and contains the food needed during the first week of embryo formation. The size change of yolk sac is one indication of the disruption of the digestive system. The food is not perfectly distributed in the embryo and resulting in the death of the embryo. The exposure of extract generated blood coagulation and heart swelling of zebrafish embryos. Blood coagulation generated heart swelling and death of larvae due to imperfect blood circulation. The flow rate of blood inhibited and cells of fish embryos did not get nutrients [16]. The same thing happened in the study of Kumar *et al.* [9] which stated that at 72nd hours after the exposure of 100 ppm methanol extract from the skin of the *Streblus asper* there was a swelling of the heart due to an increase in heart rate.

![Zebrafish embryos](image)

**Figure 3.** Zebrafish embryos which exposed to crude ethyl acetate extract of *C. freundii*(A; *B. subtilis*(B); *P. otitidis*(C); *B. cenocepacia* (D) at different concentration of: 0 ppm, control (a); 50 ppm (b); 100 ppm (c); 150 ppm (d); 200 ppm (e); 250 ppm (f).

Organ abnormalities are correlated to the extract concentration used. The application of different extract at the same concentration would generate similar abnormalities. The addition of 50 ppm ethyl acetate extract showed several abnormalities, especially egg yolk sac become bigger than control. Application of *B. subtilis* extract at 50 ppm; zebrafish embryo hatched into larvae. However, there were several abnormalities in the intensity of eye color and pigmentation (Figure 3b). Similar results
also appeared in the application of 100 ppm extract (Figure 3c). Several abnormalities occurred at low concentration and accordingly it can be conclude that the extract was toxic to zebrafish embryo.

The application of extract at 150 ppm for 72 hours, zebrafish embryo hatched into larvae with abnormalities in the axis of the body, egg yolk sac, heart, eye and pigmentation colour (Figure 3d). The abnormalities were more complex compared the concentration below. Wei et al. [16] reported that abnormalities embryos showed a different pigmentation colour compared controls. The extract of B. cenocepacia induced the most severe abnormalities in the body pigmentation, the colour of the yolk sac, and the intensity of eye colour. Black is the normal eye colour and turns white when exposed with extract of B. cenocepacia.

The exposure of 200 ppm of extract gave a significant effect on all zebrafish embryos. With the exposure of P. otitidis extract, the embryos hatched into larvae, however the embryos did not develop perfectly and the larvae immediately died. Meanwhile, the embryos were unable to hatched when exposed with B. cenocepacia extract (Figure 3e). This was different from the treatment of 250 ppm extract which caused death in all embryos. The embryos exposed with C. freundii extract were able to hatch into larvace but the larvae died because the yolk sac broke. The embryos exposed with B. subtilis, P. otitidis, and B. cenocepacia extracts coagulated after 24 hours (Figure 3f). These results indicated that the toxicity of extracts at 250 ppm were very high, as indicated from the absence of embryos that hatched into larvae.

The data was obtained showed that the ethyl acetate extract of C. freundii (BI.S.3), B. subtilis, (RB.P.1), P. otitidis (BL.S.1) and B. cenocepacia (RI.P.1) inhibited the growth of zebrafish embryos. Based on the LC50 values of toxicity test from bacterial extract, could support the view that endophytic bacteria from C. zedoaria are potential source of toxic compounds. These compound give same effect on the growth of the cancer cells, inhibit and damage as well as the organs of zebrafish embryos.

The study of Li et al. [17] reported that from 502 toxic natural compounds tested, 59 compounds were identified as toxic compound in the zebrafish and 21 of those could suppressed MCF7 breast cancer growth in vitro and caused zebrafish embryo death within 24 h. The se results indicate that FET test using zebrafish could complement the MTT assay. Therefore zebrafish is reliable and efficient tool for cancer drug screening and these result also support our finding that endophytic bacteria from C. zedoaria are source of toxic compounds with great potential as a source of anticancer drug candidate.

4. Conclusion
Endophytic bacteria from C. zedoaria showed antimicrobial and toxicity activity. Antimicrobial activity from endophytic bacteria associated with C. zedoaria were weak, however four of selected endophytic bacteria had toxic activity on zebrafish embryos. These result support that endophytic bacteria from C. zedoaria are great potential as a source of anticancer drug candidate.

5. References
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