Antimicrobial and Antioxidant Activity of Endophyte Bacteria Associated with *Curcuma longa* Rhizome

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Abstract

Most cases of bacterial resistance towards antibiotics, encourage various efforts to gain new sources of antibiotics. Endophyte bacteria is a micoorganism has important role as the producer of bioactive compounds. Endophyte bacteria from *Curcuma longa* with antimicrobial and antioxidant activities have not been studied yet. *Curcuma longa* has been utilized as the main ingridients of traditional herbal medicines (*jamu*). The objective of this research was to investigate the antimicrobial and antioxidant activity of endophyte bacteria associated with *Curcuma longa* rhizome. Based on morphological characteristics of bacterial colonies, eight endophyte bacteria was isolates from *Curcuma longa* rhizome. Screening of endophyte isolate has antimicrobial activity was done using agar well diffusion method. The culture supernatant of each endophyte isolate was dropped on agar well against pathogenic bacteria *Salmonella enterica* ser. Typhi, *Staphylococcus aureus* and yeast *Candida albicans*. Three endophyte isolates K_3, K_2 and M_{1b} showed antimicrobial activity against pathogenic bacteria and yeast. Isolate K_3 showed strong antimicrobial activity againts *C. albicans* and *S. aureus*, however isolate K_2 and isolate M_{1b} showed antimicrobial activity againts *Salmonella enterica* ser. Typhi and *S. aureus*, respectively. Those endophyte bacteria also had antioxidant activity shown by scavenging ability toward DPPH radical with consecutive percentage of isolate K_3 (72.3 %), K_2 (51.3 %) and M_{1b} (64.6 %). Isolate K_3 showed the highest antimicrobial and antioxidant activity. Based on biochemical characteristics using Microbact 24E kit, isolate K_3 was identified as *Paenibacilus alvei* and isolate K_2 as *Enterobacter agglomerans*.

Keywords: antimicrobial, antioxidant, *Curcuma longa*'s rhizome, endophyte bacteria.

INTRODUCTION

Recent main health care issues include the rise of antibiotic resistances and the rise of chronic and degenerative disease in countries throughout the world regardless of income level. The rise of antimicrobial resistance need the discovery and/or production of novel anti-microbial. Antioxidants, that have capability scavenging free radicals, are known to play important roles in preventing the degenerative, ROS-linked diseases. As the human population growth and the increase awareness on healthy life, people prefer natural compounds. Thus, the exploration of novel source of natural bioactive compound is unavoidable. One of the most promising source of natural bioactive compound is endophyte [1].

Endophytes are microorganisms, often bacteria, actinomycetes or fungi that live in healthy plant tissue intercellularly and/or intracellularly without causing any apparent symptoms of disease. Endophyte bacteria are found in roots, tubers, rhizome, nodule, stems, leaves, flowers, ovules, seeds and fruits of various plant species. In general roots have greater numbers of endophytes than above ground tissues [2]. Many evaluations of bacterial endophytes have shown that they are widespread in numerous plant kingdom. A single plant may have several different endophyte bacteria. The structure of bacterial endophyte communitas are varied, dynamic overtime, and attributed to plant source, plant age, tissue type, time of sampling, season and environment [3].

The endophyte bacteria beneficial to its host by promote plant growth and yield, suppress pathogens, help plants to tolerate biotic stress or abiotic stresses, help to remove contaminants, solubilize phosphate, or contribute in fixing nitrogen. Endophytes bacteria are also known for the production of various classes of natural products and have been reported to exhibit a broad range of biological activity. It has reported over two thousands natural products have been isolated from endophytes associated with medicinal plants, including alkaloids, flavonoids, glycosides, phenolic acid, xanthones, steroids, terpenes, tetralones, coumarins, quinones, lactone, polysaccharide, peptides. Such bioactive

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metabolites are found to apply as agrochemicals like insecticidal, growth-promoting, and their potential in the pharmaceutical like antibiotics, antioxidants, antitumor, antidiabetics, antiparasitics, antithrombotic, anticancer and immunosuppressants agents [4].

Medicinal plant is well known as source of precious bioactive compound. Endophytes that have long time associate with medicinal plant may participate in metaboloc pathway or gain some genetic information to produce specific bioactive compound similar to the host plant. Plant that have ethnobotanical history should be sourced of endophyte microbe. Therefore, it is needed and important to study and explore medicinal plants and endophyte microbes that live in [4]. *Curcuma longa*'s rhizome, commonly called as turmeric has been widely used as a spice and has a long history of medicinal use in the treatment of a variety of human diseases especially in Asia regions. This study was aimed to analyze the antimicrobial and antioxidant activity of endophyte bacteria associated with turmeric rhizome.

MATERIALS AND METHODS

Study Area

Sample of turmeric rhizomes were collected from Mondo Village, Mojo District, Kediri Regency. The soil type in the research site is aluvial, along the area of Brantas Watershed. Soil acidity in the plant site is 6.34. Optimum pH of soil for most plants range 5.5 – 7.0 and nutrient will adsorbed well in the range pH 5.5 – 6.5 [5]. Refer to the soil pH at the sample site, it has qualified for the plant to grow well. Healthy ten months old plants were selected as source of rhizome for endophyte bacteria isolation.

Surface Sterilization of Turmeric rhizome

Rhizomes of turmeric were washed with running tap water. The procedure includes sequential immersion of rhizomes parts in 70% ethanol for 3 minutes, sodium hypochlorite 2% for 5 minutes and 70% ethanol for 30 seconds, then rhizomes was washed using sterilized distilled water for five times [6]. The last twice washing solutions were plated on Nutrient Agar (NA) to confirm the effectiveness of sterilization treatments. The surface of turmeric rhizomes were pilled out using aseptic technique and the inner tissues of rhizomes were macerated using a sterile mortar and pestle [7].

Isolation of Endophyte Bacteria from *Curcuma longa* Rhizome [8,9]

Total of 10 g turmeric rhizomes were extracted then performed a serial dilution in saline solution (0.85% NaCl) and plated out in Nutrient Agar (NA) to recover endophyte bacteria present in the rhizome. All the plates were incubated at 28-30°C (room temperature) for 48 hours. The isolated bacteria were preliminary characterized according to their morphological characteristics. The distinct colony types were picked up from Nutrient Agar (NA) plates and were purified through three rounds of streaking and single colony was selected an refresh in the same medium.

Test Microorganisms

Pathogenic strain yeast of *Candida albicans*, Gram-positive bacteria *Staphylococcus aureus* and Gram-negative *Salmonella enterica* ser. Typhi clinical isolates were used as test microorganism in this study. All pathogenic strains were obtained from Department of Microbiology, Medical Faculty, University of Brawijaya. After 18-24 hours of incubation at 37^{o}C (for bacterial strains) in NA and 30^{o}C (for yeast strain) in PDA, a loopful of each test strains was suspended in sterile distilled water water until obtained 1 x 10^{6} cfu.mL^{-1} for bacteria and 10^{5} cfu.mL^{-1} for yeast.

Assays of Antimicrobial Activity

Isolated endophyte bacteria from turmeric rhizomes were cultured in 5 mL Nutrient Broth (NB) medium at room temperature (28-30^{o}C) for five days. After five days, culture medium was centrifuged at 4000 rpm for 15 minutes and supernatant was screened for antimicrobial activity by agar-well diffusion technique on NA media that was previously seeded with test pathogens. Supernatant (50µL) was added into wells (7 mm) formed by cork borer on the NA medium [10]. Sterile NB was set as control. As a positive control for antimicrobial activity towards test microorganism, we used amoxicillin antibiotic dose 10 µg.mL^{-1} [11] for *S. enterica* ser. Typhi and dose 25 µg.mL^{-1} [12] for *S. aureus*. While for the antimicrobial activity on yeast, we used anti fungal nystatin 12 µg.mL^{-1} as positive control [13]. The plates were incubated in suitable temperatures for 24-48 hours; the zone of inhibition was measured and recorded.

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Assay of Antioxidant Activity by Scavenging DPPH Free Radical

Endophyte bacteria culture (in NB medium) were centrifuged at 4000 rpm for 15 minutes, 4°C and then the supernatants were assayed their antioxidant activity by scavenging DPPH free radical methode [14] described with any modification. The supernatant (0.5 mL) was added to 3 mL of 0.1 mM DPPH in methanol solution. Methanol 1.5 mL was then added thus the final volume of solution was 5 mL. For control, supernatant of each sample was replaced by steril Nutrient Broth (NB). Methanol was used as blank. Discoloration of DPPH radical solution was measured at 517 nm in triplicate after incubation in the dark for 5 hours. Ascorbic acid was used as the positive control. Percentage of scavenged DPPH radical was calculated using following formula

$$\% \text{ Scavenging} = \left[\frac{A_0 - A_1}{A_0}\right] * 100$$

A_0 is the absorbance of control and A_1 is the absorbance of sample (supernatant of endophyte bacteria culture) or standard. Ascorbic acid was taken at various concentrations as a known antioxidant for comparative analysis. Then the percentage of scavenging were plotted against respective concentrations used, and from the graph, EC_{50} was calculated.

Statistical analysis

The experimental results of biological activity tests were expressed as mean ± standard deviation (SD) of three replicates. The results were processed using Microsoft Excel 2007 and SPSS software. The data of antimicrobial activity assay results was analyzed using Kruskal-Wallis test followed by t-test and Tukey test whereas antioxidant activity using Anova following Tukey test.

RESULTS AND DISCUSSION

Based on the morphology characteristics of colony, we obtained eight isolates K_1, K_2, K_3, K_4, M_{1a}, M_{1b}, M_5 and M_6 of endhopytes bacteria. Each pured isolate was tested further for the antimicrobial and antioxidants activities.

Antimicrobial Activity

Three of eight isolates of turmeric endophyte bacteria has inhibition activity towards pathogenic test microorganism. Isolate K_3 inhibit the pathogenic yeast *C. albicans* (Fig. 1a) and pathogenic bacteria *S. aureus* (Fig. 1b). Otherwise, isolate K_2 inhibit the pathogenic bacteria *Salmonella enterica* ser. Typhi (Fig. 1c) and isolate M_{1b} inhibit *S. aureus* (Fig. 1d).

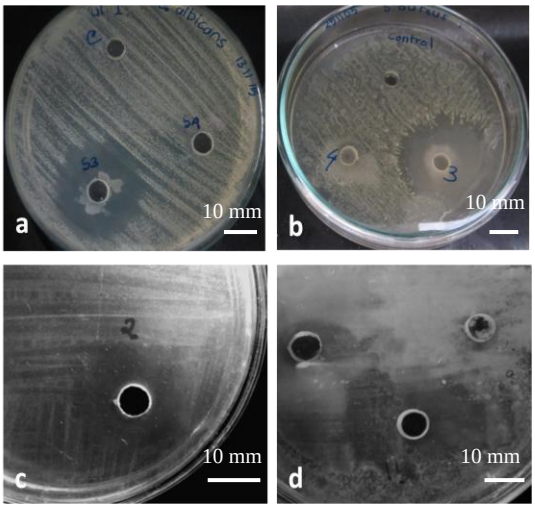

Figure 1. Inhibition zone of endophyte bacteria isolates Isolate K_3 towards *Candida albicans* (a) and *Staphylococcus aureus* (b); Isolate K_2 towards *Salmonella enterica* ser.Typhi (c); Isolate M_{1b} towards *Staphylococcus aureus* (d).

The results showed that the inhibition zone of isolate K_3 towards *C. albicans* was greater than antifungal nystatin (12 µg.mL^{-1}). It also showed similar results for inhibition zone of isolate K_3 to *S. aureus*, which is greater than amoxicilin (25 µg.mL^{-1}) and isolate K2 to *Salmonella enterica* ser. Typhi than amoxicilin (10 µg.mL^{-1}). Otherwise, the inhibition zone of isolate M_{1b} to *S. aureus* was relatively similar to the inhibition zone of amoxicillin (25 µg.mL^{-1}) (Fig 2).

Microbes produce any substance for defense systems or survival mechanism. These include antibiotics, bacteriocins, metabolic by-products, lytic agents, numerous types of protein exotoxins, and short chain fatty acid [15]. This study found that Isolate K_3 and M_{1b} (Gram positive bacteria) showed inhibition of growth towards the pathogenic bacteria *S. aureus* as Gram-positive bacteria and showed no inhibition towards Gram-negative bacteria *S. enterica* ser. Typhi. In contrast, isolate K_2 showed inhibition to Gram- negative bacteria *S. enterica* ser. Typhi and showed no inhibition to the Gram-positive bacteria *S. aureus* and yeast *C. albicans*. These properties are similar or corresponding to the nature of bacteriocins that they have a relatively narrow killing spectrum and are toxic only to bacteria closely related to the producing strain. But further test is needed to confirm that the

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substance is bacteriocin. In addition more than 99% of bacteria can produce at least one bacteriocin and within a species tens or even hundreds of different kinds of bacteriocins are present [15].

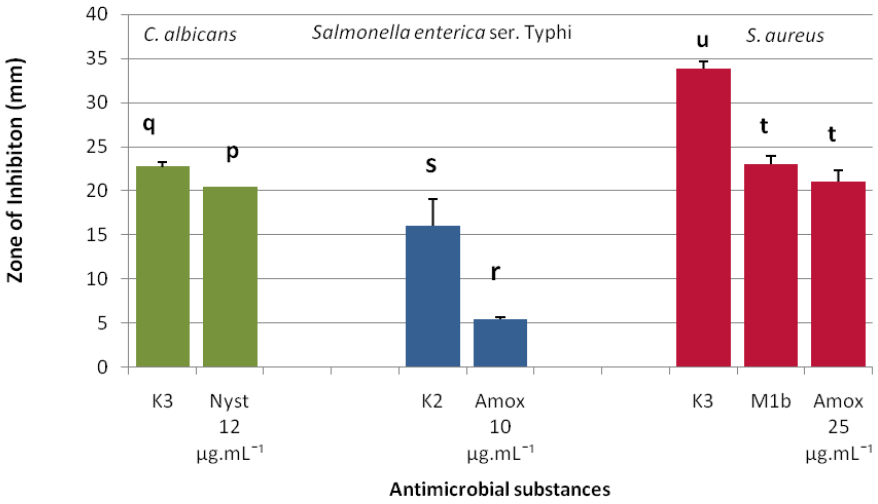

Figure 2. Antimicrobial Activity of Endophyte Bacteria Turmeric Rhizomes towards the Pathogenic Test Microorganism

Antioxidant Activity

The test of antioxidant activity on the eight isolates of endophyte bacteria showed that all isolates has the ability of scavenging to DPPH radical (Fig. 3). Three isolates with the highest antioxidant activity are isolate K_3, K_2, and M_{1b}. Bacterial growth curve were made for the three isolates to obtain optimum time for sampling to test the antioxidant activity.

Antioxidant compound produced by the endophyte bacteria, as reported by scholar, is consisted of various substances. Antioxidant substances produced by endophyte bacteria are EPS [16], surfactin [17], L-asparaginase [18], carotenoid pigment [19], and several enzymes [20]. Most of the compounds were produced maximally at the end of exponential phase. Thus the sample for antioxidant activity was collected at the 14th hour (the end of exponential phase).

Before the test of antioxidant activity, OD of liquid culture of endophyte bacteria was equated. The test of antioxidant activity showed that isolate K_3 has the highest ability of antioxidant activity compared to the other two isolates (Table 1) and then the EC_{50} of K_3 isolate was determined.

Efficient Concentration or EC_{50} value is defined as the concentration of substrate that causes 50% loss of the DPPH activity (colour)[21]. Isolate K_3 supernatant had EC_{50} value 70.26 µL.mL^{-1} and vitamin C (as standard) had EC_{50} value 3.71 µg.mL^{-1}. In this study isolate K_3 supernatant was still in original liquid and had not evaporated yet or extracted in to concentrate, so it was intelligible that the EC_{50} value was too lower than vitamin C. For next study may be required further processing of the supernatant.

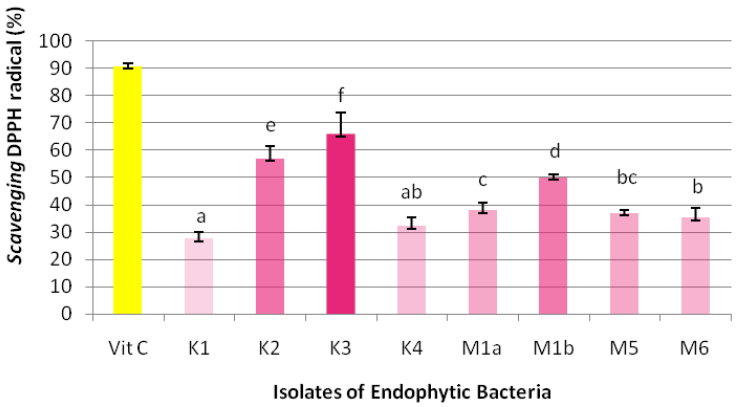

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Figure 3. Antioxidant Activity of *Curcuma longa* Endophyte Bacteria Isolates

Tabel 1. Antioxidant activity of 3 isolates

No	Isolates	Scavenging DPPH radical (%)
1	K_2	51.3 ± 3.1
2	K_3	72.0 ± 1.7
3	M_{1b}	64.7 ± 2.5

Note: Each value is represented as mean ± SD (n=3)

Species Identification

Isolates K_3 and K_2 had both antimicrobial and antioxidant activity significantly , therefore need to characterize these isolates furthermore. Isolate K_3 and K_2 was characterized based on the biochemical characteristic using Kit Microbact System 24E. Biochemical characteristics of isolate K_2 were analyzed by the software Microbact System 24E and isolate K_3 were analyzed refer to Identification flow chart from Microbiology Laboratorium, The University of Ottawa Canada base on Bergey's Manual of Determinative Bacteriology [22]. The results of characterization showed that isolate K_2 was assumed as *Enterobacter agglomerans* with 99.9% similarity*,* whereas isolate K_3 is *Bacillus alvei* or *Paenibacillus alvei* with 91.2% similarity.

Paenibacillus alvei are rod-shaped, Gram-positive, motile, spore-forming , catalase-positive bacteria and grow on simple media (NA/NB). *Paenibacillus alvei* are common found in honeybee colonies , soil , milk, mosquito larvae, the wax mot, humans and very rarely pathogenic for vertebrates. It has reported that *Paenibacillus alvei* produce antimicrobial substance: paenibacillin P and paenibacillin N [23], peptide AN5-1 [24], cyclic lipopeptides [25], depsipeptide [26]. Some of the antimicrobial substance show active againts pathogen *S. aures* and *C. albicans* [23,24,26,27] and consistent with this findings, this study showed endophyte bacteria from *Curcuma longa's* rhizome, isolate K_3 that assumed as *Paenibacillus alvei* show antimicrobial activity to *S. aureus* and *C. albicans*. Isolate K_3 also show antioxidant activity, it promote previous research that *Paenibacillus alvei*'s metabolite have antioxidant activity Exopolysaccarides (EPS) [16,28,29]. *Enterobacter agglomerans* are rod shape, Gram negative, motile, non-sporforming bacteria. These bacteria first were isolated from plants, vegetable, fruits, seeds, and they are also commonly found in the ecological niches such as water, soil, sewage, feculent material, foodstuffs, clinical specimens [30]. It has reported that *Enterobacter agglomerans* produce antimicrobial substance that have inhibition growth to any pathogen bacteria and fungi. Some of them have inhibition growth to *Salmonella sp.* like herbicolin O [31], phenazine [32], and consistent with these finding, isolate K_2 that assumed as *Enterobacter agglomerans* has antimicrobial activity to *Salmonella enterica* ser.Typhi. Isolate K_2 also has antioxidant activity, it promote previous research that *Enterobacter agglomerans* has free radicals-scavenging ability [33].

CONCLUSION

The study obtained eigth isolates of endophyte bacteria from the *Curcuma longa* rhizomes. Three isolates of endophyte bacteria have antimicrobial activity, i.e. isolate K_3 to *C. albicans* yeast and *S. aureus* bacteria; isolate K_2 to *S. enterica* ser. Typhi, and isolate M_{1b} to *S. aureus*. All isolates of endophyte bacteria from *Curcuma longa* rhizomes has the antioxidant activity. The highest antioxidant and strong antimicrobial to Gram positive pathogenic bacteria activity was showed by isolate K_3 which identified as *Paenibacillus alvei*. The strong antimicrobial activity to Gram negative pathogenic bacteria and had high relative antioxidant activity was showed by Isolate K_2 which identified as *Enterobacter aglomerans* by biochemical characterization.

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