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Research Article

Potency of Endophytic and Rhizospheric Bacteria of Akar Kucing (*Acalypha indica* Linn.) as Antibacteria against *Klebsiella pneumoniae*

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ABSTRACT

The prevalence of pneumonia in Indonesia was 2% in 2018. Treatment of pneumonia using antibiotics caused resistance to pathogenic bacteria. Endophytic and rhizospheric bacteria from the medicinal plant *Acalypha indica* Linn., is a new type of bacteria that produces antibacterial compounds against *Klebsiella pneumoniae*. This study aims to analyze the potency and identify endophytic and rhizospheric bacteria of the *A. indica* Linn as an antibacterial of *K. pneumoniae*. The research includes isolation and purification of endophytic and rhizospheric bacteria of the *A. indica* Linn, antagonistic assay of bacteria cell culture, antibacterial assay of bacteria metabolites, and identification of potential isolates based on 16S rDNA sequence similarity. The results showed that number of rhizosphere bacteria 6.83×10^5 CFU/g was more than endophytic bacteria 1.78×10^4 CFU/g. Diversity of rhizosphere bacterial 0.72 was higher than endophytic bacteria 0.62. The rhizospheric bacteria RU112B and RU315B had the highest activity to inhibit the growth of *K. pneumoniae*. Both isolates RU112B and RU315B were identified as *Staphylococcus saprophyticus* with a similarity 99.83% and *Luteimonas terrae* with a similarity 99.67% respectively.

Keywords: Acalypha indica Linn, Endophytes, Klebsiella pneumoniae, Rhizosphere

Introduction

Pneumonia is a respiratory infection that attacks the lungs. Pneumonia prevalence reaches 60% in developing countries with bacteria as an infectious agent [1]. Basic Health Research Data (Riskesdas) in 2018 stated the majority of pneumonia in Indonesia increased from 1.6% in 2013 to 2% in 2018 [2]. The most common bacterial cause of pneumonia was *Klebsiella pneumoniae* [3]. *K. pneumonia* was resistant to all β-lactam antibiotics [4]. *K. pneumoniae* becomes resistant to antibiotics due to inactivation of antibiotics by hydrolyzing it using Enzim Extended Spectrum β-Lactamase (ESBL) [5].

Akar Kucing (*Acalypha indica* Linn.) is a medicinal plant with potency as an antibacterial due to the content of phenol compounds, alkaloids, tannins, and flavon, and steroids. The antibacterial potency of *A. indica* Linn is proven by inhibition zones formed by the administration of plant extracts to Gram-positive bacteria (*Staphylococcus*, *Bacillus*, *Lactobacillus*, *Enterococcus*, and *Streptococcus*) and Gram-negative bacteria (*Klebsiella*, *Aeromonas*, *Alcaligenes*, *Enterobacter*, *Escherichia*, *Pseudomonas*, *Salmonella*, and *Shigella*). All parts of the plant *A. indica* Linn (roots, stems, leaves, flowers, and seeds) can be used as antibacterial [6].

Like the host plants, endophytic and rhizosphere bacteria can produce bioactive compounds or secondary metabolites as a result of coevolution due to genetic transfer (genetic recombination) from the host plant to the bacteria [7, 8]. Endophytic and rhizospheric bacteria's ability to produce the same metabolites as their host can be used as a producer of similar bioactive compounds. The utilization of endophytic bacteria and rhizosphere as producers of bioactive compounds can maintain wild plants' biodiversity such as *akar kucing* (*A. indica* Linn.). It does not require a long time to grow bacteria, and reduce production costs [9].

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Therefore, this study aims to (1) analyze the potency of endophytic and rhizospheric bacteria of *akar Kucing* (*A. indica* Linn.) as antibacterial of *K. pneumoniae*, and (2) identify the bacteria with the highest potency as antibacterial of *K. pneumoniae* base on the similarity of 16S rDNA sequences.

Material and Methods

Isolation of endophytic bacteria

The plants of *A. indica* Linn. with white root characteristics and about 40-60 cm high were obtained from Ketawanggede Village, Lowokwaru District, Malang City. The fresh root plants were taken, and it weighed as much as 10 g [10]. Root samples were cleaned with running water and cut 1-3 cm in length. Root samples were sterilized by immersing in 70% ethanol (1 minute), sodium hypochlorite (NaOCl) 5.25% (5 minutes), and ethanol 70% (30 seconds), then they were rinsed with sterile distilled water (3×, @ 1 minute) [11]. The roots were cultured on Nutrient Agar medium as confirmation that the root surface was sterile. Root samples 10 g were crushed using a blender. Endophytic bacteria were isolated by serial dilution techniques 10^{-1} to 10^{-6} in a physiological salt solution by comparing samples and physiological salts (1: 9). The sample suspension 1.0 mL was inoculated on Nutrient Agar medium (NA) by pour plate method. Bacterial culture was incubated at 30ºC for 48 hours, then the number of bacterial cells was calculated based on the Total Plate Count (TPC) method [11]. The diversity of root endophytic bacterial communities was determined based on the Simpson Diversity Index were calculated by the number of cells of each isolate obtained from morphological characterization [12] (equation 1).

$$D = 1 - \sum_{i=1}^{s} \left(\frac{ni(ni-1)}{N(N-1)}\right) \quad (1)$$

Information:
D = Simpson Diversity Index
n = Number of individual types i
N = Total number of individuals
s = Total number of species in the community

Isolation of rhizospheric bacteria

Three soil samples were taken from the rhizosphere of *A. indica* Linn. Each rhizosphere soil sample was composite from five plants [13]. The environment's physicochemical parameters were measured, including air temperature, soil temperature, soil pH, and soil C/N ratio.

Soil sample 25 g was diluted into 225 mL of physiological salt solution, a vortex homogenized it. Soil samples were made serial dilution 10^{-1} to 10^{-6} in a physiological salt solution. As much as 1 mL culture samples were poured into NA medium, then they were incubated at room temperature for 48 hours [13]. The number of rhizosphere bacteria was calculated based on TPC method. The diversity of the rhizosphere bacterial community was determined based on the Simpson Diversity Index were calculated by the number of cell of each isolate obtained from morphological characteri-zation [12] (equation 1).

Antibacterial assay of bacterial cell culture

One loop of endophytic and rhizosphere bacterial isolates was cultured on 20 mL NB medium then it incubated at 30°C for 24 hours. One loop of pathogenic bacteria *K. pneumoniae* originating from the Clinical Microbiology Laboratory, Faculty of Medicine, Brawijaya University was into 20 mL NB medium and it incubated at 37ºC for 24 hours. Cell density of each bacterial culture was equalized. As much as 100 µL *K. pneumoniae* culture was spread on NA medium, then 50 µL of each rhizospheric and endophytic bacteria isolates were inoculated into well (0.6 cm in diameter) on NA medium. The culture was incubated at 37°C for 24 hours. The inhibition index was measured using equation 2 [14]. Inhibition index data were carried out the one-way analysis of variance (ANOVA) with a significance level α = 0.05, and it continued with Tukey's test using the SPSS v.16 software [15].

$$\text{IB} = \frac{\text{DCZ(mm)} - \text{DTD(mm)}}{\text{DTD (mm)}} \quad (2)$$

Information:
IB = Inhibition Index
DTD = Disk diameter/well (mm)
DCZ = Diameter of clear zone (mm)

Antibacterial assay of bacteria cell metabolite

K. pneumoniae with cell density 10^6 cells/mL as much as 100 µL was spread on NA media. Each endophytic and rhizospheric bacteria isolate at logarithmic growth phase 10 mL was inoculated into 200 mL NB media and it was incubated at 30°C for 48 hours. Each bacterial culture with an

equal density of 10^8 cells/mL as much as 100 mL was centrifuged at 3000 rpm, 4°C, for 5 minutes [16]. Cell-Free Supernatant (CFS) was adjusted at pH 7 and concentrated by freeze-drying. The Minimum Inhibitory Concentration (MIC) was carried out using dilution technique (equation 3) against *K. pneumonia* with CFS concentration (90, 70, 50, and 30%); with distillation water as a negative control, while Streptomycin 500 ppm as a positive control. MIC was the lowest concentration to inhibit tested bacteria [9].

A solution of CFS sample 100 µL was filled into a well with 0.8 cm diameter on pathogen bacteria plate, then it incubated at 37°C for 24 hours [17]. The clear zone around the well was measured by a micrometer. The inhibition index was calculated using equation 2 [14]. Inhibition index data was carried out one-way analysis of variance (ANOVA) with a significance level ɑ = 0.05 and it continued with Tukey's test using SPSS v.16 software [15].

$$V1.M1 = V2.M2 \quad (3)$$

Information:
V1 = Initial volume
M1 = initial concentration
V2 = Final volume
M2 = Final concentration

Identification of Selected Isolates Based on 16S rDNA Sequences

Chromosomal DNA of bacterial isolates extracted with Zymo-Spin™ Kit (*Quick*-DNA™ Fungal/Bacterial Miniprep Kit Catalog Number D6005). [18]. Concentration and purity of DNA was measured using nanodrop [19]. The 16S rDNA sequences were amplified using the PCR method with primers 27f (5 '-AGAGTTTGATCCTGGCTCAG3') and 1492r (5 '-GGTTACCTTGTTACGACTT-3'). Composition of 50 µL PCR Mix Solution: Nuclease free water 16 µL, Go Taq Green Master Mix 25 µL, Primer 27f (10 pmol/µL) 2 µL, primer 1492r (10 pmol/µL) 2 µL, and DNA template (46 ng/µL) 5 µL. PCR Program was used: initial-denaturation (94ºC for 5 min), denaturation (94ºC for 50 sec), annealing (55°C for 50 sec), extension (72°C for 1,5 min), and post-extension (72°C for 5 min) [20]. The 16S rDNA sequence was purified and sequenced at the 1st Base Pte. Malaysia. The 16S rDNA sequences of endophytic and rhizospheric bacteria together with reference bacteria were aligned and it analyzed using the Basic Local Alignment Search Tool (BLAST) program. The phylogenetic tree was constructed based on the Neighbor-Joining algorithm [21] bootstrap 1000 with Phylogenetic and Molecular Evolutionary Analyses were conducted using MEGA version 6.0 [22].

Results and Discussions

Physico-chemical parameters of rhizosphere soil, number and diversity of endophytic and rhizospheric bacteria of Acalypha indica linn.

Rhizosphere soil samples of *A. indica* Linn. had temperature 29.7 ± 0.47°C which is not significant different from the air temperature was 28°C. This temperature is quite normal for soil microorganisms to live and growth. The rhizos-phere soil of the plant had pH 7.1 which allows microorganisms growth well. C/N ratio of rhizosphere was 12 which indicated fertile soil and good nutritional conditions for supporting the growth of plants and soil microorganisms (Table 1).

Physicochemical conditions of the environment are essential factors that determine the composition of the bacterial community. Physicochemical conditions that influence microorganism communities' formation are temperature, humidity, UV radiation, and nutrients in the form of carbon sources in the soil [23]. The physicochemical properties of soil, which also affect the structure of soil microorganism communities, are pH. At normal pH or neutral pH can increase the abundance of microorganisms in the soil while at extreme pH such as acid or that only certain species can grow. Soil acidity can influence the availability of nutrients, salinity, and organic carbon, influencing the abundance and structure of microorganism communities in the soil [24]. A good C/N ratio according to SNI 19-7030-2004 ranges from 10-20, this value indicated that soil is more fertile and increasingly supports the life of plants and soil microorganisms. The low value of the C/N ratio results in rapid decomposition with a shortage of

Tabel 1. Physico-chemical parameters of rhizosphere soils

Physico-chemical factors	Average ± SD
Air temperature (ºC)	28.0 ± 0.47
Soil temperature (ºC)	29.7± 0.47
pH	7.1± 0.08
C/N	12.0 ± 0

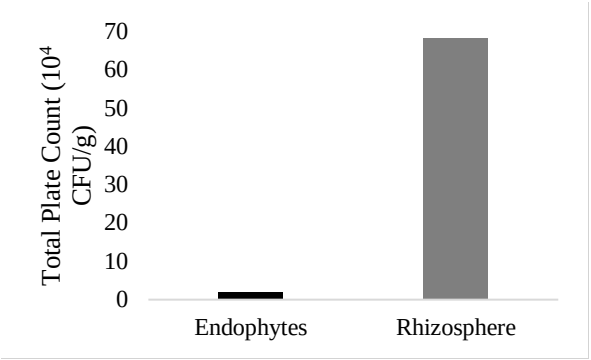

Figure 1. The density of rhizospheric and endophytic bacteria of *A. indica* Linn.

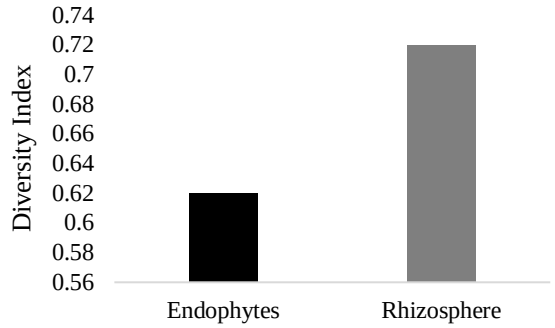

Figure 2. The diversity index of rhizospheric and endophytic bacteria of *A. indica* Linn.

C as an energy source [24].

The number of endophytic bacteria of *A. indica* Linn. (1.78×10^4 CFU/g) is lower than rhizospheric bacteria (6.827×10^5 CFU/g) (Figure 1). The number of rhizosphere bacteria correlates with the physicochemical factors of the *A. indica* Linn. habitat; the more relevant the soil's physicochemical characteristics, the greater the number of bacteria. The presence of carbon sources influences the presence of soil bacteria. In the rhizosphere, the number of carbon sources is higher than in plant roots, so that the number of bacteria in the rhizosphere is more than the roots. The presence of bacteria in plants' roots indicates the attraction of bacteria to the root exudate, which contains a carbon source from the soil around the roots.

Generally, the number of rhizospheric bacteria is more plenty than endophytic bacteria. A study to *Echinacea purpurea* and *Echinacea angustifolia* and their rhizosphere shows that the number of rhizospheric bacteria is higher than endophytic bacteria. Environmental conditions and soil nutrition influence this. The presence of rhizosphere bacteria is strongly influenced by root exudates, while the presence of endophytic bacteria is influenced by plants selection. Bacteria that conform to receptors that are produced by plant can live and grow on plant roots [26].

The diversity index of endophytic bacteria 0.62 was lower than rhizospheric bacteria 0.72 (Figure 2). The diversity index of rhizospheric bacteria was higher due to the rhizosphere's favorable physicochemical condition that supports many species of microorganisms. Diversity index 0 - 0.3 is classified low, 0.3 - 0.6 is moderate, and 0.6-1.0 is high. Rhizosphere soils have a higher bacterial diversity index compared to the inner parts of plant root tissue. The high diversity index indicates that each species in the environment was evenly distributed, whereas samples with a low diversity index indicate that one species dominates. Rhizosphere soils can provide carbon, amino acids, organic acids, and carbohydrates needed for microorganisms to obtain energy compared to inner parts of plant root tissue where nutrient conditions are limited. This condition caused the diversity of rhizospheric bacteria is higher than the endophytic bacteria. Rhizosphere soils have heterogeneous microenvironments with different microhabitats. Their chemical properties are also different, and plant exudates around rhizosphere soils play an important role in modifying dynamic and complex rhizosphere environments so that the diversity of bacterial species is higher [23]. Other studies also prove that the diversity index of rhizosphere bacteria is higher than in plant root tissue due to the attractiveness of rhizodeposition and root exudation by the host plant in the roots and colonization of rhizosphere soils that result in the formation of rich and diverse rhizosphere microbiota. Endophytic bacteria tend to do systemic colonization with plants so that bacteria that are not able to associate with plants are unable to live in plants so that diversity is low [27].

Antibacterial Potency of Bacterial Cell Culture

Among 21 isolates of rhizospheric and endophytic bacteria of *A. indica* Linn. was obtained six potential isolates were consist of two endophytic bacteria isolates (EU121, EU231) and four rhizospheric bacteria isolates (RU112B, RU313B, RU315A, and RU315B). The isolates which had highest inhibitory activity were EU121, followed by EU231, RU313B, RU112B, RU315B, and RU315A with inhibition indices 0.85; 0.57; 0.55; 0.54; 0.53; and 0.50 respectively (Figure 3). The

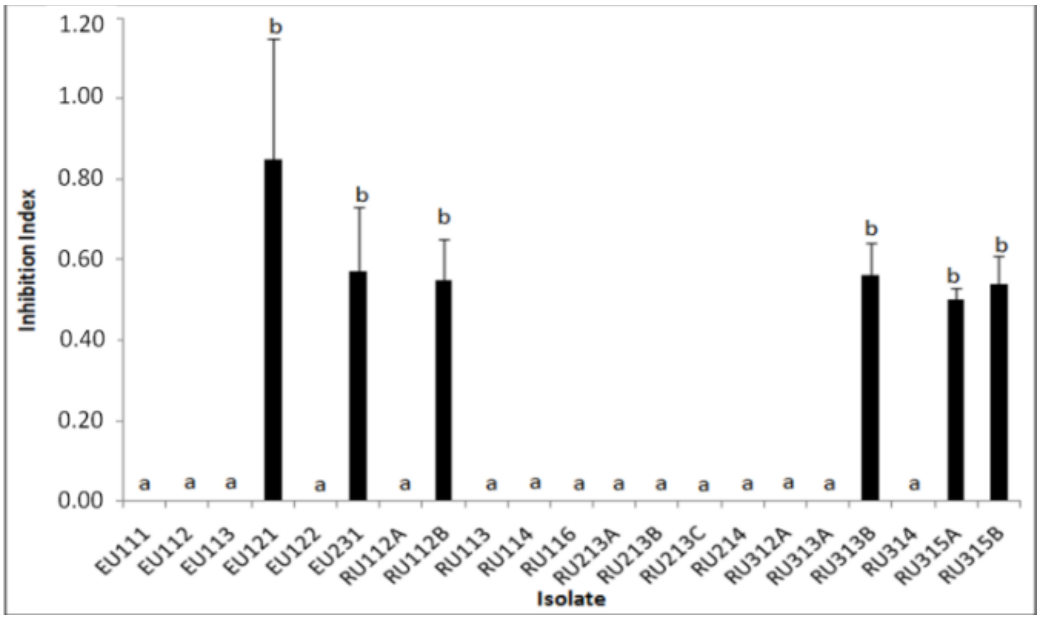

Figure 3. The potency of endophytic and rhizospheric bacterial cell culture to inhibit the growth of *K. pneumoniae* (EU: endophytic bacteria; RU: rhizospheric bacteria)

high antibacterial activity of six isolates may be due to the high concentration of secondary metabolites produced by bacteria to inhibit the growth of *K. pneumoniae*. Rhizosphere bacteria can produce antibiotic compounds, siderophore, organic acids, and lytic agents that can inhibit pathogenic bacteria.

Endophytic bacteria can live in plant tissues by symbiosis with their host plants, so it is efficiently used for biocontrol agents by producing secondary metabolites as antimicrobials agent. Endophytic bacteria associated with medicinal plants can produce the same metabolites as their host plants. The coevolution process causes the horizontal transfer of genes from the host to the endophytic bacteria [28]. Endophytic bacteria *Enterobacter cloacae* strain ACP6 from Noni fruits (*Morinda citrifolia*) can produce antibacterial compound against *Streptococcus mutans* ATCC 31987, *Staphylococcus aureus* ATCC 25323, *Escherichia coli* ATCC 25922, and *Shigella dysenteriae* ATCC 13313) [9]. Rhizosphere is a suitable habitat for microorganism growth because plant roots can release organic material. Some species of rhizosphere bacteria can act as biocontrol agents and biofertilizers [14]. Rhizospheric bacteria of Tumeric (*Curcuma longa*) has antimicrobial activity against *Staphylococcus aureus* [31]. The formation of the clear zone depends on the ability of the bacteria to release secondary metabolites. The ability of bacteria as a biocontrol agent is closely related to the power of competition between bacteria by producing anti-biotics, siderophore, and extracellular enzymes. The difference of inhibition index among isolates is associated with differences in genetic of each isolate strain. The inhibition index varies among antagonist bacteria isolates due to the differentcy of them' adaptation ability to the environment and resistance to toxins produced by pathogenic bacteria [14].

Antibacterial potency of cell-free supernatant

Antibacterial activity of CFS at pH 7 showed six potential isolates were able to inhibit *K. pneumoniae*. This assay proved that inhibitory activity was not due to the production of acid metabolites. The bacterial isolate EU121 and EU231 have inhibitory activity up to 50% and 70% concentration of crude metabolite respectively, while isolates RU112B, RU313B, RU315A, and RU315B have an inhibitory activity up to 30% (Figure 4). Each bacteria's type of metabolites influenced the differences in inhibition activity (MIC). The bacterial isolates RU112B RU315B are the most potential candidate to inhibit *K. pneumonia* even though their CFS inhibition index is lower than streptomycin 500 ppm due to streptomycin is a pure compound while crude metabolites consist of various combinations. Secondary metabolites were produced by antagonist bacteria at the stationary growth phase after

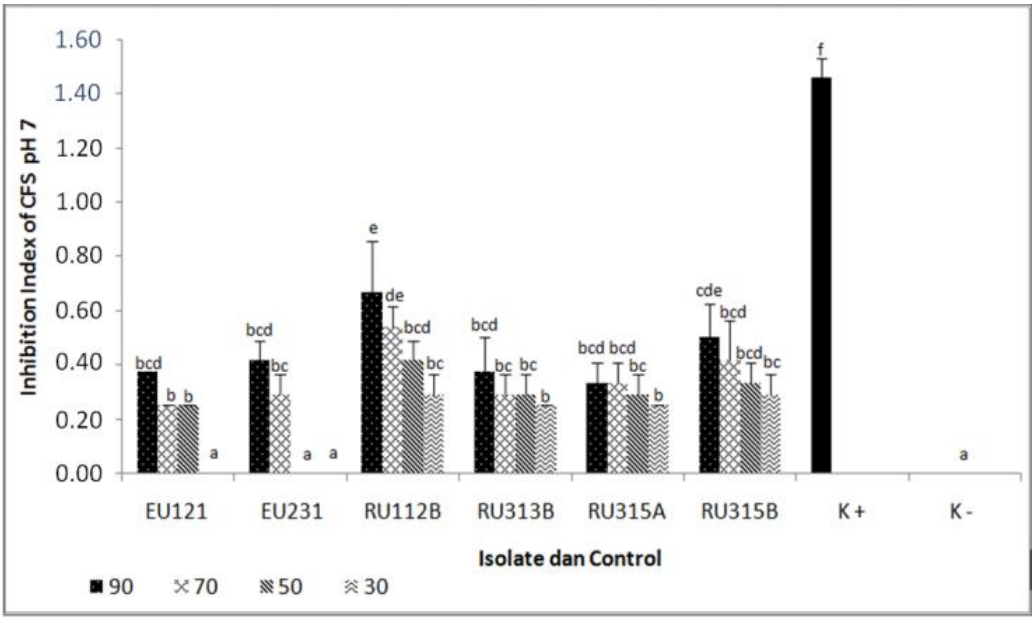

Figure 4. The potency of endophytic and rhizospheric bacterial CFS at pH 7 to inhibit the growth of *K. pneumoniae* (EU: endophytic bacteria; RU: rhizospheric bacteria; K: control)

culture incubation for 48 hours.

In the antibacterial assay of secondary metabolites, CFS must be adjusted at pH 7 to prove that secondary metabolites produce antibacterial activity [29]. Secondary metabolites produced by endophytic bacteria can be developed in the medical, agricultural, and industrial fields. Different anti-bacterial compounds of each isolate caused the different inhibition index. The MIC of each bacterial isolate varies depending on the antibacterial compounds and concentration produced by antagonist bacteria. The inhibition index of antagonist bacteria with the lowest MIC was lower than ampicillin 10 µg due to the content of antibacterial compounds of CFS was lower than the active compound of antibiotics. Antibacterial compounds can be classified based on their MIC, classified as strong, moderate, and weak inhibitors when the MIC less than 10%, 10 – 30%, and more than 30%, respectively [9].

Rhizospheric bacteria can produce antibiotic substances because of their adaptation with plants and interactions with other rhizospheric bacteria. The mechanism of inhibition of rhizospheric bacterial isolates varies depending on the type of compound produced. Rhizospheric bacteria generally carry out antibacterial mechanisms by hydrolyzing cell and genetic material [30]. Naturally, secondary and extracellular metabolites of rhizospheric bacterial isolates had higher antibacterial activity than non-rhizospheric isolates. This is related to the root exudate from plants that support rhizosphere isolates' better activity [31]. Streptomycin is not a β-Lactam class of antibiotics but an aminoglycoside group, an inhibitory mechanism against pathogenic bacteria by binding to A-site on 16S rRNA from 30S ribosomes, thus it inhibits the process of protein synthesis [32]. The antibiotic streptomycin has MIC 20 µg/mL to inhibit *K. pneumoniae* ATCC 1538. Streptomycin antibiotics from the aminoglycoside group combined with the β-Lactam antibiotic group can treat Multi-Drug Resistance (MDR) bacteria [33].

Secondary metabolites are organic compounds produced by microorganisms during the modification of primary metabolism. The metabolite is not used in the growth and development of microorganisms, so it is made during the stationary growth phase. Secondary metabolites widely used are antibiotics, whose primary source comes from soil microorganisms [34]. Antibacterial activity of *Bacillus subtilis* MK-4 at different ages of culture incubation shows that the maximum antibacterial activity occurs after incubation for 48 hours [35]. Other studies showed that *Lactococcus lactis* produce some volatile compounds of secondary metabolites at the stationary growth phase [36].

Potentials Bacteria Species Antagonist of Klebsiella pneumoniae

Based on 16S rDNA sequence similarity,the

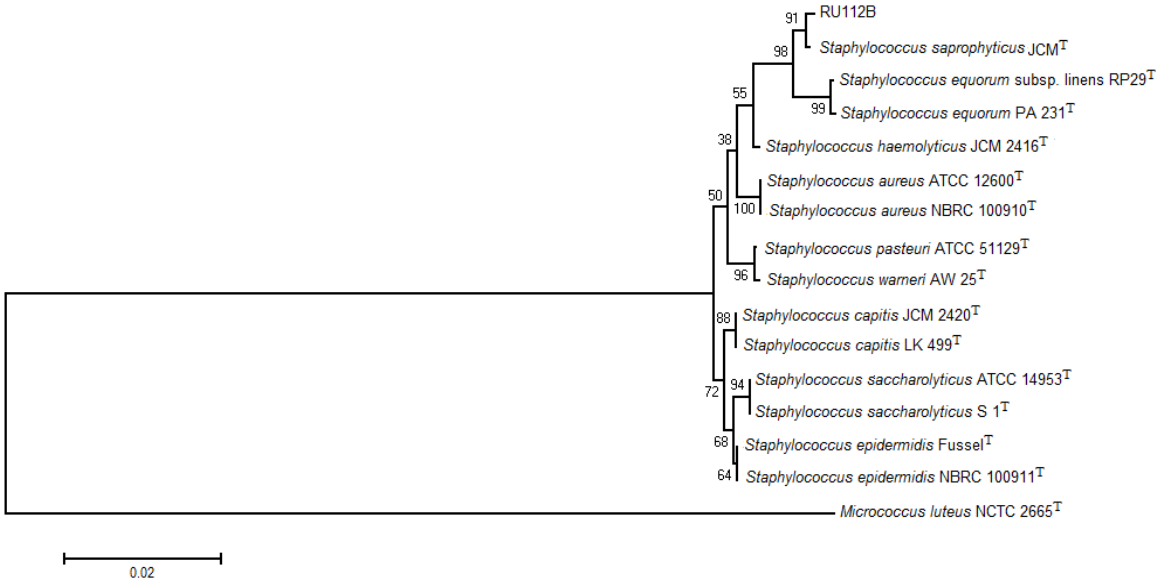

Figure 5. Phylogeny tree of antagonist bacteria RU112B and reference strains based on 16S rDNA sequence similarity with Neighbor-Joining algorithm and Tamura-Nei method

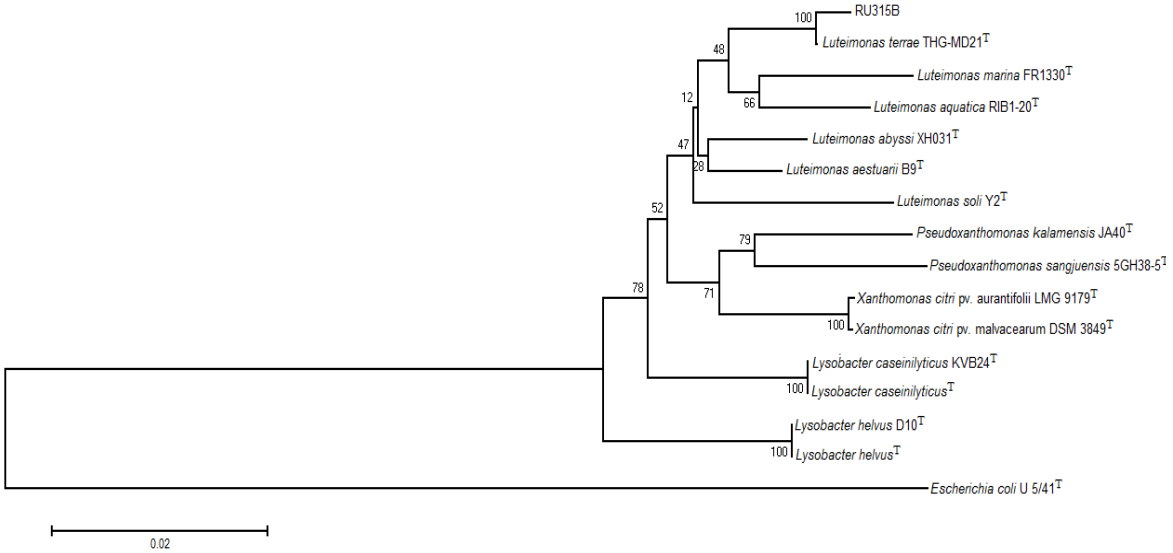

Figure 6. Phylogeny tree of antagonist bacteria RU315B and reference strains based on 16S rDNA sequence similarity with Neighbor-Joining algorithm and Tamura-Nei method

RU112B and RU315B isolate with the highest potency to inhibit Klebsiella pneumonia's growth were identified as *Staphylococcus saprophyticus* JCM^T with 99.83% similarity and *Luteimonas terrae* THG-MD21^T with 99.67% similarity respectively. This result showed that RU112B isolate is a *Staphylococcus saprophyticus* while RU315B isolate is a *Luteimonas terrae* (Figure 5 and 6).

Generally, *Staphylococcus* infects animals and humans, but some species are known as common commensals. Recent studies show that species member of the Genus *Staphylococcus* produces secondary metabolites used in biotechnology and biomedicine. *S. saprophyticus* was proven to produce biosurfactants that potential as antimicrobial agents for human pathogenic microorganisms. Biosurfactant from *S. saprophyticus* able to inhibit the growth of *K. pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Vibrio cholerae*, *Bacillus subtilis*, *Salmonella paratyphi*, *S. aureus*, *Cryptococcus neoformans*, *Candida albicans*, and *Aspergillus niger*. Lipopeptide surfactants produced by *S.*

saprophyticus have extensive antimicrobial activity [37]. *Luteimonas* is a member of the Xanthomonadaceae family, the Gammaproteo-bacteria class. *Luteimonas terrae* THG-MD21^T is a bacterium isolated from plants rhizosphere. These bacteria can hydrolyze DNA, aesculin, casein, starch, CM-cellulose, L-tyrosine, urea, gelatin, and b-D-galactoside. *L. terrae* bacterial cells are also able to produce hydrolysis enzymes [38, 39].

Conclusion

The diversity index of endophytic and rhizospheric bacteria of *Acalypha indica* Linn plant were 0.62 and 0.72 respectively. Two endophytic isolates EU121 and EU231, and four rhizospheric isolates RU112B, RU313B, RU315A, and RU315B able to inhibit the growth of *K. Pneumoniae.* Isolates of RU112B and RU315B had the highest potency to inhibit the growth of *K. Pneumoniae* and both isolates were identified as *Staphylococcus saprophyticus* and *Luteimonas terrae* respectively.

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