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Evaluation of antioxidant and cytotoxic activities of ethyl acetate extracts of bacterial isolates associated with mangrove soil from the Ngurah Rai mangrove forest, Denpasar, Bali

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Abstract. Mangrove forest is a natural ecosystem that has been designated as a conservation and tourism area. Besides its function as an ecotourism area, mangrove forests also store biological wealth in the form of bacteria associated with soil from mangrove plants. Bacteria from mangrove communities are known to synthesize various secondary metabolites. However, the bacterial biodiversity associated with soil from mangrove plants, especially those in Bali, has not been explored optimally. This study was designed to analyze the potential of antioxidant activity and cytotoxicity of five bacterial isolates namely Bacillus sp. SA1, Bacillus sp. SA4, Bacillus sp. AM23, Bacillus sp. RM10 and Bacillus sp.RM 18 which were previously isolated from mangrove soil in Ngurah Rai mangrove forest Denpasar Bali. These five bacterial isolates were grown on ISP-2 liquid media and were fermented for seven days. Subsequently, the supernantant of each bacterial isolates was extracted using 100 mL of ethyl acetate with 1:1 ratio (v:v). The antioxidant activity of each ethyl acetate extract was analyzed in vitro using DPPH (1,1-diphenyl-2-picrylhydrazil) assay and the cytotoxicity activity was evaluated by the Brine Shrimp Lethality assay. Results showed that ethyl acetate extracts of the five bacterial isolates display weak antioxidant activity with IC50 values > 150 ppm. The cytotoxicity test showed that the etyhyl acetate crude extract of bacterial isolate RM10 inhibted 70% of Artemia salina larvae, followed by crude extract of Bacillus sp. RM18 and Bacillus sp. SA4 isolate extracts which equally inhibited 60% of these larvae. This results indicated the potential of isolate Bacillus sp.RM18, Bacillus sp.RM10 and Bacillus sp. SA4 in producing cytotoxic compounds. GC-MS chemical profiling of ethly acetate Bacillus sp. RM10 showed 11 different compounds. Three compounds namely 5-hydroxymethylfurfural, 1,3,-propanediamine and 2-Aminononadecane were the most dominant compounds detected in the extract. Overall, these findings confirmed the potential bacteria associated with mangrove soil as the source of bioactive compounds.

Keywords: bacteria, mangrove, antioxidant, cytotoxicity

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1. Introduction

Indonesia as an archipelagic country is a country with the largest mangrove forest in the world, which is 42.278 km2 or 25.79% of the total global mangrove forest area [1]. Mangroves are wetland forests in the intertidal zone which are composed of halophytic plants and shrubs [2]. Mangrove ecosystems not only have very important ecological functions for coastal and terrestrial fauna, but also function in protecting coastal areas against storms, sea level rise and erosion, and mitigating the impacts of climate change by sequestering carbon [2]. In addition, mangrove plants have long been used in traditional medicine and are claimed to have activity against pathogens that attack humans, animals, and plants [2]. Harsh environmental conditions such as high salinity and low oxygen levels make mangrove plants adapt, one of which is by synthesizing unique secondary metabolites [1, 3, 4].

Mangrove forests also contain diverse microbial communities, where their interactions with other ecosystem components such as mangrove roots are very important in creating and maintaining mangrove [5]. Some bacteria develop mechanisms to survive in extreme environments, one of which is by producing secondary metabolites [6]. Bacteria naturally produce only very small amounts of secondary metabolites, increasing production can be done through several methods such as changing the composition of the media and fermentation conditions (concentrations of nitrogen, phosphate, and carbon, pH, temperature) using specific precursors, as well as genetic manipulation [7].

The Ngurah Rai Bali mangrove forest is the biggest mangrove ecosystem in Bali. The Ngurah Rai mangrove forest is located in the Benoa Bay and its surroundings in the Kuta and South Kuta Districts, Badung Regency and Serangan Island, South Denpasar District, Denpasar City [8]. More than 19 species of mangroves were identified in Ngurah Rai Bali with the dominance of four types of plants, namely *Rhizophora mucronata*, *Avicennia marina*, *Rhizophora apiculata* and *Sonneratia alba*. To date, research that focuses to explore bioactivity of natural products in the Ngurah Rai mangrove forests is rather limited. A recent study has reported antibacterial activity of mangrove root extracts collected from the Ngurah Rai Bali mangrove forest. This study reported that 3 mg/mL of chloroform crude extract of *R. apiculata* formed zone of inhibition of 19.83 mm against *Streptococcus mutans* [9].

Furthermore, another study has reported isolation of bacteria associated with mangrove soil at the Ngurah Rai mangrove forest which resulted of 22 bacterial isolates with antibacterial activity [10]. Five of these 22 isolates namely *Bacillus sp*. SA1, *Bacillus* sp. SA4, *Bacillus* sp. AM23, *Bacillus* sp. RM10 and *Bacillus* sp.RM 18 showed the highest antibacterial activities [10]. Apart from antibacterial activitity, other bioactivity that could be produced by these five isolates especially antioxidant activity and cytotoxicity are remain unknown. Therefore, this study was focused to screen for antioxidant and cytotoxicity assay of these selected isolates in order to elucidate their secondary metabolites potential.

2. Method

2.1. Refreshing bacterial isolate

Five bacterial isolates namely *Bacillus sp*. SA1, *Bacillus* sp. SA4, *Bacillus* sp. AM23, *Bacillus* sp. RM10 and *Bacillus* sp.RM 18 which were isolated from the soil rhizosphere of mangrove plants and have stored in slanted ISP-2 agar [10]. From the culture stock, 1 loop of bacterial culture loops were streaked on ISP-2 agar media and incubated in an incubator at 28°C for 2 x 24 hours until a separate single colony was obtained. These five bacterial isolates

were stained using Gram staining and were observed under light microscope complemented with emersion oil using 1000x magnifications.

2.2. Extraction of secondary metabolite

Each single colony separated from each bacterial isolate was inoculated in a 100 mL Erlemeyer flask containing sterile liquid ISP-2 broth media. Erlenmeyer flask containing this bacterial suspension will be shaken in a shaker machine at 100 rpm for 7 days. Furthermore, the bacterial biomass was separated from the supernatant by filtering using Whatman paper no 1 with diameter 125 mm. The supernatant from bacterial isolates will be extracted with ethyl acetate with a volume ratio of 1:1 which is carried out three times. The ethyl acetate extract was then evaporated to obtain a crude extract for further testing.

2.3. Antioxidant activity

The free radical scavenging activity of the bacterial extract was tested using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay method. A total of 1 ml of DPPH solution (0.1 mmol/L) in methanol was added to 3 ml of crude extract (7.5 g/ml – 25 mg/ml) in a suitable solvent, then shaken vigorously and allowed to stand at room temperature for 30 minutes. The absorbance of the solution was read at a wavelength of 517 nm on a UV-visible spectrophotometer. The low absorbance of the mixed solution indicates high free radical scavenging activity. Ascorbic acid (AA) and butylated hydroxy anisole (BHA) were used as positive controls and phosphate buffer was used as blank. The percentage of DPPH scavenging effect is calculated using the following equation:

$$\text{DPPH } scavenging\ effect\ (\%) = \frac{\text{Ac} - \text{At}}{\text{Ac}} x\ 100$$

Ac refers to the absorbance of the control reaction and At is the absorbance of the sample extract. The IC50 value was calculated using the linear regression formula of the concentration gradients of 20, 40, 60, 80, and 100 ppm.

2.4. Cytotoxicity assay

Cytotoxicity testing of crude extracts was carried out using the brine shrimp lethality assay. A total of 10 newly hatched *Artemia salina* nauplii were transferred to the bacterial extract to be tested. One extract will be tested in 7 concentrations, namely 0.15627, 0.3125, 0.625, 1.25, 2.5, 5, and 10 ppm. After 24 hours, viable A. salina was counted and lethal concentrations were analysed. The positive control used potassium dichromate with 6 concentrations of 0.15627 to 5 ppm, while the negative control was seawater with 0.1% DMSO.

2.5. GC-MS analysis

The ethyl acetate extract of Bacillus sp. RM 10 was subjected to GC-MS analysis to determine various volatile chemical compounds in the extract. To perform the analysis, 0.1 gram of the ethyl acetate was sent to Bidlabfor Polda Bali and the analysis was carried out using Agilent Technologies 7890B and Agilent Technologies 5977B MSD.

3. Result and Discussion

Bacteria associated with mangrove soils provide untapped sources of biochemical compounds with diverse bioactivities. In this study we evaluated antioxidant and cytotoxicity of five bacterial isolates that have been reported from a previous study. These five bacterial isolates were characterized by rod structure (Figure. 1).

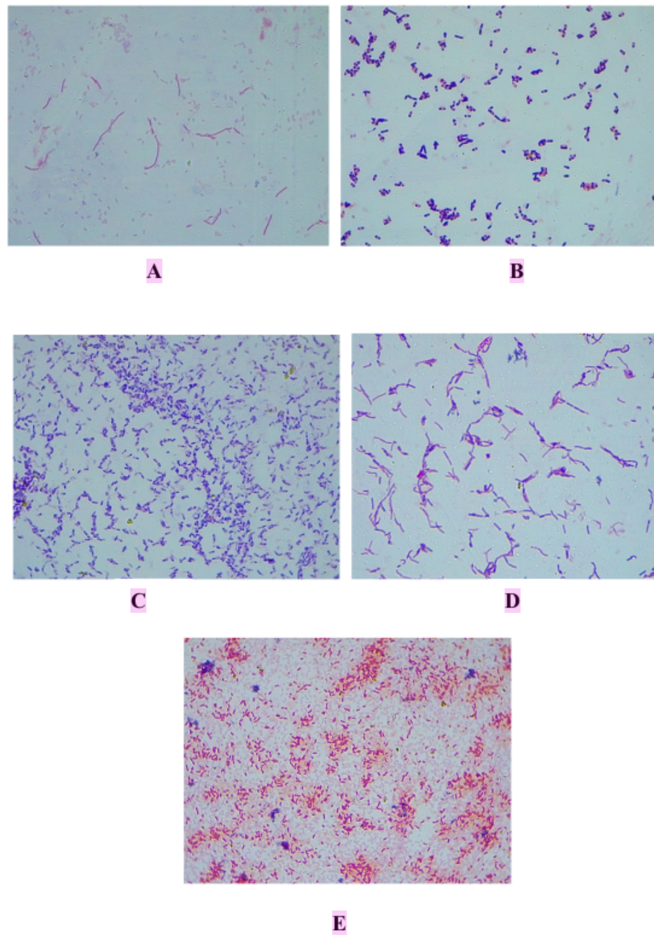

Figure 1. Cell structure of five selected bacterial isolates under light microscope. A. Bacillus sp. SA4; B. Bacillus sp. RM10; C. Bacillus sp. RM18; D. Bacillus sp. AM23; E. Bacillus sp. SA1.

Antioxidant activity refers to an ability of a compound to reduce free radical [11]. Based on the DPPH assay, antioxidant activities of an extract(s) can be classified into five categories namely very strong (IC50 < 50ppm), strong (IC50 50-100 ppm), moderate (IC50 101-150 ppm), weak (IC50, 150 – 200 ppm) and very weak (IC50 > 200 ppm) [12]. The results of antioxidant activity screening of the five isolates using the DPPH method are presented in Table 1 below. Overall, the antioxidant activity of the ethyl acetate extract of five mangrove bacterial isolates was classified as very weak (IC50 > 200 ppm).

Table 1. IC50 value of ethyl acetate extracts of five bacterial isolates from mangrove soil. The IC50 value was calculated from three replicates and presented in the form of an average IC50 value and standard deviation.

No	Sample	IC50 (ppm)
1	SA1	2711.17±244.74
2	SA4	3035.76±360.72
3	AM23	1807.13±74.01
4	RM10	1558.29±35.08
5	RM18	1861.34±64.26

The results of the toxicity screening of using the brine shrimp lethality assay (BSLA) method at a concentration of 10 ppm with two replicates is presented in Figure 1. The ethyl acetate extract from *Bacillus* sp. RM10 showed the highest toxicity activity at 70%, followed by *Bacillus* sp. RM18 and *Bacillus* sp. SA4 isolate extracts at 60%. Meanwhile, the two crude extracts with the lowest toxicity were isolates Bacillus sp. SA1 and Bacillus sp. AM23 at 50% and 30%, respectively. When compared with the positive control of potassium dichromate, the activity of the isolates of RM18 and SA4 was lower. At the same concentration, potassium dichromate was able to kill all tested shrimp larvae. While the negative control in the form of 0.1% DMSO only had a toxicity value of 10%.

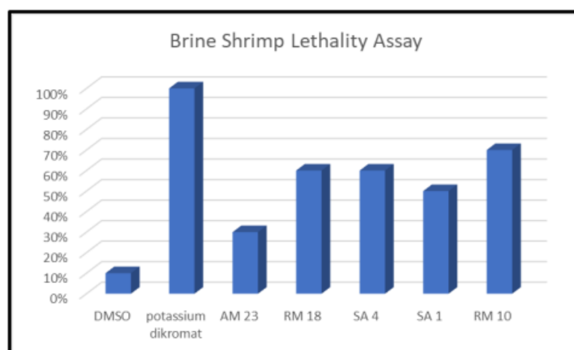

Figure 2. Percentage of toxicity of extracts of mangrove bacterial isolates to shrimp larvae.

Among five bacterial isolates, *Bacillus* sp. RM 10 displayed the highest potential in term of cytotoxicity activity. Therefore, GC-MS analysis was performed to evaluate the chemical compositions of the ethyl acetate extract of *Bacillus* sp. RM 10 as shown in Figure 3.

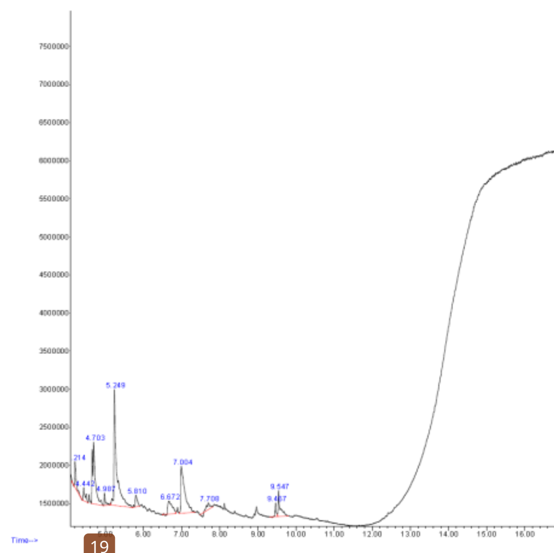

Figure 3. GC-MS profile of ethyl acetate extracts of Bacillus sp. RM 10

Based on the GC-MS profile, there were 11 different compounds that were detected which are summarized in Table 2. Among 11 compounds that were present in the ethyl acetate extract of Bacillus sp. RM10, three compounds namely 5-hydroxymethylfurfural, 1,3,-propanediamine and 2-Aminononadecane showed the highest peak area (Table 2). A previous study reported that 5-Hydroxymethylfurfural showed a negative genotoxicity based on in vivo test [13]. Meanwhile, 1,3,-propanediamine has been reported as an integral fine organic chemical intermediate which is used for wide industrial purposes such as cosmetics, surfactants, detergents and textiles [14]. 2-Aminononadecane has been reported to present in GC-MS profiling of Pediococcus acidilactici BD16 and this compound was among many compounds that were predicted to play a role to increase quality of aroma, flavor, odor, and other factors affecting the fermented buttermilk and soymilk [15]. However, from this result, it is still unknown which compound responsible for toxicity observed in brine shrimp lethality assay of ethyl acetate extract Bacillus sp. RM10.

Table 2. Compounds that are detected in ethyl acetate extract of Bacillus sp. RM10

Cyclohexanol	4.06	4.21
Metaraminol	2.72	4.44
2-Aminononadecane	19.37	4.70
Cyclopropyl carbinol	1.74	4.98
5-Hydroxymethylfurfural	33.73	5.25
Amphetamine	2.82	5.81
Acetic acid	5.99	6.67
1,3,-propanediamine	20.50	7.00
Cycloserine	1.78	7.70
1-Octadecanamine	1.49	9.47
Cyclopropyl carbinol	5.78	9.55

4. Conclusion

The ethyl acetate extract of the five isolates had very weak antioxidant activity (IC50 > 200 ppm). Ethyl acetate extract from isolate Bacillus sp. RM10 showed the highest toxicity activity at 70%, followed by extract from isolate Bacillus sp. RM18 and Bacillus sp. SA4 at 60%. Three compounds, 5-hydroxymethylfurfural, 1,3,-propanediamine and 2-Aminononadecane, were the dominant constituents of ethyl acetate extract of Bacillus sp. RM10. However, it is remained unknown which compounds play an important role for cytotoxic activity. Therefore, further study is required to fully elucidate the exact compound that is responsible for the cytotoxic activity observed in ethyl acetate extract of *Bacillus* sp. RM10

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