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ANTIMICROBIAL ACTIVITIES OF ENDOPHYTIC BACTERIA ISOLATED FROM *COTYLELOBIUM MELANOXYLON* (HOOK.F.) PIERRE.

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ABSTRACT

The current study used the culturable endophytic bacterium *Nocardiopsis* sp. BER-17 isolated from the barks of *Cotylelobium melanoxylon* to synthesize a novel antimicrobial compounds. *Cotylelobium melanoxylon* is one of therapeutic plant species used in traditional medicine. It is containing compounds with antibacterial activity and bacterial endophytes that provide bioactive metabolites for medicinal purposes, and sources of novel compounds. An attempt has been made to employ endophytic bacterium isolate from medicinal plants in the synthesis of antibacterial compounds. The objectives of this study was to investigate the antibacterial activities produced by *Nocardiopsis* sp. BER-17. Dichloromethane endophytic bacterial extract and culture broth were tested for antimicrobial activity using well diffusion method against pathogenic bacteria of *Escherichia coli* ATCC 35218, *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* FNCC 0061 and *Pseudomonas aeruginosa* FNCC 0063. The antibacterial activities were assessed based by the presence of clear zones as inhibition zones. Composition of secondary metabolites in the dichloromethane bacterial extract was spectrometrically analyzed using GC-MS. The endophytic bacterial extract activity produced with clear zones against both Gram negative and Gram positive bacterial pathogens. Based on GC-MS result, dichloromethane extract endophytic contain components antibacterial activity.

KEYWORDS: ***Cotylelobium melanoxylon*, culturable endophytic bacterium, *Nocardiopsis* sp., antibacterial activities.**

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INTRODUCTION

Tropical forests occupy approximately 7% of the world's total land areas and 50-70% of plant species are located in the area [1]. More or less 300,000 plant species become the hosts of endophytic microbes [2]. The threats of forest denudation in the tropical forest ecosystem can reduce the diversity of vegetation as habitat for endophytic microbes [3]. The exploration of endophytic microbes in the tropical forest ecosystem is necessary to do for multiple purposes such as in agricultural, health, and industrial sectors [7]. Many opportunities to recover endophytic microbes, which can be used to improve plant growth, as natural raw materials of drugs, and compound producers of secondary metabolites, are still open with the higher level of diversity in plant species in tropical forest ecosystem [4]. The diversity of plant species in tropical forests is largely not investigated its endophytic bacteria [5]. The potential diversity of endophytic microbes as well as their compound contents of secondary metabolites produced are still not identified well [6]. *Raru* (*Cotylelobium melanoxylon*) plant is a plant living in tropical regions and largely growing in Sumatera and Borneo islands as well as in Malaysia Peninsular and Thailand [8]. *C. melanoxylon* plant is a perennial plant with a tree-like form. Emergent tree up to 52 m tall and 72 cm dbh. Stem with resin. A medium-sized to tall tree reaching up to 50 m in height, frequently with a twisted bole, branchless up to 30 m and up to 160 cm in diameter. Stipules ca 3 mm long. Twigs more or less smooth. Leaves alternate, ovate-lanceolate, 5-10 x 2-6 cm, simple, penni-veined, venation inconspicous and fine, lower surface mostly very light to almost whitish. Flowers ca. 13 mm in diameter, white, placed in small panicles. Fruits ca. 10 mm long, yellow-green to reddish, nut with 2 wings up to 60 mm long, wind dispersed [9]. It is included in a red list of endangered plant according to the International Union for Conservation of Nature (IUCN) in 1998 [8]. The organ parts of *C. melanoxylon* are largely utilized for multiple purposes. For example, its stem can be used as raw material for construction and its stem bark has been used by a group of communities as the mixing materials for *tuak* (traditional alcoholic beverage) in North Sumatera. Some local communities in North Tapanuli have used the stem barks and leaves of *C. melanoxylon* plant to treat some diseases such as diarrhea, malaria, and diabetes [9]. The stem barks of *C. melanoxylon* contains compounds consisting of *ampelopsin* F, *isoampelopsin* F, ε-*viniferin*, *vaticanol* A, E, G, and *lyoniresinol* that are useful as anti-diabetic drugs [10]. Moreover, the stem barks of *C. melanoxylon* contain a group of flavonoid, tannin, and saponin compounds [11]. In order to isolate endophytic microbes as well as the potential compounds of secondary metabolite in plants, the following aspects must be considered: 1) the host plants are originated from the specific environment and have the high level of diversity; 2) they have ethno-botanical values closely related to the needs of local communities; and 3) they are endemic plants in a region [2]. Based on them, *C. melanoxylon* was included into the criteria of host plants that can produce endophytic microbes with the potential compounds of secondary metabolite.*Nocardiopsis* sp BER-17 was a culturable endophytic bacterium, isolated using specific method from the barks of *C. melanoxylon* [13]. It demonstrated their activity against Gram positive and Gram negative bacterial pathogens. Therefor, *Nocardiopsis* sp BER-17 was poromissing novel antimicrobial compound producer.

MATERIALS AND METHODS

a. Bacterial Cultures

The pure culture of endophytic bacteria *Nocardiopsis* sp of strain BER-17 was obtained from the isolation from the barks of *C. melanoxylon*. Endophytic bacteria *Nocardiopsis* sp. BER-17 is identified based on 16S rRNA gene sequences [12]. Identification of the plants *C. melanoxylon* conducted in Hebarium LIPI Cibinong, based on morphological characteristic compared with herbarium specimen data. The bacteria test used were *Escherichia coli* ATCC 35218, *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* FNCC 0061 and *P. aeruginosa* FNCC 0063 that were obtained from the Laboratory of Microbiology, Faculty of Medicine and the Laboratory of Food and Nutrient, Biotechnology, the Inter-University Center, Gadjah Mada University, Yogyakarta. All bacterial cultures were previously purified by the method of single cell culture before being used in the test [13]. Colony morphology and endophytic bacterial cells were characterized by Gram staining method.

a. *Bacterial Cultivation for Antibacterial Production*

The endophytic bacteria *Nocardiopsis* sp. of strain BER-17 were grown in 250 ml Erlenmeyer flask containing TSB 100 ml medium (three-fold repetitions) on pH 7-8. They were incubated at a temperature of 37^{o}C in shaker with speed of 180 rpm for 7 and 14 days. After the bacteria grown, the culture media was centrifugated with speed of 13,000 x g for 5 minutes and supernatants were filtered using whatman filter paper No. 5, so that filtrate media solution was gained. The supernatant were extracted with the same volume of the extracting solution for 3 times consecutively using ethyl acetate, dichloromethane and n-butanol. It was concentrated using vacuum rotary evaporator. The resultant extracts were stored in desiccators for use in further test [12].

b. Detection of Antibacterial Activity

The compounds of endophytic bacteria *Nocardiopsis* sp. of strain BER-17 that were extracted by ethyl acetate, dichloromethane, and n-butanol were tested by Kirby-Baur method [14]. The bacteria *B. subtilis*, *E. coli*, *S. aureus* and *P. aeruginosa* were grown in nutrient agar medium for 24 hours. Furthermore, using ose, pathogenic bacteria were inoculated in nutrient broth media and incubated for 5-8 hours or a night at a temperature of 37^{o}C. Furthermore, 1 ose was taken by using sterile cotton swabs that was previously inoculated by a scratch method on surface of petri cup containing nutrient agar medium and being left for 5 minutes [21]. Paper disc drops of as much as 10 µL extraction endophytic bacteria *Nocardiopsis* sp. BER-17 and dried.Paper disk was immersed into the extracts of endophytic bacteria *Nocardiopsis* sp of strain BER-17 and placed in the sterile petri cup for wind drying.

Furthermore, the paper disk was placed by pinset on the surface of nutrient agar medium previously inoculated in the bacteria and incubated for 2-3 days at a temperature of 37°C. Inhibition zone diameter was measured (mm) using a millimeter ruler [14].

b. *GC-MS Analysis*

Compounds resulted from extraction of endophytic bacteria *Nocardiopsis* sp of strain BER-17 were analyzed by using gaseous chromatography and mass spectroscopy (GC-MS). The analysis was carried out by using GC-MS QP2010S SHIMADZU, Japan. The operating procedures and conditions applied the type of capillary column AGILENT HP 5MS (30 M X 0.25 mm, I.D.). Helium was used as carrying gas with a constant rate of 0.40 mL/min, being injected for 1 μL (split ratio of 33:1) at an injector temperature of 310°C. The column was programmed at a temperature of 120°C (5 minutes) to 300°C (69 minutes) with the increase of temperature regulated by 5°C/minute. The conditions of GC-MS showed the ion source temperature of 250°C, the interface temperature of 305°C, the pressure of 13.7 kPa. The interpretation of mass spectrum GC-MS was identified by using the database from the National Institute Standard and Technology (NIST62.LIB and WILEY229.LIB). The components of compounds assayed were recognized, i.e. name, weight, and structure of molecules [20].

RESULTS

The microorganisms of *B. subtilis* (FNCC 0061), *E. Coli* (ATCC 3521), *P. aeruginosa* (FNCC 0063) and *S. aureus* (ATCC 25923) were tested by using the dichloromethane, ethyl acetate and N-butanol extracts from endophytic bacteria of strain BER-17 (Table 1). The dichloromethane extracts from endophytic bacteria of strain BER-17 were able to inhibit bacterial growth of *B. subtilis* (25 mm), *E. coli* (17 mm) and *S. aureus* (18 mm). The ethyl acetate extracts and N-butanol from endophytic bacteria of strain BER-17 were able to inhibit bacterial growth of *P. aeruginosa* (18 mm) and (17 mm). Based on the results of the analysis, the dichloromethane extracts from endophytic bacteria of strain BER-17 were selected because the extracts showed wide inhibitory zone against many pathogenic bacteria.

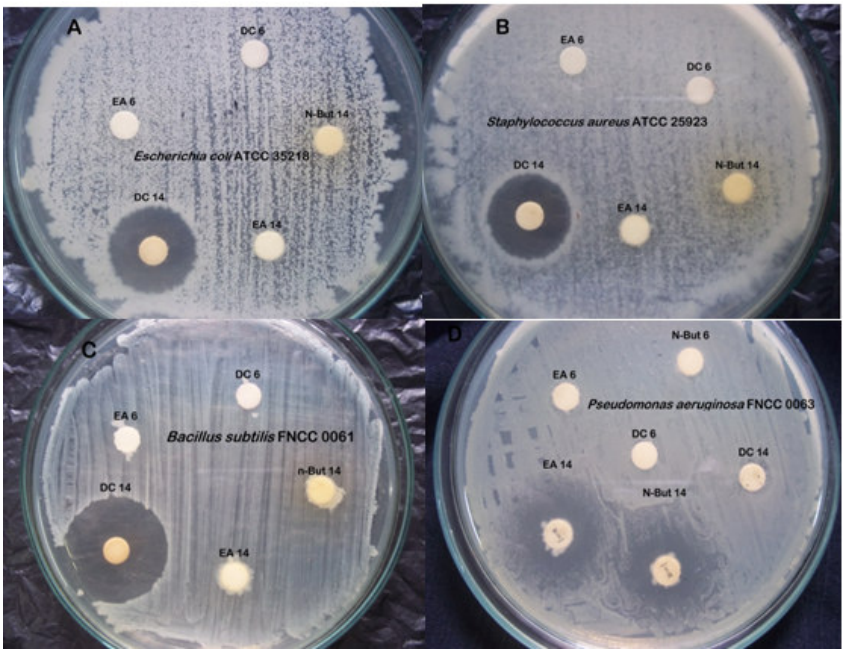

Figure 1

Antibacteri activies test of endophytic Nocardiopsis sp.BER-17 cultivationfor 7 and 14 days with extraction by diklorometan (DC), etil asetat (EA) and n-butanol (n-But). (A) Escherichia coli ATCC 35218 (DC 14 = 17 mm); (B) Staphylococcus aureus ATCC 25923 (DC 14 = 18 mm; (C) Bacillus subtilis FNCC 0061 (DC 14 = 25 mm and (D) Pseudomonas aeruginosa FNCC 0063 (EA 14 = 18 mm and n-But = 17 mm).

Table 1

***Antibacterial activity ofdichloromethane extracts from endophytic bacteriaNocardiopsis* sp. BER-17**

Bacteria test	Dichloromethane	Ethyl acetate	N-butanol
***B. subtilis* FNCC 0061**	25	-	-
***E. coli* ATCC 35218**	17	-	-
***P. aeruginosa* FNCC 0063**	-	18	17
***S. aureus* ATCC 25923**	18	-	-

The results of analysis using GC-MS showed eight peaks (Figure 2) with the retention times of 32.259, 31.533, 24.543, 24.025, 23.664, 23.268, 21.850, and 21.199. Based on the results of GCMS, eight components of compounds in the dichloromethane extracts from endophytic bacteria of strain BER-17 were found. The mass spectra of each of the compounds are shown from Figure 3 to 10.

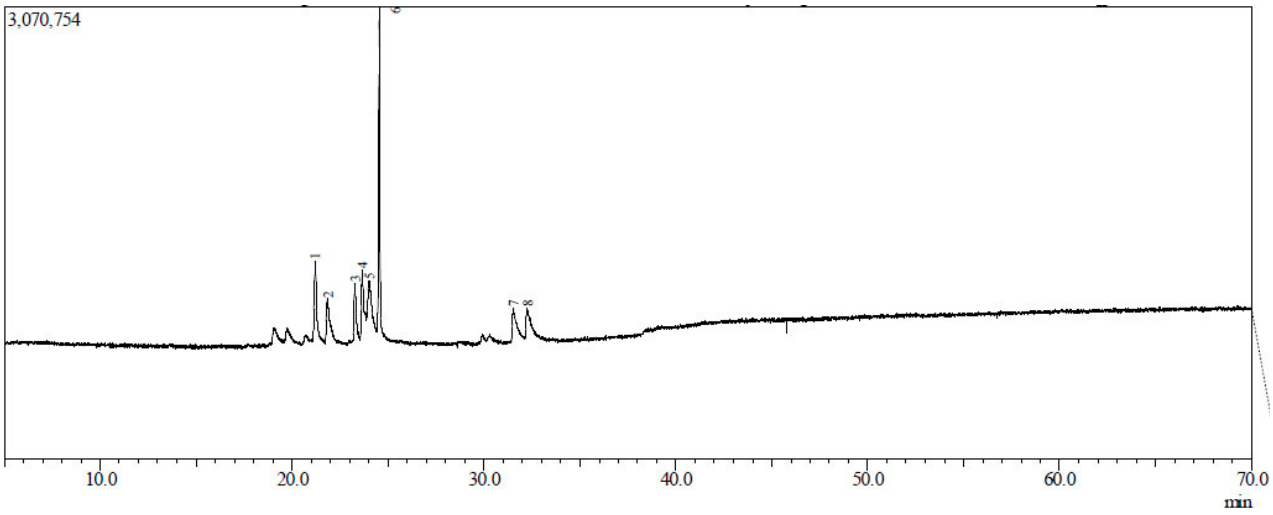

Figure 2
GC-MS chromatogram of the dicloromethane endophytic bacterial crude extract

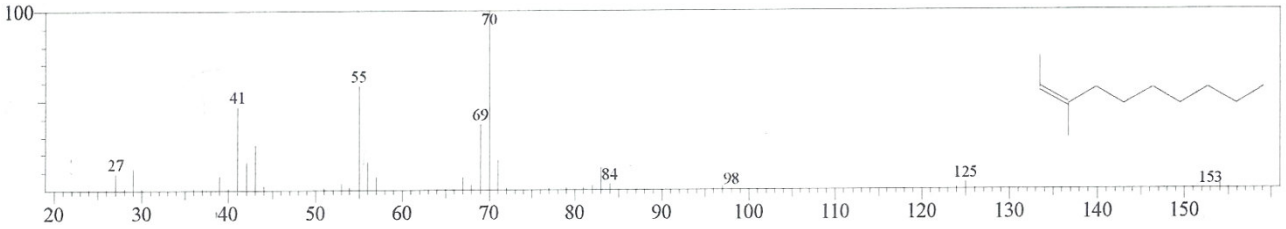

Figure 3
2-decene, 3-methyl

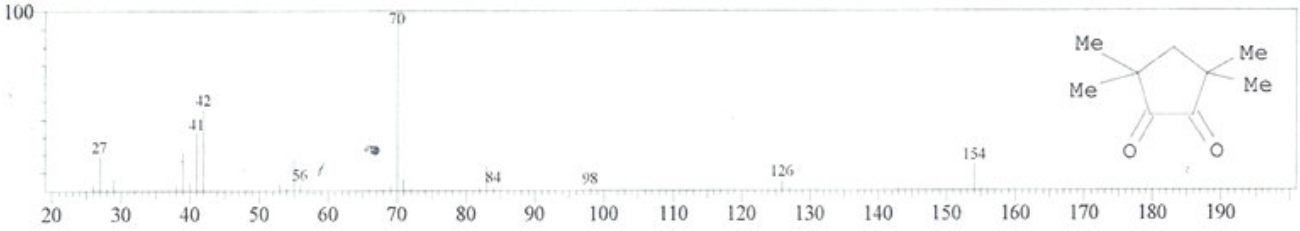

Figure 4
1.2-cyclopentanedione, 3,3,5,5,-tetramethyl

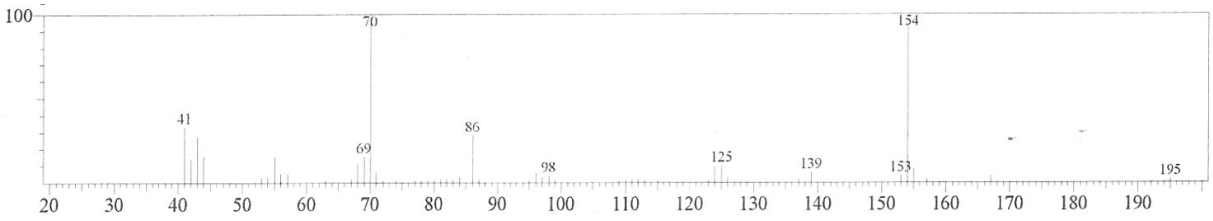

Figure 5
1,4-diaza-2,5-dioxo-3-isobutyl bicyclo[4.3.0]nonane

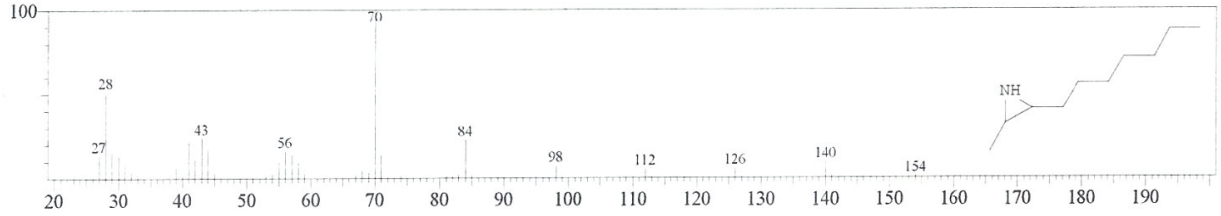

Figure 6
Aziridine, 2-heptyl-3-methyl

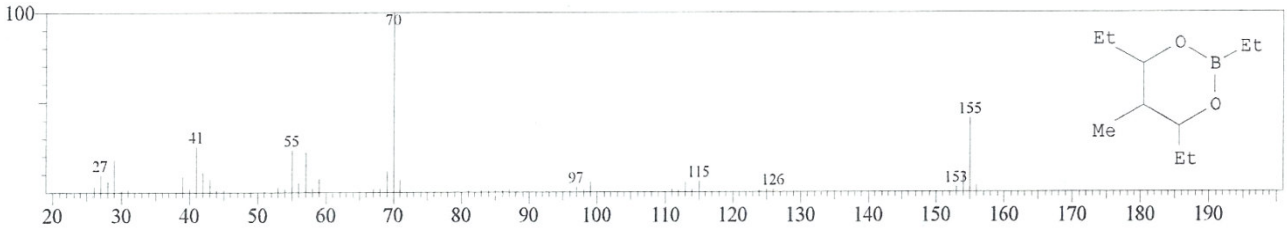

Figure 7
1,3,2-Dioxaborinane, 2,4,6-triethyl-5-methyl-

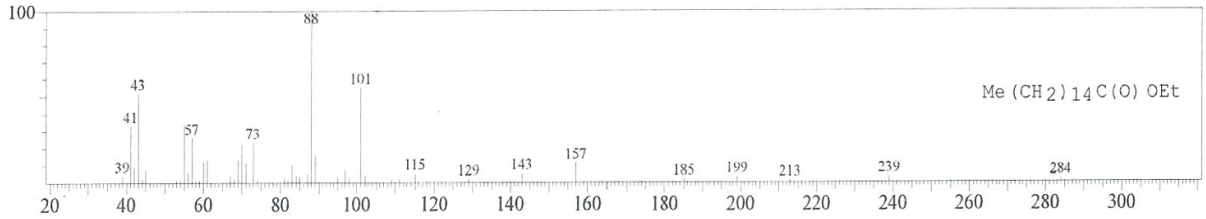

Figure 8
Hexadecanoic acid ethyl ester

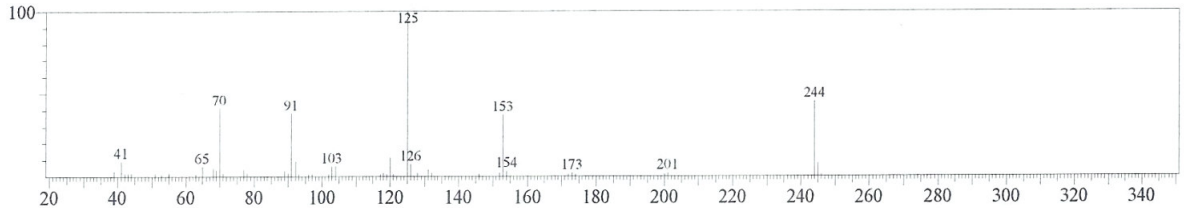

Figure 9
3-benzyl-1,4-diaza-2,5-dioxobicyclo[4.3.0]nonane

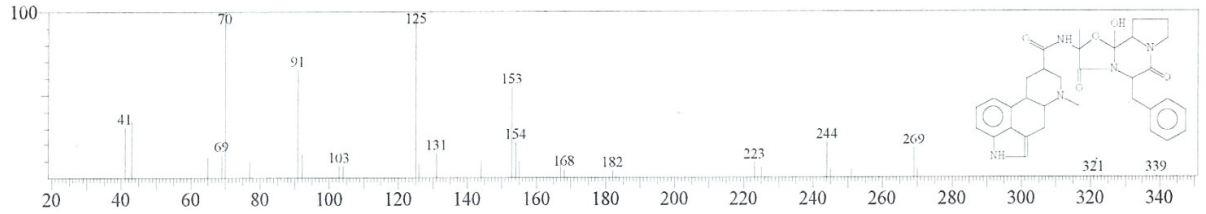

Figure 10
Dihydroergotamine

The components of compounds as the results of analysis using GC-MS: 2-decene, 3-methyl (13.80%), 1.2-cyclopentanedione, 3,3,5,5,-tetramethyl (9.76%), 1,4-diaza-2,5-dioxo-3-isobutyl bicyclo[4.3.0]nonane (9.09%), aziridine, 2-heptyl-3-(11.08%), 1,3,2-dioxaborinane, 2,4,6-triethyl-5-methyl-(20.37%), hexadecanoic acid ethyl ester (28.31%), 3-benzyl-1.4-diaza-2,5-dioxobicyclo[4.3.0]nonane (4.34%) and dihydroergotamine (3.25%) (Table 2).

Tabel 2
Compound identified in dicloromethane endophytic bacterial Nocardiopsis sp BER-17 crude extract by GC-MS

Chromatogram peak	Compound name	Molecular formula	Molecular weight	Peak area (%)	Retention time (min)	Sim. Index (%)
1	2-Decene, 3-methyl-(Z)-	$C_{11}H_{22}$	154	13.80	21.200	79
2	1,2-Cyclopentanedione, 3,3,5,5,-tetramethyl	$C_9H_{14}O_2$	154	9.76	21.850	77
3	1,4-diaza-2,5-dioxo-3-isobutyl bicyclo[4.3.0]nonane	$C_{11}H_{18}N_2O_2$	210	9.09	23.267	83
4	Aziridine, 2-heptyl-3-methyl	$C_{10}H_{21}N$	155	11.08	23.667	77
5	1,3,2-Dioxaborinane, 2,4,6-triethyl-5-methyl-	$C_{10}H_{21}BO_2$	184	20.37	24.025	80
6	Hexadecanoid acid, ethyl ester	$C_{18}H_{36}O_2$	284	28.31	24.542	92
7	3-benzyl-1,4-diaza-2,5-dioxobicyclo[4.3.0]nonane	$C_{14}H_{16}N_2O_2$	244	4.34	31.533	79
8	Dihydroergotamine	$C_{33}H_{37}N_5O_5$	244	3.25	32.259	72

DISCUSSIONS

Nocardiopsis sp. of strain BER-17 is an endophytic microbe isolated from the bark of *raru* (*Cotylelobium melanoxylon*) and identified based on the sequence of 16S rRNA gene [13]. From the results of the study conducted, *Nocardiopsis* sp as endophytic microbe has been found in other vegetations such as yam [22], mangrove [23], *Hygrophila spinosa* [24], *Ocimum sanctum* [25], and *Hibicus rosasinensis* [26]. Endophytic bacteria *Nocardiopsis* contained in some plant tissues as endophytes or surface microflora serves to produce α-amylase and antifungal enzymes against lethal plant pathogens [15]. The dichloromethane extracts of endophytic bacteria *Nocardiopsis* sp. of strain BER-17 contain compounds with the activity against Gram-negative (*E. coli* ATCC 35218 and *P. aeruginosa* FNCC 0063) bacteria as well as Gram-positive (*B. subtilis* FNCC 0061 and *S. aureus* ATCC 25923) bacteria. The classes of *Actinobacteria* with genus *Streptomyces, Micromonospora* and *Nocardiopsis* had antimicrobial activity against pathogenic bacteria [16]. Member of the genus *Nocardiopsis* is clustered into the class *Actinobacteria* consisting of several species and able to grow in extreme and contaminated environment, so that during an adaptation process they produce chemical compounds that are useful for drugs and industry [27].The components of compounds resulted from the dichloromethane extracts from endophytic bacteria of *C. melanoxylon* plant had various biological activities (Table 2). The secondary metabolite compounds resulted from several species of *Nocardiopsis* sp. include 3-trehalosamine phenazine, 1,6-dihydroxyphenazine, 1,6-dihydroxyphanazine-5,10-dioxide, indole alkaloid, pendolmycin, apoptolidins, kahakamides A dan B, neosidomycin, endo-1,3-β-glucanase, endo- β-1,4-D-glucanase, 3-hydroxybutyrate, 3-hydroxyvalerate [28]. Compounds of 1,4-diaza-2,5-dioxo-3-isobutyl bicycle[4.3.0]nonane and 3-benzyl-1,4-diaza-2,5-dioxobicyclo[4.3.0]nonane had antioxidant activity extracted by chloroform from endophytic bacteria of BSI isolate [17]. In addition, reported compounds of 3-benzyl-1,4-diaza-2,5-dioxobycyclo[4.3.0]nonane extracted from isolates as bacteria sample from sea. *Burkholderia cepacia* has antibacterial and anticancer compounds [18]. The compound of hexadecanoic acid ethyl ester has antibacterial activities that inhibit bacterial growth of *E. coli, S. typhi* and *S. aureus* [19]. The hexadecanoic acid ethyl ester also has antioxidant, *hypocholesterolemic*, nematicide, pesticide, lubricant, antiandrogenic activities [20].

CONCLUSION

The study is an effort to seek the sources of raw materials for new natural medicines from endophytic bacteria. It investigated antimicrobial activities of the extract of *Nocardiopsis* sp of strain BER-17 against pathogenic bacteria *B. subtilis* (FNCC 061), *E. Coli* (ATCC 3521), *P. aruginosa* (FNCC 063), and *S. aureus* (ATCC 15923). The results of the analysis using GC-MS showed the components of active compounds with antibacterial activities, being then assayed in vivo as a model of antibacterial drugs.

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