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WORD COUNT 4457
TIME SUBMITTED 16-JAN-2021 06:55AM
PAPER ID 67932490

Molekul, Vol. 15. No. 3, November 2020: 160 – 167

MOLEKUL
www.jmolekul.com

Articles

https://doi.org/10.20884/1.jm.2020.15.3.601

Antibacterial Activity of Essential Oil from Rosemytle Leaves (*Rhodomyrtus tomentosa* (Ait.) Hassk)

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Received November 09, 2019; **Accepted** July 04, 2020; **Available online** November 20, 2020

ABSTRACT. Rosemytle leaves (*Rhodomyrtus tomentosa* (Ait.) Hassk) have been used by society to treat various diseases related to bacterial infections, such as dysentery and typhoid fever caused by *Shigella dysenteriae* and *Salmonella typhi*, respectively. This study aimed to evaluate the antibacterial activity of essential oils from rosemytle leaves against both bacterias. Extraction was performed with a macerating device using *n*-hexane, ethyl acetate, and ethanol extracts, sequentially. This study used the agar diffusion method to test the antibacterial activity applied to the essential oils with concentrations of 1000, 500, 250, 125, 62.5, and 31.25 µg/mL. The antibacterial test results showed that *n*-hexane and ethyl acetate extracts were active against both bacterias while ethanol extract was not. Then, isolates N1 and E1 were produced respectively from *n*-hexane extract and ethyl acetate extract. The MIC values of both N1 and E1 for *S. dysenteriae*, and *S. typhi* were the same, namely 125 µg/mL. Isolate N1 was an essential oil containing menthol (59.60%), caryophyllene (25.77%), and cubenol (14.63%) while isolate E1 was an essential oil containing (73.93%), pentanone (8.30%), alpha calacorene (7.58%), and calacorene (3.78%). Rosemytle leaves have the potential to be developed as a drug to treat dysentery and typhoid fever.

Keywords: Rosemytle, *Rhodomyrtus tomentosa*, *S. dysenteriae*, *S. typhi*

INTRODUCTION

Nature provides raw materials for both traditional and modern medicines. The presence of various plants that support life has led researchers to discover their benefits to treat certain infectious diseases. WHO reported that 80% of the world population had been using conventional medicines, which are mostly plant-based, to care for their health (Kamazeri, 2012).

Plants utilize essential oils they contain to protect themselves against bacteria, viruses, fungi, and pests. People in the Middle Ages used these oils for preservative and flavoring, antibacterial, antifungal, analgesic, sedative, anti-inflammatory, spasmolytic, and local-anesthetic drugs. Currently, around 3,000 kinds of essential oils have been discovered, 300 of which are utilized for commercial use in various industries, such as pharmacy, agronomy, food, tourism, cosmetics, and perfume (Saranraj & Devi, 2017). In plants, the oils are found in leaves, fruits, flowers, stems, and roots of the families Myrtaceae, Asteraceae, Aristolochiaceae, Lamiaceae, Cupressaceae, Fabaceae, Lauraceae, Meliaceae, and Rutaceae (Shah, Jani, Shah, Chaudhary, & Shah, 2014; Raut, Sawant, & Jamge, 2014).

Found in Southeast Asia, Rhodomyrtus tomentosa (Ait.) Hassk, so-called rosemytle, comes from the family Myrtaceae. People use its roots, leaves, and fruits for conventional medicine. Extracts from the aerial part of rosemyrtle contain different bioactive phytochemicals and have been found to have antibacterial, antifungal, anti-inflammatory, antimalarial, antioxidant, and osteogenic activities (Hazrulrizawati & Zeyohannes, 2017).

Salni and Marisa (2019) found that *n*-hexane and ethyl acetate extracts from rosemytle were active against *S. typhi* and *S. dysentriae* bacteria, with MIC values of of 250 µg/mL. Rosemytle leaves contain rhodomyrtone, a natural antibiotic or antibacterial compound that is derived from phloroglucinol (Dachriyanus et al., 2002), that is used treating staphylococcal skin infections. It has a strong in vitro activity against various gram-positive and gram-negative bacterias. With ethanol extracted from the same origin, it has a strong antibacterial activity against gram-positive bacterias, including *B. cereus*, *B. subtilis*, *E. faecalis*, *S. aureus*, *S. pyogenes*, and *S. salivarius* (Limsuwan et al., 2009). Ethanol extract from rosemytle leaves is active against staphylococcal bacteria isolated from acne. Both substances are active against acne-causing bacteria *Propionibacterium acnes*. Since rhodomyrtone shows very low toxicity to skin cells, ethanol extract from rosemytle can be a candidate for a treating agent for acne (Saising & Vorayutthikunchai, 2012).

EXPERIMENTAL SECTION

Materials and Tools

Rosemytle (*Rhodomyrtus tomentosa* (Ait) Hassk) leaves were collected at Sungayang, Solok. *S. dysenteriae* and *S. typhi* were obtained from Biopharma, Bandung. The materials prepared were *n*-hexane, ethyl acetate, ethanol, dimethyl sulfoxide (DMSO) solvent, filter paper, disc paper of 6 mm, nutrient agar (NA), medium nutrient broth (NB), and silica gel GF254.

The apparatus used in this experiment were autoclaves, hot plates, incubators, thin layer chromatography, column chromatography, laminar airflow cabinet, magnetic stirrer, electric heater, water bath, capillary pipette, serological pipette, rotary evaporator, macerating tool, GC-MS, blender, measuring cups, water baths, and petri dishes.

Isolation of Active Compounds

As much as 100 grams of powdered rosemyrtle leaves were extracted using macerating tools with solvents, started with 1L *n*-hexane for 2x24 hours, ethyl acetate, and ethanol, respectively. Each extract was evaporated in a rotavapor until becoming a paste and then tested for its antibacterial activity. The most active extracts were fractionated using the vacuum liquid chromatographic method with sloping eluent consisting of *n*-hexane, ethyl acetate, and ethanol in 12 fractions. The fractionation began with non-polar solvents, 100% *n*-hexane, followed by 12 combinations of solvents in the form of n-hexane and ethyl acetate, ethyl acetate and ethanol, and, finally, 100% ethanol. The fractions were tested for antibacterial activity by the diffusion method. Furthermore, the active fractions were purified using a gravity column containing silica gel with eluent from *n*-hexane and ethyl acetate (9: 1).

Bioautography Tests

To obtain the Rf value of antibacterial compounds, the active fractions were examined using the TLC-bioautographic method. An active fraction was loaded onto two TLC plates, which were placed in vessels with a ratio of *n*-hexane and ethyl acetate of 8:2. The first chromatogram was placed with a bacterial culture on a petri dish and left attached to the media for 1 hour. The petri dish was then incubated for 24 hours. Bright spots caused by the active compound was observed to calculate the Rf value. An H_2SO_4 solution was sprayed on the second chromatogram. Based on the color formed, the class of active compounds can be estimated: yellow for phenols, purple for terpenoids, and brown for tannin groups (Farnsworth,1996).

Antibacterial activity test

n-hexane extract, ethyl acetate extract, and ethanol extract from leaves of *Rhodomyrtus tomentosa* were tested for their ability to inhibit the growth of *S. dysenteriae*, and *S. typhi* bacteria. Antibacterial activity tests were carried out on paper discs using the agar diffusion method by placing 50 µL of bacterial suspension into 10 mL of each medium that had previously been diluted in a petri dish. The media were then allowed to become stable. Paper discs with a diameter of 6 mm were placed on the surfaces of reliable media. A total of 20 µL of the tested mixture was dropped on each disc and allowed to spread for 30 minutes. Then, the discs were placed in an incubator with a temperature of 37 °C. MIC was determined using the diffusion method with various concentrations of active compounds of 1000, 500, 250, 125, 62.5, and 31.25 µg/mL. The solvent used to dissolve isolates N1 and E1 was dimethylsulfoxide.

RESULT AND DISCUSSION

Isolation of Active Compounds

The test results of antibacterial activity against bacterias causing dysentery and typhoid fever, namely *S. dysenteriae* and *S. typhi*, respectively, using *n*-hexane, ethyl acetate, and ethanol extracts indicated that only *n*-hexane and ethyl acetate extracts were active against bacteria, while ethanol extract was not. Both extracts were fractionated by the vacuum liquid chromatography method with eluent level consisting of *n*-hexane, ethyl acetate, and ethanol extracts. The results of the antibacterial activity test of *n*-hexane extract showed that the active fractions were fraction 3 (HC), fraction 6 (HG), and fraction 7 (HI). Furthermore, fraction 3 (HC) was added in the gravity column with eluent *n*-hexane and ethyl acetate with a ratio of (9:1). Active isolate (isolate N1) was obtained in bottles 6 to 8, as in **Figure 1 (a)**. The results of the antibacterial activity test of ethyl acetate extract showed that active fractions were found in fractions 2 (EB) to 6 (EF) and fractions 12 (EL) to 13 (EM). Fractions 2 (EB) to 6 (EF) were applied to a column chromatography containing eluent *n*-hexane and ethyl acetate with a ratio of 9:1. The active fractions were shown in bottles 5 to 9 (E1 isolate), **Figure 1(b)**. Isolates N1 and E1 were obtained in the forms of brownish yellow paste. Bioautography tests were performed on both isolates to determine the Rf values and the classes of their active fractions.

Bioautography Tests

Bioautography test results showed that isolates N1 and E1 had Rf values of 0.22 and 0.33, respectively. After being sprayed with 10% H_2SO_4 solution, both isolates showed purple spots, indicating the existence of terpenoids (essential oils), as shown in **Table 1** and **Figure 2**. The figure also shows the presence of obstacles (bright areas) in bacterial cultures that indicates the inability of bacteria to grow due to the presence of antibacterial compounds.

From the colors, it can be concluded that the active compounds contained by both isolates N1 and E1 were terpenoids (essential oils). Both isolates were active against *S. dysenteriae* and *S. typhi* that

respectively cause dysentery and typhoid fever. The antibacterial activity of *n*-hexane and ethyl acetate extracts was expected due to the presence of essential oils from rosemytle leaves, that had never been reported before. Thus, both extracts had the potential as raw materials for drugs to deal with dysentery and typhoid fever.

Essential oils also contain several types of terpenoid compounds. According to the results of previous studies, essential oils consisting of terpenoids showed antibacterial activity. The composition of essential oils can vary in different parts of the same plant. Research by Benchaar et al. (2008) showed that essential oils are mixtures consisting mainly of terpenoids, especially monoterpenes (C10) and sesquiterpenes (C15). However, diterpenes (C20) can also be in the forms of acids, alcohols, aldehydes, acyclic esters or lactones, and extraordinary compounds containing N- and S, coumarin, and phenylpropanoid homologs.

Essential oils that are found to have antibacterial activity contain terpenoids. The results of this study are supported by the research of Trombetta et al. (2005), which found three monoterpenes namely linalyl acetate, menthol, and thymol active against *S. aureus* and *E. coli*, which are a gram-positive and gram-negative bacterias, respectively. Research on the antibacterial activity of essential oils thymol, carvacrol, eugenol, and menthol on four strains of bacterias that cause nosocomial infections, namely *E. coli, P.aeruginosa, K. pneumonia,* and *S. aureus*, showed that thymol, carvacrol, and eugenol have significant antibacterial activity. Thymol has a high antibacterial activity against *S. aureus* and *E. coli* with MIC values of 0.35 mg/mL while menthol has low activity against all bacterias tested with MIC values greater than 6 mg/mL (Atki et al., 2019).

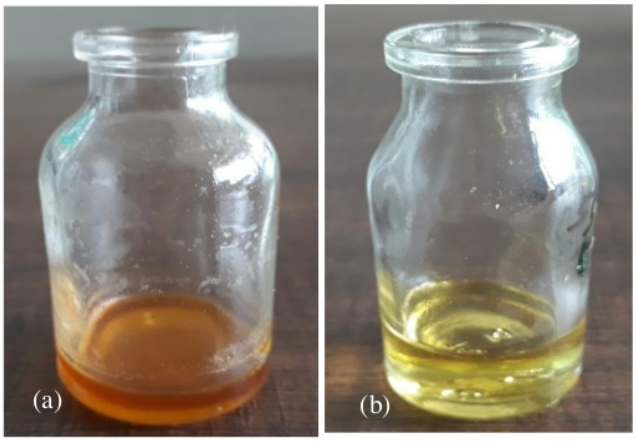

Figure 1. Isolates (a) N1 and (b) E1

Table 1. Rf values and classes of active compounds of isolates N1 and E1 from rosemytle leaves.

Compound	Rf	Color	Class
Isolate N1	0.22	Purple	Terpenoid
Isolate E1	0.33	Purple	Terpenoid

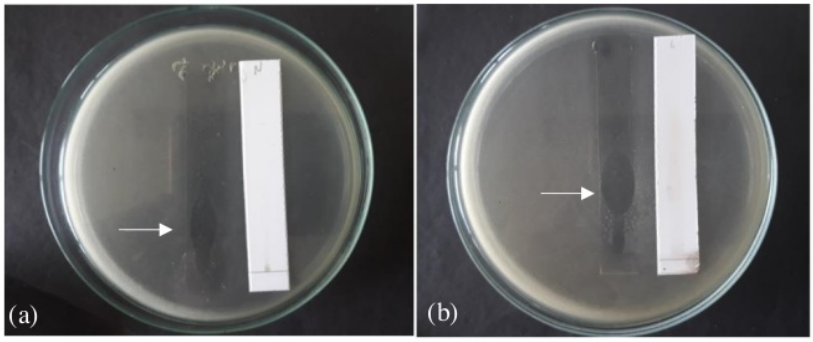

Figure 2. Bioautography test results and determination of active fraction groups of isolates (a) N1 and (b) E1

Characterization of Isolates N1 and E1

Characterization of isolate N1 was done using GC-MS. The obtained GC-MS chromatogram is shown in **Figure 3**. In the figure, there are three dominant peaks identified. The dominant peaks 16.919, 12.621, and 15.236 indicates content percentages of menthol (59.60%), caryophyllene (25.77%), and cubenol (14.63%). The contents of isolate N1 is shown completely in **Table 2**.

The essential oils contained by isolate N1 are menthol, caryophyllene, and cubenol. N1 was active against *S. dysenteriae* and *S. typhi* bacterias. Several studies were found some findings related to this research. Peppermint (*Mentha piperita* L.) was found to contain menthol (20-54%), 1-menton (15-43%), mentil acetate (1-29%), and menthofuran (1-8%). Various species of mints were also found to have antimicrobial properties against *S. aureus, E. faecalis, P. vulgaris, C. perfringens, B. brevis*, and *V. cholerae*. Peppermint oils can be used to treat digestive tracts infected by *S. enteritidis*, *S. typhimurium*, *S. sonnei*, *L. monocytogenes,* and *E. coli*. These essential oils also showed strong activity in methicillin-resistant strains of *S. aureus* and *H. pylori* genera (Sienkiewicz, Denys, & Kowalczyk, 2011).

A study revealed that essential oil of *Mentha rotundifolia* consist of, mainly, menthol (40.50%) and other predominant constituents, namely menthone, menthyl acetate, menthofuran, oxyde de piperitone, linalyl acetate, neomenthol, piperitone, isomenthone, 1,8-cineole, linalool, limonene, geraniol, myrcene, geranyl acetate, and trans sabinene hydrate. The essential oils extracted from *Mentha rotundifolia* showed the highest activity against *E. coli, S. aureus,* and *S. intermedius*, with the most potent inhibitory zones of 45, 34, and 31 mm, respectively (Derwich, Benziane, & Boukir, 2010).

Isolate E1 was characterized by GC-MS. The results obtained can be seen in **Figure 4**. **Table 3** shows the essential oils contained by isolate E1 from the characterization. Isolate E1 contains, mainly, menthol 73.93%, pentanone 8,73%, and alpha calacorene 7.58%,. Like isolate N1, the menthol compound also has the highest content percentage in E1.

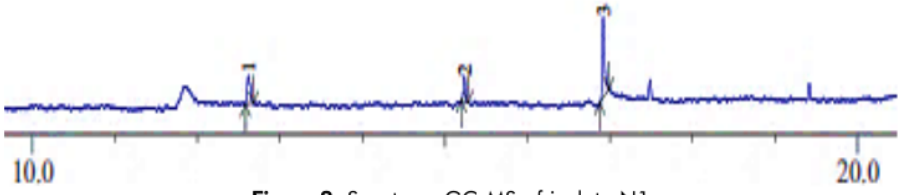

Figure 3. Spectrum GC-MS of isolate N1

Table 2. The essential oils contained by isolate N1

No.	Peaks	Percentage (%)	Essential oil
1	12.621	25.77	Caryophyllene
2	15.236	14.63	Cubenol
3	16.919	59.60	Menthol

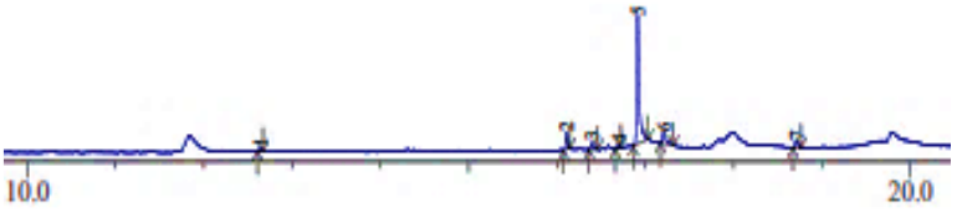

Figure 4. Spectrum of GC-MS of isolate E1

Table 3. The essential oils of isolate E1

No.	Peaks	Percentage (%)	Essential oil
1	12.642	1.16	Cyclohexene
2	16.111	7.58	Alpha Calacorene
3	16.396	3.78	Calacorene
4	16.701	1.49	Caryophyllene
5	16.918	73.93	Menthol
6	17.209	8.30	Pentanone
7	18.712	3.75	Isopropyl

Another previous study also showed contents similar to those of isolates N1 and E1. *Salvia triloba* in South Brazil has been reported to have essential oils, including *β*-caryophyllene, along with *α*-thujone, 1,8-cineole, and camphor, exhibiting extraordinary bacteriostatic and bactericidal activity against *B. cereus*, *B. megatherium*, *B. subtilis*, *A. hydrophila*, *A. sobria*, and *K. oxytoca* (Delamare, Moschen, Atti, & Echeverrigaray, 2007).

Soetjipto, Dewi & Prayitno (2008) investigated the chemical contents in antibacterial compounds from Japanese lavender (*Tithonia diversifolia* (Hemsley) A. Gray) and gave similar results to this study. They showed that the essential oils obtained 29 components consisting of, mainly, caryophyllene (27.76%), nerolidol (21.81%), caryophyllene oxide (7.06%), copaene (6.41%) and bicylogermacrene (4.90%). To determine the antibacterial activity, a bioautography test was performed. Bioautography test results of two antibacterial spots gave Rf values of 0.49 and 0.61. The spots with an Rf value of 0.49 were a mixture of 21 compounds with henisicon, nonacosana, and tetratetracontane as the main compounds, while spots with an Rf value of 0.61 consist of 22 compounds with nerolidol as the main compound.

The characterization results showed that N1 and E1 contain several compounds following research conducted on essential oils from 10 commonly consumed herbs. The main components of these essential oils are camphor, carvacrol, 1,8-cineole, linalool, linalyl acetate, limonene, menthol, a-inene, b-pinene, and thymol. The essential oils have antibacterial activity against human pathogenic bacterias, namely *B. subtilis*, *E. cloacae*, *E. coli*, *M. flavus*, *P. mirabilis*, *P. aeruginosa*, *S. enteritidis*, *S. epidermidis*, *S. typhimurium*, and. *aureus*. The highest and fullest activity was shown by *Origanum vulgare* oil. Carvacrol had the highest antibacterial activity among the components tested (Sokovic, Glamočlija, Marin, Brkić, & Griensven, 2010).

Minimum Inhibitory Concentration (MIC) of The Isolates

From the results of the isolation of the antibacterial compounds, the active compounds of isolate N1 were obtained from *n*-hexane extract, whereas, the active compounds of isolate E1 were obtained from ethyl acetate extract. Both N1 and E1 were essential oils. The results of determining the MIC value of isolate N1 can be seen in **Table 4** and **Figure 5**. The following shows the MIC value of isolate E1 in **Table 5** and **Figure 6**.

As shown in **Table 4**, isolate N1 at a concentration of 1000 µg/mL produced the largest inhibitory diameters of 19.33 mm for *S. dysenteries* and 18.33 mm for *S. typhi*. The test results in **table 4** also show that the decrease in isolate N1 concentration resulted in smaller inhibitory diameter. The smallest inhibition against *S. dysenteriae* and *S. typhi* was obtained at a concentration of 125 µg/mL, meaning that the MIC value of isolate N1 was 125 µg/mL.

Table 4. Antibacterial activity of isolate N1 against *S. dysenteriae* and *S. typhi*

No	Concentration µg/mL	Inhibitory zone diameter (mm)	
		S. dysenteriae (Mean ± SD)	*S. typhi* (Mean ± SD)
1	1000	19.33 ±0.57	18.33 ±0.57
2	500	17.33 ±0.57	13.33 ±0.57
3	250	12.33 ±0.57	10.33 ±0.57
4	125	9.33 ±0.57	8.33 ±0.57
5	62.5	0	0
6	31.25	0	0

Table 5. Antibacterial activity of isolate E1 against *S. dysenteriae* and *S. typhi*

No	Concentration µg/mL	Inhibitory zone diameter (mm)	
		S. dysenteriae (Mean ± SD)	*S. typhi* (Mean ± SD)
1	1000	17.00 ±1.00	16.00 ±1.00
2	500	11.66 ±1.52	10.66 ±1.52
3	250	10.33 ±0.57	9.66 ±1.52
4	125	9.66 ±1.52	8.33 ±0.57
5	62.5	0	0
6	31.25	0	0

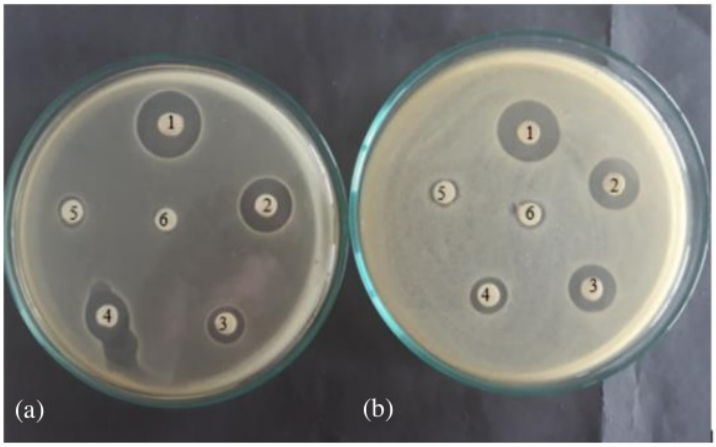

Figure 5. The inhibitory zone of isolate N1 against (a) *S. thypi* and (b) *S. dysentriae* bacterias

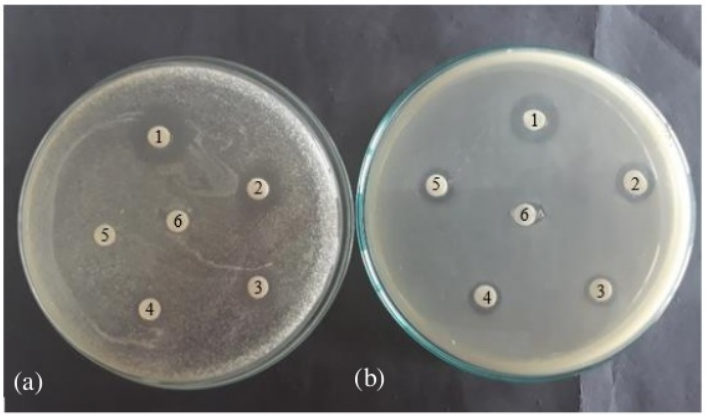

Figure 6. The inhibitory zone of isolate E1 against (a) *S. thypi* and (b) *S. dysentriae* bacterias

The antibacterial activity of isolate E1 showed that the largest inhibitory diameter of bacterial growth was found at a concentration of 1000 µg/mL with inhibitory diameters of 17.00 mm for S. *dysenteriae* and 16.00 mm for S. *typhi*. The test results also showed that the decreasing concentration of isolate E1 decreases inhibitory diameter produced. The smallest resistance against *S. dysenteriae* and *S. typhi* was obtained at a concentration of 125 µg/mL, hence it can be stated that the MIC value of E1 isolate is 125 µg/mL. The MIC values of isolates N1 and E1 containing menthol against *S. thypi* and *S. dysenteriae* bacteria were 125 µg/ smaller than MIC values of essential oils thymol against *S. aureus* and *E. coli*, which were 310 µg /mL and 500 µg/mL, respectively. Against the same bacterias, menthol had MIC values of 620 g/mL and 250 µg/mL, while linalyl acetate had M values of 125 mg/mL and 500 µg/mL (Trombetta et al., 2005).

MIC values of isolates N1 and E1 were classified as antibacterial with strong activity. Holetz et al. (2009) classified antibacterial compounds based on their MIC values. Antibacterial compounds with MIC less than 100 µg/mL are classified as very strong. mpounds are classified as strong enough when having MIC valu ranging from 100 to 500 µg/mL. Whereas, those having MIC values ranging from 500 to 1000 µg/m are classified as weak. Meanwhile, compounds are classified to have no antibacterial activity when having MIC values of more than 1000 µg/mL. Both isolates N1 and E1 isolate have the same MIC value of 125 µg/mL, thus categorized as strong enough.

MIC values of isolates N1 and E1 against both bacterias were 125 µg/mL, which are the same as that of fennel (*Foeniculum vulgare* Mill.) showed by the investigation by Diao, Hua, Zhanga, & Xuab (2014), which also revea that MIC values of essential oils from fennel showed the antibacterial activity against *S. typhimurium, S. dysenteriae* and *E. coli*. These essential oils showed the most active ction to *S. dysenteriae* when reaching the lowest MIC value of 125 µg/mL. Besides, the killing time test also showed that the essential oils of fennel were the

fastest in killing *S. dysenteriae*. Based on the results of tests and observations of using electron microscope, the essential oils worked against *S. dysenteriae* on its membrane integrity and caused its electrolyte leakage and loss of contents (protein, sugars, and materials sized 260 nm).

The mechanism of antibacterial action is carried out by three monoterpenes, namely menthol, thymol, and linalyl acetate by causing interference with the plasma membrane lipids of microorganisms that result in changes cell membrane permeability. This effect depends on the lipid content and the surface charge of the microbial cell membrane. Changes in permeability cause drug ingredients to cross the cell membrane, penetrate the interior of the cell, and interact with intracellular material. It is important for antibacterial activity (Trombetta *et al*., 2005).

The main components of essential oils N1 and E1 are the same as those found in peppermint oil. Peppermint oil with antiseptic properties derived from the family Labiatae has antibacterial activity. The MIC values of peppermint oil for various types of microorganisms are in the range of 0.125-2 µL/mL. *Candida albicans* is the most sensitive microorganism, and *Pseudomonas aeruginosa* is the least sensitive. Peppermint oil shows the same activity as vancomycin, gentamicin, and amphotericin B. It can be used as a natural antibiotic and can reduce the dose of an effective antibiotic. Menthol, menthone, and methyl acetate are the main components of peppermint oil, followed by carveone, neomenthol, 1,8-cineole, and limonene (Mahboubi & Kazempour, 2014).

CONCLUSIONS

This study aimed to evaluate essential oils derived from *Rhodomyrtus tomentosa* leaves. Two isolates were obtained from extracts, namely, N1 from *n*-hexane extract and E1 from ethyl acetate extract. The minimum inhibitory concentration values of N1 and E1 against *S. dysenteriae* and *S. typhi* bacterias were the same, namely 125 µg/mL. N1 is an essential oil containing, mainly, menthol (59.60%), caryophyllene (25.77%), and cyclopentasiloxane (14.63%) while E1, which is also an essential oil, contains, mainly, menthol (73.93%), pentanone (8.30%), alpha calacorene (7.58%), and calacorene (3.78%). Rosemytle leaves have the potential to be developed as a medicine for treating patients with dysentery and typhus.

ACKNOWLEDGMENTS

This study was funded by the grant provided for "Penelitian Terapan Unggulan Perguruan Tinggi" of 2019. The authors would like to express gratitude to the Indonesian Ministry of Higher Education Research and Technology for funding and Universitas Sriwijaya University for permitting the authors to use research facilities in its laboratory.

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