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Antimicrobial activities of isoprene compounds produced by an endophytic fungus isolated from the leaves of *Coleus amboinicus* Lour.

[Actividades antimicrobianas de compuestos de isopreno producidos por un hongo endofítico aislado de las hojas de *Coleus amboinicus* Lour.]

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Abstract

Context: Medicinal plants have been reported to produce various bioactive molecules. In this study an endophytic fungus was isolated from the leaves of *Coleus amboinicus* Lour., a medicinal plant used in Jamu and the fungus was identified as *Athelia rolfsii*.

Aims: To isolate, identify and characterize bioactive compound present in ethyl acetate extract of fermentation broth and determine its antimicrobial activities.

Methods: The compound was isolated and purified by Preparative Thin Layer Chromatography. Antimicrobial activities were conducted by determining its IC_{50} and MBC (Minimum Bactericidal Concentration) values against *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis, Salmonella typhi* and *Staphylococcus mutans*. The structure of the bioactive compound was deduced by spectroscopic data.

Results: The bioactive compound displayed antimicrobial activities with IC_{50} of 0.86, 1.35, 1.33, 2.69, 1.9, 0.24 µg/mL and MBC values of 40, 40, 40, 40, 20, 20 µg/mL against *S. aureus, E. coli, P. aeruginosa, B. subtilis, S. typhi, S. mutans*, respectively. Based on IR, LC-MS, ^{13}C-NMR, ^{1}H-NMR data, the bioactive compound was suggested to consist of two compounds, methyl hemiterpenoate as a major compound and methyl, 2,3 diene-butanoate as the minor one.

Conclusions: The endophytic fungus isolated from the leaves of *C. amboinicus* produced antimicrobial agents which could be potential for further development.

Keywords: antimicrobial; *Athelia rolfsii*; *Coleus amboinicus*; endophyte; fungus.

Resumen

Contexto: Se ha informado que las plantas medicinales producen diversas moléculas bioactivas. En este estudio, se aisló un hongo endofítico de las hojas de *Coleus amboinicus* Lour., una planta medicinal utilizada en Jamu y el hongo se identificó como *Athelia rolfsii*.

Objetivos: Aislar, identificar y caracterizar el compuesto bioactivo presente en el extracto de acetato de etilo del caldo de fermentación y determinar sus actividades antimicrobianas.

Métodos: El compuesto se aisló y purificó por cromatografía preparativa de capa fina. Las actividades antimicrobianas se llevaron a cabo determinando sus valores de IC_{50} y MBC (concentración bactericida mínima) contra *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis, Salmonella typhi* y *Staphylococcus mutans*. La estructura del compuesto bioactivo se dedujo por datos espectroscópicos.

Resultados: El compuesto bioactivo mostró actividades antimicrobianas con IC_{50} de 0.86, 1.35, 1.33, 2.69, 1.9, 0.24 µg/mL y valores de MBC de 40, 40, 40, 40, 20, 20 µg/mL contra *S. aureus, E. coli, P. aeruginosa, B. subtilis, S. typhi, S. mutans*, respectivamente. Basado en datos de IR, LC-MS, ^{13}C-NMR, ^{1}H-NMR, se sugirió que el compuesto bioactivo consistiera en dos compuestos, metil hemiterpenoate como compuesto principal y metil, 2,3 diene-butanoate como el menor.

Conclusiones: El hongo endofítico aislado de las hojas de *C. amboinicus* produjo agentes antimicrobianos que pudieran ser potenciales para un mayor desarrollo.

Palabras Clave: antimicrobiano; *Athelia rolfsii*; *Coleus amboinicus*; endófito; hongo.

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INTRODUCTION

Recently development of drug resistance microbes is increasingly observed and is becoming a global phenomenon. Increased incidence of resistance towards antibiotics were evidence such as the finding of methicillin-resistant *Staphylococcus aureus,* multiple antibiotic-resistant *Pseudomonas aeruginosa* and the emergence of *Salmonella typhimurium* and *Salmonella Kentucky*, which were resistance towards cephalosporin (Iwamoto et al., 2013; Mohamed et al., 2014; Pachori et al., 2019). Various efforts were made to find new antibiotics to overcome this resistance and endophytic fungi were potential as antibiotic producers (Strobel et al., 2004). Endophytes were reported to be able to synthesize associated plant compounds having therapeutic importance, such as camptothecin, trichodermin, podophyllotoxin and helvolic acid (Eyberger et al., 2006; Ran et al., 2017; Yang et al., 2017; Leylaie and Zafari, 2018).

Coleus amboinicus Lour. is a medicinal herb traditionally used to treat indigestion disorder, cough, cold as well as to encounter loss of appetite and as lactagogue (Damanik et al., 2006; Rout and Panda, 2010). The essential oil of this plant has been reported to have antimicrobial activities (Alankararao et al., 1991; Weli et al., 2011; Santos et al., 2015) including those towards drug-resistant *S. aureus* (Vasconcelos et al., 2017). The essential oil of this plant synergized the effect of aminoglycoside antibiotics against resistant bacterial strains (Aguiar et al., 2015). Extracts and fractions of *C. amboinicus* were also reported to exhibit antimicrobial activities (Girish, 2016). Whilst ethanol and hot water extract of the leaves of *C. amboinicus* inhibited the growth of positive and negative pathogenic bacterial strains (Subhaschandrappa et al., 2010), the fractions of this plant were found to be effective against methicillin-resistant *S. aureus* (MRSA) (Oliveira et al., 2013). This study was aimed to explore potential of endophytic fungi residing in the plant in producing bioactive molecules as anti-microbial agents.

MATERIAL AND METHODS

Materials

Pure culture of *Athelia rolfsii,* an endophytic fungus isolated from the leaves of *C. amboinicus* were grown on Potato Dextrose Agar (PDA) plates without antibiotics, maintained for routine culture and stored in glycerol stock for culture collection at the Pharmaceutical Biology Department, Faculty of Pharmacy Universitas Gadjah Mada. Potato Dextrose Agar (PDA), Potato Dextrose Broth (PDB), Nutrient Agar (NA), Nutrient Broth (NB), Mueller Hinton (Oxoid). Silica gel F_{254}, Silica gel 60 PF_{254} containing gypsum, dimethyl sulfoxide (DMSO), methanol, chloroform, n-hexane, ethyl acetate (Merck). Plant materials were collected (GPS coordinates: 7°46'3.741"N 110°22'42.704"E) from Medicinal Plant Garden, Faculty of Pharmacy, Universitas Gadjah Mada. Plant material was identified by Dr. Djoko Santosa, M.Si. A voucher specimen was deposited for future reference in the Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada. Voucher number 253.

Isolation and Identification of endophytic fungus

Endophytic fungus was isolated from the leaves according to Ding et al. (2010) with slight modification. The leaves washed with running tap water were surface-sterilized using 70% ethanol for 1 minute, immersed on 5% sodium hypochlorite for 3 minutes and drained. After 30 seconds re-soaking the leaves in 70% ethanol, the samples were rinsed three times in sterile distilled water and surface-dried with sterile filter paper. The surface sterilised samples were cut aseptically into 1 cm long segments and placed onto PDA plates containing 30 µg/mL streptomycin. The plates were incubated at 25°C for 2 to 3 days. The hyphal tip growing out from the segments were transferred into new PDA plates containing 30 µg/mL

streptomycin and further incubated for 10 – 14 days. The pure culture was obtained after several times of sub-culturing and grown on PDA plates without antibiotics. The culture was deposited for culture collection of Pharmaceutical Biology Department, Faculty of Pharmacy Universitas Gadjah Mada. Identification of endophytic fungus was conducted based on a partial genetic analysis at Internal Transcribed Spacer (ITS) of the fungal ribosomal DNA.PCR amplification using ITS primer 4: 5'- TCC TCC GCT TAT TGA TAT GC – 3' and ITS Primer 5: 5' -GGA AGT AAA AGT CGT AAC AAG G –3' (White et al., 1990; O`Donnell, 1993). Purified PCR product was precipitated using PEG precipitation method (Hiraishi et al., 1995), followed by cycle sequencing. The result was re-purified by ethanol purification method. Sequencing was conducted using automated DNA sequencer (ABI PRISM 3130 Genetic Analyzer-Applied Biosystems). Sequenced data was trimmed and assembled using BioEdit program (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) followed by BLAST alignment using genomic data in DDBJ/ DNA Data Bank of Japan (http://blast.ddbj.nig.ac.jp/) or NCBI/ National Center for Biotechnology Information(http://www.ncbi.nlm.nih.gov/BLAST/) to analyze homology/ similarity.

Fermentation and bioassay-guided isolation of bioactive compound

Five plugs of endophytic fungus culture on PDA were transferred onto 500 mL culture flasks containing potato dextrose broth and incubated at 25°C on a shaker at 160 rpm. After 14 days of incubation, mycelium was separated from supernatant using a Whatman filter paper. The supernatant was centrifuged at 4000 rpm for 5 min to obtain mycelium free supernatant followed by liquid-liquid partition using an equal volume of ethyl acetate thrice to obtain ethyl acetate fractions. The compound was separated from ethyl acetate extract using preparative thin layer chromatography (TLC) [stationary phase = silica gel 60 PF_{254}; mobile phase = chloroform: ethyl acetate [1: 1 v/v]. Fractions showing UV_{366} positive signals at hR_F of 15 were combined and further purified. A pure compound showed a single peak using HPLC.

Antimicrobial testing

The IC_{50} and MBC values of isolated compound were determined by modified microdilution method (da Silva Filho et al., 2008; Muhammad et al., 2003). Testing microorganisms like *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 11229), *Pseudomonas aeruginosa* (ATCC 27853), *Bacillus subtilis* (ATCC 6633), *Salmonella typhi* (culture collection from patient – Faculty of Medicine, Public Health and Nursing UGM) and *Staphylococcus mutans* (ATCC 25175) were grown on Mueller Hinton and the densities comparable to 0.5 McFarland standard were diluted 10× with Mueller Hinton medium prior to transfer into 96-well plate. The sample dissolved in ethanol were serially diluted and transferred into microbial inoculum in 96 well plate to final concentration from 20 – 0.16 µg/mL for determining IC_{50} and 40 – 0.16 µg/mL for determining MBC. Controls of microbial growth, solvent and media were included in testing plate and the experiment was conducted in five replicates. The plates were incubated at 37°C overnight and read at 595 nm using microplate reader (Biorad). The IC_{50} is the concentration that inhibits 50% of microbial growth and is obtained from plotting of percent growth and the tested sample concentration. The MBC is defined as the lowest concentration that shows no growth after subculture into fresh PDA media.

Structure elucidation of bioactive compound

The crystal was having melting range at 202.37°C – 203.40°C. Structure elucidation was conducted based on analysis of FT-IR (Perkin Elmer Spectrum 100, Perkin Elmer Life and Analytical Science, Shelton, USA), LC-MS (Shimadzu, Shimadzu Corporation, Kyoto, Japan), ^{1}H-NMR and ^{13}C-NMR (JEOL 500, JEOL USA Inc., Boston, USA) data.

Statistical analysis

Antimicrobial activities were performed in five replication and the results were calculated as mean ± standard deviation of mean (SD) values. Statisti-

cal significance was calculated using one-way analysis of variance (ANOVA) to test the null hypothesis. Tukey's test was done to compare the sample means. The data were considered significantly different from streptomycin control when significance level was $p<0.05$ (Haynes, 2013).

RESULTS AND DISCUSSION

The need to search and develop lead compounds having antimicrobial values comes from the occurrence of evolution of resistance towards existing antibiotics which subsequent decreased of effectivity (Sykes, 2010; WHO, 2014; Baym et al., 2016). Endophytic fungi had been reported as potential sources of various bioactive metabolites having therapeutic values (Strobel et al., 2004; Kusari and Spiteller, 2011; Uzma et al., 2018). An endophytic fungus was isolated from the leaves of *Coleus amboinicus* and identified as *Athelia rolfsii*. This fungus was found to be plant pathogen in various types of crops (Aycock, 1961); however, co-existence of other fungus such as *Trichoderma harzianum in vitro* with *A. rolfsii* has been showed to minimize the growth of this fungus (Das et al., 2000). Recently, *A. rolfsii* strain orchid was reported to be found as endophytic fungus in the stem tissue of red betel medicinal plant (Yuniati et al., 2018).

A compound showing positive signals under UV_{366} light was isolated from ethyl acetate extract of *A. rolfsii* fermentation broth. This compound was found to be major compound visualized under this detection method (Fig. 1). Infrared spectrum (KBr) of this compound (Fig. 2) displayed a weak band at 3020 cm^{-1} indicating the present of an unsaturated C-H, and an ester –C=O band at 1732 cm^{-1}. The presence of a –C-O- bond was shown by a strong band at 1215 cm^{-1} and a distinctive band at 744 cm^{-1} was identified as a terminal methylene (=CH_2). The ^{13}C-NMR (500 MHz, CD_3OD) spectra (Fig. 3, Table 1) displayed downfield carbon signal at δ, 115.4 ppm confirmed the present of terminal methylene group that was shown previously in the IR spectrum. An alkene carbon was shown by a signal at δ, 132.5 ppm and a –C=O signal was shown at δ, 161.3 ppm that confirmed the –C=O as an ester –C=O. The ^{1}H-NMR (500 MHz, CD_3OD) (Fig. 4, Table 1) did not show clearly unsaturated protons at the downfield area as shown in the IR and ^{13}C- NMR spectra, instead of up filed signals were dominant signals in the spectra.

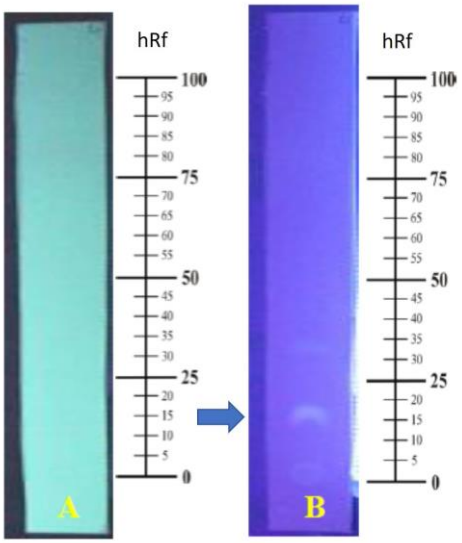

Figure 1. TLC profiles of ethyl acetate extract detected using (A) UV_{254} light, (B) UV_{366} light.

Stationary phase = silica gel F_{254}, mobile phase = chloroform: ethyl acetate (1:1 v/v).

This data suggested that the compound consist of two close related ones, major (**1**) and minor (**2**) compounds (Fig. 5). A signal (δ, 3.32; s) was a –OCH_3 signal that confirmed the data presented by IR and ^{13}C- NMR spectra, signal at δ, 1.93 ppm (s) was assigned as an allylic –CH_3 and 2 singlet of methyl signals (δ, 1.14, and 1.13 ppm). Based on the spectroscopic data above, tentatively in general both compounds were classified as hemiterpenoic type of compounds, compound **1** was identified as methyl hemiterpenoate and **2** was identified as methyl, 2,3 diene-butanoate (Fig. 5). LC-MS (Fig. 6) showed that 2 peaks were detected in the LC Chromatogram [rt.2.4 min (minor, **2**), and 4.7 min (major, **1**)]. Mass spectrum of **1** showed the highest mass at m/z 260 (73%), followed by m/z 243 (80%), 219 (100%, base peak), while mass spectrum of **2** showed the highest mass at m/z 441 (15%) and 419 (100%, base peak). LC chromatogram was

such comparable to the data shown by IR and NMR spectra, containing 2 compounds (**1** and **2**).

To evaluate the antimicrobial efficacy of this compound, testing the activity against as serial of six microorganisms *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis, Salmonella typhi* and *Staphylococcus mutans* was conducted. As shown in Table 2, this compound was most active against *S. mutans* with MBC values of 20 µg/mL, similar to that against *S. typhi*. Isoprene were known as materials led to the formation of terpenes (Zhang et al., 2011). Antimicrobial activities of terpenoids including essential oils produced by medicinal plants were widely reported (Barbieri et al., 2017). The compounds found in this study suggested that they may hemiterpenoids, the simplest form of terpenoids which were widely found in trees and herbs. Considering the IC_{50} values of this compound against all testing microorganisms, this compound was considered potential for further development (Cos et al., 2006).

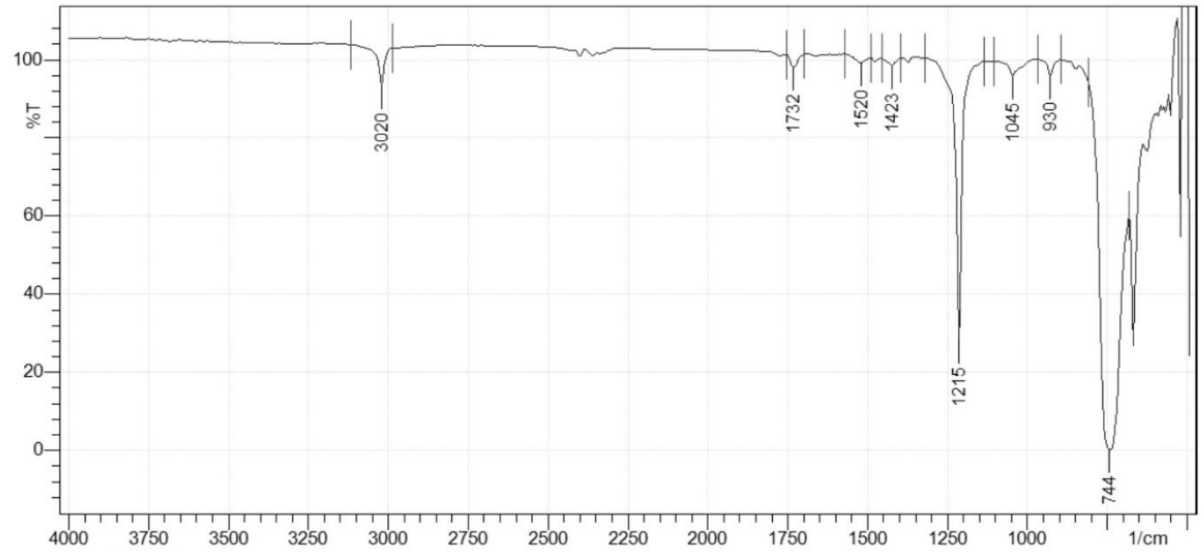

Figure 2. FT-IR (KBr) spectrum of isolated compound.

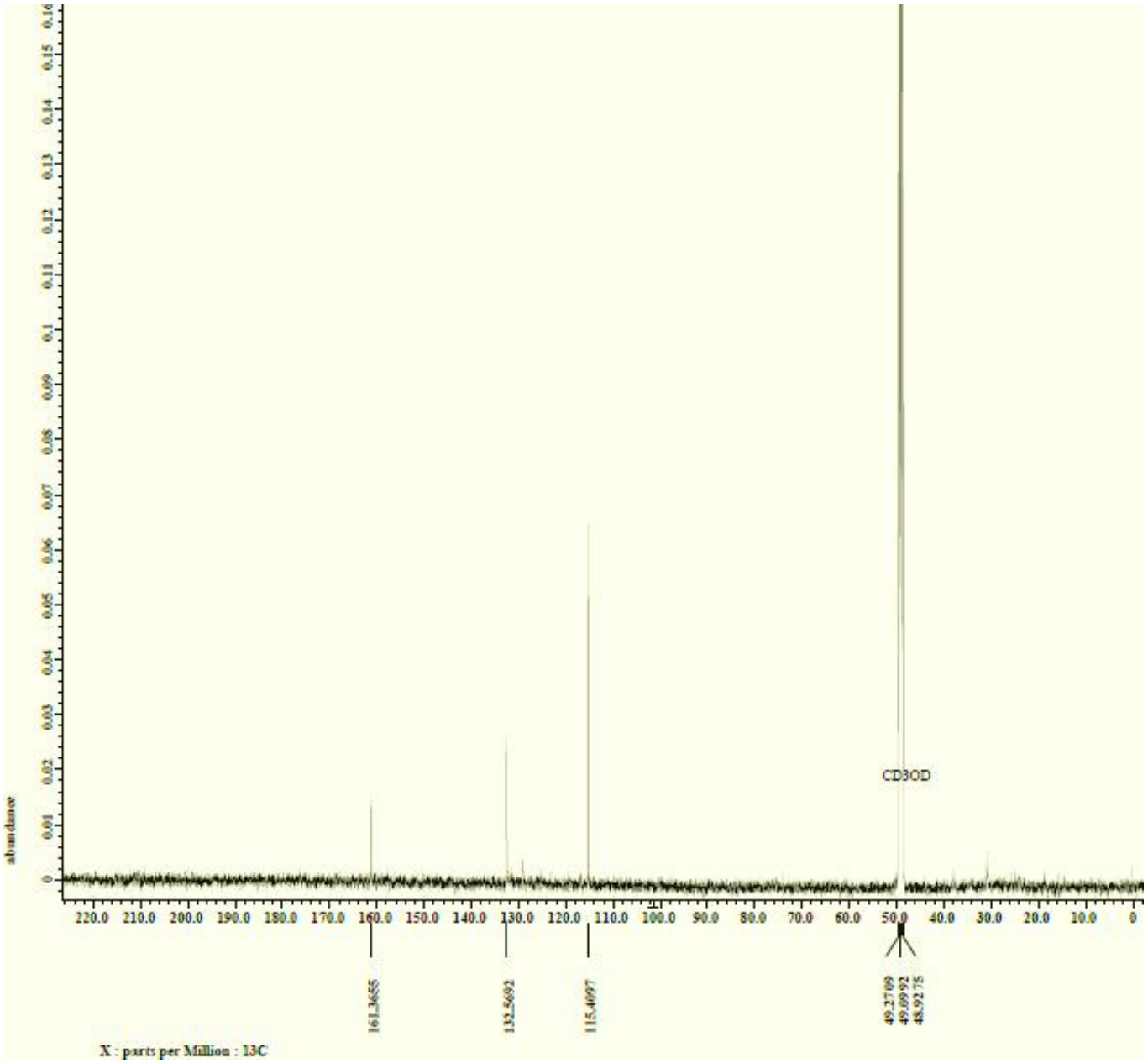

Figure 3. ^{13}C-NMR spectra (methanol-d4, 500 MHz) of isolated compound.

Table 1. The ^{1}H and ^{13}C NMR spectral data of the isolated compound.

Position	Compound 1		Compound 2	
	δ_C, Type	**δ_H, (J;Hz)**	**δ_C, Type**	**δ_H, (J;Hz)**
1	161.2, C=O	-	161.3, C	-
2	32.3, CH_2	2.10, d (6.8)	138.5, C	-
3	23.4, CH	1.14, m	132.5, =CH	6.8,t
4, 5	21.2, CH_3	1.13, d (7.4, 7.0)	117.3; 116.1, $=CH_2$	5.2 d (12)
-	65.5, $-OCH_3$	3.32, s	-65.6, OCH_3	3.32, s

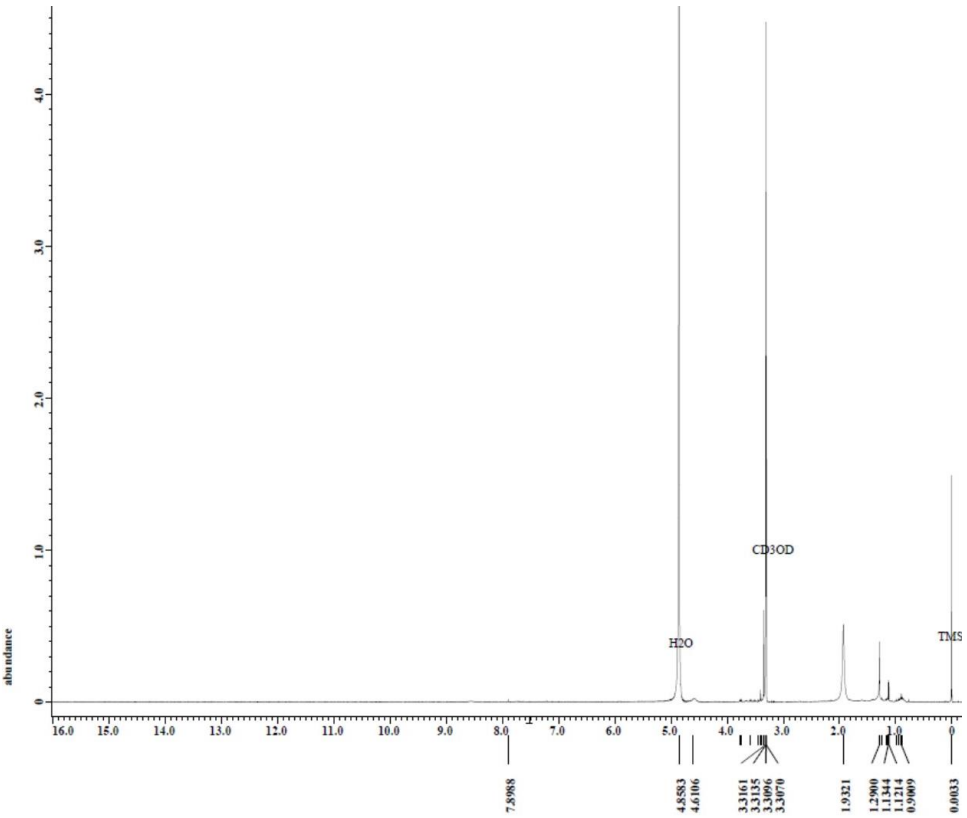

Figure 4. ^{1}H-NMR spectra of isolated compound (methanol-d4 500 MHz).

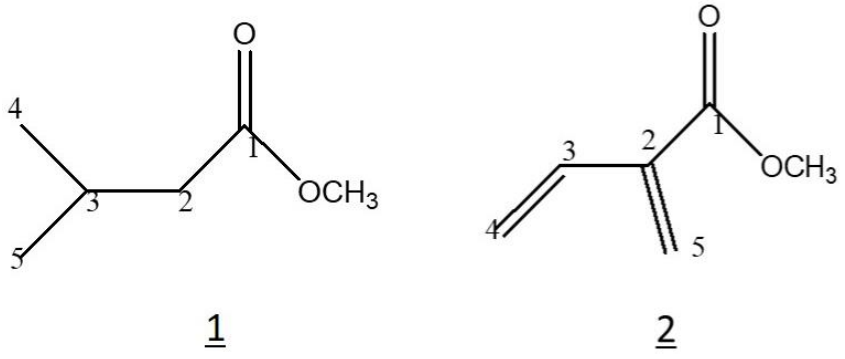

Figure 5. Isolated compound composed of compound **1,** (methyl hemiterpenoate) and compound **2**, (2 methyl, 2,3 diene-butanoate).

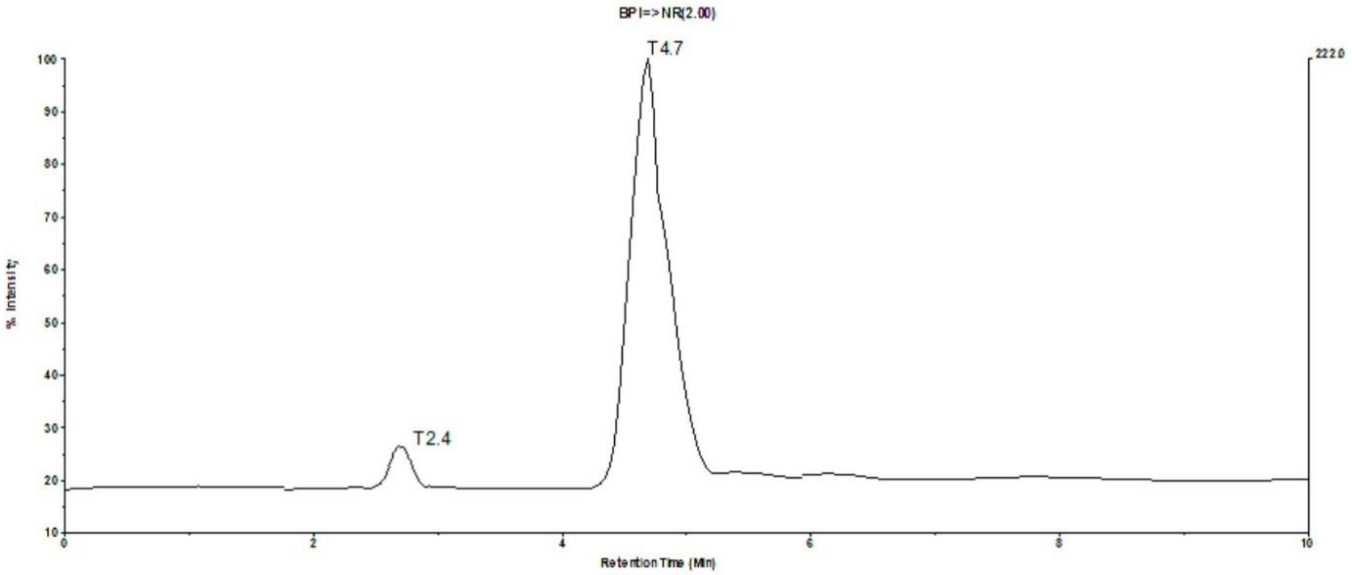

Figure 6. Liquid chromatography profile of isolated compound.
Mobile phase = methanol: H2O (90:10). Stationary phase = C18. Flow rate = 1 mL/minute.

Table 2. IC_{50} and MBC values of isolated compound.

Microorganisms	IC_{50} ± SD (µg/mL)	MBC ± SD (µg/mL)
S. aureus	0.86 ± 0.03	40.00 ± 0.00
B. subtilis	2.69 ± 0.08*	40.00 ± 5.77*
E. coli	1.35 ± 0.14*	40.00 ± 5.77*
S. mutans	0.24 ± 0.08	20.00 ± 3.45
P. aeruginosa	1.33 ± 0.64*	40.00 ± 0.00*
S. typhi	1.90 ± 0.07*	20.00 ± 3.45
Streptomycin	0.81 ± 0.02	20.00 ± 0.12

Values area means ± SD, n = 5 replicates, $p<0.05$ indicates statistically significant differences in comparison to streptomycin control.

CONCLUSIONS

Hemiterpenoid compounds isolated from an endophytic fungus *Athelia rolfsii* showed potential antimicrobial activities. Further studies on targets and mechanism of action are of importance to be explored.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTION:

Contribution	Astuti P	Rollando R	Wahyuono S	Nurrochmad A
Concepts or ideas	x	x	x	x
Design	x	x	x	x
Definition of intellectual content	x		x	x
Literature search	x	x	x	x
Experimental studies	x	x		
Data acquisition	x	x		
Data analysis	x	x	x	x
Statistical analysis	x	x		x
Manuscript preparation	x	x	x	x
Manuscript editing	x	x	x	x
Manuscript review	x	x	x	x

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