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Research Article

CHEMICAL COMPOSITION AND ANTIMICROBIAL ACTIVITY OF DITERPENE AND ESSENTIAL OILS OF *HEDYCHIUM ROXBURGHII* BLUME RHIZOME

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ABSTRACT

Objective: The objective of the present study was to isolate and determine diterpene compound and essential oils from *Hedychium roxburghii* Blume rhizome and investigated those antimicrobial activities.

Methods: The essential oils were obtained by steam distillation method, the residual was then extracted by reflux with ethanol. The content of essential oils was analyzed by gas chromatography-mass spectrometry (GC/MS) method. Ethanolic residual-distillation extract was concentrated then used to isolate compound 1 by vacuum liquid chromatography and centrifugal chromatography. It was characterized by infrared spectrophotometry, ^{1}H-nuclear magnetic resonance (NMR), ^{13}C-NMR, heteronuclear single quantum coherence-NMR, heteronuclear multiple bond correlation-NMR and carbon coupling^{13}C-NMR. The antimicrobial activity of essential oils, ethanolic residual-distillation extract and compound 1 were carried out by microdilution method.

Results: The oils exhibited antimicrobial activity against *Bacillus subtilis* American Type Culture Collection (ATCC) 6633 (minimum inhibitory concentration [MIC] 1750 µg/ml), *Staphylococcus aureus* ATCC 6538 (MIC 1750 µg/ml), *Escherichia coli* ATCC 8939 (MIC 3500 µg/ml), *Pseudomonas aeruginosa* ATCC 9027 (>3500 µg/ml) and *Candida albicans* ATCC 10231 (MIC 875 µg/ml). A phytochemical study of the rhizome essential oils of *H. roxburghii* Blume were performed by GC/MS and the result showed that fenchyl acetate (45.85%) was the main component of the oils. Compound 1 was identified as diterpene compound, coronarin E. Coronarin E have not exhibited MIC at 512 µg/ml, however, it showed inhibition profile against all of tested microbes.

Conclusion: The essential oils and ethanolic residual-distillation extract of *H. roxburghii* Blume rhizome exhibited weak antimicrobial profile. Compound 1 was identified as diterpene compound, (coronarin E), it was exhibited weak antimicrobial activity, but showed inhibition profile against all of the tested microbes.

Keywords: *Hedychium roxburghii*, Zingiberaceae, Antimicrobial, Essential oils, Coronarin E.

INTRODUCTION

The genus Hedychium consists of 80 species worldwide. There are 29 species distributed in the tropical and sub-tropical regions of China and 40 species growth in Indonesia. The genus is well-known as a group of medicinal plants. Various species were used in traditional medicines for the treatment of asthma, bronchitis, blood purification, gastric diseases, and as anti-emetics [1,2].

Zingiberaceae plants have been known as essential oils sources, there were rhizome, flower, and leaves. The main components of Hedychium essential oils were myrcene, limonene, *p*-cimene and γ-terpinene [3]. Essential oils of *Hedychium coronarium* J. Koenig rhizome were extracted by enfleurage method has been known contained ethyl hexadecanoate, tetradecanol, benzyl alcohol, α-farnesene, and linalool. Sesquiterpene essential oils from *H. coronarium* J. Koenig rhizome were identified as nerolidol; hedichyol A and hedichyol B 8,9-diacetate [4]. Medeiros *et al*. identified that essential oils component of *Hedychium gardnerianum* Sheppard ex Ker Gawl. Leaves and flower by gas chromatography-mass spectroscopy (GC/MS), the main component were α-pinene, β-pinene, caryophylene, α-cadinol and δ-cadinen [5].

In addition, the study of diterpene compound of Hedychium plants has been done. More than 10 diterpene compounds were isolated from *H. coronarium* J. Koenig rhizome, then as identified as 6-oxo-7,11,13-labdatriene-17-al-16,15-olide; 7,17-dihydroxy-6-oxo-7,11,13-labdatriene-16,15-olide; (E)-labda-8(17),12-diene-15,16-dial; (E)-15,16-bisnorlabda-8(17),11-diene-13-one; coronarin B; coronarin D; coronarin C; coronarin D methyl ether; C-14 eimers isocoronarin D; ethoxy coronarin D; coronarin F; eicosyl; docosyl-(E)-ferulate; cryptomeridiol; hedychenone; 6-oxo-7,11,13-labdatriene-16,15-olide; 9-hydroxy,15,16-epoxy-7,11,13(16)14-labdatetraene-6-one; pacovatine A; 4-hydroxy-3-metoxicinnamaldehyde; 4-hydroxy-3-methoxy ethyl cinnamate [6-14].

Antimicrobial activity of *H. coronarium* leaves had strong potency against *Candida glabrata,* followed by *Candida albicans* and *Malassezia furfur* [15]. In addition, essential oils of *H. gardnerianum* leaves exhibited antimicrobial activity against *Staphylococcus aureus* and *Staphylococcus epidermidis*, but have not shown against *Pseudomonas aeruginosa* [5]. The study of the antifungal activity of essential oils of *Hedychium spicatum* rhizome demonstrated the minimum inhibitory concentration (MIC) value at 2.5 µl/ml and MBC at 6.0 µl/ml against *Aspergillus flavus* [16].

However, the study of *Hedychium roxburghii* Blume (Zingiberaceae) insufficiently explored. The objectives of this research were to investigate antimicrobial activity of the essential oils and diterpene compound of *H. roxburghii* Blume rhizome.

METHODS

Collection of plant materials

H. roxburghii rhizomes (10 kg) were collected around Tangkuban Perahu, West Java, Indonesia then determined by a botanist from Herbarium Bandungense (Bandung Institute of Technology).

Processing of plant materials

The collected *H. roxburghii* rhizomes were cleaned thoroughly and dried by oven <50°C, grinded into powder and stored in airtight container at room temperature.

Preparation of extracts
The dried rhizome (1 kg) was steam distilled to obtain essential oils. The residual distillation was subjected to freeze-dryer, and then extracted with 96% ethanol in 3 times (5 L each time) by reflux method. The ethanolic residual extract was evaporated under reduced pressure using rotary evaporator.

Essential oils analysis
The phytochemical study of the essential oils of the *H. roxburghii* Blume rhizome was conducted by GC/MS analysis (Varian Saturn 2000®). The GC system was mobile phase: Helium; column: Capillary column VF-5 ms 3 m × 0.25 mm ID; detector: Ion Trap Detector; injection volume: 5 µl; temperature: 230°C and flow rate 1.3 ml/minute.

Isolation
The ethanolic residual extract was fractionated by using vacuum liquid chromatography (VLC) to get 33 fractions. Fraction 1-4 were then purified by centrifugal chromatography (Chromatotron®) was using silica gel GF_{254} as stationary phase and n-hexane as mobile phase to obtain compound 1, then characterized by infrared (IR) spectrophotometry (Fourier transform [FT] IR),^{1}H-nuclear magnetic resonance (NMR), ^{13}C-NMR, heteronuclear single quantum coherence (HSQC)-NMR, heteronuclear multiple bond correlation (HMBC)-NMR and coupling^{13}C-NMR (Agilent®, 500 MHz).

Microbes
S. aureus American Type Culture Collection (ATCC) 6538, *Bacillus subtilis* ATCC 6633, *P aeruginosa* ATCC 9027, *Escherichia coli* ATCC 8939, *C. albicans* ATCC 10231 were cultured at Microbiology Laboratory of School of Pharmacy, Bandung Institute of Technology.

Determination of antimicrobial activity
Antimicrobial activity was carried out using broth microdilution method adapted from CLSI [17]. The suspension for inoculation was prepared by diluting the broth culture with medium solution to obtain 0.5 McFarland (5 × 10^5 CFU/ml) suspension was then diluted 1:20 with the medium. The suspension 0.01 ml was inoculated into 96 well microwell-plate. Tetracycline, nystatine and ketoconazole were used as positive controls. Microbial inhibition profile of isolate was measured by a microplate reader. Absorbance calculated by reducing the absorption of microbe culture in the presence of isolate with a mixture of medium-sample at wavelength 625 nm.

RESULTS

Analysis of essential oils by GC/MS showed 45 peaks, whereas 43 peaks were identified a compound by comparing with the database in the instrument. The chromatogram can be seen in Fig. 1 and the components of essential oils were listed in Table 1.

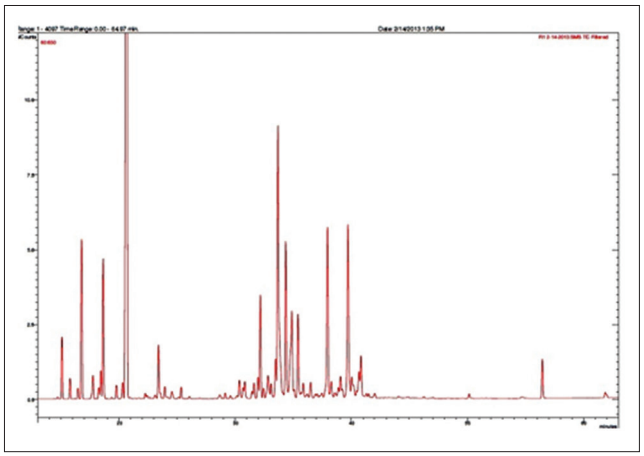

Fig. 1: Gas chromatography-mass spectrometry chromatogram of essential oils of *Hedychium roxburghii* Blume rhizome

Diterpene compound were obtained from ethanolic residual extract. The ethanolic residual extract was fractionated using VLC to get 33 fractions. Fraction 1-4 were then purified by centrifugal chromatography (Chromatotron®) using silica gel GF254 as the stationary phase and n-hexane as the mobile phase to obtain compound 1. Based on the purity test of compound 1 by two-dimensional (2D) thin layer chromatography, the isolate was pure that shown by single spot after spraying with H_2SO_4 10% in methanol (followed by heating in dry oven) (Fig. 2).

Compound 1 was then characterized by IR spectrophotometry (FTIR), the data showed in Table 2. ^{1}H-NMR, ^{13}C-NMR, and HSQC-NMR and HMBC-NMR (Agilent®, 500 MHz). The data were listed below.

Table 1: Component of essential oils of *H. roxburghii* Blume rhizome

Compound	%
α-pinene	1.37
Camphene	0.46
β-phellandrene	0.24
2-β-pinene	3.54
δ-3-carene	0.62
Phellandrene	0.26
Limonene	0.66
1,8-cineole	3.19
Trans-limonene oxide	0.32
Neril acetate	0.37
α-fenchyl acetate	45.85
Citronella	0.09
Borneol	1.24
4-terpineol	0.10
α-terpinenil acetate	0.24
Geraniol	0.25
α-cubebene	0.13
α-copaene	0.43
Calarene	0.22
Zingiberene	0.41
α-farnesene	0.16
Caryophylene oxide	0.37
α-eudesmol	0.21
Veridiflorol	0.54
Alloaromadendrene oxide	0.17
β-maaliene	4.88
Isoledene	0.55
τ-cadinol	0.32
Selina-3,7 (11)-diene	0.80
Spatulenol	4.42
β-gurjunene	0.37
Caryophylene	2.44
Germacrene D	0.22
Aromadendrene	0.82
α-gurjunene	0.38
α-humulene	0.96
Alloaromadendren	8.83
γ-cadinene	4.02
Bicyclogermacrene	3.58
α-neoclovene	0.19
δ-cadinene	2.13
Nerolidol	0.40

H. roxburghii: Hedychium roxburghii

Table 2: Functional groups of compound 1 based on IR spectrum

Wavenumber (cm^{-1})	Functional groups
1577.49	Furan
898.67	
775.24	
1778.05	α, β-unsaturated keton

IR: Infrared

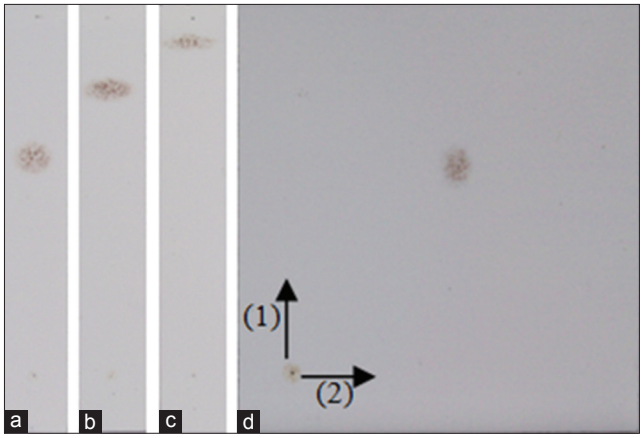

Fig. 2: Thin layer chromatogram of compound 1, stationary phase: silica gel GF_{254}, mobile phase: (a) N-hexane, (b) n-hexane-chloroform (6:0.5), (c) toluene-acetone-formic acid (9 ml: 0.2 ml: 2 drops), (d) two-dimensional thin-layer chromatography: (1) n-hexane (2) n-hexane-chloroform (7:0.5)

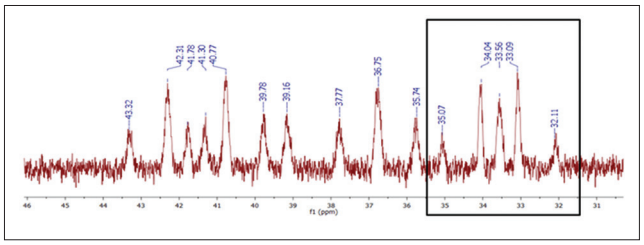

Fig. 3: Coupling^{13}C-nuclear magnetic resonance spectrum of compound 1

^{1}H-NMR (500 MHz, $CDCl_3$) showed signal at δ_H 0.85 (6H, d, *J*=2.4 Hz); 0.89 (3H, s); 1.04 (2H, m, *J*=26.8 Hz); 1.13 (1H, d, *J*=15 Hz); 1.22 (2H, m, *J*=30 Hz); 1.41 (4H, m, *J*=30,5 Hz); 1.52 (4H, m, *J*=35.77 Hz); 1.73 (1H, dd, *J*=15 Hz); 2.11 (1H, d, *J*=21 Hz); 2.44 (1H, d, *J*=10 Hz); 2.47 (1H,m, *J*=11 Hz); 3.49 (1H,s); 4.54 (1H, d, *J*=1.6 Hz); 4.76 (1H, k, *J*=1.6 Hz); 5.98 (1H, dd, *J*=9.8 Hz, *J*=15.7 Hz); 6.19 (1H, d, *J*=15.7 Hz); 6.54 (1H,d, *J*=15.7 Hz); 7.35 ppm (2H, d, *J*=3.5 Hz).

^{13}C-NMR (125 MHz, $CDCl_3$) showed signal at δ_C 15.01; 19.28; 22.12; 23.55; 33.73; 36.93; 39.31; 40.93; 42.47; 54.97; 61.63; 107.79; 108.11; 121.89; 124.65; 128.44; 139.76; 143.4; 150.4 ppm.

2D NMR (HSQC) of compound 1 were shown the correlation between C and H (Table 3), while 2D NMR (HMBC) were listed in Table 4.

Essential oils (MIC 3500 µg/ml) showed the greater activity than ethanolic residual extract and compound 1 against *E. coli*. Essential oils, ethanolic residual extract and compound 1 have not showed inhibition activity against all of the tested microbes until maximum concentration. Whereas, ethanolic residual extract (MIC 512 µg/ml) exhibited inhibition against *B. subtilis* greater than essential oils (MIC 1750 µg/ml) and compound 1 (>512 µg/ml). Antimicrobial activity of essential oils, ethanolic residual extract and compound 1 against *S. aureus* gave MIC 1750, 1024 and >512 µg/ml, respectively. Greater activity against *C. albicans* was exposed by essential oils than ethanolic residual extract. The resumes of MIC were shown in Table 5.

DISCUSSION

Essential oils distillation of *H. roxburghii* Blume rhizome was followed by extraction of the residual part were a fractionation step to separate the volatile and non-volatile compounds. Antimicrobial activity of essential oils and ethanolic residual extract had been determined

Table 3: C and H correlation of compound 1 by HSQC-NMR

Number	δ_C (ppm)	δ_H (ppm)	Interpretation
1	40.93	1.04 (2H, m, *J*=26.8 Hz) 1.52 (4H, m, *J*=35.77 Hz)	CH_2
2	19.28	1.41 (4H, m, *J*=30.5 Hz) 1.52 (4H, m, *J*=35.77 Hz)	CH_2
3	42.47	1.22 (2H, m, *J*=30 Hz) 1.41 (4H, m, *J*=30.5 Hz)	CH_2
4, 19	33.73	0.89 (6H, s)	C (quarterner)* CH_3
5	54.97	1.13 (1H, d, *J*=15 Hz)	CH
6	23.55	1.41 (4H, m, *J*=30.5 Hz) 1.73 (1H, dd, *J*=15 Hz)	CH_2
7	36.93	2.11 (1H, d, *J*=21 Hz) 2.47 (1H, m, *J*=11 Hz)	CH_2
8	150.40	-	C (quarterner)
9	61.63	2.44 (1H, d, *J*=10 Hz)	CH
10	39.31	-	C (quarterner)
11	128.44	5.98 (1H, dd, *J*=9.8 Hz, *J*=15.7 Hz)	CH
12	121.89	6.19 (1H, d, *J*=15.73 Hz)	CH
13	124.65	-	C (quarterner)
14	139.76	7.35 (2H, d, *J*=3.5 Hz)	CH
15	143.41	7.35 (2H, d, *J*=3.5 Hz)	CH
16	108.11	6.54 (1H, d, *J*=15.7 Hz)	CH
17	107.79	4.54 (1H, d, *J*=1.6 Hz) 4.76 (2H, d, *J*=1.6 Hz)	CH_2
18	15.01	0.85 (3H, s)	CH_3
20	22.12	0.89 (6H, s)	CH_3

*Confirmed by coupling ^{13}C-NMR. NMR: Nuclear magnetic resonance, HSQC: Heteronuclear single quantum coherence

Table 4: C and H of neighbor correlation of compound 1 by HMBC-NMR

Number	δ_C (ppm)	δ_H (ppm)
1	40.93	0.85
2	19.28	
3	42.47	0.85
		0.89
4, 19	33.73	0.85
5	54.97	0.85
		0.89
		1.41
		2.47
6	23.55	
7	36.93	
8	150.40	1.73
		2.11
		2.44
9	61.63	2.47
		6.21
10	39.31	
11	128.44	
12	121.89	
13	124.65	5.98
		6.19
		6.54
		7.35
14	139.76	6.19
		6.54
		7.35
15	143.41	7.35
16	107.79	6.19
17	108.11	
18	15.01	
20	22.12	1.13

NMR: Nuclear magnetic resonance, HMBC: Heteronuclear multiple bond correlation

Table 5: Antimicrobial activity of essential oils, ethanolic residual extract and compound 1 from *H. roxburghii* Blume rhizome

Sample	MIC (µg/ml)				
	E. coli	*P. aeruginosa*	*B. subtilis*	*S. aureus*	*C. albicans*
Essential oils	3500	>3500	1750	1750	875
Ethanolic residual extract	>1024	>1024	512	1024	>1024
Compound 1	>512	>512	>512	>512	>512
Tertracycline	12.5	>25	0.4	0.2	-
Nystatine	-	-	-	-	0.78
Ketoconazol	-	-	-	-	12.5

H. roxburghii: Hedychium roxburghii, MIC: Minimum inhibitory concentration, *E. coli: Escherichia coli, P. aeruginosa: Pseudomonas aeruginosa, B. subtilis: Bacillus subtilis, S. aureus: Staphylococcus aureus, C. albicans: Candida albicans*

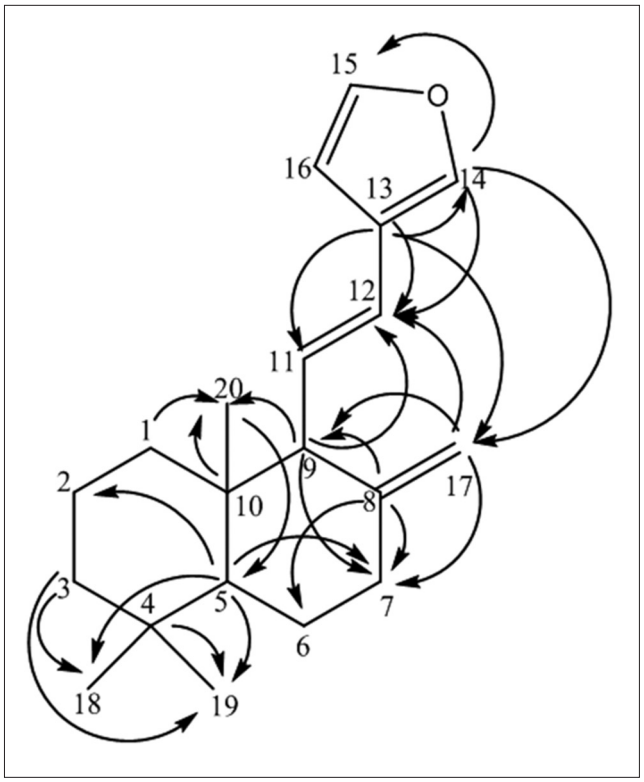

Fig. 4: C and H atom of neighbor of compound 1 based on heteronuclear multiple bond correlation-nuclear magnetic resonance data

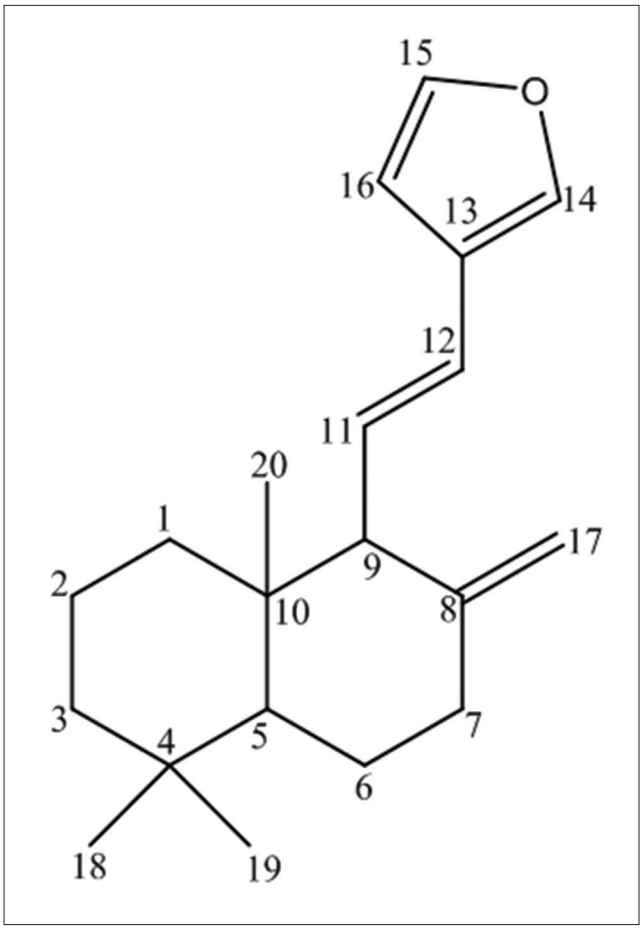

Fig. 5: Chemical structure of compound 1 (coronarin E)

by microdilution test. Based on the results, essential oils showed the growth inhibition against *C. albicans* (MIC 875 µg/ml), *B. subtilis* and *S. aureus* (MIC 1750 µg/ml) followed by activity against *E. coli* (MIC 3500 µg/ml). Ethanolic residual extract exhibited growth inhibition against *B. subtilis* (MIC 512 µg/ml) and *S. aureus* (MIC 1024 µg/ml), while have not shown inhibition against Gram-negative bacterial and *C. albicans* at the highest concentration. Some of researchers revealed that the essential oils had greater antibacterial activity compared with antibacterial activity of mixture of main components [18], therefore the effect of minor compounds could provided very important influence on the potential antimicrobial activity of essential oils [19]. This phenomenon could be occurred because of addition, antagonistic or synergistic effect between each component, such as the addition effects which was occurred in a combination of carvacrol and thymol against *S. aureus* and *P. aeruginosa* [20].

The^{1}H-NMR spectrum of the compound 1 was indicated that the compound have 28 protons. The presence of methyl groups was indicated by singlet signal at δ 0.89 and 0.91 ppm. Integration at δ 0.89 ppm (six protons) exposed the presence of two methyls and integration at δ 0.91 ppm (three protons) showed one methyl substituent. It exhibited that the structure of compound 1 had three methyl substituents.

^{13}C-NMR spectrum of compound 1 showed chemical shift at δ 107.79; 108.11; 121.89; 124.65; 128.44; 139.76; 143.41; 150.40 ppm which indicated that eight carbons of the structure were located in a cyclic chain. Based on 2D HSQC-NMR spectrum, the structure of compound 1 have three quaternary carbons at chemical shift δ 42.47; 124.65 and 150.40 ppm.

^{13}C-NMR spectrum showed 19 signals, however coupling^{13}C-NMR spectrum exhibited overlapping signal at δ 33.73 ppm. HSQC spectrum revealed that methyl substituent should bring out four signals at that chemical shift, meanwhile coupling^{13}C-NMR spectrum showed the presence of five signals (Fig. 3). The data indicated that five signals on the spectrum were carbon from methyl and quarterner carbon.

Analysis of HMBC 2D NMR spectrum of compound 1 showed the correlation between each atom C and H of neighbor (Fig. 4).

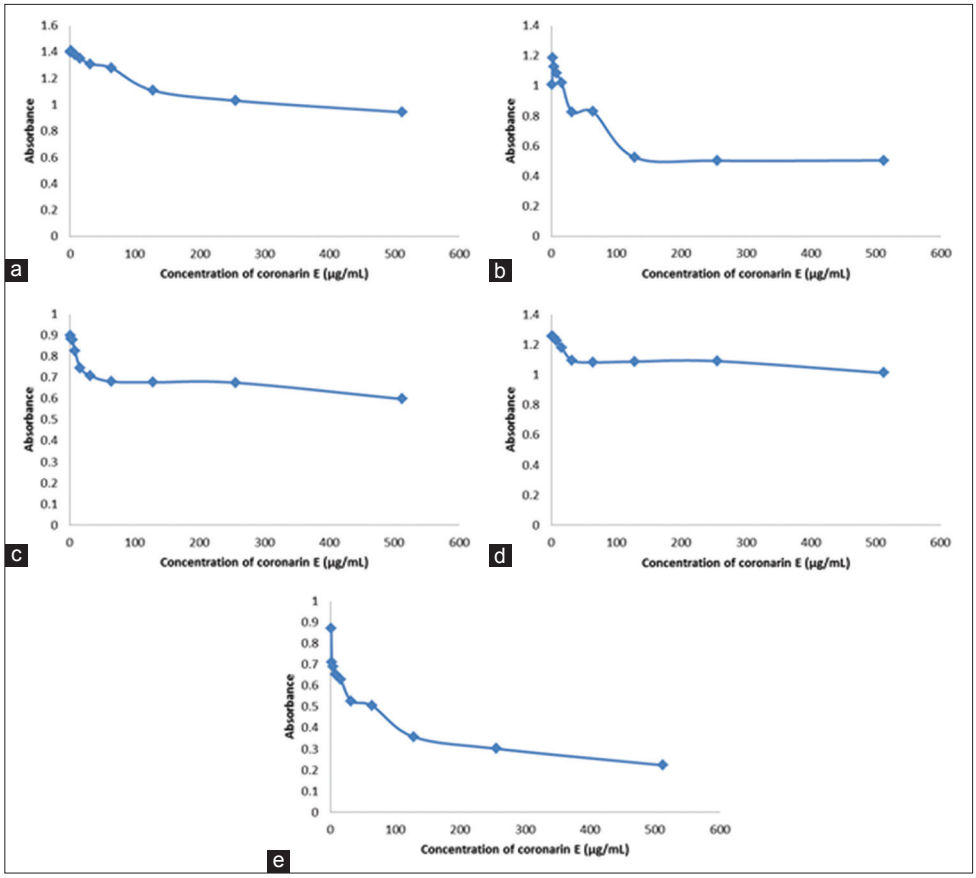

Fig. 6: Antimicrobial profile of coronarin E against: (a) *Staphylococcus aureus*; (b) *Bacillus subtilis*; (c) *Pseudomonas aeruginosa*; (d) *Escherichia coli*; (e) *Candida albicans*

Based on data above, compound 1 known as coronarin E (Fig. 5) that have not been isolated from *H. roxburghii* rhizomes yet.

Antimicrobial activity assay was carried out on compound 1 by obtaining the value of the MIC. MIC was defined as the lowest concentration of an antibacterial compound that can inhibit microbial growth with significant decreasing in viability inoculum more than 90% of the number of colonies [21,22]. MIC value indicated the potential level of antimicrobial activity of compounds. Determination of MIC can be visually observed by microbial growth, colorimetric, determination of turbidity, absorbance measurement, conductance or viable count [19].

Absorbance measurements were used to determine antimicrobial profile of coronarin E. Absorbance that was shown in Fig. 6 can be obtained by calculating the difference between maximum absorbance of microbe culture in the presence of compound 1 and mixture of medium sample of each concentration at l 625 nm. Compound 1 showed inhibition in all of tested microbes, although MIC value had not been achieved at the highest concentration (512 µg/ml).

CONCLUSION

The essential oils of *H. roxburghii* Blume that contained fenchyl acetate (45.85%) as the main component, exhibited an antimicrobial activity against *B. subtilis* ATCC 6633 (MIC 1750 µg/ml), *S. aureus* ATCC 6538 (MIC 1750 µg/ml), *E. coli* ATCC 8939 (MIC 3500 µg/ml), *P. aeruginosa* ATCC 9027 (>3500 µg/ml) and *C. albicans* ATCC 10231 (MIC 875 µg/ml). Compound 1 was isolated from ethanolic residual-distilation extract, and then identified as coronarin E. Coronarin E have not exhibited MIC at 512 µg/ml, however, it showed inhibition profile against all of tested microbes.

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