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Antibacterial and cytotoxicity activities of phenylbutanoids from *Zingiber cassumunar* Roxb.

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ABSTRACT

Bioautography was employed as the screening method for purifying bioactive substances of the crude extract of *Zingiber cassumunar* Roxb. Purification procedures included silica gel 60 column chromatography, thin layer chromatography, and medium pressure liquid chromatography. Identification of purified compounds was achieved by spectroscopic methods. Three phenylbutanoids were purified and identified as (*E*)-3-(3,4-dimethoxyphenyl)-4-[(*E*)-3,4-dimethoxystyryl] cyclohex-1-ene (**1**), (*E*)-4-(3,4-dimethoxyphenyl)-but-3-en-1-ol (**2**) and (*E*)-4-(3,4-dimethoxyphenyl)-but-3-en-1-yl acetate (**3**). Compound **1** showed high antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* with both MIC (16 µg/ml) and MBC (32 µg/ml). These were followed by the MIC values (32 µg/ml) and MBC values (128 µg/ml) for compounds **2** and **3** against the same microorganisms. These compounds revealed bacteriolytic effects on the assayed strains, causing evident damage to cell walls and membranes using SYTOX Green. The cytotoxicity activity of purified compounds was determined using MTT colorimetric assay against L929 and Vero cell lines. They showed weak cytotoxicity activity with IC_{50} values of 1263.42 to 2857.83 µg/ml and 1537.83 to 2698.45 µg/ml toward L929 and Vero cell lines, respectively.

INTRODUCTION

Zingiber cassumunar Roxb. (Family; Zingiberaceae) is used in folk remedies for the treatment of muscular and joint pain, inflammation, rheumatism, wounds, asthma (Bhuiyan *et al.*, 2008; Chaiwongsa *et al.*, 2012; 2013) in south-east Asia, especially in Thailand. It is known by the Thai name "Plai". The essential oil extracted from the rhizome of *Z. cassumunar* has various active chemical ingredients. These include: α- and β-pinene, α- and γ-terpinene, limonene, monoterpenes, myrcene, terpinen-4-ol, terpinolene, sabinene, sabmene and phenylbutenoids (Pongprayoon *et al.*, 1997; Bordoloi *et al.*, 1999; Jantan *et al.*, 2003; Bhuiyan *et al.*, 2008). These phytochemicals have various pharmaceutical properties, including, anti-inflammatory, anticancer, antifungal, and antioxidant activities (Lu *et al.*, 2008). (*E*)-4-(3', 4'-dimethoxyphenyl) but-3-en-1-ol is the main phenylbutenoid that exhibits anti-inflammatory activity (Kanjanapothi *et al.*, 1987; Panthong *et al.*, 1990; 1997; Jeenapongsa *et al.*, 2003; Han *et al.*, 2005).

In this study, three phenylbutanoids were isolated from a *Z. cassumunar* extract. This is the first Thai study of their antibacterial activity. Our research was intended to determine the antibacterial activity of phenylbutenoids isolated from a *Z. cassumunar* extract. Sytox green assays provided detailed information on cellular damage and alterations caused by the tested compounds. The results of this study may support the clinical applications of *Z. cassumunar* in treatments for bacterial infections.

MATERIALS AND METHODS

Plant material and extraction procedure

Rhizomes of *Z. cassumunar* cultured in Nakhon Pathom, Thailand were collected for use in this study. Fresh rhizomes were washed and chopped into small pieces. Three hundred grams of dry rhizomes were extracted with hexane three times, at room temperature using the maceration method for 3 days. The filtrates

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were pooled and evaporated by rotary evaporator at 40°C. The crude oil obtained was stored at 4°C in dark bottles until it was used in the experiments.

Isolation of the compounds

The crude extract was dissolved in methanol to perform the bioautography assays (Suleimana *et al.*, 2010). The major compounds were isolated by silica gel 60 (230-400 mesh, Merck) column chromatography and eluted with hexane:ethyl acetate (95:5). Fractions were monitored by thin layer chromatography (TLC) (Kieselgel 60 F254, Merck), and spots were visualized under ultraviolet light and by heating silica gel plates sprayed with 10% H_2SO_4 in ethanol. The combined fractions were eluted with 20-40% ethyl acetate in hexane by medium pressure liquid chromatography (MPLC) (400 × 40 mm column, Merck LiChroprep Si 60, 25-40 mm, UV-detection, 254 nm) to afford fraction (fr.) A (42 mg), fr. B (59 mg) and fr. C (45 mg). The fr. C had no activity against tested microorganisms. Final purification of fr. A and B was achieved by preparative TLC (Si gel 60, 0.5 mm, Merck) to afford compound **1** (31 mg) from fr. A and compounds 2 (28 mg) and 3 (21 mg) from fr. B. By using infrared, UV and nuclear magnetic resonance analyses, the chemical structures of these compounds were identical with (*E*)-3-(3,4-dimethoxyphenyl)-4-[(*E*)-3,4-dimethoxystyryl] cyclohex-1-ene (**1**), (*E*)-4-(3,4-dimethoxyphenyl)-but-3-en-1-ol (**2**) and (*E*)-4-(3,4-dimethoxyphenyl)-but-3-en-1-yl acetate (**3**).

Antibacterial assay

An *in vitro* plate assay technique was used to test the inhibitory effects of crude extract and purified compounds on the tested bacteria using the paper disk method according to the Clinical Laboratory Standard Institute (CLSI, 2012). Sterile paper discs (6 mm, Whatman 2017-006) were loaded with 50 µl of two-fold dilution of 440 mg/ml of crude extract or 1 mg/ml of purified compounds. Four bacterial species were used in this study: *S. aureus* ATCC25932, *Bacillus cereus* ATCC7064, *E. coli* ATCC10536 and *Pseudomonas aeruginosa* ATCC27853. These bacteria were cultured in nutrient broth at 37°C for 24 hrs. Dilutions of bacterial suspensions were prepared using McFarland standard tubes (1×10^8 CFU/ml). The air-dry discs with various concentrations of the crude extract and purified compounds were placed on a lawn of bacterial spread on Muller Hinton agar. The plates were incubated at 37°C for 24 hrs. The diameter of the formed inhibition zones around each disc was recorded. The experiment was carried out in triplicate using gentamicin (30 unit/disk) and chloramphenicol (30 µg/disk) (Oxoid, UK) as references for antimicrobial activity control.

Minimum inhibitory concentrations

The minimum inhibitory concentrations (MIC) of the compound was tested against microorganisms in a 96-well microtiter plate by NCCLS microbroth dilution methods (NCCLS, 2000). The compound was twofold diluted from 0.5 µg/ml to 512 µg/ml in nutrient broth supplemented with 10% glucose containing 0.01% phenol red as a color indicator (NBGP). Bacteria was adjusted to 10^5 CFU/ml for each microtiter plate. The microtiter plates were incubated at 37°C for 24 hrs. Microbial growth was determined by observing the change of color in the wells (red to yellow when there is microbial growth). The lowest concentration that showed no change of color was considered as the MIC. Minimum microbicidal concentration was determined by inoculating onto nutrient agar plates 10 µl of the medium from each of the wells from the MIC test which showed no turbidity. The plates were incubated at 37°C for 24 hrs. Minimum bactericidal concentration (MBC) was defined as the lowest concentration of the test agent at which no microbial growth was observed on the plates.

Sytox green assays

The assay was performed in microcentrifuge tubes of 0.5 ml final volumes. The crude extract and purified compounds were assayed at the concentration of 10 mg/ml and 200 mg/ml, respectively. Growth controls replaced samples with sterile water. Five µl of Sytox green solution were added to the tubes which were incubated at 37°C for 1 hr. Fifty microliters of samples were placed on glass slides, covered and observed with incident light fluorescence of a Nikon fluorescence microscope (Nikon Fluophot) equipped with an Osram HBO 200 W/2 mercury vapor lamp. An exciter filter IF 420-490 was used and the photomicrographs were taken using Olympus' cellSens imaging software (version 1.16).

Cytotoxicity activity assay

In order to evaluate the cytotoxicity activity of the crude extract and purified compounds, cytotoxicity tests were performed and the effect of the median inhibitory dose (IC_{50}) on two normal cell lines (L929, murine fibroblast cell line, and Vero, African green monkey kidney cell line) was assessed as previously described (Taechowisan *et al.*, 2017). Briefly, different concentrations (1, 2, 4, 8, 16, 32, 64, 128, 256 and 512 µg/ml) of the crude extract and purified compounds were prepared and used in the cytotoxicity tests. To measure the cytotoxicity, 5×10^4 cells were seeded in 96-well plates and incubated in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum containing different concentrations of the test agents at 37°C for 24 hrs in a 5% CO_2 incubator. The wells were washed with a serum-free medium. Vehicle control groups were added with double distilled water.

In the tetrazolium salt, 3-4,5 dimethylthiazol-2,5 diphenyl tetrazolium bromide (MTT) assay, yellow MTT was reduced to purple formazan in the mitochondria of viable cells. A quantity of 100 µl of the MTT working solution (0.5 mg/ml) was added to each well and incubated at 37°C for 5 hrs. Next, the media were removed, wells were washed with phosphate buffer saline, and 100 µl of DMSO was added to solubilize the formazan crystalline product. The absorbance was measured using a plate reader (Packard AS10000 Spectrocount, USA) at 590 nm. The production of formazan dye was proportional to the number of viable cells.

The inhibition of cytotoxicity rate of the cell lines for each test agent with different concentrations was calculated according to the following equation:

$$\%\text{Inhibition} = 100 - [(\text{Abs}_{\text{sample}} - \text{Abs}_{\text{blank}})/(\text{Abs}_{\text{control}} - \text{Abs}_{\text{blank}})] \times 100,$$

where $\text{Abs}_{\text{sample}}$ is the absorbance of the test agent and $\text{Abs}_{\text{control}}$ is the absorbance of the control reaction (containing all reagents except

the test agent). The %inhibition was plotted against a sample concentration, and a linear regression curve was established in order to calculate the IC_{50}. Tests were carried out in triplicate. Correlation coefficients were optimized.

Table 1: Diameters of inhibition zones of the crude extract and purified compounds on the tested microorganisms.

Test agents/concentrations		Diameters of inhibition zones on tested microorganisms (mm)			
		S.a.[a]	B.c.	E.c.	P.a.
Crude extract	2.75 mg/disc	NZ	NZ	NZ	NZ
	5.5 mg/disc	NZ	NZ	NZ	NZ
	11 mg/disc	16.68 ± 2.78	12.26 ± 2.33	27.70 ± 3.74	12.35 ± 1.57
	22 mg/disc	68.66 ± 4.95	44.23 ± 3.86	70.33 ± 5.78	38.47 ± 3.13
Compound **1**	1 µg/disc	NZ	NZ	NZ	NZ
	5 µg/disc	NZ	NZ	NZ	NZ
	10 µg/disc	23.21 ± 5.08	17.67 ± 3.92	27.34 ± 4.18	15.30 ± 3.26
	50 µg/disc	54.34 ± 4.53	38.88 ± 3.97	53.66 ± 5.11	33.12 ± 4.35
Compound **2**	1 µg/disc	NZ	NZ	NZ	NZ
	5 µg/disc	NZ	NZ	NZ	NZ
	10 µg/disc	NZ	NZ	NZ	NZ
	50 µg/disc	30.37 ± 2.85	14.67 ± 3.38	31.22 ± 3.60	10.81 ± 47
Compound **3**	1 µg/disc	NZ	NZ	NZ	NZ
	5 µg/disc	NZ	NZ	NZ	NZ
	10 µg/disc	NZ	NZ	NZ	NZ
	50 µg/disc	22.13 ± 3.82	15.27 ± 2.47	20.50 ± 2.66	11.50 ± 3.33
Chloramphenicol	30 µg/disc	34.61 ± 1.88	33.23 ± 1.92	44.31 ± 1.23	22.15 ± 1.62
Gentamicin	30 µg/disc	24.92 ± 1.69	23.53 ± 1.28	23.53 ± 1.65	19.38 ± 1.54

[a]S.a.; *Staphylococcus aureus* ATCC25932, B.c.; *Bacillus cereus* ATCC7064, E.c.; *Escherichia coli* ATCC10536, P.a.; *Pseudomonas aeruginosa* ATCC27853.
Results represent the mean ± SD.
NZ = No inhibition zone.

RESULTS

TLC and column chromatography on silica gel was performed with hexane : ethyl acetate as the mobile phase resulted in the separation of three major compounds. Identification of each compound was carried out by ^{1}H-NMR, ^{13}C-NMR as following.

Compound **1**: ^{1}H-NMR (500 MHz, $CDCl_3$): 1.68 (1H, m), 1.97 (1H, m), 2.17 (2H, m), 2.24 (1H, m), 3.20 (1H, m), 3.78 (3H, s), 3.80 (3H, s), 3.82 (3H, s), 3.90 (3H, s), 5.64 (1H, dd, J = 10.0, 2.1 Hz), 5.86 (1H, dt, J = 10.0, 2.3 Hz), 5.98 (1H, dd, J = 15.9, 7.3 Hz), 6.03 (^{1}H, d, J = 15.9 Hz), 6.66-6.83 (6H, m); ^{13}C-NMR (125 MHz, $CDCl_3$): 24.9 (CH_2), 28.4 (CH_2), 45.8 (CH), 48.5 (CH), 56.3 (OCH_3), 56.8 (OCH_3), 57.0 (OCH_3), 110.2 (CH), 114.0 (CH), 115.1 (CH), 116.0 (CH), 120.4 (CH), 122.5 (CH), 125,7 (CH), 131.9 (CH), 134.3 (CH), 135.6 (CH), 137.8 (C), 139.8 (C), 149.6 (C), 150.4 (C), 151.7 (C). MS *m/e*: 380.199 (M^+) (Calcd for $C_{24}H_{28}O_4$, 380.199). According to literature (Kuroyanagi *et al.*, 1980; Jitoe *et al.*, 1993; Masuda and Jitoe, 1995; Lu *et al.*, 2008), *compound* **1** was identified as (*E*)-3-(3,4-dimethoxyphenyl)-4-[(*E*)-3,4-dimethoxystyryl] cyclohex-1-ene.

Compound **2**: ^{1}H-NMR (500 MHz, CDCl3): 2.61 (2H, q, J = 6.5 Hz), 3.72 (2H, t, J = 6.5 Hz), 3.83 (3H, s), 3.85 (3H, s), 5.58 (^{1}H, dt, J = 15.5, 6.5 Hz), 6.48 (^{1}H, d, J = 15.5 Hz), 6.80 (1H, d, J = 8.5 Hz), 6.90 (1H, dd, J = 8.5, 1.5 Hz), 6.92 (1H, d, J = 1.5 Hz); ^{13}C-NMR (125 MHz, $CDCl_3$): 32.8 (CH_2), 56.2 (OCH_3), 56.8 (OCH_3), 63.0 (CH_2), 112.2 (CH), 114.2 (CH), 122.0 (CH), 128.4 (CH), 131.3 (CH), 132.2 (C), 149.8 (C), 150.2 (C). MS *m/e*: 208 (M^+) (Calcd for $C_{12}H_{16}O_3$, 208). According to literature (Kuroyanagi *et al.*, 1980; Jitoe *et al.*, 1993; Masuda and Jitoe, 1995; Lu *et al.*, 2008), compound **2** was identified as (*E*)-4-(3,4-dimethoxyphenyl)-but-3-en-1-ol.

Compound **3**: ^{1}H-NMR (500 MHz, $CDCl_3$): 2.10 (3H, s), 2.58 (2H, q, J = 6.5 Hz), 3.90 (3H, s), 3.96 (3H, s), 4.22 (2H, t, J = 6.5 Hz), 6.10 (1H, dt, J = 15.5, 6.5 Hz), 6.47 (1H, d, J = 15.5 Hz), 6.86 (1H, d, J = 8.0 Hz), 6.90-6.94 (2H, m); ^{13}C-NMR (125 MHz, $CDCl_3$): 21.8 (CH_3), 32.7 (CH_2), 56.0 (OCH_3), 56.5 (OCH_3), 64.6 (CH_2), 108.8 (CH), 111.8 (CH), 119.7 (CH), 124.2 (CH), 130.8 (CH), 133.4 (C), 149.5 (C), 150.2 (C), 172.6 (C=O). MS *m/e*: 251 (M^+) (Calcd for $C_{24}H_{28}O_4$, 251). According to literature (Kuroyanagi *et al.*, 1980; Jitoe *et al.*, 1993; Masuda and Jitoe, 1995; Lu *et al.*, 2008), compound **3** was identified as (*E*)-4-(3,4-dimethoxyphenyl)-but-3-en-1-yl acetate.

The structures of these compounds are shown in Figure 1.

The crude extract from *Z. cassumunar* rhizome showed a pale amber color. The crude extract yield was 7.25 ml/kg. The antibacterial activity of the crude extract and purified compounds are summarized in Table 1. Various concentrations of the crude extract and purified compounds were tested using agar disc diffusion assay. A zone of inhibition >8 mm in diameter was interpreted as sensitive. All of the susceptible strains were sensitive to the crude extract at 11 to 22 mg/disc. The crude extract showed the highest activity against *E. coli* and *S. aureus* at 22 mg/disc with the average zones of inhibition being 70.33 ± 5.78 mm and 68.66 ± 4.95 mm, respectively. However, this crude extract showed low activity against *B. cereus* and *P. aeruginosa* at 22 mg/disc with the average zones of inhibition 44.23 ± 3.86 mm and 38.47 ± 3.13 mm, respectively. Compound **1** showed higher activity than compounds **2** and **3**. It also showed the highest activity against *E. coli* and *S. aureus* at 50 µg/disc with the average zones of inhibition being 53.66 ± 5.11 mm and 54.34 ± 4.53 mm, respectively. Compounds **2** and **3** showed activity against all the tested microorganisms only

at 50 µg/disc, while the compound **1** showed prominent activity at 10 µg/disc. Sensitive results were not obtained with discs containing 2.75-5.5 mg/disc of the crude extract, 1-5 µg/disc of compound **1**, and 1-10 µg/disc of compounds **2** and **3**.

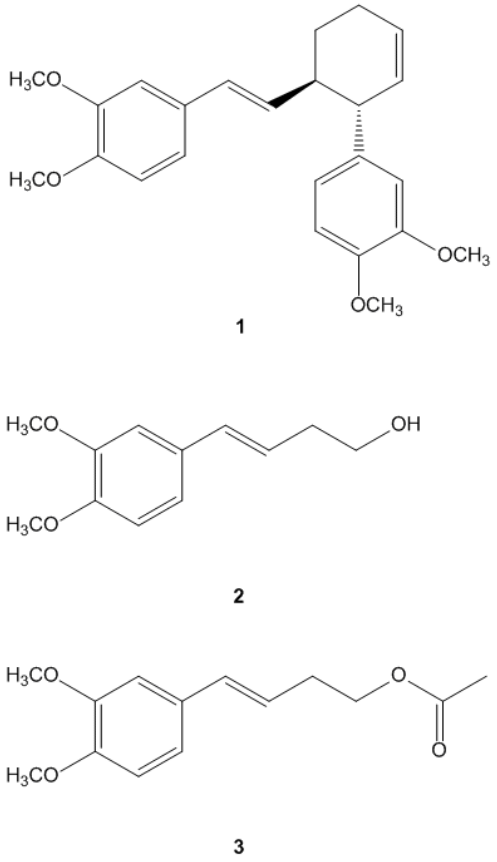

Fig. 1: The chemical structures of the purified compounds; (*E*)-3-(3,4-dimethoxyphenyl)-4-[(*E*)-3,4-dimethoxystyryl] cyclohex-1-ene (**1**), (*E*)-4-(3,4-dimethoxyphenyl)-but-3-en-1-ol (**2**), (*E*)-4-(3,4-dimethoxyphenyl)-but-3-en-1-yl acetate (**3**).

Table 2: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of crude extract, purified compounds on tested microorganisms.

Test microorganisms	Antibacterial activity of the test agents									
	Crude extract (mg/ml)		Compound 1 (µg/ml)		Compound 2 (µg/ml)		Compound 3 (µg/ml)		Chloramphenicol (µg/ml)	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
S.a.[a]	1.09	2.19	16	32	32	128	32	128	4	512
B.c.	8.74	34.97	64	128	64	256	64	256	8	>512
E.c.	2.19	4.37	16	32	32	128	32	128	4	>512
P.a.	17.49	69.94	64	256	128	512	128	512	8	>512

[a]S.a.; *Staphylococcus aureus* ATCC25932, B.c.; *Bacillus cereus* ATCC7064, E.c.; *Escherichia coli* ATCC10536, P.a.; *Pseudomonas aeruginosa* ATCC27853.

A classification based on MIC values proposed by Algiannis *et al.* (2001) was used for this study. The extract or the compounds with MIC values up to 512 µg/ml were considered strong inhibitors; with 512 µg/ml as moderate inhibitors; and those above 512 µg/ml as weak inhibitors. Because the crude extract showed MIC values greater than 512 µg/ml, it was therefore considered a weak inhibitor against all the test microorganisms. Compound **1** showed the lowest MIC (16 µg/ml) against *S. aureus* and *E. coli* (Table 2). This was followed by the MIC values (32 µg/ml) of compounds **2** and **3** against the same microorganisms. Compounds **2** and **3** had high MIC values (128 µg/ml) against *P. aeruginosa*. Compound **1** showed the lowest MBC (32 µg/ml) against *S. aureus* and *E. coli* whereas compounds **2** and **3** had high MBC values (512 µg/ml) against *P. aeruginosa*. The crude extract has weak inhibitory activity in MBC against *B. cereus* and *P. aeruginosa*. Bacteria exposed to crude extract and purified compounds showed intense fluorescence after Sytox green stain in contrast to control experiments (Figure 2). These findings suggest that these purified compounds exert antibacterial effects by damaging bacterial cell walls and membranes.

To evaluate the cytotoxicity activity of the crude extract and purified compounds against murine fibroblast cells (L929) and African green monkey kidney cells (Vero), the cell lines were incubated with different doses of two-fold dilution (1-512 µg/ml) of the crude extract and purified compounds. After 24 hrs of incubation, cell viability was determined by the MTT assay. The crude extract and purified compounds induced cell cytotoxicity in a concentration-dependent manner. The corresponding IC_{50}

was calculated, and the results are presented in Table 3. The cytotoxicity activity of the crude extract and purified compounds was observed and showed weak cytotoxicity activity with IC_{50} values of 1263.42 to 2857.83 µg/ml and 1537.83 to 2698.45 µg/ml toward L929 and Vero cell lines, respectively.

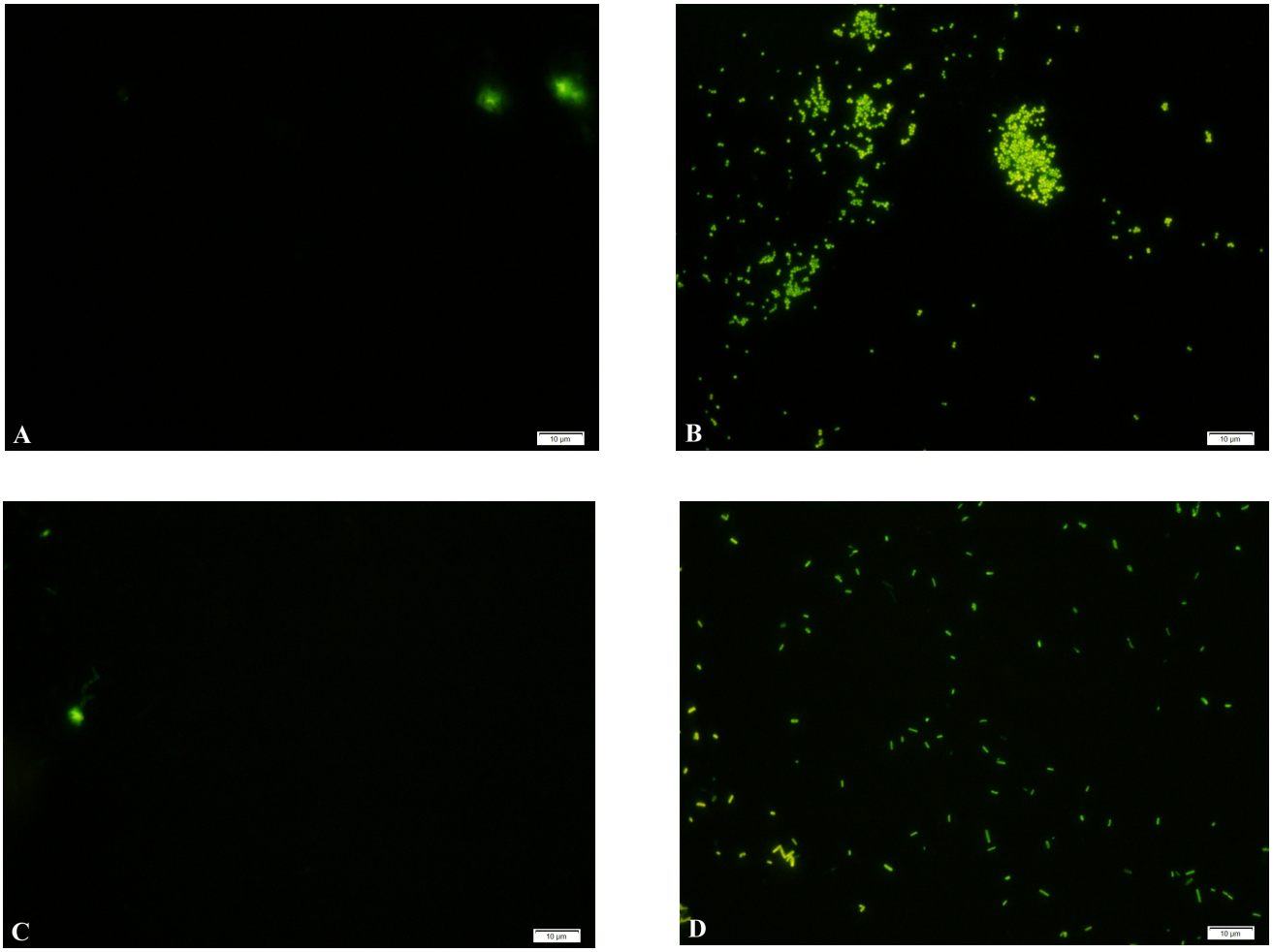

Fig. 2: Sytox green assay. (A) *Staphylococcus aureus* (control). (B) *S. aureus* (exposed to compound **1**). (C) *Escherichia coli* (control). (D) *E. coli* (exposed to compound **1**).

Table 3: IC_{50} of the crude extract, purified compounds against normal cell lines after 24 h using the MTT assay.

Test microorganisms	IC_{50}[a] values of crude extract, purified compounds on tested cell lines (µg/ml)	
	L929[b] cells	Vero cells
Crude extract	1483.65	1857.16
Compound **1**	1263.42	1537.83
Compound **2**	2640.11	2481.96
Compound **3**	2857.83	2698.45

[a]IC_{50} values represent the concentration causing 50% growth inhibition. They were determined by linear regression analysis.
[b]L929, murine fibroblast cell line; Vero, African green monkey kidney cell line.

DISCUSSION

Our research findings regarding the major compounds of *Z. cassumunar* from Thailand differ from previous reports in the literature regarding *Z. cassumunar* from other geographical regions. Bhuiyan *et al.* (2008) reported that the essential oil of *Z. cassumunar* rhizome from Bangladesh contain triquinacene 1,4-bis (methoxy), (Z)-ocimene and terpinen-4-ol as the major compounds. Taroeno and Zwaving (1991) found that the *Z. cassumunar* essential oil from Indonesia obtained by extraction with light petroleum had about 46%, with sabinene and terpinen-4-ol, *trans*-1-(3,4-dimethoxyphenyl)but-1-ene, *trans*-1-(3,4-dimethoxyphenyl)butadiene and *trans*-4-(3,4-dimethoxyphenyl) but-3-ene-1-yl acetate as the main constituents. *Z. cassumunar* from the northeast of India contained terpinen-4-ol, α- and β-pinene, sabinene, myrcene, α- and γ-terpinene, limonene, terpinolene, sabmene and mono-terpenes (Bordoloi *et al.*, 1999). In Malaysia, Kamazeri *et al.* (2012) reported the rhizome essential oil to contain 2,6,9,9-tetramethyl-2,610-cycloundecatrien-1-one and α-caryophyllene as the major compounds. *Z. cassumunar* from the northern and eastern parts of Thailand contained sabinene, terpinen-4-ol and *trans*-1-(3,4-dimethoxyphenyl)butadiene as the main component of the rhizome essential oil (Bua-in and Paisooksantivatana, 2009). In this study, therefore, *Z. cassumunar* essential oil from Thailand was found to have a significantly

different chemical composition from *Z. cassumunar* essential oil from other geographical locations. Variations in the chemical composition of the essential oils are known to differ considerably due to the existence of different subspecies. They might also be attributed to other factors such as climate, different regional geographic and seasonal conditions, metabolism of plants, stage of maturity and extraction conditions (Anwar *et al.*, 2009).

In this study, the three phenylbutenoids; were isolated from the *Z. cassumunar* crude extract. These compounds have been reported as the main active components of the essential oil isolated from the hexane extract of *Z. cassumunar* (Amatayakul, 1979). Previous research established, that the phenylbutenoid group is analgesic, anti-inflammatory, antioxidative and anticancer activities (Masuda and Jitoe, 1994; Murakami *et al.*, 2002). These phenylbutenoid compounds have phagocytosis effect that has immunostimulant activity to macrophage cells in the peritoneum of mice (Chairul and Chairul, 2009).

Phenylbutenoid compounds can resist the growth of bacteria. A phenylbutenoid is a derivative from phenol with one or more methoxy substitution. The methoxy group from the phenylbutenoid is the one that possibly interacts with bacteria. The following discussion follows the structure and sequence of a discussion of similar research by Malladi *et al.* (2017). Both projects investigated the anti-bacterial effects on specific compounds, although the compounds and geographical regions were different, the results were similar. In the present case, as in research previously undertaken by Shah and Desai (2007), the presence of methoxy groups on phenyl group improved the antibacterial activity of purified compounds, a similar observation was reported in isoxazoline derivatives (Shah and Desai, 2007). The antibacterial effects on compounds **2** and **3** were lower than on compound **1** because phenyl groups include two ortho methoxy. The lower activity was shown by compounds **2** and **3** against tested bacteria compared to compound **1** which can be attributed to the presence of only one dimethoxyphenyl group. The methoxy group affects the charge distribution which significantly improves the biological effect. It is suggested that the increased resistance noted in the presence of methoxy group is likely due to its interaction with some intracellular target. The presence of a strong electron-withdrawing group appears to alter the nature of the compound in some way which facilitates binding to the target(s) (Waring *et al*., 2002). Highest antibacterial activity was exhibited by the compound having methoxy substitution on two ortho positions.

Highest antibacterial activity was exhibited by the compound having methoxy substitution on two ortho positions. Both electrons withdrawing and donating groups on phenyl group have shown improved antimicrobial activity on the bacterial cells. The better antibacterial activity of compound **1** was observed against *S. aureus* and *E. coli*. Faqroddin *et al*. (2012) reported that electron releasing groups such as methoxy on 1-(2″,4″-dichlorophenyl)-3-(substituted aryl)-2-propene-1-ones displayed maximum antibacterial activity having against Gram-positive bacteria (*Bacillus subtilis*) and Gram-negative bacteria (*Escherichia coli*). But in this study, both Gram-positive and Gram-negative bacteria have a similar effect, this reason may be that the types of chemical compounds.

Sytox green is a cationic molecule and high-affinity nucleic acid stain that easily penetrates cells with compromised plasma membranes and yet will not cross the membranes of live cells, thus emitting intense green fluorescence after excitation between 450–490 nm radiations. These properties make Sytox green a suitable indicator for the visualization of both Gram-positive and Gram-negative bacteria with damages in cell walls or cell membranes (Langsrud and Sundheim, 1996), as those exposed to phenylbutenoid compounds.

Among the abundant bioactive constituents of *Z. cassumunar* essential oil, some previous studies have shown that sabinene and terpinene-4-ol had antibacterial activities (Wasuwat *et al.*, 1989; Giwanon *et al.*, 2000). They reveal that other compositions in rhizome extract of *Z. cassumunar* are potentially useful in medicines because they exhibit antibacterial activity.

CONCLUSION

The present study has demonstrated the comparative antibacterial activity of three purified compounds from rhizome extract of *Z. cassumunar*. Compound **1** ((*E*)-3-(3,4-dimethoxyphenyl)-4-[(*E*)-3,4-dimethoxystyryl] cyclohex-1-ene) was the most effective in comparison with the other two compounds, namely compound **2** ((*E*)-4-(3,4-dimethoxyphenyl)-but-3-en-1-ol) and compound **3** ((*E*)-4-(3,4-dimethoxyphenyl)-but-3-en-1-yl acetate). These compounds showed a bactericidal effect against tested bacteria, especially against *S. aureus* and *E. coli* with both MIC and MBC at a concentration of 16 µg/ml and 32 µg/ml, respectively. Compound **1** showed bacteriolytic effects on the tested strains, causing evident damage to cell walls and membranes.

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