Journal of Food Health and Bioenvironmental Science (September - December 2019), 12(3): 13-19

Journal of Food Health and Bioenvironmental Science
Journal homepage : http://jfhb.dusit.ac.th/

Anti-Cariogenic Activity, Cytotoxicity and Chemical Constituents of *Zingiber rubens* Roxb.

Wipawan Pukumpuang[a]* & Pratya Chaliewchalad[b]

[a] *Program of Biological Sciences, Chiang Rai Rajabhat University, Chiang Rai, 57100 Thailand*
[b] *Department of Biology, Rambhai Barni Rajabhat University, Chanthaburi, 22000 Thailand*

Article info

Article history:
Received: 9 October 2019
Revised: 6 December 2019
Accepted: 13 December 2019

Keywords:
Zingiber rubens, Antibacterial, Plant Extract, Essential Oil

Abstract

The objectives of this study were to evaluate the antibacterial activity, cytotoxicity and chemical constituents of *Zingiber rubens* Roxb. Four parts of the dried plant materials including rhizomes, stems, leaves and fruits were extracted with distilled water and 95% ethanol to obtain crude extracts. The essential oil was derived from the rhizome using steam distillation. After that, the crude extracts and essential oil were screened for antibacterial activity against *Lactobacillus casei* TISTR 390 and *Streptococcus mutans* ATCC 25175 using the agar disc diffusion and broth dilution methods. Moreover, the cytotoxicity of the plant extracts was evaluated using MTT assay on Vero cells. The results show that all ethanolic extracts could inhibit the tested bacteria with an inhibition zone ranging from 7.00–8.67 mm and MIC/MBC ranging from 6.25–50 mg/ml. Additionally, the essential oil also effectively inhibited both the bacterial strains with an inhibition zone ranging from 8.17–8.83 mm and MIC/MBC of 250 mg/ml. For *in vitro* cytotoxic properties, all plant extracts exhibited no toxicity on Vero cells with CC_{50} between 30.32 - > 1,000 µg/ml. Notably, the essential oil derived from the plant rhizome also revealed no toxicity *in vitro* with CC_{50} of 2.5 µg/ml. Furthermore, the essential oil from the rhizome identified volatile compounds using Gas Chromatography-Mass Spectrometry (GC-MS). Importantly, 2,6,10-Cycloundecatrien-1-one, 2,6,9,9-tetramethyl-, (E,E,E)- or zerumbone is the main compound in essential oil with a % peak area of 20.47 %. Thus, the crude extract and essential oil of this plant could inhibit cariogenic bacteria and display low toxicity on human cells, which may be useful in the development of an antibacterial agent in the future.

Introduction

Dental caries or tooth decay is a global public health problem. Severe caries often cause pain and infection, resulting in tooth extraction and also have an effect on general health and well–being (Veiga et al., 2016; Yadav & Prakash, 2017). The major cause of tooth decay is the colonization of tooth surfaces by cariogenic bacteria (Costa et al., 2012; Selwitz et al., 2007). Oral Streptococci, especially *Streptococcus mutans,* is one of

* Corresponding Author
e-mail: wipawan.puk@crru.ac.th

the cariogenic bacteria, most associated with initial formations of caries (Van Houte, 1994). This bacterium synthesizes extracellular polysaccharides from sucrose and produces acids, which eventually demineralizes tooth enamel (Loesche, 1986). Other microflora, such as *Streptococcus sanguis, Actinomyces* and *Lactobacillus* spp., which tolerate acidity can survive and cause severe caries (Hamada & Slade, 1980). Various chemical compounds such as alcohol, fluoride and antibiotics (chlorhexidine, erythromycin, ampicillin, penicillin) have been widely used for dental caries prevention for many years (Baker et al., 1987; Wolinsky, 1994). However, it has some side effects including brown staining of the tooth, alteration in the sensation of taste, soreness in the oral mucosa (Vieira et al., 2014). Importantly, antibiotic-resistant bacterial populations are increasing rapidly. For this reason, antimicrobial agents from natural sources may become another choice for bacterial disease treatment. Medicinal plants have long been used for treatment due to their availability, low cost, no toxic and minimal side effects compared to conventional antibiotics (Abdel-Aziz et al., 2016).

Zingiber rubens Roxb. belonging to the family Zingiberaceae, is a native plant mostly distributed over tropical and subtropical areas including Thailand, Myanmar, Vietnam, India, Bangladesh, China South-Central and East Himalaya (Ahmed, 2008). Various species are widely used as foods, spices, flavoring agents and traditional usages such as herbal drugs for the treatment of carminative, stomachache, diarrhea, stimulant and cold (Nontasit et al., 2015; Yob et al., 2011). Many plants of the Zingiberaceae family have been found to possess antibacterial properties against several pathogens. (Abdul et al., 2008; Habsah et al., 2000; Sanpa & Sanpa, 2019). Nevertheless, few studies have investigated the biological activity of *Z. rubens*. The previous studies showed that the ethanolic extract of this plant exhibited moderate antioxidant activity among other species (Kantayos & Paisooksantivatana, 2012). However, its biological capabilities in other aspects have not been much studied. Therefore, this study evaluates the antibacterial activities against some cariogenic bacteria and cytotoxic properties of crude plant extracts and essential oil of various parts of *Z. rubens*. Moreover, the chemical composition was analyzed using GC-MS analysis.

Materials and methods

1. Plant extraction

Zingiber rubens Roxb. was collected from the local area of Lamphun province, Thailand. For crude plant extraction, four parts of fresh plants including stems, rhizomes, leaves and fruits were washed and dried at 60°C. The ground plants (100 g) were extracted with distilled water at 45°C for 3 hours or macerated with 95% ethanol for 72 hours at room temperature at a proportion 1:10 (w/v). After that, the plant's extracts were filtrated, evaporated under vacuum using rotary evaporator and then lyophilized to obtain crude powder. The crude plant extracts were dissolved using dimethyl sulfoxide (DMSO) at a concentration of 100 mg/ml.

2. Essential oil extraction

Fresh rhizome was grounded (50 g) using a blender. The essential oil was obtained by steam distillation at 100°C for 3 hours with a Clevenger - type apparatus. The essential oil was separated from water using sodium sulfate anhydrous (Na_2SO_4) and stored at 4°C for further study.

3. Antibacterial activity

3.1 Agar disc diffusion assay

The antibacterial effect of plant extracts and essential oil was tested against *Lactobacillus casei* TISTR390 and *Streptococcus mutans* ATCC25175 by agar disc diffusion assay according to the guidelines of Clinical and Laboratory Standards Institute (CLSI, 2015). The tested bacteria were cultured in de Man Rogosa Sharpe broth (MRS broth) (Himedia, India) for *L. casei* and Brain heart infusion broth (BHI broth) (Himedia, India) for *S. mutans* and incubated at 37°C for 18 hours. After that, the turbidity of the bacterial cultures was adjusted to be comparable to McFarland standard No. 0.5 (Himedia, India) to obtain approximately 1.0 x 10^8 CFU/ml. The cultures of bacteria were swabbed on MRS agar for *L. casei* and BHI agar for *S. mutans*. Then, 20 µl of crude extracts (100 mg/ml) and essential oil (1,000 mg/ml) were applied on 6 mm diameter sterile paper discs (Macherey-Nagel®) and the discs were placed on the agar. 100% DMSO was used as control and chlorhexidine at a concentration of 2 mg/ml was used as a positive control. These plates were incubated at 37°C for 24 hours.

3.2 Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC)

Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) of the plant

extracts and essential oil were determined using broth dilution method with slight modifications of CLSI (2015). The plant extracts, essential oil and chlorhexidine were diluted in growth medium by two-fold serial dilutions into 96-well plate to obtain concentrations between 6.25-100 mg/ml for plant extracts, 250-1,000 mg/ml for essential oil and 0.0039-2 mg/ml for chlorhexidine. Then, the bacterial culture with 1×10^8 CFU/ml was inoculated to each well plate and incubated at 37°C for 24 hours. MIC was defined as the lowest concentration of plant extract that prevents the growth of bacterial strains.

For MBC evaluation, the well plate with no visible turbidity in MIC assay were streaked onto medium agar plates and incubated at 37°C for 24 hours. After incubation, MBC was determined as the lowest concentration of the plant extract showing no visible growth of bacterial strains.

4. Cytotoxicity assay

The cytotoxicity assay was conducted on Vero cells (African green monkey kidney cell) using the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay (Yu et al., 2004). The cells in 96-well plates were cultured in Dulbecco's modified eagle medium (D-MEM) (Gibco, USA) supplemented with 10% fetal bovine serum (HyClone, UK) and incubated at 37°C in 5% CO_2 incubator. After incubation, the growth medium was removed and replaced with 2-fold serial dilutions of plant extracts (7.81-1,000 µg/ml) and essential oil (0.078-10 µg/ml). The cell control and vehicle control dimethyl sulfoxide (DMSO) was used as a negative control. The plates were incubated at 37°C in 5% CO_2 for 72 hours. Then, the plant extracts, essential oil and growth medium were removed and the MTT reagent (5 mg/ml) (Bio Basic, Canada) was added and incubated for 4 hours. Finally, the formazan crystal blue was dissolved with 100 % DMSO and the absorbance was measured at 540 and 630 nm using microplate reader (Biochrom, UK). The percentage of viability was calculated comparing to the cell control and 50% cytotoxic dose (CC_{50}) concentration was determined using probit analysis.

5. Chemical composition analysis

The essential oil component analyses were conducted by NSTDA Characterization and Testing Service Center, National Science and Technology Development Agency (NSTDA), Thailand. The constituents of the oil were analyzed using Gas chromatography-Mass spectrometry (GC-MS). The GC-MS analysis was performed on GC-MS TQ8050 (Shimadzu, Japan) equipped with DB-5 MS columns (30 m × 0.25 mm, film thickness 0.25 µm, Agilent Technology). The analytical condition was applied by Dai et al., 2013. Helium was used as a carrier gas and was adjusted to column velocity flow of 0.69 ml/min. The injector temperature (PTV) was 250°C, detector temperature 250°C, column temperature-programmed 60°C (2 min hold) to 220°C (10 min hold) at a rate of 4°C/min. One milliliter of diluted oil sample (1:10 v/v in methanol) was injected in the split mode with split ratio 10:1 by auto-injection. Inlet pressure was 30.1 kPa. Identification of the components was achieved based on retention time and mass spectral matching with NIST/EPA/NIH Mass Spectral Library 2014.

6. Statistical analysis

The results of three replicates were reported as mean ± SD. Analysis of variance (ANOVA) was calculated using Duncan's new multiple range test at $P < 0.05$.

Results

1. Antibacterial activity

The antibacterial activity of plant extracts and essential oil were evaluated using agar disc diffusion and broth dilution method. The results were shown in Table 1 and 2. According to disc diffusion assay, all ethanolic extracts and essential oil could inhibit both tested bacteria with the inhibition zones ranging from 7.00-8.83 mm while all aqueous extracts could not inhibit any tested bacteria. However, the zones of inhibition of all plant extracts and the essential oils were smaller than that of the positive control, chlorhexidine. Moreover,

Table 1 Zone of inhibition of medicinal plant extracts against *L. casei* TISTR 390 and *S. mutans* ATCC 25175 by agar disc diffusion method

Extracts	Part used	Zone of inhibition ± SD (mm)	
		L. casei TISTR 390	*S. mutans* ATCC 25175
Aqueous (100 mg/ml)	rhizome	NZ	NZ
	Stem	NZ	NZ
	leave	NZ	NZ
	fruit	NZ	NZ
Ethanol (100 mg/ml)	rhizome	7.17 ± 0.29^{c}	7.67 ± 0.58^{cd}
	Stem	7.17 ± 0.29^{c}	8.67 ± 0.58^{b}
	leave	7.17 ± 0.29^{c}	7.17 ± 0.29^{d}
	fruit	7.00 ± 0.00^{c}	7.00 ± 0.00^{d}
Essential oil (1,000 mg/ml)	rhizome	8.83 ± 0.29^{b}	8.17 ± 0.29^{bc}
chlorhexidine (2 mg/ml)	-	27.67 ± 0.58^{a}	36.00 ± 0.00^{a}
DMSO (100%)	-	NZ	NZ

Remark: NZ; no zone of inhibition, Means with different letters[a-d] in each column are significant differences (p<0.05) for each extract

the plant extracts which inhibited the tested bacteria were further evaluated for MIC and MBC values. Table 2, shows that the ethanolic extracts of the stem had an effective antibacterial activity with MIC and MBC of 6.25 mg/ml for both bacterial species. Furthermore, the plant essential oil also shows the MIC and MBC of 250 mg/ml on *L. casei* TISTR390 and *S. mutans* ATCC25175.

Table 2 MIC and MBC values of crude extract and essential oil from *Z. rubens* against *L. casei* TISTR 390 and *S. mutans* ATCC 25175 using broth dilution method

Extracts	Part used	MIC/MBC			
		L. casei TISTR 390		*S. mutans* ATCC 25175	
		MIC	MBC	MIC	MBC
Aqueous (mg/ml)	rhizome	ND	ND	ND	ND
	stem	ND	ND	ND	ND
	leave	ND	ND	ND	ND
	fruit	ND	ND	ND	ND
Ethanol (mg/ml)	rhizome	6.25	6.25	25	25
	stem	6.25	6.25	6.25	6.25
	leave	6.25	6.25	50	50
	fruit	12.5	12.5	50	50
Essential oil (mg/ml)	rhizome	250	250	250	250
Chlorhexidine (mg/ml)	-	1	1	0.0039	0.0039

Remark: ND; not determine.

2. Cytotoxicity of plant extracts

The results of cytotoxicity of crude extracts and essential oil of various parts of *Z. rubens* to the Vero cells are presented in Table 3. For *in vitro* cytotoxic properties, the aqueous extract exhibits lower toxicity than ethanolic extract with CC_{50} > 1,000 µg/ml while the CC_{50} concentrations of ethanolic extract is between 30.32 - 377.35 µg/ml. However, all extracts show a CC_{50} more than 20 µg/ml, which is considered to be nontoxic as regarded by the US National Cancer Institute (NCI).

3. Chemical constituents of essential oil

The yield of essential oil obtained from rhizomes by steam distillation was 0.21 % (w/w) with yellow color and an agreeable smell. Eighty-seven constituents identified by GC-MS analysis are represented in Table 4. The major constituents of oils are 2,6, 10-Cycloundecatrien-1-one, 2,6,9,9-tetramethyl-, (E,E,E)-, commonly known as zerumbone (20.47%), 1,4,7,-cycloundecatriene, 1,5,9,9-tetramethyl-, Z,Z,Z- (7.31%), cyclohexene, 4-ethenyl-4-methyl-3-(1-methylethenyl)-1-(1-methylethyl)-, (3R-trans)- (6.90%), (1R,2S,6S,7S,8S)-8-Isopropyl-1-methyl-3-methylenetricyclo [4.4.0.02,7] decane-rel- (6.12%) and isospathulenol (4.11%).

Table 3 Cytotoxicity of crude extracts and essential oil of *Z. rubens* to the Vero cell

Extracts	Part used	50% cytotoxic concentration, CC_{50}
Aqueous	Rhizome	> 1,000 µg/ml
	Stem	> 1,000 µg/ml
	Leave	> 1,000 µg/ml
	Fruit	> 1,000 µg/ml
Ethanolic	Rhizome	30.32 µg/ml
	Stem	66.66 µg/ml
	Leave	377.35 µg/ml
	Fruit	147.58 µg/ml
Essential oil	Rhizome	2.50 µg/ml

Table 4 The main components and peak-area percentage (%) of essential oil of *Z. rubens*

Peak	RT (min)	% Area	Identification of the compounds
1	6.968	0.07	Bicyclo[3.1.0]hex-2-ene, 2-methyl-5-(1-methylethyl)-
2	7.220	0.95	(1R)-2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene
3	7.732	0.90	Camphene
4	8.398	0.12	Bicyclo[3.1.0]hexane, 4-methylene-1-(1-methylethyl)-
5	8.624	2.67	Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene-, (1S)-
6	8.863	0.35	.beta.-Myrcene
7	9.511	2.12	.alpha.-Phellandrene
8	9.576	0.68	Santolina triene
9	9.818	0.08	(+)-4-Carene
10	10.088	0.88	o-Cymene
11	10.248	0.61	D-Limonene
12	10.363	0.67	Eucalyptol
13	10.767	0.19	1,3,6-Octatriene, 3,7-dimethyl-, (Z)-
14	11.214	0.16	.gamma.-Terpinene
15	12.168	0.26	Cyclohexene, 3-methyl-6-(1-methylethylidene)-
16	12.328	0.05	Fenchone
17	12.637	0.06	Linalool
18	14.437	0.35	(+)-2-Bornanone
19	15.305	0.11	Bicyclo[2.2.1]heptan-2-ol, 1,7,7-trimethyl-, (1S-endo)-
20	15.592	0.10	3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)-, (R)-
21	16.107	0.12	.alpha.-Terpineol
22	17.917	0.12	5,8-Decadien-2-one, 5,9-dimethyl-, (E)-
23	19.222	0.05	Bicyclo[2.2.1]heptan-2-ol, 1,7,7-trimethyl-, acetate, (1S-endo)-
24	20.724	0.05	7-Oxabicyclo[4.1.0]heptane, 1-methyl-4-(2-methyloxiranyl)-
25	21.115	6.90	Cyclohexene, 4-ethenyl-4-methyl-3-(1-methylethenyl) -1-(1-methylethyl)-, (3R-trans)-
26	21.385	0.27	.alpha.-Cubebene
27	22.131	0.07	1,2,4-Metheno-1H-indene, octahydro-1,7a-dimethyl -5-(1-methylethyl)-, [1S-(1.alpha.,2.alpha.,3a.beta., 4.alpha.,5.alpha.,7a.beta.
28	22.358	0.82	.alfa.-Copaene
29	22.532	0.08	Cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-, [1S-(1.alpha.,2.beta.,4.beta.)]-
30	22.804	1.71	Cyclohexane, 1-ethenyl-1-methyl-2,4-bis (1-methylethenyl)-, [1S-(1.alpha.,2.beta.,4.beta.)]-
31	23.180	0.19	(1S,5S)-2-Methyl-5-((R)-6-methylhept-5-en-2-yl) bicyclo[3.1.0]hex-2-ene
32	23.387	0.30	1H-Cyclopropa[a]naphthalene, 1a, 2, 3, 3a, 4, 5, 6, 7b-octahydro-1,1,3a,7-tetramethyl-, [1aR-(1a.alpha., 3a.alpha.,7b.alpha.)]-
33	23.842	1.72	Caryophyllene
34	24.085	1.02	1,5-Cyclodecadiene, 1,5-dimethyl-8- (1-methylethylidene)-, (E,E)-
35	24.283	0.22	.alpha.-Maaliene
36	24.485	0.52	(1R,3aS,8aS)-7-Isopropyl-1,4-dimethyl-1, 2, 3, 3a, 6, 8a-hexahydroazulene
37	24.776	1.14	(1S,4S,4aS)-1-Isopropyl-4, 7-dimethyl-1, 2, 3, 4, 4a, 5-hexahydronaphthalene
38	25.135	7.31	1,4,7,-Cycloundecatriene, 1,5,9,9-tetramethyl-, Z,Z,Z-
39	25.638	1.50	.gamma.-Muurolene

Table 4 The main components and peak-area percentage (%) of essential oil of *Z. rubens* (Cont.)

Peak	RT (min)	% Area	Identification of the compounds
40	25.911	6.12	(1R,2S,6S,7S,8S)-8-Isopropyl-1-methyl-3-methylenetricyclo[4.4.0.02,7]decane-rel-
41	26.183	1.65	Benzene, 1,1'-(1,1,2,2-tetramethyl-1,2-ethanediyl) bis-
42	26.256	1.39	(1S,2E,6E,10R)-3,7,11,11-Tetramethylbicyclo[8.1.0] undeca-2,6-diene
43	26.462	0.44	isoledene
44	26.609	0.96	Cyclohexane, 1-ethenyl-1-methyl-2,4-bis (1-methylethenyl)-, [1S-(1.alpha.,2.beta.,4.beta.)]-
45	26.961	0.86	Naphthalene, 1,2,4a,5,8,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, [1S-(1.alpha.,4a.beta.,8a.alpha.)]-
46	27.268	079	Cyclohexene, 4-ethenyl-4-methyl-3-(1-methylethenyl)-1-(1-methylethyl)-, (3R-trans)-
47	27.506	0.44	Cyclohexane, 1-methyl-2,4-bis(1-methylethenyl)-, (1.alpha.,2.beta.,4.beta.)-
48	27.635	0.10	Selina-3,7(11)-diene
49	27.848	0.27	Cyclohexanemethanol, 4-ethenyl-.alpha.,.alpha., 4-trimethyl-3-(1-methylethenyl)-, [1R-(1.alpha.,3.alpha., 4.beta.)]-
50	27.995	0.23	7-epi-cis-sesquisabinene hydrate
51	28.266	3.64	1,5-Cyclodecadiene, 1,5-dimethyl-8-(1-methylethylidene)-, (E,E)-
52	28.486	0.06	1,3,12-Nonadecatriene
53	28.729	0.35	Isospathulenol
54	28.913	1.50	Caryophyllene oxide
55	29.032	0.63	Isospathulenol
56	29.444	2.90	1,4,7,-Cycloundecatriene, 1,5,9,9-tetramethyl-, Z,Z,Z-
57	29.56	0.32	Ledol
58	29.776	2.51	(1R,3E,7E,11R)-1,5,5,8-Tetramethyl-12-oxabicyclo [9.1.0]dodeca-3,7-diene
59	30.002	0.38	(3R, 3aR, 3bR, 4S,7R, 7aR)-4-Isopropyl-3, 7-dimethyloctahydro-1H-cyclopenta[1,3]cyclopropa[1,2] benzen-3-ol
60	30.323	4.41	Isospathulenol
61	30.559	1.68	(-)-Spathulenol
62	30.810	0.75	Eudesma-4(15),7-dien-1.beta. -ol
63	31.091	1.30	.tau.-Muurolol
64	31.334	4.11	Cyclohexene, 4-pentyl-1-(4-propylcyclohexyl)-
65	31.570	0.54	Cyclohexanemethanol, 4-ethenyl-.alpha.,.alpha., 4-trimethyl-3-(1-methylethenyl)-, [1R-(1.alpha., 3.alpha.,4.beta.)]-
66	32.074	0.19	3, 7-Cyclodecadien-1-one, 3, 7-dimethyl-10-(1-methylethylidene)-, (E,E)-
67	32.226	0.12	Curlone
68	33.692	20.47	2,6,10-Cycloundecatrien-1-one, 2,6,9,9-tetramethyl-, (E,E,E)-
69	33.895	0.61	(-)-Globulol
70	34.087	0.18	2-((2R,4aR,8aR)-4a,8-Dimethyl-1,2,3,4,4a,5,6, 8a-octahydronaphthalen-2-yl)prop-2-en-1-ol
71	34.291	0.72	Isospathulenol
72	34.442	0.79	Isospathulenol
73	34.700	0.41	3-Buten-2-one, 4-(6,6-dimethyl-1-cyclohexen-1-yl)-
74	34.854	0.18	11-Hydroxy-11-methyl-tricyclo[4.3.1.1(2,5)] undecan-10-one
75	34.949	0.05	Acetic acid, 1-[2-(2,2,6-trimethyl-bicyclo [4.1.0]hept-1-yl)-ethyl]-vinyl ester
76	35.080	0.15	Ambrial
77	36.066	0.47	9,19-Cyclolanostan-3-ol, acetate, (3.beta.)-
78	36.637	0.05	geranyl-.alpha.-terpinene
79	38.172	0.06	Longifolenaldehyde
80	39.511	0.05	(E)-15,16-Dinorlabda-8(17),11-dien-13-one
81	39.639	0.15	Trachylobane
82	40.171	0.04	cis-Thujopsene
83	40.586	0.12	geranyl-.alpha.-terpinene
84	41.861	0.03	Squalene
85	42.912	0.14	Coronarin E
86	43.093	0.05	4,8,13-Cyclotetradecatriene-1,3-diol, 1,5,9-trimethyl-12-(1-methylethyl)-
87	43.993	1.14	Thunbergol

Discussion

The results of antibacterial activity shows that all ethanolic extracts from rhizomes, stems, leaves and fruits of *Z. rubens* could inhibit both *L. casei* TISTR 390 and *S. mutans* ATCC 25175 at different levels. The present study demonstrates the highest antibacterial activity of stem extract against cariogenic bacteria. However, all aqueous extracts could not inhibit any bacterial strains. The difference in effects between aqueous and ethanolic extracts of the plant might be due to the polarity of solvent extraction that generated various antimicrobial components. Alcohol might solubilize the wider range of compounds in medicinal plants than water. Alcohol is mostly used for extraction of various polar compounds but a certain group of nonpolar compounds was also soluble (Tiwari et al., 2011). The antibacterial activity of *Zingiber* spp. extracts could be expected to be the compounds like flavonoids and volatile oils which dissolved in organic solvents, so the ethanolic extract could have a greater antibacterial activity. Additionally, the essential oil from *Z. rubens* rhizome also inhibited against both bacterial pathogens with inhibition zones of 8.17 and 8.83 mm. According to the GC/MS analysis in this study, zurumbone (20.47%) was found to be the major component of *Z. rubens* oil. This finding is different from the previous study that reported that the major components of *Z. rubens* root oil collected from Vietnam using water distillation were (Z)-citral (30.1%), camphene (9.7%), β-phellandrene (7.5%), 1,8-cineole (7.0%) and zingiberene (5.3%) (Dai et al., 2013). Nonetheless, the composition of the essential oil depended on parts used, geographical distribution and different stages of plant species (Chamorro et al., 2012; Johnson et al., 2004). Zurumbone was previously known for its antimicrobial activity. This compound at concentration between 0.13 - 13 mg/ml, isolated from *Z. zerumbet* could inhibit *Salmonella cholerasuis* by agar disc diffusion method (Abdul et al., 2008). Likewise, the zurumbone derived from *Z. zerumbet* showed MIC of 250 μg/ml MBC of 500 μg/ml against *S. mutans* ATCC 35668 (da Silva et al., 2018). The cytotoxicity assay is one of the reference materials for evaluating the safety screening of bioactive compounds. The current study shows that the ethanolic extracts (30.32-377.35 μg/ml) gives a higher cytotoxicity effect on Vero cells than aqueous extracts (CC_{50}>1,000 μg/ml). Also, the essential oil from rhizomes is not toxic to *in vitro* cells with CC_{50} of 2.5 μg/ml. According to the US National Cancer

Institute, a crude plant extract with CC_{50} less than 20 µg/ml is regarded as having cytotoxicity (Boik, 2001). Therefore, it is clear that the plant extracts and essential oil of *Z. rubens* has no cytotoxicity against normal mammalian cells. However, the *in vitro* cytotoxicity testing was a close system and direct exposure of the cells to bioactive molecules might lead to a high cytotoxicity (Di Nunzio et al., 2017). Furthermore, the chemical kinetics such as absorption, distribution and excretion of the compounds might affect cytotoxic properties of an *in vivo* study (Freshney, 2000). Therefore, the cytotoxicity should also be investigated *in vivo* in the animal models to confirm the effect of extracts in further studies.

Conclusion

In summary, this study suggests that the ethanolic extract and the essential oil of *Z. rubens* shows a promising antibacterial activities against cariogenic species and has a low cytotoxicity. The result suggests that *Z. rubens* might be responsible for the prevention of dental caries.

Acknowledgments

The authors are thankful to the National Research Council of Thailand (NRCT) and Chiang Rai Rajabhat University for funding. We also thank the Chiang Rai Rajabhat University Faculty of Science and Technology for providing laboratory facility.

References

Abdel-Aziz, S. M., Aeron, A., & Kahil, T. A. (2016). Health benefits and possible risks of herbal medicine. In N. Garg, S.M. Abdel-Aziz, & A. Aeron (Eds.), *Microbes in Food and Health* (pp. 97-116). Cham, Switzerland: Springer.

Abdul, A.B., Abdelwahab, S.I., Al-Zubairi, A.S., Elhassan, M.M., & Murali, S.M. (2008). Anticancer and antimicrobial activities of zerumbone from the rhizomes of *Zingiber zerumbut. International Journal of Pharmacology, 4*(4), 301-304.

Ahmed, Z.U., Begum, Z.T., Hassan, M.A., Khondker, M., Kabir, S.M.H., Ahmad, M.A.T.A., ... & Haque, E. U. (2008). Encyclopedia of flora and fauna of Bangladesh. *Asiatic Society of Bangladesh, Dhaka.*

Baker, P. J., Coburn, R. A., Genco, R. J., & Evans, R. T. (1987). Structural determinants of activity of chlorhexidine and alkyl bisbiguanides against the human oral flora. *Journal of dental research, 66*(6), 1099-1106.

Boik, J. (2001). *Natural compounds in cancer therapy* (Vol. 25). Princeton, MN: Oregon Medical Press.

Chamorro, E.R., Zambón, S.N., Morales, W.G., Sequeira, A. F., & Velasco, G.A. (2012). Study of the chemical composition of essential oils by gas chromatography. *Gas chromatography in plant science, wine technology, toxicology and some specific applications, 1,* 307-324.

Clinical and Laboratory Standards Institute, CLSI. (2015). *M02-A12 performance standards for antimicrobial disk susceptibility tests; approved standard* (12th ed.). Wayne, USA: Clinical and Laboratory Standards Institute.

Costa, S.M., Martins, C.C., Bonfim, M.D.L.C., Zina, L.G., Paiva, S.M., Pordeus, I.A., & Abreu, M.H. (2012). A systematic review of socioeconomic indicators and dental caries in adults. *International journal of environmental research and public health, 9*(10), 3540-3574.

Dai, D.N., Thang, T.D., Chau, L.T., & Ogunwande, I.A. (2013). Chemical constituents of the root essential oils of *Zingiber rubens* Roxb., and *Zingiber zerumbet* (L.) Smith. *American Journal of Plant Sciences, 4*(01), 7-10.

da Silva, T.M., Pinheiro, C.D., Orlandi, P.P., Pinheiro, C.C., & Pontes, G.S. (2018). Zerumbone from *Zingiber zerumbet* (L.) smith: a potential prophylactic and therapeutic agent against the cariogenic bacterium *Streptococcus mutans*. *BMC complementary and alternative medicine, 18*(1), 301.

Di Nunzio, M., Valli, V., Tomás-Cobos, L., Tomás-Chisbert, T., Murgui-Bosch, L., Danesi, F., & Bordoni, A. (2017). Is cytotoxicity a determinant of the different in vitro and in vivo effects of bioactives?. *BMC complementary and alternative medicine, 17*(1), 453.

Freshney, R.I. (2000). *Culture of Animal Cells: A Manual of Basic Technique.* NY, USA : John Wiley & Sons Inc.

Habsah, M., Amran, M., Mackeen, M.M., Lajis, N.H., Kikuzaki, H., Nakatani, N., ... & Ali, A.M. (2000). Screening of Zingiberaceae extracts for antimicrobial and antioxidant activities. *Journal of Ethnopharmacology, 72*(3), 403-410.

Hamada, S., & Slade, H.D. (1980). Biology, immunology, and cariogenicity of *Streptococc mutans. Microbiological reviews, 44*(2), 331.

Johnson, C.B., Kazantzis, A., Skoula, M., Mitteregger, U., & Novak, J. (2004). Seasonal, populational and ontogenic variation in the volatile oil content and composition of individuals of *Origanum vulgare* subsp. Hirtum, assessed by GC headspace analysis and by SPME sampling of individual oil glands. *Phytochemical Analysis: An International Journal of Plant Chemical and Biochemical Techniques, 15*(5), 286-292.

Kantayos, V., & Paisooksantivatana, Y. (2012). Antioxidant activity and selected chemical components of 10 *Zingiber* spp. in Thailand. *Journal of Developments in Sustainable Agriculture, 7*(1), 89-96.

Loesche, W. J. (1986). Role of *Streptococcus mutans* in human dental decay. *Microbiological reviews, 50*(4), 353-380.

Nontasit, N., Kanlayanapaphon, C., Mekanawakul, M., & Nualmangsar, O. (2015). Taxonomic studies and traditional uses of Zingiberaceae in Khao Luang national park, Nakhon Si Thammarat Province, Thailand. *Walailak Journal of Science and Technology, 12*(8), 643-658.

Sanpa, S., & Sanpa, S. (2019). Antimicrobial activity of edible plant extracts against skin infection pathogens. *Journal of Food Health and Bioenvironmental Science, 12*(2), 34-39.

Selwitz, R.H., Ismail, A.I., & Pitts, N.B. (2007). Dental caries. *The Lancet, 369*(9555), 51-59.

Tiwari, P., Kumar, B., Kaur, M., Kaur, G., & Kaur, H. (2011). Phytochemical screening and extraction: a review. *Internationale pharmaceutica sciencia, 1*(1), 98-106.

Van Houte, J. (1994). Role of micro-organisms in caries etiology. *Journal of dental research, 73*(3), 672-681.

Veiga, N., Aires, D., & Douglas, F. (2016). Dental caries: A review. *Journal of Dental and Oral Health, 3*(1), 2.

Vieira, D. R., Amaral, F. M., Maciel, M. C., Nascimento, F. R., Libério, S. A., & Rodrigues, V. P. (2014). Plant species used in dental diseases: ethnopharmacology aspects and antimicrobial activity evaluation. *Journal of ethnopharmacology, 155*(3), 1441-1449.

Wolinsky, L. E. (1994). Caries and cardiology. *Oral microbiology and immunology,* 341-359.

Yadav, K., & Prakash, S. (2017). Dental caries: A microbiological approach. *Journal of Clinical Infectious Diseases & Practice, 2*(1), 1-15.

Yob, N. J., Jofrry, S. M., Affandi, M. M. R., Teh, L.K., Salleh, M. Z., & Zakaria, Z. A. (2011). *Zingiber zerumbet* (L.) Smith: a review of its ethnomedicinal, chemical, and pharmacological uses. *Evidence-Based Complementary and Alternative Medicine, 2011, 1-12.*

Yu, Z., Li, W., & Liu, F. (2004). Inhibition of proliferation and induction of apoptosis by genistein in colon cancer HT-29 cells. *Cancer Letters, 215*(2), 159-166.