

Limonene and eucalyptol rich essential oils with their antimicrobial activity from the leaves and rhizomes of *Conamomum vietnamense* N.S. Lý & T.S. Hoang (Zingiberaceae)

Danh Duc Nguyen¹, Hieu Nguyen-Ngoc², Hieu Tran-Trung³,
Dang-Khoa Nguyen¹, Lien-Thuong Thi Nguyen¹

¹ Institute of Applied Technology, Thu Dau Mot University, 06 Tran Van On, Phu Hoa Ward, Thu Dau Mot City 75100, Binh Duong, Vietnam

² Faculty of Pharmacy, PHENIKAA University, Hanoi 12116, Vietnam

³ Department of Chemistry, College of Education, Vinh University, 182 Le Duan, Vinh City, Nghean 43000, Vietnam.

Corresponding author: Lien-Thuong Thi Nguyen (thuongntl@tdmu.edu.vn)

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Abstract

Conamomum vietnamense, a new species of Zingiberaceae family, has been discovered and described from Tay Nguyen (Central Highlands, Vietnam) in 2022. The present study described the preparation of essential oil from leaves and rhizomes of the plant by hydrodistillation process. Then, the chemical composition of these essential oils was analyzed by Gas Chromatography-Mass Spectrometry (GC-MS), which indicated that limonene (18.74 and 26.20%) and eucalyptol (40.47 and 49.49%) were the main components, respectively. The essential oils also showed moderate antimicrobial activities against Gram-positive bacterial strains (*Enterococcus faecalis* ATCC 299212, *Staphylococcus aureus* ATCC 25923, *Bacillus cereus* ATCC 14579), Gram-negative bacterial strains (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella enterica* ATCC 13076), and a pathogenic yeast (*Candida albicans* ATCC 10231) in the MIC range of 32–256 µg/mL, which was comparable to those of positive controls, streptomycin and cycloheximide. For the first time, the chemical composition and antimicrobial activity of the essential oil of *C. vietnamense* were studied.

Keywords

Conamomum vietnamense, GC-MS, essential oil, limonene, eucalyptol, antimicrobial activity

Introduction

The genus *Conamomum* Ridl. is a small genus in the Zingiberaceae family with only 12 accepted species worldwide, mainly occurring in evergreen lowland and montane forests in Southeast Asian countries, such as Vietnam, Cambodia, Thailand, Malaysia, Singapore, Sumatra and Borneo (Boer et al. 2018; Luu et al. 2019;

Ngoc-Sam et al. 2022). Before 2018, the genus *Conamomum* had been a synonym of the genus *Amomum* Roxb., however, based on the combination of molecular (nrITS and *matK*) and morphological evidence, the botanist De Boer proposed *Conamomum* as a separate genus (Boer et al. 2018). To date, there have been a limited number of studies on the essential oils of *Conamomum* species and their bioactivities. *Conamomum rubidium* (Lamxay

& N.S.Lý) Škorničk. & A.D.Poulsen was described and studied essential oil before the combination of the genus. Therefore, relevant documents mentioned this species under the old name *Amomum rubidum*. *C. rubidum* leaves mainly contained 1,8-cineole (37.7%), δ -3-carene (19.5%) and limonene (16.3%) while the stem part was found to contain δ -3-carene (21.9%), limonene (17.8%) and β -phellandrene (14.6%). The oils were also shown to inhibit the growth of *Candida albicans* and *Fusarium oxysporum* with MIC values of 50 μ g/mL (Le et al. 2021). The essential oil prepared from rhizome was found to have similar components to that of stem, specifically β -phellandrene (16.1%), limonene (14.4%), and δ -3-carene (13.9%). The rhizome oil exhibited larvicidal activity against *Aedes aegypti* (LC₅₀ values of 31.44 μ g/mL and 31.03 μ g/mL (Huong et al. 2020), and antimicrobial activities against *Escherichia coli* and *Fusarium oxysporum* with MIC values of 50 μ g/mL (Le et al. 2019). The newest species of this genus, *C. vietnamense* (Fig. 1) was described in Tay Nguyen (Central Highlands), Vietnam (Ngoc-Sam et al. 2022). The plant is most similar to *C. odorum* Luu,

Tran & Tran and *C. rubidium*, except for well-developed stilt roots, elliptic leaf blades, narrowly ovate bracts, abaxially pubescent bracteoles, longer calyx with two truncate lobes, broadly obovate to orbicular glabrous labellum, longer filament, and glabrous style (Ngoc-Sam et al. 2022). In this study, we report for the first time chemical composition of essential oil prepared from *C. vietnamense* leaves and rhizomes and their antimicrobial activity.

Materials and methods

Plant materials

The fresh leaves and rhizomes of *Conamomum vietnamense* were collected from Loc Bac, Bao Lam District (11°47'31.9"N, 107°35'47.2"E), Lam Dong Province, Vietnam in September 2022. The plant was identified by the first author based on the protologue (Ngoc-Sam et al. 2022), and a voucher specimen (HC015) was deposited at the Institute of Applied Technology, Thu Dau Mot University.

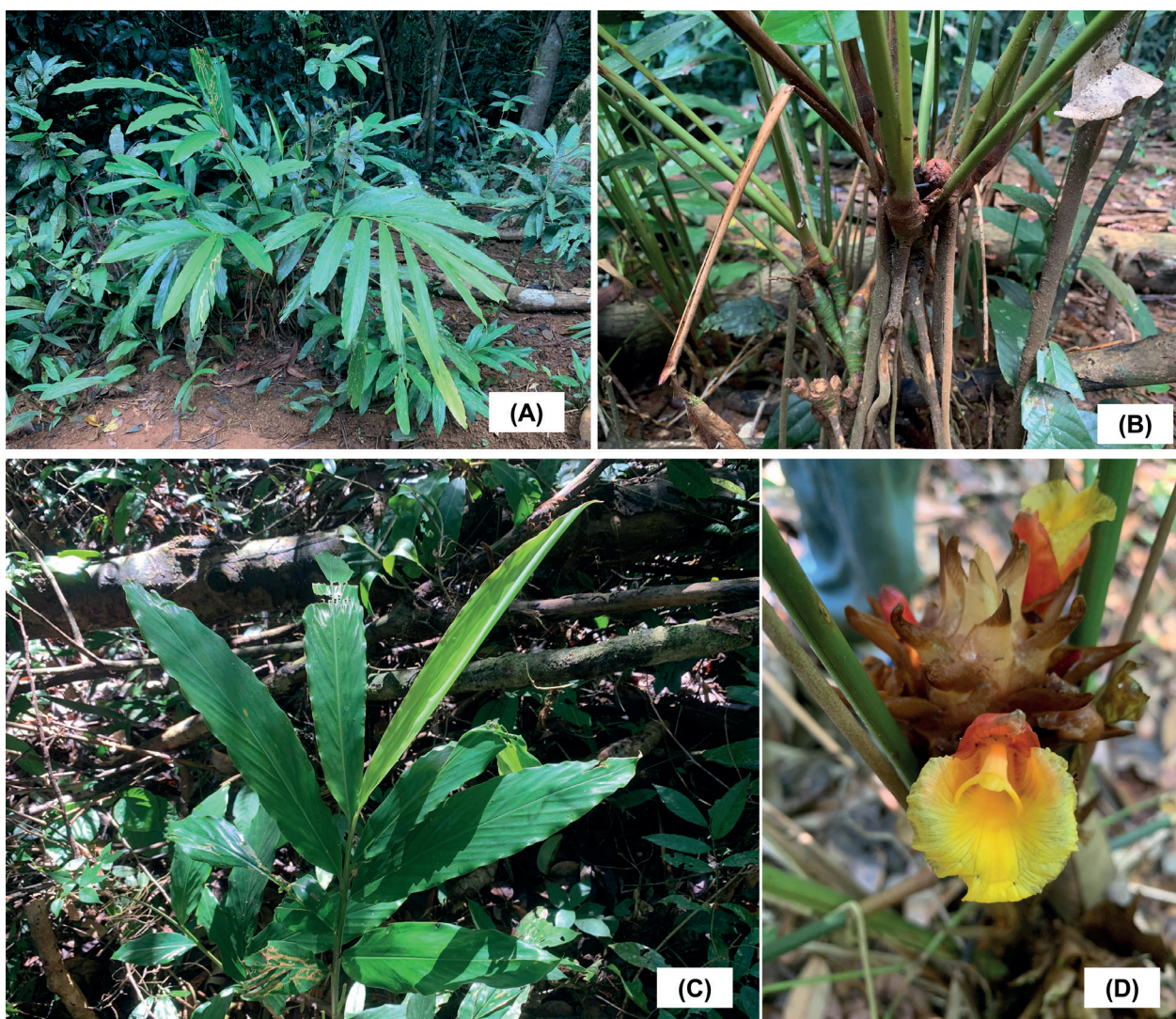


Figure 1. *Conamomum vietnamense*. A. Aerial parts; B. Rhizomes and stilt roots; C. Leaves; D. Flower. Photos taken by Cuong Quang Truong from specimen HC015.

Preparation of essential oil

The scraps of leaves (500 g) and rhizomes (500 g) of *Conamomum vietnamense* were hydro-distilled for 3 h (beginning from the water boiling point) using a Clevenger-type apparatus, according to the Vietnamese Pharmacopoeia (The Committee of Vietnamese Pharmacopoeia 2017). Then, the obtained essential oil was removed from all water traces with Na_2SO_4 and stored in sealed glass vials at 4 °C. The oil yield (%) was calculated by dividing the weight (g) of the essential oil over the weight (g) of the fresh samples.

GC-MS analysis

The essential oils of *Conamomum vietnamense* were analyzed for their constituents via Gas chromatographic/mass spectrometry using an Agilent GC-7980 linked to an Agilent MS 5977C system working in EI mode, with an HP-5MS UI column (30 m × 0.25 mm id. × 0.25 µm film thickness, Agilent Technologies). System installation: carrier gas was Helium (flow rate of 1 mL/min); injection volume: 1 µL, split ratio 1:50; the temperature was programmed from 60 °C (kept for 1 min) to 240 °C (kept for 4 min) at a rate of 4 °C/min; injector temperature: 300 °C, MS Quad temperature: 150 °C, transfer line temperature: 300 °C, MS source: 230 °C; ionization energy: 70 eV, and mass range: 50–550 amu (2.0 scan/s). Identification of the oil components was performed based on comparing their mass spectra value to that of the NIST14 library and then confirmed by comparing retention indices with reference to a homologous series of n-alkanes. The percentage of the relative peak area was used for quantification.

Antimicrobial assay

The anti-microbial bioassays were performed on Gram-positive bacterial strains (*Enterococcus faecalis* ATCC 299212, *Staphylococcus aureus* ATCC 25923, *Bacillus cereus* ATCC 14579), and Gram-negative bacterial strains (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella enterica* ATCC 13076), and a pathogenic yeast (*Candida albicans* ATCC 10231). All strains were provided by National Institute for Food Control (No. 65 Pham Than Duat Street, Mai Dich Ward, Cau Giay District, Ha Noi, Vietnam). The antimicrobial activity of the essential oil prepared from the leaves and rhizomes of *C. vietnamense* was assessed by microdilution broth susceptibility assay method, which strictly followed the standards issued by the Clinical and Laboratory Standards Institute (CLSI supplement M100, 2020) (Clinical and Laboratory Standards Institute 2020). Luria Bertani Agar (LBA) and Sabouraud Dextrose Agar (SDA) were used to culture bacteria and yeast on 96-well plates, respectively. Streptomycin and cycloheximide were used as positive controls for bacteria and yeast, respectively, at the concentration range of 2–256 µg/mL, which were provided by the Institute of Drug Quality Control in Ho Chi

Minh City, Vietnam. The experimental design was conducted as follows: (1) the essential oils were diluted in 10% DMSO and prepared in eight different concentrations (2, 4, 8, 16, 32, 64, 128, 256 µg/mL). The bacteria and fungi (yeast) were standardized with a concentration of 2×10^5 CFU/mL. The mixtures of microbiological and tested samples were incubated at 37 °C/18–24h for bacteria and at 35–37 °C/36–48h for yeast. After 24h, the minimum inhibitory concentration (MIC) value was observed and preliminarily determined as the lowest reagent concentration that completely inhibited microbial growth after 24h of culture. Additionally, the IC_{50} values were also calculated based on the turbidity measurement data by Biotek spectrophotometer (USA) and RawData software (Brussels, Belgium) (Huong et al. 2021, 2022). The experiments were performed in triplicate.

The microbial inhibition rate expressed as a percentage of inhibition was calculated using the following formula (Huong et al. 2021, 2022):

$$\% \text{ inhibition} = \frac{A_o - A_t}{A_p - A_{oc}} \times 100\%$$

$$\text{IC}_{50} = H_c - \frac{(H_i - 50\%) \times (H_c - L_c)}{(H_i - L_i)}$$

Where: A_o : absorbance of blank sample. A_{oc} : absorbance of the culture medium without cells. A_t : absorbance of the test sample. H_c and L_c : high and low concentration (%) of test agent, respectively. H_i and L_i : inhibition percentage at high and low concentrations, respectively.

Results and discussion

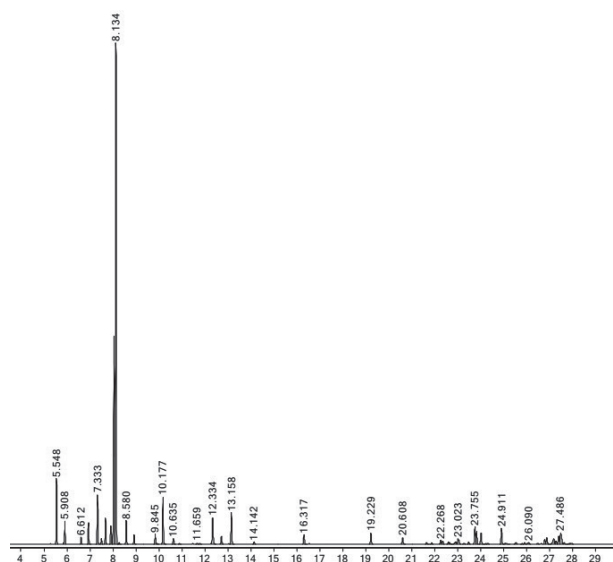
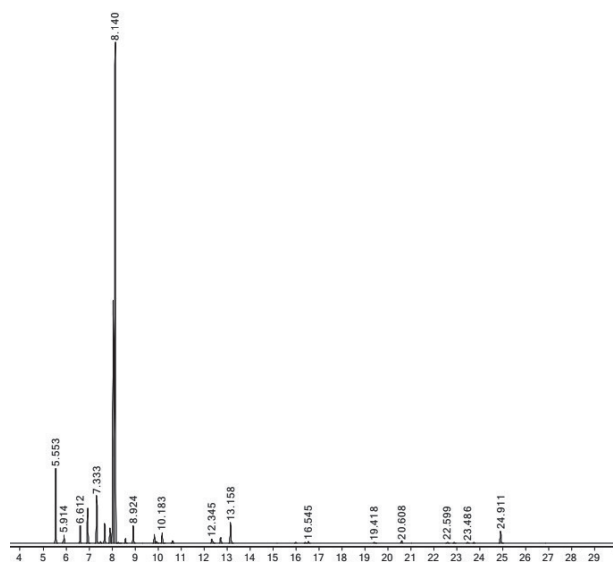
The yields of the hydrodistilled essential oils from the leaves and rhizomes of *Conamomum vietnamense* were 0.43% and 0.37% (w/w, fresh weight), respectively. The chemical composition of essential oils was analyzed and presented in Table 1, whereby there were a total of 52 (in the rhizome oil) and 28 (in the leaf oil) identifiable compounds, representing 99.35% and 99.83% of the volatile content. As can be seen from Table 1, the major compositions of both oil samples were almost similar. Specifically, it is noticeable that eucalyptol was a dominant component in rhizome and leaf oil samples with 40.47% and 49.49% of the total content, respectively. Following that, limonene was identified as the second-highest oil component with contents of 18.74% and 26.20%. Two monoterpenes, α -pinene and α -phellandrene, were also present in both oils with similar percentages (3.30–4.91%). GC-MS chromatograms of leaf and rhizome oil samples from *C. vietnamense* can be found in Figs 2, 3.

The two oil samples were then evaluated for their antimicrobial activities against several bacterial and fungal strains. The investigated strains included *Enterococcus faecalis* (causing endocarditis, sepsis, urinary tract infections, and meningitis), *Staphylococcus aureus* (causing bacteraemia, infective endocarditis), *Bacillus cereus* (causing food-

Table 1. Chemical compositions of essential oil distilled from *C. vietnamense* rhizomes and leaves.

No.	Compounds	RT (min)	RI (calc)	RI (db)	Concentration (%)	
					Rhizomes	Leaves
1	α -Pinene	5.548	939	937	3.60	4.91
2	Camphene	5.908	955	952	1.42	0.66
3	β -Pinene	6.612	981	979	0.42	1.27
4	β -Myrcene	6.938	993	991	1.25	2.36
5	α -Phellandrene	7.333	1007	1005	3.30	3.77
6	3-Carene	7.499	1013	1011	0.34	0.09
7	α -Terpinene	7.676	1020	1017	1.70	1.47
8	p-Cymene	7.905	1029	1025	1.33	1.29
9	Limonene	8.048	1034	1030	18.74	26.20
10	Eucalyptol	8.134	1037	1032	40.47	49.49
11	<i>cis</i> - β -Ocimene	8.271	1042	1038	0.11	0.07
12	<i>trans</i> - β -Ocimene	8.58	1052	1049	1.50	0.34
13	γ -Terpinene	8.924	1063	1060	0.64	1.37
14	Terpinolene	9.845	1091	1088	0.74	0.71
15	2-Nonanone	9.937	1094	1092	0.05	0.16
16	Linalool	10.177	1100	1099	3.23	0.87
17	exo-Fenchol	10.635	1116	1115	0.40	0.18
18	Camphor	11.659	1149	1145	0.10	-
19	Camphene hydrate	11.779	1153	1148	0.07	-
20	endo-Borneol	12.334	1170	1167	2.19	0.49
21	Terpinen-4-ol	12.723	1181	1177	0.58	0.46
22	α -Terpineol	13.158	1193	1189	2.43	1.84
23	Fenchyl acetate	14.142	1224	1123	0.17	-
24	Bornyl acetate	16.317	1289	1285	0.74	-
25	Dihydroedulan	16.408	1291	1293	-	0.06
26	2-Undecanone	16.545	1295	1294	-	0.15
27	α -Copaene	19.229	1382	1376	0.87	-
28	Methyl cinnamate	19.418	1385	1380	-	0.08
29	Caryophyllene	20.608	1423	1419	0.57	0.21
30	Humulene	21.661	1458	1454	0.14	-
31	Alloaromadendrene	21.884	1465	1461	0.12	-
32	Cadina-1(6),4-diene	22.268	1478	1481	0.33	-
33	γ -Muurolene	22.348	1480	1477	0.3	-
34	Aristolochene	22.599	1488	1487	0.21	0.1
35	β -Eudesmene	22.657	1490	1486	0.16	-
36	α -Selinene	22.914	1498	1494	0.35	-
37	Epizonarene	23.023	1501	1051	0.71	-
38	β -Bisabolene	23.292	1511	1509	0.10	-
39	γ -Cadinene	23.481	1518	1513	0.20	0.08
40	Cadina-3,9-diene	23.755	1527	1518	1.47	-
41	Cadina-1(10),4-diene	23.824	1530	1524	1.04	0.05
42	Cubenene	24.018	1537	1532	0.98	-
43	α -Calacorene	24.333	1547	1542	0.09	-
44	(\pm)- <i>trans</i> -Nerolidol	24.911	1567	1564	1.21	1.1
45	Palustrol	25.06	1572	1567	0.07	-
46	Caryophyllenyl alcohol	25.129	1574	1570	0.11	-
47	(-)-Globulol	25.529	1588	1580	0.19	-
48	Guaiol	25.924	1600	1596	0.11	-
49	Epicubenol	26.788	1633	1627	0.43	-
50	γ -Eudesmole	26.880	1636	1631	0.63	-
51	α -epi-Cadinol	27.183	1647	1640	0.77	-
52	δ -Cadinol	27.286	1651	1645	0.29	-
53	β -Eudesmol	27.400	1655	1649	0.68	-
54	α -Eudesmol	27.486	1658	1653	1.52	-
55	(+)-Intermedeol	27.646	1664	1667	0.18	-
Total					99.35	99.83

RT: Retention time (min). RI (calc): Retention index calculated. RI (db): Retention indices from the databases.

**Figure 2.** GC-MS chromatogram of *C. vietnamense* rhizome essential oil.**Figure 3.** GC-MS chromatogram of *C. vietnamense* leaf essential oil.

borne illnesses with symptoms of nausea, vomiting, and diarrhea), *Escherichia coli* (causing diarrhea, urinary tract infections, respiratory illnesses, and pneumonia), *Pseudomonas aeruginosa* (causing pneumonia, urinary tract infections, and wound, ear, and bloodstream infections), *Salmonella enterica* (food-borne pathogen causing gastroenteritis), and *Candida albicans* (causing hospital-acquired infections, vaginal yeast infection, and bloodstream infection). The bioassays used streptomycin and cycloheximide as positive controls (Table 2). As for antibacterial properties, the leaf oil of *C. vietnamense* displayed moderate antibacterial effects against *E. faecalis* with MIC value of 32 $\mu\text{g/mL}$ and weak activities against *S. aureus* (MIC 128 $\mu\text{g/mL}$), *B. cereus* (MIC 128 $\mu\text{g/mL}$), *E. coli* (MIC 256 $\mu\text{g/mL}$), *P. aeruginosa* (MIC 128 $\mu\text{g/mL}$), *S. enterica* (MIC 128 $\mu\text{g/mL}$). Meanwhile, the rhizome oil exhibited more

Table 2. Antimicrobial activities of essential oil distilled from *C. vietnamense* rhizomes and leaves.

Microorganisms	MIC (µg/mL)			
	Leaf oil*	Rhizome oil*	Streptomycin	Cycloheximide
<i>E. faecalis</i> ATCC 299212	32 ± 2.65	32 ± 1.89	256	NT
<i>S. aureus</i> ATCC 25923	128 ± 1.56	128 ± 2.64	256	NT
<i>B. cereus</i> ATCC 14579	128 ± 2.14	64 ± 3.24	128	NT
<i>E. coli</i> ATCC 25922	256 ± 4.35	128 ± 2.58	32	NT
<i>P. aeruginosa</i> ATCC 27853	128 ± 2.17	128 ± 1.39	256	NT
<i>S. enterica</i> ATCC 13076	128 ± 2.13	64 ± 3.25	128	NT
<i>C. albicans</i> ATCC 10231	32 ± 1.45	64 ± 2.45	NT	32 ± 0.07

NT: not tested; *: mean ± SD, n=3

potential antibacterial activities with lower MIC values, specifically against *E. faecalis* (MIC 32 µg/mL), *S. aureus* (MIC 128 µg/mL), *B. cereus* (MIC 64 µg/mL), *E. coli* (MIC 128 µg/mL), *P. aeruginosa* (MIC 128 µg/mL), and *S. enterica* (MIC 64 µg/mL). When compared to the positive control, streptomycin, both essential oil samples generally exhibited better inhibitory effects on bacterial growth of investigated strains, except for *E. coli*. The oil samples were also tested against a fungal strain, *C. albicans*. As a result, the leaf and rhizome oils showed potential activities with MIC values of 32 and 64 µg/mL, respectively, which were comparable to that of the positive control, cycloheximide (MIC 32 µg/mL).

Eucalyptol and limonene were the two main components of *C. vietnamense* leaf and rhizome essential oils, which occupied 75.69 and 59.21% of the total oil content, respectively. Eucalyptol, or 1,8-cineol, is a bicyclic terpenoid, which naturally occurs in many plants, especially *Eucalyptus* species with high-content (60–97.32%) (Mączka et al. 2021). The oxygenated monoterpene was shown to possess potential antimicrobial activities. Furthermore, eucalyptol was also shown to synergistically inhibit the growth of methicillin-resistant *Staphylococcus aureus* strains with chlorhexidine gluconate and amoxicillin/ clavulanic acid combination (Merih and Reşat

2017; Hriouech et al. 2020). Further studies indicated that eucalyptol might affect quorum sensing, a chemical signal-based system of communication between microorganisms (Sybiya Vasantha Packiavathy et al. 2012), and change the shape and size of bacterial cell (Li et al. 2014). Limonene, a well-known monoterpene with lemon-like odor, has been found mostly in the essential oil of *Citrus* plants. The compound showed moderate antimicrobial activities against various types of bacteria and fungi with MIC values ranging between 1 and 20 mg/mL dependent on bioassays. Limonene was found to affect cell respiration and electron chain activity, which leads to cellular energy depletion and intracellular ROS accumulation (Thielmann and Muranyi 2019). Therefore, the high content of eucalyptol and limonene in prepared essential oils partly explained the antimicrobial activities against investigated bacterial and fungal strains in this study.

Conclusion

The essential oils were prepared from *C. vietnamense* leaves and rhizomes by hydrodistillation method with yields of 0.43% and 0.37% (w/w, fresh weight), respectively. The chemical composition of both oil samples was identified by GC-MS analysis, indicating that eucalyptol and limonene were the two dominant components (40.47–49.49% and 18.74–26.20%, respectively). The essential oils were then evaluated for their antimicrobial activities. The leaf oil was the most active against *E. faecalis* and *C. albicans* with MIC values of 32 µg/mL while the rhizome oil showed potential inhibitory effects on *E. faecalis* (MIC 32 µg/mL), *B. cereus* (MIC 64 µg/mL), *S. enterica* (MIC 64 µg/mL), and *C. albicans* (MIC 64 µg/mL). The study results showed that the essential oil of *C. vietnamense* can be a resource for eucalyptol and limonene and possessed potential antimicrobial effects. Remarkably, this is the first report on the chemical constituents and antimicrobial activity of the essential oil prepared from *Conamomum vietnamense*.

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