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Article

Chemical compositions, larvicidal and antimicrobial activities of *Zingiber castaneum* (Škorničk. & Q.B. Nguyễn) and *Zingiber nitens* (M.F. Newman) essential oils

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In this paper, the chemical constituents, larvicidal and antimicrobial activities of hydrodistilled essential oils from *Zingiber castaneum* Škorničk. & Q.B. Nguyễn and *Zingiber nitens* M.F. Newman were reported. The main constituents of *Z. castaneum* leaf were bicyclogermacrene (24.8%), germacrene D (12.9%), *cis*-β-elemene (11.2%) and β-pinene (10.3%), while sabinene (22.9%) and camphene (21.2%) were the significant compounds in the rhizome. However, the dominant compounds in the leaf of *Z. nitens* includes β-pinene (45.8%) and α-pinene (10.7%). Terpinen-4-ol (77.9%) was the most abundant compound of the rhizome. *Z. castaneum* rhizome oil displayed larvicidal activity against *Aedes aegypti* and *Culex quinquefasciatus* with LC_{50} values of 121.43 and 88.86 µg/mL, respectively, at 24 h. The leaf oil exhibited activity with LC_{50} values of 39.30 µg/mL and 84.97 µg/mL, respectively. Also, the leaf and rhizome oils of *Z. nitens* displayed greater larvicidal action towards *Ae. aegypti* with LC_{50} values of 17.58 µg/mL and 29.60 µg/mL, respectively. Only the rhizome oil displayed toxicity against *Cx. quinquefasciatus* with LC_{50} value of 64.18 µg/mL. All the studied essential oils inhibited the growth of *Pseudomonas aeruginosa* ATCC25923 with minimum inhibitory concentration (MIC) value of 50.0 µg/mL. This paper provides information on the larvicidal and antimicrobial potentials of *Z. castaneum* and *Z. nitens* essential oils.

Keywords: *Aedes aegypti*. *Culex quinquefasciatus*. *Pseudomonas aeruginosa*. β-Pinene. Terpinen-4-ol. Rhizomes.

INTRODUCTION

Plants are part of our daily life and essential oils have been extracted from over 3000 different species. These essential oils have domestic, industrial and medicinal uses (Adorjan, Buchbauer, 2010). Essential oils have an important role in the protection of plants and are well known for their various biological and pharmacological effects. These activities are normally related to the chemical substances mostly terpenes that are present in them. Essential oils are generally recognized as environmental friendly, easily biodegradable, minimally toxic to mammals and have toxicity against different pathogens and insect pests.

Zingiber species are economically important plants. *Zingiber castaneum* Škorničk. & Q.B. Nguyễn is easily recognized among other terminally flowering species by its upright inflorescence with reflexed bracts. The plant is also a rhizomatous herb forming small clumps. The creeping aromatic rhizome which grows up to 1.5 cm in

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diameter is externally light brown and internally cream white (Leong-Škorničková *et al.*, 2015). The translucent light green leaves are glabrous. Flowering starts in July and extends to September. It was found growing in Ninh Bình Province. *Zingiber nitens* M.F. Newman is a new species in flora of Vietnam (Hung *et al.*, 2017a). It is a forming herb of about 0.5-1.5 m tall, while the rhizome being 1 cm in diameter. The leafy shoots composed of about 12 leaves and leaf sheaths are dark brownish green, especially lower ones. The flowers are white at base, pale yellow at apex while the lobes are also pale yellow (Hung *et al.*, 2017a).

Both *Z. castaneum* (Leong-Škorničková *et al.*, 2015) and *Z. nitens* (Hung *et al.*, 2017a) were recently described as new species in the genus. There is no record of the chemical constituents and biological activities of the non-volatile extracts from these *Zingiber* species. However, the chemical compositions of essential oils from the leaf of *Z. castaneum* (Huong *et al.*, 2018) revealed the abundance of β-pinene (30.6%), α-pinene (9.5%), β-caryophyllene (9.4%) and bicycloelemene (9.1%), while β-caryophyllene (14.7%), δ-cadinene (9.8%) and bicycloelemene (8.4%) occurred in higher quantity in the stem oil. In addition, camphene (15.1%), 1,8-cineole (13.6%) and linalool (11.3%) were identified as the major constituents of the root oil. The main constituents of the fruit oil were (*E*)-nerolidol (23.2%), (*Z*)-9-octadecenamide (17.3%) and β-caryophyllene (10.8%) (Huong *et al.*, 2018). Likewise the main constituents of the leaf oil of *Z. nitens* (Hung *et al.*, 2017b) were δ-elemene (17.0 %), β-pinene (12.8 %) and β-elemene (8.8 %). The compositions of stem oil comprised mainly of δ-elemene (20.1 %), germacrene D (8.6 %) and bicyclogermacrene (8.1 %) while the root oil had an abundance of β-pinene (21.0 %), δ-elemene (12.8 %) and bornyl acetate (11.8 %). The rhizome essential oil of *Z. castaneum* displayed larvicidal activity against *Aedes albopictus* with median lethal concentration (LC_{50}) values of 49.85 μg/mL and 43.93 μg/mL, respectively, at 24 h and 48 h (Huong *et al.*, 2020a).

Aedes aegypti (Skuse) (Diptera: Culicidae) are important vectors of arboviral infections, including yellow fever, chikungunya virus, dengue virus, and Zika virus (Wilder-Smith *et al.*, 2017). It is known as Asian tiger mosquito. *Culex quinquefasciatus* Say, commonly known as the southern house mosquito, is a medium-sized brown mosquito that exists throughout the tropics. It is a vector of many pathogens of humans, domestic and wild animals. Viruses transmitted by this species include lymphatic filariasis (LF), West Nile virus (WNv), St. Louis encephalitis virus (SLEv), Western equine encephalitis virus (WEEv) and Zika virus (ZIKV) (Darsie Jr, Morris, 2000). Dengue fever epidemics are frequent and widespread in Vietnam (Quyen *et al.*, 2017; Quyen *et al.*, 2018) and the outbreaks of chikungunya and Zika infections have been reported lately (Quyen *et al.*, 2017; Huy *et al.*, 2019). In April 2016, the first two confirmed cases of locally acquired ZIKV infections were reported in southern Vietnam (Quyen *et al.*, 2017). In the early year of 2017 (Figure 1), an outbreak of dengue fever was transmitted throughout the country with much higher number of cases than in previous years (Huy *et al.*, 2019). In the dengue outbreak in 2017, patients were found in all ages, from 18 to over 80 years old, and inhabited in 53/63 (84.0%) provinces in Vietnam (Huy *et al.*, 2019). The control of the vector is one of the primary approaches to reduce the spread of arboviral infections. Botanical insecticides in general and essential oils in particular have emerged as promising, environmentally friendly alternatives to synthetic pesticides for mosquito control (Benelli, 2015; Masetti, 2016).

Considering the facts that *Zingiber* plants and products are source of biologically important substances for the control of these diseases (Huong *et al.*, 2019; Huong *et al.*, 2020a-d), essential oils were hydrodistillated from *Z. castaneum* and *Z. nitens*, and their mosquito larvicidal activity were examined accordingly. The present study is a continuation of an ongoing extensive research aimed at the characterization of the chemical compositions and biological efficacies of essential oils from Vietnamese plants (Ban *et al.*, 2020; Chau *et al.*, 2020; Dai *et al.*, 2020; Chung *et al.*, 2020; Huong *et al.*, 2020e).

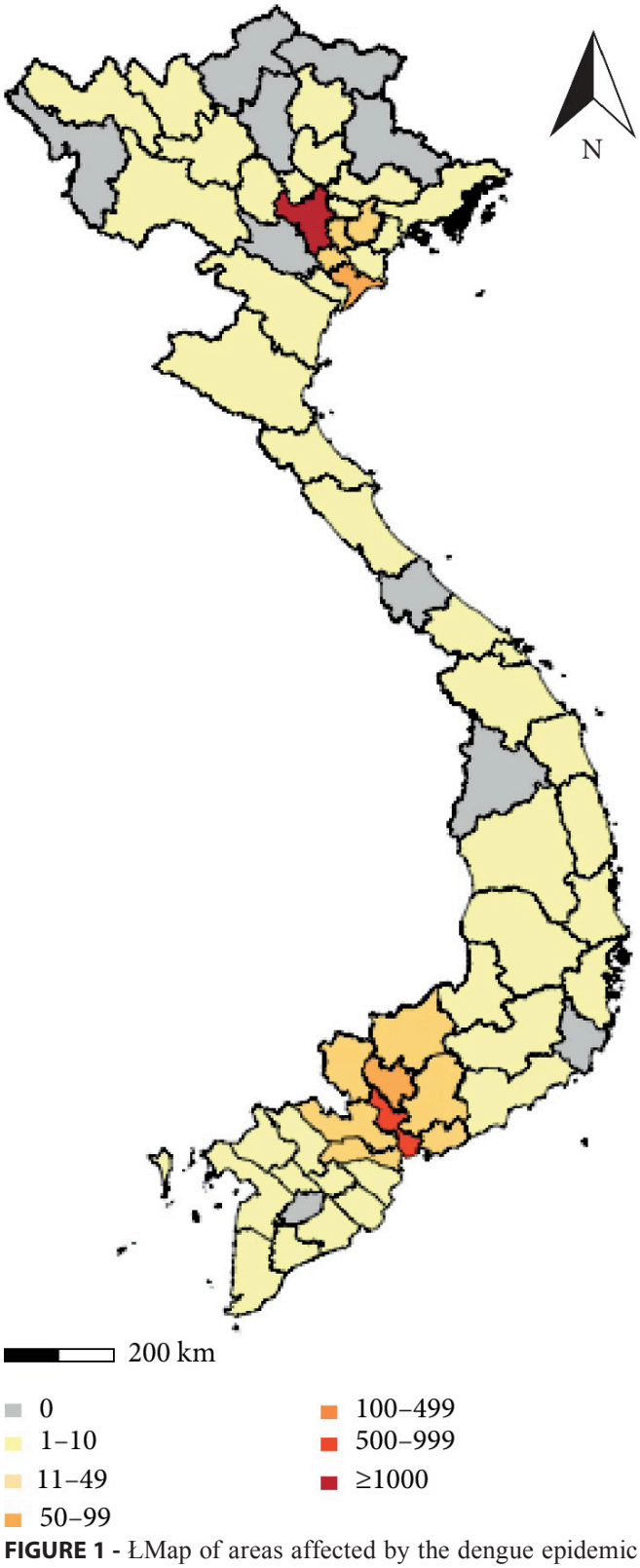

FIGURE 1 - ŁMap of areas affected by the dengue epidemic of Vietnam in 2017 (Huy et al., 2019).

MATERIAL AND METHODS

Collection of *Zingiber castaneum* and *Zingiber nitens*

The leaves and rhizomes of *Z. castaneum* and *Z. nitens* were collected from Pu Hoat Nature Reserve, Nghệ An Province, Vietnam, in August 2018. The plant samples were identified and authenticated at Botany Museum, NghệAn College of Economics, Vietnam, where voucher specimens, LTH741 and LTH750 respectively, were deposited for future references. The plant samples were air-dried (22^0C) under laboratory shade for two weeks to reduce the moisture contents. The rhizomes were dried as a whole sample by spearing on clean material. Thereafter unwanted materials were also removed from the samples by handpicking. Afterwards, the samples were pulverized to coarse powder using a locally made grinder.

Essential oil extraction

One kilogram (kg) of each of the leaf and rhizome of *Z. castaneum* and *Z. nitens* was used for the hydrodistillation experiment. Each sample was separately introduced into a 5 L flask after which distilled water was added until it covered the sample completely. Essential oils were obtained by hydrodistillation which was carried out in a Clevenger-type distillation unit designed according to an established procedure (Vietnamese Pharmacopeia, 2009) as described in previous studies (Hung *et al.*, 2017b; Huong *et al.*, 2018; Huong *et al.*, 2019; Huong *et al.*, 2020a-d). The distillation time was 3 h and conducted at normal pressure. The essential oils, which distilled over water, were collected separately into clean and previously weighed sample bottles by running through the tap in the receiver arm of the apparatus. The oils were kept under refrigeration (4^oC) until the moment of analyses. The experiment was conducted in triplicate. The essential oil yield (%) was calculated by mass (g) of the essential oil divided by the mass (g) of the dried leaf and rhizomes of the plant.

Gas chromatography (GC) analysis of the essential oils

GC analysis was performed on an Agilent Technologies HP 7890 Plus Gas chromatograph equipped with a FID and fitted with HP-5MS column (30 m x 0.25 mm, film thickness 0.25 μm, Agilent Technology). The analytical conditions were: carrier gas He (1 mL/min), injector temperature at 250°C, detector temperature 260°C, column temperature programmed from 40°C (2 min hold) to 220°C (10 min hold) at 4°C/min. Samples were injected by splitting mode, at the split ratio of 10:1. The volume of diluted oil in hexane (1: 10) injected into GC was 1.0 μL. Inlet pressure was 6.1 kPa. Each analysis was performed in triplicate. The relative amounts of individual components were determined on normalized percentages.

Gas chromatography-Mass spectrometry (GC/MS) experiment

An Agilent Technologies HP 7890N Plus Chromatograph fitted with capillary HP-5 MS column (30 m x 0.25 mm, film thickness 0.25 μm) and interfaced with a mass spectrometer HP 5973 MSD was used for this experiment. The GC conditions were the same as described above with He (1 mL/min) as carrier gas. The MS conditions were as follows: ionization voltage 70 eV; emission current 40 mA; acquisitions scan mass range of 35-350 amu at a sampling rate of 1.0 scan/s.

Identification of the components of the oils

The identification of constituents of essential oils from the GC/MS spectra of Z. *nitens* and *Z. castaneum* was performed on the basis of comparison of retention indices (RI) with reference to a homologous series of *n*-alkanes (C_4-C_{40}), under identical experimental conditions with GC. In some cases, co-injection with known compounds under the same GC conditions was employed. The mass spectral (MS) fragmentation patterns were checked with those of other essential oils of known composition in literature (NIST, 2018) as described recently (Hung *et al*., 2017; Huong *et al*., 2018; Huong *et al*., 2019; Huong *et al*., 2020a-d).

Mosquito larvae

Adults of *Cx. quinquefasciatus* and *Ae. aegypti* were collected from Hoa Khanh Nam ward, Lien Chieu district, Da Nang city (16°03'14.9"N, 108°09'31.2"E). Adult mosquitoes were maintained in entomological cages (40 x 40 x 40 cm) and fed a 10% sucrose solution and were allowed to blood feed on mice. Eggs hatching were induced with tap water. Larvae were reared in plastic trays (24×35×5 cm). The larvae were fed on dog biscuits and yeast powder in the 3:1 ratio. All stages were held at 25 ± 2°C, 65-75% relative humidity, and a 12:12 h light-dark cycle at the Center for Entomology and Parasitology Research, Duy Tan University.

Evaluation of larvicidal activity of the essential oil

The larvicidal activity of the essential oils from *Z. nitens* and *Z. castaneum* was evaluated according to established protocol (WHO, 2015) with slight modifications as described previously (Huong *et al*., 2019; Huong *et al*., 2020a-d). For the assay, aliquots of the essential oils from both samples dissolved in EtOH (1% stock solution) was placed in a 200 mL beaker and added to water that contained 20 larvae (fourth instar). With each experiment, EtOH (96%) was used as a negative control, while permethrin, a larvicidal drug, was used as a positive control. Mortality was recorded after 24 h and again after 48 h of exposure during which no nutritional supplement was added. The experiments were carried out 25 ± 2°C. The larvicidal test was conducted with four replicates under six concentrations (200, 150, 100, 50, 25 and 12.5 μg/mL).

The mortality rate was calculated according to the formula;

$$Mc = (Mo)/(Mt) \times 100$$

Mo = number of larvae dead in the treated groups, Mt = number of larvae introduced and Mc = calculated mortality

Microorganisms

Eight standardized ATCC strains from laboratory stock cultures were used in the evaluation of the antimicrobial activity of the oil samples. The Gram negative strains were *Escherichia coli* (ATCC25922) and *Pseudomonas aeruginosa* (ATCC25923). The Gram positive strains were *Bacillus subtilis* (ATCC11774), *Staphylococcus aureus* subsp. *aureus* (ATCC11632), while mycetes include *Aspergillus niger* (ATCC9763) and *Fusarium oxysporum* (ATCC48112). Two strains of yeast, *Candida albicans* (ATCC10231) and *Saccharomyces cerevisiae* (ATCC16404) were also used for the experiment. Testing media included Mueller-Hinton Agar (MHA) used for bacteria and Sabouraud Agar (SA) used for fungi. The strains were obtained from the laboratory stock of Institute of Marine Biochemistry, Vietnam Academy of Science and Technology, Hanoi, Vietnam.

Determination of minimum inhibitory concentrations

The minimum inhibitory concentration (MIC) and median inhibitory concentration (IC_{50}) values were measured by the microdilution broth susceptibility assay as described in our previous studies (Huong *et al.,* 2019; Chau *et al.*, 2020; Chung *et al.*, 2020). Stock solutions of the essential oils were prepared in dimethylsulfoxide (DMSO) and a serial dilution was prepared from 16,384 to 2 μg/mL. The choice of investigated concentrations was based on previous reports on similar reports where essential oils are active within specific concentration ranges (Huong *et al.*, 2019; Chau *et al.*, 2020; Chung *et al.*, 2020; Huong *et al.*, 2020c). Bacteria grown in double-strength Mueller-Hinton broth or double-strength tryptic soy broth, and fungi were sustained in double-strength Sabouraud dextrose broth, were standardized to 5×10^5 and 1×10^3 CFU/mL, respectively. The last row, containing only the serial dilutions of sample without microorganisms, was used as a negative control. Ampicillin and Nystatine served as positive controls for bacterial and fungal respectively. All experiments were performed in triplicate. After incubation at 37°C for 24 h, the MIC values were determined at well with the lowest concentration of agents completely inhibiting the growth of microorganisms.

Statistical analysis

The data obtained from the larvicidal test were subjected to log-probit analysis (Finney, 2009) to obtain LC_{50} values, LC_{90} values, 95% confidence limits, and chi square values using XLSTAT v. 2018.5 (Addinsoft, Paris, France). Statistical analysis (ANOVA) of the differences between mean values obtained for experimental groups were calculated as a mean of standard deviation (SD) of four independent measurements using Microsoft excel program 2003.

RESULTS

Chemical composition of the essential oils

The average yields of the essential oils of *Z. castaneum* were 0.22% and 0.31% (v/w, ± 0.01), for the leaf and rhizome, respectively. The main constituents of the leaf oil (Table I) were bicyclogermacrene (24.8%), germacrene D (12.9%), *cis*-β-elemene (11.2%), β-pinene (10.3%), α-pinene (9.6%) and δ-elemene (6.5%). The significant constituents of the rhizome oil were sabinene (22.9%), camphene (21.2%), α-pinene (7.8%), β-pinene (6.5%), bornyl acetate (6.1%) and γ-terpinene (5.5%). The essential oils from the leaf and rhizome of *Z. nitens* were obtained in yields of 0.27% and 0.54% (v/w, ± 0.01), respectively. From GC/MS spectral analysis, it was found that β-pinene (45.8%), α-pinene (10.7%), bicyclogermacrene (7.8%) and α-zingiberene (6.4%) were the main constituents of the leaf oil. However, terpinen-4-ol (77.9%) occurred as the compound occurring in highest quantity in the rhizome oil. All other compounds were identified in much lower quantity (Table I).

TABLE I - Chemical composition of *Zingiber castaneum* and *Zingiber nitens* leaf and rhizome essential oils.

Peaks	Compoundsa	RI (Cal.)	RI (Lit.)	Z. castaneum		Z. nitens	
				L	Rh	L	Rh
				Relative area %			
1	Tricyclene	928	921	-	0.4	-	-
2	α-Thujene	930	926	-	0.5	-	-
3	α-Pinene	939	932	9.6	7.8	10.7	-
4	α-Fenchene	952	948	-	0.9	-	-
5	Camphene	955	952	0.5	21.2	0.1	-
6	Sabinene	979	972	1.7	22.9	1.6	1.1
7	β-Pinene	985	978	10.3	6.5	45.8	0.6
8	Myrcene	992	988	0.3	2.7	0.5	0.3
9	α-Phellandrene	1011	1009	-	0.3	-	-
10	δ-3-Carene	1016	1017	-	-	0.2	-
11	α-Terpinene	1022	1024	0.1	3.2	-	1.2
12	o-Cymene	1030	1030	0.2	1.0	-	1.4
13	Limonene	1034	1032	0.5	3.3	1.5	-
14	β-Phellandrene	1036	1034	-	0.4	0.2	0.2
15	1,8-Cineole	1038	1036	-	0.3	0.2	0.3
16	γ-Terpinene	1064	1062	0.2	5.5	0.2	4.6
17	cis-Sabinene hydrate	1074	1073	-	-	-	0.5
18	Terpinolene	1095	1094	0.1	0.5	-	0.8
19	trans-Sabinene hydrate	1106	1110	-	-	-	0.6
20	1-Octen-3-yl acetate	1110	1112	-	0.3	-	-
21	cis-p-Menth-2-el-1-ol	1130	1130	-	-	-	2.0
22	trans-p-Menth-2-el-1-ol	1148	1148	-	-	-	1.6
23	Borneol	1178	1177	-	-	-	-
24	Terpinen-4-ol	1187	1188	0.1	-	-	77.9
25	α-Terpineol	1200	1200	-	-	-	1.9
26	cis-Piperitol	1205	1207	-	-	-	0.6
27	trans-Piperitol	1217	1218	-	-	-	1.1
28	Fenchyl acetate	1228	1229	0.1	0.3	-	-
29	2-Decanal	1265	1264	-	0.2	-	-
30	Bornyl acetate	1294	1297	0.2	0.5	-	-
31	Bicycloelemene	1345	1343	0.5	-	-	
32	δ-Elemene	1348	1350	6.5	5.4	0.4	-
33	α-Copaene	1390	1391	0.3	0.4	-	-
34	β-Bourbonene	1400	1401	-	-	0.2	-

TABLE I - Chemical composition of *Zingiber castaneum* and *Zingiber nitens* leaf and rhizome essential oils.

Peaks	Compoundsa	RI (Cal.)	RI (Lit.)	Z. castaneum		Z. nitens	
				L	Rh	L	Rh
				Relative area %			
35	cis-β-Elemene	1405	1407	11.2	9.8	2.8	-
36	cis-Thujopsene	1425	1422	-	-	0.1	-
37	β-Caryophyllene	1437	1437	0.4	1.7	1.2	-
38	γ-Elemene	1445	1445	0.4	0.8	-	-
39	allo-Aromadendrene	1457	1457	0.1	0.4	0.2	-
40	(Z)-β-Farnesene	1461	1465	-	-	0.5	-
41	α-Humulene	1472	1475	0.8	7.5	0.3	-
42	9-epi-(E)-Caryophyllene	1479	1480	2.2	2.0	1.2	-
43	β-Chamigrene	1490	1489	0.6	-	-	
44	Valencene	1491	1491	0.4	-	0.5	-
45	ar-Curcumene	1493	1494	0.4	1.6	1.4	-
46	Germacrene D	1499	1500	12.9	9.2	4.7	-
47	Aristolochene	1502	1502	-	-	1.8	-
48	α-Zingiberene	1505	1506	1.1	4.6	6.4	-
49	γ-Amorphene	1510	1508	-	-	0.3	-
50	(E,E)-α-Farnesene	1513	1511	-	-	1.8	-
51	Bicyclogermacrene	1516	1517	24.8	15.8	7.0	-
52	β-Bisabolene	1518	1520	0.2	1.3	1.3	-
53	γ-Cadinene	1531	1530	0.3	0.3	0.2	-
54	β-Sesqui[hellandrene	1536	1535	0.2	1.1	2.6	-
55	δ-Cadinene	1538	1540	1.2	1.3	0.6	-
56	Elemol	1565	1563	-	0.2	-	-
57	(E)-Nerolidol	1571	1571	0.2	0.5	0.2	-
58	Germacrene B	1578	1580	1.3	1.6	-	-
59	Germacrene-D-4-ol	1595	1594	2.4	1.8	0.2	-
60	Spathulenol	1599	1600	1.2	2.0	0.7	-
61	Caryophyllene oxide	1605	1606	-	0.6	0.4	-
62	Viridiflorol	1606	1608	0.2	-	0.1	-
63	Guaiol	1615	1618	-	0.4	-	-
64	Zingiberenol	1624	1626	-	1.0	0.3	-
65	Ledol	1626	1628	0.3	-	-	-
66	Humulene epoxide II	1632	1632	-	0.6	-	-
67	α-Acorenol	1644	1644	-	0.3	0.1	-

TABLE I - Chemical composition of *Zingiber castaneum* and *Zingiber nitens* leaf and rhizome essential oils.

Peaks	Compoundsa	RI (Cal.)	RI (Lit.)	Z. castaneum		Z. nitens	
				L	Rh	L	Rh
				Relative area %			
68	Alismol	1648	1650	1.9	-	-	-
69	1-epi-Cubenol	1649	1652	-	3.2	-	-
70	Isospathulenol	1658	1660	-	-	0.1	-
71	epi-α-Cadinol	1660	1662	0.4	-	0.1	-
72	epi-α-Muurolol	1662	1664	0.4	1.1	0.2	-
73	α-Cadinol	1675	1676	0.8	1.6	0.5	-
74	α-Turmerone	1682	1680	0.4	2.9	0.5	-
75	Curlone	1716	1720	-	1.0	-	-
76	Phytol	2120	2119	-	-0.1		-
	Total			**96.8**	**94.1**	**98.9**	**96.7**
	Monoterpene hydrocarbons			**23.5**	**10.1**	**59.0**	**10.2**
	Oxygenated monoterpenes			**0.4**	**0.8**	**0.2**	**86.5**
	Sesquiterpene hydrocarbons			**64.9**	**66.2**	**36.3**	**-**
	Oxygenated sesquiterpenes			**8.0**	**16.5**	**3.3**	**-**
	Diterpenes			**-**	**-**	**0.1**	**-**
	Non-terpenes			**-**	**0.5**	**-**	**-**

[a] Compound listed in order of elution from HP-5 column; RI (Cal.): Retention index calculated using *n*-alkane C_7 - C_{28} in HP-5 column; RI (Lit.): Identification based on retention index reported by NIST (2018) and identification based on comparison of mass spectra using NIST 11.0 library; Relative area (%): percentage of the area occupied by the compounds in the chromatogram; (-): Absent; L: Leaf; Rh: Rhizome

Mortality of the essential oils against vector mosquitoes

The leaf and rhizome oils of *Z. castaneum* exhibited 100% mortality against *Ae. aegypti* at concentrations of 100 µg/mL and 200 µg/mL respectively, under the test period of 24 h and 48 h (Table II). However, both samples showed 100% mortality against *Cx. quinquefasciatus* at concentration of 150 µg/mL. On the other hand, the leaf and rhizome oils of *Z. nitens* displayed mortality of 100% against *Ae. aegypti* at concentrations of 50 µg/mL and 100 µg/mL, respectively, at 24 h (Table III). However, only the rhizome oil exhibited mortality of 92.5% against *Cx. quinquefasciatus* at concentration of 100 µg/mL during the same period.

TABLE II - Mortality and larvicidal action of *Z. castaneum* oils

	Mortality (%)[a, b] Concentration (µg/mL)					
	12.5	**25**	**50**	**100**	**150**	**200**
Ae. Aegypti						
Leaf						
24 h	5.0 ±.816	15.0 ±.000	53.75 ± 3.304	100.0 ±.000	n.d	n.d
48 h	10.0 ±.816	22.5 ± 1.291	62.5 ± 2.517	100.0 ±.000	n.d	n.d
Rhizome						
24 h	0	0	0	10.0 ±2.708	75.0 ±2.582	100.0±.000
48 h	0	0	13.7 ±1.708	15.0 ±2. 160	64.0 ±2.944	100.0 ±.000
Cx. quinquefasciatus						
Leaf						
24 h	0	0	13.75 ±.000	57.0 ±3.916	100.0 ±.000	n.d
48 h	0	10.0 ±.000	42.5 ± 2.646	84.3 ± 1.258	100.0 ±.000	n.d
Rhizome						
24 h	0	0	3.70 ±.500	55.0 ±3.916	100.0 ±.000	n.d
48 h	0	10.0 ±1.000	42.5 ±2.6446	81.3 ±1.258	100.0 ±.000	n.d

	Minimum lethal concentration (µg/mL)[c]				
	LC_{50}	**LC_{90}**	**Regression equation**	***X*²**	***P***
Ae. Aegypti					
Leaf					
24 h	39.30	89.94	y = -5.683 + 3.564x	8.472	0.000
48 h	31.78	80.37	y = -4.778 + 3.181x	9.943	0.000
Rhizome					
24 h	121.43	145.28	y = -6.525 +0.054x	9.512	0.000
48 h	110.31	125.33	y = -9.445 +0.086x	2.497	0.000
Cx. quinquefasciatus					
Leaf					
24 h	84.97	141.45	y = -11.172 + 5.791x	7.458	0.000
48 h	47.40	92.29	y = -7.423 +4.429x	6.914	0.000
Rhizome					
24 h	88.86	117.68	y = -3.952 +0.044x	8.502	0.000
48 h	48.08	72.13	y = -2.562 +0.053x	6.871	0.000

[a]n =4; [b]no mortality in the EtOH used as negative control; n.d, not determined; [c]Permethrin, the standard drug used as positive control displayed larvicidal activity against *Cx*. quinquefasciatus and *Ae. aegypti* with LC_{50} values in the range of 2.19 - 3.43 µg/mL.

TABLE III - Mortality and larvicidal action of *Z. nitens* leaf and rhizome oil

	Mortality (%)[a, b] Concentration (µg/mL)			
	12.5	**25**	**50**	**100**
Ae. Aegypti				
Leaf				
24 h	12.5 ±.816	76.3 ±3.862	100.0 ±.000	100.0 ±.000
48 h	15.0 ±.957	82.5 ±3.317	100.0 ±.000	100.0 ±.000
Rhizome				
24 h	0	17.5 ±1.291	83.7 ± 2.872	100.0 ±.000
48 h	5.0 ±.816	35.0 ±2.651	90.0 ± 2.309	100.0 ±.000
Cx. quinquefasciatus				
Rhizome				
24 h	0	6.3 ±.500	15.0 ±.000	92.5 ±1.291
48 h	0	6.3 ±.500	15.0 ±.000	92.5 ±1.291

	Minimum lethal concentration (µg/mL)c				
	LC_{50}	**LC_{90}**	**Regression equation**	***X*²**	***P***
Ae. Aegypti					
Leaf					
24 h	17.58	23.25	y = -3.979 + 0.226x	9.343	0.000
48 h	15.12	18.70	y = -5.407 + 0.358x	2.095	0.036
Rhizome					
24 h	29.60	37.60	y = -5.688 +0.192x	2.012	0.044
48 h	26.32	36.92	y = -2.990 +0.593x	5.938	0.000
Cx. quinquefasciatus					
Rhizome					
24 h	64.18	92.68	y = -2.887 +0.045x	5.363	0.000
48 h	59.06	84.31	y = -2.998 +0.051x	5.963	0.000

[a] n= 4; bno mortality in the EtOH used as negative control; [c]Permethrin, the standard drug used as positive control displayed larvicidal activity against *Cx. quinquefasciatus* and *Ae. aegypti* with LC50 values in the range of 2.19 - 3.43 µg/mL.

Result of larvicidal tests

As seen in Table II, the leaf oil of *Z. castaneum* displayed significant larvicidal activity against *Ae. aegypti* with LC_{50} values of 39.30 µg/mL (24 h) and 31.78 µg/mL (48 h) while the rhizome exhibited moderate activity with LC_{50} values of 121.43 µg/mL and 110.31 µg/mL at 24 h and 48 h, respectively. In addition, LC_{90} values over the same test periods for the leaf oil were 89.94 µg/mL (24 h) and 80.37 µg/mL (48 h). Moreover, LC_{90}

values of 145.28 µg/mL and 125.33 µg/mL were obtained for the rhizome oil, at 24 h and 48 h, respectively. On the other hand, the leaf oil exhibited activity against *Cx. quinquefasciatus* depicted by LC_{50} values of 84.97 µg/mL at 24 h, and 47.40 µg/mL at 48 h. The LC_{90} values over the same period were 141.45 µg/mL and 92.29 µg/mL, respectively. Moreover, LC_{50} values of 88.86 µg/mL and LC_{90} of 117.68 µg/mL were recored at 24 h by the rhizome oil against *Cx. Quinquefasciatus*. The LC_{50} and LC_{90} values of 48.08 µg/mL and 72.13 µg/mL, respectively, were obtained at 48 h towards *Cx. quinquefasciatus*. From Table III, the leaf oil of *Z. nitens* displayed larvicidal activity against *Ae. aegypti* with LC_{50} value of 17.58 µg/mL and LC_{90} value of 23.25 µg/mL at 24 h, while LC_{50} and LC_{90} values of 15.12 µg/mL and 18.70 µg/mL, respectively, were obtained at 48 h. For the rhizome oil, LC_{50} value of 29.60 µg/mL and LC_{90} of 37.60 µg/mL were displayed towards *Ae. aegypti* at 24 h. Moreso, LC_{50} value of 26.32 µg/mL and LC_{90} of 36.92 µg/mL were obtained at 48 h. Only the rhizome oil of *Z. nitens* exhibited larvicidal action against *Cx. quinquefasciatus* with LC_{50} value of 64.18 µg/mL and LC_{90} value of 92.68 µg/mL at 24 h. The LC_{50} and LC_{90} values obtained at 48 h were 59.06 µg/mL and of 84.31 µg/mL, respectivelly. Permethrin, the standard drug used as control displayed larvicidal activity at much lower values.

Antimicrobial data

The leaves and rhizomes essential oils of *Z. castaneum* and *Z. nitens* displayed antibacterial activity against *P. aeruginosa,* both with MIC value of 50.0 ± 0.12 µg/mL. No activity could be found against the other tested microorganisms. Thus the leaf and rhizome essential oils of *Z. castaneum* and *Z. nitens* could only inhibit the growth of *P. aeruginosa*. The present results represent the first report on the antimicrobial action of the studied essential oils.

DISCUSSION

This is the first report on the chemical constituents of rhizome oil of *Z. castaneum*. The compositions of both the leaf and rhizome oils of *Z. castaneum* were dominated by monoterpene hydrocarbons, sesquiterpene hydrocarbons and oxygenated sesquiterpenes in varying quantities (Table I). It was well noted that the identities of these major compounds differ from one oil sample to another. For example, sabinene and camphene, the significant constituents of the rhizome oil occurred in much lower quantities in the leaf oil. However, the leaf oil contained higher contents of α-pinene, β-pinene, δ-elemene, *cis*-β-elemene, germacrene D and bicyclogermacrene when compared to the rhizome oil. A comparative analysis of the previous and present studies on the chemical constituents of *Z. castaneum* essential oils indicates some interesting analysis. Firstly, the amounts of bicyclogermacrene, germacrene D and *cis*-β-elemene in the present study on the leaf oil of *Z. castaneum* were much higher than reported previously (Huong *et al.*, 2018), while the percentages of β-pinene and β-caryophyllene in the present study were lower than reported in the previous study. Secondly, bicycloelemene, one of the main compounds of the previously analyzed oil sample, was not identified in the present study. Interestingly, the quantitative amounts of α-pinene were similar in both the previous analysis and the present study on the leaf oil of *Z. castaneum*.

In the present study on the essential oils of *Z. nitens* from Vietnam, monoterpene hydrocarbons and sesquiterpene hydrocarbons were the predominant classes of compounds in the leaf oil. On the other hand, the rhizome oil consists mainly of monoterpene hydrocarbons and oxygenated monoterpene compounds. However, sesquiterpene compounds were not identified in the rhizome oil (Table I). The main constituents of the leaf oil of *Z. nitens* namely δ-elemene, β-pinene, β-elemene, bicyclogermacrene, germacrene D and ledol, were not identified in the rhizome oil. Moreover, terpinen-4-ol, the most abundant compound of the rhizome oil of *Z. nitens*, was not identified in the leaf oil. This is the first report on the volatile constituents of the rhizome of *Z. nitens*. Moreover, ledol a significant compound in previously analysed of essential oil of the leaf oil of *Z. nitens* (Hung *et al.*, 2017b) was conspicuously absent in the present investigated oil sample. However, the composition of essential oil in

the present study contained higher amount of α-pinene when compared with the previous study.

The chemical profiling of the leaf oil of *Z. nitens* was highly dominated by monoterpene hydrocarbon and sesquiterpene hydrocarbons. The rhizome oil contained the highest quantity of oxygen-containing monoterpene compounds. However, both sesquiterpene hydrocarbons and oxygenated sesquiterpene class of compounds were not identified in the rhizome oil of *Z. nitens*. Sesquiterpene hydrocarbons were identified in sizeable amounts in the leaf and rhizome oils of *Z. castaneum*. The rhizome essential oils of both plants contained equal amount of monoterpene hydrocarbons (Table I). The essential oils of the two *Zingiber* plants exhibited chemical variability. The abundant of monoterpene and sesquiterpene compounds in the studied essential oils confer similarity with some other *Zingiber* species including *Z. officinale* (rhizome), *Z. purpureum* (leaf), *Z. nimmonii* (rhizome), *Z. roseum* (rhizome), *Z. spectabile* (inflorescences), *Z. rufopilosum* (leaf), *Z. gramineum* (leaf), *Z. collinsii* (rhizome), *Z. rubens* (rhizome) e.t.c (Hung *et al.*, 2017b; Huong *et al.*, 2018).

The observed variation in the chemical profiling between the studied essential oils and the previous studies could be attributed to some factors, which may include the ecological and climatic variation between the Pu Hoat Nature Reserve, Nghệ An Province, (this study) and Vu Quang National Park, Ha Tinh Province (previous collection site of *Z. castaneum*) as well as Pu Mat National Park, Nghean Province (previous collection site of *Z. nitens*). In addition, the harvest time, age and conditions of the plant may also account for the variations in the amount and the qualitative compositions of the bioactive substances.

As mentioned earlier, no previous information exists on the mortality of the studied essential oils towards insects pests especially *Ae. aegypti* and *Cx. quinquefasciatus*. This result was the first of its kind in this regard. The percentage mortality was dependent on the concentration of the tested oil samples. Thus, higher inhibition of mosquito larvae was observed as concentration increases. There was no mortality in the EtOH used as control for all the tested oil samples. Permethrin, the standard drug used as control displayed larvicidal activity against *Cx. quinquefasciatus* and *Ae. aegypti* with LC_{50} values in the range of 2.19 - 3.43 µg/mL. The leaf oil of *Z. castaneum* was more toxic towards *Ae. aegypti* than *Cx. quinquefasciatus*. Conversely, the rhizome oil of *Z. castaneum* exhibited higher toxicity towards *Cx. quinquefasciatus* than *Ae. aegypti* (Table II). The leaf oil of *Z. nitens* was more toxic towards *Ae. aegypti* than the rhizome oil (Table III). Overall, the leaf and rhizome oils of *Z. nitens* showed high toxicity towards *Ae. aegypti* and *Cx. quinquefasciatus* than those of *Z. castaneum*. A previous report indicated that the rhizome oil of *Z. castaneum* displayed larvicidal activity against *Ae. albopictus* (Huong *et al.*, 2020a) with LC_{50} values of 49.85 µg/mL and 43.93 µg/mL at 24 h and 48 h, respectively, slightly higher than those of *Ae. aegypti* in this study.

A comparative analysis of the larvicidal activities of the studied essential oils revealed some interesting observations. The leaf oil of *Z. castaneum* displayed higher larvicidal activity than than the rhizome oil towards *Ae. aegypti* and *Cx. quinquefasciatus* (Table II). However, the leaf of *Z. nitens* showed stronger larvicidal activity towards *Ae. aegypti* than the rhizome oil. In addition, the rhizome oil also exhibited pronounced larvicdial activity towards *Ae. aegypti* than towards *Cx. quinquefasciatus.* Therefore the order of larvicidal activity towards *Ae aegypti* was *Z. nitens* leaf > *Z. nitens* rhizome > *Z. castaneum* leaf > *Z. castaneum* rhizome. This order of activity was reinforced by the lowest LC_{50} of 17.58 and 15.12 µg/mL at 24 h, as well as LC_{90} values of 23.25 and 18.70 µg/mL at 48 h obtained for *Z. nitens* leaf oil. For *Cx. quinquefasciatus,* the order of activity was *Z. nitens* rhizome > *Z. castaneum* leaf > *Z. castaneum* rhizome. The essential oil of *Z. nitens* rhizome had the lowest LC_{50} and LC_{90} values of 64.18 and 59.03 µg/mL at both 24 h and 48 h test periods. The observed larvicidal action of *Z. castaneum* and *Z. nitens* in this study was comparable with findings from *Zingiber* plants analyzed for their larvicidal activity from Vietnam and other parts of the world (Table IV).

TABLE IV - Larvicidal activity of essential oils of some *Zingiber* plants

		LC_{50} 24 h[a]			
Plants	**Origin**	***Ae. aegypti***	***Ae. albopictus***	***Cx. quinquefasciatus***	**References**
Z. collinsii	Vietnam	-	25.51 µg/mL	50.11 µg/mL	Huong *et al*., 2020b
Z. zerumbet	‘’	-	55.75 µg/mL	33.28 µg/mL	Huong *et al*., 2019
‘’	Thailand	48.88 ppm	-	-	Sutthanont *et al*., 2010
‘’	Malaysia	102.6 µg/mL	-	-	Jantan *et al*., 2003
‘’	Malaysia	82.05 mg/L	106.5 mg/L	49.28 mg/L	Restu, Halijah and Awang, 2017
‘’	Thailand	-	-	50.78 ppm	Pushpanathan, Jebanesan and Govindarajan, 2008
Z. officinalis	Malaysia	197.2 µg/mL	-	-	Jantan *et al*., 2003
‘’	‘’	-	15.8%[a]	21.8%[a]	Rabha *et al*., 2016
‘’	India	40.5 mg/L	-	-	Kalaivani, Senthil-Nathan and Marugesan, 2012
‘’	Brazil	70.6 mg/mL	-	-	Dias, Morae, 2014
Z. officinale					
var. *rubrum*	Malaysia	120.60 mg/L	96.86 mg/L	130.50 mg/L	Restu, Halijah and Awang, 2017
Z. cernuum	India	44.88 µg/mL	55.84 µg/mL	48.44 µg/mL	Rajeswary *et al*., 2018
Z. spectabile	Malaysia	155.93 mg/L	93.35 mg/L	107.78 mg/L	Restu, Halijah and Awang, 2017
Z. nimmonii	Thailand	44.46 µg/mL	-	48.26 µg/mL	Govindarajan *et al*., 2016
‘’	Malaysia	84.95 mg/L	99.04 mg/L	176.35 mg/L	Restu, Halijah and Awang, 2017
Z. castaneum *Vietnam*	-	49.85 µg/mL	-	Huong *et al*., 2020a	
Z. montanum Vietnam	32.20 µg/mL	35.17 µg/mL	31.12 µg/mL	Huong *et al*., 2020d	
Z. cornubracteatum	‘’	16.97 µg/mL	12.72 µg/mL	24.31 µg/mL	Huong *et al*., 2020c
Z. neotruncatum	‘’	34.95 µg/mL	21.50 µg/mL	33.58 µg/mL	‘’
Z. nudicarpum[b]	‘’	19.30 µg/mL	22.33 µg/mL	12.44 µg/mL	‘’
Z. nudicarpum	‘’	23.44 µg/mL	28.05 µg/mL	11.50 µg/mL	‘’
Z. ottensii	‘’	38.16 µg/mL	19.79 µg/mL	27.19 µg/mL	‘’
Z. recurvatum	‘’	20.90 µg/mL	45.48 µg/mL	31.67 µg/mL	‘’

[a]Leaf sample; - Not mentioned

It is known that there have been no established standard criteria for determining the larvicidal activity of natural products and essential oils. This prompted some authors (Komalamisra *et al.*, 2005; Kiran *et al.*, 2006) to proposed individual criteria to establish the potency of mosquito larvicidal activity of bioactive products. In effect, products showing $LC_{50} \leq 50$ mg/L were considered to be strongly active, 50 mg/L $< LC_{50} \leq 100$ mg/L to be active, 100 mg/L $< LC_{50} \leq 750$ mg/L to be effective, and $LC_{50} > 750$ mg/L to be inactive (Kiran *et al.*, 2006). It should be noted that these criteria must be directly correlated with the time of exposure and the origin of larvae, which are variables that can alter the LC_{50} values. According to the above criterion, the studied essential oils of *Z. nitens* exhibited the strongest activities against both *Ae. aegypti* and *Cx. quinquefasciatus*.

The variations in the toxicity of essential oils against different species of mosquitoes and other insect pests have been established and this is due to differences in the nature and amount of chemical constituents identified in the oil samples. In effect, some of the chemical constituents of essential oils under in this study have been investigated for their larvicidal activity. The leaves and rhizomes oils of *Z. nitens* showed greater larvicidal potential, probably due to the presence of *β*-pinene and terpiene-4-ol, respectively. *β*-Pinene was reported previously to displayed larvicidal action against *Ae. aegypti* with LC_{50} value of 21.1 mg/mL (Lucia *et al.*, 2007) while terpinen-4-ol, which has a proven LC_{50} of 64.76 mg/mL against *Ae. aegypti* (Dias, Morae, 2014).

The recent dengue outbreak occurred in a larger scale than in the previous years in terms of time, location, and number of patients (Huy *et al.*, 2019). It occurred in 53/63 (84.0%) provinces in Vietnam, and patients in all ages were affected. The number of patients with dengue fever was 1675 (57.3%), dengue with warning signs was 914 (31.3%), and severe dengue was 333 (11.4%). For example, in high incidence years, upwards of 2,000 dengue cases were notified in Nha Trang Province, representing a substantial burden on the local health services (Quyen *et al.*, 2018). Among patients with severe dengue, severe plasma leakage and dengue shock account for 238 (8.1%), severe organ impairment rose to 73 (2.5%) while severe bleeding amounnted to 22 (0.75%). The rate of mortality increase by 0.8%, and the outcome of dengue patients was worse in the elderly and people with underlying diseases (Huy *et al.*, 2019). The studied essential oils fraction from *Z. castaneum* and *Z.nitens* and their major compounds displayed larvicidal activity against *Cx. quinquefasciatus* and *Ae. aegypti*. Therefore a probable formulation of active ingredients from these essential oils can be used for the prevention of these insects and damage they can cause to human beings especially in this endemic country like Vietnam.

The leaf and rhizome essential oils of *Z. castaneum* and *Z. nitens* could only inhibit the growth of *P. aeruginosa* at the same MIC value of 50 μg/mL. The observed antimicrobial results of *Z. castaneum* and *Z. nitens* oils differed completely from those of other *Zingiber* essential oils from Vietnam and other parts of the world, which were effective towards several other microorganisms. The ability of the studied essential oils to inhibit the growth of gram-negative bacterium is noteworthy. Majority of the reported essential oils are known to be susceptible greatly to the growth of several gram-positive microorganisms (Huong *et al.*, 2019; (Chau *et al.*, 2020; Chung *et al.*, 2020; Huong *et al.*, 2020c). Essential oil constituents were previously reported to inhibit significantly the growth and cell viability of potential infectious of broad spectrum microorganisms. For example, the antimicrobial activity of the essential oil from the leaves of *Z. nitens* may be attributed to the monoterpenic hydrocarbons α-pinene and β-pinene which previously showed antimicrobial activity against strains of *P. aeruginosa* with MIC of 10.0 μg/mL (Soković *et al.*, 2007). Also, terpinen-4-ol, the main compound of *Z. nitens* rhizome has previously shown potential bacteriocidal activity towards *P. aeruginosa* (Papadopoulos *et al.*, 2006). Essential oil with large contents of bicyclogermacrene and germacrene D have displayed antimicrobial activity against organisms such as *P. aeruginosa, C. albicans* and *S. aureus* with MIC value of 125 mg/mL (Tabanca *et al.*, 2001). The present oil essential oil constituents such sabinene, 1,8-cineole, *β*-caryophyllene, bicyclogermacrene and germacrene were previously reported to inhibit significantly the growth and cell viability of potential infectious of broad

spectrum microorganisms including *P. aeruginosa* (Ali, Chen, Sargsyan, 2014; Şener *et al*., 2017).

Pseudomonas aeruginosa and other *Pseudomonsa* spp. are notorious for their involvement in nosocomial infections and their incidence of resistance to antibiotics (Papadopoulos *et al*., 2006). *Pseudomonas aeruginosa* is an opportunistic pathogen that can cause urinary tract infections, respiratory system infections, dermatitis, soft tissue infections, bacteremia, bone and joint infections, gastrointestinal infections,and a variety of systemic infections, particularly in patients with severe burns and in cancer and AIDS patients who are immunosuppressed (Ha *et al*., 2019). Therefore a adjunct or alternative treatments for *Pseudomonas* skin and wound infections that fall outside the realm of conventional antibiotics are needed. The studied essential oils of *Z. castaneum* and *Z. nitens* may serve this purpose if properly exploited for their antimicrobial activity.

The control of adult mosquitoes and microbes commonly relies on the use of synthetic insecticides, repellents and synthetic drugs. Treatments with these chemicals are expensive, exhibit minimal efficacy and have a strong environmental impact related to human health risks. In effect, the search for safe alternative natural insecticides, repellents and herbal formulations should be novel idea to be taken into consideration in hyperendemic country like Vietnam. Essential oils and their constituentss are considered among the most promising alternative to synthetic chemicals.

CONCLUSION

The main constituents identified in the essential oils of *Z. castaneum* and *Z. nitens* were α-pinene, β-pinene, sabinene, camphene, terpinen-4-ol, *cis*-β-elemene, bicyclogermacrene and germacrene D. In the present study, essential oil from the leaf of *Z. nitens* showed greater larvicidal potential towards *Ae. aegypti*, with LC_{90} of 23.25 µg/mL at 24 h and LC_{90} of 18.70 µg/mL in 48 h of contact with the oil, and the activity may probably be due to the effect of β-pinene, the major compound of in the leaves. Also, both essential oils displayed antimicrobial action *P. aeruginosa* at MIC level comparable to other oil samples and may serves as alternative natural product against *P. aeruginosa*. Therefore, the results indicate the potentials of *Z. castaneum* and *Z. nitens* essential oils as a source of antimicrobial and larvicidal agents.

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