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FULL PAPER

Composition and bioactivity of essential oils from lolot pepper (*Piper lolot* C. DC.) extracted by microwave-assisted hydrodistillation

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Abstract

In this study, lolot pepper (*Piper lolot* C. DC.), a popular vegetable, a special spice, and effective medicine in Vietnam, was utilized for the extraction of essential oil. Chopped or grounded lolot leaves were hydro-distilled by microwaving supplemented with water levels (200 to 700 mL) in different extraction periods (30 to 120 mins) by different heating power levels (400 to 800 W). The chemical composition of the extract was analyzed by mass spectrometry gas chromatography. Then, the antibacterial and antioxidant properties of lolot essential oil were investigated. As a result, the suitable conditions for the extraction of essential oils of lolot pepper by microwaving were determined by utilizing the chopping raw material with 500 mL distilled water per 500 g of lolot pepper, through a 90-minute extraction at 800 W). The main components of the essential oils of the lolot pepper collected in Dong Thap province were 19 compounds, mainly (E)-nerolidol (18.43%), germacrene D (12.52%), β-elemene (6.7%), β-caryophyllene (4.14%), and α-selinene (4.1%). Lolot pepper essential oils had inhibitory effects on *Bacillus subtilis*, *Escherichia coli*, *Edwardsiella ictaluri*, and *Streptococcus pneumoniae*. In particular, this essential oil could significantly inhibit *E. ictaluri* and *S. pneumoniae*. The antioxidant ability of lolot pepper essential oils tested by the DPPH free radical inhibition activity indicated that the concentration of the essential oils for 50% DPPH inhibition was 16.79 µg/mL.

1. Introduction

The attraction to essential oils from natural aromatic plant materials has recently increased. Each extract has a unique aroma and fragrance that could improve relaxation, comfort, and pleasure. These distillates possessed many medicinal properties which can be applied to the production of medicine, and antimicrobial and antiviral agents (Vergis *et al*., 2015; Puškárová *et al.*, 2017; Tariq *et al*., 2019). The antibacterial activity of essential oils is effective at high vapour concentrations for a short time (Inouye *et al*., 2001). Essences possess numerous biological activities (antioxidant, anti-inflammatory, antimicrobial) required in the food and cosmetic industries, as well as in the human health field (Tongnuanchan and Benjakul, 2014; Ali *et al*., 2015; Dhifi *et al*., 2016; Aziz and Karboune, 2018; Bhavaniramya *et al*., 2019).

Microwave-assisted hydrodistillation combines microwave heating and dry distillation, performed at atmospheric pressure without adding any solvent. Previous studies illustrated that essential oil extracted by this method possesses higher amounts of valuable oxygenated compounds, and reduces costs, time, and energy by bypassing sample preparation and/or evaporation steps. Furthermore, this method produces extract with a higher concentration of active phenolic compounds compared to other techniques (Lucchesi *et al*., 2004; Chan *et al*., 2011; Thakker *et al*., 2016; Liu *et al*., 2016; Michel *et al*., 2011).

Vietnam is located in the tropical monsoon region where natural conditions are favourable for the formation and development of plants, especially high-value essential oil-bearing plants. Piper species are widely used in folk medicine to heal wounds and reduce swelling and skin irritation (Gardner, 2010). In the Mekong Delta, lolot pepper (*P. lolot*), a popular vegetable in Vietnam, is also a special spice and effective medicine. Lolot pepper leaves contribute positive health effects on exhibiting warmth and pain relief (Lau, 2017). Although lolot pepper is a good medicinal plant with many applications for treating disease, it is considered only as a cooking spice. Compared to many other medicinal plants, the

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scientific data on the lolot pepper has been inadequate with few related studies. This study aimed to investigate the factors affecting the hydro-distillation of lolot pepper essence by microwave method, to determine the composition of lolot pepper essential oils as well as assess the antibacterial and antioxidant ability of lolot pepper essential oils.

2. Materials and methods

2.1 Materials and chemicals

The leaves of lolot pepper were collected in Dong Thap province (10° 40′ 0″ N, 105° 40′ 0″ E), Vietnam. Strains of *B. subtilis*, *E. coli*, *E. ictaluri*, and *S. pneumoniae* were stored at Food Biotechnology Laboratory, Biotechnology Research and Development Institute, Can Tho University, Vietnam. Bacterial growing media were including MRS Broth, MRS Agar, Nutrient Broth, Luria Broth, and TSA (Merck, Germany). Chemicals such as anhydrous Na_2SO_4, 1,1-diphenyl-2-picrylhydrazyl (DPPH), dimethyl sulfoxide (DMSO), and diethyl ether were analytical grade.

2.2 Extraction of lolot pepper essential oils by microwave method

Leaves of lolot pepper were collected in Dong Thap province. Then, samples were identified based on Vietnam Medicinal Handbook (Loi, 2004). The collected leaves were washed, chopped, or ground. Approximately 500 g of the lolot pepper leaves were mixed with 500 mL water and transferred into a 1,000 mL flask. The first hydro-distillation was done at 800 W in 90 mins. In the following analysis, a volume of distilled water was poured into the flask so that the volume of the water did not exceed 60% volume of the flask. Microwave-assisted hydrodistillation was performed in a Milestone "DryDist" microwave reactor at 2455 MHz (Milestone Srl, Bergamo, Italy); the PTFE-coated cavity dimensions were 35×35×35 cm (Figure 1). After the extraction process, the mixture of extracted fluid consisted of *P. lolot* oil and water. The extraction of the mixture was performed using diethyl ether solvent to remove water to collect the solvent part. Dry with Na_2SO_4, then the solvent was evaporated to quantify of essential oils obtained.

During the tests, lolot pepper leaves were kept in whole leaves, shredded or pureed, and mixed with water. The classification of leaf treatment is referred to as raw state for whole leaf samples, a one-centimetre size for shredded samples, and a 2-mm size for pureed samples. The water content varied from 200 to 700 mL per 500 g of lolot pepper samples. Then, the mixture was microwaved at different extraction times including 30, 60, 90, and 120 mins. The electric capacities designed for the extraction were at different levels ranging from 400 to 800 W. After each analysis, the most appropriate conditions were applied in the following hydro-distillation.

Figure 1. Milestone "DryDist" microwave extraction system

2.3 Determination of the chemical compositions of lolot pepper essential oils

The distillates of lolot pepper obtained by microwave-assisted extractions were analyzed by mass spectrometry gas chromatography (GC/MS) for their chemical composition. Approximately 25 µL extracted samples of *P. lolot* were supplemented with 1 mL of n-hexane. The GC6890N Agilent supported by MS 5973 was installed with the HP5-MS column. The GC-MS pressure was kept at 9.3 psi. Helium was in the carrier phase and was injected at a 1.0 mL/min flow rate, 1:100 ratio, and 1.0 µL volume. The overall process started with incubation at 50°C for 120 s. Then, the heat was supplied to increase the temperature by 2°C every minute until 80°C. The heating accelerated to a 5°C increase per minute to 150°C, a 10°C increase/min to 200°C and then a 20°C heating/min for 5 mins to 300°C, subsequently.

2.4 Evaluate the antibacterial properties of lolot pepper essential oils

The antibacterial properties of lolot pepper oils were determined by the well diffusion agar method (Hernández *et al*., 2004). This method is based on the antibacterial properties of the extract against the indicator bacteria on the agar plates. The antibacterial properties of the oils were tested on 4 bacterial strains including *B. subtilis*, *E. coli*, *E. ictaluri*, and *S. pneumoniae* (10^8 cells/mL density). Essential oil was prepared at 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} dilution. The antibacterial properties were evaluated by the diameters of inhibitory zones (at least 4 mm).

2.5 Evaluation of the antioxidant properties of lolot pepper essential oils

The antioxidant of lolot pepper oils was determined by DPPH antioxidant method (Tabart *et al*., 2007). One millilitre of the extracted sample was supplemented with 2 mL DPPH (100 µmol/L), and the reaction took place for 30 mins to measure the optical absorbance at 517 nm. The negative control (A_c) was built by mixing 1 mL of methanol and 2 mL of DPPH. Besides, the blank samples were 3 mL of methanol. The percentage of inhibition for antioxidant capacities was calculated using the following formula:

$$IC_{50} = \frac{A_c - A_s}{A_c} \times 100$$

Where IC_{50} is the antioxidant capacity, A_c is the measured absorbance of the control sample, and A_s is the measured absorbance of the test sample.

2.6 Experimental data analysis

Experimental data were processed statistically by Statgraphics Centurion XV.I software (Statpoint Technologies Inc., USA) and charted by Microsoft Excel 2010 (Microsoft Corporation, USA).

3. Results and discussion

3.1 Features of lolot pepper and essential oils

The identification results indicated that the leaves of the plant used in this study were identified with the scientific name of *P. lolot* C. DC. which belonged to the family Piperaceae (Loi, 2004; Lau, 2017). The sample plant shared the same basic morphological characteristics as lolot pepper including a soft-bodied tree, the body bulges up in the patches, had vertical lines, sometimes brown, slightly covered with fur. The leaves were ovoid broad, the base of the heart is pointed, the pointed tip illuminated with points, the upper smooth surface, and the slightly hairy veins on the underside. Inflorescence in the interstitial branches out into monochromatic flowers, about 1.5 cm long. Lolot pepper essential oils extracted by the microwave-assisted method had a pale yellow colour and a pleasant aroma.

3.2 Suitable conditions for microwave extraction of the essential oils

Figure 2A indicates the effects of material size treatments (whole leaves, chopped leaves, and pureed leaves) on the amount of produced oil (g) from 500 g of material. Chopped material resulted in a statistically significantly higher number of essential oils (reaching 0.278±0.003 g from 500 g raw material) than that of whole leaves (0.15±0.009 g) and pureed leaves (0.257±0.003 g). Therefore, chopped material was selected as a suitable sample size treatment for subsequent experiments.

When increasing the volume of distilled water in oil extraction, the production of essence increased proportionally. The highest oil content (0.274±0.004 g) was achieved by adding 500 mL of water per 500 g of samples (Figure 2B). This produced oil was statistically higher than the other treatments at 95% confidence. The longer extraction times produced higher oil contents. After reaching a peak at the 90-minute extraction time, the longer times resulted in decreasing oil contents (Figure 2C). The highest oil content (0.250±0.09 g) was achieved at a 90-minute extraction. The rising power

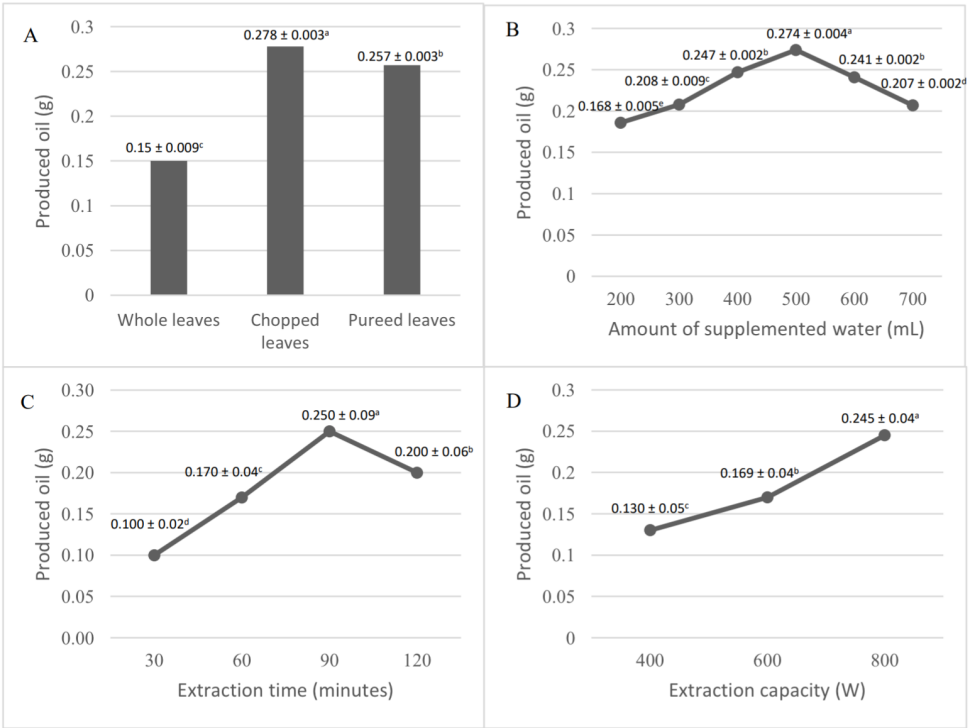

Figure 2. Conditions affected the extraction of lolot pepper essential oils

levels helped improve the amount of extracted oil. Figure 2D indicated the lowest produced oil content (0.13±0.05 g) from the 400 W power. In contrast, the highest amount of produced oil (0.245±0.04 g) was collected at 800 W power.

From the collected data, the suitable conditions for microwave extraction of the essential oils of lolot pepper were as followed: 500 g of chopped material in a 1,000 mL flask, 500 mL supplemented water content, 800 W power levels, and a 90-minute extraction time.

3.3 Chemical contents of the essential oils of lolot pepper

The chemical composition of lolot pepper essentials oil was determined by mass spectrometry (GC/MS) as shown in Figure 3 and Table 1. The results showed that the essential oils of lolot pepper leaves in Dong Thap province contained 19 compounds. In specific, the main components were identified as (E)-nerolidol (18.43%), germacrene D (12.52%), β-elemene (6.7%), β-caryophyllene (4.14 %) and α-selinene (4.1%). The number of components analyzed in *P. lolot* extracts in this study was lower than that of a study on the oil extracted from leaf, stem, and rhizome of *P. lolot* (Dung *et al*., 1996), with 35 identified compounds among which β-caryophyllene accounted for the highest ratio (26.1-30.9%) followed by bornyl acetate (10.0%). However, the majority of the extracted compounds from *P. lolot* in Table 1 were analyzed with higher ratios compared to the essential oils extracted in the study on *P. retrofractum* (Hieu *et al*., 2014) except *β*-pinene that *P. lolot* oil accounted for a lower ratio (1.2%) than *P. retrofractum* (1.6%). *P. lolot* had a higher quantity of essence composition than some other species in the genera of *Piper,* such as *P. brevicaule* (Hieu *et al*., 2015), *P. bellidifolium* (Araujo *et al*., 2018), and *P. jacquemontianum* (Cruz *et al*., 2011).

3.4 Antibacterial properties of essential oils

The extracted essential oils of lolot pepper were investigated for their antibacterial properties against *B. subtilis*, *E. coli*, *E. ictaluri* and *S. pneumoniae* by well-diffusing agar method. Figure 4 indicates the diameters of inhibitory zones created by the inhibitory effects of extracted samples. It is clear that logarithm dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}) affected inhibitory halo zones (Table 2).

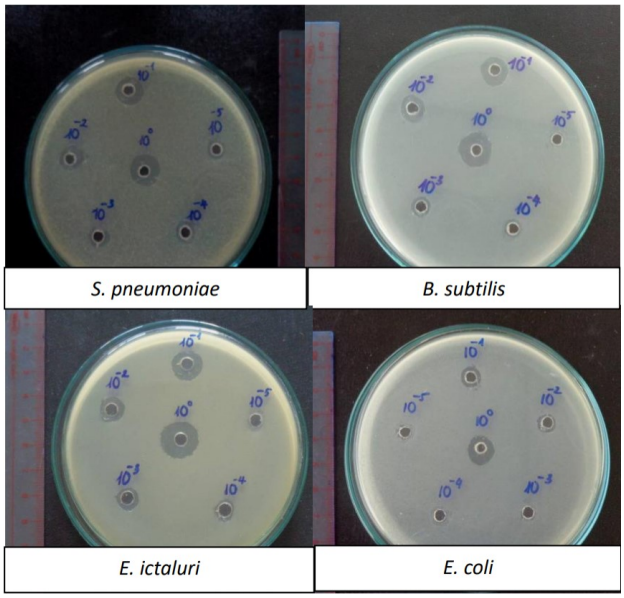

Figure 4. The inhibitory zones of bacterial strains created by lolot pepper essential oils

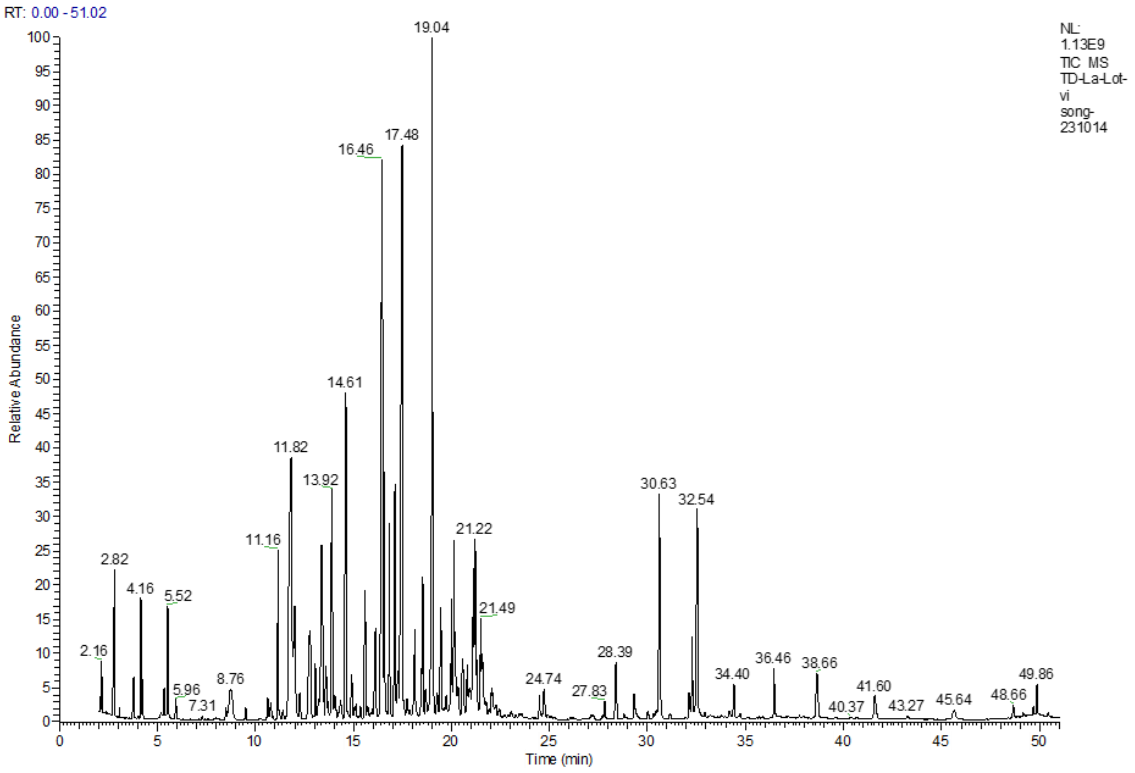

Figure 3. The graph of gas chromatography–mass spectrometry (GC/MS) of lolot pepper essential oils

Table 1. Chemical compositions of *P. lolot* essential oils

No.	Chemical components	Retention time (min)	Percentage (%)				
			P. lolot (This study)	*P. ret.* (Hieu *et al*., 2014)	*P. bre.* (Hieu *et al*., 2015)	*P. bel.* (Araujo *et al*., 2018)	*P. jac.* (Cruz *et al*., 2011)
1	(E)-Nerolidol	19.04	18.43	0.6	-	20.3	8.0
2	Germacrene D	11.82	12.52	3.3	0.8	1.7	1.0
3	β-Elemene	13.40	6.7	0.6	0.6	0.4	-
4	β-Eudesmol	21.12	5.2	-	13.8	2.3	-
5	β-Caryophyllene	15.60	4.14	5.3	0.2	-	0.4
6	α-Selinene	16.85	4.1	-	-	-	-
7	(6E,10E)-3,7,11,15-Tetramethyl-1,6,10,14-hexadecatetraen-3-ol	30.63	3.83	-	-	-	-
8	β-Eudesmene/β-Selinene	16.57	3.78	0.1	0.6	3.6	0.1
9	trans-Phytol	32.54	3.65	0.1	1.8	-	-
10	Germacrene A	17.12	2.88	-	0.8	-	-
11	α-Eudesmol	20.15	2.33	-	-	0.7	1.0
12	γ-Elemene	18.55	1.97	0.4	0.3	0.4	-
13	2-Undecanone	11.16	1.8	0.1	-	-	-
14	α-Caryophyllene	14.61	1.44	-	0.4	0.7	0.4
15	Germacrene B	16.13	1.44	0.7	-	-	0.1
16	Squalene	19.45	1.37	-	-	-	-
17	(E-E)-Farnesol	18.13	1.27	-	-	-	-
18	β-Pinene	2.82	1.2	1.6	0.9	-	2.4
19	(6Z,9Z)-6,9-Pentadecadien-1-ol	21.49	1.11	-	-	-	-

P. ret.: P. retrofractum; P. bre.: P. brevicaule; P. bel.: P. bellidifolium; P. jac.: P. jacquemontianum; "-" not identified.

Table 2. The diameters of inhibitory zones (mm) on bacterial strains created by lolot pepper essential oils

Bacterial strain	The diameters of inhibitory zones (mm)					
	10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
S. pneumoniae	12.01±0.51	10.83±0.29	9.83±0.28	9.33±0.58	7.17±0.29	5.04±0.21
B. subtilis	10.83±0.29	9.33±0.56	7.17±0.29	6.17±0.28	5.02±0.01	4.16±0.11
E. ictaluri	13.17±0.29	11.17±0.17	9.02±0.11	7.12±0.04	5.83±0.14	4.15±0.12
E. coli	10.17±0.29	7.83±0.17	6.17±0.21	4.17±0.01	4.21±0.01	4.12±0.02

Note: 100 was the original sample; 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} were the dilution at 10 times, 100 times, 1.000 times, 10.000 times. The diameter of well was 4 mm.

The concentrations of original extracts, 10^{-1} and 10^{-2} created inhibitory zones on all indicators (zone diameters > 4 mm). At concentrations of 10^{-3} and 10^{-4}, results showed inhibitory zones on plates spread with *S. pneumoniae*, *B. subtilis*, and *E. ictaluri*. At a concentration of 10^{-5}, there were only aseptic zones appeared on plates of *S. pneumoniae* that were not present in the case of the remaining indicators (zone diameter = 4 mm). The antimicrobial effects on all four indicators showed that lolot pepper oil could be promising as a supplemental component to natural human cosmetics, food preservatives, and medicines. This study was corresponding to many recent papers demonstrating the effective activities of essential oils on bacteria such as *Haemophilus influenzae*, *S. pneumoniae*, *S. pyogenes, Staphylococcus aureus, Salmonella enterica* serovar Typhimurium, *Yersinia enterocolitica, E. coli* (Inouye *et al*., 2001; Puškárová *et al*., 2017; Tariq *et al*., 2019).

3.5 Antioxidant activity of essential oils

DPPH was a stable free radical, the solution was purple and had a maximum absorbance of 517 nm. Antioxidant substances could neutralize the DPPH radical by transferring hydrogen which would further reduce the optical absorbance at the maximum wavelength along with changing the solution colour turning from violet to light yellow. When *P. lolot* essential oils and vitamin C respectively reacted with DPPH solution at concentrations of 5, 10, 15, 20, and 25 µg/mL, the solution colour faded and changed from purple to light yellow. The optical absorbances (OD_{517nm}) were shown in Table 3.

The vitamin C concentrations and percentage of inhibition were expressed as a line graph with the equation y = 2.8276x – 8.014 (where x is the content of vitamin C and y is the inhibition percentage) and the coefficient of determination R^2 = 0.9872. The extrapolation graph indicated that the IC_{50} value of

vitamin C was 20.52 µg/mL. Vitamin C was used as a standard to compare with the antioxidant of the essence of *P. lolot*. The lolot pepper essential oils concentrations and percentage of inhibition were similarly expressed as a line graph with the equation y = 3.2294x – 0.9812 and the coefficient of determination R^2 = 0.9812. The extrapolation graph suggested the IC_{50} value of lolot pepper extract was 16.79 µg/mL. Thus, the inhibitory concentration of 50% DPPH in the distilled sample was 16.79 µg/mL, while the inhibitory concentration of 50% DPPH of vitamin C is 20.52 µg/mL. The results illustrated that the concentration of antioxidant molecules of *P. lolot* essential oils was higher than vitamin C. Recent studies worked on the extraction of varieties of *P. lolot* for essence by methanol extraction (Rathee et al., 2006) and soxhlet (Dasgupta and De, 2004) showed highest IC_{50} levels at 52.43±1.24 µg/mL and 62.6 µg/mL, respectively, which are significantly lower than the activity of oil samples in this study (16.79 µg/mL). This proves the advantage of the microwave-assisted extraction method over the others. Besides, many papers have revealed that the antioxidant activity is related to the presence of phenolic compounds (such as thymol, carvacrol, ethers, ketones, aldehydes, and monoterpenes) which have significant redox properties and play important roles in neutralizing free radicals and peroxide decomposition (Aruoma, 1998; Burt, 2004; Modzelewska *et al*., 2005).

4. Conclusion

To our knowledge, this is the first report on a suitable protocol for microwave-assisted hydro-distillation of lolot pepper essence. Besides, the extract consisted of compositions with antibacterial and antioxidant properties. With those characteristics, lolot pepper essential oils are a potential source for further studies and applications in food and medicine.

Conflict of interest

The authors declare no conflict of interest.

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Table 3. Optical absorbance (OD_{517}nm) of vitamin C and the oil of lolot pepper in the reaction with DPPH

Concentration (µg/mL)	Vitamin C		Lolot pepper essential oils	
	OD_{517nm}	IC_{50}(%)	OD_{517nm}	IC_{50}(%)
5	0.199±0.003[a]	9.13	0.190±0.011[a]	12.5
10	0.179±0.007[b]	18.26	0.151±0.002[b]	32.37
15	0.150±0.011[c]	31.55	0.124±0.007[c]	45.11
20	0.113±0.005[d]	48.22	0.103±0.005[d]	56.34
25	0.077±0.001[e]	64.84	0.041±0.002[e]	81.25

Values are presented as mean±SD of triplicate repetitions. Values with different superscript within the same columns are significantly different at 95% confidence.

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