

Phytochemical analysis, antioxidative activity, cytotoxicity, and antimicrobial activity of two Vietnamese *Camellia* species: *Camellia quephongensis* and *Camellia puhoatensis*

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Abstract. *Camellia quephongensis* and *Camellia puhoatensis* are two Vietnamese perennial plants, which have engaged the attention due to their great value in beverage and pharmacology. For the first time, the phytochemical and biological evaluations of the ethanol extracts from the flowers and leaves of two Vietnamese *Camellia* species *C. puhoatensis* and *C. quephongensis* were carried out. Phytochemical screenings determined the appearance of flavonoids, tannins, saponins, free-reducing sugars, and polysaccharides. These ethanol extracts are promising antioxidants since they show strong lipid peroxidation inhibitory activity. These alcohol extracts were also superior to the positive control streptomycin in antimicrobial assays against the growth of Gram-positive bacterium *Enterococcus faecalis* ATCC299212 and Gram-negative bacteria *Pseudomonas aeruginosa* ATCC27853 and *Salmonella enterica* ATCC13076. In another approach, these extracts exhibit cytotoxicity towards seven cancer cell lines A549, HT-29, SK-Mel-2, HepG2, MCF-7, Hela, and MKN-7 at different levels of results. It needs to get more studies, focusing on the explanations by mechanism of actions.

Keywords: *Camellia quephongensis*, *Camellia puhoatensis*, antioxidative, cytotoxic, antimicrobial.

Classification numbers: 1.1.1, 1.1.6.

1. INTRODUCTION

The liver is a major organ found in vertebrates that performs many essentially biological functions such as detoxification, protein synthesis, and biochemical digestion [1]. Mostly the detoxification process occurs via oxidative reactions, which result in the increased production of ROS (reactive oxygen species) [2]. The ROS overproduction causes DNA damage, protein adduct formation, mitochondrial malfunction, and liver disease through mediating lipid peroxidation [3]. The reaction between oxygen and unsaturated fatty acids is known as lipid peroxidation, which is catalyzed by enzymes, e.g., lipoxygenases, or cyclooxygenases. Lipid peroxidation products include lipid hydroperoxides, 4-hydroxynonenals, especially malondialdehyde (MDA) [4]. These agents are mainly responsible for cellular injuries by changing lipid-lipid interactions, membrane fluidity, and membrane permeability [4]. However, this process could be balanced by antioxidants.

Cancer remains one of the most serious global health concerns, causing 7.9 million deaths in 2007 and an estimated 12 million by 2030 [5]. Among over 100 cancer types, breast cancer is the most common, followed by prostate, lung, ovarian, colon, and rectal cancers [6]. Over half of breast cancers are estrogen receptor-positive, making it the second-leading cause of cancer-related deaths in women [7]. Chemotherapy often leads to adverse effects, prompting the search for natural compounds from medicinal plants, which possess strong antioxidant properties and may help reduce oxidative damage when used alongside anticancer drugs [8].

The growing resistance of pathogenic bacteria to antibiotics, as well as continuing focuses on health care expenditures, led to create new antimicrobial agents that are potentially effective against drug resistance. In the meantime, the role of the Vietnamese tropical plants as antimicrobial ingredients in herbal drug formulations has been recognized for years. For instance, the methanol extracts of *Dalbergia tonkinensis* leaves, stem barks, and roots, at the concentration of 1.0 mg/mL, inhibited 60 - 80 % growth of seven Gram-positive microbacteria *Bacillus cereus*, *Escherichia coli*, *Staphylococcus aureus*, *S. epidermidis*, *Streptococcus pneumoniae*, and *S. pyogenes* [9]. The 96 % ethanol extract of *Phyllanthus amarus* leaves was active against the Gram-negative bacterium *Aeromonas hydrophila* with a low minimum inhibitory concentration (MIC) of 156 µg/mL [10]. Therefore, searching for antimicrobial agents from medicinal plants seems to be a good strategy for Viet Nam.

The genus *Camellia* contains about 280 species, which are distributed mainly in Asia. Viet Nam and China are recorded to have the largest number of species [11]. With the commercial goods like tea and seed oils, *C. sinensis* (L.) Kuntze, *C. japonica* L., and *C. oleifera* Abel species have received much attention in investigations on chemical compositions and biological activities [11]. The major chemical classes in *C. sinensis* were alkaloids, steroids, terpenoids, saponins, and polyphenols [12]. The previous reports have also shown that *Camellia* plants showed a variety of pharmacological activities such as antioxidant, anticancer, antimicrobial, and other health benefits [12].

The current study aims to provide new information on phytochemical analysis and biological assessments (lipid peroxidation inhibition, cytotoxicity, and antimicrobial activity) of 70 % ethanol extracts from different parts of two Vietnamese perennial *Camellia* species *Camellia puhoatensis* Ly N.S., Luong V.D., Le T.H., Nguyen D.H. & Do N.D. and *Camellia quephongensis* (Hakoda et Ninh) Le N.H.N, Luong V.D., Do N.D. The results of this study may

be used to compare with previously studied *Camellia* species, and to guide further studies on these two species.

2. MATERIALS AND METHODS

2.1. Plant materials

The fresh flowers and leaves of *C. puhoatensis* were gathered from Dong Van, Que Phong, Nghe An, Viet Nam (19°48'32" N; 105°5'44" E; 290 m) in January 2022. In the meantime, the fresh flowers and leaves of *C. quephongensis* were collected from Hanh Dich, Que Phong, Nghe An, Viet Nam (19°40'4" N; 105°55'30" E; 234 m) in January 2022. The fresh materials were washed with tap water and dried at 60°C for 2 days. The moisture of leaves and flowers was about 32 % and 37 %, respectively. Plant materials are coded by LQP (*C. quephongensis* leaves), HQP (*C. quephongensis* flowers), LPH (*C. puhoatensis* leaves), and HPH (*C. puhoatensis* flowers).

2.2. Chemicals

The standards trolox, ellipticine, streptomycin, and cycloheximide were purchased from Sigma-Aldrich. Acetonitrile and phosphoric acid were purchased from Merck. DMEM (Dulbecco's Modified Eagle Medium) supplemented with L-glutamine was purchased from Invitrogen (MA, USA). All other chemicals and reagents were of analytical grade.

2.3. Animals, cancer cell lines, and microbial organisms

Twelve-week-old BALB/c female mice (23-26 g), which were provided by the Institute of Biotechnology-Vietnam Academy of Science and Technology, were used for the lipid peroxidation inhibition assay. Mice were fed with standard food and water for three days before killing and isolating brains. Seven cancer cell lines, including A549 (human lung carcinoma), HT-29 (human colorectal carcinoma), SK-Mel-2 (human carcinoma), HepG2 (human hepatocellular cancer), MCF-7 (human breast carcinoma), Hela (cervical carcinoma), and MKN-7 (human gastric carcinoma) were provided by Prof. Chi-Ying Huang, National Yang Ming Chiao Tung University, Taiwan. Seven pathogenic bacterial strains, consisting of three Gram-positive bacterial strains *Enterococcus faecalis* ATCC299212, *Staphylococcus aureus* ATCC25923, and *Bacillus cereus* ATCC14579, three strains of Gram-negative bacterial strains *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC27853, and *Salmonella enterica* ATCC13076, and one yeast strain *Candida albicans* ATCC 10231, were provided by the Institute of Biotechnology.

2.4. Phytochemical analysis

Dried materials were pulverized to a size of 3.5 mm, and then immersed in ethanol (3 times x 3 days). These obtained extracts were evaporated using an Eyela N-1300V-W (Japan) at 50 °C. They were then dried, and stored at 4 °C for further analysis. Accurately-weighed 100 mg samples of extracted residues were added to 25 mL of the 70 % ethanol, sonicated for 20 min, filtered, and the filtrates were used for *in vitro* qualitative reactions (See Supplementary Material).

2.5. Lipid peroxidation (MDA) assay

The MDA assay was carried out using the previously described methods with a few modifications [13, 14]. Lipid peroxidation inhibition capacity of the tested sample was calculated by the content of MDA, in which MDA is of the final products of polyunsaturated fatty acid peroxidation in the cells. The MDA reacted with thiobarbituric acid to form trimethin complex (pink color) with a maximum absorption peak at $\lambda = 532$ nm.

The tested samples (four ethanol extracts HPH, HQP, LPH, and LQP) were prepared by diluting with 1 % DMSO to reach the serial concentrations of 100, 20, 4, 0.8, and 0.16 $\mu\text{g/mL}$. Mouse brains were separated and crushed homogeneous in phosphate buffer solution (pH = 7.4) in the ratio 1:10 at temperatures 0 – 4 °C. This brain homogenate (1 mL) was mixed with test sample (0.1 mL) at different concentrations, phosphate buffer (0.8 mL), and 0.1 mL of Fenton reagent [FeSO_4 0.1 mM- H_2O_2 15 mM (1:1, v/v)]. This mixture was incubated in 5 % CO_2 at 37 °C for 15 min. The reaction was stopped by 10 % trichloroacetic acid (1 mL), and was then centrifuged at 12000 rpm for 5 min, to discard supernatants. The obtained solution (2 mL) was then mixed with thiobarbituric acid (0.8 %, 1 mL) at a rate of 2:1, and incubated at 100 °C for 15 min. After cooling, the absorbance was measured at $\lambda = 532$ nm. Each experiment was repeated 3 times, while trolox was used as a positive control. The inhibitory percentage was calculated following the formula below, and the IC_{50} value (the half maximal inhibitory concentration) was calculated using Table-Curve 2D:

$$\text{Inhibitory percentage (\%)} = \frac{(\text{OD}_C - \text{OD}_T) \times 100}{\text{OD}_C}$$

Where the optical density (OD_C) stands for the optical density of the control well without the tested sample, OD_T is the optical density of the tested sample.

2.6. Cytotoxic assay

The sulforhodamine B (SRB) assay was used to assess the cytotoxicity of four ethanol extracts HPH, HQP, LPH, and LQP against seven cancer cell lines A549, HT-29, SK-Mel-2, HepG2, MCF-7, HeLa, and MKN-7, following the previously described reports [15, 16]. The tested samples were prepared by diluting with 1 % DMSO to reach the serial concentrations of 100, 20, 4, and 0.8 $\mu\text{g/mL}$. In brief, cancer cells were cultured in 5 % CO_2 at 37 °C for 48 h. The SRB method for calculating cell density, which relies on the determination of cellular protein content, was used to consider cell viability. Microtiter plates were used to plate experimental cultures, which had a density of 6000 cells, the tested sample (10 μL) in 190 μL of the growth media (10 % fetal bovine serum) per well. The adopted duration assay was 3 days. The tested plates were incubated in 5 % CO_2 at 37 °C for 72 h, whereas the incubation of the 0-day control was 1 h. After the removal of the medium, the surviving cell monolayers were fixed with a cold solution of trichloroacetic acid (20 %, w/v) for 1 hour at 4 °C and stained with 1 \times SRB staining solution for 30 min at 25 °C, by which the unbound dye was discarded by washing several times with acetic acid (1 %, v/v). The protein-bound dye was dissolved in unbuffered *Tris*-base solution (10 mM) for the OD measurement at 515 nm on an OD Plate Reader (MPP-96). The positive control was ellipticine, while the blank sample was 1 % DMSO. Experiments were performed in triplicate. The cell viability in the presence of extracted residue was determined by the formula below, and the IC_{50} value was calculated using Table-Curve 2D:

$$\text{Cell viability (\%)} = \frac{[\text{OD (reagent)} - \text{OD (day 0)}] \times 100}{\text{OD (negative control)} - \text{OD (day 0)}}$$

2.7. Antimicrobial assay

The antimicrobial effect of four ethanol extracts HPH, HQP, LPH, and LQP was carried out using the broth dilution approach. The selection of the tested concentrations was mostly relied on our previous reports [17, 18] in which the tested samples were active with specific concentration ranges. A stock solution of the tested sample was created by using DMSO (1 %). Dilution ranges (2-fold) were created from 16.384 to 2 µg/mL. After that, they were placed to 96-well plates. Bacteria growing in double-strength Mueller-Hinton medium were standardized to 5×10^5 CFU/mL. The final row of well plates containing only the serial dilutions of samples without microorganisms can be seen as a positive control. As a negative control, DMSO (1 %) was used without an antimicrobial agent. Standards for antibacterial and antifungal activities were streptomycin and cycloheximide, respectively. Experiments were run 3 times. The outcomes were demonstrated by the IC₅₀ and minimum inhibitory concentration (MIC) values.

2.8. Statistical analysis

The IC₅₀ values were determined using Table-Curve 2D computer software, version 4.0. ANOVA testing was performed using Minitab version 19 software.

3. RESULTS AND DISCUSSION

3.1. Phytochemical analysis

The yields of extraction of the studied samples are presented in Table S1, in which the ethanol extracts of the flowers achieved around 9.0 - 9.4 g/1 kg dried powder, in comparison with those of the ethanol extracts of the leaves (12.2 - 12.8 g/1 kg). Phytochemical screenings were also carried out on these extracts. The macerations of both leaves and flowers of these two *Camellia* indicated the presence of flavonoids, tannins, saponins, free reducing sugars, and polysaccharides, but coumarins, amines, amino acids, and alkaloids were absent (Table 1 and Figures S1-S8).

Table 1. Chemical classes in the EtOH extracts of the leaves and flowers of *C. quephongensis* and *C. puhoatensis*.

Chemical classes	HPH	HQP	LPH	LQP	Chemical classes	HPH	HQP	LPH	LQP
Flavonoids	+	+	+	+	Polysaccharides	+	+	+	+
Tannins	+	+	+	+	Coumarins	-	-	-	-
Saponins	+	+	+	+	Amines/amino acids	-	-	-	-
Free reducing sugars	+	+	+	+	Alkaloids	-	-	-	-

The current results matched well with the previous report [19]. By HPLC-MS/MS quantitative analysis, the EtOAc and water/ethanol extracts of *C. quephongensis* flowers were associated with the presence of various chemical classes, but flavonoids (7.38 % - 34.10 %), phenols (1.97 % - 12.20 %), and esters (10.02 % - 39.65 %) can be seen as major compounds [19]. *C. sinensis* species is always a hot subject in phytochemical analysis. Geoffrey *et al.* suggested that flavonoids, tannins, saponins, and alkaloids were characteristic of the leaves, collected from Kenya [20]. In the meantime, the fresh leaves of *C. sinensis*, cultivated in India, were reported to contain only flavonoids [21]. Therefore, it can be concluded that traditional

values and pharmacological properties of teas are mostly dependent on the appearance of polyphenols.

3.2. Lipid peroxidation inhibition

Four extracts HPH, HQP, LPH, and LQP have been subjected to the MDA assay. From Fig. 1, at the concentration of 0.16-100 $\mu\text{g/mL}$, these samples have shown a variety of inhibitory percentages, including HPH (14.48 % - 76.45 %), HQP (14.18 % - 76.45 %), LPH (1.41 - 68.48 %), and LQP (5.36 % - 85.39 %) when trolox was used as a positive control (5.23 - 84.41 %). Taking the IC_{50} values into consideration (Fig. 2), their IC_{50} values run in the order of LQP ($\text{IC}_{50} = 2.02 \pm 0.19 \mu\text{g/mL}$) < HQP ($\text{IC}_{50} = 3.65 \pm 0.36 \mu\text{g/mL}$) < HPH ($\text{IC}_{50} = 4.79 \pm 0.44 \mu\text{g/mL}$) < trolox ($\text{IC}_{50} = 8.67 \pm 0.52 \mu\text{g/mL}$) < LPH ($\text{IC}_{50} = 29.60 \pm 1.37 \mu\text{g/mL}$). As can be seen, the parts of two Vietnamese *Camellia* are outstanding candidates since they were better than the positive control trolox in the cases of the LQP, HQP, and HPH samples, or showed strong lipid peroxidation inhibition action in the case of the LPH sample. As compared with other *Camellia* species, e.g., the aqueous extract of green tea (*C. sinensis*) successfully inhibited pro-oxidants Fe (II), sodium nitroprusside, and quinolinic acid-induced lipid peroxidation in the rat's brain *in vitro* [22]. In another report, green tea is believed to have a protective effect against oxidative stress caused by hydrogen peroxide in mice via lowering biochemical parameters MDA, Ox-LDL (oxidized low-density lipoprotein), and coronary risk [23]. Thereby, this evidence, once again, confirmed the uses of *Camellia* species as well-known antioxidants in food nutrients. The potential antioxidative activities of these samples may be due to the presence of flavonoids and tannins.

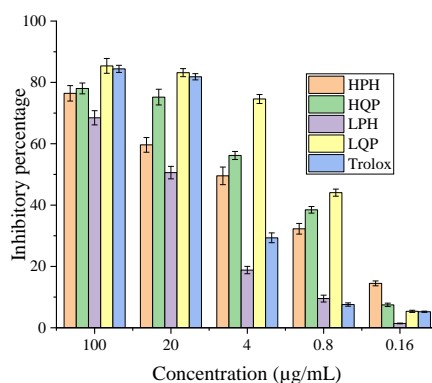


Figure 1. The MDA inhibitory percentage of the tested samples at the concentration of 0.16 - 100 $\mu\text{g/mL}$.

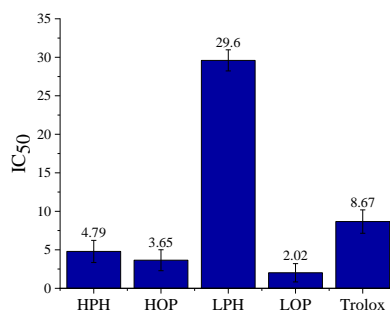


Figure 2. The IC_{50} values of the tested samples and positive control trolox in the MDA inhibitory assay.

3.3. Cytotoxicity

The results of the cytotoxic evaluation against seven cancer cell lines A549, HT-29, SK-Mel-2, HepG2, MCF-7, Hela, and MKN-7 are outlined in Tables 2 and S2. At the highest concentration of 100 µg/mL, the tested samples possessed inhibitory percentages of 37.09 - 75.18 % (Table S2). All four extracts HPH, HQP, LPH, and LQP showed moderate cytotoxicity towards HT-29, HepG2, and MKN-7, with the IC₅₀ values ranging from 48.02 to 96.43 µg/mL. In comparison between HPH and HQP, the HPH sample exhibited cytotoxic to all tested seven cancer cell lines, whereas the HQP sample was inactive to A549 and MCF-7. In contrast, LQP seems always better than the LFH in cytotoxicity, by which the LQP sample was associated with the IC₅₀ values of 48.20 - 81.67 µg/mL against the growth of A549, SK-Mel-2, and Hela, while the LFH failed to do so (IC₅₀ > 100 µg/mL). The previous reports revealed that the alcoholic extracts of *C. sinensis* have cytotoxic effects on A549, HT-29, HepG2, and MCF-7 with the IC₅₀ values of 72 - 324.5 µg/mL [24 - 27]. The leaf extract of *C. chrysanth* caused the IC₅₀ values of 48 - 103 µg/mL against SK-Mel-2 MCF-7, and HepG2 [28]. Collectively, these findings argued that *Camellia* plants are appropriate for anticancer treatments.

Table 2. Cytotoxic activity of the EtOH extracts of the leaves and flowers of *C. quephongensis* and *C. puhoatensis*. Data are presented as mean ± SD (*P* < 0.05 as compared with the control group).

Samples	Cancer cell lines (IC ₅₀ µg/mL)						
	A549	HT-29	SK-Mel-2	MCF-7	HepG2	Hela	MKN-7
HPH	82.63 ± 4.07	56.03 ± 5.02	68.79 ± 3.72	92.92 ± 2.33	62.24 ± 2.65	68.89 ± 2.82	82.78 ± 5.14
HQP	>100	96.43 ± 3.59	92.72 ± 3.23	>100	83.19 ± 3.59	91.64 ± 6.60	87.48 ± 4.62
LFH	>100	82.50 ± 8.11	>100	> 100	93.11 ± 3.69	>100	94.92 ± 7.86
LQP	59.19 ± 4.25	72.94 ± 1.73	48.20 ± 1.83	>100	77.73 ± 5.33	81.67 ± 4.57	89.47 ± 2.70
Ellipticine	0.43 ± 0.02	0.32 ± 0.02	0.31 ± 0.04	0.44 ± 0.05	0.34 ± 0.03	0.32 ± 0.03	0.42 ± 0.05

3.4. Antimicrobial activity

Table 3. Antimicrobial activity of the EtOH extracts of the leaves and flowers of *C. quephongensis* and *C. puhoatensis*.

Microbial strains		IC ₅₀ /MIC (µg/mL)	HQP	LQP	HPH	LPH	Streptomycin	Cycloheximide
Gram (+)	<i>E. faecalis</i>	IC ₅₀	45.36	50.23	46.32	50.21	50.34	
		MIC	128	128	128	128	256	
	<i>S. aureus</i>	IC ₅₀	-	-	-	-	45.24	
		MIC	-	-	-	-	256	
	<i>B. cereus</i>	IC ₅₀	78.35	87.34	75.36	84.56	20.45	
		MIC	256	256	256	256	128	
Gram (-)	<i>E. coli</i>	IC ₅₀	42.14	38.67	43.32	41.21	9.45	
		MIC	128	128	128	128	32	
	<i>P. aeruginosa</i>	IC ₅₀	41.46	39.23	40.23	38.21	41.46	
		MIC	128	128	128	128	256	
	<i>S. enterica</i>	IC ₅₀	20.34	21.58	20.31	21.21	45.67	
		MIC	64	64	64	64	128	

Fungus	<i>C. albicans</i>	IC ₅₀	34.68	52.67	33.12	51.25		10.46
		MIC	128	128	128	128		32

Four extracts HPH, HQP, LPH, and LQP were further evaluated for antimicrobial activity against three Gram-positive bacterial strains *E. faecalis*, *S. aureus*, and *B. cereus*, three strains of Gram-negative bacterial strains *E. coli*, *P. aeruginosa*, and *S. enterica*, and one yeast strain *C. albicans*. From Table 3, both four samples with the IC₅₀ = 45.36 - 50.21 µg/mL/MIC = 128 µg/mL were better than the positive control streptomycin (IC₅₀ = 50.34 µg/mL/MIC = 256 µg/mL) against Gram-positive bacterium *E. faecalis*. Four tested samples also moderately inhibited the growth of *B. cereus* with the IC₅₀ = 75.36 - 84.56 µg/mL/MIC = 256 µg/mL, but they failed to control bacterium *S. aureus*. Similarly, these alcoholic extracts are comparable or superior to the standard streptomycin against the Gram-negative bacteria *P. aeruginosa* (IC₅₀ = 38.21 - 41.46 µg/mL/MIC = 128 µg/mL), and *S. enterica* (IC₅₀ = 20.31 - 21.58 µg/mL/MIC = 64 µg/mL), as well as they were moderately repellent to *E. coli* (IC₅₀ = 38.67 - 43.32 µg/mL/MIC = 128 µg/mL). Last but not least, these four extracts were observed to moderately suppress the growth of fungus *C. albicans* with the IC₅₀ = 33.12 - 52.67 µg/mL/MIC = 128 µg/mL (Table 3). Feas *et al.* suggested that the seed oil extracts of *C. oleifera*, *C. reticulata*, and *C. sasanqua* were better than the positive control gentamicin in an antimicrobial assay against *E. coli*, as well as showing moderate activity against *B. cereus*, and *C. albicans* [29]. It turned out that the methanol extract of *C. sinensis* leaves was found to be the most effective against *B. cereus* [30]. Hence, it can be safe to deal with the application of *Camellia* extracts to treat bacterial infections.

4. CONCLUSIONS

Phytochemical screenings identified that flavonoids, tannins, saponins, free reducing sugars, and polysaccharides are the chemical classes of two Vietnamese *Camellia* species *C. puhoatensis* and *C. quephongensis*. The 70 % ethanol extracts of *C. puhoatensis* flowers and *C. quephongensis* flowers and leaves exhibited lipid peroxidation inhibition with the better IC₅₀ values of 2.02-4.79 µg/mL than that of the positive control trolox (IC₅₀ = 8.67 µg/mL). In the cytotoxic assay, the flower and leaf ethanol extracts of these two medicinal plants moderately inhibited HT-29, HepG2, and MKN-7 with the IC₅₀ values of 56.03-96.43 µg/mL. Regarding antimicrobial assay, all four extracts were better than the positive control streptomycin against the growth of Gram-positive bacterium *E. faecalis* (IC₅₀ = 45.36-50.21 µg/mL/MIC = 128 µg/mL) and Gram-negative bacteria *P. aeruginosa* (IC₅₀ = 38.21 - 41.46 µg/mL/MIC = 128 µg/mL), and *S. enterica* (IC₅₀ = 20.31 - 21.58 µg/mL/MIC = 64 µg/mL). Besides that, they were moderately repellent to Gram-positive bacterium *B. cereus*, Gram-negative bacteria *E. coli*, and fungus *C. albicans*. The current study can be seen as basic knowledge for further research.

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CRedit authorship contribution statement. Ninh The Son and Do Ngoc Dai: Methodology, writing the manuscript and supervision. Nguyen Thi Giang An, Nguyen Thi To Trang and Dao Thi Minh Chau: Formal analysis. Le Thi Huong and Vu Van Khoa: Sample collection and methodology. Nguyen Ngoc Hoa and Huynh Thi Ngoc Ni: Investigation and formal analysis.

Declaration of competing interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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