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RESEARCH ARTICLE

Chemical composition and anti-*Helicobacter pylori* activity of essential oils from rhizomes and tuberous roots of *Boesenbergia rotunda* (L.) Mansf.

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Abstract

Boesenbergia rotunda (Zingiberaceae) is a well-known traditional medicinal and culinary plant found in Southeast Asia and China. The extracts of this plant have been shown to have antibacterial potential against several pathogenic bacteria, including *Helicobacter pylori*. However, the antibacterial activity of the essential oils (EOs) from *B. rotunda* toward *H. pylori* has not been reported. Hence, the present study investigated the chemical compositions and antibacterial properties of the EOs extracted from the rhizomes and tuberous roots of *B. rotunda* against *H. pylori*. GC–MS analysis revealed that the rhizome EO was rich in camphor (28.6 %), 1,8-cineole (16.0 %), geraniol (15.5 %) and *E*-β-ocimene (12.8 %), while the tuberous root EO predominantly contained *E*-β-ocimene (31.9 %), camphor (19.9 %), geraniol (16.3 %), limonene (7.5 %) and 1,8-cineole (4.3 %). Both of the EOs exhibited strong anti-*H. pylori* activity, with minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of 0.31 and 2.50 mg/mL for the rhizome EO and 0.16 and 1.25 mg/mL for the tuberous root EO, respectively. *In vitro* urease inhibition assay revealed that these EOs exhibited comparable and significant inhibitory action on the urease activity of *H. pylori*. Mechanism of action showed that the tuberous root EO had a stronger antibiofilm effect than the rhizome EO and both of the EOs demonstrated potent membrane-permeabilising properties resulting in the leakage of intracellular proteins from *H. pylori* cells. These findings support the potential application of *B. rotunda* EOs as effective natural agents for the prevention and control of *H. pylori* infections.

Keywords: antibacterial effect; *Boesenbergia rotunda*; essential oil; *Helicobacter pylori*; mechanism of action; urease inhibition

Introduction

Helicobacter pylori is widely recognised as a major cause of various gastric pathologies, including gastritis, peptic ulcers, gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma (1, 2). Although combinations of antibiotics and proton pump inhibitors are effective in treating *H. pylori* infections (2), the increasing prevalence of antibiotic resistance poses a considerable challenge to successful eradication of the infectious pathogen, limiting treatment options and contributing to higher morbidity and healthcare costs (2-7). Therefore, the exploration of alternative therapeutic strategies is an urgent need. Plant EOs have attracted considerable attention as potential sources of novel antimicrobial agents due to their diversity in chemical compositions and broad spectrum of biological activities (8). Many plant EOs and their constituents have been reported to possess significant antibacterial activities against *H. pylori* in various studies (9-12).

Boesenbergia rotunda, a rhizomatous herb belonging to the Zingiberaceae family, is commonly known as fingerroot due to its characteristic rhizomes with finger-like roots. It is a well-known traditional medicinal and spice plant from Southeast Asia and China (13). The plant is used to treat a spectrum of conditions, including fever, rheumatism, muscular discomfort, peptic ulcers, gastrointestinal disturbances and bacterial infections (14). *B. rotunda* was reported to exhibit broad bioactivity, such as anticancer, anti-inflammatory, antimicrobial, antioxidative, anti-viral, anti-insect, anti-diabetic, anti-allergic, anti-obesity, hepatoprotective, neuroprotective, renoprot-ective, gastroprotective, skin protective, vasorelaxant and aphrodisiac activities (13, 15). The ethyl acetate and methanolic extracts have also demonstrated their potent antibacterial activity against various bacteria, including *Escherichia coli, Staphylococcus aureus, Serratia marcescens, Streptococcus mutans*, *S. sobrinus* and different strains of *H. pylori* (16- 20).

B. rotunda yields 0.20 - 0.39 % EO from its rhizomes and 0.18 - 0.22 % from its tuberous roots (21-23). *The* major constituents reported to be present in both EOs include 1,8-cineole, camphor, camphene, geraniol, ocimene and limonene (21, 23). The *B. rotunda* rhizome EO was found to possess more potent antibacterial activity against Gram-positive bacteria, including *S. aureus* and *Bacillus*

cereus and Gram-negative bacteria, including *Pseudomonas aeruginosa* and *E. coli,* compared to two other aromatic plants of the Zingiberaceae Family in Malaysia, including *Curcuma mangga* and *Kaempferia galanga* (22). This EO also exhibited a bactericidal effect against oral bacterial pathogens, encompassing *S. pyogenes, S. mutans and S. sobrinus* (23). The rhizome EO demonstrated strong antibacterial and antibiofilm effects against foodborne pathogens (*E. coli, Salmonella typhi* and *B. cereus) (*24). The *B. rotunda rhizome* EO had antibacterial activity toward methicillin-resistant S. aureus (MRSA) and showed synergistic interaction with cloxacillin against MRSA (25). The rhizome EO was also reported to exert multiple anti-MRSA mechanisms, including inhibition of biofilm formation, increase of membrane permeability, alterations of cell wall and cytoplasmic membrane and induction of intracellular component leakage in MRSA *(25).* However, there is a lack of available data about these mechanisms of action of the *B. rotunda* EOs against *H. pylori. Therefore, the current* study investigates the anti-*H. pylori* activity of the EOs extracted from *B. rotunda* rhizomes and tuberous roots and provides potential mechanisms of actions of the EOs against *H. pylori.*

Materials and Methods

Chemicals and reagents

Amoxicillin (≥98 %) was procured from Santa Cruz Biotechnology Inc. Alkane standard solution (C_8–C_{20}) was supplied by Merck. Brucella broth (BB) and brain–heart infusion (BHI) broth used for bacterial culture and antimicrobial tests were purchased from Becton, Dickinson and Company (Sparks, MD, USA) and Newborn bovine serum (NBS) was sourced from Hyclone (Logan, UT, USA). All additional chemicals and reagents used in the study were of analytical grade.

Plant samples

Samples of *Boesenbergia rotunda* (L.) Mansf. were collected in April 2023 at My An Hung commune, Dong Thap province, Vietnam (14°44'07.8" N, 108°16'46.9" E). Species identification was carried out by Tran Thanh Hung at the Biotechnology Laboratory, Institute of Engineering Technology, Thu Dau Mot University, Binh Duong Province, based on morphological characteristics. The plant samples are perennial herbaceous species with pseudo-stems formed by tightly packed, overlapping leaf sheaths exhibiting a purple hue and reaching 60-80 cm in height. Leaf blades are elliptic, measuring 25-30 cm in length and 9-12 cm in width, with entire margins (Fig. 1A). The inflorescences emerge from the central portion of the pseudo-stems, typically bearing two flowers with pink corolla per cluster (Fig. 1B). The plants are distinguished by short rhizomes and multiple elongated, finger-like roots that are yellowish in color (Fig. 1C). A voucher specimen (TTH23.01) of the plant was deposited in the Biotechnology Laboratory, Institute of Engineering Technology, Thu Dau Mot University, Ho Chi Minh City.

Essential oil extraction

Essential oils were extracted following the method described (11). The fresh *rhizomes* (487 g) and tuberous roots (1440 g) were ground and subjected to hydro-distillation at 100 °C for 3 hr using a Clevenger-type apparatus with a 5 L capacity. The EOs obtained were dried over anhydrous sodium sulfate and the yields (%, w/w) were measured based on the fresh weight of the samples. The EOs were stored in dark glass vials at –20 °C for further use.

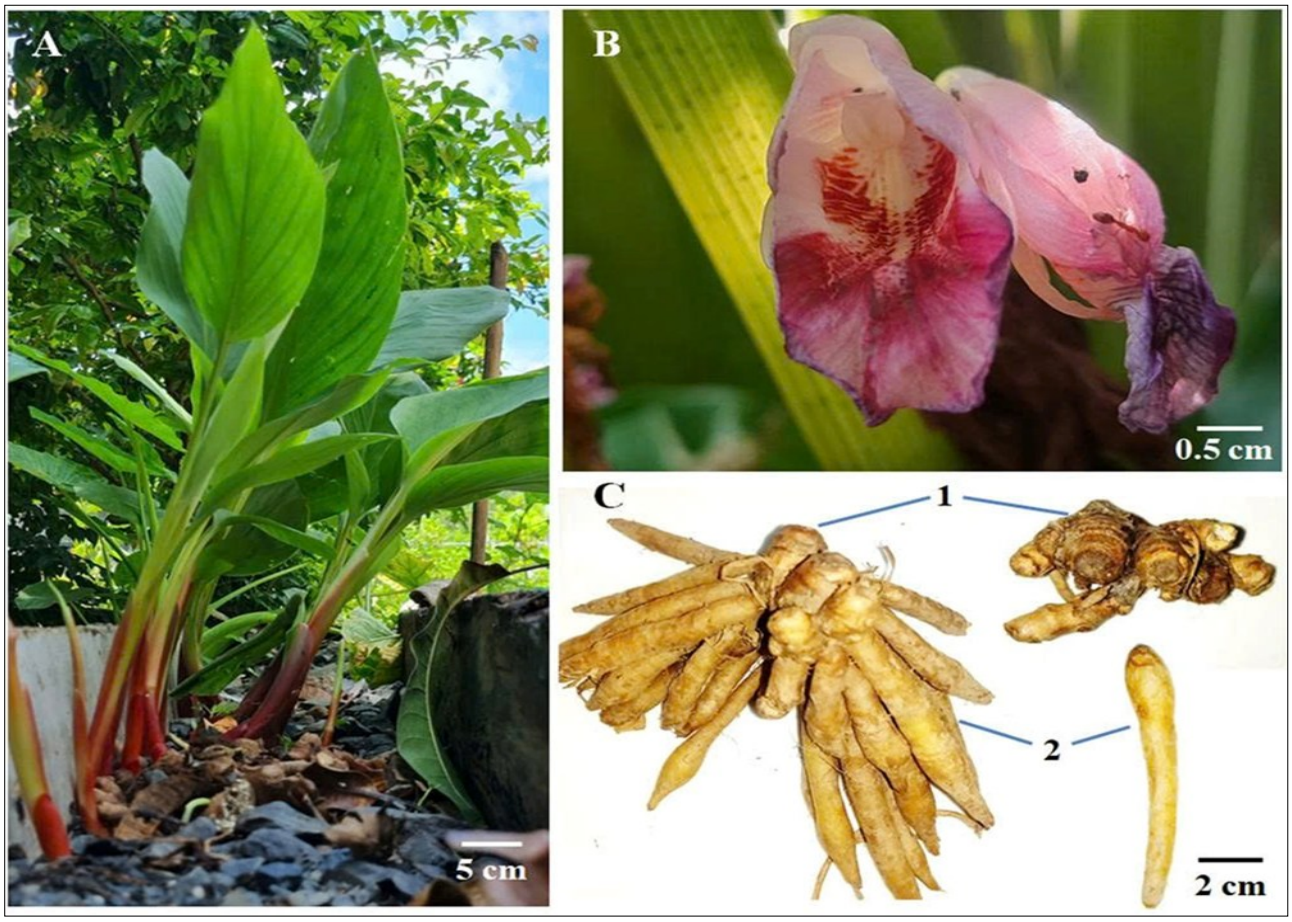

Fig. 1. The morphology *of B. rotunda.* **A.** Whole plant, **B.** Flower, **C.** Fingerroot, **1.** Rhizomes and **2.** tuberous roots.

H. pylori strain and culture condition

The reference strain *Helicobacter pylori* ATCC 43504 was obtained from the Department of Genetics, Faculty of Biology and Biotechnology, VNUHCM-University of Science. Bacterial preservation and culture methods followed previously established protocols (26, 27). In brief, the strain was maintained in BHI broth containing 25 % glycerol and stored in a liquid nitrogen container. For cultivation, the bacteria were grown on Brucella agar supplemented with 10 % NBS and incubated at 37 °C for 72 hr under microaerophilic conditions generated using Oxoid campy gen gas packs (Thermo Scientific, UK).

Essential oil analysis

Gas chromatography (GC) and GC–mass spectrometry (GC–MS) analyses of the EOs were performed at the Physical Chemical Analysis Laboratory, Institute of Applied Materials Science, Ho Chi Minh City, Vietnam. A 2 µL aliquot of the EOs was diluted in 1 mL of n-hexane and a 1 µL portion of this solution was injected into an Agilent 6890N GC system (Santa Clara, CA, USA), equipped with a split injector and flame ionisation detector (FID). Separation was achieved using an HP-5 MS fused silica capillary column (30 m × 0.25 mm internal diameter, 0.25 µm film thickness) coated with polydimethylsiloxane. The oven temperature program was initiated at 50 °C (held for 2 min), then increased at a rate of 2 °C/min to 80 °C, 5 °C/min to 150 °C, 10 ° C/min to 200 °C and finally ramped at 20 °C/min to 300°C, where it was held for 5 min. The split injection ratio was 1:40 and helium served as the carrier gas at a constant flow rate of 1.0 mL/min.

GC–MS analysis was carried out using the same GC system interfaced with an Agilent 5973 inert mass selective detector (MSD). GC conditions were consistent with those used in the FID analysis. MS parameters included an ionisation energy of 70 eV and an electron multiplier voltage of 1024 V. The injector, transfer line and ion source temperatures were set at 280 °C, 280 °C and 230 °C, respectively. Retention indices (RIs) were calculated by analysing a C_8–C_{20} n-alkane standard mixture under identical chromatographic conditions. Identification of EO constituents was accomplished by comparing the obtained mass spectra with those from the National Institute of Standards and Technology (NIST) MS library and further validated by comparing the calculated RIs with published literature values (28).

Anti-*H. pylori* activity

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the EOs against *H. pylori* were determined using the broth microdilution method in sterile 96-well microtiter plates, following the procedure described by a previous study (29). Stock solutions of the EOs were prepared by dissolving them in 50 % Dimethyl sulfoxide (DMSO) at a concentration of 100 mg/mL. Serial dilutions of each stock solution of the EOs were prepared in the wells to yield final concentrations ranging from 0 to 10 mg/mL. Each test well received 50µL of a standardised bacterial suspension (10^6 CFU/mL) and 100µL of the EO solution at the designated concentrations. Negative control wells contained bacterial suspension, Brucella broth supplemented with NBS and dimethyl sulfoxide (5 % DMSO), while amoxicillin was used as a positive control under identical conditions. Blank wells containing 5 % DMSO in the culture medium without bacteria were also included.

The plates were incubated at 37 °C for 48 hr under microaerophilic conditions with shaking at 70 rpm. Following incubation, 10 µL of 0.01 % resazurin solution was added to each well and incubated for an additional hr at 37 °C. MIC values were recorded as the lowest concentrations showing no bacterial growth (no resazurin colour change). To determine MBC values, 20 µL aliquots from MIC wells that showed no resazurin colour change were spread on the Brucella agar well surface of 24-well plates. The plates were incubated for 72 hr at 37 °C under microaerophilic conditions. MBC was defined as the lowest concentration of the EO at which no visible bacterial growth was observed on the agar surface.

Inhibition of *H. pylori* urease activity

Crude urease was extracted and its protein content quantified following the previous method (11). The urease inhibitory activity of the EOs was assessed using the salicylate–hypochlorite method in 96-well microplates, as previously reported (27, 30). Stock solutions of the EOs were prepared by dissolving in 80 % ethanol at a concentration of 100 mg/mL and subsequently diluted in sodium phosphate–EDTA buffer (pH 7.3) to yield final concentrations ranging from 0 to 1.25 mg/mL per well. Each well was loaded with 10µL of crude urease solution (equivalent to 0.04 urease units) and 40µL of the EO solution. Thiourea, prepared in the same manner with final concentrations ranging from 0 to 0.125 mg/mL, served as the positive control.

Negative control wells contained crude urease and buffer with or without 4 % ethanol. Background and blank wells were similarly prepared but employed heat-inactivated urease (100°C for 1 hr) to account for non-enzymatic reactions. The mixtures were incubated at 37 °C with shaking at 50 rpm for 90 minutes, followed by the addition of 50 µL of 5mM urea in sodium phosphate–EDTA buffer. After an additional 30-minute incubation at 37 °C, a stop reagent consisting of 35 µL of Solution A (14.6 % sodium salicylate and 0.1 % sodium nitroprusside) and 65 µL of Solution B (1.78 % NaOH, 11.57 % sodium citrate and 0.54 % active NaOCl) was added sequentially to each well. The plate was incubated at 37 °C for 30 mint to allow for colour development.

$$\text{Inhibition (\%)} = \left(1 - \frac{\text{OD}_{625}\text{ tested sample} - \text{OD}_{625}\text{ corresponding background}}{\text{OD}_{625}\text{ control} - \text{OD}_{625}\text{ blank}}\right) \times 100$$

Ammonia produced by the enzymatic hydrolysis of urea was quantified by measuring absorbance at 625 nm using a microplate reader, with ammonium chloride serving as the standard. Urease inhibition (%) was calculated using the formula:

(Eqn. 1)

The urease inhibitory activity of the EOs was expressed as the IC_{50} value, defined as the concentration required to inhibit 50 % of urease activity relative to the untreated control.

Inhibition of *H. pylori* biofilm formation

The biofilm inhibition assay was carried out using a 96-well microtiter plate, following the crystal violet staining method as previously described (27). The procedure was similar to the MIC assay, with the EO solution applied at sub-MICs. Negative control wells contained 2.5 % DMSO and a standardised bacterial suspension. Blank wells (containing 2.5 % DMSO in the culture

medium without bacteria) and background wells (containing the EO in the culture medium without living bacteria) were included to account for absorbance interference.

Following a 48-hr incubation period under microaerophilic conditions, wells were stained with 0.1 % crystal violet solution to assess biofilm formation. Absorbance was measured at 595 nm using a Microlisa Plus microplate reader (Micro Lab Instruments, India). The percentage of biofilm inhibition was calculated using the formula:

$$\text{Biofilm inhibition (\%)} = \left(100 - 100 \times \frac{OD_{595}\ \text{treatment} - OD_{595}\ \text{background}}{OD_{595}\ \text{control} - OD_{595}\ \text{blank}}\right) \quad \text{(Eqn. 2)}$$

Effect on cell membrane permeability of *H. pylori*

The effect of the EOs on *H. pylori* membrane permeability was evaluated using a crystal violet uptake assay, based on previously published protocols with minor modifications (31, 32). In each treatment group, 900 µL of a bacterial suspension (10^8 CFU/mL) was mixed with 100 µL of the EO solution to reach final concentrations corresponding to the MBC, 4xMIC, 2xMIC, 1xMIC, ½×MIC and ¼ xMIC levels. Amoxicillin at MBC of 0.13 µg/mL was included as a positive control under the same conditions. Negative control groups received 900µL of bacterial suspension and 100 µL of 0.01 M phosphate-buffered saline (PBS, pH 7.3) with or without 2.5 % DMSO.

All samples were vortexed thoroughly and incubated under microaerobic conditions at 37 °C with shaking at 150 rpm for 2 hr. Following incubation, bacterial cells were pelleted by centrifugation at 14,000 rpm for 1 minute at room temperature, washed twice with 500 µL of PBS (pH 7.3) and resuspended in 500 µL of 0.005 % (w/v) crystal violet solution in PBS. The suspensions were incubated for 30 seconds, then centrifuged again under the same conditions. Subsequently, 150µL of the resulting supernatant was transferred to a 96-well plate for absorbance measurement.

$$\%\ \text{Absorption} = \left(1 - \frac{OD_{595}\ \text{sample} - OD_{595}\ \text{blank}}{OD_{595}\ \text{crystal violet} - OD_{595}\ \text{blank}}\right) \times 100 \quad \text{(Eqn. 3)}$$

Blank wells containing only PBS and wells containing 0.005 % crystal violet solution were also prepared. Membrane permeability was assessed by measuring the absorbance at 595 nm using a microplate reader. The percentage of dye uptake was calculated according to the following equation:

Effect on cell membrane integrity of *H. pylori*

Membrane integrity was evaluated based on protein release into the supernatant, using the Bradford protein assay, following protocols described in previous studies (31-33). To assess protein leakage, *H. pylori* cells were first washed and resuspended in PBS, with turbidity adjusted to approximately 10^8 CFU/mL. The suspensions (900 µL) were then exposed to the EO solutions (100 µL) at their respective MIC and MBC concentrations and incubated for 4 hr. Amoxicillin served as a positive control, while untreated samples and those containing 2.5 % DMSO functioned as negative controls. After 4 hr of incubation under microaerobic conditions at 37 °C with shaking at 150 rpm, samples were centrifuged at 14000 rpm for 1 minute at room temperature. Supernatants were collected and analysed for protein content using the Bradford method (34).

Data analysis

All bioassays were repeated three times in triplicate and mean values ± SE were presented. The IC_{50} values were determined using the GraphPad Prism 5 software program (La Jolla, CA). The Bonferroni multiple comparison method was used to test for significant differences among the treatments (GraphPad Prism 5 software program). MIC values of < 0.1, 0.1–0.62, 0.62–1.25, 1.25–2.5 and > 2.5 mg/mL were classified as extremely high, high, moderate, low and no growth inhibition, respectively (35).

Results and Discussion

Chemical constituents of the rhizome and tuberous root EOs

Clear, light-yellow EOs were obtained from the fresh rhizomes and the tuberous roots of *B. rotunda* collected in Dong Thap (Vietnam), with yields of 0.48 % and 0.25 % (w/w), respectively. GC-MS analysis identified 34 components in the EOs from both the rhizomes and the tuberous roots, representing 98.28 % and 99.42 % of the total EO content, respectively (Table 1). In the rhizome EO, oxygenated monoterpenes (67.54 %) were the predominant components, followed by monoterpene hydrocarbons (25.41 %). The oxygenated monoterpenes such as camphor (28.57 %), 1,8-cineole (16.00 %) and geraniol (15.47 %) were the most widespread components, followed by linalool (2.14 %), *α*-terpineol (1.21 %), camphene hydrate (1.19 %) and geranial (1.03 %). *E*-*β*-ocimene (12.87 %), camphene (6.16 %), *Z*-*β*-ocimene (2.21 %) and limonene (2.12 %) were the major monoterpene hydrocarbons, whereas *E*-methyl cinnamate (4.05 %) is the methyl ester of cinnamic acid, belonging to the phenylpropanoid group. In addition to the rhizome EO, the EO obtained from the tuberous roots was found to contain oxygenated monoterpenes and monoterpene hydrocarbons in similar proportions (48.45 and 49.21 %, respectively). The most dominant oxygenated monoterpenes were camphor (19.88 %), geraniol (16.34 %), 1,8-cineole (4.25 %), geranial (2.51 %), neral (1.71 %) and linalool (1.65 %), while *E*-*β*-ocimene (31.92 %), limonene (7.45 %) and camphene (5.21 %) were the prevalent monoterpene hydrocarbons, followed by *o*-cymene (1.73 %) and myrcene (1.06 %). Sesquiterpenoids were detected in trace amounts in both EOs of the rhizomes and tuberous roots (Table 1).

The rhizomes and tuberous roots in the present study showed higher EO yields than those collected in Thailand, Malaysia and Indonesia, which produced EO yields ranging from 0.20 to 0.39 % and from 0.18 to 0.22 %, respectively *(21-23)*. Many studies have reported the EO compositions of the whole rhizome of *B. rotunda* (22-24). However, a few reports have separately analysed the compositions of the rhizome EO and tuberous root EO. There were many differences between the constituents of the rhizome EO and the tuberous root EO in our study, as shown in Table 1. The EO components from the rhizome collected in Dong Thap were found to be most similar to those of the rhizome in Pathum Thani, both rich in camphor, 1,8-cineole, geraniol and camphene (Table 2). *B. rotunda* rhizome and tuberous root EOs contained significantly high levels of camphor (41.12 and 22.39 %, respectively) in Chiang Mai, 1,8-cineole (65.87 and 67.89 %, respectively) in Ratchaburi and geraniol (9.32 – 15.47 % and 16.28 – 18.45 %, respectively) in Dong Thap, Pathum Thani and Chiang Mai, but only 4.49 and 2.93 %, respectively, in Ratchaburi. While camphene and *α*-pinene were not detected in the Ratchaburi EOs, both compounds were found in the

Table 1. Chemical constituents of EOs from rhizomes and tuberous roots of *B. rotunda* collected in Dong Thap (Vietnam)

Peak	Identified components	Molecular formula	RI_L[1]	RI_C[2]	Rhizome EO (%)	Tuberous root EO (%)
1	Butyl acetate	$C_6H_{12}O_2$	811	810	0.06	0.09
2	2,4-Dimethylheptane	C_9H_{20}	837[Δ]	842	0.07	
3	2,3-Dimethylheptane	C_9H_{20}	858[Δ]	853	0.11	
4	*o*-Xylene	C_8H_{10}	863[Δ]	866		0.08
5	Tricyclene	$C_{10}H_{16}$	926	919	0.26	0.23
6	*α*-Pinene	$C_{10}H_{16}$	939	930	0.99	0.90
7	Camphene	$C_{10}H_{16}$	954	946	6.16	5.21
8	Benzaldehyde	C_7H_6O	960	957	0.13	0.10
9	Butyl methacrylate	$C_8H_{14}O_2$	967[Δ]	977	0.07	0.09
10	6-Methyl-5-heptene-2-one	$C_8H_{14}O$	985	984	0.16	0.11
11	Myrcene	$C_{10}H_{16}$	990	989	0.68	1.06
12	*o*-Cymene	$C_{10}H_{14}$	1026	1025	0.12	1.73
13	Limonene	$C_{10}H_{16}$	1029	1029	2.12	7.45
14	1,8-Cineole	$C_{10}H_{18}O$	1031	1031	16.00	4.25
15	*Z*-*β*-Ocimene	$C_{10}H_{16}$	1037	1039	2.21	
16	*E*-*β*-Ocimene	$C_{10}H_{16}$	1050	1050	12.87	31.92
17	*γ*-Terpinene	$C_{10}H_{16}$	1059	1055		0.07
18	*α*-Terpinolene	$C_{10}H_{16}$	1088	1085		0.12
19	Rosefuran	$C_{10}H_{14}O$	1090[Δ]	1093		0.07
20	Linalool	$C_{10}H_{18}O$	1096	1095	2.14	1.65
21	*α*-Pinene oxide	$C_{10}H_{16}O$	1099	1097	0.25	0.07
22	5,6-Dimethyldecane	$C_{12}H_{26}$	1126[Δ]	1121	0.15	
23	Neo-allo-ocimene	$C_{10}H_{16}$	1144	1136		0.14
24	(3*Z*,5*Z*)-2,7-Dimethyl-3,5-octadiene	$C_{10}H_{18}$		1142		0.38
25	Camphor	$C_{10}H_{16}O$	1146	1144	28.57	19.88
26	Camphene hydrate	$C_{10}H_{18}O$	1149	1152	1.19	0.71
27	Isoborneol	$C_{10}H_{18}O$	1160	1161	0.15	
28	Borneol	$C_{10}H_{18}O$	1169	1169	0.79	0.44
29	Terpinen-4-ol	$C_{10}H_{18}O$	1177	1177	0.23	0.19
30	*α*-Terpineol	$C_{10}H_{18}O$	1188	1189	1.21	0.63
31	Neral	$C_{10}H_{16}O$	1238	1234	0.51	1.71
32	Geraniol	$C_{10}H_{18}O$	1252	1250	15.47	16.34
33	Geranial	$C_{10}H_{16}O$	1267	1264	1.03	2.51
34	*E*-Methyl cinnamate	$C_{10}H_{10}O_2$	1378	1376	4.05	0.50
35	Geranyl propinoate	$C_{13}H_{22}O_2$	1477	1475	0.12	0.09
36	*α*-Zingiberene	$C_{15}H_{24}$	1493	1485	0.06	0.24
37	*α*-Farnesene	$C_{15}H_{24}$	1505	1496		0.08
38	Geranyl butanoate	$C_{14}H_{24}O_2$	1564	1552	0.07	0.09
39	2-Ethylhexyl cyclohexanecarboxylate	$C_{15}H_{28}O_2$		1659	0.13	
40	Oleyl alcohol	$C_{18}H_{36}O$		2047	0.10	0.29
41	Pinostrobin chalcone	$C_{16}H_{24}O_4$	2402[Δ]	2394	0.05	
Total percentage of identified components					98.28	99.42
Monoterpene hydrocarbons					25.41	49.21
Oxygenated monoterpenes					67.54	48.45
Sesquiterpene hydrocarbons					0.06	0.32
Oxygenated sesquiterpenes					0.13	0
Others					5.14	1.44

[1]RI_L: Retention index (Literature); [2]RI_C: Retention index (Calculated); The rhizomes and tuberous roots of Fingeroot (*B. rotunda*) collected in Apr. 2023 at My An Hung commune, Dong Thap Province, Vietnam and RI_C identified on a HP-5MS column compared with RI_L reported in the literature (28); [Δ] From NIST.

Dong Thap, Pathum Thani and Chiang Mai EOs at concentrations of 5.21 – 6.88 % and 0.90 – 1.27 %, respectively, except for the tuberous root EO in Chiang Mai with higher percentages up to 16.02 % and 4.20 %. Limonene, linalool and α-terpineol were also commonly present in these EOs, except limonene, which was absent in the rhizome EOs in Ratchaburi and Chiang Mai. In particular, limonene was present at a relatively high concentration (7.45 %) in the tuberous root EO from Dong Thap, while α-terpineol was abundant (6.54 %) in the rhizome EO from Ratchaburi. The rhizome EO in Dong Thap was found to be rich in both *E*-β-ocimene (12.87 %) and *Z*-β-ocimene (2.21 %), but only *Z*-β-ocimene was present in high levels in the rhizome and tuberous root EOs (17.26 and 27.74 %, respectively) in Pathum Thani and (16.25 and 7.25 %, respectively) in Chiang Mai. In contrast, *E*-β-ocimene was the predominant compound, accounting for 31.92 % in the tuberous root EO from Dong Thap, followed by 7.83 % in the EO from Chiang Mai. Camphene hydrate, neral, geranial and *o*-cymene were also characteristic components found only in the rhizome and tuberous root EOs in Dong Thap. *p*-Cymene, carvone, caryophyllene and methyl 3-phenylpropanoate were exclusively detected in the tuberous root EO, while *Z*-linalool oxide and *p*-menth-8-en-1,2-diol were present only in the rhizome EO in Ratchaburi (Table 2). The variation in the chemical composition of essential oils may be due to differences in environmental and climatic conditions, geographical location and plant chemotypes (36, 37).

Antibacterial activity of the rhizome and root EO against *H. pylori*

The antibacterial potential of the rhizome and tuberous root EOs of *B. rotunda* in Dong Thap was assessed against *H. pylori* ATCC 43504 using the broth microdilution method. As shown in Table 3, both the rhizome and tuberous root EOs exhibited inhibitory and bactericidal activities, despite notable differences in their potency. The tuberous root EO exhibited a markedly lower MIC value (0.16 mg/mL) than that of the rhizome EO (0.31 mg/mL), indicating a stronger anti-*H. pylori* effect. Similarly, the MBC value of the tuberous root EO (1.25 mg/mL) was lower than that of the rhizome EO (2.50 mg/mL). Despite this, both of the EOs exhibited identical MBC/MIC values of 8, suggesting a bacteriostatic action at lower concentrations and a bactericidal effect at elevated doses.

The rhizomes and tuberous roots of *B. rotunda* have been widely utilised as culinary ingredients in various countries (13). Beyond their dietary use, different parts of the plant have been traditionally employed in the treatment of peptic ulcers, gastrointestinal disorders and bacterial infections (14). Previous studies have demonstrated that extracts and isolated constituents of *B. rotunda* exhibited antibacterial activities against multiple strains of *H. pylori* (19, 20). In the present study, the EOs derived from the rhizomes and tuberous roots of *B. rotunda* displayed potent antibacterial effects against *H. pylori*, further supporting their therapeutic potential.

The anti - *H. pylori* activities of the rhizome EO and the tuberous root EO could be attributed to the presence of key constituents such as camphor, 1,8-cineole, geraniol, *E*-β-ocimene and limonene. Previous studies have demonstrated that plant EOs rich in these compounds could exhibit notable antibacterial effects. The EO of *Tanacetum parthenium*, which contains 49–60.8 % camphor, showed strong inhibitory activity against *Bacillus subtilis* and *Staphylococcus aureus*, with a MIC value of 125 µg/mL (38). Camphor has also been reported to exert synergistic and additive effects, enhancing the antibacterial activity of *Lavandula latifolia* EO against *Listeria monocytogenes* and *S. aureus* (39). Similarly, EO from *Cinnamomum glanduliferum*, dominated by 1,8-cineole (65.9 %), exhibited potent antibacterial activity against both Gram-positive and Gram-negative bacteria, including *H. pylori*, with MIC values ranging from 0.49 to 31.3 µg/mL (40). Geraniol was proven to possess

Table 2. Comparative analysis of chemical constituents of *B. rotunda* rhizome and tuberous root EOs collected in Dong Thap (Vietnam) and different regions in Thailand

Components	Rhizome (%)				Tuberous root (%)			
	Dong thap	Pathum thani	Ratchaburi	Chiang mai	Dong thap	Pathum thani	Ratchaburi	Chiang mai
α-Pinene	0.99	1.05		1.02	0.90	1.27		4.20
Camphene	6.16	6.88		6.82	5.21	6.34		16.02
Myrcene	0.68	0.70			1.06	1.40		0.54
p-Cymene		0.18					4.87	0.20
o-Cymene	0.12				1.73			
Limonene	2.12	2.66			7.45	2.39	3.60	3.11
1,8-Cineole	16.00	20.11	65.87	13.18	4.25	13.87	67.89	9.46
Z-β-Ocimene	2.21	17.26		16.25		27.74		7.25
E-β-Ocimene	12.87				31.92			7.83
Z-Linalool oxide			3.55					
Linalool	2.14	2.42	1.83	2.36	1.65	2.00	1.15	1.51
Camphor	28.57	25.78	7.93	41.12	19.88	20.09	2.59	22.39
Camphene hydrate	1.19				0.71			
Isoborneol	0.15	0.20		2.38		0.26		2.69
Borneol	0.79	0.89		1.73	0.44	0.64		1.10
α-Terpineol	1.21	1.46	6.54	1.40	0.63	1.20	2.04	1.46
Neral	0.51				1.71			
Carvone							3.25	
Geraniol	15.47	11.83	4.49	9.32	16.34	16.28	2.93	18.45
Geranial	1.03				2.51			
p-Menth-8-en-1,2-diol			7.61					
Methyl 3-phenylpropanoate		0.13					2.38	
E-Methyl cinnamate	4.05	5.87			0.50	1.64		
Caryophyllene							7.57	

The rhizomes and tuberous roots of Fingeroot collected Dong Thap Province, Vietnam compared with those collected in Jun. 2015 at Pathum Thani, Ratchaburi and Chiang Mai Provinces, Thailand (21).

strong bactericidal activity toward *H. pylori*, with an MBC of 100 µg/mL (9). The EO from *Neolitsea sericea* twigs, containing 73.7 % *E*-β-ocimene, showed potent antibacterial activity against several Gram-positive (*B. cereus*, *S. aureus and S. epidermidis*) and Gram-negative bacteria (*E. coli*, *Enterobacter aerogenes* and *Vibrio parahaemolyticus*), with MIC values ranging from 128 to 1024 µg/mL and MBC values from 256 to 1024 µg/mL (41). EOs from *Citrus paradisi* and *C. limon*, rich in limonene (58.1 % to 92.3 %), also showed inhibitory effects on the growth of *H. pylori,* with MIC (MBC) values of 100 (125) µg/mL and 125 (250) µg/mL, respectively (9, 12). Limonene alone exhibited anti-*H. pylori* activity with a MIC of 75 µg/mL (42). As plant EOs are complex mixtures of volatile terpenoids, the unique combination of constituents likely plays a critical role in their overall antibacterial effects. The anti-*H. pylori* activity of the rhizome and tuberous root EOs of *B. rotunda* may result from synergistic interactions among both major and minor constituents. Therefore, further investigations into the individual and combined antibacterial effects of these components and their mode of action against *H. pylori* are needed.

Inhibition of *H. pylori* urease activity

The inhibitory effects of *B. rotunda* rhizome and tuberous root EOs against *H. pylori* urease were evaluated *in vitro* and the results are presented in Table 3. Both of the EOs exhibited dose-dependent urease inhibition, with the tuberous root EO showing slightly greater inhibitory potency than the rhizome EO. Specifically, the IC_{50} value of the tuberous root EO was 0.191 mg/mL (95 % CI: 0.138 – 0.266), whereas the rhizome EO showed an IC_{50} value of 0.277 mg/mL (95 % CI: 0.215 – 0.356). The 95 % confidence intervals indicated that there was no statistically significant difference in the *H. pylori* urease inhibitory activity of the two EOs. In comparison, the reference inhibitor thiourea exhibited significantly stronger activity, with an IC_{50} of 0.039 mg/mL (95 % CI: 0.034–0.044).

Numerous natural extracts have been identified as potent *H. pylori* urease inhibitors (43). However, studies focusing on the *H. pylori* urease inhibitory activity of EOs remain limited. Previous investigations have demonstrated that EOs from *Juniperus virginiana*, *Pinus silvestris*, *Citrus limon*, *Abies alba*, *Melaleuca alternifolia*, *Cymbopogon schoenanthus*, *Origanum vulgare* and *Thymus vulgaris* exhibited urease inhibition with IC_{50} values ranging from 0.005 to 0.248 mg/mL (12). Similarly, EOs extracted from several *Nepeta* species, including *N. aristata*, *N. baytopii*, *N. italica*, *N. nuda* and *N. stenantha*, have shown strong inhibitory effects on urease activity with IC_{50} values between 0.004 and 0.07 mg/mL (44). The EOs from fresh fruits of *Litsea cubeba* showed the *H. pylori* urease inhibitory activity with an IC_{50} value of 0.120 mg/mL (11). In the present study, the rhizome and tuberous root EOs of *B. rotunda* exhibited comparable and significant inhibitory action on *H. pylori* urease. This inhibitory effect is of particular interest considering the central role of urease in *H. pylori* pathogenicity. Urease hydrolyses urea to produce ammonia, which buffers gastric acidity and facilitates bacterial survival and colonisation within the acidic environment of the stomach (45). By neutralizing the surrounding gastric acid, the enzyme enables *H. pylori* to persist and establish infection (1). Therefore, inhibition of *H. pylori* urease activity represents a promising therapeutic strategy for combating the bacterial infection. The observed inhibitory effects of the two EOs on the enzyme underscore their potential as natural agents for the development of alternative or adjunct treatments targeting *H. pylori*.

Inhibition of *H. pylori* biofilm formation

The inhibitory effects of *B. rotunda* rhizome and root EOs on *H. pylori* biofilm formation were assessed at sub-MIC levels, revealing a concentration-dependent inhibition (Fig. 2). The rhizome EO exhibited moderate antibiofilm activity, with inhibition increasing from 41.33 % at 1/8×MIC to 69.78 % at 3/4×MIC with significant differences at $p < 0.01 – 0.001$. In contrast, the tuberous root EO demonstrated a more potent antibiofilm activity, achieving 76.12 % and 79.03 % inhibition at 1/2×MIC and 3/4×MIC, respectively, with nonsignificant differences at $p > 0.05$. Moderate antibiofilm activity of the EO (52.35 and 57.55 %) was found at 1/8×MIC and 1/4×MIC, respectively.

Biofilms, structured microbial communities embedded in a matrix of extracellular polymeric substances (EPS), play a critical role in bacterial survival by protecting host immune defences and antimicrobial agents (46). The formation of biofilm by *H. pylori* has been recognised as a major factor contributing to antibiotic treatment failure and recurrent infections (46). Therefore, the inhibition of biofilm formation represents a promising strategy for controlling *H. pylori* associated diseases.

Previous studies have shown that the EO extracted from the rhizome of *B. rotunda* exhibits strong antibiofilm activity against various foodborne pathogens, including *E. coli*, *S. typhi* and *B. cereus* (24), as well as methicillin-resistant *Staphylococcus aureus* (MRSA) (25). In the present study, the EO from tuberous roots of *B. rotunda* displayed a potent inhibitory effect on *H. pylori* biofilm formation. These findings underscore the potential of *B. rotunda* EO as a natural antibiofilm agent for the prevention and treatment of *H. pylori* infections.

Effect on cell membrane permeability of *H. pylori*

The results in Fig. 3 demonstrated that the membrane of *H. pylori* cell walls significantly increased in the crystal violet uptake after 2 hr of treatment with the rhizome and tuberous root EOs of *B. rotunda.* Both of the EOs induced a dose-dependent increase in membrane permeability. The rhizome EO significantly increased the membrane permeability of *H. pylori,* with the highest percentages of crystal violet uptake (94.0 and 97.7 %, respectively) at 4 × MIC and MBC, with no significant difference between the two concentrations ($p > 0.05$) and showed 74.24 % uptake at 2 × MIC, which was significantly lower ($p < 0.001$). Similarly, the tuberous root EO also caused high crystal violet uptake (83.36 and 91.06 %, respectively) at 4 × MIC and MBC, with no significant difference ($p > 0.05$), but induced a slight increase in membrane permeability (45.68 %) at 2 x MIC. In addition, the EOs exhibited either a slight increase (36.35 – 43.53 %) or no significant change (28.75 -31.83 %) in the membrane permeability of *H. pylori* at

Table 3. *In vitro* MIC and MBC values and urease inhibitory activity of *B. rotunda* rhizome and tuberous root EOs against *H. pylori*

Sample	MIC (mg/mL)	MBC (mg/mL)	MBC/MIC	Slope±SE[1]	IC_{50}[2], mg/mL (95 % CI[3])
Rhizome EO	0.31	2.50	8	0.338 ± 0.029	0.277 (0.215 – 0.356)
Tuberous root EO	0.16	1.25	8	0.294 ± 0.034	0.191 (0.138 – 0.266)
Amoxicillin (µg/mL)	0.06	0.13	2		
Thiourea				1.236 ± 0.108	0.039 (0.034 – 0.044)

[1]Slope ± standard error (SE); [2]IC_{50}: 50 % inhibitory concentration; [3]CI: confidence interval

¼ ×, ½ × and 1 × MIC, whereas the control groups gave only minimal membrane permeability to crystal violet (15.84 - 22.25 %). The positive control, amoxicillin, was found to show 58.99 % uptake at MBC.

Plant EOs have been shown to increase cell membrane permeability in a variety of bacterial species. Treatment of *E. coli* with 200 µL/L of *Cinnamomum camphora* var. *linaloolifera* EO resulted in approximately 70 % crystal violet uptake after 3.5 hr (47). Similarly, *Acinetobacter baumannii* treated with *L. cubeba* EO at MIC (1.04 mg/mL) and 2×MIC exhibited a notable increase in relative electrical conductivity after 2 hr, suggesting alterations in cell membrane permeability (48). A previous study had also reported that the EO of *B. rotunda* rhizome can increase in membrane of MRSA (25). In the present study, both the rhizome and root EOs of *B. rotunda* exhibited strong membrane-permeabilising effects against *H. pylori*. Crystal violet typically shows low permeability across intact cell membranes, but it can rapidly penetrate cells when the membrane is damaged (32). This disruption likely contributes to bacterial cell death by facilitating the entry of harmful substances

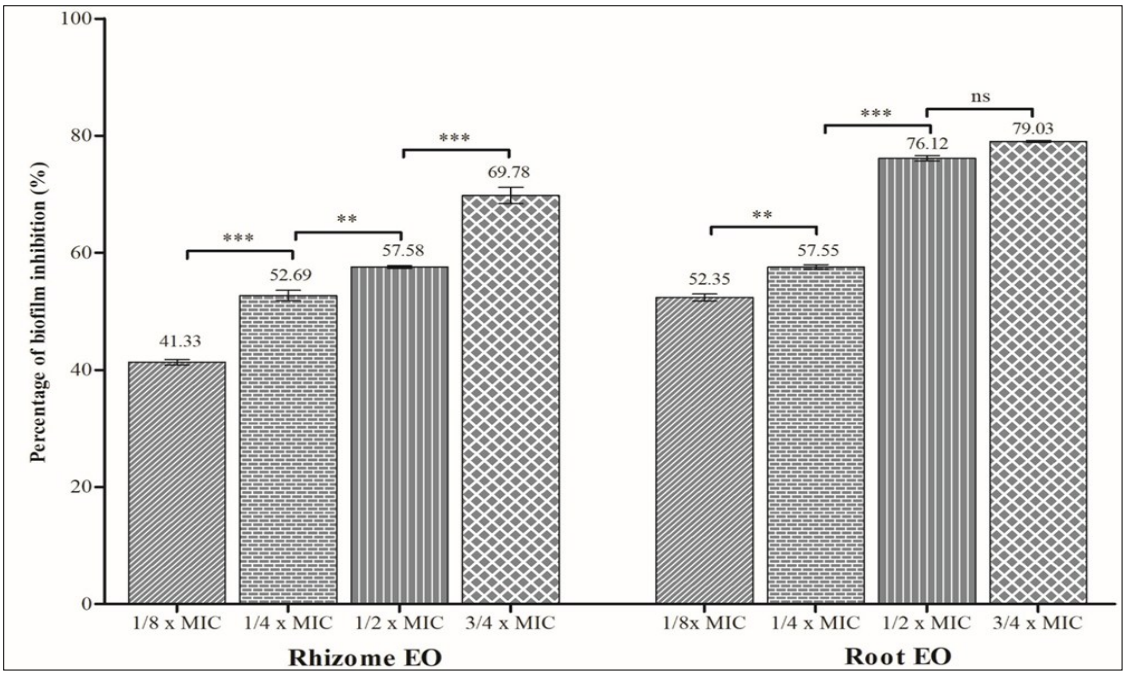

Fig. 2. Inhibitory effects of *B. rotunda* rhizome and tuberous root EOs at sub-MICs after 48 h-treatment on *H. pylori* biofilm formation. 'ns' depicts no significant difference at $P > 0.05$; **indicates significant difference at $P < 0.01$ and ***indicates significant difference at $P < 0.001$ (Bonferroni test).

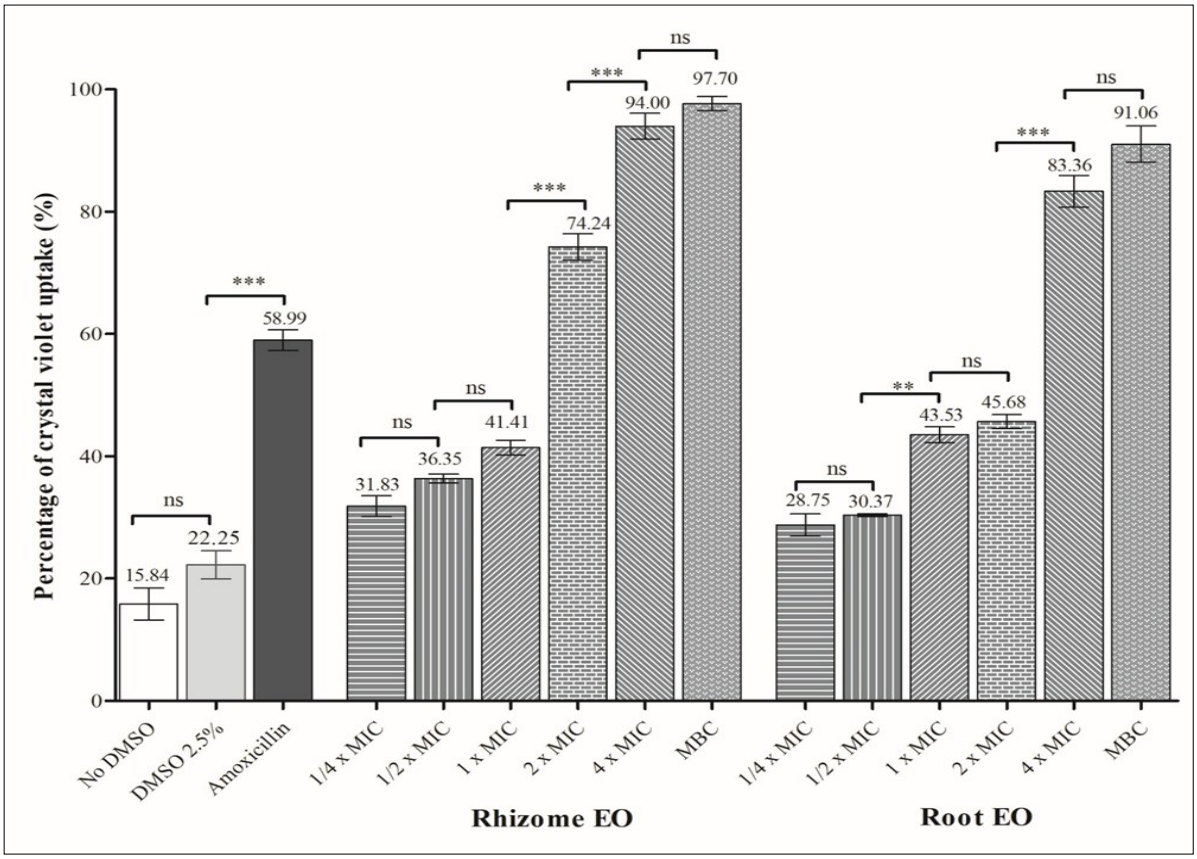

Fig. 3. Effects of *B. rotunda* rhizome and tuber root EOs at sub-MICs after 2 h-treatment on cell membrane permeability of *H. pylori*. 'ns' depicts no significant difference at $P > 0.05$; **indicates significant difference at $P < 0.01$ and ***indicates significant difference at $P < 0.001$ (Bonferroni test).

into the cytoplasm. These findings indicate the EOs possess significant antimicrobial potential and may represent a promising strategy for the treatment and control of *H. pylori* infections in humans when used individually or in combination with conventional antibiotics.

Effect on cell membrane integrity of *H. pylori*

The effect of *B. rotunda* EOs on the cell membrane integrity of *H. pylori* was evaluated by measuring the leakage of intracellular proteins after 4 hr of treatment. As shown in Fig. 4, the treatments with both of the rhizome and tuberous root EOs at their MBCs led to significantly increased protein leakage. At MBC, the rhizome EO induced protein leakage levels of 6.71 µg/mL, while the root EO resulted in significantly greater protein leakage at 9.11 µg/mL ($P < 0.001$), suggesting that the root EO exerted a membrane-damaging effect comparable to that of amoxicillin (9.95 µg/mL). At MIC, the rhizome and root EOs showed remarkably low levels of protein leakage at 4.31 and 4.77 µg/mL, respectively. These protein leakage levels were not significantly different from those of the control groups without and with DMSO (3.86 and 4.24 µg/mL, respectively) ($p > 0.05$). The limited protein leakage implies that the EOs at MIC levels had minimal impact on the membrane integrity within 4 hr of treatment. As expected, the positive control (liquid nitrogen) caused the highest protein release (17.79 µg/mL), consistent with complete cell lysis. These results suggest that the disruptive effects of *B. rotunda* EOs on *H. pylori* membranes were concentration-dependent, with membrane integrity significantly compromised at bactericidal concentrations.

Protein leakage induced by EOs has been widely reported as a marker of membrane damage in bacterial cells. *E. coli* treated with *Origanum compactum* EO at concentrations ranging from 0.31 to 1.24 mg/mL exhibited protein leakage levels between 2.62 and 5.47 µg/mL after 24 hr of exposure (49). Similarly, in *B. subtilis*, treatment with *O. compactum* EO at concentrations of 3.125–12.5 mg/mL resulted in protein concentrations in the supernatant ranging from 3.02 to 5.25 µg/mL after 24 hr (49). In addition, the EO of *B. rotunda* rhizome has previously been shown to cause morphological alterations in the cell wall and cytoplasmic membrane of MRSA, accompanied by leakage of intracellular contents *(25)*. In the present study, both rhizome and root EOs of *B. rotunda* induced significant protein leakage from *H. pylori* cells at their MBCs. Protein leakage compromises essential cellular processes by causing the loss of vital intracellular components, ultimately leading to bacterial cell death. These findings support the membrane-damaging properties of *B. rotunda* EOs as a key mechanism underlying their antibacterial activity.

Conclusion

This study demonstrated that EOs extracted from the rhizome and tuberous root of *B. rotunda* exhibit notable bacteriostatic activity against *H. pylori*. The antibacterial effects of these EOs are associated with multiple mechanisms, including significant inhibition of urease activity, disruption of biofilm formation and impairment of both membrane permeability and integrity. Both the EOs demonstrated potent membrane-permeabilising properties, while the root EO

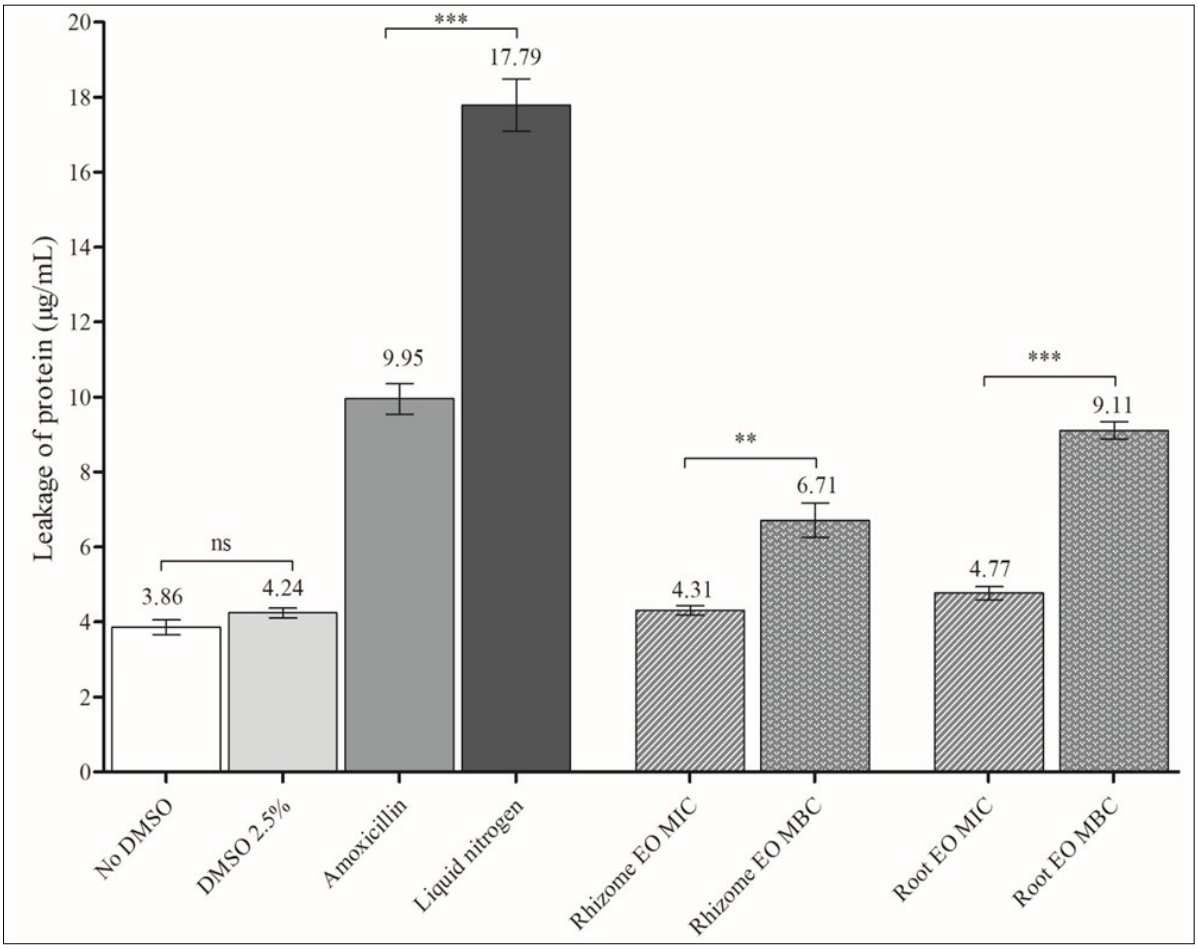

Fig. 4. Protein leakage from *H. pylori* after 4 h of treatment with the B. rotunda rhizome and tuberous root EOs at both MIC and MBC levels. 'ns' depicts no significant difference at $P > 0.05$; **indicates significantly different at $P < 0.01$ and ***indicates significantly different at $P < 0.001$ (Bonferroni test).

exerted profound biofilm-inhibitory and membrane-damaging effects. These findings highlight the potential of *B. rotunda* EOs as promising natural agents for the management of *H. pylori* infections. Nevertheless, further direct investigations into the effects of the EOs on morphology, structure of the cell membrane, gene expression of *H. pylori*, along with *in vivo* studies, are required.

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Authors' contributions

TTH[1], PAT, MTT, LAD, NTNN, LTMN and TTH[2] contributed equally in composing the manuscript. All authors read and approved the final manuscript. [TTH[1] stands for Tran Thanh Hung and TTH[2] stands for Tran Trung Hieu].

Compliance with ethical standards

Conflict of interest: The Authors have declared that no competing interests.

Ethical issues: None

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