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Original Research Article

Chemical Composition of *Plectranthus amboinicus* Leaf Essential Oil from Phetchabun, Thailand, and Its Potential to Combat Enteropathogenic Bacteria and Regulate Blood Pressure

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ABSTRACT

The increasing demand for natural therapeutics has driven interest in plant-derived essential oils as potential alternatives to synthetic drugs for managing infectious and chronic diseases. *Plectranthus amboinicus* is a traditional medicinal plant known for its diverse therapeutic uses, particularly in Asian and African folk medicine. In this study, the chemical composition and biological activities of *P. amboinicus* essential oil (PAEO), derived from plants grown in Phetchabun, Thailand, were investigated. Gas chromatography–mass spectrometry (GC–MS) analysis identified 142 volatile compounds, with shyobunol (9.1%), germacrene D (8.3%), α-cadinol (7.7%), and carvacrol (7.5%) being the predominant constituents. Antibacterial testing revealed that PAEO exhibited moderate to strong bactericidal activity against *Escherichia coli*, *Salmonella Typhimurium*, and *Shigella flexneri*, with minimum inhibitory concentrations (MIC) ranging from 1 to 2 mg/mL. Time-kill kinetics assays demonstrated a rapid bactericidal effect, whereas post-antibiotic effect (PAE) analysis showed prolonged suppression of bacterial regrowth compared with standard antibiotics. Scanning electron microscopy (SEM) and biochemical studies confirmed that PAEO disrupts bacterial cell membranes, leading to the leakage of nucleic acids and proteins. In addition, PAEO significantly inhibited the activity of angiotensin-converting enzyme 1 (ACE1) *in vitro* in a dose-dependent manner, achieving over 70% inhibition at 1 mg/mL, with a median inhibitory concentration (IC_{50}) of approximately 0.51 mg/mL. These results highlight the potential of PAEO as a multifunctional agent for the treatment of microbial infections and hypertension warranting further preclinical and clinical investigation.

Keywords: *Plectranthus amboinicus*, hypertension, angiotensin-converting enzyme 1, essential oil, antibacterial, enteropathogenic bacteria

Introduction

Medicinal plants have long served as a rich source of bioactive compounds for the treatment and prevention of a various of human diseases. *Plectranthus amboinicus* (Lour.) Spreng, commonly known as, Cuban oregano, Mexican mint or Indian borage, is a medicinal plant widely used in traditional medicine across Asia, Africa and South America.[1] In Thai folklore medicine, the leaves of *P. amboinicus* have traditionally been used to relieve symptoms of colds, coughs, digestive disturbances, and skin infections.[2] In addition to their medicinal applications, the leaves are also consumed as a culinary herb in several countries, indicating their low toxicity and general safety for human consumption.[1] These therapeutic effects are largely attributed to the essential oil extracted from the plant, which contains a complex mixture of bioactive constituents, particularly terpenoids, that are believed underlie its diverse pharmacological properties.[3,4]

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However, the chemical profile and biological potency of essential oils can vary depending on geographical and environmental factors, highlighting the need for localized studies to capture region-specific phytochemical variations.[5,6] The composition of essential oils is known to be influenced by several factors, including soil type,[5] environmental conditions,[6] the plant's genetic background,[7] time of harvest,[8] altitude and climate.[7] Consequently, it is crucial to investigate region-specific chemical profiles to better understand their unique pharmacological effects. Despite growing interest in *P. amboinicus*, the chemical constituents and biological activities of its essential oil (PAEO) from Phetchabun Province in Thailand remain poorly studied. The unique agricultural practices and climatic conditions in Phetchabun may influence biosynthesis of specific compounds, in the plant,
potentially altering the chemical makeup and enhancing the functional properties of the essential oil.

Hypertension and enteropathogenic bacterial infections continue to pose major global health challenges.[9,10] Hypertension is a leading risk factor for cardiovascular diseases the foremost cause of death worldwide.[9] Simultaneously, infections caused by enteric pathogenic bacteria, particularly those associated with gastrointestinal illnesses, continue to strain healthcare systems, due to the rising prevalence of antibiotic-resistant strains.[11] The potential interplay between these two health concerns hypertension and microbial infections has attracted increasing scientific interest, as chronic inflammation and gut dysbiosis are increasingly implicated in the pathogenesis of hypertension. The traditional use of *P. amboinicus* for treating both infections and hypertension-related symptoms suggests that it may exert dual therapeutic effects.[12-13] Previous studies have shown that essential oils from various plants may exhibit both vasodilatory [14-16] and antimicrobial activity,[17] indicating that PAEO may serve as a

multifunctional therapeutic agent. However, there is limited data on the essential oil of *P. amboinicus* grown in Phetchabun and its potential to regulated blood pressure or inhibit enteric bacterial pathogens. This study aims to elucidate the chemical composition of the essential oil extracted from *P. amboinicus* leaves cultivated in Phetchabun, Thailand, and to evaluate its potential to regulate blood pressure and inhibit the growth of enteropathogenic bacteria. By integrating phytochemical profiling with biological assays, this research provides novel insights into the pharmacological potential of PAEO. Its dual bioactivities, antihypertensive and antibacterial effects, were investigated to support its application in natural health products. Chemical constituents were identified using gas chromatography–mass spectrometry (GC–MS); antihypertensive activity was assessed via ACE1 inhibitory assay; and antibacterial effects were evaluated against clinically relevant enteropathogenic strains. These finding aim to provide scientific validation for the medicinal use of this plant, support its development for pharmaceutical and food industry applications, promote the sustainable use of local herbal resources, and enhance the economic value of Thai medicinal plants.

Materials and Methods

Chemicals and antimicrobial agents

All solvents utilized in the experiments were acquired from Labscan (Labscan Asia Co., Ltd., Thailand). All other analytical-grade chemicals were sourced from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany), exhibiting purities of 98% or higher unless stated otherwise. Ciprofloxacin (purity ≥98%) and gentamicin sulfate (purity ≥89.8%) were sourced from Dr. Ehrenstorfer GmbH (LGC Labor GmbH, Augsburg, Germany). Mueller-Hinton Broth (MHB) and Mueller-Hinton Agar (MHA) utilized for antibacterial susceptibility testing, were obtained from HiMedia Laboratories Pvt. Ltd. (Mumbai, India) and prepared following the manufacturer's guidelines.

Extraction of P. amboinicus leaves essential oil

In October 2023, leaves of *P. amboinicus* were collected from Phetchabun, Thailand (16°53'11.2"N 101°11'29.3"E), and a voucher specimen was registered on CRI No. 804 at the Chulabhorn Research Institute in Bangkok. The freshly harvested leaves (1 kg per batch) were subjected to a 1.5 h steam distillation in a simple distillation apparatus. The essential oil obtained was isolated by partitioning with dichloromethane (CH_2Cl_2; ≥99.8%, AR grade, Labscan, Thailand). The dichloromethane layer was then dried over anhydrous sodium sulfate (≥99%, ACS reagent, Merck, Germany) to remove residual moisture. CH_2Cl_2 was removed under evaporation under reduced pressure using a rotary evaporator (Buchi R-300, Switzerland) at 40°C. The resulting PAEO appeared as a light-yellow oil and was stored in amber vials at -4°C in the dark until further analysis.

Essential oil analysis

The chemical constituents were analyzed using GC-MS with Shimadzu GC-2030 instrument coupled with a QP2020 NX quadrupole mass detector (Shimadzu Corp., Kyoto, Japan). The electron ionization (EI) source operated at 70 eV with a 220°C ion source temperature. A 4 mg oil sample was diluted in 1 mL of CH_2Cl_2 (≥99.8%, AR grade, Labscan, Thailand), and 1 µL was introduced into the system using a 1:5 split ratio. The injector and transfer line temperature were maintained at 230°C. Separation was performed using a DB-5 capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness; Agilent J&W, USA). Helium (99.999% purity, Linde Thailand) served as the carrier gas at a constant flow rate of 1.0 mL/min. The oven temperature was programmed to increase from 40°C to 240°C at a constant rate of 4°C/min. Compound identification involved comparison with spectra in the NIST 2017 library and calculation of retention indices according to Adams (2009).[18]

Microorganisms

The pure cultures of *Escherichia coli* (*E. coli*) ATCC8739, *Salmonella Typhimurium* (*S. Typhimurium*) ATCC13311, and *Shigella flexneri* (*S. flexneri*) ATCC12022 were sourced from the American Type Culture Collection (ATCC, USA). All bacterial strains were cultivated on MHA or MHB at 37°C for for a duration of 18 to 24 h. Following incubation, the bacterial cultures were diluted to attain a concentration of 10^8 colony forming units (CFU)/mL. The cultures were collected and suspended in sterile normal saline solution (0.85% NaCl, NSS; prepared from analytical-grade NaCl, Merck, Germany) for use in subsequent antibacterial assays.

Determination of minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC)

The microdilution method was used to determine this experiment, following the guidelines established by the Clinical and Laboratory Standards Institute (CLSI).[19] The MICs were established as the lowest concentrations of compounds that entirely inhibited visible microbial growth, whereas the MBCs indicated the minimum concentrations necessary to eradicate ≥99.9% of the initial bacterial population. Antibiotic and PAEO stock solutions were prepared in DMSO. Ciprofloxacin and gentamicin were utilized as positive controls, while DMSO alone functioned as a negative control. Antibiotic concentrations varied between 0.0015 and 200 µg/mL, whereas PAEO was evaluated at concentrations from 0.015 to 16 mg/mL. The experiment was carried out in sterile 96-well microtiter plates (Corning, USA). Bacterial suspensions were standardized to approximately 1.5×10^8 CFU/mL (0.5 McFarland) and subsequently diluted to 5×10^5 CFU/mL in Mueller-Hinton broth. Following incubation at 37 °C for 24 h, MIC values were visually assessed by comparing with control wells, while MBC values were determined by sub-culturing 10 µL aliquots from wells without visible growth onto MHA plates and incubating for another 24 h. Colonies were counted to confirm bacterial lethality. To characterize the antibacterial mode of action, the MBC/MIC ratio was calculated, with values <4 indicating bactericidal activity and values between 4 and 32 indicating bacteriostatic activity, as described by Radhakrishnan et al.[20]

Determination of bacteria growth curves

The effect of PAEO on the killing kinetics of enteropathogenic bacteria was investigated using a viable cell counting approach based on a protocol modified by Achukwu et al.[21] Bacterial killing kinetics were assessed based on MIC findings by monitoring the response of selected bacterial strains over 24 h. Overnight cultures were standardized to approximately 10^6 CFU/mL and exposed to concentrations from one and four times the MIC of the test compound. DMSO containing no test sample was used as a negative control. From each treated culture, 10 µL were taken and serially diluted in NSS. These dilutions were then plated onto MHA at specific time points: 0, 5 sec, 10 sec, 15 sec, 30 sec, 1 min, 5 min, 10 min, 20 min, 30 min and 1 h. The plates were incubated for 24 h at 37°C, after which the number of colonies forming units (CFU) was determined. Time-kill curves were generated from the results.

Post-antibiotic effect (PAE)

PAE refers to the phenomenon where bacterial growth is inhibited after the removal of an antibiotic, despite the absence of the drug. This effect is significant in understanding antibiotic efficacy and resistance mechanisms. PAE of PAEO, ciprofloxacin, and gentamicin on residual enteropathogenic bacteria was assessed using the viable plate count method, as established in prior studies.[22] Two milliliters of an overnight bacterial culture were added to 40 mL of fresh MHB and incubated at 37°C with shaking at 200 rpm for 1 to 2 h until the bacteria reached the active growth phase. Following incubation, bacterial cells were harvested by centrifugation at 10,000 × g for 5 minutes at 4°C using a Beckman Coulter Avanti J-E centrifuge (USA). The cells were washed twice with phosphate-buffered saline (PBS; pH 7.4, Gibco, USA) containing 0.05% Tween 80 (analytical grade, Sigma-Aldrich, Germany) and subsequently resuspended in PBS-T. The cell suspension was modified to an optical density (OD_{620}) of 0.3 utilizing a SYNERGY H1 Hybrid Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT, USA).

PAEO, ciprofloxacin, and gentamicin were added at their MIC concentration. The negative control consisted of 10 % (v/v) DMSO. Treated suspensions were shaken at 37°C for 30 min, then centrifuged at 10,000 × g for 10 min at 10°C, washed twice with PBS-T, and resuspended in 20 mL of fresh MHB. The cultures were incubated at 37°C with shaking at 200 rpm. At selected intervals (0, 1, 2, 3, 4, 5, 6,

7, and 8 h), 25 µL samples were serially diluted in sterile NSS and plated onto MHA using the drop plate technique. After incubation 18-24 h at 37°C, colonies were counted from plates counaining 3-30 colonies.[23] The calculation of PAE was performed using the subsequent equation: PAE = T - C, where T represents the duration needed for the treated culture to achieve a 1 $\log_{10}$ increase in CFU relative to baseline post-treatment, and C denotes the equivalent duration for the untreated control.

Scanning electron microscopy (SEM)

The morphological effects of PAEO on enteropathogenic bacterial cells were examined using SEM with a modified protocol derived from Hao's method.[24] Sterile 13 mm Thermanox™ plastic coverslips (NUNCTM, NY, USA) were positioned in the wells of a 24-well sterile tissue culture plate. Each well received 1 mL of a bacterial suspension standardized to 10^6 CFU/mL and was incubated at 37°C for 24 h to facilitate cell attachment. Following incubation, the bacterial cultures underwent treatment with PAEO at its MIC for 30 minutes at 37°C. A 10% (v/v) DMSO solution devoid of PAEO served as the negative control. Post-treatment, the cells had three to five washes with PBS (pH 7.4) to eliminate residual compounds and unbound cells. The samples were fixed using 2.5% glutaraldehyde (Grade II, 25% in H2O; Merck, Germany) at 4°C for a duration of 16 to 24 h. Following fixation, the cells received three rinses with sterile distilled water and were dehydrated at room temperature using a graded series of ethanol (30%-100%; analytical grade, Merck, Germany). Dehydrated samples were sputter-coated with gold using a Leica EM ACE200 coater (Leica Microsystems, Washington, D.C., USA). Imaging was performed using a FEI Quanta 250 SEM (FEI, Eindhoven, Netherlands) at an accelerating voltage of 30 kV.

LIVE/DEAD BacLight assay

The bacterial membrane's integrity was assessed using the LIVE/DEAD *Bac*Light™ Bacterial Viability Kit (Invitrogen™ L7012; Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's protocol.[25] Bacterial cultures were cultivated to logarithmic phase and subsequently harvested via centrifugation at 10,000 × g for 5 minutes at 4°C. The cell pellets were washed twice with PBS-T and were subsequently resuspended in PBS-T to achieve an optical density (OD_{620}) of 0.3. To assess the impact of the test compounds on cell viability, PAEO and gentamicin were used at concentrations equivalent to twice the MIC. A 10% (v/v) DMSO solution was utilized as a negative control. The mixtures were incubated at 37°C for one hour with moderate stirring. Following treatment, cells were harvested via centrifugation at 10,000 × g for 10 minutes at 10°C, washed twice with PBS-T, and resuspended in 1 mL of PBS-T. To prepare the staining solution, 6 µL of SYTO® 9 and 6 µL of propidium iodide (PI) were combined with 2 mL of sterile filtered water. Following that, 100 µL of this solution was added to each well containing the resuspended cells in a 96-well microplate. The plate was incubated for 15 min in darkness at room temperature. Fluorescence measurements were conducted using a Synergy H1 Hybrid Multi-Mode Microplate Reader, with excitation set at 485 nm and emission wavelengths of 530 nm (green fluorescence; live cells) and 630 nm (red fluorescence; dead cells).

Leakage of 260 nm absorbing material

Membrane disruption was further evaluated by measuring the release of intracellular nucleic acids, absorbing at 260 nm.[26] Bacterial cultures were cultivated to logarithmic phase, subsequently harvested via centrifugation at 10,000 × g for 10 min, and washed twice with PBS-T. The cells were subsequently resuspended in PBS-T and adjusted to an optical density (OD_{620}) of 0.3. To assess the treatment effects, PAEO and gentamicin were added to the cell suspensions at concentrations that matched their MIC. A 10% (v/v) DMSO solution served as the negative control. Samples were incubated at 37°C, with aliquots collected at 0, 4, and 8 h. At each time point, the suspensions were filtered through a 0.2 µm syringe filter to yield cell-free supernatants. Filtrate absorbance was assessed at 260 nm with a SYNERGY H1 Hybrid. Nucleic acid leakage was quantified by measuring the OD_{260} at each time point.

Protein leakage assay

To assess the disruption of bacterial membrane integrity, the release of intracellular proteins was analyzed using the Pierce™ BCA Protein Assay Kit (Thermo Scientific, IL, USA).[27] Bacterial cultures were grown to the logarithmic phase and harvested by centrifugation at 10,000 × g for 5 min at 4°C. The cell pellets were subsequently washed twice with PBS-T to remove residual media. After washing, cells were resuspended in PBS-T and the turbidity was adjusted to an OD_{620} of 0.3. PAEO, ciprofloxacin, and gentamycin were added to each bacterial suspension at concentrations equivalent to their respective MICs. A 10% (v/v) DMSO solution served as the negative control. Samples were incubated at 37°C, and aliquots were collected at specific time points (0, 15, 30, 60, and 120 min). Each sample was filtered through a 0.2 µm syringe filter to eliminate intact cells, and the resulting cell-free supernatants were subjected to protein quantification using the BCA assay according to the manufacturer's protocol. Absorbance was recorded at 562 nm using a SYNERGY H1 Hybrid. The detected protein concentration reflected the extent of membrane damage.

ACE1 inhibitory activity assay

The inhibitory activity of PAEO against angiotensin-converting enzyme 1 (ACE1) was assessed using an ACE1 Inhibitor Screening Kit[28] (Merck KGaA, Darmstadt, Germany) with slight modifications. A stock solution was prepared by dissolving 100 mg of the oil sample in 1 mL of DMSO (100 mg/mL). The solution was mixed thoroughly to ensure complete dissolution. Serial dilutions of the stock solution were prepared to obtain final concentrations range between 0.01 to 1.0 mg/mL for the inhibition assay. Captopril (0.02 mg/mL) was used as the positive control. In a 96-well microplate, 10 µL of sample or control was mixed with the ACE1 enzyme and assay buffer. Control wells contained only enzyme, buffer and DMSO. The control groups comprised wells containing only enzyme, buffer and DMSO. The plate was incubated at 37°C for 15 min to allow interaction between enzyme and inhibitor. After incubation, 40 µL of ACE1-specific substrate was added to each well. The reaction mixture was then incubated at 37°C for 90 min. Fluorescence was measured using a microplate reader at excitation/emission wavelengths of 330/430 nm. The percentage inhibition of ACE1 activity was calculated by comparing the fluorescence intensities of treated and untreated wells. All measurements were performed in triplicate for accuracy.

Statistical analysis

Results are presented as mean ± standard deviation (SD) derived from three independent experiments. All experiments were conducted in triplicate to ensure reproducibility. Statistical analyzes were performed using GraphPad Prism software (version 10.1.2, GraphPad Software, San Diego, CA, USA). Specific statistical tests and significance thresholds are described in figure legends or the Results section.

Results and Discussion

Chemical composition

The essential oil extracted via hydro-distillation yielded 0.062% (w/w) from 21 kg of fresh *P. amboinicus* leaves and was analyzed using GC-MS with a DB-5 column. Compound identification was based on comparisons with the NIST Mass Spectra Library and retention indices. A total of 142 compounds were identified, with the major constituents being shyobunol (9.1%), germacrene D (8.3%), α-cadinol (7.7%), carvacrol (7.5%), α-humulene (5.5%), β-caryophyllene (5.0%), δ-cadinene (4.7%), α-cadinol isomer I (4.2%), linalool (3.0%), and α-copaene (2.87%) (Table 1). *P. amboinicus* is classified into several chemotypes based on its principal essential oil components, such as thymol-rich and carvacrol-rich chemotypes. Thymol-rich chemotypes typically contain thymol concentrations ranging from 35% to 94%, while carvacrol-rich chemotypes range from 32% to 98%.[29]

In contrast, present study found relatively low levels of both thymol and carvacrol. However, sesquiterpenes and oxygenated sesquiterpenes were more abundant, consistent with findings El-Hawary et al.,[30] who also reported low carvacrol and thymol levels with high concentration of sesquiterpene hydrocarbons and their oxygenated derivatives.

Table 1: Compounds identified from *P. amboinicus* leaf essential oils (Only compounds with %Area greater than 0.5% are shown).

Peak No.	Compounds	Retention Time (min)	Retention Index	Retention Index (Reference)[a]	%Area
1.	1-Octen-3-ol	9.93	978	979	1.36
2.	Fenchone	13.79	1087	1086	1.56
3.	Linalool	14.22	1099	1096	2.95
4.	Terpinen-4-ol	17.00	1177	1177	1.04
5.	Ascaridole	19.13	1238	1237	1.15
6.	Carvacrol	21.31	1302	1299	7.49
7.	α-Copaene	23.85	1379	1376	2.66
8.	β-Caryophyllene	25.26	1424	1419	5.04
9.	α-Humulene	26.33	1458	1454	5.54
10.	Germacrene D	27.19	1486	1481	8.34
11.	δ-Cadinene	28.44	1457	1545	4.70
12.	NID	29.17	1552		0.71
13.	Germacrene D-4-ol	29.98	1580	1575	1.82
14.	Spathulenol	30.03	1582	1578	1.09
15.	Caryophyllene oxide	30.21	1588	1583	1.70
16.	NID	30.79	1608		0.56
17.	Humulene epoxide II	30.97	1614	1608	1.36
18.	1-epi-Cubenol	31.48	1633	1628	1.00
19.	Humulenol-II	31.60	1637	MS only	0.52
20.	Tau-Cadinol	31.83	1645	1640	Coelute with cpd 21
21.	α-Cadinol isomer I	31.87	1646	Tentative from mass spectrum	4.15
22.	α-Muurolol	31.98	1650	1646	1.07
23.	α-Cadinol	32.23	1660	1654	7.72
24.	Germacra-4(15),5,10(14)-trien-1-α-ol	33.11	1691	1686	1.91
25.	Shyobunol	33.28	1697 (tentative identification)	1688[a] 1697[b] 1707[c]	9.14
26.	NID	33.90	1720		1.52
27.	NID	35.82	1792		1.18
28.	NID	36.29	1811		0.80
29.	NID	36.38	1814		0.85
30.	NID	38.97	1917		0.56

[a]Adams RP. Identification of essential oil components by gas chromatography/mass spectrometry. 4th ed. Illinois: Allured Pub. Corp.; 2009. 804 p. [b]Figuerédo G, Cabassu P, Chalchat J-C, Pasquier B. Studies of Mediterranean oregano populations. VIII—Chemical composition of essential oils of oreganos of various origins. Flavour Fragr. J. 2006; 21:134-139. [c]Blacio S, Avecillas G, Maldonado J, Gadvay K, Porras M, Leon W, Calva J. Comparative study of the chemical composition of the essential oil of Plectranthus amboinicus from different sectors of Southern Ecuador. Horticulturae 2025; 11(2):173. NID = not identified

Shyobunol, which is rarely reported in *P. amboinicus*, emerged as a major component in this study. Its dominance may indicate either genetic variation or unique environmental influences. Notably, shyobunol was also recently reported in relatively high amounts in *P. amboinicus* samples from southern Ecuador.[31] Similarly, α-cadinol, although generally uncommon in this species, was identified in high quantities, echoing the seasonal findings by El-Hawary et al.[30] These observations highlight the chemical diversity of PAEO and suggest potential industrial applications, particularly in pharmaceuticals and food preservation, owing to the high sesquiterpene content. Shyobunol and α-cadinol, in particular, have been associated with antimicrobial and anti-inflammatory properties.[32,33]

Antibacterial activity and post-antibiotic effect of PAEO

The antibacterial properties of PAEO were tested against three clinically relevant enteropathogens: *S. Typhimurium*, *S. Flexneri* and *E. coli*. The results for MIC, MBC, and PAE determinations are presented in Table 2. PAEO exhibited moderate but consistent bactericidal activity, with MIC and MBC values of 2 mg/mL for *S. Typhimurium* and *E. coli* and a stronger effect against *S. flexneri* with an MIC of 1 mg/mL. In contrast, gentamicin and ciprofloxacin demonstrated significantly higher antibacterial efficacy with MIC values ranging from 0.006 to 3.125 µg/mL, consistent with their broad-spectrum clinical profiles.[34] Table 2 also shows the effects of PAEO on PAE, defined as the time (in hours) during which bacterial regrowth remains suppressed after brief exposure to an antimicrobial agent.[22] The PAE results demonstrated a remarkable advantage for PAEO in terms of prolonged suppression of bacterial growth following the removal of the agent. PAEO exhibited the longest PAE against *E. coli* (3.33 ± 0.57 h), followed by *S. flexneri* (2.67 ± 0.57 h), and *S. Typhimurium* (1.33 ± 1.16 h). In comparison, gentamicin produced a PAE of 1.33 ± 0.57 h for all tested strains, while ciprofloxacin ranged from 1.00 to 1.33 h, suggesting that PAEO may exert a more persistent effect after treatment. Previous studies have reported similar effects with essential oils and herbal antimicrobial agents, indicating that their multi-target mode of action may lead to prolonged stress responses in bacterial cells.[35]

Table 2: Antibacterial activity and post-antibiotic effect (PAE) of PAEO, gentamicin, and ciprofloxacin against enteropathogenic bacteria.

Strains	*Escherichia coli*			*Salmonella Typhimurium*			*Shigella flexneri*		
Substance	MIC	MBC	PAE	MIC	MBC	PAE	MIC	MBC	PAE
PAEO (mg/mL)	2	2	3.33±0.57	2	2	1.33±1.16	1	1	2.67±0.57
Gentamicin (µg/mL)	3.125	3.125	1.33±0.57	1.56	1.56	1.33±0.57	3.125	3.125	1.33±0.57
Ciprofloxacin (µg/mL)	0.006	0.024	1±0	0.006	0.024	1±1	0.012	0.024	1.33±0.57

MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration. PAE: post-antibiotic effect, 0.25 = 15 min; 0.50 = 30 min; 0.75 = 45 min; 1.0 = 60 min. Values represent means of three independent replicates ± standard deviation.

From a clinical and therapeutic perspective, a prolonged PAE is beneficial as it may reduce the need for frequent dosing and potentially slow the development of antimicrobial resistance.[36,37] Although PAEO is less effective than conventional antibiotics, its prolonged suppressive effect on bacterial regrowth could be valuable in adjunctive therapy, particularly for the treatment of persistent or recurrent infections.

Time-kill kinetics of PAEO against enteropathogenic bacteria

Figure 1 demonstrates the efficacy of PAEO in inhibiting the growth of enteropathogenic bacteria by quantifying the population changes of microorganisms within 1 h. The results indicate a dose- and time-dependent antibacterial effect of PAEO. The control group showed no significant change in the number of microorganisms during the same period, while the bacteria treated with PAEO exhibited a rapid decrease in population. At a concentration of 1× MIC, PAEO reduced the number of *S. Typhimurium* and *E. coli* by more than 99.99% within 5 min and that of *S. flexneri* within 10 min. At 2× MIC, PAEO eliminated *E. coli*, *S. Typhimurium* and *S. flexneri* within 30 s, 1 min, and 5 min respectively. At 4× MIC, PAEO demonstrated the highest efficiency, killing the tested microorganisms within 10-15 s. These rapid kill times are particularly significant in light of the typical incubation periods of these pathogens: 1-3 days for *E. coli*,[38] 6-72 for *S. Typhimurium*,[39] and 12 h to 7 days for *S. flexneri*.[40] Clinical symptoms typically manifest during bacterial replication and toxin production. Therefore, the rapid bactericidal action of PAEO may significantly reduce the incidence and severity of infections caused by these pathogens, especially in the early stages of disease when timely intervention is critical. This effect underscores the therapeutic potential of PAEO in treating acute bacterial intestinal infections, particularly in resource-limited settings where fast-acting alternatives to synthetic antibiotics are urgently needed.

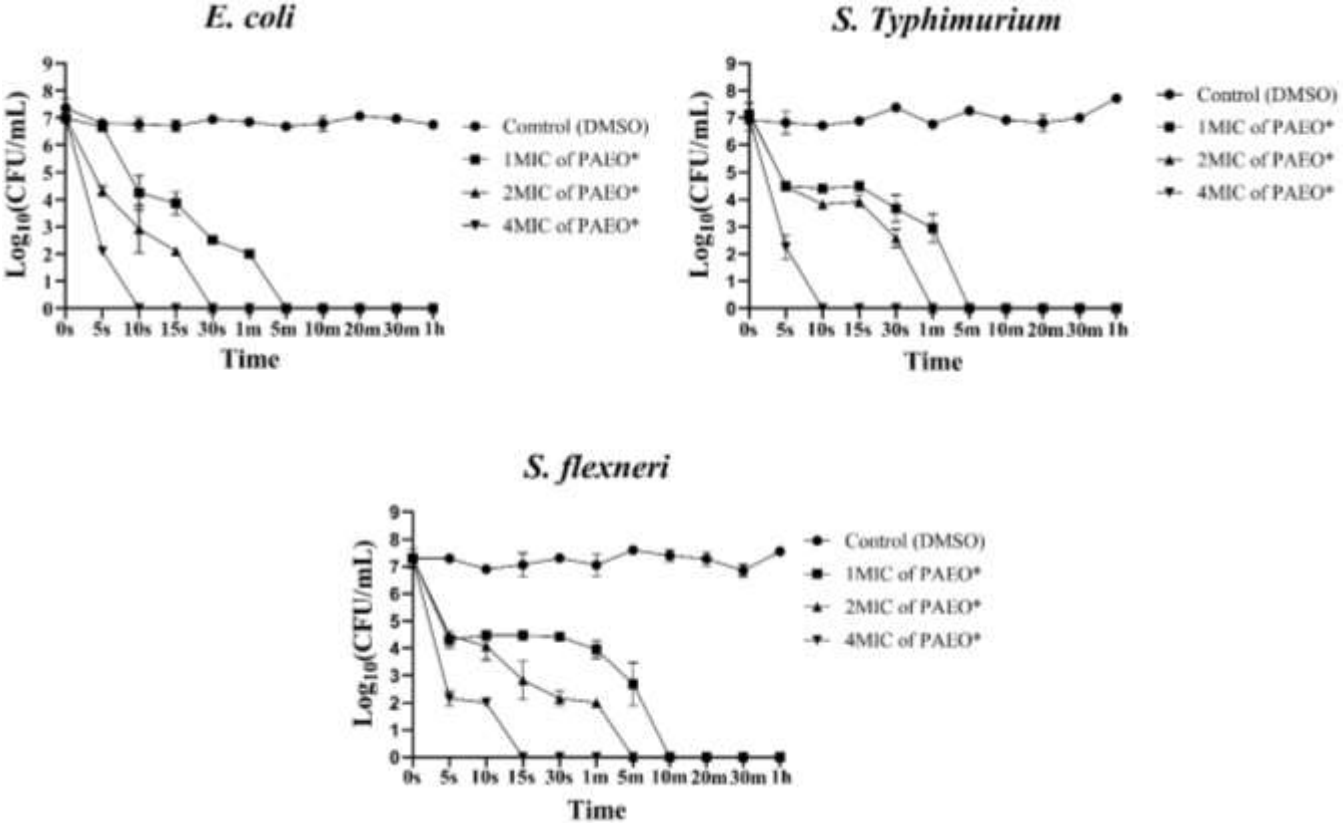

Figure 1: Effect of PAEO on the growth curves of enteropathogenic bacteria. Significant differences compared to the control group are indicated (* $p<0.0001$).

Effect of PAEO on morphological change

The effects of PAEO on bacterial cell morphology were investigated using SEM (see Figure 2). The untreated bacterial cells exhibited a regular morphology characterized by intact cell walls, a smooth and plump surface, and uniform size and shape. In contrast, cells exposed to PAEO at a concentration of 1× MIC for 30 min showed significant morphological alterations. These treated cells displayed damaged cell walls, visible shrinkage, and an irregularly shrunken appearance. SEM analysis revealed that PAEO strongly effects the structural integrity of bacterial cells, as evidenced by the marked morphological changes in observed after treatment. While the untreated cells maintained a regular

and intact morphology, indicative of a healthy physiological state, those treated with PAEO exhibited severe cell wall disruption, including shrinkage and deformation, suggesting compromised membrane integrity.

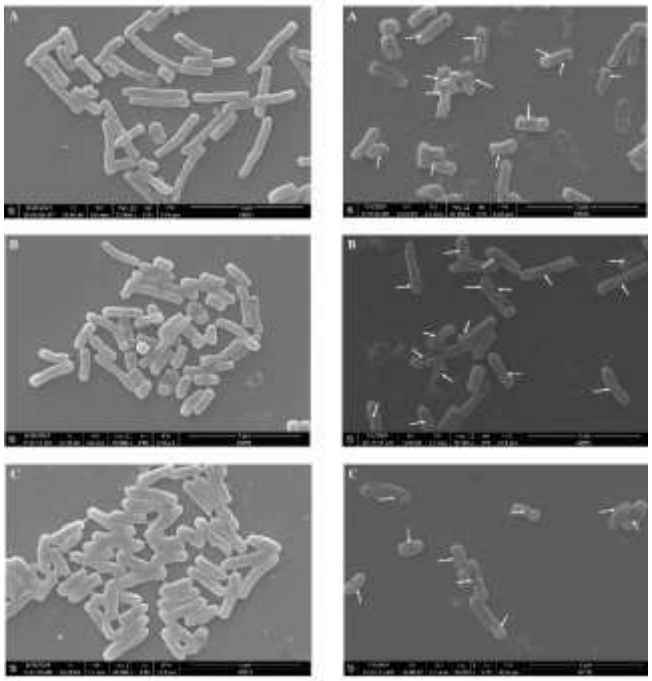

Figure 2: Morphology of *E. coli* (A), *S. Typhimurium* (B), and *S. flexneri* (C) in the absence (control; left) and presence of PAEO at 1× MIC (right). Arrows indicate a shriveled appearance and holes on the bacterial cell surface.

This structural damage indicates that PAEO targets bacterial cell walls and membranes, impairing both their integrity and function. Such damage likely contributes to the antibacterial activity of the essential oil, as compromised cell walls can lead to cell lysis and eventual bacterial deat.[24,41] The rapid onset of these morphological changes, observed within 30 min of exposure, underscores the potential of PAEO as an effective antimicrobial agent. These findings suggest that PAEO's ability to disrupt bacterial morphology plays a key role in its rapid bactericidal action, as previously described. The extent of visible damage supports the hypothesis that PAEO compromises membrane integrity and cellular architecture, aligning with its observed post-antibiotic and time-kill effects. This reinforces the potential for incorporating PAEO into topical formulations or surface disinfectants targeting enteropathogenic bacteria. These results are consistent with other studies[42,43] indicating that essential oils commonly exert antimicrobial effects by disrupting bacterial membranes, leading to increased permeability and subsequent cell death. The ability of PAEO to induce such pronounced morphological changes highlights its promise as an alternative or complementary antimicrobial therapy, particularly in response to rising antibiotic resistance.

Effects of PAEO on the permeability of the cytoplasmic membrane

The effects of PAEO on the cytoplasmic membrane permeability of *E. coli*, *S. Typhimurium*, and *S. flexneri* were evaluated after a 1-h exposure to PAEO at a concentration of 1× MIC. Green fluorescence intensity (excitation/emission; 485/530 nm) represents the proportion of live bacterial cells stained with SYTO 9. Values were normalized to the untreated control group (set at 100%). The results indicate that all strains treated with PAEO exhibited significant changes in membrane permeability, as reflected by lower fluorescence ratios compared to untreated controls. Specifically, *E. coli* showed a 91.84% decrease in green fluorescence, *S. Typhimurium* an 87.02% decrease, and *S. flexneri* an 89.04% decrease (Table3). In contrast, the gentamicin-treated samples showed no significant changes, with fluorescence intensities remaining comparable to the untreated control, suggesting that gentamicin does not disrupt membrane integrity.

These findings demonstrate that PAEO compromises cytoplasmic membrane integrity in *E. coli*, *S. Typhimurium*, and *S. flexneri*, as evidenced by the reduced fluorescence ratios following treatment. The decrease in fluorescence indicated increased membrane permeability, implying that PAEO disrupts the membrane's barrier function.[25] Changes in membrane permeability are a common indicator of cell wall damage, which often results in leakage of cellular contents, impaired metabolic processes, and eventual cell death.[44] In contrast, the stable fluorescence observed in gentamicin-treated cells highlights a key difference in the mechanisms of action between the two agents. While gentamicin primarily inhibits protein synthesis,[45] PAEO appears to exert a direct physical effect on the bacterial membrane, causing structural damage that compromises membrane integrity. These results support a membrane-targeted mechanism of action for PAEO, consistent with the previously observed bactericidal kinetics and morphological disruption. This membrane activity may offer a distinct advantage in overcoming resistance mechanisms that target intracellular pathways, the potential of PAEO as a novel, membrane-active antimicrobial agent.

Effect of PAEO on integrity of cell membrane

To elucidate the antibacterial mechanism of PAEO, its effects bacterial membrane integrity was evaluated using two complementary indicators: leakage of nucleic acid material (measured as absorbance at 260 nm, A_{260}) and leakage of proteins (quantified using the BCA assay). Protein quantification was calibrated using a BSA standard curve, following the manufacturer's protocol. The tests were conducted on three gram-negative pathogens: *E. coli*, *S. Typhimurium*, and *S. flexneri*. Gentamicin and ciprofloxacin were used as reference antibiotics, while drug-free samples served as negative controls. Ciprofloxacin inhibits DNA gyrase, while gentamicin targets bacterial protein synthesis by binding to the 30S ribosomal subunit.[45,46] These antibiotics served as mechanistic controls to distinguish membrane-specific effects of PAEO from other mode of action.

The leakage of UV-absorbing materials at 260 nm, primarily nucleotides and nucleic acids, was used as the first indicator of membrane damage. As shown in Figure 3, all three bacterial species exhibited a time-dependent increase in A_{260} after treatment with PAEO. In *E. coli*, absorbance increased significantly within the first hour ($p < 0.001$) and continued to rise over 2 h ($p < 0.0001$). Similar trends were observed for *S. Typhimurium* and *S. flexneri*, with statistically significant increases in A_{260} from the first hour onward. These results indicate that PAEO compromises bacterial membranes and, facilitating the efflux of intracellular components. In contrast, gentamicin-treated and untreated samples showed minimal changes in A_{260}, highlighting PAEO's unique effect on membrane integrity. This observation aligns with previous reports suggesting that essential oils increase membrane permeability due to their high content of hydrophobic monoterpenes and phenolic compounds, which integrate into lipid bilayers and disrupt structural integrity.[47,48] To further confirm membrane disruption, we quantified the release of cytoplasmic proteins into the extracellular medium. As shown in Figure 4, PAEO treatment induced a rapid and substantial increase in protein leakage in all tested bacteria. In *E. coli*, protein concentration in the supernatant exceeded 600 µg/mL within 30 min and approached 900 µg/mL after 120 min ($p < 0.01$). Similar patterns were observed for *S. Typhimurium* and *S. flexneri*, with peak protein levels ranging from 600 to 700 µg/mL. Neither ciprofloxacin nor gentamicin treatments resulted in significant protein leakage, underscoring that these antibiotics, which target intracellular processes such as protein synthesis and DNA replication, do not cause acute membrane disruption. The significant leakage observed only in PAEO-treated samples provides further evidence its membrane-targeting mechanism. These findings are consistent with prior studies on the bactericidal activity of essential oils. For example,[49] it has been reported that carvacrol and thymol two monoterpenoids structurally related to PAEO components-induce membrane depolarization and cytoplasmic leakage in *Bacillus cereus* Similarly,[50] other essential oils have been shown to rupture membranes, leading to the release of DNA, RNA, and proteins.

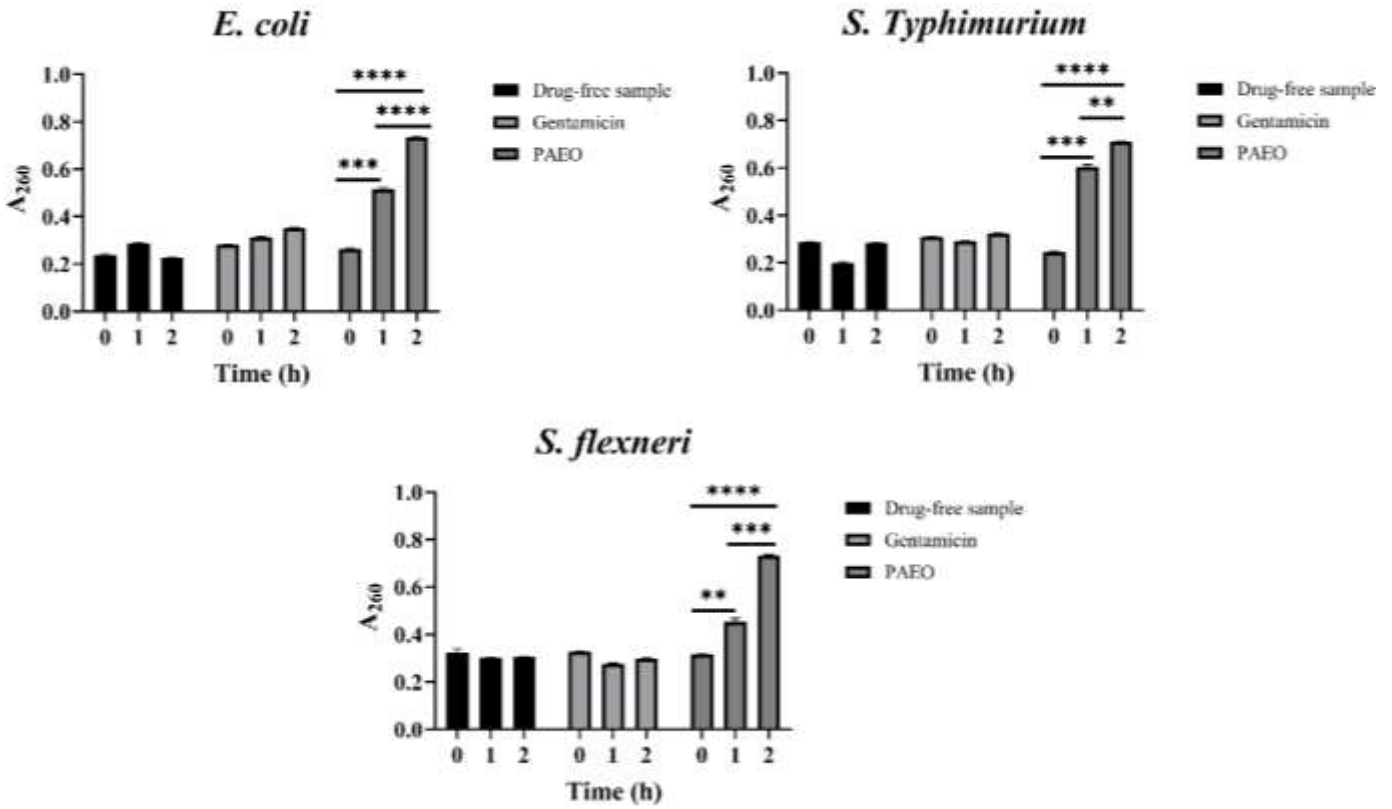

Figure 3: The release of intracellular materials that absorb at 260 nm (A_{260}) occurred in *E. coli*, *S. Typhimurium*, and *S. flexneri* after treatment with PAEO. Statistical significance compared to each time point is indicated as: **($p < 0.01$), ***($p < 0.001$), ****($p < 0.0001$).

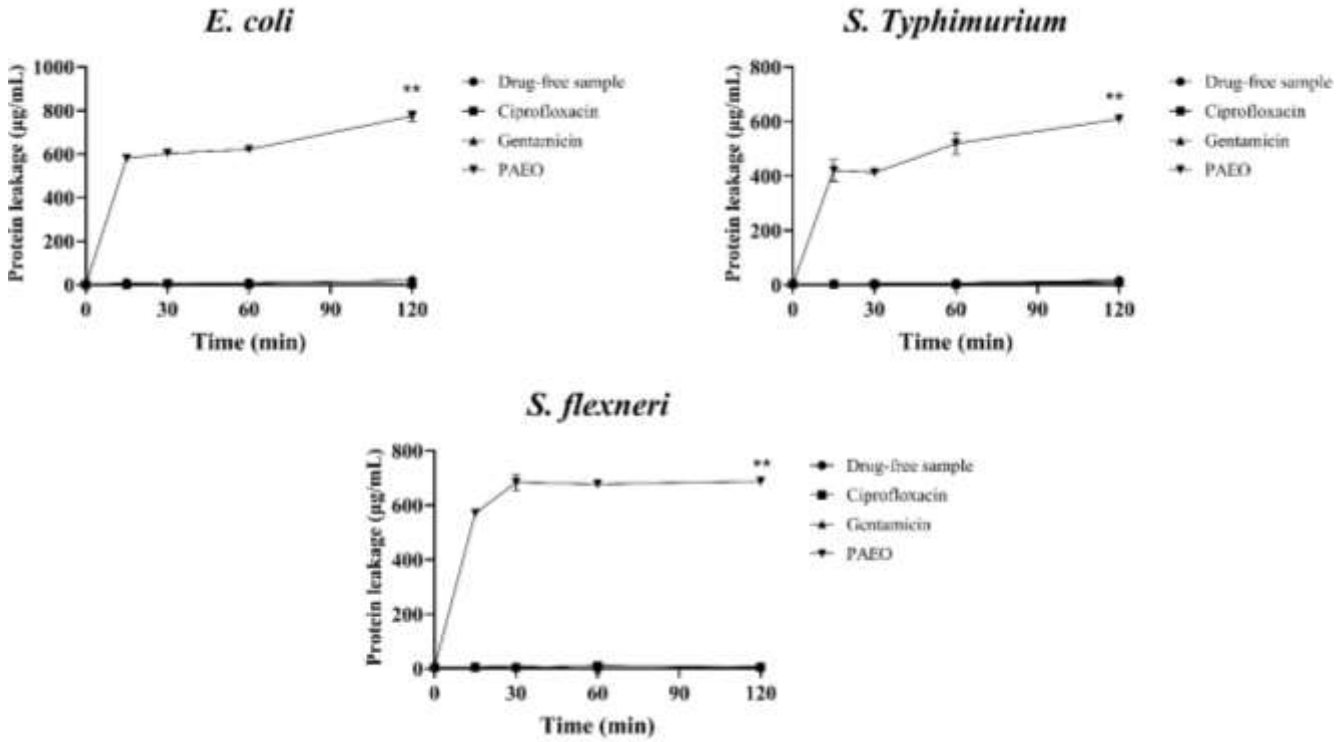

Figure 4: Effect of the essential oil of PAEO on protein leakage in enteropathogenic bacteria. **$p < 0.01$ compared to the drug-free control at the same time point

Table 3: Effect of PAEO and gentamycin antibiotic on the membrane integrity of *E. coli*, *S. Typhimurium*, and *S. flexneri* assessed by *Bac*Light bacterial viability assay.

Compounds	Green Fluorescence Intensity (% ± SD)		
	Escherichia coli	*Salmonella Typhimurium*	*Shigella flexneri*
Drug-free sample	100	100	100
2MIC of PAEO	8.16 ± 0.10	12.98 ± 0.18	10.96 ± 0.19
2MIC of gentamicin	116.59 ± 39.24	101.81 ± 1.49	90.81 ± 1.66

Values represent means of three independent replicates ± standard deviation.

The simultaneous release of nucleic acids and proteins strongly suggests that PAEO disrupts both the outer and inner membranes of gram-negative bacteria. This dual membrane damage compromises structural integrity and leads to cell lysis or irreversible metabolic dysfunction. Given the complex and multi-component nature of essential oils, it is likely that synergistic interactions among the various volatile compounds enhance this membrane-lytic activity. This mechanism is particularly significant in the context of rising antibiotic resistance. Many conventional antibiotics target specific intracellular pathways and are thus prone to resistance development. In contrast, membrane-active agents such as PAEO exert non-specific physical damage, reducing the likelihood of resistanc.[51] Furthermore, essential oils sensitize drug-resistant bacteria to antibiotics by increasing membrane permeability,[24,52] suggesting their potential for use in combination therapies.

Inhibitory effect of PAEO on angiotensin-converting enzyme 1 (ACE1) activity

ACE1 is a key enzyme in the renin–angiotensin system, significantly contributing to blood pressure regulation through the conversion of angiotensin I to angiotensin II, an effective vasoconstrictor. Inhibitors of this enzyme are therefore frequently used as therapeutic agents for the treatment of high blood pressure.[53] Essential oils from plants such as *Amomum compactum*, *Citrus limon* and *Citrus sinensis* have shown ACE inhibitory activity in vitro. Inhibition is thought to occur through competitive blinding to the active site of the enzyme or through chelation of essential metal ions such as zinc. Furthermore, the overall activity may result from synergistic interactions between several bioactive components and not only from individual compounds.[54,55] Notably, several species from the Lamiaceae family have also shown ACE1-inhibiting effects. For example, *Thymus vulgaris* (thyme)[56] and *Ocimum basilicum* (basil)[57] have been shown to inhibit ACE1 activity, possibly contributing to their traditional use in the treatment of hypertension. These research studies suggest that essential oils from Lamiaceae plants may serve as promising natural sources of ACE1 inhibitors for the development of functional foods that help lower blood pressure or herbal treatments. ACE1 activity was measured using a commercially available ACE1 Inhibitor Screening Kit (Colorimetric) (Sigma-Aldrich, MAK422), following the manufacturer's protocol. Captopril was used as a positive control for comparative evaluation. The inhibitory effect of PAEO on ACE1 activity was assessed in vitro, with results shown in Table 4 and Figure 5, and expressed as mean ± SD (n = 3). The reference inhibitor captopril used as a positive control showed significant ACE1 inhibition at a concentration of 0.02 mg/mL, with a percentage inhibition of 50.72 ± 0.50%. This provided a benchmark for evaluating the inhibitory potential of PAEO. PAEO demonstrated a dose-dependent inhibitory effect on ACE1 activity. At the highest concentration tested (1.0 mg/mL), inhibition reached 70.69 ± 0.60 %, while the lowest concentration (0.01 mg/mL) still showed notable inhibition at 62.02 ± 0.10%. Inhibition values remained consistently above 60% across all tested concentrations, with minimal SD, indicating reliable and reproducible activity. From the plotted inhibition curve (Figure 5), the half-maximal inhibitory concentration (IC_{50}) of PAEO was estimated at approximately 0.51 mg/mL. While this IC_{50} indicates moderate potency compared to synthetic inhibitors like captopril, the strong inhibition across a wide concentration range highlights PAEO's potential as a natural ACE1 inhibitor. These findings support the broader therapeutic promise of essential oils as adjunctive agents in the treatment of hypertension and underscore the need for further studies to clarify structure–activity relationships and confirm in vivo efficacy.

Table 4: Inhibitory effect of PAEO on angiotensin-converting enzyme 1 (ACE1) activity at various concentrations.

Samples	Concentration (mg/mL)	% Inhibition (Mean ± SD)
Enzyme control	-	-
Inhibitor control (Captopri)	0.02	50.72 ± 0.50
PAEO	1	70.69 ± 0.60
	0.5	66.89 ± 0.56
	0.25	63.20 ± 1.10
	0.13	63.88 ± 0.14
	0.06	65.97 ± 0.86
	0.03	63.89 ± 0.85
	0.01	62.02 ± 0.10

Values represent means of three independent replicates ± standard deviation.

One of the major bioactive components identified in PAEO is alpha-cadinol, a sesquiterpenoid compound. The role of alpha-cadinol in ACE inhibition has been confirmed by recent in silico and in vitro studies with volatile extracts of *Phaseolus vulgaris* (common bean). In molecular docking analysis, alpha-cadinol bound more effectively to ACE (−7.27 kcal/mol) than the reference drug captopril (−6.41 kcal/mol). Molecular dynamics simulations also revealed that alpha-cadinol forms a strong and stable bond with the active site of ACE, mainly by interacting with key amino acids such as Tyr523 and Phe457. The binding energy of the ACE-alpha-cadinol complex averaged −42 kJ/mol,[58] indicating a strong and stable interaction in a physiological environment. These computational findings are consistent with the in vitro results for PAEO, suggesting that the significant ACE1 inhibition observed may be, at least in part, due to the presence of alpha-cadinol. In addition, drug-likeness predictions suggest that alpha-cadinol is likely safe for human use, as no hepatotoxicity or mutagenicity (AMES toxicity)[58] was predicted, further enhancing its therapeutic potential. These findings highlight the promise of PAEO and its constituent alpha-cadinol as natural candidates for the development of functional foods or phytopharmaceuticals aimed at managing hypertension. However, *In vivo* studies and clinical trials are necessary to confirm efficacy and safety profiles.

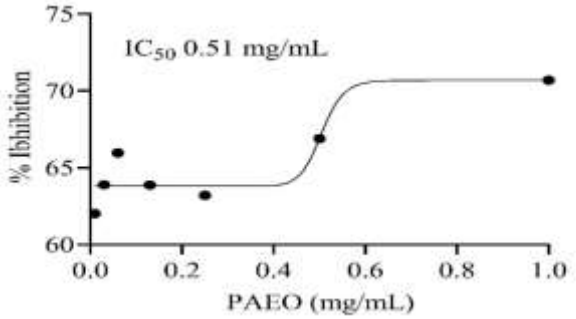

Figure 5: Inhibitory effect of PAEO on ACE1 activity at 50% inhibition (IC_{50}).

Conclusion

This study highlights the promising pharmacological potential of PAEO, in particularly its rapid and effective antimicrobial activity against enteropathogenic bacteria and its significant inhibitory effect on ACE1. This combination of bioactivities supports the potential application of PAEO as a multifunctional natural agent managing both infectious diseases and hypertension. To advance its therapeutic development, further research is warranted, especially in the areas of formulation design, *in vivo* validation, and clinical trials to assess safety, efficacy, and pharmacokinetic properties.

Conflict of Interest

The author`s declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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