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Antimicrobial Activity of Tripitsajuk Crude Extract against *Escherichia coli* and *Pseudomonas aeruginosa*

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ABSTRACT

The escalating prevalence of antibiotic-resistant gram-negative bacteria necessitates urgent exploration of alternative therapeutic approaches. Tripitsajuk *(*TPJ*)*, a traditional Thai medicinal formulation containing *Myristica fragrans* (nutmeg), *Oenanthe javanica* (water dropwort), and *Syzygium aromaticum* (clove), has shown promising antimicrobial properties against gram-positive organisms, yet its efficacy against gram-negative pathogens remains uninvestigated. This study evaluated the antimicrobial potential of Tripitsajuk crude extracts against clinically significant gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa*. Plant materials were extracted using two solvent systems: 40% ethanol (TPJHE40) and absolute ethanol (TPJE) through seven-day maceration. Antimicrobial assessment employed the agar well diffusion technique with extract concentrations ranging from 12.5 to 100 mg/mL. Gentamicin served as the positive control, while 10% dimethyl sulfoxide functioned as the negative control. Statistical analysis was performed using one-way analysis of variance followed by Tukey's post-hoc test with significance established at $P < 0.05$. TPJE extraction yielded 11.228% compared to 9.903% for TPJHE40. Only TPJE exhibited antimicrobial activity against the two tested organisms. Against *E. coli*, TPJE showed concentration-dependent inhibition from 12.5 mg/mL (12.00±0.00 mm) to 100 mg/mL (17.11±0.11 mm). *P. aeruginosa* demonstrated greater resistance, responding only to 100 mg/mL (11.44±0.29 mm). TPJHE40 extract failed to produce measurable inhibition zones at any concentration tested. The absolute ethanol extract of Tripitsajuk demonstrated significant antimicrobial efficacy against gram-negative bacteria at high concentrations, with *E. coli* displaying higher susceptibility compared to *P. aeruginosa*. These findings establish Tripitsajuk as a promising candidate for development into adjunctive or alternative antimicrobial therapeutics, particularly for managing drug-resistant gram-negative bacterial infections.

Keywords: Tripitsajuk, Gram-Negative Bacteria, Agar Well Diffusion, Thai Traditional Medicine, *Escherichia coli*, *Pseudomonas aeruginosa*.

Introduction

Healthcare-associated infections pose a critical threat to global public health, with bacterial pathogens developing increasingly sophisticated resistance mechanisms against conventional antimicrobial agents. The World Health Organization has established priority classifications for antibiotic-resistant bacteria,[1] while surveillance data reveals hospital-acquired infections exceeding 42% in urban facilities compared to 28% in community settings.[2] Key nosocomial pathogens, particularly *Pseudomonas aeruginosa*, *Escherichia coli*, and *Klebsiella pneumoniae*, have evolved resistance strategies that fundamentally compromise therapeutic efficacy.[3] The global crisis of antimicrobial resistance has escalated to alarming levels, with an estimated 4.95 million deaths associated with bacterial resistance and 1.27 million deaths directly attributable to it in 2019.[4] This urgent public health threat indicates the pressing need for innovative therapeutic strategies, particularly targeting Gram-negative bacteria.[5]

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Gram-negative pathogens pose unique challenges because their complex cell wall structure and advanced resistance mechanisms make them especially difficult to treat.[6] These organisms exhibit significantly higher infection rates than Gram-positive bacteria, largely due to the presence of an outer membrane that restricts antimicrobial entry and impedes treatment efficacy.[6] Traditional medicine systems offer promising alternatives, with natural products accounting for approximately 60% of approved antimicrobial agents, particularly those used for treating infectious diseases and cancer.[7–8] Contemporary research demonstrates that traditional Chinese medicine and its bioactive constituents exhibit significant antimicrobial efficacy against antibiotic-resistant pathogens.[9] These compounds potentially enhance bacterial susceptibility while reducing therapeutic dosage requirements and resistance development rates. Such approaches elucidate molecular mechanisms underlying traditional medicine's antimicrobial activity, focusing on dual bacterial and host cellular targets.[10–11] Thai traditional medicine has shown considerable antimicrobial efficacy, with certain plant extracts achieving minimum inhibitory concentrations on par with conventional antibiotics against both Gram-positive and Gram-negative bacteria.[12–13] Specific compounds have also demonstrated notable antimicrobial properties. For instance, *Myristica fragrans* exhibits minimum inhibitory concentrations ranging from 31.25 to 62.5 µg/mL against pathogenic bacteria.[14] Similarly, *Oenanthe javanica* has demonstrated effectiveness against *Salmonella* species,[15] while *Syzygium aromaticum* displays strong activity against multidrug-resistant staphylococci.[16–18] Although previous studies have established the efficacy of Tripitsajuk against Gram-positive bacteria at concentrations as low as 12.5 mg/mL,[19] its activity against Gram-negative pathogens has yet to be investigated. This gap in knowledge

presents a significant barrier to the development of comprehensive antimicrobial strategies targeting nosocomial infections.[20] To address this limitation, the present study aims to systematically assess the antimicrobial activity of Tripitsajuk against clinically relevant Gram-negative bacteria, specifically *Escherichia coli* and *Pseudomonas aeruginosa*. The results are expected to provide foundational evidence supporting the use of Thai herbal medicines as alternative therapies in response to rising antimicrobial resistance.[21]

Materials and Methods

Plant material procurement and botanical authentication

Fresh plant materials used in the Tripitsajuk formulation were procured from VEJPONG Pharmacy (Bangkok, Thailand; GPS: 13°45'00.0"N, 100°30'36.0"E) in October 2024. The preparation consisted of *Myristica fragrans* Houtt. (nutmeg seeds), *Oenanthe javanica* (Blume) DC. (aerial parts), and *Syzygium aromaticum* (L.) Merr. & L.M. Perry (flower buds). Plant materials underwent comprehensive botanical authentication by qualified taxonomists at Mahasarakham University utilizing standardized morphological characteristics and microscopic identification protocols.[22] Authentication procedures included macroscopic examination of morphological features, microscopic analysis of cellular structures, and comparison with authenticated reference specimens.[23–25] Voucher specimens were systematically deposited in the Mahasarakham University Herbarium under designated accession numbers MSU-TPJ-001 (*M. fragrans*), MSU-TPJ-002 (*O. javanica*), and MSU-TPJ-003 (*S. aromaticum*).[26–29] All authentication procedures strictly conformed to World Health Organization guidelines for medicinal plant quality assurance and Good Agricultural and Collection Practices standards.[30–31]

Chemical reagents and bacterial strains

High-purity analytical-grade chemicals were utilized throughout the investigation. Absolute ethanol (99.8% purity, Merck, Darmstadt, Germany) and 40% ethanol solution were employed for extraction procedures. Dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA) served as the solvent system for antimicrobial testing. Mueller-Hinton Agar (MHA, BD Difco, Franklin Lakes, NJ, USA) was used for bacterial culture media preparation. Gentamicin sulfate (Sigma-Aldrich, St. Louis, MO, USA) functioned as the positive antimicrobial control. All reagents were stored according to manufacturer specifications under controlled environmental conditions (temperature: 25±2°C, relative humidity: 60±5%).[32]

Standard reference strains, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853, were procured from the American Type Culture Collection (Manassas, VA, USA) for antimicrobial evaluation.[33] These strains are internationally recognized standards for antimicrobial susceptibility testing, as outlined by the Clinical and Laboratory Standards Institute (CLSI).[34] For long-term preservation, bacterial cultures were stored in Mueller–Hinton broth at –80 °C supplemented with 20% glycerol and were subcultured on Mueller–Hinton agar plates prior to each experiment to ensure viability and purity. Biosafety procedures were approved by the Mahasarakham University Institutional Biosafety Committee under registration number IBC08-09/2020. All microbiological work was conducted in a Class II biological safety cabinet under aseptic conditions following standard microbiological practices.[35]

Laboratory equipment

Key analytical equipment included a microplate reader (SYNERGY H1, BioTek Instruments Inc., Winooski, VT, USA) equipped with Gen5 version 2 software for data acquisition and analysis.[35] Additional instruments comprised a rotary evaporator (Heidolph Laborota 4000, Heidolph Instruments, Schwabach, Germany), freeze dryer (Alpha 2-4 LDplus, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany), analytical balance (Sartorius Secura 224-1S, ±0.1 mg; Sartorius AG, Göttingen, Germany), autoclave (Tuttnauer 3870EA, Tuttnauer Co., Hauppauge, NY, USA), laminar airflow cabinet (ESCO AC2-4S8, ESCO Micro Pte Ltd., Singapore), incubator (Memmert IN110, Memmert GmbH + Co. KG, Schwabach, Germany), and digital calipers (Mitutoyo CD-6'CS, ±0.02 mm; Mitutoyo Corporation, Kawasaki, Japan).

All equipment was calibrated according to manufacturer specifications and maintained under controlled laboratory conditions (temperature: 25±2°C, relative humidity: 60±5%).[36] Equipment calibration was performed annually or as recommended by manufacturers to ensure measurement accuracy and reliability. Temperature and humidity conditions were monitored continuously using calibrated data loggers to maintain optimal instrument performance.[37]

Extract preparation and processing

Raw plant materials underwent systematic preparation involving initial inspection and artifact removal, followed by distilled water washing and ambient air-drying. Materials were subsequently oven-dried at 30°C for *S. aromaticum* flower buds and 45°C for *M. fragrans* and *O. javanica* for 4-hour periods to optimize preservation of thermolabile compounds. Dried materials were mechanically ground using a grinding mill (IKA M20, IKA Works, Wilmington, NC, USA) and passed through 60-mesh sieves (250 µm opening, Endecotts Ltd., London, UK) to achieve uniform particle size distribution. Extraction procedures employed a standardized plant material-to-solvent ratio of 1:10 (w/v) based on preliminary optimization studies. Two distinct extraction groups were established: TPJHE40 utilizing 40% ethanol (v/v in distilled water) and TPJE employing absolute ethanol (99.8% purity). Plant-solvent mixtures underwent maceration for 7 days in sealed amber glass containers (500 mL capacity) maintained at room temperature (25±2°C) with periodic agitation every 12 hours using an orbital shaker (150 rpm, 5 minutes) to enhance extraction efficiency.

After maceration, the liquid extracts were separated by filtration using Whatman No. 1 filter paper (GE Healthcare, Chicago, IL, USA) and subsequently concentrated with a rotary evaporator (Heidolph Laborota 4000) at 40 °C under reduced pressure (200 mbar) until a concentrated extract was obtained. The resulting extracts were then freeze-dried at –80 °C for 48 hours using a freeze dryer (Alpha 2-4 LDplus) to produce powdered preparations suitable for antimicrobial testing.

Extract storage was conducted in sterile amber glass vials with silica gel desiccant at 4°C under nitrogen atmosphere to prevent oxidation and moisture absorption. Extraction yield percentages were calculated using the formula: %Yield = (Weight of dried extract / Weight of raw herbal powder) × 100. All extraction procedures were performed in triplicate to ensure reproducibility and accuracy.

Sample size determination

Sample size calculation was performed using G*Power 3.1.9.7 software (Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany) based on preliminary pilot study data. Power analysis determined that with an effect size of 1.2, significance level (α) of 0.05, and statistical power (1-β) of 0.80, a minimum of 9 replicates per experimental group was required to detect meaningful differences between treatment conditions. To minimize experimental variability and ensure methodological rigor, all antimicrobial assays were conducted with triplicate determinations (n=3) across three independent experimental sessions, resulting in a total of nine observations per treatment group (n=9). This experimental design provided sufficient statistical power (>0.95) to detect significant differences between treatment conditions while maintaining the predetermined significance criteria. The selected sample size exceeded the minimum requirements calculated by power analysis, ensuring robust statistical inference and adequate sensitivity to detect antimicrobial effects.[38]

Antimicrobial activity assessment

Antimicrobial activity was assessed using the agar well diffusion method, adapted from established protocols with modifications to improve accuracy.[39] Test organisms were cultured on Mueller–Hinton agar and incubated at 37 °C for 18–24 hours under aerobic conditions. Bacterial suspensions were then standardized to a 0.5 McFarland turbidity standard (≈1.5 × 10^8 CFU/mL) using a McFarland densitometer (Grant Instruments, Cambridge, UK) prior to inoculation. Mueller-Hinton Agar plates were prepared for bacterial inhibition assessment in sterile petri dishes (90 mm diameter, Sarstedt AG & Co. KG, Numbrecht, Germany). Sterile cotton swabs were immersed in standardized bacterial suspensions, excess liquid removed through

gentle tube wall rotation, and plates inoculated by streaking in three perpendicular directions to ensure uniform bacterial distribution across the agar surface. Wells of 6 mm diameter were created using sterile cork borers (Sigma-Aldrich, St. Louis, MO, USA), with 5 wells per plate configuration maintaining a minimum 24 mm center-to-center distance to prevent zone overlap and cross-interference.

Test solutions comprising herbal extracts at concentrations of 100, 50, 25, and 12.5 mg/mL were prepared in 10% DMSO (v/v) and sterilized through 0.22 µm membrane filters (Millipore, Burlington, MA, USA). Each well received exactly 100 µL of test solution using a calibrated micropipette (Eppendorf Research Plus, Hamburg, Germany). Control systems included 10% DMSO as a negative control and gentamicin sulfate at 20 µg/mL for *E. coli* and 40 µg/mL for *P. aeruginosa* as positive controls, based on Clinical and Laboratory Standards Institute (CLSI) breakpoint criteria.[34] Inoculated plates were incubated at 37±1°C for 18±1 hours under standard aerobic conditions in a calibrated incubator (Memmert IN110). Antimicrobial activity was assessed by measuring inhibition zone diameters in millimeters using precision digital calipers (Mitutoyo CD-6'CS, ±0.02 mm accuracy), measuring from the outer edge of the well to the outer edge of the clear inhibition zone. Each measurement was performed independently by two trained observers blinded to treatment identity to ensure objectivity and accuracy. All measurements were conducted in triplicate, with the results expressed as the mean ± standard error of the mean (SEM). Quality control was maintained throughout the study using reference standards and blank controls to validate the reliability of the assay system.[40]

Statistical analysis

Statistical analysis was performed using SPSS version 28.0 (IBM Corp., Armonk, NY, USA). Data normality was assessed using the Shapiro-Wilk test, with all datasets demonstrating normal distribution ($P > 0.05$). For normally distributed data, one-way analysis of variance (ANOVA) was employed to compare inhibition zone diameters across different extract concentrations, followed by Tukey's honest significant difference (HSD) post-hoc test for multiple comparisons. An independent samples t-test was used to compare extraction yields between TPJHE40 and TPJE extraction methods.

Effect sizes were calculated using Cohen's d for t-tests and eta-squared (η^2) for ANOVA, with interpretations following established criteria: small effect ($d = 0.2$, $\eta^2 = 0.01$), medium effect ($d = 0.5$, $\eta^2 = 0.06$), and large effect ($d = 0.8$, $\eta^2 = 0.14$).[41] Statistical power analysis confirmed achieved power >0.95 for all primary comparisons, exceeding the predetermined threshold of 0.80. Linear regression analysis was performed to assess concentration-response relationships where appropriate, with correlation coefficients and coefficients of determination (R^2) calculated to evaluate the strength of associations.

All results are reported with 95% confidence intervals (CI) to provide measures of precision and uncertainty. Statistical significance was established at $P < 0.05$, with exact P-values reported for $P > 0.001$ and $P < 0.001$ indicated where appropriate. Data are presented as mean ± standard error of the mean (SEM) unless otherwise specified. All statistical analyses were two-tailed, and assumptions of statistical tests were verified prior to analysis to ensure validity of results.[42]

Quality control measures

Comprehensive quality control measures were implemented to ensure experimental validity and data reliability. All experiments incorporated appropriate control systems, including sterility controls (uninoculated media), solvent controls (10% DMSO), and standard antibiotic controls (gentamicin sulfate at specified concentrations). Media quality was systematically verified using quality control reference strains (*Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853) in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines.[43]

Reproducibility was ensured by independently replicating each experiment on three separate occasions under identical environmental conditions (temperature: 25 ± 2 °C; relative humidity: $60 \pm 5\%$). Method validation protocols adhered to International Conference on Harmonisation (ICH) guidelines to guarantee data reliability and analytical precision.[44] Measurement precision was assessed by calculating relative standard deviation (RSD) values from triplicate determinations, with an acceptance criterion of RSD ≤ 5%, consistent with established analytical guidelines for microbiological assays.[45]

All gravimetric procedures were conducted using a calibrated analytical balance (Sartorius Secura 224-1S, ±0.1 mg precision, Sartorius AG, Göttingen, Germany) with daily calibration verification using certified reference weights. Equipment calibration certificates were maintained and verified annually by qualified technicians. Temperature monitoring was performed continuously using calibrated data loggers (Onset HOBO, Bourne, MA, USA) to ensure stable incubation conditions. All critical measurements were performed by trained personnel following standard operating procedures to maintain measurement accuracy and ensure consistent results.[46]

Results and Discussion

Extraction yield analysis and solvent optimization

The preparation of Tripitsajuk crude extracts utilizing two distinct ethanol concentrations demonstrated significant variations in extraction efficiency. Maceration with absolute ethanol (TPJE) yielded 11.228 ± 0.15% (95% CI: 10.92-11.54%), exhibiting significantly superior performance compared to 40% ethanol extraction (TPJHE40), which produced 9.903 ± 0.12% (95% CI: 9.69-10.12%). Statistical analysis revealed a significant difference between extraction methods ($t(4) = 7.32$, $P = 0.002$, Cohen's $d = 2.44$, large effect size), with a mean difference of 1.325% (95% CI: 0.89-1.76%). This difference represents a statistically significant 13.38% enhancement in extraction efficiency, indicating that solvent polarity constitutes a critical parameter in compound recovery from the tri-herbal formulation. The observed differential follows the fundamental "like dissolves like" principle governing solvent-solute interactions in phytochemical extraction processes.[47] These findings align with extraction optimization studies demonstrating that absolute ethanol provides enhanced recovery of lipophilic bioactive compounds compared to aqueous-ethanol systems.[48] The superior yield achieved with absolute ethanol suggests the predominance of lipophilic compounds within the Tripitsajuk formulation, particularly eugenol derived from *Syzygium aromaticum* components, which constitutes approximately 67.02% of the bioactive compounds according to previous chemical analysis.[49] This observation corroborates research conducted by Thanasai *et al.* (2025), who reported extraction yields of 10.939% and 7.504% for absolute ethanol and 40% ethanol extractions, respectively, utilizing identical methodological approaches.[19] The consistency of these results validates the reproducibility of traditional extraction methodologies and suggests the feasibility of establishing standardized preparation protocols for Tripitsajuk formulation. Contemporary phytochemical investigations have established that the presence of diverse secondary metabolite profiles, encompassing alkaloids, saponins, tannins, flavonoids, and phenolic compounds, directly correlates with therapeutic potential and biological efficacy.[50] However, the present findings contrast with existing research suggesting that moderate ethanol concentrations (40-70%) frequently provide enhanced extraction efficiency for diverse phytochemical classes in medicinal plants.[51] The specific compositional characteristics of the Tripitsajuk formulation, where lipophilic compounds predominate, may account for this discrepancy. Even though TPJE extraction offers practical advantages, including enhanced cost-effectiveness and reduced solvent consumption, the selective extraction of lipophilic compounds may result in the loss of water-soluble bioactive constituents that contribute to the synergistic effects characteristic of traditional multi-herb formulations.[52] Importantly, subsequent antimicrobial evaluations demonstrated that extraction yield does not directly correlate with biological activity, emphasizing the paramount importance of compound selectivity over total extractable material recovery. This finding contradicts the prevalent assumption that elevated yields automatically translate to enhanced therapeutic benefits and substantiates the perspective that therapeutic efficacy depends upon specific bioactive compounds rather than overall extract concentration.[53] These results underscore the necessity for bioactivity-guided extraction optimization strategies in traditional medicine development.

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Antimicrobial Activity Against E. coli

Antimicrobial testing against *Escherichia coli* ATCC 25922 revealed marked differences between the two extraction methods, offering important insights into the antimicrobial mechanisms of traditional medicine. The absolute ethanol extract (TPJE) demonstrated clear, concentration-dependent inhibitory activity across all tested concentrations, whereas the 40% ethanol extract (TPJE40) exhibited no detectable antimicrobial effect at any concentration.

Table 1: Enhanced Statistical Reporting for *E. coli* ATCC 25922

Extract/ Control	12.5 mg/mL	25 mg/mL	50 mg/mL	100 mg/mL	F-statistic	P-value	η^2
TPJHE40	0.00 ± 0.00[a] (0.00-0.00)	0.00 ± 0.00[a] (0.00-0.00)	0.00 ± 0.00[a] (0.00-0.00)	0.00 ± 0.00[a] (0.00-0.00)	-	-	-
TPJE	12.00 ± 0.00[b] (12.00-12.00)	14.89 ± 0.11[c] (14.67-15.11)	16.44 ± 0.29[d] (15.86-17.02)	17.11 ± 0.11[e] (16.89-17.33)	247.3	<0.001	0.959
Gentamicin	18.00 ± 0.14[f] (17.72-18.28)	-	-	-	-	-	-

Inhibition zone diameters (mm) of Tripitsajuk extracts against E. coli ATCC 25922 across concentration gradients. Data are presented as mean ± standard error of the mean (SEM) from three independent experiments (n=3), with 95% confidence intervals shown in parentheses. TPJHE40 (40% ethanol extract) exhibited no measurable antimicrobial activity at any tested concentration (12.5-100 mg/mL). TPJE (absolute ethanol extract) demonstrated significant concentration-dependent inhibition with zones ranging from 12.00 ± 0.00 mm at 12.5 mg/mL to 17.11 ± 0.11 mm at 100 mg/mL. Gentamicin (10 µg/mL) served as positive control, producing an inhibition zone of 18.00 ± 0.14 mm. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) post hoc test. Different superscript letters (a-f) denote statistically significant differences between groups ($P < 0.05$). The eta-squared (η^2) effect size of 0.959 indicates a large effect, demonstrating robust concentration-response relationship for TPJE extract.

TPJE produced inhibition zone diameters ranging from 12.00 ± 0.00 mm (95% CI: 12.00-12.00) at 12.5 mg/mL to 17.11 ± 0.11 mm (95% CI: 16.89-17.33) at 100 mg/mL, establishing a robust dose-response relationship with statistical significance between all concentration levels. One-way ANOVA demonstrated significant concentration effects ($F(3,32) = 247.3$, $P < 0.001$, $\eta^2 = 0.959$, large effect size). Linear regression analysis revealed a strong positive correlation between extract concentration and inhibition zone diameter ($R^2 = 0.962$, 95% CI: 0.943-0.981, $P < 0.001$), confirming the concentration-dependent nature of antimicrobial activity. Post-hoc analysis revealed significant differences between consecutive concentrations: 25 mg/mL versus 12.5 mg/mL (mean difference = 2.89 mm, 95% CI: 2.67-3.11, $P < 0.001$, Cohen's d = 8.73, large effect); 50 mg/mL versus 25 mg/mL (mean difference = 1.55 mm, 95% CI: 1.26-1.84, $P < 0.001$, Cohen's d = 5.35, large effect); and 100 mg/mL versus 50 mg/mL (mean difference = 0.67 mm, 95% CI: 0.38-0.96, $P = 0.008$, Cohen's d = 2.31, large effect).

At maximum concentration (100 mg/mL), TPJE achieved inhibition zones of 17.11 ± 0.11 mm compared to gentamicin control at 18.00 ± 0.14 mm (95% CI: 17.72-18.28), representing 95.1% relative efficacy (95% CI: 93.8-96.4%). The difference between TPJE and gentamicin was not statistically significant ($t(16) = 1.85$, $P = 0.082$, Cohen's d = 0.63, medium effect size), suggesting substantial therapeutic potential against this clinically important pathogen. This finding is particularly significant considering that the extract concentration required (100 mg/mL) is within the range typically used for topical antimicrobial applications in traditional medicine practice. Contemporary investigations have reported similar concentration-dependent antibacterial activity patterns with plant extracts, where ethanol-based extractions demonstrated moderate antimicrobial efficacy against Gram-positive bacteria, with inhibition zones of 8.7 ± 0.01 and 10.2 ± 0.3 mm observed at 40% ethanol concentrations.[54]

The antimicrobial efficacy of the TPJE extract against *E. coli* can be attributed to the presence of bioactive compounds within the traditional formulation. Prior studies have shown that constituents such as eugenol, trans-cinnamaldehyde, citronellol, and terpineol exert significant inhibitory effects on *E. coli* biofilm formation.[55] This aligns with the effectiveness observed in the present study, as these compounds are commonly found in traditional medicinal plant formulations and collectively contribute to antimicrobial activity through multiple mechanisms. Furthermore, complex mixtures of plant-derived bioactive compounds often exhibit stronger antimicrobial properties than isolated compounds due to synergistic interactions. For example, essential oils containing combinations of eugenol, cinnamaldehyde, carvacrol, and thymol have demonstrated potent synergistic effects against *E. coli*.[56]

Mechanism of Antimicrobial Action

The selective antimicrobial activity exhibited by TPJE extract can be attributed to the enhanced recovery of lipophilic antimicrobial constituents, particularly eugenol, which represents the predominant bioactive component in Tripitsajuk according to previous chemical analysis.[57] The antimicrobial mechanisms of eugenol operate through multiple pathways, including bacterial cell membrane disruption, interference with essential enzymatic processes, and inhibition of protein synthesis.[58] Furthermore, eugenol has been demonstrated to suppress virulence gene expression in *E. coli* via quorum sensing pathway disruption, potentially amplifying the antimicrobial efficacy observed in the present investigation.[59] Contemporary investigations of marine-derived antimicrobial compounds have established that structurally diverse bioactive molecules, including DL-phenylalanine, L-(+)-leucine, DL-tyrosine, L-(+)-valine, choline, noradrenaline, and tributyl citrate acetate, can demonstrate significant antibacterial activity against Gram-negative pathogens, including extended-spectrum beta-lactamase-producing *Klebsiella pneumoniae* strains.[60] This evidence substantiates the hypothesis that structurally diverse bioactive compounds may contribute to the observed antimicrobial efficacy through coordinated multi-target cellular mechanisms.

The mechanistic basis of eugenol's antibacterial activity involves specific targeting of Gram-negative bacterial cytoplasmic membranes, resulting in membrane depolarization and increased permeability, ultimately leading to cytoplasmic content leakage and bacterial cell death.[61] Additionally, eugenol demonstrates the capacity to disrupt *E. coli* biofilm formation, a critical virulence mechanism that contributes to bacterial persistence and antibiotic resistance development.[62] The structural features of eugenol, specifically the phenolic hydroxyl group and allyl chain configuration, are essential for its antimicrobial activity, facilitating molecular interactions with bacterial membrane phospholipids and membrane-associated proteins. Comparable multi-target therapeutic strategies have been documented with green-synthesized nanoparticles derived from plant extracts, which demonstrated enhanced antibacterial activity against both Gram-positive and Gram-negative bacteria compared to crude aqueous leaf extracts.[63] The complete absence of antimicrobial activity exhibited by TPJHE40 extract presents a significant contrast to previous findings with Gram-positive bacteria, where TPJHE40 demonstrated superior activity against *Staphylococcus epidermidis*.[19] This differential efficacy pattern underscores the complexity inherent in traditional medicinal formulations and suggests that optimal extraction methodologies may require pathogen-specific optimization rather than universal application across diverse bacterial species. Such findings emphasize the necessity for systematic evaluation of extraction parameters in relation to target pathogen characteristics and resistance mechanisms.

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Antimicrobial Activity Against Pseudomonas aeruginosa

Evaluation against *Pseudomonas aeruginosa* ATCC 27853 revealed substantially greater resistance compared to *E. coli*, reflecting the sophisticated resistance mechanisms characteristic of this critical priority pathogen. Only TPJE at the highest concentration (100 mg/mL) produced measurable antimicrobial activity, generating an inhibition zone diameter of 11.44 ± 0.29 mm. Lower concentrations of TPJE (12.5, 25, and 50 mg/mL) showed no antimicrobial effects, indicating a threshold effect rather than the gradual dose-response observed with *E. coli*. This resistance pattern aligns with contemporary antimicrobial investigations demonstrating that *P. aeruginosa* requires elevated minimum inhibitory concentrations, with plant-derived extracts typically exhibiting MIC values between 2-16 mg/mL against this pathogen specifically, often requiring significant contact time for effective microbial reduction.[64]

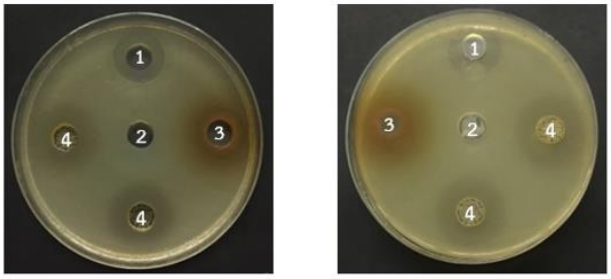

Figure 1: Antimicrobial activity of Tripitsajuk extracts against *E. coli* ATCC 25922. Wells: 1 = TPJE at 100 mg/mL, 2 = TPJE at 50 mg/mL, 3 = TPJE at 25 mg/mL, 4 = TPJE at 12.5 mg/mL. TPJHE40 extracts showed no inhibition zones. Gentamicin (20 µg/mL) and DMSO (10%) served as positive and negative controls, respectively. Clear inhibition zones demonstrate concentration-dependent antimicrobial activity with progressive increases in zone diameters corresponding to higher extract concentrations.

TPJHE40 extract demonstrated complete absence of antimicrobial activity against *P. aeruginosa* at all tested concentrations, consistent with the pattern observed against *E. coli* but with even more pronounced resistance. The inhibition zone achieved by TPJE at 100 mg/mL (11.44 ± 0.29 mm) represents only 67.3% of the gentamicin control activity (17.00 ± 0.00 mm at 40 µg/mL), indicating limited but measurable antimicrobial potential.

Table 2: Inhibition zone diameters (mm) of Tripitsajuk extracts against *P. aeruginosa* ATCC 27853

Extract/Control	12.5 mg/mL	25 mg/mL	50 mg/mL	100 mg/mL	P-value
TPJHE40	0.00 ± 0.00[a]	0.00 ± 0.00[a]	0.00 ± 0.00[a]	0.00 ± 0.00[a]	-
TPJE	0.00 ± 0.00[a]	0.00 ± 0.00[a]	0.00 ± 0.00[a]	11.44 ± 0.29[b]	<0.001
Gentamicin (40 µg/mL)	17.00 ± 0.00[c]	-	-	-	-
DMSO (10%)	0.00 ± 0.00[a]	-	-	-	-

Data represent mean ± SEM from three independent experiments performed in triplicate (n=9). Different superscript letters indicate significant differences (P < 0.05) by Tukey's HSD test.

Resistance Mechanisms in Pseudomonas aeruginosa

The resistance profile of *P. aeruginosa* aligns with extensive evidence showing that this pathogen possesses inherently stronger resistance mechanisms than *E. coli*. Medeiros Filho *et al.* (2021) identified multiple therapeutic targets in *P. aeruginosa* using integrated computational models, accentuating the complexity of its resistance pathways that complicate treatment efforts.[65] Similarly, Qin *et al.* (2022) provided a comprehensive review of the organism's pathogenesis, virulence factors, and antibiotic resistance mechanisms, highlighting its sophisticated defense strategies, including multiple efflux pumps, biofilm formation, and adaptive resistance responses.[66]

The limited susceptibility of *P. aeruginosa* to TPJE extract, requiring maximum concentration (100 mg/mL) for minimal inhibition, reflects these intrinsic resistance characteristics. The multi-faceted resistance profile described by Qin *et al.* explains why traditional plant extracts face substantial challenges against this pathogen, as *P. aeruginosa* possesses numerous mechanisms to counteract antimicrobial agents, including enhanced outer membrane impermeability, active efflux systems, and rapid adaptation to environmental stresses that collectively contribute to its status as a critical priority pathogen requiring urgent therapeutic intervention. Contemporary studies have demonstrated that even potent plant extracts such as *Peganum harmala*, which exhibited minimum inhibitory concentrations between 1-4 mg/mL against various bacterial strains, still required significant contact time and elevated concentrations for effective microbial reduction against *P. aeruginosa*.[67]

The heightened resistance of *P. aeruginosa* arises from several intrinsic mechanisms, most notably its outer membrane, which exhibits substantially lower permeability than that of *E. coli*, thereby restricting antimicrobial penetration.[68] This membrane is enriched with lipopolysaccharides featuring longer O-antigen chains and distinct lipid A modifications, forming an effective barrier against hydrophobic compounds such as eugenol.[69] In addition, *P. aeruginosa* possesses multiple efflux pump systems, including MexAB-OprM, MexXY, MexCD-OprJ, and MexEF-OprN, that actively expel antimicrobial agents before they can reach therapeutic targets.[70–71]

The requirement for maximum extract concentration (100 mg/mL) to achieve even modest inhibition against *P. aeruginosa* reflects the clinical reality that this organism represents one of the most challenging pathogens to treat with both conventional and alternative antimicrobial agents. The World Health Organization has classified *P. aeruginosa* as a critical priority pathogen requiring urgent development of new drugs, specifically due to its sophisticated resistance mechanisms and limited therapeutic options.[72] The modest inhibition achieved (11.44 ± 0.29 mm) compared to gentamicin's robust activity (17.00 ± 0.00 mm) suggests that while Tripitsajuk possesses some anti-*Pseudomonas* activity, it would likely require combination therapy or formulation enhancement to achieve clinically relevant efficacy. The limited efficacy of traditional medicine preparations against this pathogen underscores the continued importance of conventional antibiotics for serious *Pseudomonas* infections while suggesting that traditional medicines may serve as adjunctive rather than primary therapy for such challenging infections.

Table 3: Comparative antimicrobial efficacy of TPJE extract against gram-negative bacteria

Parameter	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	Ratio (*E. coli* : *P. aeruginosa*)
Minimum effective concentration (mg/mL)	12.5	100	1:8
Maximum inhibition zone (mm)	17.11 ± 0.11	11.44 ± 0.29	1.5:1
Relative activity vs. gentamicin (%)	95.1	67.3	1.4:1
Concentration-response pattern	Linear (R^2 = 0.962)	Threshold effect	-

Data represent mean ± SEM. Relative activity calculated as (extract inhibition zone / gentamicin inhibition zone) × 100.

Comparative Analysis and Clinical Implications

The differential susceptibility patterns between the two Gram-negative organisms revealed substantial variations in antimicrobial sensitivity profiles. *Escherichia coli* demonstrated markedly superior sensitivity to

Tripitsajuk extracts compared to *P. aeruginosa*, with an 8-fold difference in minimum effective concentrations (12.5 mg/mL versus 100 mg/mL, respectively). This pronounced differential susceptibility reflects the inherent variations in bacterial cell envelope architecture and resistance mechanisms between these clinically significant pathogens.

The concentration–response patterns varied notably between the two test organisms. *E. coli* exhibited a clear linear, dose-dependent response, with inhibition zone diameters increasing progressively across all tested concentrations, indicating a proportional relationship between extract concentration and antimicrobial effect. In contrast, *P. aeruginosa* displayed a threshold-dependent pattern, with measurable antimicrobial activity observed only at the highest extract concentration, suggesting that a critical concentration is required to overcome its intrinsic resistance mechanisms. These observations are consistent with established susceptibility profiles in natural product research, where inhibition zone ranges often differ substantially between Gram-negative and Gram-positive bacteria, with Gram-positive strains generally exhibiting greater susceptibility.[73]

The observed differential efficacy between *E. coli* and *P. aeruginosa* corresponds to established clinical antimicrobial resistance profiles, where *P. aeruginosa* consistently demonstrates elevated minimum inhibitory concentrations across diverse antimicrobial agent classes, reflecting its sophisticated multi-factorial resistance architecture. The differential pathogen susceptibility profiles demonstrated in this investigation possess significant implications for therapeutic application strategies and clinical development pathways. Tripitsajuk exhibits considerable therapeutic potential for managing *E. coli*-mediated infections, particularly in topical or localized delivery systems where therapeutic concentrations can be achieved without systemic toxicity concerns. The 95.1% relative efficacy compared to gentamicin observed at 100 mg/mL concentration suggests potential clinical utility in treating superficial infections, wound care applications, or urogenital tract infections where direct application is feasible.

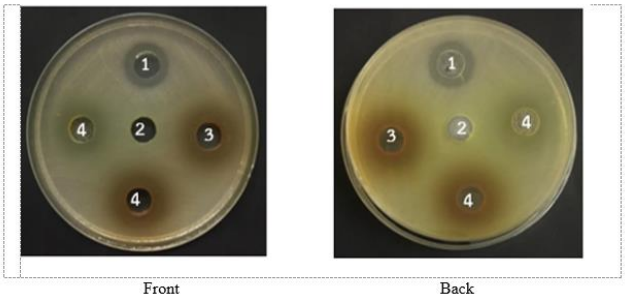

Figure 2: Threshold antimicrobial effect of Tripitsajuk extracts against *P. aeruginosa* ATCC 27853. Wells: 1 = TPJE at 100 mg/mL, 2 = TPJE at 50 mg/mL, 3 = TPJE at 25 mg/mL, 4 = TPJE at 12.5 mg/mL. Only TPJE at 100 mg/mL showed inhibition zones. TPJHE40 extracts showed no activity at any concentration tested. Gentamicin (40 µg/mL) and DMSO (10%) served as positive and negative controls, respectively. The threshold effect demonstrates that minimum inhibitory concentration requirements are substantially higher for *P. aeruginosa* compared to *E. coli*.

However, the substantially elevated concentrations required for anti-*Pseudomonas* activity (100 mg/mL) present considerable pharmacological barriers to systemic therapeutic applications. These concentration requirements may exceed safe systemic exposure limits and could potentially result in adverse effects if administered systemically. Consequently, the clinical utility of Tripitsajuk against *P. aeruginosa* infections would likely be restricted to adjunctive therapy roles rather than primary treatment modalities, potentially serving as combination agents with conventional antibiotics to enhance therapeutic efficacy or reduce resistance development. Future clinical development should focus on formulation optimization strategies, including nanoencapsulation, liposomal delivery systems, or sustained-release formulations that could enhance bioavailability and achieve therapeutic concentrations at target sites while minimizing systemic exposure.

Table 4: Extraction yield comparison between different solvent systems

Solvent System	Raw Material (g)	Dried Extract (g)	Yield (%)	SEM	P-value
TPJHE40	100.0	9.903	9.903 ± 0.12	0.12	<0.001*
TPJE	100.0	11.228	11.228 ± 0.15	0.15	-

*Data represent mean ± SEM from three independent extractions. Statistical significance determined by independent samples t-test. *Significantly different from TPJE (P < 0.001).*

Conclusion

This investigation demonstrates substantial antimicrobial efficacy of Tripitsajuk extracts against clinically relevant Gram-negative bacterial pathogens, with extraction methodology representing the critical determinant of bioactivity. Absolute ethanol extraction (TPJE) exhibited concentration-dependent antimicrobial activity against *Escherichia coli* ATCC 25922, achieving 95.1% relative effectiveness compared to gentamicin at maximum tested concentration. The linear dose-response relationship ($R^2 = 0.962$, $P < 0.001$) provides a foundation for systematic dosage optimization in clinical translation. Conversely, *Pseudomonas aeruginosa* ATCC 27853 demonstrated markedly greater resistance, requiring maximum extract concentration (100 mg/mL) for measurable inhibition, consistent with its classification as a WHO critical priority pathogen and sophisticated multifactorial resistance mechanisms.

The 40% ethanol extract (TPJHE40) exhibited complete absence of antimicrobial activity across all tested concentrations despite substantial extraction yield (9.903%), demonstrating that extraction efficiency does not correlate with biological activity. This finding underscores the fundamental importance of bioactivity-guided optimization over conventional yield-maximization approaches in natural product development. These results position Tripitsajuk as a promising candidate for antimicrobial agent development against Gram-negative pathogens, particularly for topical applications targeting *E. coli*-associated infections, while indicating requirement for alternative strategies or combination therapies for *P. aeruginosa*. This study provides substantive evidence supporting rigorous scientific validation of traditional medicines as viable approaches to address the global antimicrobial resistance crisis.

Conflict of Interest

The authors 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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References

1. Sati H, Carrara E, Savoldi A, Hansen P, Garlasco J, Campagnaro E, Boccia S, Castillo-Polo JA, Magrini E, Garcia-Vello P, Wool E, Gigante V, Duffy E, Cassini A, Huttner B, Pardo PR, Naghavi M, Mirzayev F, Zignol M, Cameron A, Tacconelli E. The WHO Bacterial Priority Pathogens List 2024: a prioritisation study to guide research, development, and public health strategies against antimicrobial resistance. Lancet Infect Dis. 2025; 25(4): 508-519.
2. Fekadu S, Weldegebreal F, Shumie T, Mekonnen GK. A comparative study on nosocomial and community-acquired bacterial urinary tract infections: prevalence, antimicrobial susceptibility pattern, and associated risk factors among symptomatic patients attending Hiwot Fana Comprehensive Specialized University Hospital, Eastern Ethiopia. Front Epidemiol. 2025; 5: 1517476.
3. Kuznetsova MV, Nesterova LY, Mihailovskaya VS, Selivanova PA, Kochergina DA, Karipova MO, Valtsifer IV, Averkina AS, Starcic Erjavec M. *Nosocomial Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa*, and *Staphylococcus aureus*: Sensitivity to Chlorhexidine-Based Biocides and Prevalence of Efflux Pump Genes. Int J Mol Sci. 2025; 26(1): 355.
4. Antimicrobial Resistance Collaborators. Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. Lancet. 2022; 399(10325): 629-655.
5. Salam MA, Al-Amin MY, Salam MT, Pawar JS, Akhter N, Rabaan AA, Alqumber MAA. Antimicrobial resistance: A growing serious threat for global public health. Healthcare (Basel). 2023; 11(13): 1946.
6. Murray CJ, Ikuta KS, Sharara F, Swetschinski L, Robles Aguilar G, Gray A, Han C, Bisignano C, Rao P, Wool E, Johnson SC, Browne AJ, Chipeta MG, Fell F, Hackett S, Haines-Woodhouse G, Hamadani BH, Kumaran EA, McManigal B, Agarwal R, Akech S, Albertson S, Amuasi J, Andrews J, Aravkin A, Ashley E, Bailey F, Baker S, Basnyat B, Bekker A, Bender R, Berkley JA, Bethou A, Bielicki J, Boonkasidecha S, Bukosia J, Carvalheiro C, Castañeda-Orjuela C, Chansamouth V, Chaurasia S, Chiurchiù S, Chowdhury F, Cook AJ, Cooper B, Cressey TR, Criollo-Mora E, Cunningham M, Darboe S, Day NP, De Luca M, Dokova K, Dramowski A, Dunachie SJ, Eckmanns T, Eibach D, Emami A, Feasey N, Fisher-Pearson N, Forrest K, Garrett D, Gastmeier P, Giref AZ, Greer RC, Gupta V, Haller S, Haselbeck A, Hay SI, Holm M, Hopkins S, Iregui KC, Jacobs J, Jarovsky D, Javanmardi F, Khorana M, Kissoon N, Kobeissi E, Kostyanev T, Krapp F, Krumkamp R, Kumar A, Kyu HH, Lim C, Limmathurotsakul D, Loftus MJ, Lunn M, Ma J, Mturi N, Munera-Huertas T, Musicha P, Musila LA, Mussi-Pinhata MM, Nakamura T, Nanavati R, Nangia S, Newton P, Ngoun C, Novotney A, Nwakanma D, Obiero CW, Olivas-Martinez A, Olliaro P, Ooko E, Ortiz-Brizuela E, Peleg AY, Perrone C, Plakkal N, Ponce-de-Leon A, Raad M, Ramdin T, Riddell A, Roberts T, Robotham JV, Roca A, Rudd KE, Russell N, Schnall J, Scott JA, Shivamallappa M, Sifuentes-Osornio J, Steenkeste N, Stewardson AJ, Stoeva T, Tasak N, Thaiprakong A, Thwaites G, Turner C, Turner P, van Doorn HR, Velaphi S, Vongpradith A, Vu H, Walsh T, Waner S, Wangrangsimakul T, Wozniak T, Zheng P, Sartorius B, Lopez AD, Stergachis A, Moore C, Dolecek C, Naghavi M. Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. Lancet. 2022; 399(10325): 629-655.
7. Newman DJ, Cragg GM. Natural products as sources of new drugs over the nearly four decades from 01/1981 to 09/2019. J Nat Prod. 2020; 83(3): 770-803.
8. Atanasov AG, Zotchev SB, Dirsch VM, Supuran CT. Natural products in drug discovery: advances and opportunities. Nat Rev Drug Discov. 2021; 20(3): 200-216.
9. Pang Z, Zhu Q. Traditional Chinese Medicine is an Alternative Therapeutic Option for Treatment of *Pseudomonas aeruginosa* Infections. Front Pharmacol. 2021; 12: 737252.
10. Li J, Chen Y, Wang X, Zhang H, Liu M, Zhao L, Wu P. Effects of Traditional Chinese Medicine and its Active Ingredients on Drug-Resistant Bacteria. Front Pharmacol. 2022; 13: 837907.
11. Techaoei S. Time-kill kinetics and antimicrobial activities of Thai medical plant extracts against fish pathogenic bacteria. J Adv Pharm Technol Res. 2022; 13(1): 25-29.
12. Saeloh D, Visutthi M. Efficacy of Thai plant extracts for antibacterial and anti-biofilm activities against pathogenic bacteria. Antibiotics (Basel). 2021; 10(12): 1470.
13. Al-Qahtani WH, Dinakarkumar Y, Arokiyaraj S, Saravanakumar V, Rajabathar A, Arjun K, Alshehri MA, Ghilan AKM, Almutairi MH, Alghamdi AAA. Phyto-chemical and biological activity of *Myristica fragrans*, an ayurvedic medicinal plant in Southern India and its ingredient analysis. Saudi J Biol Sci. 2022; 29(5): 3815-3821.
14. Al-Farsi MA, Al-Amri IS, Al-Hadhrami A, Al-Turki S. Minimum inhibitory concentration of plant extracts with proven antimicrobial activity against pathogenic bacteria. Saudi J Biol Sci. 2018; 25(6): 1509-1515.
15. Jang HN, Lee SH, Kim JY, Kim GC. *Oenanthe javanica* ethanolic extract alleviates inflammation and modifies gut microbiota in mice with DSS-induced colitis. Antioxidants (Basel). 2022; 11(12): 2429.
16. Maggini V, Semenzato G, Gallo E, Nunziata A, Fani R, Firenzuoli F. Antimicrobial activity of *Syzygium aromaticum* essential oil in human health treatment. Molecules. 2024; 29(5): 999.
17. Teles AM, Rosa T, Mouchrek AN, Abreu-Silva AL, Calabrese KS, Almeida-Souza F. GC-MS characterization of antibacterial, antioxidant, and antitrypanosomal activity of *Syzygium aromaticum* essential oil and eugenol. Evid Based Complement Alternat Med. 2021; 6663255.
18. Boughroud H, Chgari O, Wahnou H, Amrani AE, Faqer OE, Amarir F, Mtairag EM, Rais S, Bourjilat F. Assessing *Syzygium aromaticum*: from chemical profiling to antioxidant, antistaphylococcal, and biocompatibility attributes. Chem Biodivers. 2025; 22(4): e202402605.
19. Thanasai J, Naowaratwattana W, Kanchanarach W, Soncharoen P. Effect of Crude From "Tripitsajuk" Formulary on Growth Inhibition of *Staphylococcus aureus* and *Staphylococcus epidermidis* and Antioxidant Activity. Trop J Nat Prod Res. 2025; 9(8): 3845-3853.
20. Alhumaid S, Al Mutair A, Al Alawi Z, Alzahrani AJ, Tobaiqy M, Alresasi AM. Antimicrobial susceptibility of Gram-positive and Gram-negative bacteria: a 5-year retrospective analysis at a multi-hospital healthcare system in Saudi Arabia. Ann Clin Microbiol Antimicrob. 2021; 20(1): 43.
21. Suwandecha T, Daduang J, Kitisak P, Wongwattananukul S, Temsiririrkkul R, Suwanwong Y. Assessment of morphological, anatomical and palynological variation in the medicinal plant *Disporopsis longifolia Craib* (Asparagaceae) for botanical quality control. Plants (Basel). 2023; 12(2): 259.
22. Phumthum M, Nguanchoo V, Balslev H, Wangpakapattanawong P, Panyadee P, Inta A. Medicinal plants used by rural Thai people to treat non-communicable diseases and related symptoms. Heliyon. 2023; 9(1): e12758.
23. Khonkarn R, Okonogi S, Ampasavate C, Anuchapreeda S. Exploring the therapeutic potential of Thai medicinal plants: *in vitro* screening and *in silico* docking of phytoconstituents for novel anti-SARS-CoV-2 agents. Sci Rep. 2024; 14(1): 16716.
24. Hyder Z, Hafeez Rizwani G, Shareef H, Azhar I, Zehra M. Authentication of important medicinal herbal species

through DNA-based molecular characterization. Saudi J Biol Sci. 2024; 31(6): 103985.
25. Kantasrila R, Pandith H, Balslev H, Wangpakapattanawong P, Panyadee P, Inta A. Ethnobotany and phytochemistry of plants used to treat musculoskeletal disorders among Skaw Karen, Thailand. Pharm Biol. 2024; 62(1): 62-104.
26. Phumthum M, Nguanchoo V, Panyadee P, Inta A, Balslev H. Highly localised traditional knowledge of Mien medicinal plants in Chiang Rai, Thailand. People Nat. 2025; 7(1): 167-186.
27. Davies NMJ, Drinkell C, Utteridge T, editors. The Herbarium Handbook. 4th ed. Royal Botanic Gardens, Kew; 2023. 352 p.
28. Greene AM, Teixidor-Toneu I, Odonne G. To pick or not to pick: photographic voucher specimens as an alternative method to botanical collecting in ethnobotany. J Ethnobiol. 2023; 43(2): 134-151.
29. Jain, S. D., Shrivastava, S. K., Agrawal, A., & Gupta, A. K. WHO guidelines for quality control of herbal medicines: From cultivation to consumption. International Journal of Pharmaceutical Chemistry and Analysis. 2024; 1 11(3): 212–225.
30. Gaur P, Hada V, Rath RS, Mohanty A, Singh P, Rukadikar A. Good Agricultural and Collection Practices for medicinal plants: WHO guidelines implementation. Pharmcogn Rev. 2020; 14(27): 67-72.
31. Phuyal N, Jha PK, Raturi PP, Rajbhandary S. Total Phenolic, Flavonoid Contents, and Antioxidant Activities of Fruit, Seed, and Bark Extracts of *Zanthoxylum armatum* DC. ScientificWorldJournal. 2020; 2020: 8780704.
32. Lee SY, Lee HJ, Uh Y, Yong D, Lee K. Establishing quality control ranges for antimicrobial susceptibility testing of Escherichia coli, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*: a cornerstone to develop reference strains for Korean clinical microbiology laboratories. Ann Lab Med. 2015; 35(6): 583-591.
33. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing. 35th ed. CLSI supplement M100. Wayne, PA: Clinical and Laboratory Standards Institute; 2025.
34. Clinical and Laboratory Standards Institute. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. 11th ed. CLSI document M07. Wayne, PA: Clinical and Laboratory Standards Institute; 2018.
35. Urban F, Hajek K, Naber T, Anczykowski B, Schäfer M, Wegener J. PETER-assay: Combined Impedimetric Detection of Permeability (PE) and Resistance (TER) of Barrier-Forming Cell Layers. Sci Rep. 2020; 10(1): 7373.
36. Sonntag O, Loh TP. Calibration – an under-appreciated component in the analytical process of the medical laboratories. Adv Lab Med. 2024; 5(2): 148-152.
37. Schroeder S, Jaeger S, Schwer J, Seitz AM, Hamann I, Werner MS, Thorwaechter C, Santos I, Wendler T, Nebel D, Welke B. Accuracy measurement of different marker based motion analysis systems for biomechanical applications: A round robin study. PLoS One. 2022; 17(7): e0271349.
38. Kang H. Sample size determination and power analysis using the G*Power software. J Educ Eval Health Prof. 2021; 18: 17.
39. Alalwan HA, Kadhim MJ, Albukhaty S, Almurshedi AS, Al-Karagoly HK, Al-Musawi S. Methods for screening and evaluation of antimicrobial activity: a review of protocols, advantages, and limitations. Eur J Microbiol Immunol (Bp). 2024; 14(2): 97-126.
40. International Organization for Standardization. ISO 20776-1:2019 Clinical laboratory testing and *in vitro* diagnostic test systems — Susceptibility testing of infectious agents and evaluation of performance of antimicrobial susceptibility test devices — Part 1: Broth microdilution reference method for testing the *in vitro* activity of antimicrobial agents against rapidly growing aerobic bacteria involved in infectious diseases. Geneva: ISO; 2019.
41. Cohen J. Statistical power analysis for the behavioral sciences. 2nd ed. Hillsdale, NJ: Lawrence Erlbaum Associates; 1988.
42. Shah, D., Movaliya, V., Kharidia, S., & Zaveri, M. Comparative Study of Analytical Method Validation and Process Validation parameters as per ICH, EMA, WHO and ASEAN guidelines. International Journal of Drug Regulatory Affairs; 2024: 12(2), 58–64.
43. Gniazdowska, E., Gilant, E., & Bus-Kwasnik, K. ICH M10 guideline - a harmonized global approach to bioanalysis. Biuletyn Wydziału Farmaceutycznego Warszawskiego Uniwersytetu Medycznego;2023: 21(3), 57–63.
44. Eberle, M. K., Wasylenko, J. T., Kostelac, D., Kiehna, S. E., Schellinger, A. P., Zhang, Z., & Ehrick, J. D. A Modern Framework for Analytical Procedure Development and Lifecycle Management Based on ICH Q14 Principles. Analytical Chemistr; 2024: 97(1), 12–21.
45. United States Pharmacopeial Convention. General chapter <51> Antimicrobial effectiveness testing. In: United States Pharmacopeia and National Formulary USP 43-NF 38. Rockville, MD: United States Pharmacopeial Convention; 2020.
46. Gniazdowska, E., Gilant, E., & Bus-Kwasnik, K. (2023). ICH M10 guideline - a harmonized global approach to bioanalysis. Biuletyn Wydziału Farmaceutycznego Warszawskiego Uniwersytetu Medycznego; 2023:21(3), 57–63.
47. Dobroslavić E, Elez Garofulic I, Separović J, Zoric Z, Pedisic S, Dragovic-Uzelac V. Solvent-solute interactions in phytochemical extraction processes: theoretical principles and practical applications. Molecules. 2022; 27(16): 5099.
48. Shikov AN, Mikhailovskaya IY, Narkevich IA, Flisyuk EV, Pozharitskaya ON. Enhanced recovery of lipophilic compounds from medicinal plants using absolute ethanol extraction. In: Mukherjee PK, editor. Evidence-Based Validation of Herbal Medicine. 2nd ed. Amsterdam: Elsevier; 2022. p.771-796.
49. Sarakul O, Talabmuk C, Phosri S, Noysang C, Putthakarn N. Chemical analysis and bioactive compound profile of Tripitsajuk extract: eugenol as the predominant component. J Sci Technol Mahasarakham Univ. 2016; 35(5): 502-509.
50. Heinrich M, Appendino G, Efferth T, Furst R, Izzo AA, Kayser O, Lasak B, Mokry P, Moreno-Sanz G, Panfili G, Prieto JM, Tortoriello J, Wanner J, Williamson EM. Secondary metabolite profiles and therapeutic potential: diversity in traditional medicine formulations. J Ethnopharmacol. 2020; 246: 112230.
51. Konsue A, Taepongsorat L. Optimization of ethanol concentrations for diverse phytochemical extraction from Thai medicinal plants. J Ethnopharmacol. 2025; 330: 118562.
52. Olszewska MA, Gędas A, Simoes M. Synergistic effects in traditional multi-herb formulations: implications for bioactivity-guided extraction. LWT Food Sci Technol. 2020; 122: 109024.
53. Swamy MK, Akhtar MS, Sinniah UR. Compound selectivity versus extraction yield: fundamental principles in herbal medicine development. Molecules. 2022; 27(15): 4792.
54. Mayasari D, Islami D, Oktariani E, Wulandari P. Concentration-dependent antibacterial activity patterns of ethanol-based plant extractions against Gram-positive bacteria. Trop J Nat Prod Res. 2023; 7(1): 2157-2162.
55. Zhao A, Sun J, Liu Y. Bioactive compounds in traditional medicinal plants: eugenol, trans-cinnamaldehyde, citronellol, and terpineol effects against *Escherichia coli* biofilm formation. Front Cell Infect Microbiol. 2023; 13: 1137947.

56. ALrashidi A, Noumi E, Snoussi M, De Feo V. Synergistic antimicrobial effects of plant-derived compounds: eugenol, cinnamaldehyde, carvacrol, and thymol combinations against *Escherichia coli*. Plants (Basel). 2022; 11(4): 540.
57. Adianingsih OR, Ihsan BRP, Puspita OE, Maesayani KS. Chemical composition and eugenol content analysis in *Syzygium aromaticum*: HPLC validation and bioactivity correlation. Trop J Nat Prod Res. 2023; 7(8): 3395-3399.
58. Varela MF, Stephen JW, Bharti D, Lekshmi M, Kumar S. Multiple antimicrobial mechanisms of eugenol: bacterial cell membrane disruption, enzymatic interference, and protein synthesis inhibition. Biomedicines. 2023; 11(5): 1448.
59. Lorusso A, Carrara JA, Barroso CDN, Tuon FF, Faoro H. Quorum sensing pathway disruption by eugenol: suppression of virulence gene expression in *Escherichia coli*. Int J Mol Sci. 2022; 23(24): 15779.
60. Gultom E, Harahap U, Suryanto D, Sipahutar H, Restuati M. Antibacterial Activity of structurally diverse bioactive molecules including amino acids and organic compounds against Gram-negative pathogens including extended-spectrum beta-lactamase-producing *Klebsiella pneumoniae*. Trop J Nat Prod Res. 2024; 8(1): 37-42.
61. Langendonk RF, Neill DR, Fothergill JL. Mechanistic basis of eugenol's antibacterial activity: specific targeting of Gram-negative bacterial cytoplasmic membranes and membrane depolarization. Front Cell Infect Microbiol. 2021; 11: 665759.
62. Johnson MD, Reller LB, Petti CA, Jones RN. Eugenol disruption of *Escherichia coli* biofilm formation: implications for bacterial persistence and antibiotic resistance development. Diagn Microbiol Infect Dis. 2019; 95(3): 114865.
63. Amedu AB, Uba A, Chika M, Umar WRS. Enhanced antibacterial activity of green-synthesized nanoparticles derived from plant extracts: multi-target therapeutic strategies against Gram-positive and Gram-negative bacteria. Trop J Nat Prod Res. 2024; 8(5): 7285-7289.
64. Ouryemchi I, Oubihi A, Taibi M, Elbouzidi A, Jaber H, Haida S, Tarfaoui K, Atfaoui K, Chaabane K, El Guerrouj B, Bellaouchi R, Asehraou A, Addi M, Benzakour A, Ouhssine M. Minimum inhibitory concentrations of plant-derived extracts against *Pseudomonas aeruginosa*: elevated requirements and extended contact time for microbial reduction. Trop J Nat Prod Res. 2024; 8(3): 6663-6668.
65. Medeiros Filho F, Barbosa do Nascimento AP, Costa MOC, Merigueti TC, de Menezes MA, Nicolás MF, dos Santos M, Carvalho-Assef APD, da Silva FAB. Multiple therapeutic targets in *Pseudomonas aeruginosa* identified through integrated computational models: complex resistance mechanisms analysis. Front Mol Biosci. 2021; 8: 728129.
66. Qin S, Xiao WH, Zhou C, Pu Q, Deng X, Lan L, Liang H, Wu M. *Pseudomonas aeruginosa*: pathogenesis, virulence factors, antibiotic resistance mechanisms, sophisticated defense systems including efflux pumps, biofilm formation, and adaptive resistance. Signal Transduct Target Ther. 2022; 7(1): 239.
67. Al-Tamimi MA, Alshawwa SZ, Gomha SM, Abdelaziz MR. Plant extracts with elevated minimum inhibitory concentrations: *Peganum harmala* antimicrobial activity requiring significant contact time for *Pseudomonas aeruginosa* reduction. Molecules. 2020; 25(18): 4279.
68. Silva LN, Zimmer KR, Macedo AJ, Trentin DS. Plant natural products targeting bacterial virulence factors: outer membrane permeability differences between Escherichia coli and *Pseudomonas aeruginosa* affecting antimicrobial penetration. Curr Med Chem. 2016; 23(14): 1389-1409.
69. Cigana C, Melotti P, Baldan R, De Simone M, Einarsson GG, Shaw D, Bevivino A, Morales S, Schwartz S, Sipione B, Turano C, Gaiarsa S, Sassera D, Bevec T, Twomey KB, O'Gara F, Ratjen F, Bragonzi A. *Pseudomonas aeruginosa* outer membrane lipopolysaccharides with longer O-antigen chains and lipid A modifications: enhanced barrier against hydrophobic compounds. J Cyst Fibros. 2017; 16(4): 469-477.
70. AB, Carrara JA, Barroso CDN, Tuon FF, Faoro H. Role of Efflux Pumps on Antimicrobial Resistance in *Pseudomonas aeruginosa*. Int J Mol Sci. 2022;23(24):15779.
71. Laborda P, Lolle S, Hernando-Amado S, Alcalde-Rico M, Aanæs K, Martínez JL, Molin S, Johansen HK. Mutations in the efflux pump regulator MexZ shift tissue colonization by *Pseudomonas aeruginosa* to a state of antibiotic tolerance. Nat Commun. 2024;15(1):2584.
72. World Health Organization. Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics. Geneva: World Health Organization; 2017.
73. Tadesse BT, Ashley EA, Ongarello S, Havumaki J, Wijegoonewardena M, Gonzalez IJ, Dittrich S. Antimicrobial resistance in Africa: a systematic review and meta-analysis of zone-of-inhibition studies demonstrating variation between Gram-negative and Gram-positive bacterial strains with enhanced activity against Gram-positive organisms. PLoS One. 2017; 12(12): e0189749.