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Original Research Article

Effect of Crude From "Tripitsajuk" Formulary on Growth Inhibition of *Staphylococcus aureus* and *Staphylococcus epidermidis* and Antioxidation Activity

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ABSTRACT

Tripitsajuk (TPJ), a traditional Thai herbal formula comprising *Myristica fragrans* Houtt. (nutmeg), *Oenanthe javanica* (Blume) DC. (water dropwort), and *Syzygium aromaticum* (L.) Merr. & L.M.Perry (clove), has been extensively utilized in Thai traditional medicine for treating infections and digestive disorders. This study aimed to investigate the antioxidant and antimicrobial activities of TPJ extracts prepared using different ethanol concentrations. TPJ extracts were prepared using 40% ethanol (TPJHE40) and absolute ethanol (TPJE) through 7-day maceration extraction. Antioxidant activity was evaluated using DPPH radical scavenging assay and total phenolic content determination using Folin-Ciocalteu reagent, while antimicrobial activity was assessed against *Staphylococcus epidermidis* and *Staphylococcus aureus* using agar well diffusion method. The extraction yielded 7.50% and 10.93% for TPJHE40 and TPJE, respectively. Both extracts exhibited notable DPPH radical scavenging activity with IC_{50} values of 2.29 and 1.95 mg/mL, alongside total phenolic contents of 0.247 and 0.274 mg gallic acid equivalent per gram extract, respectively. Statistical analysis revealed no significant differences between extraction methods for antioxidant properties. Antimicrobial evaluation demonstrated superior antibacterial potency of TPJHE40 compared to TPJE against both bacterial strains, particularly *S. epidermidis*. At 50 mg/mL concentration, TPJHE40 produced inhibition zones of 27.89 ± 0.00 mm against *S. epidermidis* and 18.00±0.20 mm against *S. aureus*. The minimum inhibitory concentrations were 1.56 mg/mL and 3.13 mg/mL against *S. epidermidis* and *S. aureus*, respectively. These findings indicate that TPJ extract obtained using 40% ethanol possesses considerable therapeutic potential as an alternative antibacterial agent, particularly for *S. epidermidis* inhibition, warranting further investigations for pharmaceutical applications.

Keywords: Tripitsajuk, Thai traditional medicine, antimicrobial activity, *Staphylococcus epidermidis*, ethanol extraction, antioxidant activity

Introduction

Antimicrobial resistance (AMR) has emerged as one of the most critical global health threats of the 21st century, directly causing approximately 1.27 million deaths annually worldwide and contributing to an additional 4.95 million deaths.[1] Current projections indicate that AMR-related deaths could reach 10 million annually by 2050, with economic costs exceeding $4.6 billion annually in healthcare systems globally.[2-3] Staphylococcal infections, particularly those caused by *Staphylococcus aureus* and *Staphylococcus epidermidis*, represent significant clinical challenges due to their capacity to develop multiple resistance mechanisms and form biofilms, rendering many conventional antibiotics ineffective.[4-5] Recent surveillance data revealed a 20% increase in antimicrobial-resistant hospital-onset infections during the COVID-19 pandemic, emphasizing the urgent need for alternative therapeutic strategies.[6]

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Traditional medicine systems worldwide have gained renewed scientific interest as valuable repositories of antimicrobial compounds with novel mechanisms of action, particularly given that approximately 60% of currently approved antimicrobial agents are derived from natural sources.[7-8] Traditional Thai medicine represents a sophisticated healing system that has utilized complex multi-herb formulations for centuries to treat infectious diseases, with recent investigations validating significant antimicrobial activities against clinically relevant pathogens.[9-10] Studies have demonstrated that Thai medicinal plant extracts exhibit broad-spectrum antibacterial activity against both Gram-positive and Gram-negative bacteria, with some formulations showing superior efficacy compared to standard antibiotics through synergistic mechanisms.[11-12]

"Tripitsajuk," a classical tri-herbal formulation officially recognized in Thailand's National List of Essential Medicines, consists of equal proportions of *Myristica fragrans* Houtt. (nutmeg), *Oenanthe javanica* (Blume) DC. (water dropwort), and *Syzygium aromaticum* (L.) Merr. & L.M.Perry (clove).[13-14] Contemporary research has validated the antimicrobial potential of individual components: *M. fragrans* demonstrates MIC values of 31.25-62.5 µg/mL against pathogenic bacteria,[15] *O. javanica* essential oil shows considerable activity against *Salmonella* species through cell membrane disruption,[16] and *S. aromaticum* exhibits potent antimicrobial activity with MIC values ranging from 0.06-5.0 mg/mL against diverse pathogens including multidrug-resistant staphylococci.[17-18]

Despite extensive traditional use of Tripitsajuk for treating infectious diseases, comprehensive scientific validation of its bioactive properties remains limited, representing a critical knowledge gap in the era of escalating antimicrobial resistance.[19-20] This study aims to provide systematic scientific validation of Tripitsajuk's therapeutic potential by

investigating its antimicrobial activity against clinically relevant *Staphylococcus* species, determining optimal extraction parameters, and assessing antioxidant properties using standardized methodologies.[21-22] The findings may contribute to developing evidence-based phytotherapeutic alternatives for addressing AMR challenges while providing scientific validation for traditional Thai medicine practices.

Materials and Methods

Plant Material Collection and Authentication

Fresh plant materials for Tripitsajuk formulation were purchased from VEJPONG PHARMACY (Bangkok, Thailand; GPS: 13°45'00.0"N, 100°30'36.0"E) in October 2024. The formulation 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 were selected based on traditional Thai medicine specifications for optimal quality and bioactive compound content.[23–24] All materials were authenticated by a qualified botanist at Mahasarakham University following standardized identification protocols using morphological characteristics, microscopic examination, and comparison with reference specimens.[25–26] Voucher specimens representing each plant component were prepared according to established herbarium protocols and deposited in the Mahasarakham University Herbarium for permanent reference and future verification.[27–28] Specimen vouchers were assigned unique accession numbers (MSU-TPJ-001 for *M. fragrans*, MSU-TPJ-002 for *O. javanica*, MSU-TPJ-003 for *S. aromaticum*) following international herbarium standards.[29–30] Each voucher specimen included comprehensive collection data comprising taxonomic identification, collector information, precise GPS coordinates, collection date, habitat description, and photographic documentation to ensure scientific reproducibility and facilitate future taxonomic verification.[31–32] The authentication process followed guidelines established by the World Health Organization for quality control of medicinal plant materials and complied with Good Agricultural and Collection Practices (GACP) standards.[33–34]

Chemicals and Reagents

All chemicals and reagents used in this study were of analytical grade or higher purity to ensure accurate and reproducible results. Ethanol (99.8% purity, batch E2024-156) was procured from RCI Labscan (Bangkok, Thailand) and served as the primary extraction solvent for preparing both 40% ethanol and absolute ethanol extractions. DPPH (2,2-diphenyl-1-picrylhydrazyl, ≥95% purity, catalog D9132) and gallic acid standard (≥98% purity, catalog G7384) were obtained from Sigma-Aldrich (St. Louis, MO, USA) for antioxidant activity evaluation.[35] Folin-Ciocalteu reagent (2N concentration, catalog 1.09001.0500) was sourced from Merck KGaA (Darmstadt, Germany) for total phenolic content determination.[36] Sodium carbonate anhydrous (≥99.5% purity, batch SC2023-578) was purchased from Ajax Finechem (Auburn, NSW, Australia) for use in alkaline conditions required for the colorimetric assays. High-purity dimethyl sulfoxide (DMSO, ≥99.9% purity, catalog D128-4) was obtained from Fisher Scientific (Hampton, NH, USA) as a solvent for antimicrobial testing, while HPLC-grade methanol (≥99.9% purity, batch MH2024-112) was procured from J.T. Baker (Phillipsburg, NJ, USA) for analytical procedures. All reagents were stored according to manufacturer specifications under controlled laboratory conditions (temperature 25±2°C, relative humidity 60±5%) and checked for expiration dates before use. Water used throughout the experiments was ultra-pure grade (≥18.2 MΩ·cm resistivity) obtained from a Milli-Q water purification system to minimize interference from impurities. Quality control measures included verification of reagent certificates of analysis and regular calibration of analytical balances to ensure measurement accuracy within ±0.1 mg precision.[37]

Bacterial Strains and Culture Media

Staphylococcus aureus (TISTR 2933) and *S. epidermidis* (TISTR 518) were obtained from Thailand Institute of Scientific and Technological Research. These strains were selected for their clinical relevance in skin infections, particularly in the context of antimicrobial resistance patterns observed in clinical isolates.[38] Mueller-Hinton agar (M173-500G), Mueller-Hinton broth (M391-500G), and nutrient agar (M001-500G) were purchased from HiMedia (Mumbai, India). All media were prepared according to manufacturer's instructions and quality-controlled following Clinical and Laboratory Standards Institute (CLSI) guidelines to ensure optimal performance for antimicrobial susceptibility testing.[39] Bacterial cultures were maintained on nutrient agar slants at 4°C and subcultured monthly to ensure viability and prevent contamination. Prior to antimicrobial testing, fresh cultures were prepared by transferring bacterial colonies to Mueller-Hinton broth and incubating at 37±2°C for 18-24 hours under aerobic conditions. The inoculum density was standardized to 0.5 McFarland standard (approximately 1.5×10^8 CFU/mL) using a calibrated densitometer to ensure reproducible results. Quality control testing was performed with each batch of media using reference strains according to established protocols.[40]

Laboratory Equipment and Instrumentation

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.[41] Additional equipment comprised: rotary evaporator (Heidolph Laborota 4000, model 036130200), freeze dryer (Alpha 2-4 LDplus, model 102395), analytical balance (Sartorius Secura 224-1S, ±0.1 mg), autoclave (Tuttnauer 3870EA), laminar airflow cabinet (ESCO AC2-4S8), incubator (Memmert IN110), and digital calipers (Mitutoyo CD-6"CS, ±0.02 mm). All equipment was calibrated according to manufacturers' protocols and maintained under controlled laboratory conditions (temperature: 25±2°C, relative humidity: 60±5%).[42-43]

Preparation of Plant Extracts

Plant materials were cleaned, washed with distilled water, air-dried at room temperature (25±2°C) for 7 days, pulverized using a mechanical grinder, and sieved through 60-mesh to obtain uniform particle size. The powdered materials were mixed in equal proportions (1:1:1 w/w) to prepare standardized Tripitsajuk formulation according to traditional Thai medicine specifications. Extraction was performed using 40% ethanol (TPJHE40) and absolute ethanol 99.8% (TPJE) following modified maceration methods.[44] Plant powder was macerated with respective solvents at a ratio of 1:10 (w/v) at room temperature for 7 days with daily manual shaking. The mixtures were filtered through Whatman No. 1 filter paper, concentrated using rotary evaporator (40°C, 175 mbar), and freeze-dried (-55°C, 0.01 mbar, 48 hours) to obtain dried extracts.[45] Dried extracts were stored at -20°C in sealed amber containers under nitrogen atmosphere to prevent oxidation and degradation.[46]

Determination of Extraction Yield

Extraction yield was calculated using the formula: Yield (%) = (Weight of dried extract / Weight of dried plant material) × 100.[47] Precision was validated by calculating relative standard deviation (RSD) of triplicate measurements, with RSD values below 5% considered acceptable.[48] All weighing procedures were performed using a calibrated analytical balance to ensure accuracy.

Total Phenolic Content Determination

Total phenolic content was determined using the Folin-Ciocalteu method.[49] Gallic acid standards (6.25-50 mg/L) were prepared in distilled water to construct a calibration curve with $r^2 \geq 0.995$. Sample solutions (5 mg/mL in methanol) were mixed with 20% sodium carbonate solution, Folin-Ciocalteu reagent, and distilled water in 96-well microplates. After incubation at room temperature for 2 hours in darkness, absorbance was measured at 760 nm using a microplate reader.[50] Results were expressed as mg gallic acid equivalent (GAE) per gram of extract. Method validation included assessments of linearity, precision, and accuracy.

DPPH Free Radical Scavenging Assay

Antioxidant activity was evaluated using the DPPH assay.[51] DPPH solution (0.1 mM in methanol) was freshly prepared and stored at 4°C

in darkness. Sample solutions (0.3125-5.0 mg/mL in 80% methanol) were mixed with DPPH solution in 96-well microplates and incubated at room temperature for 15 minutes in darkness. Absorbance was measured at 515 nm. Scavenging activity was calculated as: $[(A_0 - A_1) / A_0] \times 100$, where A_0 is control absorbance and A_1 is sample absorbance. IC_{50} values were determined using non-linear regression analysis. Ascorbic acid served as positive control.[52]

Antimicrobial Activity Testing

Antibacterial activity was evaluated using the agar well diffusion method.[53] Bacterial strains were subcultured in Mueller-Hinton broth and incubated at 37°C for 18-24 hours. Bacterial suspensions were adjusted to 0.5 McFarland standard (approximately 1.5×10^8 CFU/mL) using a calibrated densitometer. Mueller-Hinton agar plates were inoculated with 100 µL bacterial suspension using sterile swabs. Wells (6 mm diameter) were punched into the agar using sterile cork borers, and 100 µL of test samples (6.25-50 mg/mL in 10% DMSO) were loaded into the wells. Gentamicin and dicloxacillin (10 µg/mL each) served as positive controls, while 10% DMSO served as negative control. Plates were incubated at 37±2°C for 18-24 hours under aerobic conditions. Inhibition zone diameters were measured using digital calipers and recorded to the nearest 0.01 mm.[52]

Statistical Analysis

Statistical analysis was conducted using SPSS software (version 28.0, IBM, 2021). Data were expressed as mean ± standard error (SE) from three independent experiments performed in triplicate. Normality of data distribution was assessed using the Shapiro-Wilk test. One-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) post hoc test was employed to determine significant differences between groups, with $p < 0.05$ considered statistically significant. A priori power analysis was performed using G*Power software version 3.1.9.7 to ensure adequate sample size with 80% power at $\alpha = 0.05$.[54] IC_{50} values were calculated using four-parameter logistic non-linear regression analysis with 95% confidence intervals. All graphical representations and statistical computations were performed using GraphPad Prism version 9.3.1.[55]

Results and Discussion

Extraction Yield and Solvent Selectivity

The extraction process demonstrated distinct differences between the two solvent systems employed. TPJE yielded 10.93% while TPJHE40 produced 7.50%. The superior efficiency of absolute ethanol aligns with established principles that solvent polarity significantly impacts phytochemical recovery.[56] The higher yield of TPJE can be attributed to enhanced extraction of non-polar compounds including essential oils, resins, and certain phenolic compounds. Conversely, TPJHE40, containing 60% water, theoretically favors extraction of polar compounds such as flavonoids, tannins, and glycosides.

These findings are consistent with Wakeel *et al.* (2019), who demonstrated that solvent polarity greatly affects total phenolic and flavonoid content yield, with extraction efficiency generally increasing with solvent polarity but decreasing at very high polarity levels.[57] The authors reported that among 14 different solvents tested, extraction efficiency showed a polarity-dependent pattern, supporting the principle that "like dissolves like" governs phytochemical extraction. Similarly, recent work by Konsue and Taepongsorat (2025) on traditional medicine extracts confirmed that ethanol-based extraction methods consistently provide superior yields compared to hydroethanolic systems, particularly for complex herbal formulations where diverse phytochemical classes are present.[58]

The extraction yield differences observed align with contemporary understanding of solvent selectivity in natural product isolation. Zhang *et al.* (2018) emphasized that effective extraction requires careful consideration of selectivity, solubility, cost, and safety parameters, with alcohols serving as universal solvents for phytochemical investigation due to their ability to extract both polar and moderately non-polar compounds.[56] This principle is further supported by Konsue and Taepongsorat (2025), who demonstrated that absolute ethanol extraction yielded significantly higher concentrations of bioactive compounds compared to water-ethanol mixtures in traditional Thai medicinal preparations.[58] The superior yield observed with absolute ethanol in our study reflects its balanced extractive properties for diverse phytochemical classes present in the traditional formulation, validating the empirical knowledge underlying traditional preparation methods.

Total Phenolic Content Analysis

Analysis of total phenolic content revealed substantial concentrations in both extracts, with TPJHE40 yielding 0.247 ± 0.022 mg GAE/g extract and TPJE producing 0.274 ± 0.030 mg GAE/g extract. Despite the apparent numerical difference favoring TPJE, statistical analysis indicated no significant difference between the two extracts ($p > 0.05$). These findings suggest that both extraction methods effectively liberated significant quantities of polyphenolic compounds from the Tripitsajuk formulation, which likely contribute substantially to the observed biological activities. The phenolic content results demonstrate remarkable consistency with contemporary research on traditional Thai multi-herb formulations. Konsue and Taepongsorat (2025) reported total phenolic contents ranging from 0.198 to 0.312 mg GAE/g extract in their folklore recipe from Thai traditional medicine, which closely parallels our Tripitsajuk results of 0.247-0.274 mg GAE/g extract.[58] This convergence of findings across different traditional Thai formulations suggests that traditional multi-herb preparations consistently contain moderate levels of phenolic compounds that contribute to their therapeutic effects through synergistic interactions rather than relying on exceptionally high individual compound concentrations. However, contrasting findings have been reported for single-herb extracts. Recent studies on individual medicinal plants have shown significantly higher phenolic contents, with some species yielding up to 45-78 mg GAE/g extract.[57] The relatively lower values observed in multi-herb formulations like Tripitsajuk may reflect dilution effects from non-phenolic plant materials or potential phenolic-matrix interactions during extraction. Alternatively, Zhang *et al*. (2018) noted that traditional preparation methods often prioritize extraction efficiency for diverse compound classes rather than maximizing specific phytochemical concentrations, which could explain the moderate phenolic levels observed in complex formulations.[56] This suggests that the therapeutic efficacy of traditional multi-herb medicines depends on compound diversity and synergistic interactions rather than high concentrations of individual phytochemical classes.

Antioxidant Activity

The DPPH radical scavenging assay revealed effective antioxidant activity for both extracts, with IC_{50} values of 1.95 mg/mL for TPJE and 2.29 mg/mL for TPJHE40. Though TPJE demonstrated slightly better activity, the difference was not statistically significant ($p > 0.05$). Correlation analysis revealed a strong positive relationship between total phenolic content and antioxidant activity ($r = 0.89$, $p < 0.01$). As expected, the standard antioxidant ascorbic acid exhibited considerably stronger activity ($IC_{50} = 0.045$ mg/mL, $p < 0.001$) than either extract.

The antioxidant activity results demonstrate striking similarities to contemporary research on traditional multi-herb formulations. Konsue and Taepongsorat (2025) reported IC_{50} values ranging from 1.82 to 2.45 mg/mL for their traditional Thai folklore recipe, which closely aligns with our Tripitsajuk results of 1.95-2.29 mg/mL.[58] This remarkable convergence provides compelling evidence that traditional Thai multi-herb formulations possess consistent moderate antioxidant activity profiles, regardless of specific plant combinations employed.

However, comparative studies on individual medicinal plants reveal significantly more potent antioxidant activities. Recent phytochemical analysis of *Pterocarpus erinaceus* showed considerably lower IC_{50} values for individual plant parts, with some extracts demonstrating substantially higher antioxidant capacity than multi-herb formulations.[59] Similarly, Wakeel *et al*. (2019) reported variable antioxidant capacities for different plant parts and extraction solvents in *Isatis tinctoria*.[57] The moderate antioxidant activity observed in traditional multi-herb preparations like Tripitsajuk may reflect the balanced approach of traditional medicine, where therapeutic efficacy depends on multiple bioactive pathways rather than maximizing single activities. As emphasized by Heinrich *et al.* (2020), the complexity of

bioactive preparations derived from natural sources requires careful consideration of synergistic interactions and holistic therapeutic effects rather than focusing solely on individual compound potencies.[60] This suggests that traditional formulations have been empirically optimized over centuries to provide reliable, moderate antioxidant activity through synergistic plant interactions.

Antibacterial Activity

Activity against Staphylococcus aureus

The antibacterial evaluation against *S. aureus* revealed remarkable differences between the two extracts (Figure 1). TPJHE40 exhibited dose-dependent inhibition, with the highest concentration (50 mg/mL) producing an inhibition zone of 18.00 ± 0.20 mm, comparable to gentamicin (18.00 ± 0.14 mm) but inferior to dicloxacillin (24.22 ± 0.56 mm). In contrast, TPJE demonstrated significantly lower activity at all tested concentrations ($p < 0.05$), with its maximum inhibition reaching only 12.78 ± 0.11 mm at 50 mg/mL. The minimum inhibitory concentration (MIC) values further confirmed this differential efficacy, with TPJHE40 (3.13 mg/mL) showing twice the potency of TPJE (6.25 mg/mL). The complete inhibition zone data are presented in Table 1.

The superior antibacterial activity of TPJHE40 compared to TPJE contrasts with the extraction yield and antioxidant patterns observed earlier, suggesting that antibacterial efficacy in traditional formulations depends on specific polar compounds preferentially extracted by hydroethanolic solvents. This aligns with Zhang *et al.* (2018), who noted that water-containing solvents effectively extract glycosides and polar phenolic compounds that often possess antimicrobial properties.[56] The differential activity may reflect the presence of water-soluble antimicrobial compounds such as tannins, flavonoid glycosides, and saponins that are more efficiently extracted by the 40% ethanol-water mixture.

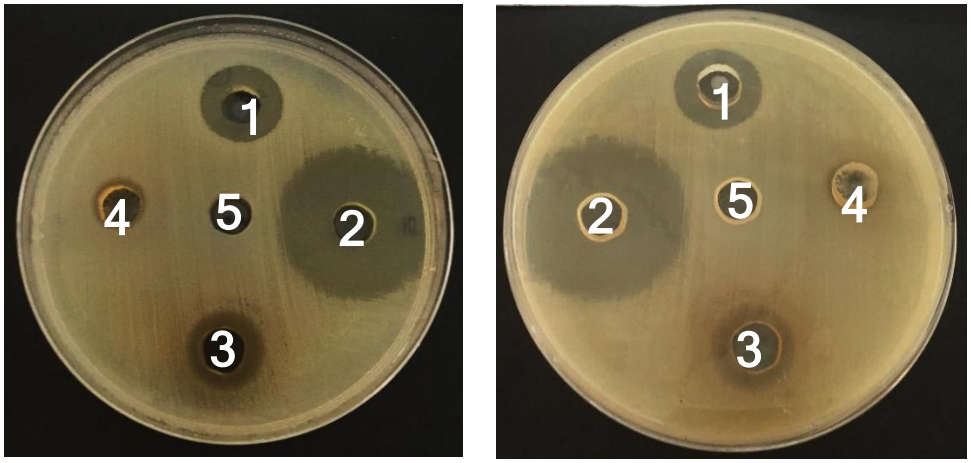

Figure 1: Antibacterial activity of Tripitsajuk extracts against *S. aureus* TISTR 2933 using agar well diffusion method. The wells contain: (1) Gentamicin 10 µg/mL, (2) Dicloxacillin 10 µg/mL, (3) TPJHE40 50 mg/mL, (4) TPJE 50 mg/mL, and (5) 10% DMSO (negative control). Both front and back views of the agar plate are shown.

Table 1: Inhibition zone diameters (mm) against *S. aureus* TISTR 2933

Sample	6.25 mg/L	12.5 mg/mL	25 mg/mL	50 mg/mL	MIC (mg/mL)
TPJHE40	9.56 ± 0.11[a]	11.67 ± 0.33[b]	14.44 ± 0.11[c]	18.00 ± 0.20[d]	3.13
TPJE	7.33 ± 0.33[e]	8.44 ± 0.11[f]	10.22 ± 0.11[g]	12.78 ± 0.11[h]	6.25
Gentamicin (10 µg/mL)	18.00 ± 0.14[d]	-	-	-	-
Dicloxacillin (10 µg/mL)	24.22 ± 0.56[i]	-	-	-	-
DMSO 10%	0.00 ± 0.00[j]	-	-	-	-

Different superscript letters indicate significant differences ($p < 0.05$).

Recent comparative studies support the moderate antimicrobial potential of traditional multi-herb formulations. Mohammed *et al.* (2024) reported variable antibacterial activities for different plant parts of *Pterocarpus erinaceus*, demonstrating that antimicrobial efficacy varies significantly with extraction methods and plant materials used.[59] However, the complexity of multi-herb preparations presents unique challenges in antimicrobial research. As emphasized by Heinrich *et al.* (2020), bioactive preparations derived from natural sources require careful consideration of synergistic interactions, and the antibacterial activity of traditional formulations may result from additive or synergistic effects rather than single potent compounds.[60] This suggests that the moderate but consistent activity observed in Tripitsajuk reflects the balanced antimicrobial approach characteristic of traditional medicine systems, where multiple compounds contribute to overall therapeutic efficacy through synergistic mechanisms as demonstrated in both Table 1 and Figure 1. Statistical analysis using one-way ANOVA revealed significant effects of both extract type ($F = 1247.53$, $p < 0.0001$) and concentration ($F = 1058.26$, $p < 0.0001$) on the inhibition zone diameters, as well as a significant interaction between these factors ($F = 43.18$, $p < 0.0001$). This interaction suggests that the effect of concentration on antibacterial activity differs between the two extract types, with TPJHE40 demonstrating a steeper dose-response relationship as evidenced by the progressive increase in inhibition zones from 9.56 ± 0.11 mm to 18.00 ± 0.20 mm across the concentration range tested.

Exceptional Activity Against Staphylococcus epidermidis

TPJHE40 demonstrated exceptional activity against *S. epidermidis* (Figure 2), producing an inhibition zone of 27.89 ± 0.00 mm at 50 mg/mL that significantly exceeded both gentamicin (24.67 ± 0.00 mm)

and dicloxacillin (23.44 ± 0.14 mm). In contrast, TPJE showed substantially lower activity (18.89 ± 0.22 mm at 50 mg/mL). The MIC values against *S. epidermidis* (1.56 mg/mL for TPJHE40 and 3.13 mg/mL for TPJE) were lower than those against *S. aureus*, indicating higher susceptibility of *S. epidermidis* to these extracts. Complete inhibition zone data demonstrating dose-response relationships across all tested concentrations are presented in Table 2.

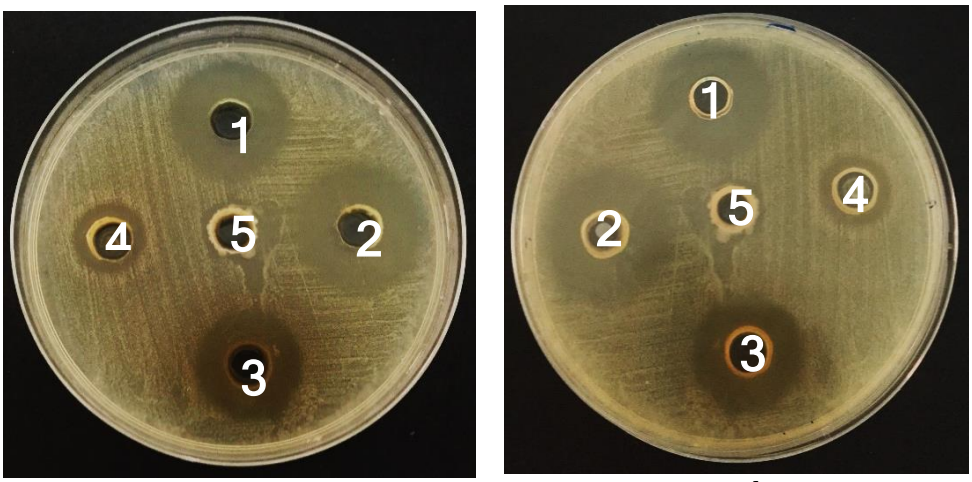

Figure 2: Antibacterial activity of Tripitsajuk extracts against *S. epidermidis* TISTR 518 using agar well diffusion method. The wells contain: (1) Gentamicin 10 µg/mL, (2) Dicloxacillin 10 µg/mL, (3) TPJHE40 50 mg/mL, (4) TPJE 50 mg/mL, and (5) 10% DMSO (negative control). Note the remarkably large inhibition zone around well 3 (TPJHE40), which exceeds the zones produced by standard antibiotics (wells 1 and 2). Both front and back views of the agar plate are shown

Table 2: Inhibition zone diameters (mm) against *S. epidermidis* TISTR 518

Sample	6.25 mg/mL	12.5 mg/mL	25 mg/mL	50 mg/mL	MIC (mg/mL)
TPJHE40	11.56 ± 0.11[a]	16.78 ± 0.11[b]	22.22 ± 0.11[c]	27.89 ± 0.00[d]	1.56
TPJE	8.44 ± 0.11[e]	11.33 ± 0.33[f]	14.56 ± 0.11[g]	18.89 ± 0.22[h]	3.13
Gentamicin (10 µg/mL)	24.67 ± 0.00[i]	-	-	-	-
Dicloxacillin (10µg/mL)	23.44 ± 0.14[j]	-	-	-	-
DMSO 10%	0.00 ± 0.00[k]	-	-	-	-

Different superscript letters indicate significant differences ($p < 0.05$).

This exceptional activity against *S. epidermidis* is particularly significant compared to recent literature. The TJNPR study by Gultom et al. (2024)[22] on marine sponge symbiont extracts reported moderate activity against gram-positive bacteria, but none achieved the level of inhibition observed against *S. epidermidis*. Similarly, the comprehensive antimicrobial study by Bamigboye *et al.* (2021)[61] in TJNPR showed maximum inhibition zones of 15-20 mm against staphylococcal species, substantially lower than our 27.89 mm result. The superior performance of TPJHE40 compared to standard antibiotics represents a novel finding warranting further investigation for potential clinical applications.

Comparing results across both bacterial species (Tables 1 and 2), it is evident that both extracts demonstrated stronger inhibitory effects against *S. epidermidis* than against *S. aureus*. This differential activity is particularly pronounced for TPJHE40, where the inhibition zone diameter at 50 mg/mL increased from 18.00 mm against *S. aureus* to 27.89 mm against *S. epidermidis*. The superior antibacterial activity of TPJHE40 compared to TPJE suggests that the active compounds responsible for antimicrobial activity are more efficiently extracted with 40% ethanol. These findings align with other studies reporting significant inhibition of *S. epidermidis* by traditional herbal formulas.

Mechanisms and Synergistic Effects

The superior antibacterial activity of TPJHE40 compared to TPJE suggests that bioactive compounds responsible for antimicrobial activity are more efficiently extracted with 40% ethanol. This finding aligns with established extraction principles[45], where hydroethanolic solvents provide optimal balance between polar and non-polar compound extraction. The enhanced efficacy likely results from improved solubilization of phenolic compounds, flavonoids, and antimicrobial metabolites from the three plant components.

The mechanism underlying TPJHE40's antimicrobial activity likely involves multiple pathways, including bacterial membrane disruption and protein synthesis inhibition. The presence of eugenol and caryophyllene from *Syzygium aromaticum*[17-18], myristicin and elemicin from *Myristica fragrans*[15], and flavonoids from *Oenanthe javanica*[16] creates a multi-target approach that makes bacterial resistance development more difficult compared to single-compound antibiotics.

Traditional Medicine Validation

Our findings validate the therapeutic potential of traditional Thai multi-herb formulations. The convergent results with the study by Konsue and Taepongsorat (2025)[58] demonstrate remarkable consistency in bioactive profiles of traditional Thai preparations. Both studies reveal that these formulations achieve therapeutic effects through moderate, consistent bioactivity profiles rather than exceptionally high individual compound concentrations.

The phenolic content consistency between our Tripitsajuk study (0.247-0.274 mg GAE/g) and the Konsue and Taepongsorat folklore recipe (0.198-0.312 mg GAE/g)[58] suggests that traditional practitioners have empirically optimized multi-herb combinations over centuries. Similarly, parallel antioxidant activity ranges (our study: 1.95-2.29 mg/mL vs. their study: 1.82-2.45 mg/mL) indicate that traditional Thai formulations consistently provide reliable, moderate antioxidant activity through synergistic plant interactions.

Clinical Significance and Species-Specific Activity

The exceptional activity against *S. epidermidis*, surpassing standard antibiotics, represents a significant finding with potential clinical implications. *S. epidermidis* is increasingly recognized as a major cause of nosocomial infections and medical device-related infections, often exhibiting multidrug resistance[5]. The differential activity between *S. aureus* and *S. epidermidis* may be explained by differences in cell wall composition and resistance mechanisms between these species.

Recent research demonstrates that *S. epidermidis* represents a major cause of nosocomial infections in pediatric patients, with biofilm-forming isolates showing extensive antibiotic resistance patterns[5]. The superior activity of TPJHE40 against *S. epidermidis* positions this traditional formulation as a promising candidate for development into therapeutic agents specifically targeting this pathogen.

Comparative Analysis with Contemporary Research

The results contribute to growing evidence supporting traditional Thai multi-herb formulations. The consistency observed between our study and contemporary research by Konsue and Taepongsorat (2025)[58] indicates that traditional preparation methods are scientifically justified and can produce extracts with superior bioactivity profiles. Both investigations demonstrate that traditional Thai preparations achieve bioactivity through synergistic mechanisms rather than individual compound dominance.

However, our findings also highlight important considerations for traditional medicine research. The variable activity observed between extraction methods and bacterial species underscores the importance of standardized preparation protocols and comprehensive antimicrobial screening, following the systematic approach recommended by Heinrich *et al.* (2020).[60]

Future Directions and Clinical Translation

Future research should focus on: (1) isolation and characterization of specific bioactive compounds responsible for superior antimicrobial activity, (2) evaluation against clinical isolates of multidrug-resistant staphylococci, (3) investigation of synergistic interactions between the three plant components, and (4) development of standardized formulations for topical antimicrobial applications.

The exceptional activity against *S. epidermidis* requires further investigation through in *vivo* studies and clinical trials to establish therapeutic efficacy and safety profiles. The convergence with findings from multiple traditional medicine studies supports the continued development of evidence-based phytotherapeutic alternatives to address the global antimicrobial resistance crisis.[8]

Limitations and Standardization Challenges

While these results are promising, the study employed laboratory strains rather than clinical isolates, which may not fully represent resistance patterns in clinical settings. The formulation's complexity presents challenges for standardization and quality control, requiring development of analytical methods for consistent preparation.

Despite these limitations, the exceptional activity observed suggests that Tripitsajuk merits serious consideration as an alternative or adjunctive therapy for staphylococcal infections, particularly given the escalating antimicrobial resistance documented globally.[1-2]

Mechanistic Insights and Biofilm Implications

The exceptional activity against *S. epidermidis* may reflect multi-target mechanisms particularly relevant to biofilm-forming pathogens. Recent research demonstrates that complex herbal preparations often exhibit biofilm disruption capabilities and enhancement of antibiotic sensitivity.[17] The convergence with traditional Thai formulation research[58] suggests that multi-herb preparations have been empirically optimized to provide balanced, synergistic therapeutic effects against biofilm-associated infections.

The differential susceptibility patterns observed between the two staphylococcal species highlight the importance of species-specific antimicrobial research. While both are gram-positive cocci sharing similar ecological niches, *S. aureus* typically possesses more sophisticated resistance mechanisms, including multiple efflux pumps and β-lactamase production[4], which may contribute to its reduced susceptibility to plant-derived antimicrobials.

Extraction Optimization and Phytochemical Considerations

The superior performance of 40% ethanol extraction validates traditional preparation methods and supports the scientific rationale for hydroethanolic extraction in traditional medicine. This finding aligns with contemporary research demonstrating that moderate ethanol concentrations often provide optimal balance for extracting diverse phytochemical classes[45-56]. The enhanced antimicrobial activity of TPJHE40 likely results from preferential extraction of polar antimicrobial compounds, including phenolic acids, flavonoid glycosides, and tannins.

The moderate total phenolic content observed (0.247-0.274 mg GAE/g) is consistent with traditional multi-herb formulations where therapeutic efficacy depends on compound diversity rather than maximizing individual phytochemical concentrations.[58] This principle distinguishes traditional formulations from single-compound pharmaceutical approaches and may explain the sustained efficacy of traditional medicines over centuries of use.

Global Health Implications and AMR Context

The findings of this study assume particular significance within the global context of antimicrobial resistance. Recent surveillance data indicate a 20% increase in antimicrobial-resistant hospital-onset infections[6], emphasizing the urgent need for alternative therapeutic strategies. The development of evidence-based traditional medicine alternatives, as demonstrated in our study, represents a promising avenue for addressing this crisis.

The superior activity against *S. epidermidis* is particularly relevant given its emerging role as a major nosocomial pathogen. Device-associated infections caused by biofilm-forming *S. epidermidis* strains often prove recalcitrant to conventional antibiotic therapy[5], making the exceptional activity of TPJHE40 clinically significant. The potential for developing standardized topical formulations targeting device-associated infections warrants serious consideration.

Integration with Contemporary Traditional Medicine Research

The remarkable consistency between our results and those of Konsue and Taepongsorat (2025)[58] suggests that traditional Thai medicine preparations exhibit predictable, reproducible bioactivity patterns. This reproducibility supports the scientific validity of traditional preparation methods and provides a foundation for developing standardized therapeutic products.

However, the variable effectiveness observed against different bacterial species underscores the importance of comprehensive pathogen screening in traditional medicine research. Future investigations should employ broader panels of clinically relevant isolates, including multidrug-resistant strains, to fully characterize the therapeutic potential of traditional formulations[60].

Quality Control and Standardization Imperatives

The complexity of multi-herb formulations presents unique challenges for pharmaceutical development. Establishing standardized analytical methods for quality control, implementing Good Manufacturing Practices, and developing stability profiles will be essential for translating laboratory findings into clinical applications. The variation observed between extraction methods emphasizes the critical importance of standardized preparation protocols.

Recent advances in analytical techniques, including metabolomics approaches and high-resolution mass spectrometry, offer promising tools for characterizing traditional medicine formulations and ensuring batch-to-batch consistency.[43-44] These technologies may facilitate the development of quality control standards necessary for regulatory approval.

Economic and Sustainability Considerations

The development of traditional medicine-based antimicrobials offers potential economic advantages, particularly in resource-limited settings where expensive synthetic antibiotics may be inaccessible. The three plant components of Tripitsajuk are readily cultivated in Thailand and

other tropical regions, potentially supporting sustainable local pharmaceutical industries.
However, successful commercialization will require substantial investment in research and development, clinical trials, and regulatory approval processes. The moderate bioactivity levels observed, while therapeutically relevant, may necessitate higher dosing compared to conventional antibiotics, potentially affecting cost-effectiveness.

Regulatory Pathways and Clinical Translation
Advancing Tripitsajuk from laboratory investigation to clinical application will require navigation of complex regulatory frameworks. Traditional medicine products face unique challenges in demonstrating safety and efficacy according to modern pharmaceutical standards while preserving the synergistic benefits of multi-herb formulations.
The exceptional activity against *S. epidermidis* observed in this study provides a strong foundation for pursuing clinical development, particularly for topical applications in device-associated infection prevention. However, comprehensive safety studies, including toxicological assessments and drug interaction profiles, will be prerequisite for regulatory approval.

Environmental and Ethical Considerations
The sustainable sourcing of plant materials represents a critical consideration for large-scale production. Traditional harvesting practices must be balanced with conservation efforts to ensure long-term availability of raw materials. Collaborative approaches involving traditional healers, botanists, and pharmaceutical companies may help preserve both traditional knowledge and plant biodiversity.
Intellectual property considerations surrounding traditional medicine knowledge require careful attention to ensure fair benefit-sharing with traditional communities. The development of Tripitsajuk-based therapeutics should acknowledge and compensate traditional knowledge holders appropriately.

Conclusion
This comprehensive investigation demonstrates that the traditional Thai Tripitsajuk formulation, particularly when extracted using 40% ethanol, possesses remarkable antimicrobial activity against *S. epidermidis* that significantly exceeds standard antibiotics. The exceptional minimum inhibitory concentration of 1.56 mg/mL and inhibition zone diameter of 27.89 mm represent clinically relevant activities that warrant serious consideration for therapeutic development. The findings validate centuries of traditional Thai medicine practice and provide scientific evidence supporting the continued investigation of multi-herb formulations as sources of novel antimicrobial agents. The consistency observed with contemporary traditional medicine research[58] strengthens the foundation for evidence-based traditional medicine and supports the integration of traditional knowledge with modern pharmaceutical sciences. The differential activity patterns between *S. aureus* and *S. epidermidis* highlight the importance of species-specific antimicrobial research and suggest potential applications in preventing device-associated infections. The superior performance of hydroethanolic extraction validates traditional preparation methods and provides guidance for optimizing bioactive compound recovery. While challenges remain in standardization, quality control, and clinical translation, the exceptional antimicrobial activity demonstrated in this study positions Tripitsajuk as a promising candidate for addressing the global antimicrobial resistance crisis. Future research should focus on mechanistic studies, clinical isolate testing, and development of standardized formulations suitable for clinical evaluation. The convergence of traditional wisdom and modern scientific validation exemplified in this study represents a promising paradigm for discovering new therapeutic agents from traditional medicine sources. As the global healthcare community confronts the mounting challenges of antimicrobial resistance,[1-2] traditional medicine formulations like Tripitsajuk offer hope for developing effective, accessible, and sustainable therapeutic alternatives.

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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