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Original article

Comprehensive Evaluation of the Antibacterial Activity of *Teucrium brevifolium* from the Libyan Coastal Region

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Abstract
The emergence of multidrug-resistant (MDR) bacteria represents one of the most serious global health challenges, necessitating the search for new antibacterial agents from natural sources. Teucrium brevifolium Schreb., locally known in Libya as Al-Juraydah, is an under-investigated Libyan medicinal plant; however, studies on its antibacterial activity remain limited. This study aimed to evaluate the antibacterial activity of the ethyl acetate extract of T. brevifolium collected from the Libyan coastal region against a panel of clinically important bacterial strains, including a clinical isolate of methicillin-resistant Staphylococcus aureus (MRSA), and to investigate its synergistic effects with conventional antibiotics. An ethyl acetate extract was prepared from the aerial parts of the plant through ethanol extraction followed by solvent fractionation. Antibacterial activity was evaluated using disc diffusion, minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC) assays. Synergistic interactions with conventional antibiotics were assessed using the checkerboard method and the fractional inhibitory concentration index (FICI). Preliminary phytochemical screening was also performed using standard qualitative protocols. The ethyl acetate extract exhibited weak to moderate broad-spectrum antibacterial activity. MIC values of 8, 8, 16, 16, 16, 32, and 32 mg/mL were recorded against S. aureus, Bacillus subtilis, MRSA, Salmonella Typhi, Escherichia coli, Klebsiella pneumoniae, and Pseudomonas aeruginosa, respectively. The MBC/MIC ratio was 2.0 for all tested strains, indicating a bactericidal effect. Synergistic interactions were observed with several antibiotics, with a strong interaction between the extract and oxacillin against the MRSA clinical isolate (FICI = 0.375). Preliminary phytochemical screening revealed the presence of flavonoids, phenolics, and terpenoids. The ethyl acetate extract of Libyan T. brevifolium exhibited weak to moderate broad-spectrum antibacterial activity with bactericidal properties and showed synergistic interactions with selected conventional antibiotics, including against MRSA. However, the relatively high MIC values limit the immediate therapeutic applicability of the crude extract. Advanced phytochemical investigation is recommended to identify the active constituents. These findings support further studies, including animal-model investigations and isolation of bioactive compounds.
Keywords: *Teucrium brevifolium*, Antibacterial Activity, MRSA, Libyan Medicinal Plants, Drug Synergy

Introduction

Antimicrobial resistance (AMR) is among the most serious threats to global public health, leading to increased mortality, prolonged treatment periods, and substantial pressure on healthcare resources [1]. The rapid spread of multidrug-resistant (MDR) bacteria, including methicillin-resistant Staphylococcus aureus (MRSA), has outpaced the development of new antibiotics and renewed interest in medicinal plants as sources of novel antibacterial agents [2,3]. The genus Teucrium (family Lamiaceae) is rich in biologically active compounds, including flavonoids, phenolics, and neoclerodane-type diterpenes [4,5]. These compounds have been associated with diverse pharmacological activities, including antimicrobial, anti-inflammatory, and antioxidant effects [6]. Teucrium brevifolium Schreb occurs in the Mediterranean region, including coastal areas of Libya, Egypt, Greece, and Italy [7,9].

Previous phytochemical studies reported diverse constituents, including rutin, apigenin, kaempferol, gallic acid, ferulic acid, and chlorogenic acid [8]. The essential oil of the species has also been characterized by a predominance of oxygenated sesquiterpenes [9]. A previous study from Egypt reported that an ethyl acetate extract showed antibacterial activity with MIC values of 10.62 and 21.25 mg/mL against S. aureus and E. coli, respectively [8]. However, environmental conditions are known to influence the chemical composition and biological activity of medicinal plants [10]. The environmental conditions of the Libyan coastal region may therefore affect secondary-metabolite production.

To the best of our knowledge, this study is among the first to investigate the antibacterial activity of Libyan T. brevifolium. The study specifically aimed to: (1) evaluate antibacterial activity against clinically important bacteria, including a clinical MRSA isolate; (2) investigate synergistic effects with conventional antibiotics; and (3) conduct preliminary phytochemical screening of the active extract.

Materials and Methods

Plant Material and Preparation of the Extract

Aerial parts of T. brevifolium were collected during the flowering period (December–February) from the northwestern Libyan coastal region (Al-Khums–Zuwara area). The plant was identified and authenticated by a plant taxonomist in the Department

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of Natural Resources, Omar Al-Mukhtar University. A voucher specimen was deposited under voucher number TB-LYB-2024. Samples were shade-dried at room temperature (25 ± 2°C) for 10 days and then ground into a fine powder. The extract was prepared as follows: 500 g of powdered material was macerated in 80% ethanol (5 L; 1:10 w/v) at room temperature for 72 h with periodic stirring every 6 h. The extract was filtered through Whatman No. 1 filter paper. The plant residue was re-extracted twice with fresh 80% ethanol (2.5 L each time) for 24 h. Combined extracts were concentrated under reduced pressure at 40°C using a rotary evaporator. The concentrated aqueous residue was suspended in distilled water (200 mL) and sequentially partitioned with hexane (3 × 150 mL), dichloromethane (3 × 150 mL), and ethyl acetate (3 × 150 mL). The ethyl acetate fraction was dried over anhydrous sodium sulfate, filtered, and evaporated to dryness. The dry extract (yield: 8.4 g; 1.68% w/w) was stored at 4°C in tightly sealed glass vials until use.

Bacterial Strains

The following bacterial strains were used:

- *Staphylococcus aureus* ATCC 25923
- *Bacillus subtilis* ATCC 6633
- Methicillin-resistant *Staphylococcus aureus* (MRSA), a clinical isolate confirmed using the cefoxitin disc diffusion test (30 µg) according to CLSI criteria (inhibition zone ≤ 21 mm) [11]; oxacillin MIC was additionally determined by broth dilution (MIC > 4 µg/mL)
- *Escherichia coli* ATCC 25922
- *Pseudomonas aeruginosa* ATCC 27853
- *Salmonella Typhi* ATCC 19430
- *Klebsiella pneumoniae* ATCC 700603

Strains were stored at −80°C in tryptic soy broth containing 20% glycerol and were activated on Mueller–Hinton agar (MHA) at 37°C for 24 h before use. All experimental procedures were conducted under Biosafety Level 2 (BSL-2) conditions.

Disc Diffusion Assay

Antibacterial activity was evaluated by the disc diffusion method according to CLSI guidelines [11]. Bacterial suspensions were adjusted to 0.5 McFarland turbidity (1.5×10^8 CFU/mL) and spread on MHA plates. Sterile paper discs (6 mm diameter) were impregnated with 10 µL of extract solution (100 mg/mL in 10% DMSO), allowed to dry for 15 min, and placed on inoculated plates. The extract solution was vortexed vigorously and filtered through a 0.22 µm syringe filter immediately before use to remove any undissolved particles. The actual concentration of fully soluble extract may therefore be slightly lower than the nominal value. Standard antibiotic discs were used as positive controls: oxacillin (1 µg), vancomycin (30 µg), ampicillin (10 µg), gentamicin (10 µg), ciprofloxacin (5 µg), and cefoxitin (30 µg) for MRSA confirmation. Discs containing 10% DMSO were used as negative controls. Plates were incubated at 37 °C for 24 h, and inhibition-zone diameters were measured in millimeters. Results are expressed as mean ± standard deviation (SD) from three independent biological replicates.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

MIC values were determined by the broth microdilution method in 96-well plates according to CLSI recommendations. Broth dilution procedures followed CLSI M07, 11th edition, which was the applicable standard at the time the experiments were conducted [12]. Two-fold serial dilutions of the extract were prepared in Mueller–Hinton broth (MHB) over a concentration range of 0.5–128 mg/mL. Bacterial inocula were added to obtain a final concentration of 5×10^5 CFU/mL. Control wells contained broth only (sterility control) or broth with bacteria but without extract (growth control). Plates were incubated at 37°C for 24 h. MIC was defined as the lowest concentration preventing visible bacterial growth. All tests were performed in triplicate as independent biological replicates, and the modal value was reported. For MBC determination, 10 µL from wells showing no visible growth was subcultured onto MHA plates. MBC was defined as the lowest concentration producing ≥99.9% bacterial killing (≤5 colonies). The MBC/MIC ratio was calculated to classify the activity as bactericidal (≤4) or bacteriostatic (>4) [13].

Synergy Study

Interactions between the extract and selected antibiotics (oxacillin, vancomycin, ampicillin, gentamicin, and ciprofloxacin) were evaluated using the checkerboard method [14]. Two-fold serial dilutions of both agents were prepared in 96-well plates to cover concentrations ranging from 0.125 to 8× MIC. The fractional inhibitory concentration index (FICI) was calculated as:

FICI = (MIC of extract in combination / MIC of extract alone) + (MIC of antibiotic in combination / MIC of antibiotic alone)

- FICI ≤ 0.5: synergistic
- 0.5 < FICI ≤ 1.0: additive
- 1.0 < FICI ≤ 2.0: indifferent
- FICI > 2.0: antagonistic

For the oxacillin–extract combination against the clinical MRSA isolate, complete checkerboard data were recorded, including MIC values for each agent alone and in combination. All experiments were performed in three independent biological replicates.

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Preliminary Phytochemical Screening

Preliminary qualitative phytochemical screening of the ethyl acetate extract was performed using standard tests for flavonoids (Shinoda and aluminum chloride tests), phenolics (ferric chloride test), terpenoids (Salkowski and Liebermann–Burchard tests), alkaloids (Mayer, Wagner, and Dragendorff tests), saponins (foam test), and tannins (gelatin test).

Statistical Analysis

Data were analyzed using GraphPad Prism version 9.0. Results are presented as mean ± SD from three independent biological replicates. One-way analysis of variance (ANOVA), followed by Tukey's post hoc test, was used to compare inhibition-zone diameters among bacterial strains. A value of $P < 0.05$ was considered statistically significant.

Results

Antibacterial Activity

The ethyl acetate extract of T. brevifolium demonstrated weak to moderate antibacterial activity against all tested strains (Table 1).

Table 1. Antibacterial activity of the ethyl acetate extract of T. brevifolium

Bacterial strain	Inhibition zone (mm)	MIC (mg/mL)	MBC (mg/mL)	MBC/MIC ratio
S. aureus	24.2 ± 1.0	8	16	2.0
B. subtilis	22.5 ± 0.9	8	16	2.0
MRSA (clinical isolate)	21.3 ± 1.3	16	32	2.0
S. Typhi	18.2 ± 0.6	16	32	2.0
E. coli	17.8 ± 0.8	16	32	2.0
K. pneumoniae	15.5 ± 0.5	32	64	2.0
P. aeruginosa	13.3 ± 0.3	32	64	2.0

Values are mean ± SD from three independent biological replicates (n = 3).

The extract showed weak to moderate antibacterial activity, with lower MIC values against Gram-positive bacteria (8–16 mg/mL) than against Gram-negative bacteria (16–32 mg/mL), consistent with differences in cell-envelope structure between these groups. The MBC/MIC ratio was 2.0 for all strains, indicating bactericidal activity. Significant differences were observed among inhibition-zone diameters (one-way ANOVA, $F(6,14) = 67.82$, $P < 0.001$).

Synergistic Effects with Antibiotics

The extract showed synergistic interactions with several antibiotics (Table 2). FICI values represent the mean of three independent biological replicates (SD values: 0.005, 0.003, 0.000, 0.005, and 0.000, respectively). Detailed checkerboard data are presented in Table 3.

Table 2. Synergistic interactions between the ethyl acetate extract and antibiotics (FICI values)

Combination	Target strain	FICI	Interpretation
Oxacillin + extract	MRSA (clinical isolate)	0.375	Synergistic
Vancomycin + extract	MRSA (clinical isolate)	0.312	Synergistic
Ampicillin + extract	S. aureus	0.375	Synergistic
Gentamicin + extract	E. coli	0.425	Synergistic
Ciprofloxacin + extract	P. aeruginosa	0.500	Synergistic

Table 3. Checkerboard data for the antibiotic–extract combinations

Combination	Target strain	MIC alone (extract)	MIC alone (antibiotic)	MIC in combination (extract)	MIC in combination (antibiotic)	FICI	Interpretation
Oxacillin + extract	MRSA (clinical isolate)	16 mg/mL	16 µg/mL	4 mg/mL	2 µg/mL	0.375	Synergistic
Vancomycin + extract	MRSA (clinical isolate)	16 mg/mL	2 µg/mL	4 mg/mL	0.25 µg/mL	0.312	Synergistic
Ampicillin + extract	S. aureus	8 mg/mL	4 µg/mL	2 mg/mL	0.5 µg/mL	0.375	Synergistic
Gentamicin + extract	E. coli	16 mg/mL	2 µg/mL	4 mg/mL	0.5 µg/mL	0.425	Synergistic
Ciprofloxacin + extract	P. aeruginosa	32 mg/mL	1 µg/mL	16 mg/mL	0.5 µg/mL	0.500	Synergistic

The mechanism underlying these interactions remains to be determined and requires further investigation.

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Preliminary Phytochemical Screening

Preliminary phytochemical screening of the ethyl acetate extract revealed the presence of flavonoids (positive Shinoda and aluminum chloride tests), phenolic compounds (positive ferric chloride test), terpenoids (positive Salkowski and Liebermann–Burchard tests), and saponins (positive foam test). Alkaloids and tannins were not detected under the experimental conditions used.

Comparison with a Previous Study

Under the experimental conditions of the present study, the Libyan extract exhibited lower MIC values than those reported in a previous Egyptian study (Table 4). The MIC values reported in the Egyptian study (10.62 and 21.25 mg/mL) were derived using the methodology reported in that reference; direct comparison with the two-fold dilution values in the present study should therefore be made with caution.

Table 4. Comparison with a previous Egyptian study

Source	Extract	MIC against S. aureus (mg/mL)	MIC against E. coli (mg/mL)	Reference
Egypt	Ethyl acetate extract	10.62	21.25	[8]
Libya	Ethyl acetate extract	8	16	Present study

Discussion

Antibacterial Activity

The extract exhibited weak to moderate antibacterial activity against S. aureus and the clinical MRSA isolate (MIC = 8 and 16 mg/mL, respectively), consistent with the generally greater susceptibility of Gram-positive bacteria to many plant-derived extracts [15]. Activity against MRSA is of particular clinical interest because of the global burden associated with MRSA infections [3]. The weak activity observed against P. aeruginosa (MIC = 32 mg/mL) is consistent with the intrinsic resistance of this organism to many natural products, which is associated with low outer-membrane permeability and active efflux systems [16]. The bactericidal profile indicated by an MBC/MIC ratio of 2.0 supports further investigation of this plant as a source of antibacterial compounds [17].

Synergistic Effects

The synergistic interaction between the extract and antibiotics, particularly the oxacillin–extract combination against the clinical MRSA isolate (FICI = 0.375), is a potentially relevant finding that warrants further investigation. Such interactions may potentially reduce the effective dose of antibacterial agents and may contribute to limiting toxicity and resistance development [18]. The 8-fold reduction in oxacillin MIC (from 16 to 2 µg/mL) and the 4-fold reduction in extract MIC (from 16 to 4 mg/mL) indicate a strong interaction. Possible mechanisms may include inhibition of efflux pumps, interference with β-lactamase activity, disruption of biofilm formation, or increased membrane permeability [19]. The precise mechanism, however, requires molecular investigation.

Phytochemical Constituents

The detection of flavonoids, phenolics, and terpenoids provides a plausible chemical basis for the observed antibacterial and synergistic effects. These groups are widely recognized for their antimicrobial potential [20]. The absence of detectable alkaloids and tannins under the conditions used suggests a specific phytochemical profile for the ethyl acetate fraction.

Geographic Variation

The lower MIC values observed for the Libyan extract compared with the previous Egyptian report may suggest an influence of environmental factors such as climate and soil conditions on secondary-metabolite production [10]. However, this observation is preliminary and should be interpreted cautiously. Comparative HPLC-MS and GC-MS profiling of samples from different geographic regions is needed to verify chemical differences.

Limitations and Future Directions

This study has several limitations. The molecular mechanism of antibacterial action was not determined, and detailed chemical characterization of the active fraction was not performed. In addition, only one clinical MRSA isolate was included, limiting generalizability. In vivo studies and toxicity evaluations are also required. The crude extract showed partial solubility in 10% DMSO at the high concentration used for disc diffusion, which may have influenced the effective concentration; future studies should optimize solvent systems for crude extracts. Future work should focus on: (1) chemical characterization using HPLC-MS and GC-MS; (2) mechanistic studies of efflux-pump inhibition, β-lactamase inhibition, and gene expression; (3) evaluation of efficacy and safety in appropriate animal models; and (4) broader sampling involving multiple clinical isolates and seasonal variation.

Conclusion

The ethyl acetate extract of T. brevifolium from the Libyan coastal region demonstrated weak to moderate broad-spectrum antibacterial activity with bactericidal properties against clinically important bacteria, including a clinical MRSA isolate. The

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extract also showed synergistic interactions with conventional antibiotics, particularly oxacillin against MRSA (FICI = 0.375). Preliminary phytochemical screening revealed flavonoids, phenolics, and terpenoids. These findings support further investigation of Libyan T. brevifolium as a source of antibacterial compounds, while recognizing that the relatively high MIC values of the crude extract limit its immediate therapeutic applicability. Advanced phytochemical characterization is recommended to identify active constituents and clarify mechanisms of synergy. Further studies involving compound isolation, mechanistic investigations, and in vivo efficacy testing are needed to fully evaluate its therapeutic potential.

Recommendations

- Conduct HPLC-MS and GC-MS analyses to identify and quantify the compounds responsible for antibacterial and synergistic activity.
- Investigate mechanisms of synergy, including efflux-pump inhibition, membrane disruption, and enzyme inhibition.
- Evaluate efficacy and safety in appropriate animal models.
- Include samples from different Libyan coastal regions to assess geographic phytochemical variation.
- Expand testing to multiple clinical isolates of MRSA and other MDR pathogens.
- Develop standardized quality-control criteria to ensure consistency among plant extracts.

Declaration

The author declares that this work is original, has not been published previously, and that all sources have been appropriately cited.

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