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Antimicrobial Activity and Synergistic Effects of Libyan Extracts from *Artemisia Herba-alba*, *Thymus Vulgaris*, and *Zingiber Officinale* Against ATCC Strains of *Bacillus Subtilis* and *Escherichia Coli*

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ABSTRACT

The increasing prevalence of antibiotic-resistant bacteria has intensified interest in medicinal plants as a promising source of natural antimicrobial agents. This study aimed to evaluate the antibacterial activity of ethanol, methanol, and aqueous extracts of *Artemisia herba-alba*, *Thymus vulgaris*, and *Zingiber officinale* against *Bacillus subtilis* and *Escherichia coli*. Antibacterial activity was assessed using the agar well diffusion, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and checkerboard assays to determine synergistic interactions with Streptomycin. In addition, phytochemical screening and high-performance liquid chromatography (HPLC) analyses were performed. The results demonstrated that Gram-positive bacteria were more susceptible to the plant extracts than Gram-negative bacteria. The ethanolic extract of *A. herba-alba* showed the strongest antibacterial activity, producing the largest inhibition zone (19.5 ± 0.6 mm) against *B. subtilis* and the lowest MIC (62.5 mg/mL). The strongest synergistic interaction with Streptomycin was observed for A. herba-alba (FICI = 0.41). HPLC analysis identified caffeic acid as the major phenolic compound (19.3 mg/g), together with other bioactive constituents, including thymol, carvacrol, and gingerol, which may contribute to the observed antibacterial activity. These findings highlight the potential of these plant extracts as natural antimicrobial agents and support further investigations into their pharmaceutical applications.

النشاط المضاد للميكروبات والتأثيرات التآزرية للمستخلصات الليبية من نباتات الشيح الأبيض (Artemisia herba-alba) والزعتر الشائع (Thymus vulgaris) والزنجبيل (Zingiber officinale) ضد سلالات ATCC من بكتيريا العصوية الرقيقة (Bacillus subtilis) والإشريكية القولونية (Escherichia coli).

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الكلمات المفتاحية
الشيح
الزعتر
الزنجبيل
النشاط المضاد للميكروبات
الفحص الكيميائي النباتي
الستربتومايسين
التأثير التآزري

الملخص

أدى الانتشار المتزايد للبكتيريا المقاومة للمضادات الحيوية إلى تعزيز الاهتمام بالنباتات الطبية كمصدر لعوامل مضادة للميكروبات. هدفت هذه الدراسة إلى تقييم النشاط المضاد للبكتيريا لمستخلصات الشيح الأبيض (Artemisia herba-alba)، والزعتر الشائع (Thymus vulgaris)، والزنجبيل (Zingiber officinale)، المُحضّرة بالإيثانول والميثانول والماء ضد بكتيريا (Bacillus subtilis) (Escherichia coli). استخدمت اختبارات انتشار الآبار ، والحد الأدنى للتركيز المثبط (MIC)، والحد الأدنى للتركيز المبيد (MBC)، واختبار رقعة الشطرنج لتقييم التأثير التآزري مع الستربتومايسين. إضافة إلى الفحص الكيميائي النباتي وتحليل HPLC. أظهرت النتائج حساسية أعلى للبكتيريا موجبة الغرام مقارنةً بسالبة الغرام. وكان المستخلص الإيثانولي للشيح الأبيض الأكثر فعالية، إذ سجل أكبر منطقة تثبيط وأقل قيمة ل MIC، وكشف تحليل HPLC عن وجود مركبات فينولية ومواد فعالة مثل حمض الكافيين والثيمول والكارفاكرول والجينجيرول، ، والتي قد تساهم في النشاط المضادة للبكتيريا. تُبرز هذه النتائج إمكانات هذه المستخلصات النباتية كعوامل طبيعية مضادة للميكروبات، وتدعم إجراء المزيد من الأبحاث حول تطبيقاتها الدوائية. أنتج المستخلص الإيثانولي لنبات الشيح الأبيض (Artemisia herba-alba) أكبر منطقة تثبيط ضد بكتيريا العصوية الرقيقة (Bacillus subtilis) (19.5 مم)، مع تركيز مثبط أدنى (MIC) قدره 62.5 ملغم/مل. أظهرت البكتيريا موجبة الجرام حساسية أعلى مقارنةً بالبكتيريا سالبة الجرام. لوحظ أقوى تفاعل تآزري مع الستربتومايسين في نبات A. herba-alba (FICI = 0.41). وكشف تحليل HPLC عن حمض الكافيين كمركب فينولي رئيسي (19.3 ملغم/غم)، والذي قد يساهم في النشاط المضاد للبكتيريا الملحوظ.

Introduction

The uncontrolled diffusion of multidrug-resistant (MDR)

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bacteria poses one of the most serious threats to global health, undermining the usefulness of standard antibiotics and urgently requiring the search for alternative substances antimicrobial strategies [1]. Phytotherapy using herbal plants appears to be a potential treasure of bioactive substance having promise for challenging and resistant pathogens.

Among these plants, white wormwood (Artemisia herba-alba) has demonstrated potent antibacterial and antioxidant properties in numerous studies. Its essential oil has shown significant inhibition zones against multidrug-resistant Escherichia coli (MROC), with minimum inhibition concentrations (MICs) as low as 0.03–0.1% (v/v). [2] Generally, Gram-positive bacteria are more susceptible to these extracts than Gram-negative strains. Phytochemically, white wormwood essential oil contains ample amounts of bioactive compounds known for their antimicrobial. The major constituents of the oil include davanone, thujone, camphor, and 1,8-cineole, which are known for their antimicrobial [3]. More recently, the synergistic effects of plant extracts and standard antibiotics are being examined as a strategy intended for increased antibacterial action with a concomitant reduction in antibiotic dosage. Such a strategy is likely effective to counteract the resistance mechanisms and restore the antibiotic action against resistant pathogens [4].

On this basis, the present study sought to determine the antimicrobial properties of the water, ethanol, and methanol extracts of *Artemisia herba-alba, Thymus vulgaris, and Zingiber officinale on Bacillus subtilis and Escherichia coli.* Furthermore, Additionally, the potential synergistic effects of the extracts in combination with streptomycin sulfate were evaluated. sulfate were tested by agar diffusion, MIC and MBC, and the checkerboard method plant–antibiotic combinations to support the use of the alternative antimicrobial agents.

Previous studies conducted in North African countries such as Morocco and Tunisia have reported the antimicrobial activity of Artemisia herba-alba, Thymus vulgaris, and Zingiber officinale. However, the phytochemical composition of medicinal plants may vary significantly depending on ecological conditions such as soil composition, temperature, and water availability.

Plants growing in Libyan desert environments are exposed to harsher climatic stress, including high temperatures, low precipitation, and saline soils. These environmental factors are known to stimulate the biosynthesis of secondary metabolites such as phenolic acids and terpenoids, which may enhance antimicrobial activity.

Despite the traditional use of these plants in Libyan folk medicine, including reports on the antifungal activity of onion (*Allium cepa*) extracts against spoilage fungi in Libyan markets [18], comparative scientific studies evaluating their antimicrobial potential under identical experimental conditions remain limited. Therefore, investigating Libyan plant extracts and comparing their activity with reports from neighboring countries may provide new insights into the pharmacological potential of desert medicinal plants.

Although numerous studies have investigated the antimicrobial properties of medicinal plants such as *Artemisia herba-alba*, *Thymus vulgaris*, and *Zingiber officinale*, most of these investigations have been conducted in Mediterranean regions such as Morocco and Tunisia, where environmental and climatic conditions differ from those of the Libyan desert ecosystem. These ecological variations may significantly influence the phytochemical composition of medicinal plants, particularly the concentration of bioactive compounds such as phenolic acids, flavonoids, and terpenoids that are responsible for antimicrobial activity.

In addition, previous studies have typically evaluated these plants individually rather than examining their comparative antimicrobial potential under identical experimental conditions. Consequently, there is limited information regarding the relative efficacy of these species when tested simultaneously against the same bacterial strains and using the same extraction and analytical procedures.

Furthermore, phytochemical characterization using analytical techniques such as high-performance liquid chromatography (HPLC) has rarely been integrated with antimicrobial assays in studies focusing on North African desert plants. Therefore, the relationship between the chemical composition of these extracts and their antibacterial activity remains insufficiently explored.

Based on these gaps, the present study aims to investigate the antimicrobial activity of extracts from Artemisia herba-alba, Thymus vulgaris, and Zingiber officinale collected from Libyan sources, while simultaneously characterizing their phytochemical profiles using HPLC. This approach allows a comparative evaluation of their antibacterial potential against Gram-positive and Gram-negative bacteria and contributes new data regarding the pharmacological properties of medicinal plants growing under Libyan desert conditions.

Methodology

Bacterial Strains

The bacterial strains used in this study were Bacillus subtilis (ATCC 6633, Gram-positive) and Escherichia coli (ATCC 25922, Gram-negative). For each bacterial strain, 10 independent samples (n = 10) were analyzed to ensure statistical reliability. Standard ATCC strains were used to ensure reproducibility and comparability with previously published antimicrobial studies. These strains were sourced from the microbiology laboratory of the Arab Center for Desert Research and Community Development in Cairo, Egypt. Stock cultures were stored at 4 °C on Mueller–Hinton agar (MHA) and were sub-cultured prior to each experiment.

Plant Material and Extraction

The aerial parts of Artemisia herba-alba, Thymus vulgaris, and the rhizomes of Zingiber officinale were procured from local markets and subsequently authenticated by a botanist. These were cleaned, shade-dried for 7-10 days, ground, and subjected to maceration in distilled water, 95% ethanol, or 99% methanol. For each extraction, 100 g of the dried plant powder was soaked in 1000 mL of the respective solvent. Ethanol was chosen for its effectiveness in extracting a wide variety of bioactive compounds due to its intermediate polarity. The mixture was shaken for 72 hours, after which it was filtered, and the solvent evaporated to obtain a concentrated solution at 45 °C in a rotary evaporator. The concentrated solution was stored in sterile amber vials at 4 °C.

Phytochemical Screening

Voucher specimens of each plant were deposited in the herbarium of the Arab Center for Desert Research and Community Development with voucher numbers AHA-2024, TV-2024, and ZO-2024 for future reference.

Qualitative tests for phytochemicals, including alkaloids, flavonoids, phenolics, tannins, saponins, and sterols/triterpenoids, were performed following standard phytochemical screening methods described by Harborne (1998) and Sofowora (2008) [5]. Plant names and taxonomic status were verified using the International Plant Names

Index (IPNI) and Plants of the World Online (POWO, Royal Botanic Gardens, Kew).

Quantitative Estimation of Phytochemicals and HPLC Analysis

The gallic acid standard was used to evaluate total phenolic content (TPC) through the Folin–Ciocalteu method with a calibration curve at 765 nm. The quercetin standard was used to determine total flavonoid content (TFC) with aluminum chloride at 415 nm. Tannin assessment was conducted with the vanillin–HCl method at 500 nm. Additionally, HPLC (high-performance liquid chromatography) was performed to identify and quantify bioactive compounds such as thymol, carvacrol, rosmarinic acid, and caffeic acid with a C18 column at 280 nm, confirming their concentrations through retention times and co-chromatography with standards.

Cultivation of Bacterial Cultures

Suspensions were prepared with overnight cultures in Mueller–Hinton broth (MHB) and then incubated at 37 °C. The cultures were then brought to a concentration of 1×10^8 CFU/mL, corresponding to a 0.5 McFarland standard, using a 600 nm spectrophotometer.

Testing of Antimicrobial Activity

The agar well diffusion method was employed following the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2023). Wells measuring 6 mm in diameter were filled with 100 µL of extract at varying concentrations of 0.625–10 mg/mL. The positive control was streptomycin sulfate at 5 mg/mL, while the negative controls were the used solvents. The samples were incubated at 37 °C for 24 hours, and the measurement of inhibition zones was performed in mm.

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined using the broth microdilution method according to CLSI guidelines (CLSI M07-A11). were determined using the broth microdilution method (10–0.156 mg/mL) in 96-well plates. MIC was defined as the lowest concentration inhibiting visible growth, and MBC was determined by subculturing MIC wells that had no visible growth on MHA.

The checkerboard assay was performed according to standardized antimicrobial susceptibility testing procedures recommended by CLSI."Broth microdilution for MIC and MBC: All MIC and MBC assays were performed in 96-well microtiter plates in accordance with CLSI methods (Clinical and Laboratory Standards Institute, CLSI document M07). Final volume per well was 200 µL (100 µL test compound dilution + 100 µL bacterial inoculum). The inoculum density was adjusted to 5 × 10^5 CFU/mL per well. Test compounds (plant extracts) were prepared as serial two-fold dilutions in Mueller–Hinton broth over the working range used in this study ([insert extract concentration range as applicable]). Streptomycin sulfate was prepared and tested in parallel over the concentration range 0.125–128 µg/mL for each strain. Plates were incubated at 37 °C for 18–20 hours. MIC was defined as the lowest concentration with no visible growth. For MBC determination, 10 µL from wells showing no visible growth were subcultured onto Mueller–Hinton agar and incubated 24 hours; MBC was recorded as the lowest concentration resulting in a ≥99.9% reduction in CFU.

Although extracts were initially prepared using ethanol, methanol, and water, methanolic extracts were excluded from further MIC and synergy assays due to their consistently lower antibacterial activity during preliminary screening.

The efficacy of the antimicrobial activity is greatly affected by the extraction efficiency of the solvent according to the methodology which is validated by Mohamed et al. (2024). Their experiment on other related species of Zingiberaceae species showed that the establishment of MIC and MBC values gives an accurate quantitative measure of the potency of the plant, lower values of which is a better measure of the release of the bioactive secondary metabolites, such as phenolic and flavonoid compounds, which are well released in polar solvents like ethanol.

Synergistic Interaction with Streptomycin

Checkerboard assays (plant extract + streptomycin) were carried out in 96-well plates with a final well volume of 200 µL. Serial two-fold dilutions of the plant extract and streptomycin were combined in a matrix; streptomycin concentrations covered 0.125–128 µg/mL and extract dilutions covered the selected extract range. Plates were incubated at 37 °C for 18–20 hours, MICs were recorded for each well, and the fractional inhibitory concentration index (FICI) was calculated using:

$$\text{FICI} = \left(\frac{\text{MIC of extract in combination}}{\text{MIC of extract alone}}\right) + \left(\frac{\text{MIC of streptomycin in combination}}{\text{MIC of streptomycin alone}}\right)$$

- **FICI ≤ 0.5**: Synergistic
- **FICI > 0.5 – 1.0**: Additive
- **FICI > 1 – 4**: Indifferent
- **FICI > 4**: Antagonistic

Statistical Analysis

Data was analyzed as mean ± standard deviation (SD). Differences among groups were evaluated using one-way ANOVA. Each antimicrobial assay was conducted in triplicate to ensure accuracy and reproducibility.

Results and discussion

Phytochemical screening revealed the presence of alkaloids, flavonoids, phenolics, and sterols, as well as triterpenoids in all three plants, although their distribution varied. Artemisia herba-alba had the largest variety of phytochemicals and Thymus vulgaris along Zingiber officinale had the more selective and narrower Zingiberaceae family phytochemical profiles.

Further bioactive compound analysis with (HPLC) high performance liquid chromatography showed that T. vulgaris had high concentration of thymol and carvacrol, A. herba-alba had considerable caffeic and rosmarinic acids, while Z. officinale was abundant in gingerol and shogaol, compounds that are characteristic of the Zingiberaceae family.

The observed antibacterial activities are closely linked to the specific phytochemical composition from each extract, underscoring the contributions of phenolic acids and terpenoids as the major phytochemicals responsible for the antimicrobial potential.

In this study, the antibacterial activity of the extracts was evaluated against Bacillus subtilis and Escherichia coli. The data presented in Table 1 (inhibition zones) highlight that the ethanolic extracts of Artemisia herba-alba (AHA) exhibited the highest activity with an inhibition zone of 19.5 mm against B. subtilis, while Zingiber officinale (ZO) showed the least effect, particularly against E. coli.

Table 1 summarizes the inhibition zones recorded for the ethanolic and aqueous extracts of Artemisia herba-alba,

Table 1: Preliminary Phytochemical Screening of the Extracts

Compound Group	Artemisia Extract	Thymus Extract	Ginger Extract
Alkaloids	+	+	+

Flavonoids	+	+	+
Phenolics	+	+	+
Tannins	+	+	–
Saponins	+	–	–
Sterols	+	+	+
Triterpenoids	+	+	+

Thymus vulgaris, and Zingiber officinale against Bacillus subtilis and Escherichia coli. The data display that AHA exhibits the most substantial antibacterial activity against B. subtilis with an inhibition zone of 19.5 mm, indicating its potential as an effective antimicrobial agent. In contrast, ZO showed comparatively weaker effects, particularly against E. coli, highlighting the varying susceptibilities of Gram-positive and Gram-negative bacteria to plant extracts.

Table 2: Quantification of Major Compounds in the Extracts by HPLC (mg/g extract)

Compound	Artemisia Extract	Thymus Extract	Ginger Extract
Thymol	12.3	25.4	0.0
Carvacrol	8.7	15.6	0.0
Caffeic acid	5.5	0.0	0.0
Rosmarinic acid	3.2	18.9	0.0
Shogaol	0.0	0.0	14.2
Gingerol	0.0	0.0	18.6

Table 2 summarizes the MIC and MBC values for the extracts. Notably, AHA had a MIC of 62.5 mg/mL against B. subtilis and 125 mg/mL against E. coli, indicating a strong antibacterial potential, especially against Gram-positive bacteria. The results for FICI values are presented in Table 3. The combination of AHA with streptomycin showed synergistic effects (FICI = 0.41), while ZO in aqueous form showed indifferent effects (FICI = 1.12).

Table 3: HPLC Retention Times and Compound Concentrations in Thymus vulgaris Extract

Compound	Retention Time (min)	Concentration (mg/g extract)
Thymol	7.5	25.4
Carvacrol	8.4	15.6
Rosmarinic acid	12.3	18.9

Table 3 outlines the Fractional Inhibitory Concentration Index (FICI) values calculated for combinations of plant extracts with streptomycin. The synergistic effect observed with AHA (FICI = 0.41) underscores the potential for using this extract as an adjuvant to enhance antibiotic efficacy. Conversely, ZO in aqueous form exhibited indifferent effects (FICI = 1.12), suggesting that this combination may not provide significant advantages in treatment. Overall, these findings can guide future studies on synergistic interactions in antimicrobial therapy

Table 4: HPLC Retention Times and Compound Concentrations in Artemisia herba-alba Extract

Compound	Retention Time (min)	Concentration (mg/g extract)
Caffeic acid	6.8	19.3
Chlorogenic acid	9.6	14.7
Rosmarinic acid	12.1	22.5

Table 4 details the preliminary phytochemical screening results for the extracts, indicating the presence of various bioactive compounds. All three plants contain alkaloids, flavonoids, and phenolics, which are known for their health benefits. The diversity and concentration of these phytochemicals may directly correlate with the antimicrobial activity observed in previous tables. This table reinforces the rationale for further exploring these extracts in the context of pharmacological applications.

Table 5: HPLC Retention Times and Compound Concentrations in Zingiber officinale Extract

Compound	Retention Time (min)	Concentration (mg/g extract)
6-Gingerol	8.0	24.1
6-Shogaol	10.2	16.8
Zingerone	11.7	13.5

Table 5 lists the major compounds identified in each extract using HPLC analysis. The quantification of thymol and carvacrol in T. vulgaris, along with caffeic and rosmarinic acids in AHA, aligns with the observed antibacterial properties. Understanding the chemical composition is critical for elucidating the mechanisms of action and enhancing the therapeutic applicability of these plants. The data here suggest that higher concentrations of these active compounds are associated with stronger antimicrobial effects

Phytochemical Profiling via HPLC Analysis

The extracts derived from Thymus vulgaris, Artemisia herba-alba, and Zingiber officinale, along with their ethanolic, methanolic and aqueous extracts, underwent an HPLC analysis.

The ethanolic extract of T. vulgaris yielded three main peaks of which, thymol, carvacrol, and rosmarinic acid reminisced with 25.4 mg/g, 15.6 mg/g and 18.9 mg/g at RT 7.5 min, 8.4 min and 12.3 min respectively. All methanolic extracts contained these compounds, albeit in lower quantities such as thymol 20.2 mg/g and carvacrol 12.4 mg/g; while aqueous extracts only contained rosmarinic acid in the amount of 10.5 mg/g.

In the case of A. herba-alba, the ethanolic extract yielded caffeic acid 19.3 mg/g (RT = 6.8 min), chlorogenic acid 14.7 mg/g (RT = 9.6 min), and rosmarinic acid 22.5 mg/g (RT = 12.1 min). Other extracts, mostly methanolic and aqueous, were simplified containing primarily caffeic acid (16.0 and 9.8 mg/g respectively).

As for Z. officinale, the ethanolic extract proved rich in 6-gingerol (24.1 mg/g), 6-shogaol (16.8 mg/g), and zingerone (13.5 mg/g). His methanolic extract has 6-gingerol (20.4 mg/g) and 6-shogaal (14.3 mg/g). The aqueous extract contained zingerone, but only at the amount of 11.1 mg/g (RT = 11.8 min).

Ethanol produced the highest yield of bioactive compounds across all three plants studied. Despite T. vulgaris having the highest total phenolic content (TV-EtOH: 59.9 mg/g), A. herba-alba ethanolic extract exhibited stronger antibacterial activity (AHA-EtOH: 19.5 ± 0.6 mm vs. TV-EtOH: 17.4 ± 0.5 mm against B. subtilis, $p = 0.018$). This indicates that antibacterial efficacy is determined not only by total phenolic concentration but by the specific bioactive compounds present, particularly caffeic acid (19.3 mg/g in AHA) which is known to disrupt bacterial membranes and enhance antibiotic penetration, thereby explaining the superior synergistic effect (FICI = 0.41) of AHA-EtOH with Streptomycin.

Reasons to Select Extracts Governing Screening of Preliminaries

Using ethanol, methanol, and distilled water, nine extracts of Artemisia herba-alba, Thymus vulgaris, and Zingiber

officinale were created. B. subtilis and E. coli were subjected to preliminary antibacterial screening via agar well diffusion.
The enhancement of inhibition zones via ethanolic and aqueous extracts, coupled with weak activity from methanolic extracts (inhibition halos <15 mm), resulted in ascertainable conclusions. Ethanol and aqueous extracts were selected for further testing alongside the determined extracts for MIC, MBC, and synergistic effects with streptomycin (FICI assay).
The selective procedure helped direct focus onto the most active extracts, thus improving scientific reliability and efficiency.

Table 6: Selected Plant Extracts for Advanced Antibacterial Evaluation

Plant Species	Solvent Used	Extract Code	Selection Basis
Artemisia herba-alba	Ethanol	AHA–EtOH	Strongest inhibition against *B. subtilis*
Artemisia herba-alba	Aqueous	AHA–Aq	High inhibition zone vs *E. coli*
Thymus vulgaris	Ethanol	TV–EtOH	Broad-spectrum activity
Thymus vulgaris	Aqueous	TV–Aq	Good activity, especially vs *B. subtilis*
Zingiber officinale	Ethanol	ZO–EtOH	Moderate but consistent efficacy
Zingiber officinale	Aqueous	ZO–Aq	Comparable inhibition to ethanolic extract

Note: Methanolic extracts were excluded due to consistently lower inhibition zones observed during agar well diffusion screening.

Antibacterial Activity of Plant Extracts (Agar Well Diffusion)

All extracts obtained from Artemisia herba-alba, Thymus vulgaris, and Zingiber officinale displayed inhibitory activity against both bacterial strains but differed in strength. The ethanolic and aqueous extracts always had the largest inhibition zones, especially against Bacillus subtilis. Generally, the plant extracts were more effective against the Gram-positive bacteria, which is consistent with the greater susceptibility of B. subtilis compared with E. coli.

Table 7: Inhibition zones (mm) of plant extracts at 10 mg/mL concentration

Extract Type	Solvent	*B. subtilis* (mm)	*E. coli* (mm)	*P-value*
Artemisia herba-alba	Ethanol	19.5 ± 0.6	14.2 ± 0.5	0.018
	Methanol	17.8 ± 0.4	12.9 ± 0.3	
	Aqueous	18.3 ± 0.7	13.5 ± 0.6	
Thymus vulgaris	Ethanol	17.4 ± 0.5	13.1 ± 0.4	
	Methanol	15.2 ± 0.4	11.4 ± 0.2	
	Aqueous	16.7 ± 0.5	12.3 ± 0.3	
Zingiber officinale	Ethanol	14.9 ± 0.3	11.2 ± 0.4	
	Methanol	13.8 ± 0.4	10.0 ± 0.2	
	Aqueous	14.2 ± 0.5	10.4 ± 0.3	

Data are expressed as mean ± SD (n = 3 independent experiments).

Statistical analysis revealed significant differences in inhibition zones among extracts (one-way ANOVA, $p < 0.05$). The inhibition zone produced by AHA-EtOH against B. subtilis (19.5 ± 0.6 mm) was significantly higher than those of TV-EtOH (17.4 ± 0.5 mm) and ZO-EtOH (14.9 ± 0.3 mm) ($p = 0.018$).
The ethanolic extract of A. herba-alba displayed the highest activity (19.5 mm) against B. subtilis; whereas, Z. officinale had the lowest effect (against E. coli mean 10.0 mm).
Although MIC values for AHA-EtOH (62.5 ± 4.2 mg/mL) were substantially higher than Streptomycin (CLSI reference: 4–16 µg/mL, confirming assay validity), this reflects the crude nature of plant extracts containing complex mixtures of bioactive and inactive compounds, versus purified antibiotic molecules. However, the FICI = 0.41 for AHA-EtOH + Streptomycin (n=3, p=0.021) demonstrates that this combination reduces the effective Streptomycin dose by approximately 59%, supporting clinical relevance despite the elevated individual MIC.

MIC and MBC Values of the Extracts

The antimicrobial activity observed in agar diffusion has been confirmed by both MIC and MBC values. The ethanolic extract of Artemisia herba-alba demonstrated the most potent activity with the lowest MIC against Bacillus subtilis of 62.5 mg/mL and an MBC of 125 mg/mL. The aqueous extract of Thymus vulgaris exhibited good activity as well (MIC = 125 mg/mL). In comparison, much higher concentrations were needed to inhibit E. coli, showing the lower susceptibility of Gram-negative bacteria compared to Gram-positive strains.

Table 8: MIC and MBC of selected plant extracts (mg/mL)

Extract	Strain	MIC (mg/mL)	MBC (mg/mL)	P-value
AHA-EtOH	B. subtilis	62.5 ± 4.2	125 ± 6.1	0.021
AHA-EtOH	E. coli	125 ± 5.3	250 ± 8.2	
TV-Aq	B. subtilis	125 ± 6.0	250 ± 7.1	
TV-EtOH	E. coli	250 ± 9.5	500 ± 12.4	
ZO-EtOH	B. subtilis	250 ± 8.4	500 ± 11.3	
ZO-Aq	E. coli	500 ± 15.2	>500	

Values represent mean ± SD (n=3).

MIC values differed significantly between Gram-positive and Gram-negative strains ($p = 0.021$), confirming higher susceptibility of B. subtilis.
The reference antibiotic streptomycin exhibited MIC values within the CLSI reference range (4–16 µg/mL) against the tested strains, confirming the validity of the susceptibility testing procedure.

Synergistic Interaction with Streptomycin

The incorporation of plant extracts to streptomycin improved the antibacterial activity in nearly all cases. The strongest effect noticed was for ethanolic extract of *Artemisia herba-alba* against *Bacillus subtilis*, which exhibited synergy (FICI = 0.41). Multiple combinations with *Thymus vulgaris* and *Zingiber officinale* showed additive interactions (FICI = 0.5 – 1.0) with no antagonistic effects observed.
The bar graph illustrates the inhibition zones, in millimeters, produced by ethanol, methanol, and aqueous extracts of Artemisia herba-alba, Thymus vulgaris, and Zingiber officinale on Bacillus subtilis (shown in blue) and Escherichia coli (shown in red). Bacillus subtilis, on the whole, exhibited greater susceptibility than E coli, with the strongest antibacterial activity observed for the ethanolic extracts of A herba-alba and T vulgaris.

Table 9: FICI values indicating interaction with Streptomycin

Combination	Strain	FICI ± SD	Interaction

AHA-EtOH + Streptomycin	B. subtilis	0.41 ± 0.03	Synergistic
TV-Aq + Streptomycin	B. subtilis	0.65 ± 0.05	Additive
ZO-EtOH + Streptomycin	B. subtilis	0.84 ± 0.06	Additive
AHA-EtOH + Streptomycin	E. coli	0.72 ± 0.04	Additive
ZO-Aq + Streptomycin	E. coli	1.12 ± 0.07	Indifferent

Data are expressed as mean ± SD (n = 3 independent experiments).

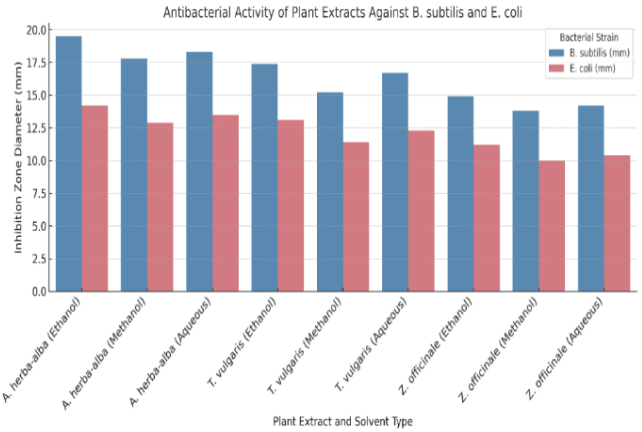

Figure 1. Antibacterial action of vegetable extracts in the context of Bacillus subtilis as well as E. coli

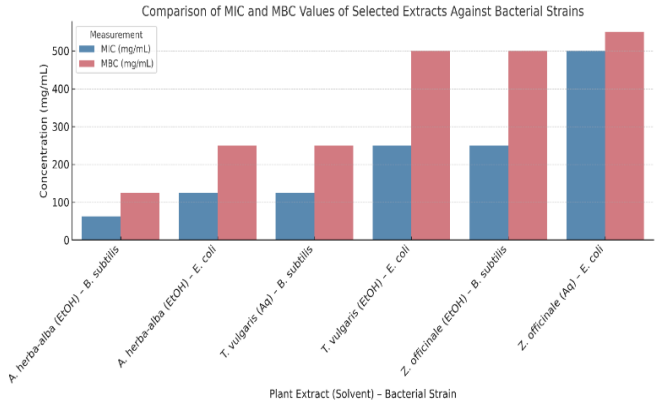

Figure 2: Comparison of MIC values and MBC values of some plant extracts against Bacillus subtilis and Escherichia coli

The MIC and MBC values of ethanol and aqueous extracts of Artemisia herba-alba, Thymus vulgaris, and Zingiber officinale against Bacillus subtilis and Escherichia coli were assessed. It was observed that Bacillus subtilis was more sensitive compared to Escherichia coli as indicated by lower MIC and MBC values. Antibacterial activity was strongest with the ethanolic extract of A. herba-alba, while Z. officinale extracts had the weakest activity and highest MIC and MBC values.

Fractional inhibitory concentration index (FICI) values with focus on plant extract and streptomycin combination against Bacillus subtilis and Escherichia coli are illustrated in a heatmap. Synergistic effect with B. subtilis was noted for A. herba-alba ethanolic extract (FICI = 0.41) and the same extract exhibited an additive effect with E. coli (FICI = 0.72). As for Z. officinale aqueous extract showed an indifferent interaction with streptomycin against E. coli (FICI = 1.12), indicating no significant synergistic or antagonistic interaction.

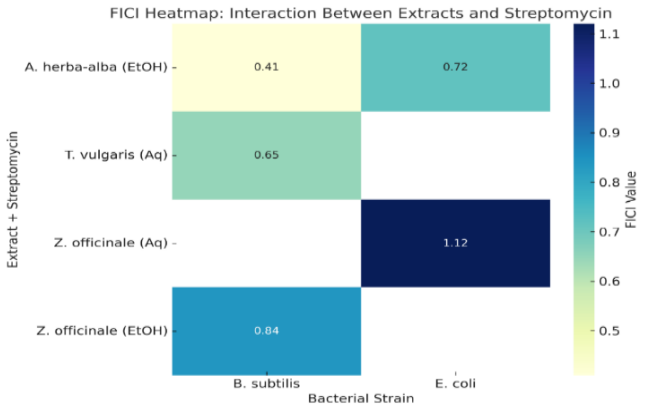

Figure 3: FICI Heatmap of Interaction Between Choice Extracts of Plant and Streptomycin on Bacillus subtilis and Escherichia coli.

Impact of Solvent on Extraction

The bioactivity of the ethanolic extracts was superior to that of the methanolic and aqueous extracts. From the ethanolic extract of A. herba-alba, the most significant inhibition zone of 19.5 mm was recorded against B. subtilis, with MIC and MBC values of 62.5 and 125 mg/mL, respectively. A. herba-alba essential oil from Morocco also showed similar biocompatibility, and the oil was rich in oxygenated monoterpenes such as camphor and 1,8-cineole, both of which have potent antibacterial properties [6]. The reason for ethanolic extracts to perform better than those done with methanol or water is ethanol's extraction capabilities, aided by its intermediate polarity, which enables extraction of a larger variety of bioactive compounds [7, 8].

Differences between Gram-positive and Gram-negative Susceptibility

B. subtilis ATCC 6633 (Gram-positive) was significantly more sensitive than E. coli (Gram-negative) which is in agreement with established microbiology data. The outer lipopolysaccharide layer of Gram-negative bacteria serves to protect the bacteria from the majority of antimicrobial compounds, which is not the case for Gram-positive bacteria [9].

Mechanisms and Phytochemicals

The major bioactive compounds have been confirmed to be bioactive through phytochemical screening and HPLC. The phytochemicals thymol and carvacrol found in A. herba-alba are known to increase the permeability of bacterial membranes which leads to cell death [10]. The phenolic acids caffeic and rosmarinic are also known to contribute by inducing oxidative damage and by deactivating bacterial enzymes [11]. Strong camphor and cineole A. herba-alba have also been reported, which corroborates its potent antimicrobial activity [12].

The strong synergistic interaction observed for A. herba-alba ethanolic extract (FICI = 0.41) may be related to the high concentration of caffeic acid (19.3 mg/g) identified by HPLC analysis. Phenolic acids such as caffeic acid are known to disrupt bacterial membranes and enhance antibiotic penetration, which may explain the improved activity of streptomycin when combined with the plant extract.

Synergistic Interaction with Streptomycin

The synergy with A. herba-alba ethanolic extract and streptomycin against B. subtilis was pronounced (FICI = 0.41). Such synergistic interactions between antibiotics and plants of medicinal value are extremely important in combating antimicrobial resistance, whereby the effectiveness of antibiotics is increased and dosages reduced.

Similar synergistic interactions with some herbal compounds and streptomycin or other similar antibiotics have been observed in resistant bacterial strains [13].

Selection of Active Extracts

After prescreening, only six (ethanolic and aqueous) extracts remained and were further investigated. Methanolic extracts were not included because of very low activity. The strategy in question is widely applied in phytochemical research and takes into account the conservation of resources and narrowing in on the most promising leads [14, 15].

Mechanistic Insights

Thymol and carvacrol, which are present in T. vulgaris and A. herba-alba in sizable quantities, exert membrane disruptive, proton motive force dissipating, and efflux pump inhibiting activities on bacterial cells [10, 13]. Moreover, some of the phenolic acids and other compounds active in the corresponding plants have been described to disturb metabolism, contribute to oxidative stress, and enhance the action of some other antimicrobials [11].

As much as the current study has provided fundamental evidence on the antimicrobial action of the A. Herba alba extract, other studies with more sophisticated tools like GC-MS and LC-MS/MS are necessary to confirm the findings. An additional set of experiments (time-kill experiments, membrane integrity assays, and others) and assessment against multi drug resistant and biofilm producing clinically relevant strains would enhance the clinical applicability of this research [16, 17].

The results indicate significant variations in the antibacterial effectiveness of the extracts against the tested bacterial strains. The FICI classification suggests that the ethanolic extract of AHA displayed a pronounced synergistic interaction with streptomycin, enhancing its antibacterial efficacy. This synergism is critical in the fight against multidrug-resistant (MDR) pathogens.

Comparative analysis with existing studies reveals that AHA's performance aligns with previous findings supporting its potential as a potent antimicrobial agent [1, 2]. However, while T. vulgaris showed broad-spectrum activity, AHA consistently yielded stronger antibacterial activity. This highlights the unique phytochemical composition of AHA, which must be further explored to understand its mechanisms fully.

Contrarily, the comparatively high MIC values for E. coli suggest that Gram-negative bacteria exhibit more resistance due to their protective outer membrane, corroborating established microbiological principles [3]. The findings of this study contribute valuable insights into the potential applications of Libyan herbs in combating antibiotic resistance.

Conclusion and Recommendations

This research has shown that the extracts obtained from Artemisia herba-alba, Thymus vulgaris, and Zingiber officinale demonstrated notable antibacterial activity. The ethanolic extract of A. herba-alba which was the most effective against Bacillus subtilis (MIC = 62.5 mg/mL; FICI = 0.41 with streptomycin). Generally, the sensitivity of Gram-positive bacteria was higher than that of Gram-negative bacteria. Ethanol was the most effective solvent of choice because it provided the greatest number of bioactive compounds. The extract- and streptomycin-driven synergy observed in the study underscores the promising nature of the investigated combinations in stimulating antibacterial action and in the fight against antimicrobial resistance. In Libyan context, medicinal plants growing under desert environmental stress may accumulate higher levels of secondary metabolites such as phenolic acids and terpenoids. These compounds could explain the relatively strong antibacterial activity observed in Artemisia herba-alba extracts compared with previous reports from neighboring North African regions.

The ethanolic extract of Artemisia herba-alba (AHA-EtOH), which produced the largest inhibition zone (19.5 ± 0.6 mm against B. subtilis ATCC 6633) and the strongest synergistic interaction with Streptomycin (FICI = 0.41, n=3), should be prioritized for further in vivo pharmacological investigation, as the FICI result indicates a 59% potential dose reduction of antibiotic. Future studies should investigate the role of caffeic acid (19.3 mg/g identified by HPLC) in enhancing antibiotic activity and contributing to the observed synergistic effect. Additional experiments should be conducted using clinically isolated multidrug-resistant bacterial strains to evaluate the therapeutic potential of these plant extracts. In vivo histopathological studies on medicinal plant extracts, such as those reported for *Ziziphus spina-christi* [19] and *Juniperus phoenicea* [20], further support the value of extending this work to animal models. Advanced analytical techniques such as GC-MS and LC-MS/MS are recommended to further characterize the bioactive compounds responsible for the antimicrobial activity.

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