



Chemical Composition, Antibacterial and Antibiofilm Activities of *Nepeta curviflora* Essential Oil Against Drug-Resistant Uropathogen

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Abstract

The global rise of multidrug-resistant (MDR) bacteria and biofilm-associated infections necessitates alternative therapeutic strategies. This study investigates the chemical composition, antibacterial, and antibiofilm activities of *Nepeta curviflora* essential oil (EO) against Gram-positive and Gram-negative clinical uropathogens, including Extended-Spectrum β -Lactamase (ESBL)-producing strains. The EO was obtained by hydro-distillation and analyzed using GC-MS, which identified 28 compounds representing 94.06% of the total oil—mainly oxygenated monoterpenes (37.26%) and sesquiterpenes (24.11%). Major components included 4 α ,7 α ,7 β -nepetalactone (15.09%) and 4 α ,7 α ,7 β -nepetalactone (10.04%). The EO showed low to moderate antibacterial activity (Minimum Inhibitory Concentration (MIC) = 12.5–50 μ L/mL) and significantly inhibited biofilm formation at sub-MIC levels (Minimum Biofilm inhibitory concentration (MBIC₅₀) = 1.5–6.25 μ L/mL), especially in ESBL *Escherichia coli* and *Pseudomonas aeruginosa*. The ability to inhibit biofilms at sub-inhibitory concentrations suggests potential interference with quorum sensing. In conclusion, *Nepeta curviflora* EO exhibits promising antibacterial and antibiofilm activities, particularly against resistant Gram-negative uropathogens. The novelty of this study lies in its comprehensive evaluation of both planktonic and biofilm-inhibitory effects on clinically significant and drug-resistant urinary isolates, supporting the EO's potential as a natural therapeutic agent for managing MDR and biofilm-associated infections.

Keywords: Nepetalactone, virulence factors, bacteriostatic, multidrug resistant, GC-MS

1. Introduction

The global emergence of antimicrobial resistance (AMR) continues to challenge the effectiveness of conventional antibiotics, especially in the management of urinary tract infections (UTIs), where multidrug-resistant (MDR) and extended-spectrum β -lactamase (ESBL)-producing pathogens are increasingly prevalent [1]. These infections are frequently complicated by the capacity of bacteria to form biofilms—structured microbial communities that adhere to surfaces and provide protection from antibiotics and host immune responses [2]. Biofilm-associated bacteria can be up to 1,000 times more resistant to antibiotics than their planktonic counterparts, contributing to chronicity and therapeutic failure in clinical settings [3].

In response to the growing threat of AMR, there has been a resurgence of interest in the therapeutic potential of natural products, particularly plant-derived essential oils (EOs), due to their broad-spectrum antimicrobial and antibiofilm properties [4]. Among these, members of the Lamiaceae family, including species from the genus *Nepeta*, have attracted attention for their complex phytochemical profiles and historical use in traditional medicine for treating microbial infections [5]. Essential oils from *Nepeta* species are rich in bioactive constituents such as nepetalactones, monoterpenes, and sesquiterpenes—compounds known to exert bactericidal effects via membrane disruption, enzyme inhibition, and quorum sensing interference [6].

Nepeta curviflora (Boiss.), commonly known as “Zaatar Farsi” in local Jordanian dialects, is a lesser-studied species endemic to the Eastern Mediterranean region. It belongs to the family Lamiaceae, genus *Nepeta*, which comprises over 250 species known for their medicinal and aromatic properties. *N. curviflora* has been traditionally used for its antispasmodic and anti-inflammatory effects [6]. Recent investigations, including the work of Barhoumi et al. [7], have begun to elucidate its essential oil composition and variability across Jordanian regions, confirming the predominance of nepetalactones among other bioactive constituents. However, scientific validation of its antimicrobial and antibiofilm potential, especially against clinically relevant pathogens, remains limited. The present study aims to evaluate the antibacterial and antibiofilm activities of *N. curviflora* essential oil against a panel of Gram-positive and Gram-negative bacteria, including MDR and ESBL-producing uropathogens. Furthermore, the study investigates the oil's chemical composition using gas chromatography–mass spectrometry (GC-MS) to identify potential bioactive constituents responsible for its observed bioactivity.

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2. Material and Methods

2.1. Plant materials and extraction of the essential oil

N. curviflora was collected in March 2020 from the Al-Karak region in Jordan. Dr. Feryal Al-Khresat from the Department of Biology at Mu'tah University in Al-Karak, Jordan successfully determined the taxonomic classification of the plant. The voucher specimens (NO: MU2020-07) were stored in the Department of Biology, Faculty of Science, Mutah University, Mutah, Jordan.

A 100 g sample of the plant material's fresh leaves subjected to hydro-distillation for 3 h utilizing simple Clevenger equipment. The aforementioned technique was repeated more than 10 times. The oil was separated from the water phase (1 L) using diethyl ether (100 mL). The diethyl ether was volatilized, and the oil was dehydrated using anhydrous sodium sulfate. Ultimately, the obtained oil was preserved at a temperature of 4 °C until it could be further examined.

2.1. GC-MS analysis

The GCMS investigation was performed using a Shimadzu qp2010 Plus (Kyoto, Japan). A column of DB5-SMS (30 m × 0.25 mm ID × 0.25 μm of film thickness) was utilized. The volume of injection was 1.0 in a ratio of 1:100. Helium was used as a mobile phase, and it was operated at a flow rate of 1 mL/min. The heating rate was programmed at 4°C/min starting from 50°C (initial temperature) to 290°C (final temperature) and held at 50°C for five minutes with a total run time of 68 min. Both the injector and transfer-line temperatures were set at 250°C. Electron ionization was the mode used in the MS with a 70 electron volt (eV) electron energy and a 250°C ion source temperature. The identification of the eluted peaks was made based on the retention time and the linear index of standard alkane compounds (C8–C20). In addition, the MS spectra of the candidate compounds were compared with their analogs in the NIST library and published data [24,31,72]. In addition, co-chromatography was performed for certain compounds under similar circumstances. The percentage of each identified compound was calculated based on the peak area of the identified compound as a proportion of the total area of all detected peaks.

2.2. Antibacterial activity

2.2.1. Bacterial species

A total of fourteen bacterial strains were used in this study, comprising both Gram-positive and Gram-negative bacteria. Clinical isolates were obtained from urine samples of patients diagnosed with urinary tract infections at Al-Karak Government Hospital (Karak, Jordan) and Al Bashir Hospital (Amman, Jordan). Identification and characterization of the bacterial species, including determination of their antibiotic resistance profiles (e.g., methicillin-sensitive, beta-lactamase-producing, and extended-spectrum beta-lactamase-producing strains), were conducted using the Biomérieux VITEK® 2 automated system (Marcy-l'Étoile, France). In addition to the clinical isolates, two reference strains—*Pseudomonas aeruginosa* ATCC 10145 and *Escherichia coli* ATCC 27922—were acquired from the American Type Culture Collection (ATCC, Manassas, VA, USA) to serve as quality controls for antimicrobial susceptibility testing.

2.2.2. Disc diffusion method:

The antibacterial activity of *N. curviflora* essential oil was evaluated initially using disc diffusion method [8]. In brief, Muller Hinton agar was inoculated with 100 μL bacterial suspension adjusted to 1×10^8 (0.5 McFarland solution). Then, 6 mm sterilized disc containing 1 μL of the essential oil was transferred to the surface of the inoculated plate. Standard antibiotic was processed similarly as positive control. The plates were then incubated at 37°C for 24h. The inhibition zone was measured in mm and the test was performed in triplicates.

2.2.3. Minimum Inhibitory Concentration (MIC)

The MIC of *N. curviflora* essential oil was determined using 96 well plate, Muller Hinton broth, and a stock solution of essential oil of 100 μL/mL DMSO (10%) [9]. The essential oil was tested at concentration equal to 50, 25, 12.5, 6.25, 3, 1.5, 0.75, 0.40, and 0.20 μL/mL. 10 μL of bacterial suspension adjusted to 1×10^8 (0.5 McFarland solution) was added to each tested well. Bacterial culture treated with equivalent concentration of DMSO and untreated culture were prepared as positive control. the plate was incubated at 37C for 24h and the absorbance at 600nm was measured using Eliza reader. The lowest concentration that prevent the visible growth of the bacterial was reported as MIC.

2.3. Antibiofilm activity

The antibiofilm activity of *N. curviflora* essential oils was evaluated using crystal violet assay [10]. A 96 well plate was prepared similar to the protocol of MIC. At the end of the incubation period (24h), the content of the wells was discarded, the well was washed, and filled with crystal violet for 15 min. Then, the crystal violet content was discarded, the well was washed, and filled with 96% ethanol for 15 min. Then, the content was

transferred to new 96 well plate and the absorbance was measured at 590 nm using Eliza reader. The percentage of biofilm inhibition was calculated relative to the untreated control, and the concentration of EO that resulted in 50% biofilm inhibition was identified as the minimum biofilm inhibitory concentration (MBIC_{50%}).

3. Results

3.1. Chemical composition of *N. curviflora*'s leaves essential oils

The chemical composition of *N. curviflora*'s leaves essential oils was analyzed using GC-MS. As shown in Table 1, where 28 compounds have been identified, representing 94.06% of the total identified compounds. The results showed that the essential oil is rich in oxygenated compounds, as indicated by the high percentage of oxygenated monoterpenes (37.26%) and oxygenated sesquiterpenes (24.11%), together accounting for more than 61% of the total composition. In contrast, the non-oxygenated compounds, including monoterpenes and sesquiterpene hydrocarbons, were present in much lower amount, reaching approximately 32%.

Additionally, 4 α ,7 α ,7 β -nepetalactone (15.9%) was identified as the most abundant compound, followed by 4 α ,7 α ,7 α -nepetalactone (10.04), E- β -ocimene (8.95), and germacrene D (8.82). The oil also contained a notable composition of Z- β -ocimene (7.89), caryophyllene (6.18%), bicyclogermacrene (5.94%), 4 α ,7 β ,7 α -nepetalactone (5.06%), α -cadinol (4.97%), and 1,8-cineole (4.58%).

Table 1: The chemical composition (GC-MS) of *N. curviflora*'s leaves essential oils

Peak	KI _{let} [11]	KI _{cal}	Compounds	%
1.	917	911	α -Pinene	1.34
2.	969	963	Sabinene	0.43
3.	989	985	β -Myrcene	0.1
4.	1010	1022	Limonene	0.15
5.	1031	1042	1,8-Cineole	4.58
6.	1037	1050	Z- β -Ocimene	7.89
7.	1050	1055	E- β -Ocimene	8.95
8.	1150	1155	Nerol oxide	0.4
9.	1180	1178	Terpinen-4-ol	2.09
10.	1364	1353	4 α ,7 α ,7 α -Nepetalactone	10.04
11.	1381	1390	4 α ,7 α ,7 β -Nepetalactone	15.09
12.	1394	1403	4 α ,7 β ,7 α -Nepetalactone	5.06
13.	1420	1421	Caryophyllene	6.18
14.	1440	1444	Aromadendrene	0.16
15.	1450	1455	(-)-Aristolene	1.65
16.	1500	1498	α -Selinene	0.41
17.	1500	1505	Germacrene D	8.82
18.	1505	1507	Bicyclogermacrene	5.94
19.	1516	1521	γ -Cadinene	0.95
20.	1556	1559	Ledol	1.07
21.	1575	1579	Spathulenol	0.56
22.	1580	1584	Caryophyllene oxide	4.3
23.	1638	1642	Cubenol	0.43
24.	1650	1652	Eudesmol	1.0
25.	1653	1655	α -Cadinol	4.97
26.	1713	1721	Farnesol	0.4
27.	1860	1860	Lanceol acetate	0.83
28.	1900	1902	Sclareoloxide	0.25
Total				94.06
Monoterpenes hydrocarbons				18.86
Oxygenated monoterpenes				37.26
Sesquiterpene hydrocarbons				13.81
Oxygenated sesquiterpenes				24.11

KI_{cal} refers to the computed KI concerning a combination of n-alkanes (C8–C31) on a DB5-SMS column. KI_{let}: KI values of the detected compounds as documented in the NIST library.

1.2. Antibacterial activity

The disc diffusion assay revealed that the essential oil of *N. curviflora* exhibited varying degrees of antibacterial activity against the tested bacterial strains. The diameter of inhibition zones ranged from 0.00 mm to 10.83 mm, indicating low to moderate activity. Among the Gram-positive bacteria, both methicillin-sensitive *Staphylococcus aureus* (MSSA) and beta-lactamase-producing *S. aureus* showed equal inhibition zones of 7.17 mm, while *Staphylococcus epidermidis* (BL) demonstrated complete resistance with a zone of 0.00 mm, indicating no detectable antibacterial activity. In contrast, Gram-negative bacteria generally showed greater susceptibility. The largest inhibition zone was observed in *Enterobacter aerogenes*, which exhibited a mean zone of 10.83 ± 0.58 mm, followed closely by ESBL-producing *Escherichia coli* and *Pseudomonas aeruginosa* ATCC 10145, each with zones exceeding 8 mm. Most other Gram-negative strains displayed inhibition zones in the range of 7 to 8 mm, except *E. coli* ATCC 27922, which was completely resistant to the essential oil.

Table 2: Inhibition zone of *N. curviflora* Essential oil (1 μ L/disc) and the positive control cefotaxime (30 μ g/disc)

		Bacteria Strain name	Essential oil	Cefotaxime mm $\pm$ SD
Gram positive bacteria		MSSA <i>Staphylococcus aureus</i>	7.17 $\pm$ 0.29	15.5 $\pm$ 1.8
		BL <i>Staphylococcus aureus</i>	7.17 $\pm$ 0.29	14.7 $\pm$ 1.2
		BL <i>Staphylococcus epidermidis</i>	0.00 $\pm$ 0.00	6.3 $\pm$ 0.6
		<i>Enterobacter agglomerans</i>	7.00 $\pm$ 0.00	8.8 $\pm$ 0.3
		ESBL <i>Escherichia coli</i>	7.00 $\pm$ 0.00	19.0 $\pm$ 0.5
		ESBL <i>Klebsiella pneumonia</i>	7.17 $\pm$ 0.29	24.2 $\pm$ 0.8
		<i>Escherichia coli</i>	7.00 $\pm$ 0.00	16.8 $\pm$ 0.6
Gram negative bacteria		ESBL <i>Escherichia coli</i>	10.67 $\pm$ 0.76	19.3 $\pm$ 0.6
		<i>Enterobacter aerogenes</i>	10.83 $\pm$ 0.58	19.3 $\pm$ 0.8
		BL <i>Pseudomonas aeruginosa</i>	7.17 $\pm$ 0.29	15.2 $\pm$ 1.0
		ESBL <i>Proteus mirabilis</i>	8.33 $\pm$ 0.29	14.8 $\pm$ 2.5
		<i>Pseudomonas aeruginosa</i> ATCC 10145	8.33 $\pm$ 0.58	20.5 $\pm$ 1.3
		<i>Escherichia coli</i> ATCC 27922	0.00 $\pm$ 0.00	18.7 $\pm$ 0.6

MSSS: Methicillin-Sensitive, BL: Beta-lactamase, ESBL: Extended Spectrum Beta-lactamase.

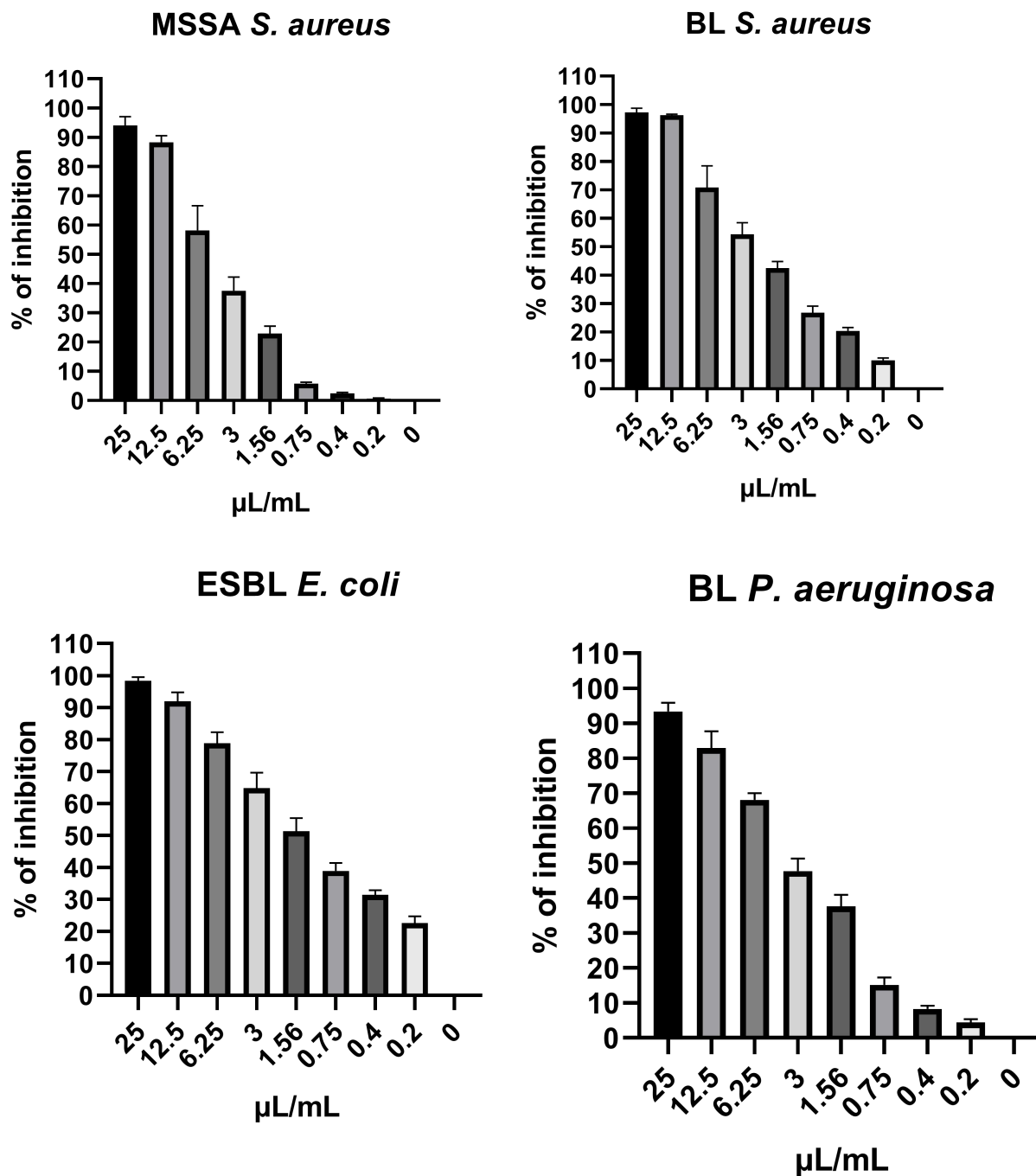
The MIC results further confirmed the antibacterial potential of *N. curviflora* essential oil, showing a range of MIC from 12.5 to 50 μ L/mL. Among the Gram-positive bacteria, both MSSA and BL *S. aureus* showed an MIC of 25 μ L/mL, whereas BL *S. epidermidis* required a higher concentration of 50 μ L/mL to inhibit growth, confirming its lower susceptibility. In contrast, most Gram-negative bacteria had MIC values at or below 25 μ L/mL, with three strains—ESBL *E. coli*, *E. aerogenes*, and BL *P. aeruginosa*—showing the lowest MICs at 12.5 μ L/mL, making them the most sensitive to the essential oil. Other Gram-negative strains such as *E. coli* ATCC 27922, *P. aeruginosa* ATCC 10145, and ESBL *P. mirabilis* exhibited MICs of 25 μ L/mL, aligning with the moderate inhibitory profile observed in the disc diffusion assay.

Table 3: MIC (μ L/mL) of *N. curviflora* essential oil

		Bacteria Strain name	MIC (μ L/mL)
Gram positive bacteria		MSSA <i>S. aureus</i>	25
		BL <i>S. aureus</i>	25
		BL <i>S. epidermidis</i>	50
Gram negative bacteria		<i>E. agglomerans</i>	50
		ESBL <i>E. coli</i>	25
		<i>E. coli</i>	25
		ESBL <i>E. coli</i>	12.5
		ESBL <i>K. pneumonia</i>	25
		<i>E. aerogenes</i>	12.5
		BL <i>P. aeruginosa</i>	12.5
		ESBL <i>P. mirabilis</i>	25
		<i>P. aeruginosa</i> ATCC 10145	25
		<i>E. coli</i> ATCC 27922	25

1.3. Antibiofilm activity

The antibiofilm efficacy of *N. curviflora* essential oil was assessed against six selected bacterial strains using the crystal violet staining assay. The tested strains included MSSA *S. aureus*, BL *S. aureus*, ESBL *E. coli*, BL *P. aeruginosa*, *P. aeruginosa* ATCC 10145, and *E. coli* ATCC 27922. The essential oil demonstrated a concentration-dependent inhibition of biofilm formation in all tested strains (Figure 1). The percentage of biofilm inhibition declined progressively with decreasing concentrations of the essential oil. The minimum biofilm inhibitory concentration (MBIC_{50%}), defined as the lowest concentration causing at least 50% inhibition of biofilm formation, was identified for each strain.



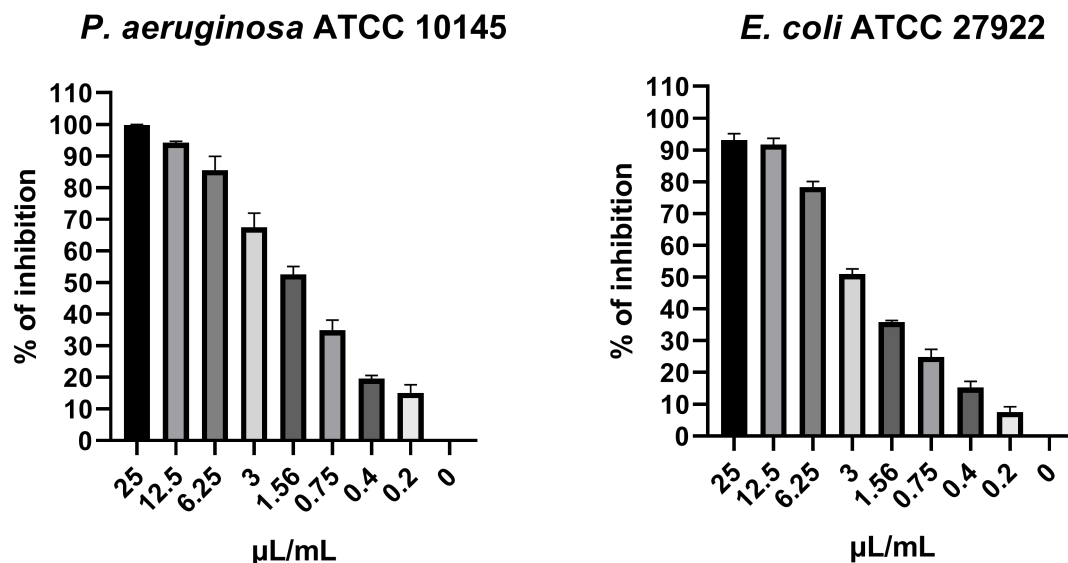


Figure 1: Percentage of biofilm inhibition in bacterial strains treated with *Nepeta curviflora* essential oil at sub-MIC concentrations.

For MSSA *S. aureus*, the MBIC_{50%} was recorded at 6.25 µL/mL, where 58.2% inhibition was observed. Similarly, BL *S. aureus* showed an MBIC_{50%} of 3 µL/mL, with 54.3% inhibition. Among Gram-negative bacteria, ESBL *E. coli* demonstrated higher susceptibility, with a 51.3% inhibition at 1.5 µL/mL. The BL *P. aeruginosa* strain had an MBIC_{50%} of µL/mL, while *P. aeruginosa* ATCC 10145 exhibited 52.6% inhibition at 1.5 µL/mL. Finally, *E. coli* ATCC 27922 had its MBIC_{50%} at 3 µL/mL, where the inhibition percentage was 50.9%.

Table 4: MBIC_{50%} of *N. curviflora* essential oil

Bacterial Strain name	MBIC _{50%} (µL/mL)	% Inhibition at MBIC _{50%}
MSSA <i>S. aureus</i>	6.25	58.2
BL <i>S. aureus</i>	3	54.3
ESBL <i>E. coli</i>	1.5	51.3
BL <i>P. aeruginosa</i>	3	47.7
<i>P. aeruginosa</i> ATCC 10145	1.5	52.6
<i>E. coli</i> ATCC 27922	3	50.9

4. Discussion

The chemical analysis of *Nepeta curviflora* essential oil (EO) by GC-MS revealed a complex phytochemical profile, rich in oxygenated monoterpenes (37.26%) and oxygenated sesquiterpenes (24.11%). The principal constituents were the nepetalactone isomers 4α,7α,7β-nepetalactone (15.09%) and 4α,7α,7α-nepetalactone (10.04%), compounds known for their antimicrobial, insect-repelling, and cytotoxic properties [12–14]. Other notable constituents included E-β-ocimene (8.95%), germacrene D (8.82%), Z-β-ocimene (7.89%), caryophyllene (6.18%), 1,8-cineole (4.58%), and caryophyllene oxide (4.30%)—all previously associated with antibacterial and anti-inflammatory effects [15–17].

Comparison with previously published data highlights clear chemotypic variability within *N. curviflora* EO across different geographical and environmental contexts. Al-Maharik et al. (2020) reported an EO from *N. curviflora* collected in Palestine that was dominated by 1,6-dimethylspiro [4.5] decane (27.51%), caryophyllene oxide (20.08%), and β-caryophyllene (18.28%), with no nepetalactones detected. The high presence of spiroalkanes and sesquiterpenes in their study suggests a different chemotype, likely resulting from ecological or genetic variation. In contrast, Al-Qudah (2016) analyzed Jordanian *N. curviflora* and found that air-dried samples had a composition similar to ours—rich in oxygenated monoterpenes (50.31%), and dominated by 4α,7α,7α-

nepetalactone (43.85%)—supporting the conclusion that post-harvest processing and drying significantly influence oil chemistry, enhancing bioactive compound levels [18,19].

The antibacterial assessment of our EO revealed greater activity against Gram-negative bacteria, with inhibition zones up to 10.83 mm and MICs as low as 12.5 $\mu\text{L/mL}$, particularly against *E. aerogenes*, *ESBL E. coli*, and *P. aeruginosa* ATCC 10145. These findings challenge the typical assumption that Gram-negative bacteria are more resistant due to their impermeable outer membrane. Interestingly, Al-Maharik et al. (2020) reported bacterial inhibition at lower concentrations (0.125–6.25 $\mu\text{L/mL}$), using a well diffusion method and testing different strains. While their EO showed strong activity, the absence of nepetalactones and the presence of spiroalkanes in their oil may suggest differences in the primary mechanisms of action [18].

Crucially, our EO retained potent activity against ESBL-producing strains, which are commonly resistant to β -lactam antibiotics. This strongly suggests that the antimicrobial mechanisms of *N. curviflora* EO are independent of β -lactam targets—likely involving membrane disruption, metabolic interference, or quorum sensing modulation. Such mechanisms offer distinct advantages in treating persistent, biofilm-associated infections and represent a promising alternative to conventional antibiotic therapies [20].

The EO also exhibited notable antibiofilm activity, with MBIC₅₀ % values ranging from 1.5 to 6.25 $\mu\text{L/mL}$ across resistant Gram-positive and Gram-negative strains. The highest inhibition was observed against *ESBL E. coli* and *P. aeruginosa* ATCC 10145, both achieving over 50% inhibition at just 1.5 $\mu\text{L/mL}$. These findings align with those of Qaralleh (2023), who reported a dose-dependent reduction in biofilm formation by *P. aeruginosa* treated with *N. curviflora* methanolic extract—showing 66.3%, 37.7%, and 18.3% inhibition at 2.5, 1.25, and 0.6 mg/mL, respectively. While different extraction methods were used (methanol vs. hydrodistilled EO), the consistency across studies underscores the robust antibiofilm potential of this species [21].

The lipophilic nature of essential oils likely contributes to their superior biofilm inhibition, facilitating penetration of the biofilm matrix and disruption of bacterial membranes [22]. Compounds such as nepetalactones, 1,8-cineole, and caryophyllene oxide, which are abundant in our EO, have been implicated in quorum sensing inhibition, early adhesion interference, and biofilm dispersal [23,24]. These effects operate independently of bacterial growth inhibition, allowing the EO to impair pathogenicity at sub-MIC levels [25].

The ability of *N. curviflora* essential oil to inhibit biofilm formation at concentrations below the MIC is particularly noteworthy, as it indicates a mechanism beyond bactericidal action. Such sub-lethal effects strongly imply disruption of biofilm-regulating pathways, such as cell-cell communication, surface adhesion, and quorum sensing. This approach holds significant therapeutic promise, as it could reduce the emergence of resistance by attenuating virulence rather than exerting direct killing pressure [26].

5. Conclusion

The study confirms the broad-spectrum antimicrobial and antibiofilm efficacy of *N. curviflora* essential oil and attributes this bioactivity to its rich composition in nepetalactones and oxygenated terpenes. These findings not only support the traditional use of *Nepeta* species in infection management but also underscore their potential as natural alternatives or adjuncts in combating drug-resistant pathogens.

6. Conflicts of interest

There are no conflicts to declare.

7. References

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