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GC-MS Analysis and Antibacterial Activity of *Terminalia chebula* Against β-lactam-resistant *Kelebsiella pneumoniae*

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ABSTRACT

Background: The increasing resistance of *Klebsiella pneumoniae* to β-lactam antibiotics poses a major challenge in treating hospital-acquired infections. Plant-derived compounds with antibacterial properties offer promising alternatives or complementary therapies. This study aimed to evaluate the antibacterial activity of the hydroalcoholic extract of *Terminalia chebula* fruit against β-lactam-resistant *K. pneumoniae* strains and identify its bioactive constituents.

Materials and Methods: Fruits of *T. chebula* were collected from the Minab region, Hormozgan Province, Iran, and extracted using 70% ethanol via maceration. Antibacterial activity was assessed using disk diffusion, well diffusion, minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC) assays. The chemical composition of the extract was analyzed by gas chromatography–mass spectrometry (GC-MS).

Results: The hydroalcoholic extract demonstrated significant inhibitory activity against β-lactam-resistant *K. pneumoniae*. GC-MS analysis identified major bioactive compounds, including 1,2,3-Benzenetriol (64.19%), phenols (5.15%), benzoic acid (4.43%), and aldehyde derivatives (3.84%). In total, 86.89% of detected compounds are known for antibacterial activity.

Conclusion: The hydroalcoholic extract of *T. chebula* exhibits promising antibacterial activity against β-lactam-resistant *K. pneumoniae* and represents a potential natural source for the development of novel antibacterial agents.

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Introduction

The increasing antibiotic resistance among pathogenic bacteria has become one of the most serious public health threats in recent decades. The emergence of bacterial strains, such as *Klebsiella pneumoniae*, which are resistant to most available antibiotics, including the β-lactam class, particularly in developing and underdeveloped countries, has created significant treatment challenges for healthcare systems and raised global concerns. The World Health Organization (WHO) has repeatedly warned about the global spread of multidrug-resistant (MDR) and even untreatable pathogens, stressing the urgent need for novel antimicrobial agents and alternative therapeutic approaches [1, 2].

In recent years, natural products, particularly medicinal plants, have gained attention as promising candidates in the search for new antimicrobial agents. Plants represent a rich source of structurally diverse bioactive compounds with pharmacological potential, many of which have already contributed to the development of modern medicines. Their accessibility, cost-effectiveness, and relatively lower side effects make them especially important for countries where access to expensive synthetic drugs is limited [3].

Among the many plants studied for their therapeutic potential, *Terminalia chebula* has been traditionally recognized in Ayurvedic, Unani, and other systems of medicine as a "king of medicines" due to its wide spectrum of healing properties. Various parts of *T. chebula* are used to treat digestive disorders, respiratory diseases, skin infections, and inflammatory conditions. Modern phytochemical investigations have revealed that the plant contains diverse secondary metabolites, including phenols, flavonoids, tannins, and glycosides, which are known to possess strong antioxidant, anti-inflammatory, and antimicrobial effects [4, 5].

The potential of *T. chebula* in combating antibiotic-resistant bacteria has attracted increasing scientific interest. Several reports suggest that its phytoconstituents interfere with bacterial cell walls, protein synthesis, or enzymatic activity, thereby suppressing the growth of resistant strains [4]. Moreover, the use of advanced analytical techniques, such as gas chromatography–mass spectrometry (GC-MS), has facilitated the identification of bioactive compounds in plant extracts, enabling a deeper understanding of their pharmacological significance.

Taken together, these considerations underscore the importance of further investigating *T. chebula* as a natural source of antibacterial agents. Strengthening the scientific evidence for its effectiveness may not only validate its traditional use but also contribute to the global efforts in finding alternative strategies against MDR pathogens.

Objective

The objective of this study was to evaluate the antibacterial activity of the hydroalcoholic extract of *T. chebula* against β-lactam-resistant *K. pneumoniae* and to identify the major phytochemical compounds through GC-MS to explore their potential role in antibacterial action.

Materials and Methods

Setting

This research was conducted in Minab County, Hormozgan Province, Iran, located approximately 90 km southeast of Bandar Abbas (27°06′ N, 57°08′ E). The region lies at an average altitude of 200 m above sea level and has a hot, arid climate with scorching summers and mild winters. Annual precipitation ranges between 100 and 200 mm, primarily in winter. These environmental conditions support the growth of several medicinal plant species, including *T. chebula*, which was the focus of this study [4].

Plant identification and collection

Fieldwork was carried out in 2024 in the Minab region to locate and collect *T. chebula* from its natural habitat. Specimens were authenticated by taxonomists at the Herbarium of the Islamic Azad University, Gorgan Branch. The fruits were dried in a dark, cool, and well-ventilated environment, ground into fine powder using a Panasonic MJ-J176P grinder (Japan), and stored in airtight containers at 4 °C until extraction [4].

Extract preparation

The powdered fruit (5 g) was extracted by cold maceration in 50 mL of 70% ethanol (Merck, Germany) at 4 °C for 14 days. The mixture was centrifuged at 4000 rpm for 20 min (Behdad BH-1200, Iran), and the supernatant was concentrated using a rotary evaporator under reduced pressure. The extract was further dried at room temperature for 48 h and stored at 4 °C in sealed containers. The final yield was 1.5 g of crude extract [4].

GC–MS analysis

Chemical constituents of the extract were analyzed by GC–MS (Agilent 6890N GC coupled with Agilent 5973 MS, USA). Helium was used as the carrier gas at a flow rate of 1.0 mL/min with a CP-5 capillary column (30 m × 0.25 mm ID×0.25 µm film thickness). The oven program was set to 60 ˚C for 1 min, then ramped to 280 ˚C at a rate of 3 ˚C/min and held for 10 min. The injection volume was 1–2 µL in splitless mode. Mass spectra were acquired using EI at 70 eV with a scan range of m/z 40–550. Compounds were identified by comparing retention indices and mass spectra with published data and the Wiley spectral library [4].

Bacterial strain and culture conditions

K. pneumoniae (PTCC 1290) was obtained in lyophilized form from the Iranian Research Organization for Science and Technology. The strain was revived in brain heart infusion (BHI) broth (Merck, Germany) at 37 ˚C for 24 h. Fresh colonies were suspended in Nutrient Broth (Himedia, India) and adjusted to the 0.5 McFarland standard (~1.5×10^8 CFU/mL) [4, 6, 7].

Antibacterial assays

Well diffusion method

The antibacterial activity of *T. chebula* extract was determined using the agar well diffusion assay. A standardized inoculum (0.5 McFarland) was spread onto Mueller–Hinton Agar (Ibresco, Iran). Wells (7 mm diameter) were aseptically punched and filled with 100 µL of the extract. The plates were incubated at 37 ˚C for 24–48 h, and the inhibition zones were measured. Tests were performed in triplicate. Inhibition diameters of <9 mm were interpreted as resistant, 9–12 mm as moderately sensitive, and >12 mm as sensitive [4, 8, 9].

Disk diffusion method

The antibiotic susceptibility of *K. pneumoniae* was evaluated using the standard disk diffusion method on MHA, following CLSI guidelines. Antibiotic disks (Padtan Teb, Iran) included penicillin G (10 U), ampicillin (10 µg), penicillin (10 U), and ceftriaxone (30 µg). The plates were incubated at 37 ˚C for 24–48 h, and the inhibition zones were measured [4, 8, 9].

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

MIC and MBC were determined by using the broth microdilution method. Serial dilutions of the extract were prepared in 96-well plates to obtain concentrations of 100, 50, 25, 12.5, 6.25, 3.12, 1.56, and 0.78 mg/mL using 10% DMSO as the solvent. Each well received 100 µL of the diluted extract and 100 µL of bacterial suspension (final inoculum: 5×10^5 CFU/mL) (Equations 1 and 2) [4, 7, 10].

1. Positive control: Bacterial suspension + 10% DMSO (without extract)

2. Negative control: Broth + Extract (without bacteria)

Plates were incubated at 37 ˚C for 24 h. MIC was defined as the lowest concentration without visible turbidity. For MBC, aliquots from wells with no turbidity were cultured on MHA and incubated at 37 ˚C for 24 h. The lowest concentration showing no bacterial growth was recorded as MBC [4, 7, 9].

Statistical analysis

All experiments were performed in triplicate. Data are expressed as Mean±SD. Statistical analyses were performed using SPSS software, version 26 (IBM Corp., Armonk, NY, USA). Independent t-tests were applied, and differences with $P<0.05$ were considered significant [4, 10].

Results

Antibiotic susceptibility testing of bacterial isolates

Antibiotic susceptibility testing for *K. pneumoniae* isolates was performed using the disk diffusion method. None of the tested antibiotics showed any inhibition zones against *K. pneumoniae*, indicating complete resistance to all antibiotics used (Table 1).

Assessment of the antibacterial activity of *T. chebula* extract via agar well diffusion assay

The antibacterial effect of the hydroalcoholic extract of *T. chebula* was assessed using the agar well diffusion method at two concentrations. The extract exhibited dose-dependent inhibitory activity against *K. pneumoniae*, with the inhibition zone decreasing from 18.50±0.50000 mm at 100 mg/mL to 11.66667±1.25831 mm at 50 mg/mL.

Table 1. Results of the antibiotic disk diffusion assay

Antibiotic	*K. pneumoniae*
Ampicilin	R
Ceftriaxon	R
Penicillin G	R
Penicillin	R

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R: Resistance, indicating the absence of any measurable inhibition zone for the corresponding antibiotic against the tested bacterial strain.

Table 2. Inhibition zone diameters of *T. chebula* extract against *K. pneumoniae*

Extract Concentration (mg/mL)	Inhibition Zone Diameter (mm)
100	18.5000±0.50000[a]
50	11.6667±1.25831[b]

[a]100 mg/mL, [b]50 mg/mL.

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An independent t-test confirmed a statistically significant difference between the two concentrations (P=0.002). Zones of inhibition were categorized into different statistical groups: "a" for 100 mg/mL and "b" for 50 mg/mL. The inhibition zone at 100 mg/mL was more than 1.5-fold greater than that at 50 mg/mL, confirming the dose-dependent antibacterial activity of the extract (Table 2).

Values represent Mean±SD from three independent replicates. Different superscript letters indicate statistically significant differences (P<0.05).

Determination MIC and MBC

The extract antibacterial activity test at different dilutions showed that the MIC of the *T. chebula* extract against *K. pneumoniae* was 12.5 mg/mL, at which partial inhibition of bacterial growth was observed. The MBC value was determined to be 25 mg/mL, where complete bacterial killing occurred.

These findings suggest that the extract possesses moderate antibacterial activity, demonstrating a clear bacteriostatic effect at lower concentrations and a bactericidal effect at higher concentrations. The MIC and MBC values provide useful benchmarks for future comparative studies and indicate potential therapeutic relevance.

Analysis of essential phytoconstituents in *T. chebula* by GC-MS

The GC-MS analysis of *T. chebula* extract revealed 1,2,3-benzenetriol (CAS No. 000087-66-1) at 64.19%, phenol (CAS No. 108-95-2) at 5.15%, benzoic acid (CAS No. 000099-06-9) at 4.43%, 2-furancarboxaldehyde, 5- (CAS No. 000067-47-0) at 3.84%, and hexadecanoic acid (CAS No. 000057-10-3) at 3.53%. Together, these compounds comprised over 80% of the identified constituents in the extract (Figure 1).

Discussion

The biological activity of plant extracts largely stems from their chemically diverse bioactive constituents, which may act alone or synergistically to produce pharmacological effects [11]. *T. chebula* was chosen for this study due to its high tannin content (30–40%) and rich phytochemical composition. Tannins are known for their antibacterial activity by penetrating bacterial membranes and disrupting internal structures [11, 12]. Other notable constituents include chebulic acid, gallic acid, ellagic acid, amino acids, and flavonoids, all linked to various therapeutic properties, such as antioxidant, antimicrobial, and anticancer effects [13, 14].

In our study, the hydroalcoholic extract of *T. chebula* exhibited notable antibacterial activity, specifically against β-lactam-resistant *K. pneumoniae*. This observation supports previous reports that highlight the antimicrobial potential of *T. chebula*, particularly due to

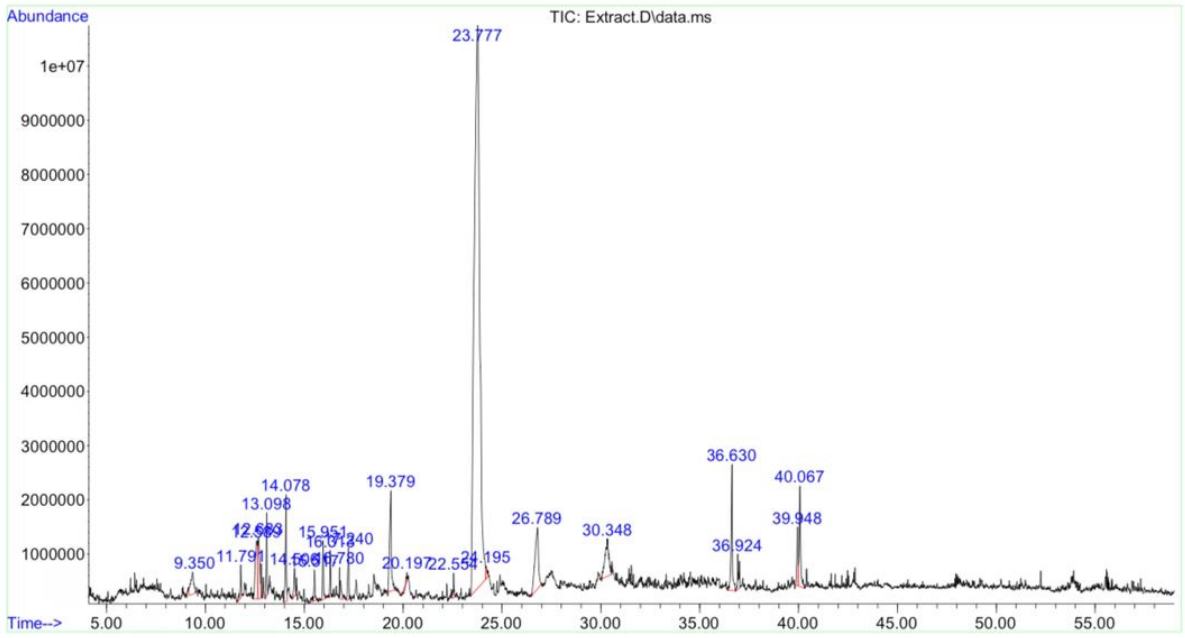

Figure 1. GC-MS chromatogram showing the chemical profile of the plant extract and the retention times of its major bioactive compounds

its phenolic compounds and hydrolyzable tannins. The results suggest that the extract may interfere with bacterial survival mechanisms, making it a promising natural agent against resistant *K. pneumoniae* strains.

GC-MS analysis identified 1,2,3-benzenetriol (pyrogallol) as the dominant compound (over 64%), a notably higher concentration than the 21–43% reported by Thoithoisana Devi et al. [15]. This compound has been associated with antioxidant and anticancer properties, including apoptosis induction in colon cancer cells [15]. Its multifunctionality is further supported by the study by Wang et al. which demonstrated its role in oxidative stress modulation and activation of antioxidant defenses [16]. Additionally, chebulinic acid—a major component—has shown inhibitory activity against *Mycobacterium tuberculosis* DNA gyrase, including resistant strains, by interfering with DNA binding [17].

Overall, the synergistic effects of the phytochemical compounds in *T. chebula* underpin its antibacterial and antioxidant potential, positioning it as a promising candidate for further pharmacological research and development of natural therapeutics.

Importantly, the MIC and MBC determinations provide deeper insights into the potency of the extract. The MIC of 12.5 mg/mL indicates that bacterial growth can be inhibited at relatively moderate concentrations, while the MBC of 25 mg/mL demonstrates the extract's ability to achieve bactericidal effects at higher concentrations. The relatively narrow gap between MIC and MBC suggests that only a small increase in concentration is required to shift from bacteriostatic to bactericidal action. Clinically, this dual behavior is relevant, as it allows flexibility in using the extract either as a suppressive agent or as a direct bactericidal treatment depending on the therapeutic need. Furthermore, considering that the strain tested was resistant to all conventional antibiotics used in the study, these findings underscore the potential of *T. chebula* as an alternative treatment option where standard therapies fail.

Moreover, Mahmood Janlou et al. showed that both ethanolic and propanolic extracts of *T. chebula* exhibit significant antibacterial activity against β-lactam-resistant gastrointestinal pathogens, with key compounds, such as 1,2,3-benzenetriol (pyrogallol) and propanoic acid, likely acting by disrupting bacterial cell walls and metabolic functions. These findings further support the antibacterial potential of *T. chebula* and align with our observations, reinforcing its role as a promising natural therapeutic agent [4].

The GC-MS analysis provided further insights into the chemical profile of the extract. The dominant compound identified was 1, 2, 3-benzenetriol (pyrogallol), which represented over 64% of the composition—substantially higher than the 21–43% previously reported by Thoithois-

ana Devi et al. [15]. This compound has been associated not only with antioxidant and anticancer properties, such as apoptosis induction in colon cancer cells [15], but also with modulation of oxidative stress and activation of cellular antioxidant defenses [16]. Additionally, chebulinic acid—a key phytochemical in *T. chebula*—has demonstrated inhibitory activity against *M. tuberculosis* DNA gyrase, including resistant strains, by disrupting DNA binding [17]. These findings highlight the multifunctionality of *T. chebula* constituents and their possible synergistic roles in antimicrobial action.

Overall, the synergistic effects of the phytochemical compounds in *T. chebula* underpin its antibacterial and antioxidant potential, positioning it as a promising candidate for pharmacological development.

Future research should aim to: 1) isolate and test individual active compounds to identify the specific agents responsible for antibacterial activity, 2) explore synergistic interactions between *T. chebula* extract and conventional antibiotics to assess its potential role as an adjuvant therapy, 3) conduct in vivo studies to confirm antibacterial efficacy, safety, and pharmacokinetics, and 4) investigate formulation strategies (e.g. nanoencapsulation) to enhance bioavailability and reduce the effective MIC and MBC values in clinical applications. By building on these findings, *T. chebula* could play an important role in addressing the urgent challenge of MDR bacterial infections.

Conclusion

The hydroalcoholic extract of *T. chebula* contains a high proportion of bioactive compounds, including 1, 2, 3-benzenetriol, phenol, benzoic acid, and hexadecanoic acid, which together account for over 80% of the identified constituents. This extract exhibited considerable antibacterial activity against β-lactam-resistant *K. pneumoniae*, underscoring its potential as a natural antibacterial agent. Given its significant inhibitory effects, *T. chebula* may be considered a promising complementary or alternative therapy to conventional antibiotics for managing infections caused by resistant *K. pneumoniae*.

Ethical Considerations

Compliance with ethical guidelines

There were no ethical considerations to be considered in this research.

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Authors' contributions

Conceptualization: Hadi Koohsari and Aryan Sateei; Investigation: Hadi Koohsari and Mohammad Kordkatouli; Data curation: Hadi Koohsari, Mohammad Kordkatoul, and Aryan Sateei; Software and resources: Mehr Ali Mahmood Janlou and Mohammad Kordkatouli; Validation: Hadi Koohsari and Mehr Ali Mahmood Janlou; Writing the original draft: Mohammad Kordkatouli and Aryan Sateei; Supervision, project administration, review and editing: Hadi Koohsari, Aryan Sateei, and Mehr Ali Mahmood Janlou; Methodology, formal analysis, visualization, and funding acquisition: All authors.

Conflicts of interest

The authors declared no conflict of interest.

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