Research Paper

Catalase Inhibition by *Terminalia chebula* Extracts: A Phytochemical Profiling Study on Resistant *Klebsiella pneumoniae*

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Citation Koohsari H, Maroufi M, Kordkatouli M, Sateei A. Catalase Inhibition by *Terminalia chebula* Extracts: A Phytochemical Profiling Study on Resistant *Klebsiella pneumoniae*. Immunoregulation. 2025; 8:E21. http://dx.doi.org/10.32598/Immunoregulation.8.21

doi: **http://dx.doi.org/10.32598/Immunoregulation.8.21**

Article info:
Received: 21 Mar 2025
Accepted: 15 Apr 2025
Available Online: 10 Jun 2025

Keywords:
Antibacterial, Catalase inhibition, Iran, *Klebsiella pneumoniae*, *Terminalia chebula*

ABSTRACT

Background: The rise of antibiotic resistance among pathogenic bacteria, particularly *Klebsiella pneumoniae* resistant to β-lactam antibiotics, poses a major challenge to global healthcare systems. Natural products with antibacterial properties may offer alternative therapeutic options.

Materials and Methods: This study evaluated the antibacterial activity of ethanolic and propanolic extracts of *Terminalia chebula* fruit against a β-lactam-resistant *K. pneumoniae* strain. Catalase inhibitory activity was assessed qualitatively, and phytochemical profiles were determined using gas chromatography–mass spectrometry (GC–MS). Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined using standard assays.

Results: Antibiotic susceptibility tests confirmed complete resistance of the bacterial strain to all tested β-lactam antibiotics. Both extracts inhibited bacterial growth at a concentration of 12.5 mg/mL dilution (MIC) and eradicated the bacteria at a 25 mg/mL dilution (MBC). Catalase activity was fully inhibited at concentrations as low as 6.25 mg/mL. Gas GC–MS analysis revealed that 1, 2, 3-benzenetriol (pyrogallol) was the dominant compound in the ethanolic extract, while the propanolic extract contained several bioactive compounds, notably D-limonene. Both compounds are known catalase inhibitors, promoting reactive oxygen species (ROS) accumulation and impairing bacterial antioxidant defenses.

Conclusion: *T. chebula* extracts exhibit potent antibacterial effects against β-lactam-resistant *K. pneumoniae*, primarily by inhibiting catalase and inducing oxidative stress. The presence of bioactive phytochemicals such as pyrogallol and D-limonene likely contributes to these effects, either individually or synergistically. These findings highlight the potential of *T. chebula* as a natural adjunct therapy for combating drug-resistant bacterial infections.

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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 exhibit resistance to most available antibiotics, including the β-lactam class, particularly in low- and middle-income countries, has created significant treatment challenges for healthcare systems and raised global concerns. The World Health Organization (WHO) has repeatedly warned about the spread of multidrug-resistant extensively drug-resistant or untreatable pathogens [1, 2].

In response to this crisis, extensive research has focused on utilizing natural resources, especially medicinal plants. One such plant is *Terminalia chebula*, traditionally recognized in Ayurvedic medicine as one of the most effective medicinal plants. It contains various bioactive compounds, such as phenols and tannins, which exhibit anti-inflammatory, antioxidant, digestive, respiratory, and antibacterial properties [3].

In recent years, detailed investigations into the antibacterial mechanisms of this plant have intensified, including its effects on bacterial defense enzymes, such as catalase. The catalase enzyme plays a vital role in neutralizing reactive oxygen species (ROS) and contributes to bacterial resistance against oxidative stress. Inhibiting this enzyme may be an effective strategy to weaken resistant bacteria [4, 5].

Objective

This study aimed to evaluate the antibacterial activity of the hydroalcoholic extract of *T. chebula* against β-lactam-resistant *K. pneumoniae* and to assess the effect of this extract on the catalase enzyme activity of the bacterium. Additionally, phytochemical compounds in the extract were identified using gas chromatography–mass spectrometry (GC-MS) analysis to explore the relationship between bioactive compounds and their inhibitory effects. This research could contribute to introducing natural alternatives for combating resistant pathogens and developing novel therapeutic strategies.

Materials and Methods

Study area and climatic description

The study was conducted in Minab County, Hormozgan Province, southeastern Iran (27.107 °N, 57.089 °E; elevation 29.6 m), approximately 90 km from Bandar Abbas. The region has a hot semi-arid climate with extremely hot summers, mild winters, scarce seasonal rainfall (<75 mm/year), and high solar radiation. In 2024, daily maximum temperatures ranged from 26.8 °C in January to 49.7 °C in June, while daily minimum temperatures varied from 6.5 °C in December to 30.1 °C in July. Relative humidity peaked in winter (up to 100%) and reached its lowest level in late spring and early summer (≈33%). Winds were predominantly from the southwest (225°) with speeds of 1.4–3.2 m/s, gusting up to 14.2 m/s. These climatic extremes—high temperatures, drought, and intense solar radiation—represent key ecological stressors that can influence the biosynthesis of secondary metabolites in medicinal plants such as *T. chebula* [1, 6].

Plant identification, collection, and preparation

Field work was carried out from early 2024 to early 2025 in various natural habitats across the Minab region, with several trips conducted in different seasons. The main collection took place in late autumn and early winter, which corresponds to the optimal period for harvesting specimens with the highest levels of phytochemical compounds. The collected plant species were identified and botanically authenticated by taxonomists, and a voucher specimen (Voucher No. MPRC 1424) was deposited at the herbarium of the Medicinal Plants Research Center, Islamic Azad University, Gorgan, Iran.

Specifically, fruits of *T. chebula* were harvested in their mature stage. Post-collection, the plant materials were stored under optimal drying conditions (cool, dark, and low-humidity environment). Once fully dehydrated, the fruits were finely ground using a Panasonic MJ-J176P grinder (Japan) to obtain a homogeneous powder suitable for further extraction and phytochemical analysis. The prepared powder was stored in airtight containers in a cool, dry place to maintain sample integrity and avoid degradation or contamination prior to laboratory experimentation [1, 6].

Preparation of *T. chebula* extract

The extraction process was performed using the cold maceration method. A 70% ethanol solution (Merck, Germany) was prepared and used as the extraction solvent. First, 5 g of *T. chebula* fruit powder were macerated with 50 mL of the ethanol solution and stored at 4 °C for two weeks. After the extraction period, the mixture was centrifuged (Behdad BH-1200, Iran) at 4000 rpm for 20 minutes, and the supernatant was carefully collected. The solvent was then removed by vacuum distil-

lation using a rotary evaporator (Heidolph Hei-VAP Expert, Germany) under vacuum at 55–65 °C and 90–120 rpm for 10–15 minutes. The remaining extract was completely dried at room temperature for 48 hours and stored in a sealed container at 4°C until further analysis. A total of 1.50 g of ethanol extract was obtained from the initial 5 g of *T. chebula* powder [1, 6].

Following ethanol extraction, 96% (v/v) n-propanol (Merck, Germany) was used to further extract bioactive compounds remaining in the plant residue. After collecting the ethanol extract, 50 mL of propanol was added to the residual plant powder, and the mixture was macerated at 4 °C for two weeks. After this period, the extract was subjected to the same centrifugation, concentration, and drying process as the ethanol extract. A total of 0.7 g of propanol extract was obtained.

Both the ethanol and n-propanol extracts were subjected to GC-MS analysis to identify phytochemical components. This allowed for comparative evaluation of the extraction efficiency and diversity of compounds obtained with each solvent.

Analysis of *T. chebula* extracts using gas chromatography–mass spectrometry

The chemical constituents of *T. chebula* extracts were analyzed using GC-MS. For this, 2 µL of the concentrated extract was injected into the GC-MS system. The analysis was conducted using an Agilent 6890 Series gas chromatograph and an Agilent 5973 Network mass selective detector.

Compound separation was performed on an Agilent HP-5MS capillary column (30 m×0.25 mm×1.00 µm). Helium (He) of 99.999% purity was used as the carrier gas at a constant flow rate of 1.0 mL/min. The injection was performed in split mode (split ratio 1:5), and the injector temperature was set at 280 °C.

The oven temperature was programmed as follows: 60 °C (held for 2 minutes), then increased at 5 °C/min to 280°C and held for 20 minutes. The total run time was 66 minutes.

Mass spectrometry was conducted using a quadrupole analyzer with electron impact ionization at 70 eV energy. The mass range analyzed was m/z 40–500, and the solvent delay was set to 3 minutes. The ion source temperature was maintained at 230 °C, and the quadrupole temperature was set at 150 °C [6].

Compound identification was carried out by comparing the retention times (RT) and mass spectra with reference data from the literature, the National Institute of Standards and Technology (NIST) mass spectral library, and the Wiley mass spectral library.

Bacterial strain

In this study, *K. pneumoniae* (PTCC 1290) was used as the test strain. The lyophilized strain was obtained from the Iranian Research Organization for Science and Technology. For activation, the strain was inoculated into Brain Heart Infusion medium (Merck, Germany) and incubated at 37 °C for 24 h. After incubation, several colonies were collected from the fresh culture and transferred to Nutrient Broth (Himedia, India), followed by additional incubation for 1–2 h until turbidity reached 0.5 McFarland (approximately 1.5×10^8 CFU/mL) [6, 7, 8].

Susceptibility and resistance testing to β-lactam antibiotics

This study aimed to assess the susceptibility and resistance profile of the *K. pneumoniae* strain against selected β-lactam antibiotics. For this purpose, a bacterial suspension adjusted to 0.5 McFarland standard was spread on Mueller-Hinton Agar. Standard β-lactam antibiotic discs (Padtan Teb Co., Iran) were applied, including penicillin G (10 U), ampicillin (10 µg), penicillin V (10 U), cefixime (10 µg) and ceftriaxone (30 µg). The discs were evenly placed on the agar surface and incubated at 37 °C for 24–48 h. After incubation, the diameters of inhibition zones around each disc were measured, and susceptibility was interpreted based on the guidelines of the clinical and laboratory standards institute (CLSI) [1, 9, 10]. This allowed for a systematic evaluation of resistance or sensitivity to β-lactam antibiotics in the test strain.

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

A stock solution of the pure extract was prepared at a concentration of 100 mg/mL in 10% dimethyl sulfoxide (Merck, Germany). Serial dilutions of this stock were prepared in nutrient broth to obtain a range of test concentrations. In a sterile 96-well microplate (Costar®, Cat. No. 3599, Corning Inc., NY, USA), 100 µL of each dilution was dispensed, and a bacterial suspension adjusted to 5×10^5 CFU/mL was added.

The standard broth microdilution method was used to determine the MIC and MBC. The microplates were incubated at 37 °C for 24 hours. In addition to the experimental groups, positive control (bacterial suspension without extract) and negative control (Mueller-Hinton broth [Ibresco, Italy] without bacteria) were included [1, 6-9].

After incubation, turbidity changes in each well were visually assessed. The lowest concentration showing no visible turbidity was recorded as the MIC. Samples with concentrations equal to or higher than the MIC that showed no turbidity were sub-cultured on Mueller-Hinton Agar and incubated at 37 °C for another 24 h. The lowest concentration with no colony growth was determined as the MBC. All tests were performed in triplicate, and results were presented as the average values [1, 6-10].

Qualitative assessment of catalase enzyme activity in the presence of propanol and ethanol extracts of *T. chebula*

To investigate the effect of n-propanol and ethanol extracts of *T. chebula* on catalase enzyme activity in bacteria, two methods were employed. All experiments were performed in triplicate to ensure reproducibility. In both methods, enzyme activity was assessed separately for bacterial suspension alone, extract alone, and the mixture of both.

The bacterial suspension was first adjusted to 0.5 McFarland. Then, 300 µL of propanol and ethanol extracts at concentrations of 100, 50, 25, 12.5, 6.25, 3.12, 1.56, and 0.78 mg/mL were mixed with 1500 µL of the bacterial suspension. The mixtures were incubated at 37 °C for 4 hours. After incubation, 10 µL of each sample was placed on a sterile glass slide, followed by the addition of 10 µL of 3% hydrogen peroxide (H_2O_2; Merck, Germany). Bubble formation was observed to qualitatively assess catalase enzyme activity [11].

Results

Analysis of *T. chebula* extracts using gas chromatography–mass spectrometry

The major chemical constituents of the ethanol and propanol extracts of *T. chebula* were identified using GC–MS. Compound identification was performed by comparison with the Wiley and NIST spectral libraries.

For the ethanol extract, the main compounds identified included phenol, 5-(hydroxymethyl) furfural, 1, 2, 3-benzenetriol (pyrogallol), benzoic acid, imperialine, hexadecanoic acid, 9, 12-octadecadienoic acid, and 9, 17-octadecadienal (Table 1; Figure 1). These compounds are primarily phenolics, terpenoids, and fatty acids, which may contribute to the biological activities of the plant.

In the propanol extract, the identified compounds included propyl propionate, D-limonene, heptadecane, ethyl palmitate, ethyl linoleate, and petroselinic acid (Table 2; Figure 2). This extract also contained phenolic and fatty acid compounds, although their relative abndances differed from those in the ethanol extract, likely due to differences in solvent polarity during extraction.

Table 1. Gas chromatography-mass spectrometry identified compounds in the ethanolic extract of *T. chebula*, arranged according to peak number and RT

Peak No.	RT Minute	Area (%)	CAS No.	Qual (%)	Compound Name	Molecular Formula
3, 4	12.590, 12.681	2.66+2.49	000108-95-2	91	Phenol	C_6H6_O
13, 14	19.382, 20.200	3.82+0.02	000067-47-0	87	5-(Hydroxymethyl) furfural	$C_6H_6O_3$
16, 17	23.776, 24.194	63.83+0.36	000087-66-1	95	1,2,3-Benzenetriol	$C_6H_6O_3$
18	26.792	4.43	000099-06-9	97	Benzoic acid	$C_7H_6O_2$
19	30.351	2.87	999206-18-6	74	Imperialine	$C_{27}H_{43}NO_3$
20	36.628	3.53	000057-10-3	99	Hexadecanoic acid	$C_{16}H_{32}O_2$
22	39.946	1.33	000060-33-3	93	9,12-Octadecadienoic acid	$C_{18}H3_2O_2$
23	40.066	2.88	056554-35-0	95	9,17-Octadecadienal	$C_{18}H_{32}O$

RT: Retention time.

Compounds marked were confirmed based on reference standards.

Table 2. Gas chromatography-mass spectrometry identified compounds in the propanolic extract of *T. chebula*, arranged according to peak number and RT

Peak No.	RT Minute	Area (%)	CAS No.	Qual (%)	Compound Name	Molecular Formula
1	6.51	40.46	000106-36-5	78	Propyl propionate	$C_6H_{12}O_2$
3	13.17	4.28	005989-27-5	97	D-limonene	$C_{10}H_{16}$
8	31.08	1.20	000629-78-7	87	Heptadecane	$C_{17}H_{36}$
13	37.23	3.05	000638-97-7	89	Ethyl palmitate	$C_{18}H_{36}O_2$
17	40.50	2.63	000544-35-4	96	Ethyl Linoleate	$C_{20}H_{36}O_2$
18	40.58	3.85	000593-39-5	95	Petroselinic Acid	$C_{18}H_{34}O_2$

ImmunoRegulation

RT: Retention time.

Table 3. Results of antibiotics disk diffusion assay for *K. pneumoniae*

Antibiotic	***K. pneumoniae*** **Result**
Ampicillin	– (No inhibition)
Ceftriaxone	– (No inhibition)
Penicillin G	– (No inhibition)
Penicillin V	– (No inhibition)
Cefixime	– (No inhibition)

ImmunoRegulation

Table 4. Catalase activity test controls

Condition	Description	Catalase Activity (Bubble Formation)
Positive Control (*K. pneumoniae* only)	Bacteria without *T. chebula* extract	+ (Clear bubbles observed)
Negative Control (*T. chebula* extracts only)	No bacteria present	– (No bubbles observed)

ImmunoRegulation

Table 5. Catalase activity of *K. pneumoniae* in the presence of *T. chebula* extracts

Catalase Activity (Bubble Formation)	Extract Concentration (mg/mL)
– – – (No bubbling, complete inhibition)	100, 50, 25, 12.5, 6.25
± (Weak activity, slight bubbling)	3.12
+++ (Full activity, strong bubbling)	<3.12

ImmunoRegulation

+++: Full catalase activity (strong bubble formation); ±: Weak activity (slight bubbling); – – –: Complete inhibition (no bubbling). These results were consistent across both ethanol and propanol extracts, with no significant differences observed

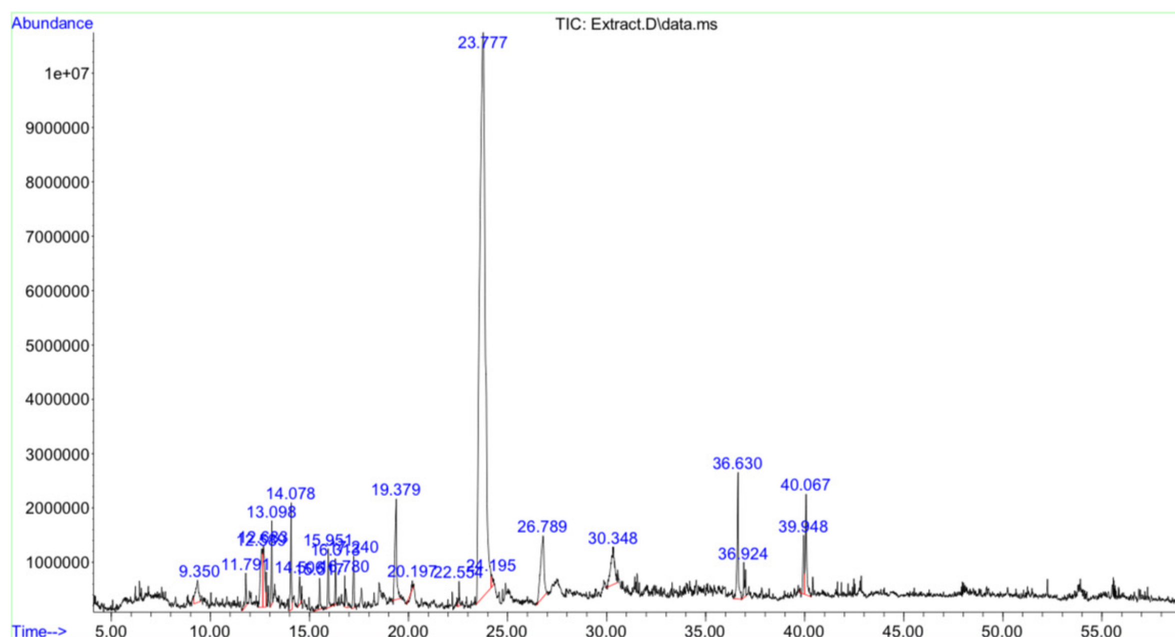


Figure 1. Gas GC-MS chromatogram showing RT and chemical profiles of major bioactive compounds in the ethanol extract of *T. chebula*

The identification of compounds using the Wiley and NIST libraries ensures the reliability of the results and allows for accurate characterization of the plant's active constituents.

Antibiotic susceptibility testing on the bacterial isolate

Antibiotics susceptibility testing of *K. pneumoniae* was performed using the disk diffusion method. The results showed that the strain was classified as resistant (R) to all examined antibiotics according to the CLSI guidelines (2023) (Table 3).

Determination of MIC and MBC

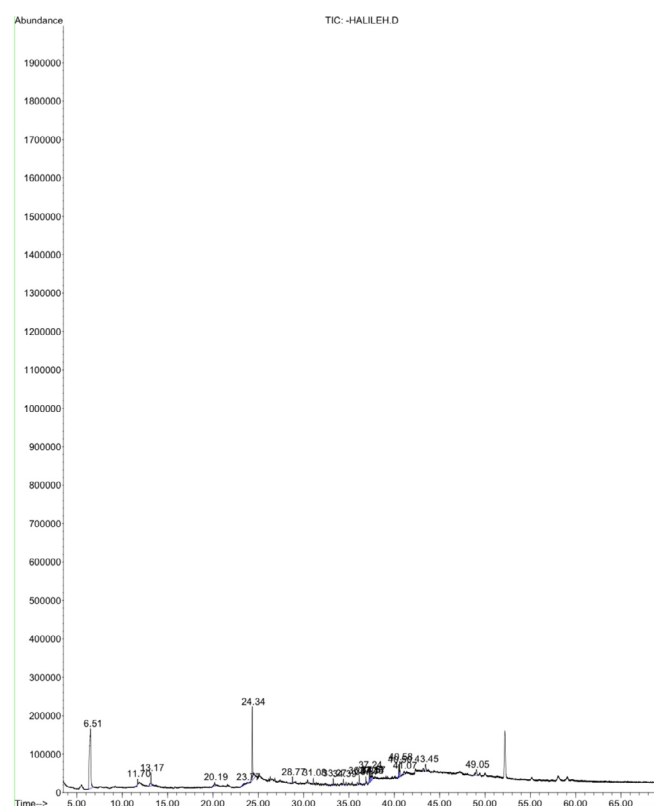
Antibacterial activity testing at various dilutions of the ethanol and n-propanol extracts showed that the MIC against *K. pneumoniae* was observed at a concentration of 12.5 mg/mL for both extracts. The MBC, at which complete bacterial death occurred, was observed at a concentration of 25 mg/mL for both extracts. These results indicate that both extracts possess moderate antibacterial activity against *K. pneumoniae*, with reduced effectiveness at lower concentrations and pronounced bactericidal activity at higher doses.

Qualitative assessment of catalase activity in the presence of *T. chebula* extracts

The qualitative effect of ethanol and n-propanol extracts of *T. chebula* on catalase activity in *K. pneumoniae* was evaluated using two methods, each repeated three times with consistent results. The extracts alone showed no bubble formation in the absence of bacteria, confirming that the observed inhibition resulted from interactions. In the positive control (bacteria only), strong bubble formation indicated full catalase activity.

In both methods, catalase activity was completely inhibited at concentrations of 100, 50, 25, 12.5, and 6.25 mg/mL, as evidenced by the absence of bubble formation. Notably, even at 6.25 mg/mL (half of the MIC value), catalase was fully inactivated. At 3.12 mg/mL (one-quarter of the MIC value), weak catalase activity (slight bubbling) was observed, and at lower concentrations, enzyme activity returned to baseline.

These findings suggest that *T. chebula* extracts effectively inhibit catalase activity in *K. pneumoniae* at relatively high concentrations, but inhibition diminishes at lower concentrations. Both extracts yielded the same pattern of inhibition, with no significant difference between ethanol and propanol extracts (Tables 4 and 5).



IMMUNOREGULATION

Figure 2. Gas GC-MS chromatogram showing RT and chemical profiles of major bioactive compounds in the n-propanol extract of *T. chebula*

The positive control confirmed the presence of active bacterial catalase, while the negative control ensured that extract alone did not cause bubble formation, verifying that inhibition is due to interaction with catalase.

These consistent results obtained using both qualitative methods showed no significant differences between ethanol and propanol extracts. All concentrations were tested under the same conditions.

Discussion

The biological effects of plant extracts are attributed to the presence of bioactive phytochemicals, which may act individually or synergistically [12]. *T. chebula* was selected for this study due to its high tannin content (30–40%) and rich phytochemical profile. Tannins are known to penetrate bacterial cell walls, interfere with metabolism, and damage intracellular structures, thereby contributing to their strong antimicrobial properties [12, 13]. In addition, the extracts contain chebulic acid, gallic acid, ellagic acid, amino acids, and flavonoids, which have demonstrated antioxidant, antimicrobial, and anticancer activities [13, 14].

The dominant compound identified in the ethanol extract was pyrogallol, comprising over 64%, significantly higher than the 21–43% range reported by Thoithoisana Devi et al. (2023). That study found that this compound induced caspase-dependent apoptosis in HCT-116 colorectal cancer cells and exhibited potent antioxidant activity. Its higher concentration here suggests potentially greater therapeutic effects, particularly in anticancer applications [15].

In the study conducted by Liu et al. (China, 2013), the cellular response of Saccharomyces cerevisiae to D-limonene exposure was investigated. The results demonstrated that D-limonene treatment led to significant intracellular accumulation of ROS, contributing to membrane damage and growth inhibition. In response, yeast cells activated antioxidant defense mechanisms, including increased expression of antioxidant enzymes and enhanced NADPH regeneration. Notably, it was suggested that D-limonene might impair the function of key enzymes, such as catalase, thereby reducing the cell's ability to neutralize hydrogen peroxide (H_2O_2) and exacerbating oxidative stress [16].

This study showed that both extracts significantly inhibited catalase activity in *K. pneumoniae*. Catalase protects bacteria from oxidative stress by breaking down hydrogen peroxide; thus, its inhibition increases bacterial susceptibility. The extracts fully suppressed catalase activity at 6.25 mg/mL and showed decreased inhibition at lower concentrations.

In a study by Aher and Wahi (2011), 100 mg/kg of ethanol extract of *T. chebula* increased catalase and superoxide dismutase (SOD) activity in liver mitochondria while reducing lipid peroxidation. It also elevated pineal melatonin levels and enhanced lymphocyte proliferation. Reverse transcription polymerase chain reaction (RT-PCR) analysis showed increased expression of interleukin (IL)-2, IL-10, and tumor necrosis factoralpha (TNF-α) cytokines, indicating immunomodulatory activity [17].

Roese et al. (2023) demonstrated that pyrogallol, a phenolic compound, effectively inhibits biofilm formation by *Staphylococcus* spp., especially in catalase-deficient strains. This effect, induced by oxidative stress, was reversed by exogenous catalase, supporting the role of hydrogen peroxide as a biofilm inhibitor. Hence, pyrogallol in the ethanol extract may help suppress drug-resistant bacteria by increasing oxidative stress and reducing biofilm formation [18].

Khan et al. (2009) reported that mice pretreated orally with 500 mg/kg aqueous *T. chebula* extract for 30 days were fully protected against intraperitoneal injection of 100,000 CFU of *Salmonella typhimurium*. Infected mice showed a 5.6% increase in catalase activity, a 97.69% decrease in lipid peroxidation, and a 62% increase in reduced glutathione, suggesting the extract can prevent oxidative stress and reduce typhoid risk [19].

Conclusion

The present study demonstrated that the hydroalcoholic extract of *T. chebula* possesses considerable antibacterial activity against β-lactam–resistant *K. pneumoniae*. The extract showed a clear inhibitory effect on bacterial growth, with a MIC of 12.5 mg/mL and an MBC of 25 mg/mL. Moreover, qualitative catalase assays revealed that both extracts of *T. chebula* completely inhibited catalase activity in *K. pneumoniae* at concentrations equal to or above 6.25 mg/mL. This suggests that one of the possible antibacterial mechanisms of the extract is through suppression of oxidative defense systems, thereby increasing bacterial vulnerability to oxidative stress.

GC–MS analysis identified 1, 2, 3-benzenetriol (pyrogallol) as the major constituent, along with several other phenolic, aldehydic, fatty acid, and alkaloid compounds. The predominance of pyrogallol—known for its strong antioxidant and antimicrobial properties—highlights the potential contribution of phenolic compounds to the observed biological activities.

Overall, these findings support the traditional medicinal value of *T. chebula* and provide scientific evidence for its potential use as a natural antimicrobial agent, especially against MDR pathogens. By targeting enzymes such as catalase, this plant extract may serve as a promising lead for developing novel therapeutic agents aimed at enhancing bacterial susceptibility to oxidative damage. Future studies should focus on isolating and characterizing the active compounds responsible for catalase inhibition, evaluating their synergistic interactions, and exploring their in vivo efficacy and safety profiles.

Ethical Considerations

Compliance with ethical guidelines

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

Funding

This research did not receive any grant from funding agencies in the public, commercial, or non-profit sectors.

Authors' contributions

Conceptualization, supervision, review & editing: Hadi Koohsari and Aryan Sateei; Methodology: Hadi Koohsari, Mohammad Kordkatouli; Investigation and Data analysis: Hadi Koohsari, Aryan Sateei and Mohammad Kordkatouli; Writing the original draft and funding Administration: Mohammad Maroufi and Mohammad Kordkatouli; Data collection: Mohammad Kordkatouli and Aryan Sateei.

Conflicts of interest

The authors declared no conflicts of interest.

Acknowledgements

The authors express their appreciation to the Islamic Republic of Iran Meteorological Organization for providing valuable meteorological data for Minab County, Hormozgan Province, Iran. The authors also gratefully acknowledge the Islamic Azad University for its moral support, provision of laboratory facilities, and partial access to equipment essential for the execution of this research.

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