Biodiversity and molecular characterization of Klebsiella pneumoniae isolated from some soil invertebrates at high altitudes and the potential activities of Rhazya stricta extracts.

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Abstract

Background

Klebsiella is a common pathogen that is dangerous to humans and other animals, including invertebrates, and is widely present in the digestive system. It also appears that the genus *Klebsiella* is ubiquitous, as it is endemic to surface water, soil, and sewage.

Methods

In this study, 70 samples were obtained from soil-dwelling invertebrates from September 2021 to March 2022 from Taif and Shafa in different altitudinal regions. Fifteen of these samples contained *Klebsiella* spp. *The Klebsiella* isolates were genetically identified as *Klebsiella pneumonia*e using rDNA sequencing. The antimicrobial susceptibility of *the Klebsiella* isolates was determined. Amplification of virulence genes was performed using PCR.

Results

In this study, 16S rDNA sequencing showed similarity from 98–100% with related *K. pneumonia* from the NCBI database, and the sequences were deposited in NCBI GenBank under accession numbers ON077036 to ON077050. We then screened the growth inhibition properties of ethanolic and methanolic extracts of the medicinal plant *Rhazya stricta* leaves against some *K. pneumoniae* strains using minimum inhibitory concentration (MIC) methods and disc diffusion. In addition, the biofilm inhibitory potential of these extracts was investigated using crystal violet. HPLC analysis identified 19 components divided into six flavonoids, 11 phenolic acids, stilbene (resveratrol), and quinol, and revealed variations in the number of components and their quantities between extracts. Both extracts demonstrated interesting antibacterial properties against *the K. pneumoniae* isolates. The two extracts also showed strong biofilm inhibitory activities, with percentages of inhibition extending from 81.5–98.7% and from 35.1–85.8% for the ethanolic and methanolic extracts, respectively.

Conclusion

Rhazya stricta leaves extract revealed powerful antibacterial and antibiofilm activities against *K. pneumoniae* isolates and could be good candidates for the treatment or prevention of *K. pneumonia*-related infections.

Introduction

The gastrointestinal tract of invertebrates is an ideal region for microflora; the stomach is rich in bacteria, while the proctodaeal region is rich in fungi (Sridhar and Ashwini, 2016). From the hindgut of *Glomeris*

species six bacteria, six actinomycetes and two fungal strains were isolated (Yang et al. 2019). *Pseudomonas stutzeri* and *P. putida* survive passage through the gut of a millipede *Pachyiulus flavipes* and increase fresh excrement (Byzov et al. 1996). Bacteria recovered from the gut of millipedes (*Ommatoiulus sabulosus*) include *Klebsiella*, *Bacillus*, and *Corynebacterium* species. Actinomycetes (*Micromonospora* sp.) are known to accumulate in the hindgut and are likely to participate in the breakdown of chitin (Zenova et al. 1996), Fecal pellets consist of dense populations of micro-organisms (Yang et al. 2019). The genus *Klebsiella*, a severe opportunistic pathogen belonging to the family Enterobacteriaceae, is a major pathogen associated with urinary, respiratory, gastrointestinal and skin infections in humans (Alsanie 2020; José et al. 2019). *Klebsiella pneumoniae* control is impaired by the frequent multidrug-resistant phenotype and genotype, representing a major threat to neonates, the elderly, and immuno-compromised patients (Alzahrani et al. 2016; Ranjbar et al. 2019). *Klebsiella* is ubiquitous in terms of habitat association. It is also a resident or transient flora, particularly in the gastrointestinal tract of some invertebrates (Yang et al. 2019). In addition, *Klebsiella* species frequently acquire antibiotic resistance genes for all classes of antibiotics and are considered the first microorganism to help in spreading resistance and virulence genes (Navon-Venezia et al. 2017; Liu et al. 2019; Zhou et al. 2020). Similar to other opportunistic pathogens, *K. pneumoniae* is a ubiquitous bacterium that thrives in environmental compartments (e.g., soil, plants, and waterways) (Bagley et al. 1985; Yang et al. 2019). While sometimes *K. pneumoniae* bacteria are present from human and animal waste sources and therefore can be considered environmental pollutants, and at other times these *K. pneumoniae* strains are environmental strains that appear in their natural habitat (Podschun and Ullmann, 1998; Chi et al. 2019). Water, vegetation, and soil have been described as the native environments for *K. pneumoniae* (Yang et al. 2019). Phenotypic and genotypic traits were compared between isolates of *K. pneumoniae* obtained from hospitals and those obtained from the natural environment (Rocha et al. 2022). It is important to investigate the common and distinct genomic traits of clinical and environmental strains of *K. pneumoniae* (Wyres and Holt, 2018). Although the distinction between clinical strains and environmental strains is difficult, characterization of strains from both origins is crucial to assess the harmful effect of both origins and to study the evolution and acquisition of new genetic traits from one source to the other, as well as to infer pathways of transmission from the environment to humans (Rocha et al. 2022).

K. pneumoniae isolates were subjected to a medicinal plant extract, *Rhazya stricta (R. stricta)*, which is an economically important medicinal plant (Albeshri, et al. 2021). In Saudi Arabia and many Asian countries, *R. stricta* and its metabolites are traditionally used for treatment of cancer, skin diseases, hypertension, rheumatism, sore throat, syphilis, parasitic infections, inflammatory conditions, and fever (El-Tarass et al. 2013). Various parts of *R. stricta* contain many phytochemical constituents, such as alkaloids, flavonoids, triterpenes, and volatile bases, (Marwat et al. 2012; Albeshri, et al. 2021) which display potential antimicrobial and biological activities (Albeshri, et al. 2021). Furthermore, leaf and fruit extracts of *R. stricta* have shown antimicrobial properties against many multidrug-resistant human pathogens (El-Tarass et al. 2013; Raziuddin et al. 2018). However, the antibiofilm activity of *R. stricta* has not been explored.

In the present study, *K. pneumoniae* strains were isolated from invertebrate animals collected from different regions of Taif. Therefore, the main aim of this study was to classify and characterize *K. pneumoniae* isolates obtained from invertebrate animals collected from the Taif region in Saudi Arabia, and to evaluate the antibacterial and antibiofilm properties of the phenolic components in *R. stricta* leaf ethanolic and methanolic extracts against *K. pneumoniae* isolates and their virulence gene profiles.

Materials And Methods

K. pneumoniae strains

Seventy samples were isolated from soil-dwelling invertebrates between September 2021 and March 2022. Digestive tracts were obtained from *Archispirostreptus syriacus* (millipede), *Porcellio laevis*, and *Armadillidium* sp. (isopods). The bacterial isolates were obtained using the dilution method, where gut contents were diluted and spread on MacConkey agar media and incubated for 24 h at 37 ° C, morphologically identified *Klebsiella* isolates were also genetically identified using 16S rDNA sequencing.

16s Rdna Gene Sequencing

Genomic DNA was isolated from all *K. pneumoniae* isolates using a DNA extraction kit (Gena Bioscience, Germany), according to the manufacturer's instructions. One fragment of the DNA (approximately 1465 bp) was amplified from the 16S rDNA gene, according to the method described by Alsanie et al. (2018). The pieces were punctuated using a QIAquick PCR purification kit (QIAGEN, Valencia, CA, USA) and sequenced using a DNA Analyzer 3146 Applied Bioscience (Applied Biosystems, USA). The sequencing texts were edited and compiled using the DNASTAR software (Laser gene, Madison, WI, USA). BLAST searches were performed using the National Center for Biotechnology Information server (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

Antimicrobial Susceptibility Test

The antibiotic sensitivity of *K. pneumoniae* strains was studied using the disc diffusion method, according to Clinical and Laboratory Standards Institute (CLSI) guidelines (Wayne, 2018). This study was carried out using 12 commercially available antibiotics: sulfamethoxazole/trimethoprim (25 µg), ampicillin (10 µg), carbecillin (100 µg), amkacillin (30 µg), Cefatrizine (10 µg), oxacillin (5 µg), penicillin (10 µg), ciprofloxacin (5 µg), gentamicin (10µg), cefoxitin (30 µg), erythromycin (15 µg), and amoxicillin/clavulanic acid (30 µg).

R. strictaleaves collection and extraction procedure

Fresh leaves of *R. stricta* were collected in September 2020 from their natural habitat at Taif-Makkah Road. The plant’s fresh leaves were air dried and ground into fine powder; then extracted using 100 ml of

95% ethanol and methanol separately at room temperature for 3 days. Each extract was centrifuged at 7000 rpm for 15 min and filtered three times with Whatman filter paper No.1 to obtain a pure filtrate. The filtrate was passed through a Buchner funnel using a rotary vacuum evaporator at 30°C, then the extracts (pellets) were dissolved in an aqueous solution of dimethylsulfoxide 1% (DMSO) (Hassan et al. 2022). The extracts were subjected to HPLC analysis to separate their components.

Hplc Analysis

Phenolic compounds were detected in the tested extracts as previously described (Hassan et al. 2022), with fine modifications, using an Agilent 1260 infinity HPLC Series (Agilent, USA) equipped with a quaternary pump. Kinetex® 5 µm EVO C18 100 mm × 4.6 mm, (Phenomenex, USA) was used as the column and operated at 30°C. The separation was carried out using a ternary linear elution gradient with (A) HPLC grade water 0.2% and H_3PO_4 (v/v), (B) methanol, and (C) acetonitrile. Subsequently, 20 µL of the extract was injected. The AVWD detector was set at 284 nm to detect phenols and flavonoids.

Antibacterial activity of R. stricta extracts

Disc Diffusion

The antibacterial properties of the *R. stricta* leaf extracts were assessed in triplicate using the agar disc diffusion method (Lagha et al. 2019). *K. pneumoniae* cells were allowed to grow for 24 h at 37°C in a liquid medium. The *K. pneumoniae* suspension was prepared in saline water, adjusted to 0.5 turbidity standards and distributed in Mueller–Hinton agar. A sterile filter disc was impregnated with *R. stricta* leaf extract (10 µl /disc) placed on the agar surface. The MHA plates were kept for 2 h at 4°C before their incubation at 37°C for 24 h. The antimicrobial properties were categorized by measuring the zone of cell growth inhibition around the discs. The inhibitory activity was evaluated as previously described (El-Tarass et al. 2013).

Determination Of Minimal Inhibitory Concentrations (Mics) And Minimal Bactericidal Concentrations (Mbcs)

MIC is the lowest concentration of the extract at which the growth of *K. pneumoniae* cells is inhibited. However, MBC have the lowest concentrations of the extract that killed ≥ 99.9% of the initial *K. pneumoniae* cells. MICs and MBCs were carried out three times using a 96-well microtiter plate (Nunc, Roskilde, Denmark) according to Lagha et al. (2019). *The K. pneumoniae* suspension was prepared from an overnight culture diluted to 0.5 McFarland. Then, serial dilutions of both methanolic and ethanolic *R. stricta* leaf extracts were prepared in 5 ml of nutrient broth with concentrations extended from 0.012-50 mg/ml. Microtiter plates were prepared by placing 95 µl of nutrient broth and 5 µl of the *K. pneumoniae*

inoculum in addition to 100 µl of the respective dilutions of the extracts. The negative control contained 5 µl of bacterial inoculum and 195 µl of nutrient broth without the *R. stricta* extract. After incubation of the plates at 37°C for 18–24 h, the MICs and MBCs were determined according to Ben Abdallah et al. (2020). MBC was determined by subculturing 20 µl of the clear wells of the MIC test on MHA.

Biofilm Formation

The potential of *K. pneumoniae* strains to develop biofilms on U-bottomed 96-well microtiter polystyrene plates was tested using a crystal violet assay, as described by Ben Abdallah et al. (2020). Briefly, *K. pneumoniae* cells were grown in a Trypticase Soy broth media overnight at 37°C. Then, 200 µl of the diluted culture (1:100) in TSB, supplemented with 2% (w/v) glucose, was transferred to a microtiter plate with wells containing sterile TSB as controls. After 24h of incubation at 37°C, the cultures were removed, and the plates were washed two times with phosphate buffer saline and dried in an inverted position. Adherent cells were fixed with 95% ethanol and stained with 100 µl of 1% crystal violet (Merck, France) for 5 min. The wells were then washed with 300 µl of sterile distilled water and left to dry in air. The biofilm produced was determined by measuring the optical density of the wells at 570.

Biofilm Inhibition

R. stricta leaf methanolic and ethanolic extracts were tested for their ability to reduce the development of biofilms by *K. pneumoniae* isolates at MICs concentrations. 100 µl of the extracts in TSB (2% glucose) were added to microtiter plate wells containing 100 µl of bacterial suspension (10^8 CFU/mL) in each well. The negative control wells contained tryptic TSB and sterile water. Biofilm formation was determined using the crystal violet assay according to Ben Abdallah et al. (2020). The percentage biofilm inhibition was calculated using the formula described by Hassan et al. (2022). This analysis was repeated three times.

% Inhibition = 100 – ((OD570 sample)/ (OD570 control) × 100)

Detection of virulence and antibiotic resistance genes of K. pneumoniae

Twelve PCR reactions were performed to detect the presence of virulence genes (*acrAB*, *tolC*, *mdtk, Ompk35, fimH*, *rmpA*, *aea, k1, k2*,) in *K. pneumoniae* isolates according to Alsanie (2020), antibiotic resistance genes according to Hassan et al. (2018), primer sequences, amplification conditions, and amplicon sizes according to Alsanie et al. (2018). PCR was performed using the GoTaq ® Green Master Mix (Promega, USA). Expected sizes of the amplicons were ascertained by electrophoresis on 1.5% agarose gel with an appropriate molecular size marker (100-bp DNA ladder, MBI, Fermentas, Lithuania, USA).

Statistical analysis

Three replicates were used for each of the tretaments and in each replicate, at least four plants were used and the significance of the difference between the mean values was calculated. One-way analysis of variance (ANOVA) was used to perform the analysis of all data and the significance of difference among the treatments, was determined according to the least significant difference (LSD)(Gomez and Gomez, 1984).

Results

Isolation of K. pneumoniae

Fifteen isolates were obtained from different invertebrate animals (millipedes and isopods) collected from the Taif governorate and identified as *K. pneumoniae*. The location and names of the animals are presented in (Table 1). Nine *K. pneumoniae* isolates were obtained from millipede guts, three of which (KTU-10, KTU-11, and KTU-12) were collected from Wady Ghazal, Taif, and six (KTU-1, KTU-2, KTU-3, KTU-13, KTU-14, and KTU-15) from Al-Shafa, Taif. Five *K. pneumoniae* isolates (KTU-4, KTU-5, KTU-6, KTU-7, KTU-8, and KTU-9) were isolated from the gut of an isopod, *Porcellio laevis*, collected from the Taif University Garden in Hawia, Taif, Saudi Arabia.

Table 1
The source and locations of *Klebsiella pneumoniae* that were isolated from some invertebrates animals in Taif, Saudi Arabia

Isolates	Species	source	Locations
KTU-1	*Klebsiella pneumoniae*	millipedes	Shafa, Taif
KTU-2	*Klebsiella pneumoniae*	millipedes	Shafa, Taif
KTU-3	*Klebsiella pneumoniae*	millipedes	Shafa, Taif
KTU-4	*Klebsiella pneumoniae*	soft isopods	Hawia, Taif
KTU-5	*Klebsiella pneumoniae*	soft isopods	Hawia, Taif
KTU-6	*Klebsiella pneumoniae*	soft isopods	Hawia, Taif
KTU-7	*Klebsiella pneumoniae*	soft isopods	Hawia, Taif
KTU-8	*Klebsiella pneumoniae*	soft isopods	Hawia, Taif
KTU-9	*Klebsiella pneumoniae*	hard isopods	Hawia, Taif
KTU-10	*Klebsiella pneumoniae*	millipedes	Wady Ghazal, Taif
KTU-11	*Klebsiella pneumoniae*	millipedes	Wady Ghazal, Taif
KTU-12	*Klebsiella pneumoniae*	millipedes	Wady Ghazal, Taif
KTU-13	*Klebsiella pneumoniae*	millipedes	Shafa, Taif
KTU-14	*Klebsiella pneumoniae*	millipedes	Shafa, Taif
KTU-15	*Klebsiella pneumoniae*	millipedes	Shafa, Taif

Molecular genotyping of K. pneumoniae **isolates according to 16S-rRNA gene**

16S-rRNA gene of all *K. pneumoniae* isolates was amplified and sequenced, and specific fragments were aligned and compared with the available 16S-rRNA sequences for other *K. pneumoniae* isolates in the NCBI database. The sequences of *K. pneumoniae* isolates were deposited in the NCBI GenBank under accession numbers ON077036 to ON077050. BLAST results showed that the partial 16S rRNA sequences were more similar to other sequences from the NCBI database. The similarity matrix among *K. pneumoniae* isolates and related strains from the NCBI database ranged from 98 to 100%, with zero E value. For example, *the K. pneumoniae* KTU-11 isolate with accession number ON077046 has low similarity to *K. pneumoniae* strains. *The K. pneumoniae* KTU-1 isolate with accession number ON077036 is moderately similar to *K. pneumoniae* strain MT-379622 and *K. pneumoniae* strain MN-314311. The *K. pneumoniae* KTU-15 isolate with accession number ON077050 has high similarity with *K. pneumoniae* strain MN749610, with approximately 100% similarity (Table 2, Fig. 1).

Table 2
The NCBI BLAST query for *Klebsiella pneumoniae* isolated from invertebrates animals in Taif, Saudi Arabia.

Isolates	Species	Query coverage %	E value	Ident %	Accession number
KTU-1	*Klebsiella pneumoniae*	100.00	0.00	99.00	ON077036
KTU-2	*Klebsiella pneumoniae*	100.00	0.00	100.00	ON077037
KTU-3	*Klebsiella pneumoniae*	99.00	0.00	99.00	ON077038
KTU-4	*Klebsiella pneumoniae*	100.00	0.00	100.00	ON077039
KTU-5	*Klebsiella pneumoniae*	99.00	0.00	99.00	ON077040
KTU-6	*Klebsiella pneumoniae*	100.00	0.00	100.00	ON077041
KTU-7	*Klebsiella pneumoniae*	99.00	0.00	100.00	ON077042
KTU-8	*Klebsiella pneumoniae*	100.00	0.00	99.00	ON077043
KTU-9	*Klebsiella pneumoniae*	100.00	0.00	99.00	ON077044
KTU-10	*Klebsiella pneumoniae*	99.00	0.00	100.00	ON077045
KTU-11	*Klebsiella pneumoniae*	98.00	0.00	99.00	ON077046
KTU-12	*Klebsiella pneumoniae*	100.00	0.00	99.00	ON077047
KTU-13	*Klebsiella pneumoniae*	100.00	0.00	99.00	ON077048
KTU-14	*Klebsiella pneumoniae*	99.00	0.00	100.00	ON077049
KTU-15	*Klebsiella pneumoniae*	100.00	0.00	100.00	ON077050

Antimicrobial Susceptibility

Klebsiella pneumoniae was tested for antimicrobial susceptibility to 12 types of antibiotics. The overall susceptibility, intermediate susceptibility, and resistance values were determined (Table 3). Most *K. pneumoniae* strains showed a high percentage of resistance against carbecillin (100%), oxacillin (100%), cefoxitin (100%), amoxicillin (100%), and penicillin (93.3%). Erythromycin (80%), amkacillin (53.3%), ampicillin (40%), and cefrizine (40%) were moderately susceptible. On other hand, intermediate resistance was found against sulfamethoxazole / Trimethoprim (26.7%). Moreover, all *the K. pneumoniae* isolates were sensitive to ciprofloxacin and gentamicin.

Table 3
Antibiotic resistance profile of *Klebsilla pneumoniae* isolates

Isolates	Antibiotic Profile
KTU-1	*Amp, Car, Caz, Oxa, Pen, Fox, Eth, Amc*
KTU-2	*Amp, Car, Caz, Oxa, Pen, Fox, Eth, Amc*
KTU-3	*Amp, Car, Caz, Oxa, Pen, Fox, Eth, Amc*
KTU-4	*Sxt, Amp, Car, Caz, Oxa, Pen, Fox, Amc*
KTU-5	*Amp, Car, Caz, Oxa, Pen, Fox, Amc*
KTU-6	*Car, Amk, Oxa, Pen, Fox, Eth, Amc*
KTU-7	*Car, Amk, Oxa, Pen, Fox, Eth, Amc*
KTU-8	*Car, Amk, Caz, Oxa, Pen, Fox, Amc*
KTU-9	*Amp, Car, Oxa, Pen, Fox, Eth, Amc*
KTU-10	*Car, Amk, Oxa, Fox, Eth, Amc*
KTU-11	*Car, Amk, Caz, Oxa, Pen, Fox, Eth, Amc*
KTU-12	*Car, Amk, Caz, Oxa, Pen, Fox, Eth, Amc*
KTU-13	*Sxt, Car, Amk, Caz, Oxa, Pen, Fox, Eth, Amc*
KTU-14	*Sxt, Car, Amk, Oxa, Pen, Fox, Eth, Amc*
KTU-15	*Sxt, Car, Amk, Oxa, Pen, Fox, Eth, Amc*

Whereas, Sxt = sulfamethoxazole / Trimethoprim (25µg), Amp = Ampicillin (10µg), Car = Carbecillin (100 µg), Amk = Amkacillin (30µg), Caz = Cefatrizine (10µg), Oxa = Oxacillin (5 µg), Pen = Pencillin (10 µg), Cip = Ciprofloxacin (5 µg), Gen = Gentamicin (10µg), Fox = Cefoxitin (30 µg), Eth = Erythromycin (15 µg), and Amc = Amoxicillin (30 µg).

Table 4
Chemical compositions of *R. stricta* leaves extracts (mg/kg)

Compounds	*R. stricta* ethanolic extract	*R. stricta* methanolic extract
Quinol	596.7	-
Resveratrol	823.35	1286.6
Chlorogenic acid	17.59	-
Vanillic acid	-	14.4
Caffeic acid	14.89	67.98
Syringic acid	58.4	109.5
p-Coumaric acid	662.9	10.43
Benzoic acid	1030.3	6334.8
Ferulic acid	424.6	1568.26
Ellagic acid	-	693,3
o-Coumaric acid	219.7	677.09
Cinnamic acid	139.6	445.06
Rosmarinic acid	200.59	485.3
Catechin	11.12	135.6
Rutin	793.8	1859.46
Quercitin	1256.7	2452.34
Neringein	316.15	8361
Myricetin	761.8	300.4
Kaempferol	7964.7	8249.13
Totals	15292.89	33050.65

Chemical composition of R. stricta **leaves extracts**

The chemical composition of the ethanolic and methanolic extracts of *R. stricta* are listed in Table (4). Nineteen components were obtained from the HPLC analysis of these extracts; they were divided into six flavonoids, 11 phenolic acids, stilbene (resveratrol), and quinol. In total, 17 compounds were detected in each extract with a quantity of 15292.89 mg/kg and 33050.65 mg/kg for ethanolic and methanolic extract respectively, indicating that methanolic extract is richer in phenolic compounds than ethanolic extract.

The results revealed variations between the two extracts in terms of the number and quantity of components. The major compounds of *R. stricta* ethanolic extract are Quinol, Resveratrol, p-Coumaric acid, Benzoic acid, Rutin, Quercitin, Myricetin, and Kaempferol. However, the major compounds of methanolic extract are Resveratrol, Benzoic acid, Ferulic acid, Rutin, Quercitin, Neringein and Kaempferol. Both *R. stricta* extracts are rich in flavonoids (11104.27 mg/kg and 21357.93 mg/kg for ethanolic and methanolic extracts, respectively) compared to other phenolic compounds (4188.62 mg/kg and 11692.72 mg/kg for ethanolic and methanolic extracts, respectively).

Antibacterial activity of R. stricta **extracts against** *K. pneumoniae*

Disc Diffusion

The ethanolic and methanolic extracts of *R. stricta* leaves were examined for their antimicrobial activity against *K. pneumoniae* isolates (Table 5). First, the disc diffusion method showed that the two extracts were active against all isolates despite the variation in the type of inhibitory action. *Rhazya stricta* ethanolic extract has demonstrated strong inhibitory activity against 40% of the strains, compared to the methanolic extract, which showed a strong inhibitory action on 33.3% of the isolates. As shown in Table (5), the ethanolic extract was slightly more effective than the methanolic extract against *K. pneumoniae* isolates.

Table 5
Antimicrobial activity of *R. stricta* leaves extract against *Klebsiella* isolates

***R. stricta* extract**	***Klebsilla* isolates**				
	(+ + + +)	**(+ + +)**	**(+ +)**	**(+)**	**(-)**
	***n* (%)**	***n* (%)**	***n* (%)**	***n* (%)**	***n* (%)**
Ethanolic extract	6 (40.0)	5 (33.3)	3 (20.0)	1 (6.7)	-
Methanolic extract	5 (33.3)	4 (26.7)	4 (26.7)	2 (13.3)	-

Strong inhibitory action (+ + + +), Complete inhibitory action (+ + +), Partial inhibitory action (+ +), Slight inhibitory action (+) and no inhibitory action (-), n: number of isolates

Determination Of (Mics) And (Mbcs)

The antimicrobial activities of the ethanolic and methanolic extracts of *R. stricta* leaves were also investigated using MICs and MBCs for the 15 *K. pneumoniae* isolates. For the ethanolic extract, the MICs ranged from 0.122 to 0.970 mg/ml, whereas the MBCs ranged from 0.224 mg/ml to 1.9 mg/ml. For the methanolic extract of *R. stricta* leaves, the MIC values varied from 0.224 mg/ml to 1.9 mg/ml, while the

MBC values ranged from 0.448 mg/ml to 3.9 mg/ml. Accordingly, the ethanolic extract had the greatest antibacterial activity against *K. pneumoniae* isolates compared with the methanolic extract.

Biofilm Formation On Polystyrene Surface

The bacterial isolates were inspected for their ability to produce biofilms on polystyrene surfaces (Table 6). Results showed that all *K. pneumoniae* strains were capable of producing biofilms and were allocated as follows: 26.7% were highly positive biofilm producers with OD570 values varying from 1.015 to 1.060, and 73.3% were low-grade positive with OD570 values ranging from 0.442 to 0.808.

Table 6
Antibiofilm potentialities of ethanolic and methanolic *R. stricta* leaves extracts against *Klebsiella* isolates

Isolates	Biofilm formation OD570 ± SD	Ethanolic extract OD570 ± SD	Inhibition (%)	Methanolic extract OD570 ± SD	Inhibition (%)
KTU-1	0.812 ± 0.081	0.150 ± 0.006*	81.5	0.169 ± 0.073*	79.2
KTU-2	0.448 ± 0.102	0.034 ± 0.021*	92.4	0.195 ± 0.103*	56.4
KTU-3	0.588 ± 0.319	0.031 ± 0.009*	94.7	0.084 ± 0.011*	85.7
KTU-4	0.808 ± 0.109	0.036 ± 0.019*	95.5	0.192 ± 0.146*	76.2
KTU-5	0.744 ± 0.286	0.070 ± 0.031*	90.5	0.149 ± 0.057*	79.7
KTU-6	0.799 ± 0.818	0.089 ± 0.024*	88.9	0.123 ± 0.002*	84.6
KTU-7	0.625 ± 0.350	0.027 ± 0.014*	95.6	0.132 ± 0.106*	78.8
KTU-8	1.015 ± 0.158	0.028 ± 0.024***	94.3	0.211 ± 0.089**	79.2
KTU-9	0.987 ± 0.025	0.072 ± 0.046*	91.1	0.265 ± 0.063**	67.2
KTU-10	1.053 ± 0.041	0.120 ± 0.066**	97.2	0.237 ± 0.230**	85.8
KTU-11	0.971 ± 0.226	0.042 ± 0.079**	95.7	0.204 ± 0.253**	78.9
KTU-12	0.802 ± 0.444	0.038 ± 0.016*	95.2	0.194 ± 0.260*	75.8
KTU-13	1.060 ± 0.006	0.014 ± 0.132***	98.7	0.206 ± 0.097**	80.4
KTU-14	0.422 ± 0.115	0.030 ± 0.001*	92.8	0.274 ± 0.385*	35.1
KTU-15	0.631 ± 0.014	0.037 ± 0.001*	94.1	0.190 ± 0.111*	69.8

* Isolates changed from low-grade positive to biofilm negative. ** Isolates changed from highly positive to low-grade positive. *** Isolates changed from highly positive to biofilm negative

Table 7
Virulence genes in *Klebsiella pneumoniae* isolates

Isolates	Virulence genes
KTU-1	*AcrAB, TolC, mdtk, Ompk35, FimH, RmpA, Aea, SHV, TEM*
KTU-2	*AcrAB, TolC, mdtk, Ompk35, FimH, RmpA, Aea, SHV, TEM*
KTU-3	*AcrAB, TolC, mdtk, Ompk35, FimH, RmpA, Aea, SHV, TEM*
KTU-4	*AcrAB, TolC, mdtk, Ompk35, FimH, RmpA, Aea, SHV, TEM*
KTU-5	*AcrAB, TolC, mdtk, Ompk35, FimH, RmpA, SHV, TEM*
KTU-6	*AcrAB, TolC, mdtk, Ompk35, FimH, RmpA, Aea, SHV, TEM*
KTU-7	*AcrAB, TolC, mdtk, Ompk35, FimH, RmpA, Aea, K1, SHV, TEM*
KTU-8	*AcrAB, TolC, mdtk, Ompk35, FimH, RmpA, K1, SHV, TEM, CTX*
KTU-9	*AcrAB, mdtk, Ompk35, FimH, RmpA, K1, SHV, TEM, CTX*
KTU-10	*AcrAB, TolC, mdtk, Ompk35, FimH, RmpA, Aea, SHV, TEM, CTX*
KTU-11	*AcrAB, mdtk, Ompk35, FimH, RmpA, K1, SHV, TEM*
KTU-12	*AcrAB, TolC, mdtk, Ompk35, FimH, RmpA, Aea, SHV, TEM*
KTU-13	*AcrAB, TolC, mdtk, Ompk35, FimH, RmpA, Aea, SHV, TEM*
KTU-14	*AcrAB, TolC, mdtk, Ompk35, FimH, RmpA, Aea, SHV, TEM*
KTU-15	*AcrAB, TolC, mdtk, Ompk35, FimH, RmpA, Aea, SHV, TEM*

Biofilm Inhibition

The ability of *R. stricta* ethanolic and methanolic extracts to inhibit biofilm formation by *K. pneumoniae* isolates is shown in Table (6). Isolates showing potential for biofilm formation were selected for this experiment. Fifteen strains were classified as low-grade and highly positive biofilms, and both extracts demonstrated strong biofilm inhibition activity.

Antibiofilm Activity

The present investigation revealed that ethanolic extract of *R. stricta* leaves has strong biofilm inhibition activity on all the isolates (15 strains), with the percentage of inhibition extending from 81.5–98.7%. Four out of five highly positive isolates (80%) were biofilm negative. In addition, 10 low-grade positive isolates (75%) changed to biofilm negative after treatment.

Biofilm inhibitory activities were also observed for methanolic extract, with most isolates ranging from 35.1–85.8%. Furthermore, the same results observed for the four highly positive biofilm isolates treated with the ethanolic extract were obtained after treatment with the methanolic extract. However, four low-grade positive isolates (26.7%) were biofilm negative. Table (6) shows that isolate No.10 conserved its initial biofilm phenotype after treatment with the two extracts, despite the large decrease in the amount of biofilm. However, the methanolic extract did not affect the ability of isolate No.1 to form a biofilm. No significant correlation was detected between the MIC and antibiofilm activity for either methanolic or ethanolic extracts of *R. stricta* leaves.

Detection of virulence genes in K. pneumoniae

The existence of antibiotic-resistant genes is shown in Figure (2) and Table (7). Virulence genes *AcrAB, mdtk, OmpK35, FimH*, and *RmpA* were recorded in all *K. pneumoniae* isolates (Table 8). *K1* gene which is responsible for the formation of capsule and K genotypes was found in only three isolates of *K. pneumoniae* KTU-7, KTU-8 and KTU-11 representing 15% of the isolates. The *K. pneumoniae* KTU-8 and KTU-10 isolates possessed the most investigated virulence genes. *OmpK35* plays a role in *K. pneumoniae* infection and virulence. The *Aea* gene was found in all *K. pneumoniae* isolates except KTU-5, KTU-8, KTU-9, and KTU-11. *TolC* was also found in all *K. pneumoniae* isolates, except *K. pneumoniae* KTU-9 and KTU-11. Moreover, *SHV* and *TEM* genes were found in all *K. pneumoniae* isolates, whereas the *CTX* gene was found in two isolates: *K. pneumoniae* KTU-8 and KTU-10.

Discussion

16S rRNA gene sequencing has recently been used as an alternative method for the molecular detection of various microbes, including *K. pneumoniae* (Alsanie et al. 2020). This gene is found in all bacteria and hence ensures the accurate identification of bacteria at the genus and species levels (Hassan et al. 2014; Hassan et al. 2022). Thus, sequencing can be reasonably applied to the preparation of many microbes, especially those isolated from the external environment or from other animals. In the present study, 16S rRNA gene sequencing displayed similarities between *K. pneumoniae* isolated from invertebrates and those obtained from GenBank, indicating that sequencing has the potential to be more sensitive than culture-dependent morphological and microscopic identification (Hassan et al. 2014; Alsanie et al. 2018).

Klebsiella pneumoniae is a public health problem worldwide. This bacterium is the most prominent antibiotic-resistant acquired pathogen. Infections can spread from person to person through the respiratory system, the environment, or by using contaminated medical equipment (Alsanie et al. 2020). *K. pneumoniae* infections are commonly associated with significant morbidity and mortality (Hassan et al. 2018). Therefore, the discovery of new therapeutic agents, especially natural products, against *K. pneumoniae* is highly important.

Currently, plant compounds have emerged as potential candidates, given the interest of scientists in the search for antimicrobial and antibiofilm drugs. Among these, *R. stricta* has gained attention because of its medicinal uses (El-Tarass et al. 2013). In this study, the potential antibacterial properties of ethanolic

and methanolic extracts of *R. stricta* against *K. pneumoniae* isolated from invertebrates from different regions of the Taif governorate were investigated. The isolates were investigated by growth inhibition assays. Experiments showed that the extracts of *R. stricta* leaves have strong antibacterial activity, which is in agreement with the findings of Beigomi et al. (2021) and Marwat et al. (2012).

The high ability of *R. stricta* leaf extracts to prevent the growth and multiplication of this bacterium, observed in this study, may be attributed to the phenols and flavonoid compounds found in these extracts (Macé et al. 2017; Xie et al. 2021). It has been shown that flavonoids such as quercetin (Macé et al. 2017), kaempferol, and catechin (El-Tarass et al. 2013), exhibit great growth inhibition activity against *K. pneumoniae* isolates. Flavonoids, which are the major components of these extracts, are responsible for the inhibition of nucleic acid synthesis (El-Tarass et al. 2013), damage to the cytoplasmic membrane through the alteration of its function (Macé et al. 2017; Xie et al. 2021), inhibition of energy metabolism by the alteration of the cytoplasmic membrane, and inhibition of the energy supply for bacteria (El-Tarass et al. 2013). In addition, the inhibition of cell membrane synthesis and the aggregatory effect on whole bacterial cells have also been reported (Beigomi et al. 2021). Several studies have demonstrated the antibacterial properties of phenolic acids, especially caffeic acid, ferulic acid, coumaric acid, and chlorogenic acid which have antibacterial activities (Zhao et al. 2015; Lima et al., 2016). Phenolic acids damage the *K. pneumoniae* cell wall, leading to cytoplasmic leakage and changes in bacterial cell morphology (El-Tarass et al. 2013; Zhao et al. 2015; Lima et al. 2016). Moreover, the high *K. pneumoniae* growth inhibition activity seems to be due to the synergetic effect of flavonoids and other phenolic compounds present in the *R. stricta* leaf extracts.

In the present study, the ethanolic extract of *R. stricta* leaves was more effective against *K. pneumoniae* isolates than the methanolic extract, despite its lower abundance of flavonoids and phenols. This can be attributed to quinol and chlorogenic acid, which do not exist in the methanolic extract, and/or to myricetin and p-coumaric acid, which are more abundant in the ethanolic extract. Accordingly, Xie et al. (2017) reported that myricetin displayed the most significant antimicrobial activity of all the flavonoids, and exhibited extensive activity against *K. pneumoniae* (Nielsen et al. 2018) and many other pathogenic bacteria (El-Tarass et al. 2013). Furthermore, myricetin inhibits *Escherichia coli* DnaB helicase, which plays a major role in DNA replication and elongation (Zaixiang et al. 2012). In addition, p-coumaric acid effectively inhibited the growth of *K. pneumoniae* and other pathogenic bacteria. p-Coumaric acid is responsible for the disruption of bacterial cell membranes and the inhibition of cellular functions by binding to bacterial genomic DNA (Zaixiang et al. 2012). Ma et al. (2019) reported that quinol exhibited relatively strong antibacterial activity against *K. pneumoniae* by destroying the bacterial cell membrane and cell wall, increasing permeability, and influencing the expression of genes. However, chlorogenic acid does not show significant antibacterial activity (El-Tarass et al. 2013).

Klebsiella pneumoniae isolates were examined for their ability to develop biofilms on polystyrene surfaces, and the experiment demonstrated that 23.33% of the isolates were strong biofilm producers, and 50% were low-grade positive producers. This finding demonstrates the high potential of *K. pneumoniae* strains to produce biofilms, confirming that *K. pneumoniae* is the most prevalent bacterium

in biofilm-associated infections (Ben Abdallah et al. 2020). Biofilm, as an important virulence factor, is responsible for more than 65% of nosocomial infections and 80% of microbial infections (Römling and Balsalobre, 2012). Biofilms are associated with nasal colonization of the respiratory system, endocarditis, soft tissue infections, urinary tract infections, and other diseases (Al-Sanei, 2020). Biofilms are also a severe issue in the field of urology because they are responsible for the persistence of bacteria in the genitourinary tract over the long term (Nielsen et al. 2018). *Klebsiella pneumoniae* biofilms have been associated with medical equipment and chronic infections, and the presence of biofilms makes bacteria more resistant to antibiotics and phagocytosis, making their treatment more difficult (Nielsen et al. (2018). Therefore, the discovery of novel therapeutic strategies for biofilm inhibition is important. Extracts of *R. stricta* leaves were tested for their ability to inhibit biofilm formation by *K. pneumoniae*. Antibiofilm examination showed that both plant extracts displayed strong biofilm inhibitory activity, with a 98.7% reduction in the amount of biofilm produced. This activity is largely due to flavonoids as a major component, in addition to other phenolic compounds found in the extracts. This result emphasizes the findings of Nielsen et al. (2018) and Donadio et al. (2021), who reported that flavonoids are responsible for the reduction of bacterial adhesion, biofilm formation and the inhibition of quorum-sensing (cell-to-cell communication system in biofilm formation signal receptors TraR and RhlR). Furthermore, a decrease in the amount of biofilm could be considered a reduction in *K. pneumoniae* virulence, which is in agreement with the study by Saadatian et al. (2018), who mentioned that flavonoids inhibit bacterial virulence factors. Moreover, Xie et al. (2021) showed that flavonoids, such as quercetin, kaempferol, naringenin, and apigenin, suppress the activity of autoinducer-2, which is responsible for cell-to-cell communication and consequently reduces biofilm synthesis. In this study, the ethanolic extract also displayed biofilm inhibitory properties more than the methanolic extract, in addition to its *K. pneumoniae* growth inhibition activity, indicating that the components involved in growth inhibition are the same as those associated with biofilm inhibition, and that myricetin inhibits biofilm formation by *K. pneumoniae* (Xie et al. 2017). Additionally, Saadatian et al. (2018) revealed that flavonoids efficiently inhibited the bacterial biofilm matrix by targeting Bap-like amyloids. Myricetin also inhibits curli-dependent biofilm formation in *E. coli* (Nielsen et al. 2018).

Deletion of OmpK36 or OmpK35/OmpK36 can reduce the virulence of highly contagious *K. pneumoniae* strains and increase their susceptibility to neutrophil phagocytosis (Aljanaby et al. 2017). In our study, Ompk 35 porin-coding genes were simultaneously detected in all *K. pneumoniae* isolates. A direct correlation between efflux pumps and the virulence of pathogenic bacteria was reported by Padilla et al. (2010). In some mutant strains, several genes essential for intracellular invasion and survival are downregulated in strains without acrAB-tolC efflux pumps (Webber et al. 2008). Most strains of intestinal bacteria contain genes that encode iron absorption systems such as enterochelin or aerobactin. Iron plays an important role, as it can inhibit T-cell proliferation, in addition to promoting iron absorption. The *rmpA, wabG, uge, Ycfm, fimh1, EntB, Ybt-irp2*, and *kfu* genes have been reported in most antibiotic-resistant *K. pneumoniae* isolates (Aljanaby et al. 2017). The most pathogenic genes lead to high-pathogenicity strains that contain virulence genes prevalent in *Klebsiella* species (Hassan and Belal, 2016; Alzahrani et al. 2016). ESBLs are now found in all *Enterobacteriaceae* species worldwide (Gharrah

et al. 2017). The ESBL genes TEM and SHV were found in all *K. pneumoniae* isolates in this investigation, and only three of them harbored the CTX gene. The majority of ESBL enzymes in *K. pneumoniae* are derived from two classical enzyme types (TEM and SHV) encoded by plasmids (Hassan et al. 2018). Moreover, the number of CTX-M-producing *K. pneumoniae* strains has also increased (Alzahrani et al. 2016; Gharrah et al. 2017).

Conclusion

Soil invertebrates are important organisms harbouring a lot of internal microflorae in their digestive tract need to be intensively studied. They already have useful microflora for the soil but they may harbour pathogenic bacteria through their feeding habits that may be harmful for human. So, we used Leaves extract of the wild plant *R. stricta* that showed strong antimicrobial activities against the pathogen *K. pneumoniae*.

Abbreviations

16S rRNA- 16S ribosomal ribonucleic acid

MIC- minimum inhibitory concentration

MBC- minimal bactericidal concentrations

DMSO- dimethylsulfoxide

ANOVA- Analysis of variance

Declarations

Ethical responsibility: Our manuscript is original research and it is not submitted to full or in parts to another journal for publication.

Availability of data: Not applicable

Conflict of interest: The authors declare that they do not have any actual or potential conflict of interest.

Informed consent: Not applicable

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Authors' contributions

MMH, AM and MH conceived and designed the experiments. TM, BA and MFA performed the experiments, analyzed the results, did the statistical analyses and MMH, RK and MH wrote the manuscript. All authors read and approved the final manuscript.

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Ethics approval and consent to participate

This study was done in High-Altitude Research Centre at Taif University as an original research project (1-442-43). The project was ethically approved by the ethics committee of Taif University, Taif, Saudi Arabia.

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Figures

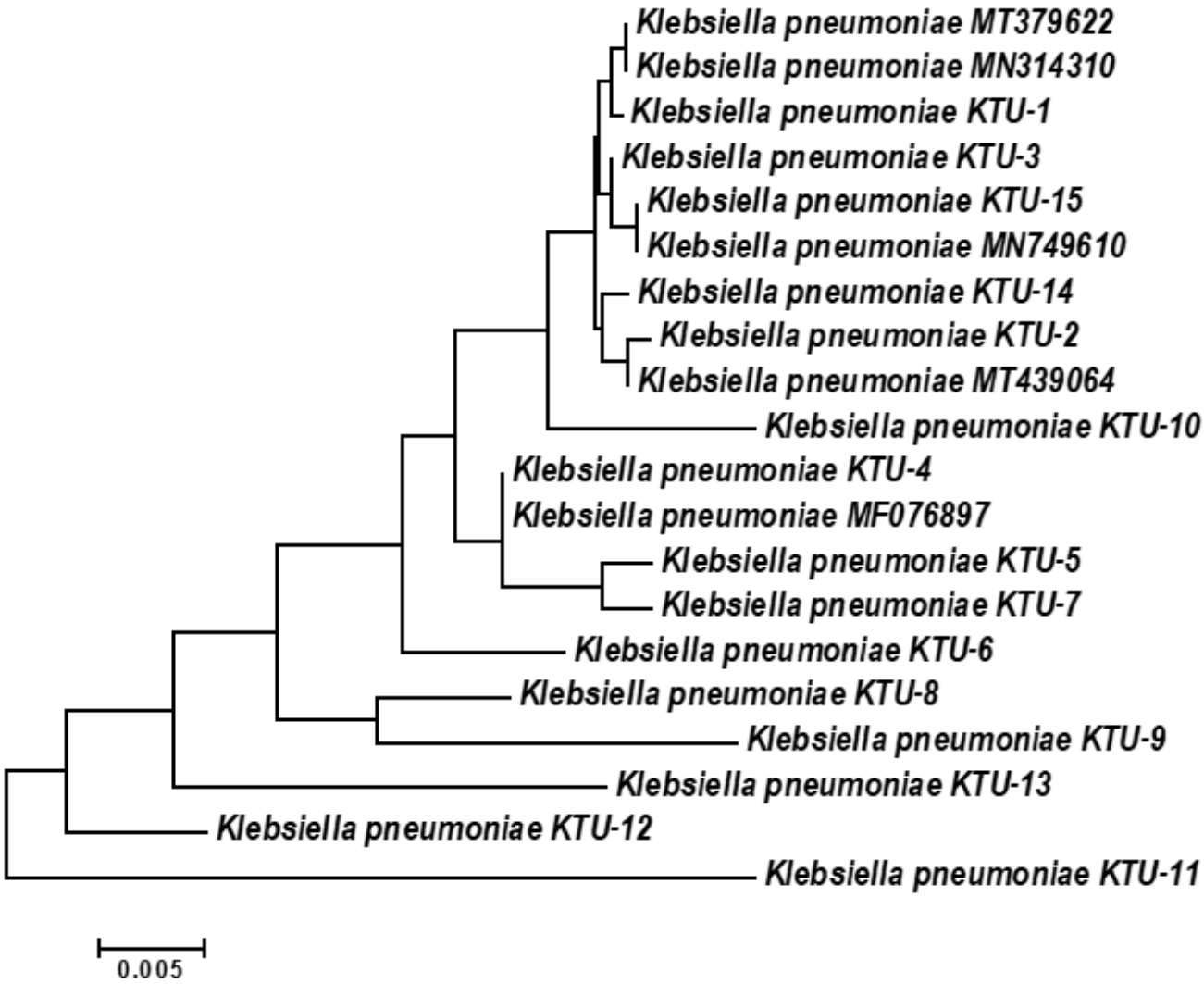

Figure 1

Neighbor-joining phylogeny tree based on 16S rDNA gene sequences of *Klebsiella pneumoniae* isolates collected from some invertebrates in Taif region, Saudi Arabia.

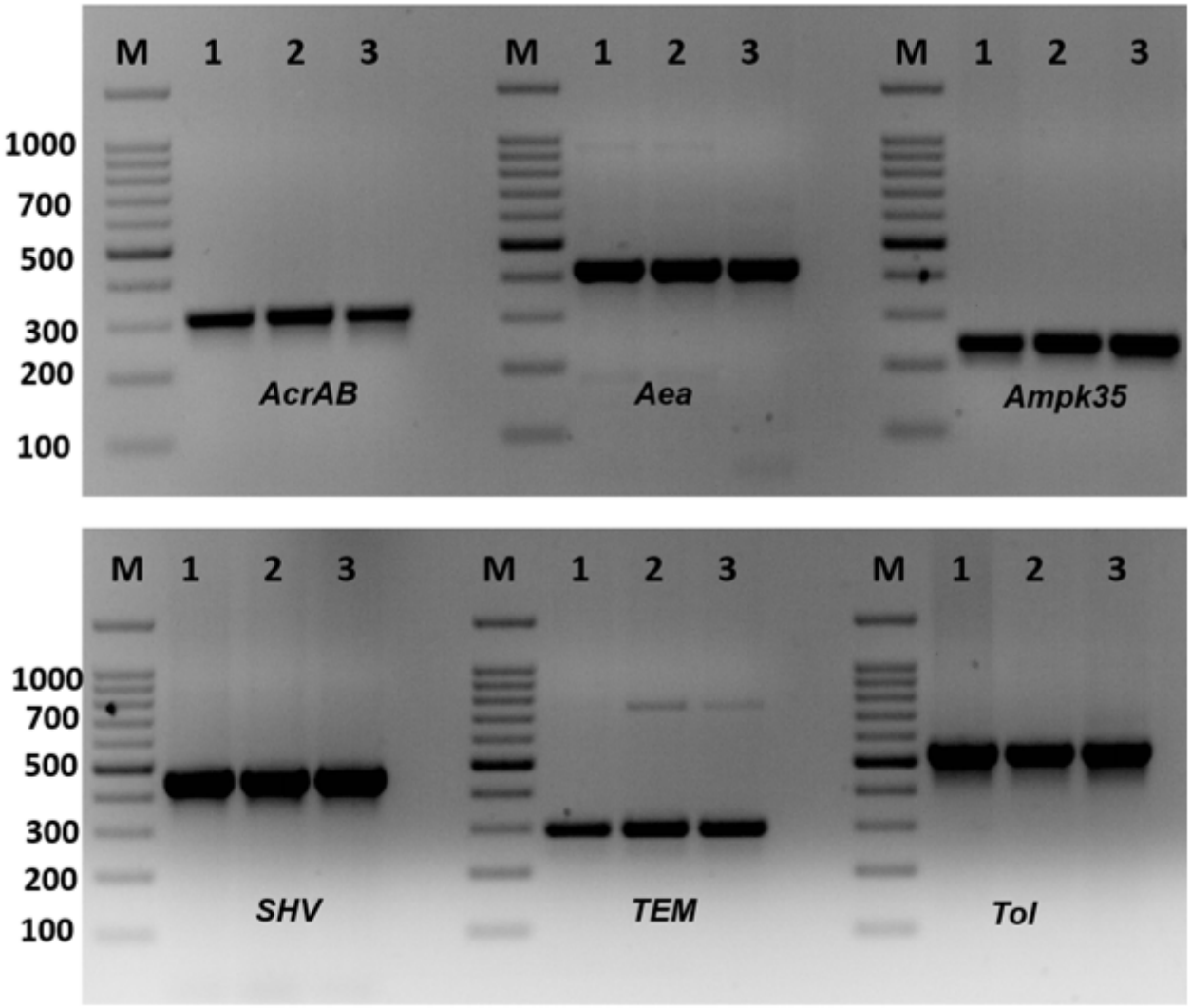

Figure 2

Amplification of virulence genes of *Klebsiella pneumoniae* isolates by single PCR. Amplification of *AcrAB* gene (312 bp), Amplification of *Aea* gene (410 bp), Amplification of *Ompk35* gene (241 bp), Amplification of *SHV*gene (436 bp), Amplification of *TEM* gene (295) and *Tol* genes (527). M: 100-bp DNA ladder.