ORIGINAL ARTICLE

Antibacterial Activity of *Solenostemma argel* Extracts Against Extended-spectrum β-lactamase Producing *Klebsiella pneumoniae*

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ABSTRACT

Background: *Extended-spectrum β-lactamases (ESBLs) can hydrolyze various types of β-lactam antibiotics. Therefore, infections caused by ESBL-producing bacteria pose a significant challenge to clinical patient care.* ***Objective****: This study aimed to assess the prevalence of ESBL-producing Klebsiella pneumoniae (ESBL-Kp) and evaluate the antibacterial potential of Solenostemma argel extracts against these isolates.* ***Methodology:*** *200 clinical Klebsiella spp. isolates were identified by MALDI-TOF, and the Vitek2 system determined the antibiograms of K. pneumoniae. Phenotypic detection of ESBL was conducted using CHROMagar ESBL and the molecular characterization of* bla_{TEM}, bla_{SHV}, *and* bla_{CTX-M} *genes was carried out by polymerase chain reaction (PCR). In addition, methanolic and ethanolic extracts of S. argel were prepared and their antibacterial activity was investigated.* ***Results:*** *K. pneumoniae isolates exhibited remarkable resistance to cefuroxime (83.9%), ciprofloxacin (58.3%), ceftazidime (55.3%), aztreonam (44%), and cefepime (36.3%), respectively. Moreover, results of the phenotypic detection of ESBL revealed that 28.5% of K. pneumoniae isolates produced ESBLs and the molecular characterization showed that 100, 87.5, and 45.8% of ESBL-Kp isolates were positive for* bla_{SHV}, bla_{CTX-M} *and* bla_{TEM} *genes, respectively. Furthermore, the methanolic extract of S. argel exhibited promising antioxidant and antibacterial activity against ESBL-Kp with minimum inhibitory concentration (MIC) values ranged from 1 to 16 µg/mL.* ***Conclusion:*** *This investigation shed light on the elevated levels of resistance among clinical K. pneumoniae isolates accompanied by the acquisition of the* bla_{SHV} *gene and verified the potent antibacterial activity of S. argel against ESBL-producing K. pneumoniae, suggesting the potential application of its methanolic extract in combating ESBL-Kp.*

INTRODUCTION

In recent years, enterobacteria have emerged as one of the main causes of nosocomial and community-acquired infections[1]. Though, being an integral part of the intestinal microbiota, *Klebsiella pneumoniae* can easily become a pathogen causing various extraintestinal infections, such as bacteremia, pneumonia, urinary tract, central nervous system, soft tissue, wound, and other invasive infections[2].Generally, aminoglycosides, cephalosporins, monobactams, and carbapenems are among the first choices to treat these infections[3]. Unfortunately, the spread of ESBL strains diminishes the efficacy of β-lactams and threatens public health[4]. It has been thought that the excessive use and misuse of antibiotics and the genetic plasticity of these bacteria have caused the emergence of highly resistant strains[5]. The ability of these bacteria to resist the existing antibiotics by changing their structures and lifestyle has caused an emerging threat to combat their infections[6]. It has been reported that the presence of bla_{TEM} and bla_{CTX-M} genes in ESBL-producing bacterial isolates are associated with considerable resistance to β-lactams and third-generation cephalosporins[7].

For these reasons, natural alternatives to antibiotics are being explored as potential treatments for bacterial infections as a potential effective strategy against the multidrug-resistant bacteria crisis[8]. Alternatives to conventional antibiotics could reduce the selective pressure for the development of antibiotic resistance. Many substitute techniques can be used to eradicate antibiotic-resistant bacteria, including phage therapy, antimicrobial peptides (AMPs), antibody therapies, and plant-based compounds[9]. In this regard, medicinal plants have been utilized in the ancient folk medicine to cure a wide range of illnesses because they contain active ingredients that may have antibacterial properties[10]. Numerous plant species, extracts, and secondary metabolites have been described as promising alternatives for inhibiting the growth of pathogenic bacteria[10]. The main advantage of using phytochemicals for antimicrobial purposes is their molecular

promiscuity, or their capacity to interact with several variables[11]. A variety of phytochemical substances, including alkaloids, coumarins, flavonoids, essential oils, phenolics, polypeptides, terpenoids, and tannins, are abundant in medicinal plants[11]. Among the medicinal plants, argel (*Solenostemma argel* Hayne) is an interesting herbal plant abundantly spread throughout North Africa. Argel's leaves, bark, and stems have a variety of medicinal uses and have long been used to cure various illnesses, including diabetes, pain, respiratory tract infections, heart conditions, gastrointestinal issues, urinary tract infections, and kidney, and liver diseases[12]. Previous studies have explored the powerful antimicrobial efficacy of argel extracts against wide array of pathogenic bacteria, including *Staphylococcus aureus*, *Porphyromonas gingivalis*, *Salmonella typhimurium*, and *Escherichia coli*[13]. Even though argel has high quantities of phenolics with antioxidant and antimicrobial potentials, reports showing its activity against ESBL-*Kp* isolates are scarce. Hence, investigation was carried out to uncover the prevalence of ESBL-coding genes in *K. pneumoniae* and determine the antibacterial efficacy of ethanolic and methanolic extracts of argel against ESBL-*Kp*.

METHODOLOGY

Identification of bacterial isolates

In this study, we investigated 200 non-repetitive clinical *Klebsiella* spp. strains that were obtained from the Kasr Al Ainy Hospital's Microbiological Laboratory, Egypt, and identified to the genus level in our previous work[14]. The matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/MS) was utilized to identify *Klebsiella* isolates as described previously[15].

Antimicrobial susceptibility testing

The antibiogram of all *K. pneumoniae* isolates was determined by the VITEK 2® automated equipment (bioMérieux, France) with Vitek2® Gram Negative Susceptibility cards (AST-GN83). The tested antimicrobials were ampicillin, piperacillin-tazobactum, amoxicillin-clavulanate, ampicillin–sulbactam, cefoxitin, cefuroxime, cefotaxime, ceftazidime, cefepime, aztreonam, amikacin, gentamicin, ciprofloxacin, co-trimoxazole, meropenem, and nitrofurantoin.

Phenotypic detection of ESBLs using CHROMagar ESBL

Each *K. pneumoniae* isolate was inoculated on CHROMagar ESBL, incubated at 37 °C for 18–24 h. Subsequently, the developed colonies with metallic blue coloration were considered ESBL-producers[16].

Detection of TEM, SHV and CTX-M genes

The prevalence of the ESBL-encoding genes (bla_{TEM}, bla_{SHV}, and bla_{CTX-M}) was investigated using conventional PCR. Briefly, DNA extraction was conducted by boiling lysis method as previously described[17]. Amplification of DNA was performed in a GeneAmp 9700 PCR system (Applied Biosystems, USA) with primer sets listed in Table 1, according to previously described cycling parameters. After electrophoresis in a 1.5% agarose gel and DNA bands of expected sizes were gel-purified and sequenced[18].

Table 1: Primer sequences for detection of ESBL genes

Target gene	Primer sequence (5′ → 3′)	Amplicon size (bp)	Reference
bla_{SHV}	F: TCGTTATGCGTTATATTCGCC	861	[19]
	R: GGTTAGCGTTGCCAGTGCT		
bla_{TEM}	F: TCGCCGCATACACTATTCTCAGAATGA	445	[20]
	R: ACGCTCACCGGCTCCAGATTTAT		
bla_{CTX-M}	F: ATGTGCAGYACCAGTAARGTKATGGC	593	[21]
	R: TGGGTRAARTARGTSACCAGAAYCAGCGG		

Plant materials and extraction process

The plant materials (*Solenostemma argel*) were purchased from the local market in Egypt. After being washed with water, the argel leaves were dried and ground into a fine powder. Fifty grams of the powdered vegetative parts were added to 500 mL of ethanol and methanol, as separate treatments, and extracted using an ultrasonic homogenizer for 8 h at 25 °C. The extracts were subsequently filtered under a vacuum and concentrated using a rotary evaporator[22]. The concentrated extracts were stored at −4 °C until use.

Determination of total phenolics

The total phenolic content (TPC) in the argel extracts was quantified spectrometrically as described previously[23]. In brief, 200 µL of the extract was mixed with 400 µL of Folin-Ciocalteu reagent and 4.0 mL distilled water. After incubation for 10 min at 25°C, 2 mL of saturated Na_2CO_3 solution was added, mixed, and incubated for 30 min. The absorbance was recorded at 765 nm using a Jenway 6300 spectrophotometer (Jenway, UK), and TPC was determined against a gallic acid calibration curve.

Determination of antioxidant activity

The antioxidant activity of the argel extracts was determined by estimating the 1,1-diphenyl-2-Picrylhydrazyl (DPPH) radical scavenging activity according to a previously reported procedure[24,25]. Briefly, 200 µL the extract was mixed with 1 mL of DPPH solution, and the mixture was incubated in the dark for 15 min. Subsequently, the absorbance was determined at 517 nm and the obtained results were expressed as percent inhibition (DPPH radical scavenging %).

Antibacterial activity against ESBL-producing *K. pneumoniae*

The MIC of each extract against ESBL-*Kp* was determined by the resazurin microtiter broth dilution method[26]. The control strain *Klebsiella pneumoniae* ATCC 700603 was used in this test. Stock solutions of each plant extract were prepared and subjected to bifold serial dilution (ranging from 0.25 to 256 µg/mL) using Mueller–Hinton broth (Condalab, Spain) in 96-well microplates. Subsequently, a bacterial suspension (5×10^5 colony-forming units/mL) was prepared and 100 µL aliquots were dispensed into each well. The microtiter plates were incubated at 37 °C, for 24 h. Subsequently, 10 µL resazurin (0.5 mg/mL) were added to each well, and the plates were further incubated for 3 hrs at 37 °C. Afterward, MIC values were visually determined by observing the color change, the lowest concentration of each extract that prevented the change in the color of resazurin was recorded as the MIC[26].

RESULTS

In this study, 200 non-repetitive *Klebsiella* spp. isolates were identified by MALDI-TOF/MS. Results revealed that the vast majority (168/200; 84%) of the isolates were identified as *K. pneumoniae*, followed by *K. variicola* (19/200; 9.5%) and *K. oxytoca* (13/200; 6.5%). Because the main aim of this work is emphasis on *K. pneumoniae*, we selected *K. pneumoniae* isolates (n = 168) for further investigations and neglected the other *Klebsiella* species (n = 32).

The susceptibility of all *K. pneumoniae* isolates (n = 168) against 16 antibiotics was investigated using the Vitek 2 system and the antibiograms were tabulated (Table 2). The investigated *K. pneumoniae* isolates exhibited high resistance against most of the assessed antibiotics, especially, ampicillin, cefuroxime, and co-trimoxazole. Regarding aminoglycosides, 106 (63%) and 21 (12.5%) of the investigated isolates were resistant to gentamicin and amikacin, respectively. In addition, 71.4% of the isolates exhibited resistance to cefotaxime. Furthermore, the isolates showed remarkable resistance to cefuroxime (83.9%), ampicillin–sulbactam (72%), ciprofloxacin (58.3%), ceftazidime (55.3%), aztreonam (44%), and cefepime (36.3%), respectively. The low resistance rates were observed against cefoxitin (11.9%) and meropenem (11.3%).

Table 2: Antibiotic susceptibility pattern of *K. pneumoniae* isolates (n = 168)

Antibiotics class/Category		Antibiotic	Antibiotic susceptibility		
			Resistant % (n)	Intermediate % (n)	Susceptible % (n)
Beta-lactams	Penicillin	Ampicillin	91 % (153)	0	8.9% (15)
	Beta-lactam combination agents	Piperacillin-Tazobactum	17.8 % (30)	14.2 % (24)	67.8 (114)
		Amoxicillin-clavulanate	41.6 % (70)	22.6 % (38)	35.7 % (60)
		Ampicillin–Sulbactam	72 % (121)	3.5 % (6)	24.4 % (41)
	Cephamycins	Cefoxitin	11.9 % (20)	1.8 % (3)	68.3 (145)
	2nd generation cephalosporins	Cefuroxime	83.9 % (141)	5.3 % (9)	10.7 % (18)
	3rd generation cephalosporins	Cefotaxime	71.4 % (120)	7.7 % (13)	20.8% (35)
		Ceftazidime	55.3% (93)	2.3 (4)	42.2 % (71)
	4th generation cephalosporins	Cefepime	36.3 % (61)	16 % (27)	47.6 % (80)
	Monobactams	Aztreonam	44 % (74)	1.2 % (2)	54.7 % (92)
	Aminoglycosides	Amikacin	12.5 % (21)	2.3 % (4)	85.1 % (143)
		Gentamicin	63 % (106)	1.8 % (3)	35.1 % (59)
	Quinolones	Ciprofloxacin	58.3 % (98)	3.5 % (6)	38.1 % (64)
	Folate pathway antagonist	Co-trimoxazole	96.4 % (162)	0	3.5 % (6)
	Carbapenems	Meropenem	11.3 % (19)	1.2 % (2)	87.5 % (147)
	Nitrofurans	Nitrofurantoin	38.7 % (65)	18.4 % (31)	42.8 % (72)

In this work, the phenotypic detection of ESBLs among the *K. pneumoniae* isolates was performed by CHROMagar ESBL. Of the investigated 168 isolates, 48 (28.5%) exhibited good growth with metallic blue coloration indicating the production of ESBL (Figure 1). Accordingly, ESBL-*Kp* isolates were selected and subjected to molecular studies. The phenotypically confirmed ESBL-*Kp* isolates (n = 48) were further characterized using conventional PCR to detect the presence of ESBLs-coding genes (Figure 2). Results revealed that all the investigated ESBL-*Kp* harbor the bla_{SHV} gene. The majority of isolates (42/48; 87.5%) were positive for bla_{CTX-M} while bla_{TEM} gene was detected in 45.8% of the investigated ESBL-*Kp* isolates (22/48).

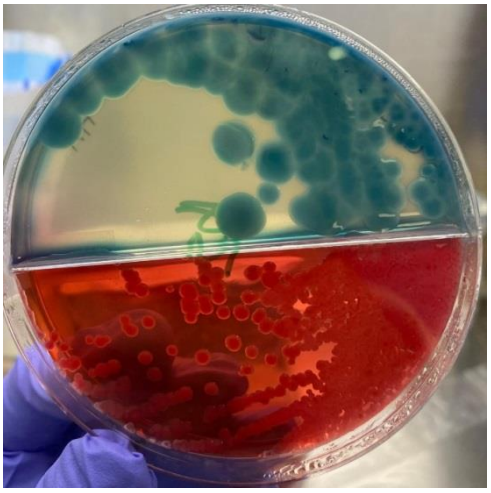

Fig. 1: Phenotypic detection of ESBL using CHROMagar ESBL. The upper part shows the typical growth of ESBL-*Kp* with the characteristic blue color. The lower part shows typical growth of *K. pneumoniae* on MacConkey agar.

Fig. 2: Agarose gel electrophoresis showing bla_{SHV}, bla_{CTX-M} and bla_{TEM}

PCR products amplified from ESBL-producing *K. pneumoniae* isolates

Herein, we performed ultrasonic-assisted methanolic and ethanolic extraction of argel leaves, and the chemical and biological properties of the extracts were assessed. The TPC of the ethanolic and methanolic extracts were 57.3 and 79.5 mg/g, respectively. Both extracts demonstrated considerable antioxidant activity, however the methanolic extract has superior antioxidant activity, compared with that of the ethanolic one (Table 3).

Table 3: Antioxidant activity of ethanolic and methanolic extracts of argel leaves

Concentration (µg/mL)	DPPH radical scavenging activities (%)	
	Ethanolic extract	Methanolic extract
20	35.6	55.1
40	54.6	69.1
60	66.3	78.2
80	73.4	84.6
100	82.4	95.3

Concerning the antimicrobial activity, we found that *S. argel* extracts had potent antibacterial activity against ESBL-*Kp* isolates. Results demonstrated that the MIC of the ethanolic extract against various ESBL-*Kp* isolates ranged from 8 to 32 µg/mL while that of the methanolic extract ranged from 1 to 16 µg/mL. The ethanolic and methanolic extracts of *S. argel* leaves inhibited *Klebsiella pneumoniae* ATCC 700603 with MIC values of 16 and 2 µg/mL, respectively.

DISCUSSION

Monitoring antibiotic resistance rates is crucial for developing empirical treatment techniques and evaluating the current guidelines. Moreover, searching for green and natural antimicrobial agents as potential alternatives to antibiotics could be a promising approach to combat the rapid emergence of the antibiotic resistance crisis. This study addresses the antibiogram of *K. pneumoniae* isolates and the phenotypic and genotypic assessment of ESBL-producing isolates. Furthermore, we investigated the antibacterial efficacy of ethanolic and methanolic extracts of argel leaves against ESBL-*Kp* isolates. In this study, *K. pneumoniae* isolates exhibited notable resistance levels to β-lactam antibiotics including penicillin (91%), cefuroxime (83.9%), ciprofloxacin (58.3%), ceftazidime (55.3%), aztreonam (44%), and cefepime (36.3%). These results agree with previous studies reporting the high resistance levels of *K. pneumoniae* isolates against β-lactam antibiotics[27,28]. It is worth noting that 11.3% of our isolates were carbapenem-resistant, exhibiting resistance against meropenem. The carbapenems are usually considered as last line of defence against severe bacterial infections, especially with MDR bacteria[29]. In an attempt to characterize the ESBL-*Kp* isolates at

molecular level, we investigated the prevalence of three ESBL-codding genes. We found that most isolates (87.5%) were positive for bla_{CTX-M} while the bla_{TEM} gene was detected in 45.8% of the investigated ESBL-*Kp* isolates. These results harmony recent studies reporting that bla_{SHV} and bla_{CTX-M} were the most prevalent ESBL-coding genes acquired by almost all ESBL-*Kp* isolates with a relatively low prevalence of bla_{TEM} gene[30]. In a recent study conducted on *K. pneumoniae* isolated from patients admitted to the Urology & Nephrology Center in Egypt, the prevalence of bla_{TEM}, bla_{SHV} and bla_{CTX-M} were 80, 70, and 63.3%, respectively[31]. The sulfhydryl reagent variable (SHV) β-lactamases have the ability to hydrolyze oxacillin and cloxacillin and are not inhibited by clavulanate acid and provide extraordinary resistance against β-lactam antibiotics[32]. Antibiotic-resistant bacteria that are challenging to treat require developing of novel therapeutics and approaches, such as using adjuvants, natural products, bacteriophages, antimicrobial peptides, and nanoparticles.

In this work, the TPC extracted from argel leaves is relatively higher than those reported in previous studies[33]. The improved recovery of phenolic content by the ultrasonic-assisted extraction approach could be due to the breakage of plant cells by ultrasound waves, resulting in the easier release of the cellular contents into the solvent. Also, the discrepancy in the phenolic content of extracts could be attributed to the differences in the growing environment, growth stage, and harvesting time[34]. Moreover, we observed the increased scavenging potency with the increasing argel concentration, and the highest antioxidant activity (95.3%) was achieved by using methanolic extract of argel leaves (100 µg/mL). These observations are consistent with previous investigation that stated the efficacy of methanol as a promising solvent to extract phenolics and antioxidant compounds from *S. argel*[35]. It has been proposed that the antioxidant activity of *S. argel* extracts is because of the existence of phenolic acids such as sinapic acid, p-coumaric, ferulic, and 4-hydroxybenzoic acids as well as glycosylated flavonoids, polyphenols, β-carotene, and monoterpenes[22,36]. Besides the powerful antioxidant activity, this investigation sheds light on the antibacterial effects of the argel extracts against ESBL-*Kp*. Likewise, extracts of argel were reported as promising antibacterial agents against *E. coli*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterobacter cloacae*, *Acinetobacter baumannii*[33]. Argel is known to have diverse phenolics including caffeoylquinic acid, feruloylquinic acid and vanillate glucoside which could be play a crucial role in inhibiting these microbes[37]. According to previous reports, argel extracts contain various flavonoidal compounds with antimicrobial activity such as catechin and kaempferol[38]. Along with phenolics and flavonoids, a recent study demonstrated the antibacterial antibiofilm activity of argel against *Salmonella enterica* serotype *typhimurium* is attributed to the presence of a steroidal glycoside called stemmoside C[39]. In a recent report, argel extract has demonstrated activity against *Salmonella typhimurium*, *Bacillus subtilis*, *E. coli*, *P. aeruginosa*, and *Listeria innocua*[40]. In a comparable investigation, it was found that argel extract displayed significant antibacterial properties and reduced the formation of biofilms in *S. aureus*[13]. One limitation of our study was that the cytotoxicity of the argel extract was not assessed. Also, the antibacterial mechanism was not investigated in comparison to standard antibiotics.

CONCLUSION

This investigation shed light on the elevated levels of resistance among clinical *K. pneumoniae* isolates and highlighted the presence of bla_{SHV} gene by all inspected ESBL-*Kp*. Moreover, the study verified the potent antibacterial activity of the methanolic and ethanolic extracts of argel against ESBL-*Kp* and indicated the superior antibacterial activity of the methanolic extract with MIC values ranging from 1 to 16 µg/mL. These findings suggested that methanolic extract of argel is a potential candidate for combating ESBL-*Kp*. In conclusion, using argel extract as a sustainable antimicrobial agent to counteract ESBL would mitigate antibiotic resistance and promote its future therapy implementation.

Declarations:

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Consent for publication: Not applicable

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