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Andrographis paniculata extract inhibit growth, biofilm formation in multidrug resistant strains of *Klebsiella pneumoniae*

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ABSTRACT

Background and aim: *Andrographis paniculata* (Kalmegh), a valuable ancient medicinal herb is used in the treatment of several diseases in most Asian countries including India. *Klebsiella pneumoniae* is an opportunistic pathogen causing nosocomial infections in human. We have investigated the antimicrobial susceptibility and the presence of AmpC gene in *K. pneumoniae* strain isolated from the sputum of the patient.

Experimental procedure: Antibiotic susceptibility test and phenotypic detection of AmpC/ESBL beta-lactamase were performed by combined disc diffusion test. The CEA of *A. paniculata* was analyzed for its antibacterial potential against susceptible and resistant strains of *K. pneumoniae* through the broth microdilution method. Molecular detection of AmpC gene was carried by polymerase chain reaction (PCR).

Results: Antibiotic susceptibility test displayed that the clinical isolate of *K. pneumoniae* were resistant towards cephalosporins, quinolone and monobactam but susceptible to carbapenems. Combined disk diffusion demonstrated AmpC^{+ve}/ESBL^{-ve} beta-lactamase. 250 μg/ml of CEA extract confirmed the inhibition of bacterial growth and biofilm formation compared to the antibiotic. CEA treated *K. pneumoniae* displayed a reduction of AmpC by polymerase chain reaction.

Conclusion: The present study illustrates that CEA extract of *A. paniculata* demonstrated potentiality to control *K. pneumoniae* growth and biofilm formation. CEA was able to suppress the expression of gene encoding AmpC. This study proves to be an economical approach to control the growth of *K. pneumoniae* which causes serious infections.

1. Introduction

A. paniculata is an annual herb belonging to the family Acanthaceae and is being used as a traditional medicine. This plant has been mentioned in Charka Samhita, Holy Quran and Holy Bible for its immense medicinal properties.[1–3] *A. paniculata*, also known as Kalmegh, Bhunimba (Sanskrit) or Nilavembu (Tamil/Telugu) or Kirayat/Kalpnath (Hindi) or king of bitters (English) is used for the treatment of diseases including common cold, diarrhea, upper respiratory infection (common cold, flu), liver disease (enlargement of lever, jaundice), fever, cardiovascular disease, dyspepsia, skin infection, colic dysentery and is also consumed as health tonic because of its antioxidant property.[4,5] Previously it has been reported that this plant possesses anti-inflammatory, antipyretic, antiviral, antiprotozoan and anti-cancer properties.[6,7]

Phytocompounds in the methanol extract of *A. paniculata* especially, 3-O-β-D- glucosyl-14 deoxyandrographolide and 14-deoxyandrogropholide exhibited antibacterial efficiency.[8] Another researcher investigated the antibacterial potential of *A. paniculata* against Gram-positive and Gram-negative bacteria. Out of 26 bioactive compounds present in *A. paniculata*, Andrographolide is a major bioactive compound.[9]

Extended-spectrum beta-lactamase (ESBL), Metallo beta-lactamase or ampicillinase (AmpC) related Gram-negative bacteria have developed broad resistance towards antibiotics. Plasmid-mediated AmpC beta-lactamase hydrolyzes the beta-lactams and cephalosporins including oxyimino-beta-lactam.[10] Multi-drug resistant *K. pneumoniae*, which is one of the major reason for

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Abbreviations

AZM	Azithromycin
AMP	Ampicillin
AMC	Amoxyclav
AT	Aztreonam
CZ	Cefazolin
CTX	Cefotaxime
CTN	Cefotetan
CAZ	Ceftazidime
CPD	Cefpodoxime
CTR	Ceftriaxone
CIP	Ciprofloxacin
IPM	Imipenem
LE	Levofloxacin
MRP	Meropenem
VA	Vancomycin
CX	Cefoxitin
CXX	Cefoxitin + Cloxacillin
CXX	Ceftazidime + Clavulanic acid
MHA	Mueller Hinton Agar
ESBL	Extended-spectrum beta-lactamase
AmpC	Ampicillinase
MARI	Multiple antibiotic resistance indexes
CEA	Crude Ethyl Extract
ATCC	American Type of Culture Collection
PCR	Polymerase Chain Reaction
DNA	Deoxyribonucleic acid
EtBr	Ethidium bromide
OD	Optical Density
μg	Microgram
mg	Milligram
HOD	Head of the Department
BSACIST	B. S. Abdur Rahman Crescent Institute of Science and Technology

nosocomial infections, poses a serious threat in the clinical practice because of the difficulties in the diagnosis due to the production of Carbapenemase and ESBLs/AmpC enzymes.[11]

The present study focused on the inhibitory effect of CEA of *A. paniculata* on multi-drug resistant *K. pneumoniae* growth and biofilm formation. Furthermore, the effect of CEA on the expression of the gene encoding AmpC beta-lactamase was studied.

2. Material and methods

2.1. Collection of isolates and A. paniculata

The clinical strain of *Klebsiella pneumoniae* (n = 1) used in this study was isolated from patient's sputum and was provided by the Department of Microbiology, Tagore Medical College and Hospital, Tamilnadu, India. The clinical isolate was confirmed through differential culture media and standard biochemical techniques.[12] Glycerol stocks of *K. pneumoniae* were stored at −80 °C for further use. ATCC strain of *K. pneumoniae* (ATCC 35657) was used as a control. Our previous studies on *A. paniculata* have been reported by Rasool et al. (2018).[13]

2.2. Antimicrobial susceptibility and phenotypic detection for AmpC and ESBL

The antimicrobial susceptibility analysis of a hospital isolates was performed by disc diffusion method on Mueller-Hinton Agar plates according to Clinical and Laboratory Standards Institute recommendations.[14] Following antibiotics (Hi-Media Mumbai) were used: Azithromycin (AZM; 15 μg/disc), Ampicillin (AMP; 10 μg/disc), Aztreonam (AT; 30 μg/disc), Amoxiclav (AMC; 20/10 μg/disc), Cefazolin (CZ; 30 μg/disc), Cefotaxime (CTX; 30 μg/disc), Cefotetan (CTN; 30 μg/disc), Ceftazidime (CAZ; 30 μg/disc), Cefpodoxime (CPD; 30 μg/disc), Ceftriaxone (CTR; 30 μg/disc), Ciprofloxacin (CIP; 5 μg/disc), Imipenem (IPM; 10 μg/disc), Levofloxacin (LE; 5 μg/disc), Meropenem (MRP; 10 μg/disc), and Vancomycin (VA; 30 μg/disc). These antibiotic discs were utilized to screen the resistance patterns of the strain. The Cefoxitin-Cloxacillin double disc synergy test was performed as explained by Tan Y. et al. (2009). This test is based on the inhibitory effect of Cloxacillin on AmpC. Discs containing 30 μg of Cefoxitin or 30 μg of Cefoxitin plus 200 μg Cloxacillin were used. The clinical isolates were considered to be positive for AmpC production when a zone diameter of approximately ≥4 mm for the discs containing CX/CXX versus the Cefoxitin alone was observed[15] on MHA plate. ESBL production was tested by double disc diffusion test where discs containing 30 μg of ceftazidime or 30 μg of ceftazidime plus 10 μg of clavulanic acid were used. The clinical isolates were considered to be positive for ESBL production when a zone diameter of approximately ≥5 mm for the discs containing CAZ/CAC versus ceftazidime alone was observed. The susceptibility results, AmpC detection and ESBL confirmation were observed after 18–24 h of incubation at 37 C.

2.3. Multiple antibiotic resistance indexes (MARI) determination

MARI calculations for clinical isolates and ATCC strain were calculated by using the following formula MARI = (a/b) Where 'a' denotes the number of antibiotics resistance, 'b' denotes the number of antibiotics used for susceptibility evaluation Krumperman (1983).[16]

2.4. Antibacterial activity (microdilution method)

CEA was tested for antibacterial efficacy against a clinical isolate (AmpC beta-lactamase producing strain) and ATCC strain of *K. pneumoniae* through broth serial microdilution.[17] Four different dilutions of the extract were made (62, 125, 250, and 500 μg/ml) in Lysogeny broth. The cultures (0.5 McFarland) were grown in Lysogeny broth and incubated overnight at 37 °C with the treatment. After the completion of the incubation period, the density was measured at 600 nm by using multimode plate reader (EnSpire™ Multilabel Reader 2300, S. No. 2300096). All the experiments were conducted in triplicates and the mean values were considered as final outcomes.

2.5. Biofilm inhibition assay (microtiter plate)

CEA was tested for its antibiofilm potential against clinical isolate (AmpC beta-lactamase producing strain) and ATCC strain of *K. pneumoniae*.[18] Four different dilutions of the extract were made (62, 125, 250, and 500 μg/ml) in Lysogeny broth. The cultures (0.5 McFarland) were grown in Lysogeny broth and incubated for 48 h at 37 °C (Rotary shaker) with the treatment. Control was maintained. After the incubation, 150 μl of 0.1% solution of crystal violet was added in the wells followed by incubation at room temperature for 10–15 min. Excess dye was washed and 150 μl of the 30% glacial

Table 1
Base sequence of primers used for gene expression.

Target gene		Sequence (5′–3′)	Amplicon size (bp)
AmpC	forward	ATTCCGGGTATGGCCGT	835
	reverse	GGGTTTACCTCAACGGC	
ß-Actin	forward	CCCAGCACAATGAAGATCAAGATCAT	110
	reverse	ATCTGCTGGAAGGTGGACAGC	

acetic acid in water was added to each well, OD was measured at 570 nm by using multimode plate reader. All the experiments were conducted in triplicates and the mean values were considered as final outcomes.

2.6. Molecular detection of AmpC gene by PCR

As per the protocol, the DNA was isolated from the treated samples (250 μg/ml of CEA was used for the treatment) of the clinical isolate and ATCC strain of *K. pneumoniae*.[19] Gel electrophoresis was performed to identify the DNA followed by PCR amplification of AmpC gene and ß actin gene (Master cycler; Eppendorf USA). As per the protocol, 20 μl reactions were prepared, containing 10 μl of the 2x-Redeye master mixture (Amplicon -iii), 2 μl of forward and reverse primers of AmpC[20] and ß actin (Table 1) and 6.0 μl of template DNA. The reaction protocol required an initial step of 5 min at 95 °C, followed by 30 cycles of 95 °C for 1 min, 58 °C for 1 min, 72 °C for 45 s and the final extension at 15 min. The amplified products were electrophoresed (1.5% agarose gel) with 100 bp ladder. Finally, the DNA was stained with EtBr and visualized (ChemiDoc MP System; Bio-Rad, USA 2013).

2.7. Statistics analysis

All the experiments were performed in triplicates, and the mean values, standard deviations and *t*-test have been calculated by using

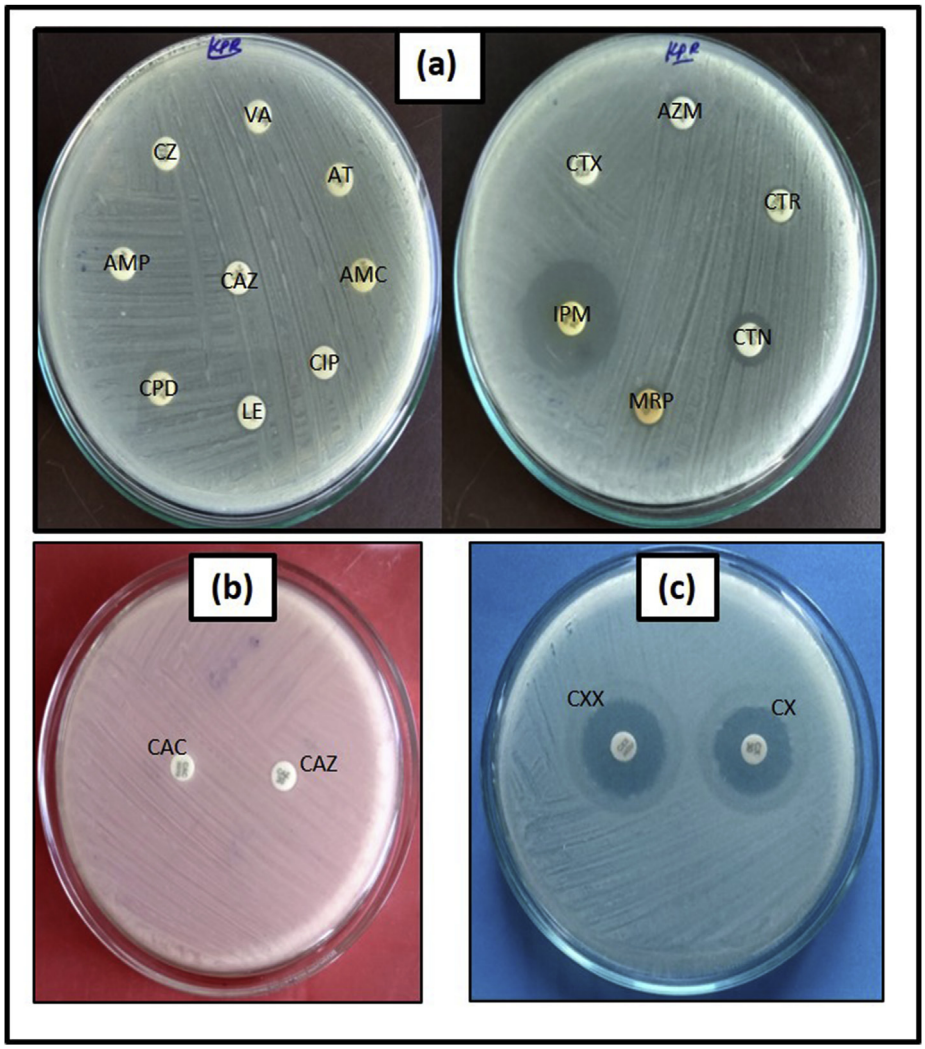

Fig. 1. (a) Susceptibility details of *K. pneumoniae* isolates towards different antibiotics (b) ESBL detection by double disk diffusion test Ceftazidime (CAZ 30 μg/disc), Ceftazidime + clavulanic acid (CAC 30/10 μg/disc). A zone diameter difference of ≥5 mm between Ceftazidime 30 μg discs & Ceftazidime-Clavulanic acid 30-10 μg discs should be interpreted as ESBL positive (c) AmpC-beta-lactamase detection by double disk diffusion test: Cefoxitin (CX 30 μg/disc), Cefoxitin + Cloxacillin (CXX 30/200 μg/disc). A zone diameter difference of ≥4 mm between Cefoxitin 30 μg discs & Cefoxitin-Cloxacillin 30–200 μg discs should be interpreted as AmpC positive.

Table 2
Susceptibility profile of *K. pneumoniae* isolate towards different antibiotics.

Strain	Source of isolate	Resistance details	Sensitive	MARI Calculations
K. Pneumoniae	Sputum	AZM, CTR, CTN, MRP, CTX, VA, AT, AMC, CIP, LE, CPD, AMP, CZ,CAZ	IPM	0.94

Where: Azithromycin (AZM 15 μg/disc), Ampicillin (AMP 10 μg/disc), Amoxyclav (AMC 20/30 μg/disc), Aztreonam (AT 30 μg/disc), Cefazolin (CZ 30 μg/disc), Cefotaxime (CTX 30 μg/disc), Cefotetan (CTN 30 μg/disc), Ceftazidime (CAZ 30 μg/disc), Cefpodoxime (CPD 30 μg/disc), Ceftriaxone (CTR 30 μg/disc), Ciprofloxacin (CIP 5 μg/disc), Imipenem (IPM 10 μg/disc), Levofloxacin (LE 5 μg/disc), Meropenem (MRP 10 μg), Vancomycin (VA 30 μg/disc).

Microsoft Excel 2007.

3. Results

3.1. Antibiotic susceptibility testing and phenotypic AmpC beta-lactamase and ESBL detection

The antibiotic resistance profiles and MAR index of the clinical strain of *K. pneumoniae* are presented in Fig. 1a and Table 2. The clinical isolate was resistant towards several antibiotics (multi-drug resistant) but susceptible to Imipenem. MAR index was 0.94 which indicates the misuse of antibiotics in society. The phenotypic confirmatory test of ESBL production by combined disk diffusion test showed no ESBL production by the clinical strain of *K. pneumoniae* (Fig. 1b). Furthermore, the same strain displayed AmpC beta-lactamase production phenotypically. A zone diameter difference of ≥4 mm between Cefoxitin 30 μg discs & Cefoxitin-Cloxacillin 30–200 μg discs was interpreted as AmpC positive (Fig. 1c). The results indicated that due to overproduction of ampicillinase, the clavulanic acid (inhibitor) was unable to inhibit the growth.

3.2. Antimicrobial activity through broth microdilution technique

Among the four different concentrations of CEA (62, 125, 250 and 500 μg/ml), 250 & 500 μg/ml of CEA was more effective against the *K. pneumoniae* strain compared to other two concentrations and the effect was comparable with the control antibiotic. 62 & 125 μg/ml of CEA was not much effective and the OD values were similar to the untreated strains (Fig. 2a and b).

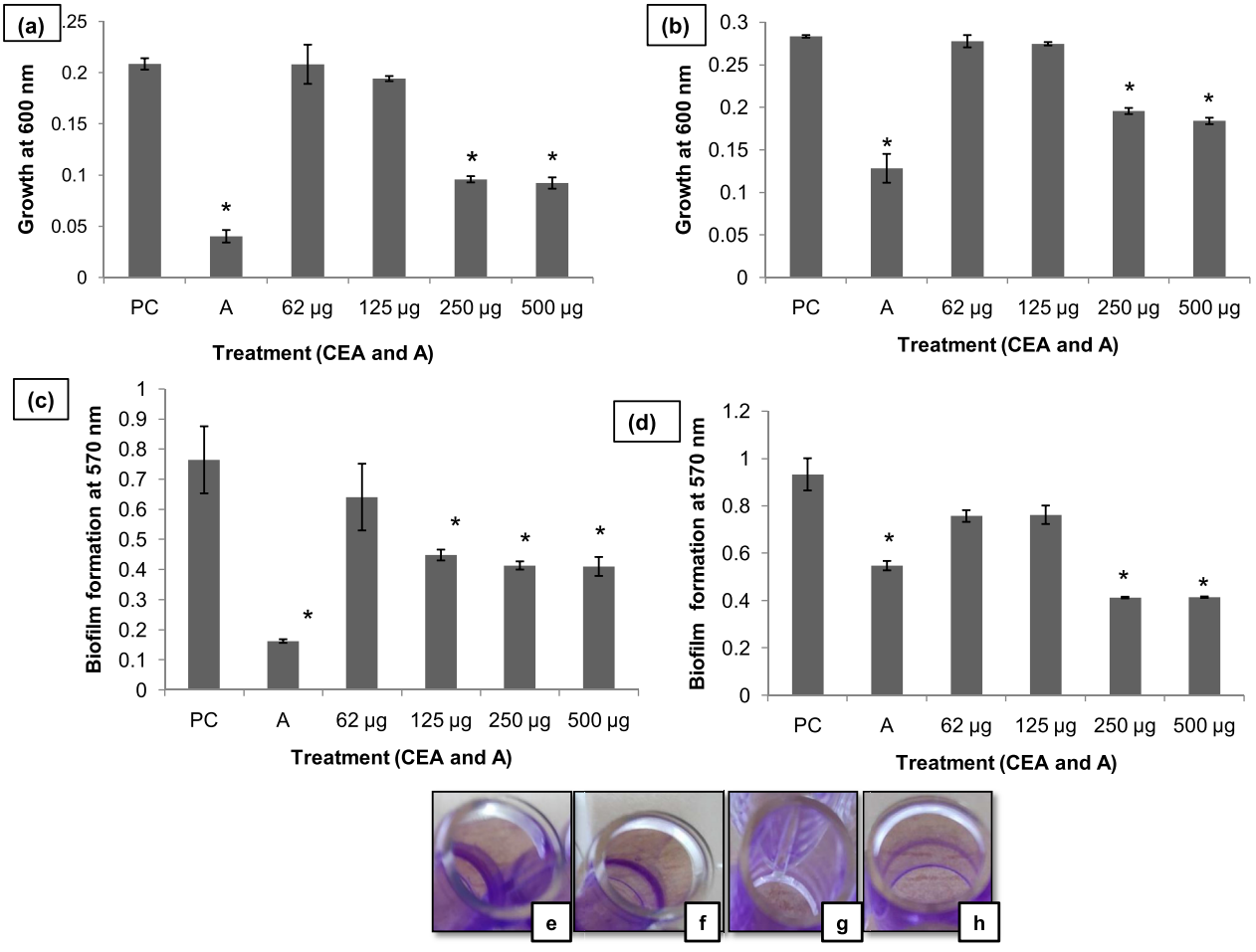

Fig. 2. Antimicrobial activity of CEA on the growth of (a) ATCC 35657 and (b) MDR strain of *K. pneumoniae*, (c) Effect of CEA on the biofilm formation of (c) ATCC 35657 and (d) MDR strain of *K. pneumoniae*. (e, f, g, & h) The biofilm at various concentrations around the walls in microtiter plate. Bar represents mean value and the error bars shows standard error. * Denotes significant difference at ($p < 0.05$).

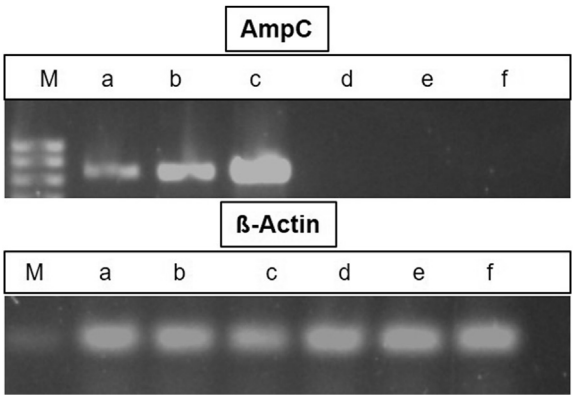

Fig. 3. Electrophoretogram of polymerase chain reaction product of AmpC gene (a) *K. pneumoniae* strain treated with CEA (b) *K. pneumoniae* strain treated with antibiotic (c) *K. pneumoniae* strain untreated as control (d) *K. pneumoniae* ATCC 35657 control untreated (e) *K. pneumoniae* ATCC 35657 treated with antibiotic (f) *K. pneumoniae* ATCC 35657 treated with CEA. M: 100 bp DNA ladder, β-actin gene was used as control.

3.3. Inhibition of biofilm

Biofilm inhibition assay was performed using four different concentration of CEA against both the strains of *K. pneumoniae*. 250 & 500 μg/ml concentrations are effective in inhibition of biofilm when compared to the commercial antibiotics. 62 mcg/ml of CEA was slightly effective against both strains and the OD values were similar to the untreated control (Fig. 2c–h).

3.4. Molecular detection of AmpC gene

DNA was isolated from AmpC beta-lactamase producing a clinical strain of *K. pneumoniae* with and without the treatment of CEA and PCR amplification was performed with AmpC beta-lactamase gene. β-Actin gene was used as the control. The PCR results were compared with the control and the intensity of the bands were evaluated, where the ATCC strain of *K. pneumoniae* was found to be negative for AmpC gene. On the other hand, the clinical isolate of *K. pneumoniae* treated with the CEA exhibited downregulation of AmpC gene when compared to the antibiotic-treated strain (Fig. 3).

4. Discussion

Nosocomial infection is a big challenge worldwide. *K. pneumoniae* is one of prominent causative agent of it. Since 1982, the hypervirulent and drug-resistant strains are spreading rapidly which causes morbidity and mortality.[21] AmpC beta-lactamase producing strains are resistant to a variety of antibiotics through the hydrolysis of beta-lactam bond and are not inhibited by clavulanic acid.[22] *A. paniculata* is one of the traditional valuable medicinal plants in India and the genus *Andrographis* consists of around 40 species.[23] Geetha and co-workers (2017)[24] researched on 100, 150 and 200 mg/ml of methanol and chloroform extracts of *A. paniculata* against nine bacterial strains and showed effective zone formations (10–16 mm zone) in *Escherichia coli, Aeromonas hydrophila, Proteus vulgaris, Staphylococcus aureus, Streptococcus pyogenes, Bacillus subtilis, K. pneumoniae* and *Salmonella typhi*, but *Pseudomonas aeruginosa* did not show any zone of inhibition. In the present study, CEA of *A. paniculata* leaves was tested for antimicrobial and antibiofilm formation towards an AmpC beta-lactamase producing clinical strain along with the ATCC (35657) strain of *K. pneumoniae*. Previously, *A. paniculata* (leaves) extract was tested against *S. aureus, Enterococcus faecalis* and *Mycobacterium tuberculosis* and 3.0 mg/ml were found effective against *S. aureus*.[25] One more study by Suparna and co-workers (2014) used 100–200 μg/ml of leaf extract of *A. paniculata* and reported the effectiveness against Gram-positive bacteria.[26] In the present study, the antibiotic susceptibility test of the clinical isolate showed resistance towards cephalosporins, beta-lactamase inhibitor (clavulanic acid), monobactam and a carbapenem (Meropenem). The effect of CEA revealed the inhibition/suppression of AmpC amplification, a gene which is most commonly found in the ampicillinase positive isolates and which provides resistance toward the beta-lactam antibiotics. The current study found the unique antibacterial potential of *A. paniculata* against AmpC producing *K. pneumoniae* that can be an alternative to the antibiotics towards which MDR pathogens.

5. Conclusion

Antibiotic susceptibility test confirmed the resistance pattern of *K. pneumoniae* strain towards the different formulation of antibiotics. Outcomes of phenotypic study confirmed the ampicillinase production by bacteria. The present study illustrated that CEA from *A. paniculata* has the potential to control both the growth and biofilm formation of *K. pneumoniae*. CEA was capable of suppressing the expression of gene encoding AmpC. The application of CEA may provide an alternative approach for the patient's health and further studies are required to identify the mechanism of action.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtcme.2019.02.006.

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