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Efficacy of *Andrographis paniculata* against AmpC producing multi drug resistant *E. coli*

Saroj Kumar Sah, Ubaid Rasool, D. Mubarak Ali, S. Hemalatha*

School of Life Sciences, B.S. Abdur Rahman Crescent Institute of Science and Technology, Chennai, 600048, Tamil Nadu, India

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ABSTRACT

Andrographis paniculata (Acanthaceae) is a well-known medicinal plant and is conventionally used in herbal medicine for the treatment of several diseases such as cancer, metabolic disorders, skin infections, respiratory tract infections, etc. In this present study, ethyl acetate extract of *A. paniculata* was used to test its efficacy on controlling growth and biofilm formation in multi drug resistant pathogenic strains of *Escherichia coli* through micro-dilution method. Antibiotic susceptibility screening through disc diffusion revealed that these *E. coli* strains were resistant towards 13 out of 15 antibiotics used. It was found that 250 and 500 μg/ml of crude ethyl acetate (CEA) extract effectively reduced the growth and biofilm formation as compared to 62 and 125 μg/ml. The strains treated with CEA extract of *A. paniculata* reduced extracellular polysaccharide (EPS) production and downregulated the expression of AmpC gene. The results suggested that CEA extract of *A. paniculata* can be explored to utilize as an alternative to antibiotics.

1. Introduction

A. paniculata is used in traditional medicine to cure diseases such as common cold, diarrhea, dengue, malaria, diabetes, liver dysfunction, insect bite, sexually transmitted disease and is also used as health tonic to induce immunity (Snower et al., 2014). *A. paniculata* is known as Kalmegh (Sanskrit) or Nilavembu (Tamil) or Kalpnath (Hindi) or king of bitter (English). This plant is growing in tropical areas of India, Nepal, China, Bangladesh, Pakistan, Malaysia, Bhutan and France (Talei et al., 2012). The bioactive compounds present in *A. paniculata* enhance the antimicrobial property (Rasool et al., 2018). Ethanol extract of *A. paniculata* has been reported to possess antibacterial activity against pathogenic and nonpathogenic bacteria including *E. coli*, *S. typhimurium*, *P. vulgaris*, *B. subtilis* and *S. aureus* (Mishra et al., 2009). Bioactive compounds, 14- deoxyandrographolide and 3-O beta-D-glucosyl-14-deoxyandrographolide, from methanol extract of *A. paniculata* have been reported to possess antibacterial activity (Sule et al., 2011). Xia and coworkers reported the anti-inflammatory activity of *A. paniculata* which is mediated through NF-$_K$B (Xia et al., 2004). *E. coli* is a facultative anaerobic bacterium which causes nosocomial infections and has the potential to form biofilm through the extracellular poly saccharide (EPS) which help in colonization and for surface attachment which is a key factor for developing antibiotic resistance. Chromosome-mediated AmpC beta-lactamase enzyme present in *E.coli* is responsible for resistance against antibiotics including extended spectrum beta-lactamase cephalosporins, narrow spectrum cephalosporins (George and Jacoby, 2009). The current study was focused on evaluating the antibacterial efficacy, biofilm inhibition potential, EPS inhibition potential and gene expression of crude ethyl acetate (CEA) extract from *A. paniculata.*

2. Materials and methods

2.1. Materials

Bacterial culture media and antibiotic disk, DNA ladder were purchased from Hi-Media Mumbai, India. Susceptible strain of *E. coli* 25922 was purchased from American type of culture collection (ATCC). Commercial available ethanol, KH_2PO_4, MgSo4.7H_2O, NaCL, H_2SO_4, and glacial acetic acid was purchased from Hi-Media, Mumbai, India. Ethyl acetate, glycerol and Phenol were purchased from MERK-Mumbai, India.

2.2. Collection of clinical isolate and extraction of A. paniculata

E. coli clinical isolate was provided by the Department of Microbiology, Tagore Medical College and Hospital, Tamilnadu, India and was confirmed through differential culture media and standard

* Corresponding author.
E-mail address: hemalatha.sls@crescent.education (S. Hemalatha).

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biochemical techniques (Farmer, 2003). Glycerol stocks of *E. coli* were stored at −80 °C for further use. A susceptible strain of (ATCC 25922) *E. coli* was used as a control. The extract of *A. paniculata* was prepared as previously described (Rasool et al., 2018). The extract was analysed through Fourier-transform infrared spectroscopy (FTIR) to identify the functional groups present in the crude extract.

2.3. Antimicrobial susceptibility and phenotypic detection of AmpC

The antimicrobial susceptibility test of *E. coli* clinical isolate was performed via disk diffusion method on Mueller-Hinton Agar plates following Clinical and Laboratory Standards Institute (CLSI, 2012) guidelines. Following antibiotic in µg per disk (Hi-Media Mumbai) were used: Azithromycin (AZM) 15 µg, Ampicillin (AMP) 10 µg, Aztreonam (AT) 30 µg, Amoxiclav (AMC) 20/10 µg, Cefazolin (CZ) 30 µg, Cefotaxime (CTX) 30 µg, Cefotetan (CTN) 30 µg, Ceftazidime (CAZ) 30 µg, Cefpodoxime (CPD) 30 µg, Ceftriaxone (CTR) 30 µg, Ciprofloxacin (CIP) 5 µg, Imipenem (IPM) 10 µg, Levofloxacin (LE) 5 µg, Meropenem (MRP) 10 µg, and Vancomycin (VA) 30 µg. The Cefoxitin-Cloxacillin double disc synergy test was performed (Tan, 2009). This test is based on the inhibitory effect of Cloxacillin on AmpC. Discs contain 30 µg of Cefoxitin or 30 µg of Cefoxitin plus 200 µg Cloxacillin were used. The clinical isolate was considered to be positive for AmpC production when a zone diameter of approximately ≥ 4 mm for the disks containing CX/CXX versus the comparative and Cefoxitin alone was observed (Tan, 2009) on MHA plate. The susceptibility results and AmpC detection were observed after 18–24 h of incubation.

2.4. Determination of multi antibiotic resistance index (MARI)

Multi antibiotic resistance index (MARI) calculations for both the strains were estimated 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 (Chika et al., 2017).

2.5. Antibacterial activity by microdilution method

A. paniculata extract was tested for antibacterial efficacy against a clinical isolate (AmpC beta-lactamase producing strain) and susceptible strain of *E. coli* through serial microdilution (Chuah et al., 2014). Four different concentrations of the extracts were (62, 125, 250, and 500 µg/ml) utilized to treat the cells and incubated at overnight at 37 °C with the treatment. After the completion of the incubation period, the OD was measured at A600 by using multimode plate reader (EnSpire™ Multilabel Reader # 2300096). All the experiments were carried out in triplicates.

2.6. Biofilm inhibition assay (Microtiter plate)

The plant extract of *A. paniculata* was tested for its antibiofilm potential against a clinical isolate (AmpC beta-lactamase producing strain) and susceptible strain of *E. coli* (Toole, 2011). Four different dilutions of the extract were made (62, 125, 250 and 500 µg/ml) in LB broth. The cultures (0.5 McFarland) were grown in LB broth and incubated for 48 h at 37 °C (Rotary shaker) with and without the extracts. After incubation the plate was washed with distilled water and air dried. Subsequently 150 µl of (0.1%) crystal violet was added in all the wells and left it for 10 min. 150 µl of the (30%) glacial acetic acid in water was added to well and OD was measured at A570 by using multimode plate reader. All the experiments were conducted in triplicates.

2.7. EPS estimation and quantification

A slightly modified version of the method proposed by Sankar and Rai (2015) was adopted for the extraction and quantification of EPS. 250 µg/ml of CEA extract was used to treat the *E. coli* strains in test tubes containing LB broth and the untreated strains served as a control. This was followed by incubation at 37 °C for 20 h in a rotatory shaker. The supernatant were discarded after the centrifugation and the pellets were re-suspended in salt buffer pH7.5 (10 m M KH_2PO_4, 2.5 Mm MgSo4.$7H_2O$, 200 mM NaCl) for 15 min and tubes were centrifuged for 20 min at 12000 rpm. Further, 500 µl of ethanol and 500 µl of collected supernatant (1:1) were added and centrifuged for 20 min at 12,000 rpm. Furthermore, 1 ml of 5% cold phenol and 1 ml of precipitated EPS were mixed. Finally, 5 ml of concentrated H_2SO_4 was added to develop a red color. Intensity of the color was measured at A490. This test was performed in triplicates.

2.8. Analysis of AmpC gene expression by PCR

As per the protocol, the DNA was isolated from the treated samples (250 mcg/ml of the plant extract was used for the treatment) of a clinical isolate and susceptible strain of *E. coli* followed by PCR amplification of AmpC gene (Master cycler; Eppendorf USA). As per the protocol, 20 µl reactions were prepared, containing 10 µl of the 2x-Redeye master mixture, 2 µl of forward and reverse primers of AmpC and beta-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 (Jones, 2010; Gianluca et al., 2003). The amplified products were run (1.5% agarose gel) along with 100 bp ladder as standard. Finally, the DNA was stained with EtBr and visualized under gel doc (ChemiDoc™ MP System; Bio-Rad, USA 2013).

2.9. Statistical analysis

All the experiments were carried out in triplicates. Mean, standard deviation and *t*-test were analysed using the Microsoft Excel 2007 to find the significant difference among different treatments and AmpC gene expression was analysed by image J software.

3. Results and discussion

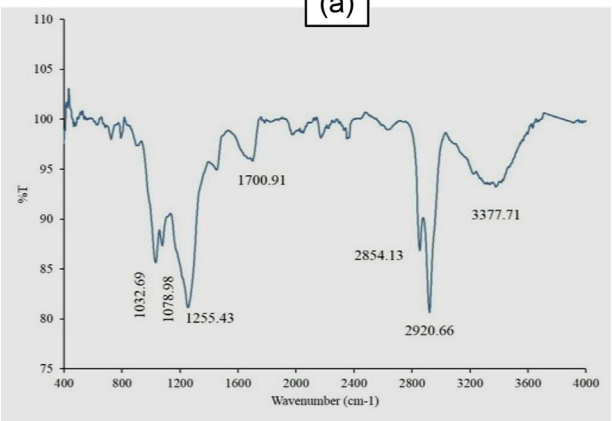
FTIR spectroscopy provided the characteristic absorptions which correspond to different functional groups (Fig. 1a and b). FTIR analysis of CEA extract provided information about various biomolecules that can enhance the antibacterial activity of the extract. Previous reports have showed the presence of amines, alkanes, alkenes, esters, alcohol, etc. in the FTIR spectra of *A. paniculata* (Rasool et al., 2018). The antibacterial activity of *A. paniculata* has been attributed to its functional groups. Roy et al. (2010) reported the presence of phenols, aromatic carboxylic acids and esters as active antibacterial in *A. paniculata*. In the present study, FTIR analysis of *A. paniculata* revealed the presence of different functional groups viz., amine, aeromatic, ester, amide, alkene and alcohol which can play an important role in enhancing the antibacterial activity of *A. paniculata*.

The antibiotic susceptibility of *E. coli* strains was carried out through Kirby Bauer (disk diffusion) method. The susceptible strain of *E. coli* was sensitive towards 15 antibiotics, the antibiotic susceptibility revealed that the clinical strain of *E. coli* was resistant towards 13 of the antibiotics used and showed sensitivity towards ceftriaxone and

Table 1
Base sequence of primers used for gene expression.

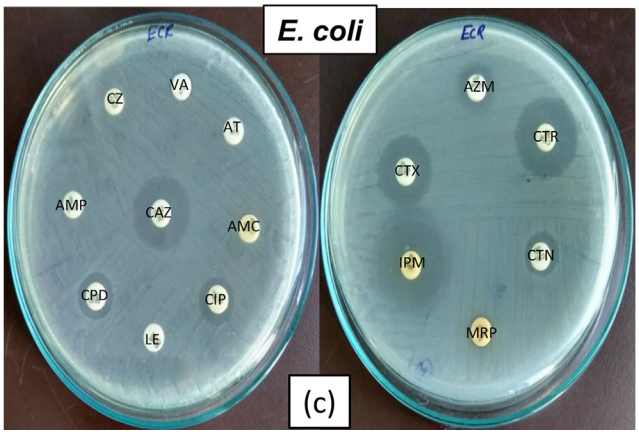
Gene		Sequence (5′-3′)	Amplicon size (bp)
AmpC	forward	ATTCCGGGTATGGCCGT	835
	reverse	GGGTTTACCTCAACGGC	
β-Actin	forward	CTGGGAYGAYATGGARAAGAT	353
	reverse	GYTCRGCCAGGATCTTCAT	

(a)



(b)

Characteristic absorptions (cm^{-1})	Functional group
1032.69, 1078.98	C-N stretching (Amine)
1255.43	C-O stretching (Aromatic ester)
1700.91	C=O (Amides)
2854.13, 2920.66	C-H (Alkene)
3377.71	O-H (Alcohol)



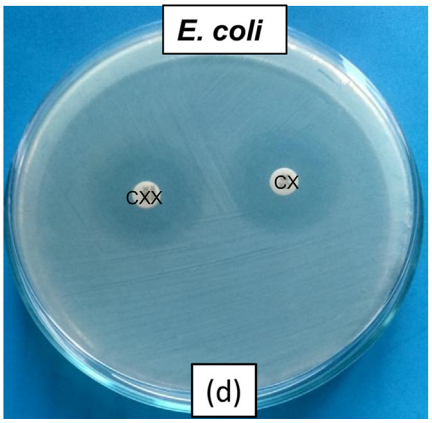


Fig. 1. (a) FTIR spectra of CEA extract (b) FTIR table displaying the characteristic absorptions and their corresponding functional groups (c) Antibiotic susceptibility through disk diffusion method displaying the zones of inhibition of different antibiotics towards *E. coli* clinical strain (d) Phenotypic detection of AmpC beta-lactamase through double disk diffusion method.

Table 2
Resistance and sensitivity details of *E. coli* clinical strain.

Strain	Source of isolates	Resistance details[a]	Sensitive	MARI Calculations
E. coli	Urine	AZM, CTN, MRP, CTX, VA, AT, AMC, CIP, LE, CPD, AMP, CZ, CAZ	CTR, IPM	0.87

[a] Azithromycin (AZM 15 µg/disc), Ampicillin (AMP 10 µg/disc), Amoxiclav (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 5µg/disc), Meropenem (MRP 10 µg), Vancomycin (VA 30 µg/disc).

imipenem. Fig. 1c shows the zones of inhibition of different antibiotics against *E. coli* clinical strain and Table 2 displays the MARI calculations obtained from antibiotic susceptibility data. The Cefoxitin-Cloxacillin double disc synergy test used for phenotypic detection of AmpC showed that the *E. coli* clinical strain was positive for AmpC. A zone diameter difference of ≥4 mm between Cefoxitin 30 mcg discs & Cefoxitin-Cloxacillin 30-200 mcg discs was interpreted as AmpC positive (Fig. 1d).

CEA extract of *A. paniculata* was utilized to check its effect on the growth of susceptible and a clinical strain of *E. coli* and it was observed that 250 µg and 500 µg were more effective compared to other two concentrations. The effect of 250 µg and 500 µg of CEA extract was comparable to that of the antibiotic treatment. In susceptible strain, 250 µg and 500 µg of CEA extract decreased the growth of *E. coli* up to 75% compared to the untreated control, whereas 62 µg and 125 µg reduced the growth up to 50% compared to the untreated control. In clinical strain, 250/500 µg also reduced the growth up to an approximate of 75% compared to the untreated control, whereas 62 µg and 125 µg of CEA extract displayed a least antibacterial potential (Fig. 2a). Previous studies have reported the effect of *A. paniculata* extracts on different bacteria, for example, crude ethanolic extract of *A. paniculata* against twelve pathogenic bacteria and found that ≥200 µg/ml was the most effective (Mishra et al., 2009) concentration when compared to other concentrations. Similarly, another *in vitro* study performed with aqueous ethanol extract of *A. paniculata* extract against two pathogenic bacteria *Legionella pneumophila* and *Bordetella pertusis* showed that the extract was effective at 0.5 mg/ml (Youhong et al., 2006). Therefore, *A. paniculata* can be considered as a strong candidate against drug-resistant bacteria.

Biofilm formation assay of *E. coli* strains was observed in presence of CEA extract, biofilm formation in *E. coli* clinical strain was inhibited to greater extent compared to susceptible strain. All four concentrations of AP extract had least effect on the biofilm formation in susceptible strain of *E. coli* contrasts to the antibiotic treatment, whereas 250 µg and 500 µg were effective in inhibiting the biofilm in *E. coli* clinical strain and the effect was comparable to that of the antibiotic (Fig. 2b). Crude extract was also tested for its action on EPS production in both strains. 250 µg of CEA extract was used to treat the strains and it was observed that CEA extract reduced the production of EPS when compared to the untreated control (Fig. 2c). The effect of the extract was comparable to the antibiotics in reducing the EPS production in both the strains of *E.*

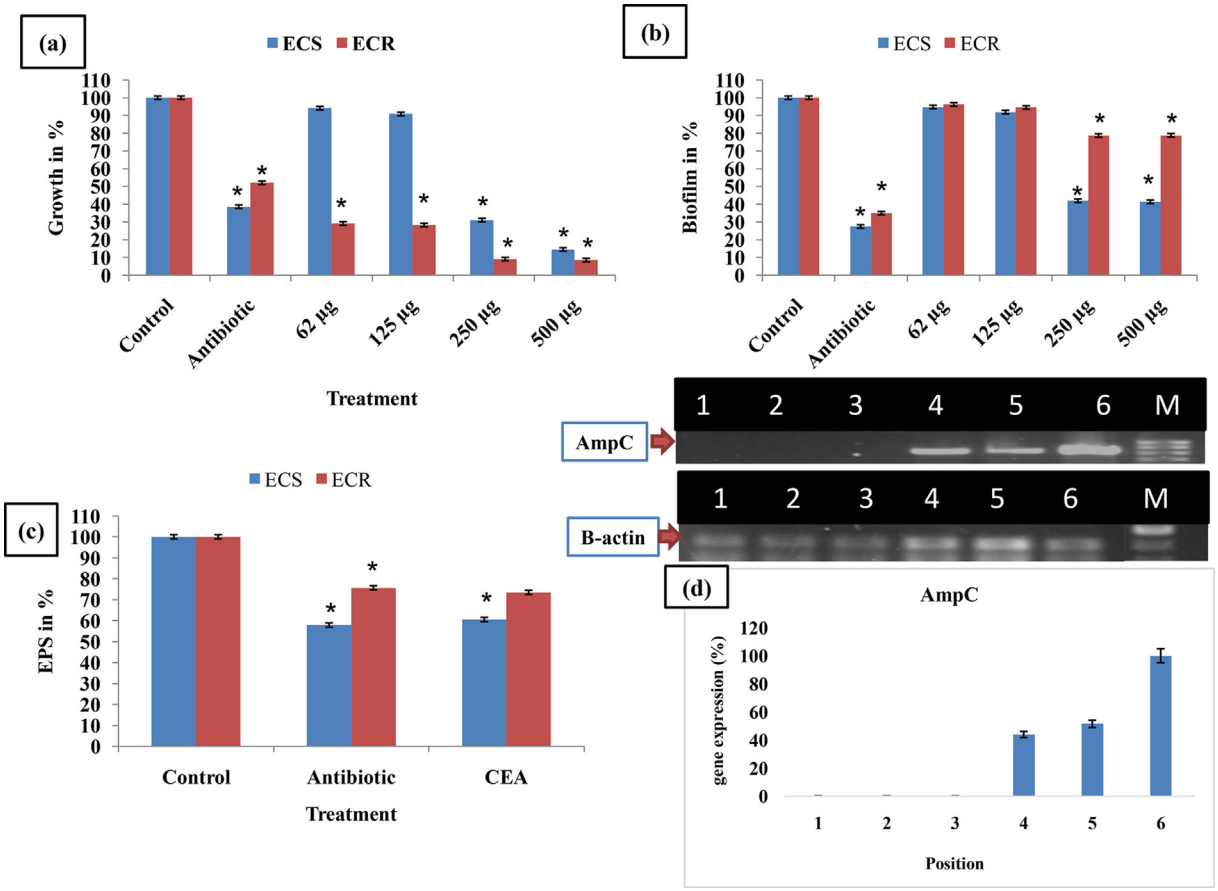


Fig. 2. (a) Antimicrobial activity of CEA extract through the broth microdilution method. The figure shows the effect of 62, 125, 250 and 500 µg/ml of CEA extract on the growth (%) of Susceptible and resistant strain of *E. coli* (b) Effect of 62, 125, 250 and 500 µg/ml of CEA extract on the biofilm formation (%) of susceptible strain of *E. coli* and resistant strain of *E. coli* (c) EPS production (%) in susceptible and resistant strain of *E. coli* with and without the treatment of CEA extract (d) The figure shows the expression of a gene encoding AmpC at various conditions (1) *E. coli* ATCC untreated (2) *E. coli* ATCC treated with CEA extract (3) *E. coli* ATCC treated with antibiotic (4) Resistant strain of *E. coli* treated with antibiotic (5) Resistant strain of *E. coli* treated with CEA extract (6) Resistant strain of *E. coli* untreated. Beta-actin was used as reference gene. Error bars were representing means ± standard errors. * denotes *t*-test ($P < 0.05$).

coli. Biofilm formation can make most of the bacterial species resistant to antibiotics. In this present study, 250 µg/ml of CEA extract reduced the biofilm formation and decreased the EPS production, therefore, *A. paniculata* has the potential to prevent the bacterial surface attachment. Previous studies demonstrated that methanol/chloroform/aqueous extracts of *A. paniculata* reduced quorum sensing in *P. aeruginosa* (Banerjee et al., 2017).

Finally, *A. paniculata* extract was used to determine its effect on the chromosomal-mediated AmpC expression. DNA was isolated from AmpC beta-lactamase producing clinical strain and sensitive strain with and without the treatment of the extract 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. In susceptible strain of *E. coli* the expression was completely abolished within *A. paniculata* treated cells when compared to control. The clinical isolate of *E. coli* treated with the extract displayed a lesser expression of AmpC compared to the antibiotic-treated strain (Fig. 2d).

4. Conclusion

To summarize, multi drug resistant *E. coli* was investigated for its antibiotic resistant pattern and observed that the strain showed resistance to 13 antibiotics. The resistant pattern was due to the production of ampicillinase by *E. coli* and was confirmed by AmpC gene expression level. The CEA extract from *A. paniculata* found a potential to control *E. coli* growth and biofilm formation through the reduction of EPS production. Thus, CEA extract could be used as crude in the traditional medicine to treat many ailments. It can be further studied and applied as potential drug for multi-drug resistant pathogens.

Conflicts of interest

The authors declare that they have no conflict of interest.

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