Original Article

Antibacterial and antibiofilm formation of ESBL resistant *K.pneumonia* by *B.diffusa* leaf extract

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Abstract:

Objectives: Antimicrobial resistance represents a huge global health crisis and is one of the most serious threats humans face today. Therefore, new antibacterial agents are needed to treat resistant bacteria. So this study aims to evaluate the antibacterial activity of *B.diffusa* leaf extract and whether it is useful against ESBL-resistant *K. pneumonia* biofilm formation.

Methods: ESBL resistant *K.pneumonia* isolates was procured from the central laboratory, Sree Balaji Medical College. Extraction was carried out using cold percolation method. Antibacterial activity was studied by disc diffusion method. Biofilm inhibition assay was performed using crystal violet staining.

Results: The extracts showed good antibacterial activity against the ESBL resistant *K.pneumonia.* Further the inhibition of biofilm was observed at the concentration of 250, 500, 750 and 1000 µg/ml.

Conclusion: Further, identification of the target anti-biofilm agent is warranted and is planned for future studies.

Keywords: *Biofilm, ESBL resistant, B.diffusa*

Introduction:

Bacteria constantly accumulates mutational changes, and their environment exerts strong selective pressure on them, in order that they are constantly and rapidly evolve. Additionally, they exchange genetic information, usually between members of an equivalent species, also between members of various species. The Gram-negative bacterium *Klebsiella pneumoniae* (K. pneumoniae) is one among the main causes of nosocomial infections in humans, but also can be acquired within the community[1]. As an opportunistic pathogen, *Klebsiella spp*. primarily targets immunocompromised people who are hospitalized and have serious underlying medical conditions such as diabetes or chronic obstructive pulmonary disease. It can be asymptomatic or cause various infections, such as pneumonia; wound in soft tissue, urinary tract and blood infections[2]. Due to their ability to spread rapidly in the hospital environment, these bacteria tend to cause nosocomial outbreaks. Hospital epidemic *Klebsiella* spp Multidrug- resistant strains, particularly in children, are frequently brought about by new strains that produce broadened range β-lactamases (ESBLs). ESBL- producing organisms present extraordinary difficulties to microbiologists,

clinicians, contamination control experts, and anti-infection disclosure scientists. ESBLs are enzymes capable of hydrolyzing penicillins, extended spectrum cephalosporins, and monobactams. These organisms mostly obtain JEM and SHV genes and shown resistance against first line of ESBLs typically situated on plasmids will be moved among strains and between bacterial species. The prevalence of ESBL is not well documented, it appears to be increasing and in many regions of the world 100 percent of *Escherichia coli* and *Klebsiella pneumoniae* strains express ESBL. Since these life forms shows protection from most anti-microbials, the development of the most recent medications is very much required.

Plant compounds possess antibacterial activity and the development of lead compounds from the plant is almost attracted the researchers because of the good activity and its less side effects. Search for novel efficacy drug is very challenging and most of the industries are looking forward to it[3]. In the present study, *Boerhavia diffusa* plant is chosen and it belongs to the family of Nyctaginaceae. The leaf of this plant was proved to have antibacterial activity against *K.pneumonia* and the results show that extract has good activity when compared to the other parts of the plant[4]. Hume the present study is aimed to evaluate the antibacterial activity of the various extracts of *B.diffusa* leaves.

Materials & Methods:

The plant material was collected from various parts in villages of Tamil Nadu. Plant material has been authenticated by Prof. P. Jayaraman, Plant Anatomy Research Centre (PARC). The collected leaves ware separated, washed with cool water and dried in a shady region. Once the leaf is dried completely, the it was coarsely grinded and used for further purpose.

Extraction:

100 gms of the leaf powder was sequentially extracted with hexane, chloroform and ethylacetate and kept for 72 hours with intermittent shaking. The extract was filtered and kept in the water bath for the solvent evaporation. The extract was collected and stored at 4^{o}C for further use.

Antibacterial activity

The test organism, ESBL resistant *K.pneumonia* was procured from the Central Laboratory after obtaining the ethical clearance. The bacteria was cultured in the nutrient broth at 37°C and maintained on nutrient agar slants at 4°C. To perform the disc diffusion method, different conc of extract namely 250, 500, 750 and 1000 µg/ml was prepared. Overnight culture of organism was grown in nutrient broth and the inoculum was adjusted to 10^6 CFU/ml. The culture was swabbed on the nutrient agar plates. Wells with 6-mm diameters was made and 50 µl of the each extract was added to the respective wells. In the center well, the standard antibiotic disc was kept. The plates incubated for 24 hours and the zone of inhibition was measured.

Antibiofilm assay:

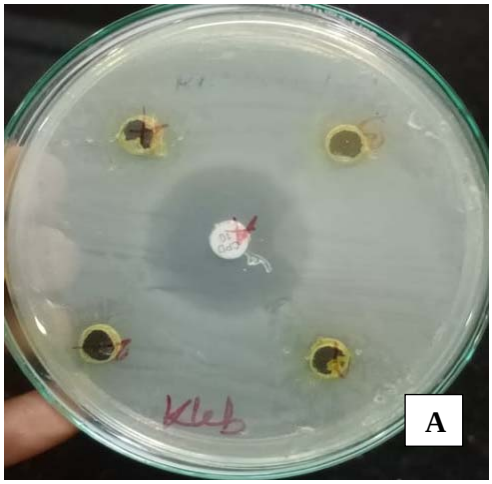
Inhibition of biofilm formation was quantitatively measured using crystal violet staining. Organism was cultured in the Muller Hinton broth (MHB) medium and incubated for overnight. The bacterial suspension was diluted to $5x10^6$ CFU. In the 96 well plate, add 180 µl of MHB with varying concentration of extracts and 20 µl of the diluted bacterial suspension and incubated at 37^{o}C for 24 hours. After the incubation period, plates well washed twice with Phosphate Buffer Saline

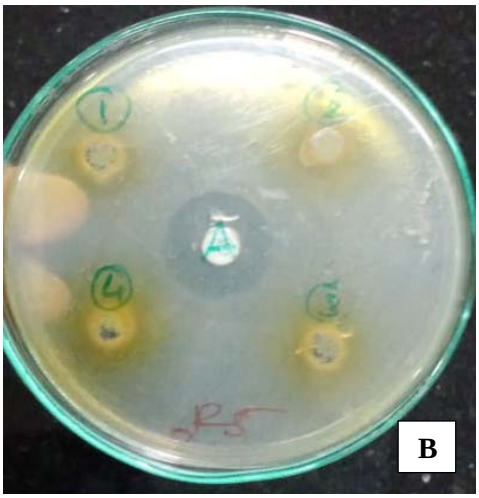
(PBS) and added 125 µL of a 0.1% solution of crystal violet in water to each well of the microtiter plate and incubated for 10-15 minutes. After the incubation period, the plates were washed with water and 125 µL of 30% acetic acid was added. Incubate the plates for 10-15 minutes and measure the absorbance at 595 nm.

Results:

Disc diffusion method:

Among the different extracts, hexane extracts at any concentration did not show antimicrobial activity. Chloroform extract at 1000 µg/ml showed zone of inhibition. Ethylacetate extract at all the concentration showed zone of inhibition at 250 a conc. of µg/ml.





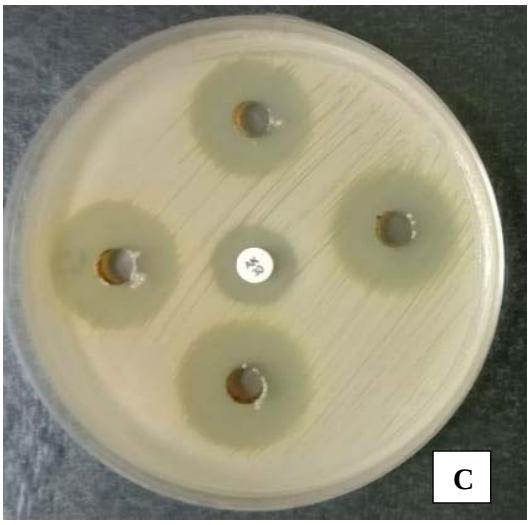


Fig 2: Zone of inhibition of various extracts against ESBL resistant *K.pneumonia*. Various concentrations of extract (250, 500, 750 and 1000 µg/ml) was used. Amikacin 30 µg/ml was used as a standard antibiotic disk in the centre. A) Hexane extract; B) Chloroform extract; C) Ethylacetate extract.

Different extract	Zone Of Growth Inhibition In (mm) Klebseilla			
	250 (µg/ml)	500 (µg/ml)	750 (µg/ml)	1000 (µg/ml)
Hexane	0	0	0	0
Chloroform	0	1	1.5	3.4
Ethylacetate	6	6.2	6.9	7.4

Determination of Biofilm inhibition activity:

Ethylactate extract showed better anti-activity biofilm inhibition activity when compared with the other two extracts.

Concentraction of extract (µg/ml)	Hexane extract	Chloroform extract	Ethylacetate extract
250	6.7	18.65	29.57
500	9.8	20.87	31.37
750	10.1	21.97	45.13
1000	11.3	23.07	55.41

Discussion:

Emerging antibiotic resistance among the microorganism made the effort to develop the

new antimicrobial agent with efficacy against the resistant strain[5]. *K.pneumonia*is one among the bacteria enlisted by WHO as ESKAPE pathogens. ESKAPE represents a group of bacteria, including both Gram-positive and Gram-negative species, including *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter* species. These pathogens can cause life threating infections in ICU patients and immunocompromised patients. These organisms can able to develop drug resistance by different mechanisms[6]. The development of antibiotic resistance, urge to develop the new drugs to invade the resistant developed microorganism. The majority of the drug developed recently was mainly based on the chemical modifications of the previously discovered drugs. Therefore development of new drug against for the antimicrobial activity is needed.

In this study, ethylacetate extract of *B.diffusa* leaf showed good anti-bacterial activity against ESBL-resistant *K.pneumonia* when compared to other extract. This might be due to the phytoconstituents present in the extract because due to the polarity of the solvent it can able to extract the phenolic compound from the samples[7]. Apart from the antibacterial activity the present study also focused on the inhibition of biofilm formation. The development of antibiotic resistance is not

Because of the genetic associated changes, but also due the organisms ability to form communities known as *biofilms* and develop more resistant to the antibiotic than in the normal planktonic state[8]. Therefore, inhibition of biofilm formation is one of the potential markers of drug development.

Conclusion:

The ethylacetate extract of *B.diffusa* leaves showed antibacterial activity and biofilm inhibiting activity. Further study should be carried out to isolate the compounds involved in the antibacterial activity.

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