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Antibiofilm activity of *Morinda tinctoria* fruit extracts against AmpC β-lactamase positive *Klebsiella pneumoniae*

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ABSTRACT

The objective of the study is to evaluate the effect of methanol extracts of Morinda tinctoria fruits on biofilm formation of clinically important AmpC β-lactamases producing K. pneumoniae. K. pneumoniae clinical isolates were screened for the production of AmpC β-lactamases by AmpC disc test. Biofilm inhibition studies were conducted on 24-well polystyrene well plates. Four uropathogenic isolates of K. pneumoniae were shown to produce AmpC β-lactamases. The methanol extracts at Biofilm inhibitory concentration (BIC - 0.06 mg ml^{-1}) of M. tinctoria fruits (immature, midmature and mature) were revealed to inhibit the biofilms formed by K. pneumoniae. The finding of the present study describes M. tinctoria fruit extracts as a promising source for biofilm inhibition in K. pneumoniae which have acquired resistance to third and fourth generation cephalosporins. This is the first report on biofilm inhibition of AmpC producing K. pneumoniae using fruit extracts of M. tinctoria at different maturity stages.

Keywords: Biofilm inhibition, AmpC β-lactamases, *K. pneumoniae*, *Morinda tinctoria* Roxb.

INTRODUCTION

AmpC β-lactamase is one of the resistant mechanisms acquired by certain species of Enterobacteriaceae which confer resistance to a range of β-lactam antibiotics and third generation cephalosphorines [1]. Plasmid mediated AmpC β-lactamases (PMABLs), evolved by horizontal gene transfer of inducible chromosomal genes have emerged as a threat to antibiotic therapy. Persistent treatments with commonly prescribed antibiotics gradually lead to the genesis of these enzymes, which are often concealed in hospitalized patients. The most common genotypes include ACC, FOX, MOX, DHA, CIT and EBC, which are frequently detected by disc diffusion techniques and multiplex PCR [2-4]. These enzymes are frequently detected in uropathogenic isolates of *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella* spp, *Proteus mirabilis*, *Citrobacter freundii*, and *Enterobacter aerogenes* which acquire resistance to diverse beta-lactam/beta-lactamase inhibitor combination [5-8]. These clinical pathogens are highly resistant to cephalosphorines, cephamycins, penicillins [9-11]. *Klebsiella* spp. is a renowned genera shown to produce AmpC β-lactamase [12]. Among *Klebsiella* spp., *Klebsiella pneumoniae* is a common nosocomial pathogen reported to cause urinary tract infections (UTIs), pneumonia and intra abdominal infections. *K. pneumoniae* is also known to cause several unusual invasive presentation infection, primary bacteremic liver abscess and severe respiratory tract infections [13, 14]. Recent reports have shown the predominance of OXA-48-producing *K. pneumoniae* (O48KP) in hospitalized patients [15-17]. This variant fabricates cluster of infections in the infected patients, which are accompanied by complications in detection and disease management [18-21]. Antibiotics for treating *K. pneumoniae*

are extremely restricted due to the emergence of multi-drug resistant strains, hence the crisis necessitate an alternative therapeutic medicine which can be derived from certain traditional folk medicinal plants.

Morinda tinctoria Roxb. is a traditional folk medicinal plant, grouped under the family, Rubiaceae. The plant is widely dispersed all over the tropical regions of Southeast Asia. It is commonly known as Nunaa and several historic records deem the plant for its medicinal values. The leaves and roots of *M. tinctoria* are used as astringent, deobstrent and as pain relievers in acute inflammatory arthritis leading to gout [22]. *M. tinctoria* leaves also possess anti-convulsant, analgesic, anti-inflammatory, antioxidant activity and antimicrobial properties [23-26].

In the present study, we have investigated the effect of various maturity stages (immature, midmature and mature) of *M. tinctoria* fruit extracts on *in vitro* biofilm formation of clinically important AmpC β-lactamase producing uropathogenic isolates of *Klebsiella pneumoniae*.

MATERIALS AND METHODS

Strains and cultures

Reference strains of *E. coli* (ATCC 25922) and *K. pneumoniae* (MTCC 432) were procured from American Type culture collection (ATCC) and Microbial Type culture collection (MTCC). The clinical isolates were generous gifts from Sanghamitra Hospital, Ongole, Andhra Pradesh

AmpC disc test

The test is based on ability of Tris-EDTA to permeabilize a bacterial cell and discharge β-lactamases into the external environment. The surface of a Mueller-Hinton agar plate was inoculated with a lawn culture of cefoxitin-susceptible *E. coli* ATCC 25922. A 30 µg cefoxitin disc was placed on the surface of the agar. A sterile plain disc containing test organism was placed adjacent to the cefoxitin disc roughly touching it, with the inoculated disc face in contact with the agar surface. The plates were incubated overnight at 37°C for 24 hours. The plates were examined for either an serration or a flattening of the zone of inhibition, indicating enzymatic inactivation of cefoxitin (positive result) or the absence of a distortion, indicating no significant inactivation of cefoxitin (negative result)[27].

Collection and preparation of fruit extracts

Different maturity stages (immature, midmature and mature) of *M. tinctoria* fruits were collected from Thanjavur, Tamil Nadu. The fruits were peeled and the core was cut into small pieces and kept for shade drying. The dried fruits were powdered using a mixer and the powdered fruit materials (40 g) were soaked in 100 ml of 90% methanol. The contents were kept in room temperature for 48 hours with constant stirring at regular intervals. The contents were then filtered with Whatmann No.1 filter paper. Filtrates were concentrated and stored at 4°C. The residues were dissolved in methanol and used for further assays.

Antibacterial activity test

Antibacterial activity of the fruit extracts was tested using agar well diffusion method. The cultures were spread on the nutrient agar plates using sterile cotton swabs. Wells were cut on the agar plates with 6 mm using a cork borer. 100 µl (7 mg/ml) of the extracts were pipetted into the well using sterile micropipette. Methanol was used as a negative control. The plates were incubated at 37°C for 24 hours. The antibacterial potency of the test organisms was determined by measuring the diameter of the zone of inhibition in millimeter.

Biofilm inhibition assay

The effects of the methanol extracts on clinical isolates of *K. pneumoniae* were tested on 24-well polystyrene plates. Methanol extracts of all the maturity stages at concentration of 0.06 mg ml^{-1} were added in nutrient broth supplemented with 0.5 % glucose containing the bacterial suspension at 10^6 CFU ml^{-1}. The polystyrene plates were incubated for 24 h at 37ºC. Following incubation, the plates were washed with distilled water to remove the unbound bacterial cells and the biofilm was stained with 0.4 % crystal violet. The wells were destained for 30 min with 95 % ethanol and then the contents of the wells were quantified at 575 nm in a UV spectrophotometer. The biofilms on glass surfaces stained with crystal violet solution were imaged using a bright-field microscope [28]

RESULTS

Phenotypic detection of AmpC producers

The secretion of AmpC β-lactamases in uropathogenic isolates of *K. pneumoniae* was initially confirmed by AmpC disc test (Figure 1). AmpC β-lactamases positive *K. pneumoniae* were further used for antimicrobial and biofilm inhibition assays.

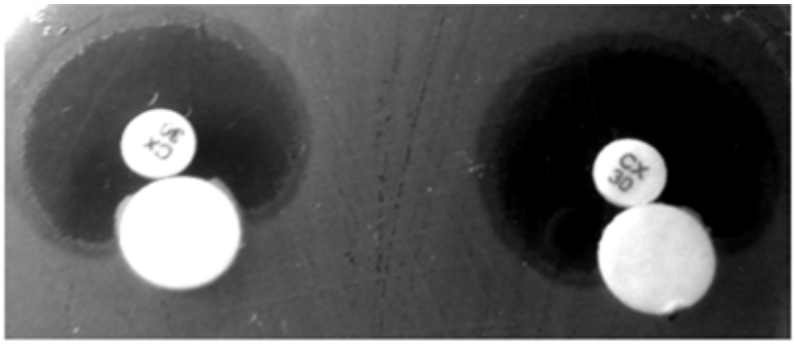

Figure 1: AmpC β-lactamase positive *K. pneumonia* isolates

Effect of fruit extracts on growth and biofilm formation

Methanol extracts of the *M. tinctoria* fruits exhibited profound antibacterial activity with 14 mm zone of inhibition against *K. pneumoniae* clinical isolate (Figure 2).

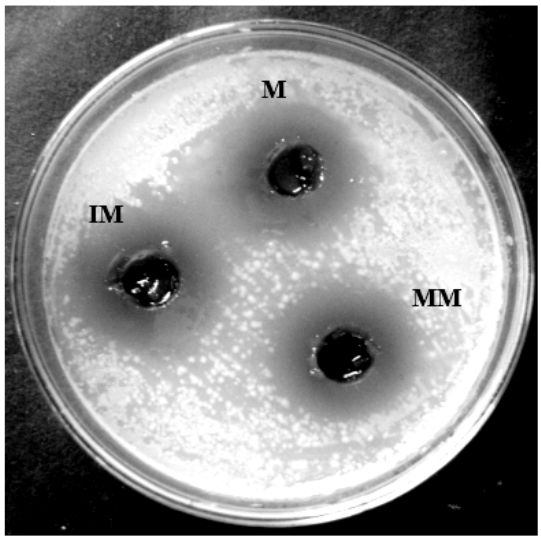

Figure 2: Antibacterial activity of methanol extracts (7 mg ml^{-1}) of *M. tinctoria* fruit. (IM-Immature, MM-Midmature, M-Mature)

The minimum inhibitory concentration (MIC) of the fruit extracts were in the range of 65-75 µg. Further, the extract of the *M. tinctoria* fruits at different maturity stages (immature, midmature and mature) showed drastic reduction in *K. pneumoniae* biofilms. The quantitative reduction of the *K. pneumoniae* biofilms on polystyrene plates were confirmed by UV spectrophotometer reading at 570 nm and BIC was found to be 0.06 mg ml^{-1}. The extracts at BIC values did not affect the growth of *K. pneumoniae* in the broth culture (Figure 3), but showed drastic decline in biofilm cells attached to the surface of the polystyrene plate (Figure 4).

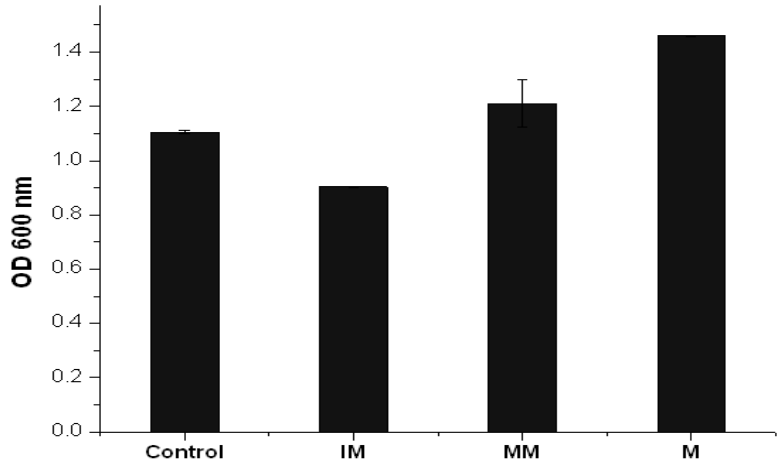

Figure 3: Effect of methanol extracts (0.06 mg ml^{-1}) on growth of *K. pneumoniae*. No significant changes decline in the planktonic cells noted

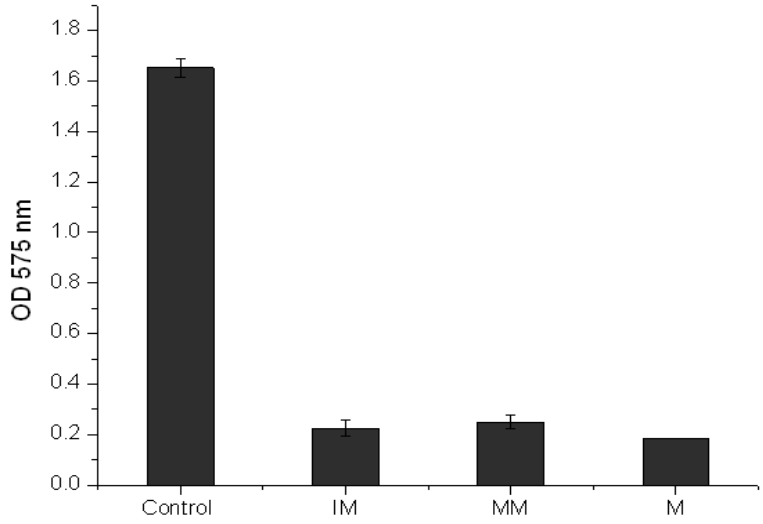

Figure 4: Effect of methanol extracts (0.06 mg ml^{-1}) on biofilm formation of *K. pneumoniae* (IM-Immature, MM-Midmature, M-Mature). Significant decline in the biofilm cells noted

The light microscopic images of *K. pneumoniae* biofilms on glass slides also showed significant inhibition of *K. pneumoniae* biofilms in extract treated slides (Figure 5B,C and D) when compared to control (Figure 5A).

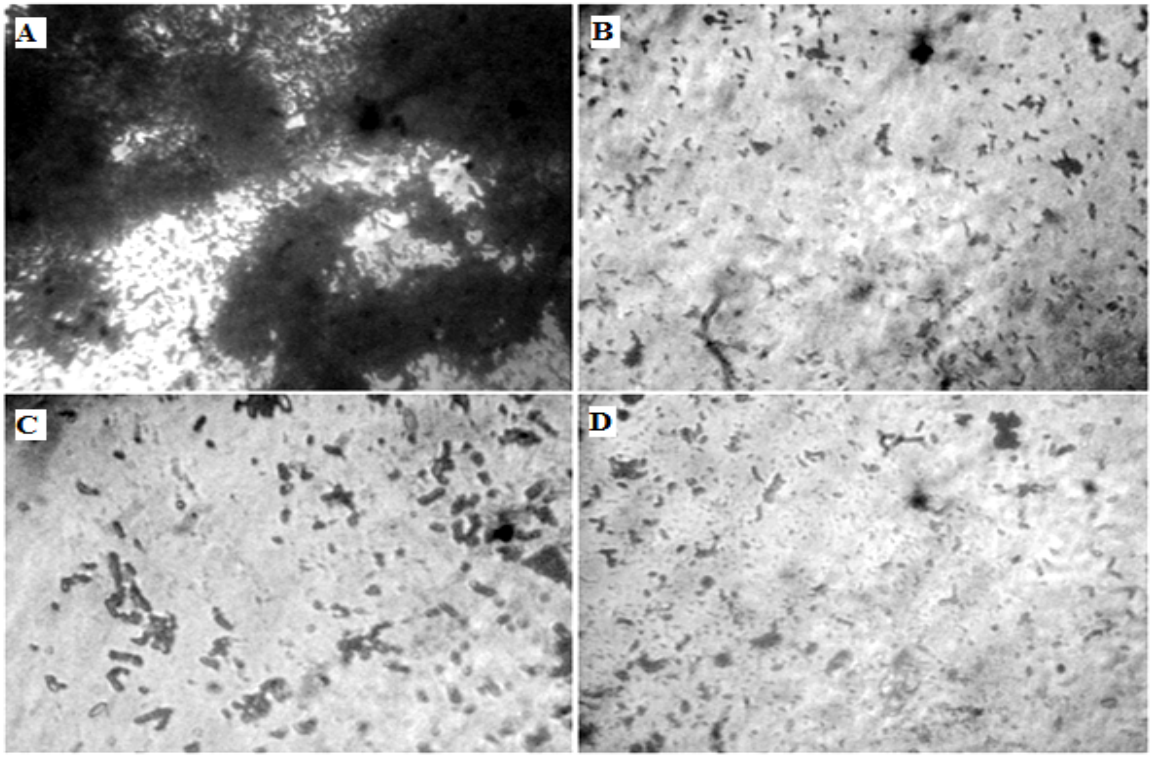

Figure 5: Microscope images of biofilms formed by an AmpC β-lactamase producing *K. pneumoniae* clinical isolate
A: Control, B: Methanol extracts (Immature). C: Methanol extracts (Midmature), D: Methanol extracts (Mature).

DISCUSSION

The evolution of multi-drug resistant strains has contributed to unrestrained spread of the causative organism and therapeutic letdown. AmpC beta-lactamases are clinically important cephalosporinases produced in several Enterobacteriaceae which confer resistance to cephamycins, penicillins, β-lactam-β-lactamase inhibitor combinations and third/fourth generation cephalosporins [1]. In spite of the discovery of AmpC β-lactamases a decade ago, uncertainty exists about their clinical importance. Rapid emergence of drug-resistant pathogens highlights the need for regular surveillance for efficient disease management and control.

The genesis of drug resistant strains in the current scenario consequently leads to the screening of various plant sources to offer reliable and cost effective antibiotic therapy. The medicinal plants are rich in various secondary metabolites and play a vital role in discovering new compounds for antimicrobial applications. The extracts of diverse plant materials are highly influential and effective against clinical bacterial pathogens. The present study was conducted to study the effect of *M. tinctoria* fruit extracts on growth and biofilm formation of uropathogenic isolates of *K. pneumoniae*. Since *K. pneumoniae* develops rapid resistance to antimicrobials and other commercially available antibiotics, the study was mainly focused on biofilm inhibition. The methanol extracts of *M. tinctoria* fruits at various maturity stages (immature, midmature and mature) were administered at a concentration below the MIC values to estimate the effect of the extracts on the biofilm formation of *K. pneumoniae*. The methanol extracts at a BIC of 0.06 mg ml^{-1} were shown to inhibit the biofilm formation. The results of the present study apparently reflect the ability of fruit extracts to diminish the biofilms formed by *K. pneumoniae*. Future study is warranted to identify the plant compound responsible for biofilm inhibition in *K. pneumoniae* clinical isolates.

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