

EFFECT OF AQUEOUS EXTRACT OF *TERMINALIA CHEBULA* ON METALLOBETALACTAMASE

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ABSTRACT

ESBL mediated resistance in Gram negative organisms poses a serious problem. Also AmpC may coexist with other types of ESBL and mask each other, thus making detection even more difficult. The aim of this study was to find Gram negative organisms from clinical isolates, analyze their resistance patterns, identify the various types of ESBLs produced and study synergistic action of the aqueous extract of *Terminalia chebula* with cefotaxime. Fifteen isolates each of *Pseudomonas aeruginosa* and *Acinetobacter baumannii* were obtained from hospitals in Mumbai. The resistance pattern towards various cephalosporins was studied. The types of enzymes responsible for antibiotic resistance were also established. Various crude phytochemical extracts were evaluated for their effect on the ESBL produced. Aqueous extract from *Terminalia chebula* was found to be effective on MBL which were produced by eleven isolates of *Pseudomonas* and eight isolates of *Acinetobacter*.

Keywords: Metallobetalactamase, *Terminalia chebula* aqueous extract, ESBL

INTRODUCTION

Extended spectrum of β lactamases (ESBLs) are considered one of the most important resistance mechanisms for β lactam antibiotics like penicillins and cephalosporins produced by *Enterobacteriaceae* family. The genes coding for these enzymes are often carried by chromosomal or multidrug resistant plasmids which can be transferred to different species of the family¹. ESBLs have also been isolated from *P. aeruginosa*, *Campylobacter jejuni* and *Acinetobacter* species^{2,3}. The appearance of these organisms in a hospital setting creates a situation in which ESBL producing organisms need to be identified quickly so that appropriate antibiotic usage and containment measures can be implemented⁴.

ESBL producing bacteria can cause serious therapeutic failure if not detected on time. The approach used currently for treating such organisms is the use of antibiotic and inhibitor combination. Clavulanic acid acts as an inhibitor to many β lactamases. But in case of inducible AmpC β lactamases, drugs which have a combination like amoxicillin and clavulanic acid can cause more harm than help. Metallobetalactamase (MBL) is another type of β lactamase reported widely in *P. aeruginosa* and *Acinetobacter* species. EDTA is the only known inhibitor used for its detection. No antibiotic inhibitor combinations are known to be effective against organisms producing this type of enzymes.

The use of plant material to prevent and treat infections successfully over the years has attracted the attention of scientists worldwide⁵. Plant based antimicrobial compounds have great therapeutic potential as they have lesser side effects as compared with synthetic drugs and also little chance of development of resistance. There is also a possibility that herbs act as inhibitors of enzymes. Moreover, the plant extract can have synergistic effect with an antibiotic. Medicinal plants have long been utilized as a source of therapeutic agent. Plants are known to produce different secondary metabolites which are naturally toxic to bacteria⁶. Lin et al proposed the combination of antibiotics and flavonoids as a potential new strategy for development of new therapies in the future, for infections caused by ESBL producing bacteria⁷.

The focus of this study was to find the effect of phytochemicals on enzyme activity. Since tannins are known to possess antibacterial activity, screening for a herb having high tannin content was carried out. The selection was done keeping in mind the non-toxicity of the herb consumed across the country. Of all the screened herbs, aqueous extract of *Terminalia chebula* was found to be effective in preliminary studies.

MATERIALS AND METHODS

Fifteen isolates each of *Pseudomonas aeruginosa* and *Acinetobacter baumannii* were collected from various hospitals in Mumbai. These isolates were from indoor patients as well as out patients.

Antimicrobial susceptibility

Disc diffusion method for checking antibiotic resistance was performed as per CLSI standards. The antibiotics used were imipenem, cefotaxime cefoxitin, ceftazidime, ceftazidime+clavulanic acid, aztreonam, ceftriaxone and cefepime. All the antibiotic discs were obtained from Hi-media, Mumbai.

Plant material

Terminalia chebula is a plant species belonging to the genus *Terminalia*, family *Combretaceae*. It is a flowering evergreen tree called the black Myrobalan in English. *Terminalia chebula* is rich in tannin. The chief constituents of tannin are chebulic acid, chebulagic acid, corilagin and gallic acid.

Extraction procedure

The dry fruits of *Terminalia chebula* obtained from local market were extracted with distilled water at 70°C, filtered and the supernatant was concentrated and spray dried to get the dry powder of the extract. This dry powder was used in the study.

Minimum inhibitory concentration

The MIC of cefotaxime was determined by two fold serial broth dilution method as per CLSI standards using 10-640 μ g/ml concentration. MIC was also performed using 10-640 μ g/ml of cefotaxime and 2.5mg/ml of aqueous extract of *Terminalia chebula*.

 β lactamase preparation

Crude enzyme extracts were prepared from 20ml cultures grown at 37°C under vigorous agitation overnight. The culture density was adjusted to 0.5 McFarland standard and 50 μ l of this culture was inoculated into 12ml of Nutrient broth and the culture was grown for 4h at 37°C. The cells were concentrated by centrifugation and crude enzyme preparations were made by sonicating the pellets for 15seconds (two cycles) with 10 seconds cooling in between sonications; this was repeated four times. The lysates were centrifuged at 100,000 x g for 30 minutes. The supernatants were used for further analysis.

Characterization of ESBL

Three Dimensional Extract Test (TDET) for detecting AmpC was carried out as described elsewhere⁸. Enhanced growth of the surface organism at the point where the slit intersected the zone of inhibition due to cefoxitin was considered a positive TDET result and was interpreted as evidence for the presence of AmpC β lactamase⁹.

Various methods have been recommended for screening MBL¹⁰⁻¹³. In this study we used zone enhancement with EDTA impregnated imipenem and ceftazidime discs. All MBL producers showed a

significant zone enhancement with one and/or both imipenem and ceftazidime.

Bioassay method to confirm synergy

A 0.5 McFarland bacterial saline suspension was prepared from overnight culture in nutrient broth. 0.1ml of this was inoculated in molten nutrient agar along with aqueous extract of *Terminalia chebula* keeping the final concentration in the plate as 2.5mg/ml. After the plates solidified, a well was punched in the centre and was filled with cefotaxime (100µg). A control was kept with no aqueous extract in the plate.

RESULTS AND DISCUSSION

The isolates used in this study are shown in the table. 73% of *P. aeruginosa* showed production of MBL while 37% of these showed

the presence of both MBL and AmpC type enzyme. Four isolates were sensitive to the cephalosporins tested. *Acinetobacter baumannii* showed greater incidence of AmpC production. 86% showed production of AmpC of which 62% showed production of both AmpC and MBL.

Two isolates were sensitive to the antibiotics tested. There are frequent reports of MBL production from Asian and Pacific countries. A study from Chennai reported 87.5% MBL mediated resistance in *Pseudomonas*¹⁴. MBL production in *Acinetobacter* species has been reported as high as 70.9% in a study conducted at Pondicherry Institute of Medical Sciences¹⁵. A study conducted at Guru Tegh Bahadur hospital, Delhi has reported 42.8% *Acinetobacter* species producing AmpC¹⁶. Another study has reported co-production of AmpC with MBL in *Pseudomonas aeruginosa* as 46.6%¹⁷.

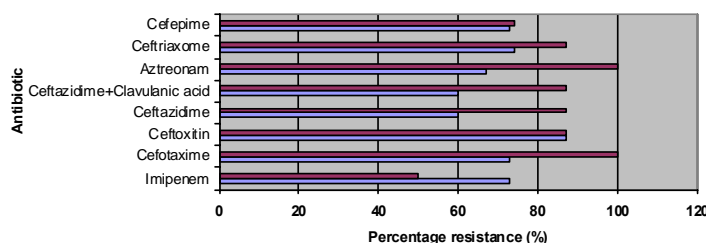


Fig. 1: Antibiotic resistance pattern

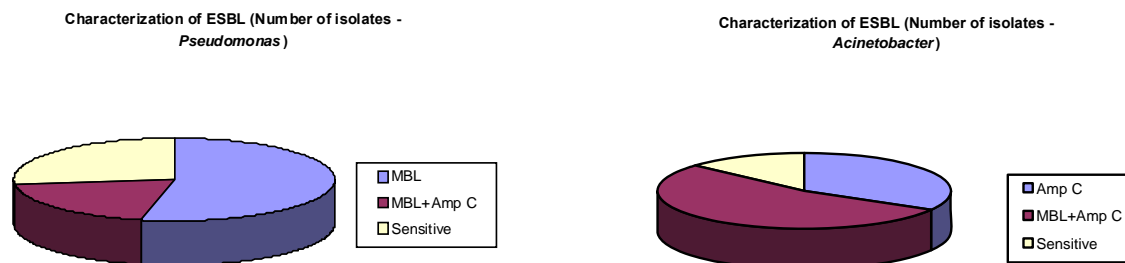


Fig. 2: ESBL Characterization

MIC of all the isolates of *Pseudomonas* and *Acinetobacter* against the aqueous extract of *Terminalia chebula* alone was between 3-5%. MIC of cefotaxime alone using broth dilution method was found to be in the range of 40-320µg/ml. A reduction in MIC of at least two-fold dilutions of cefotaxime was observed in the presence of aqueous

extract of *Terminalia chebula* of all the isolates that were MBL positive. The isolates that were producing both MBL and AmpC also showed a reduction in MIC of cefotaxime, but to a lesser extent. However, no reduction in MIC was noted for cultures producing AmpC only.

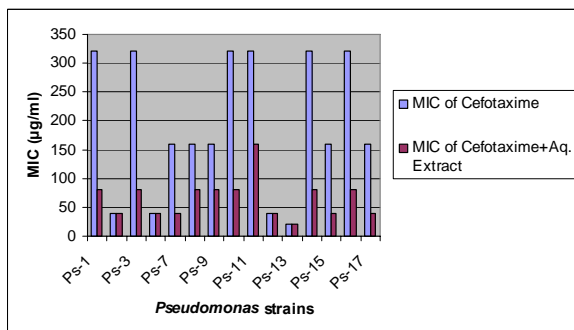


Fig. 3: MIC of cefotaxime with and without aqueous extract for *Pseudomonas*

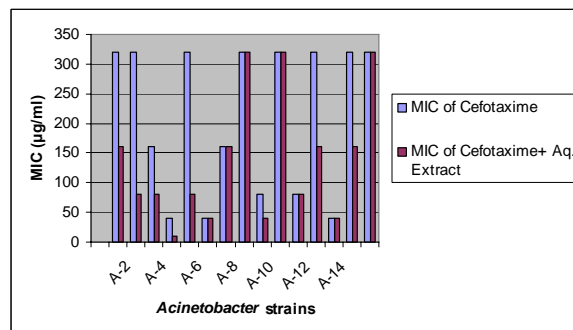


Fig. 4: MIC of cefotaxime with and without aqueous extract for *Acinetobacter*

Bioassay method was used to confirm the above results. The control plates containing cefotaxime (100µg) but no aqueous extract showed no zone of inhibition, but in the presence of the aqueous

extract, the organisms showed a zone enhancement of more than 5mm as against cefotaxime alone making it sensitive to the antibiotic.

Table 1: Zones of inhibition of *Pseudomonas* by bioassay method

Organism	Ps1	Ps2	Ps3	Ps4	Ps7	Ps8	Ps9	Ps10	Ps11	Ps12	Ps13	Ps14	Ps15	Ps16	Ps17
Control (zone of inhibition) mm	R	18	R	15	R	R	R	10	R	20	18	R	R	R	R
N.A+Aq. Ext+Cefotaxime (zone of inhibition) mm	17	18	18	15	15	13	18	20	15	20	18	13	15	18	17

R: Resistant

Table 2: Zones of Inhibition of *Acinetobacter* by bioassay method

Organism	A 1	A 2	A 3	A 4	A 5	A 6	A 7	A 8	A 9	A 10	A 11	A 12	A 13	A 14	A 15
Control (zone of inhibition) mm	10	R	R	R	R	18	10	8	R	R	R	20	10	8	R
N.A+Aq. Ext+Cefotaxime (zone of inhibition) mm	20	15	16	18	18	18	20	15	22	23	17	20	15	15	22

R: Resistant

The aqueous extract of *Terminalia chebula* acts as an inhibitor of MBL enzyme. To the best of our knowledge, synergistic activity of the aqueous extract of *Terminalia chebula* against MBL producing *Pseudomonas* and *Acinetobacter* has not been reported earlier. Research on synergism is very limited and few studies have been reported^{18,19,20}. The secondary metabolites from plants are good sources for combination therapy. There are reports available listing the synergistic interactions of β lactam antibiotics with natural compounds to overcome resistant microorganisms. These natural compounds include catechins (*Camellia sinensis*)²¹, EGCG (Epigallocatechin gallate) from green tea²² tellimagrandin 1 and rugosin B from rose red (*Rosa canina*)²³.

Plants are rich in wide variety of secondary metabolites such as tannins, terpenoids, alkaloids and flavonoids which have been found to have antimicrobial properties *in vitro*²⁴. Tannin is a general descriptive name for a group of polymeric phenolic substances. Many physiological activities such as stimulation of phagocytic cells, host mediated tumor activity and a wide range of anti infective actions have been assigned to tannins. Their mode of antimicrobial action may be related to their ability to inactivate microbial enzymes and transport proteins²⁵. As reported in this study, the synergistic action of the aqueous extract of *Terminalia chebula* which is high in tannin is due to inhibition of MBL which is an enzyme that requires Zn²⁺ for its mode of action. Tannins act similar to EDTA which is a metal chelator²⁶. Despite the lack of thorough knowledge for the underlying mechanism of the synergistic effect of these combinations, there is potential for their clinical use. Combination therapy has earlier been reported to increase activity and prevent the development of resistance²⁷. Since phytochemicals are structurally different from antibiotics and often have different modes of action, they provide novel means of studying the mechanisms of bacterial control at a molecular level. With the increased prevalence of MDR strains especially *P. aeruginosa*, synergism testing using various combinations of phytochemicals with antibiotics could be a powerful tool to aid selection of appropriate antibiotic therapy²⁸. These data encourage further studies of these agents plus other antimicrobial classes and *in vivo* animal experiments to validate this finding.

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