

STUDY OF SYNERGISTIC ACTION OF CEFOTAXIME AND *TERMINALIA CHEBULA* ON *ACINETOBACTER BAUMANNII* USING CHECKERBOARD ASSAY

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ABSTRACT

Objective: Infections caused by drug resistant Gram-negative bacteria are increasing day-by-day. An alternative mode of treatment needs to be considered to overcome the problem.

Methods: With this aim in mind, the effect of Cefotaxime and aqueous extract of *Terminalia chebula* was checked on *Acinetobacter baumannii* by using Disc Diffusion Method and Checkerboard Assay.

Results: Disc Diffusion Method showed synergistic interaction between Cefotaxime and the aqueous extract of *Terminalia chebula* against *Acinetobacter baumannii*. The FIC value obtained by Checkerboard Assay was 0.5 for *Acinetobacter baumannii* tested in combination, thus indicating synergistic interaction between Cefotaxime and the aqueous extract of *Terminalia chebula*.

Conclusion: These results lead us to believe that the active components present in the aqueous extract of *Terminalia chebula* should be an area of further *in-vivo* research in order to find leads of compounds which can act in conjunction with antibiotics.

Keywords: Cefotaxime, *Terminalia chebula*, Disc Diffusion Method, Checkerboard Assay, Synergistic interaction.

INTRODUCTION

Throughout history, there has been a continual battle between humans and the multitude of micro-organisms that cause infection and disease. However, the euphoria over the potential conquest of infectious diseases was short lived. Almost as soon as antibacterial drugs were deployed, bacteria responded by manifesting various forms of resistance. As antimicrobial usage increased, so did the level and complexity of the resistance mechanisms exhibited by bacterial pathogens [1].

Both the amount of antibiotics used and how they are used contribute to the development of resistance. The use of broad-spectrum antibiotics rather than narrow-spectrum drugs is known to favour the emergence of resistance by broadly eliminating competing susceptible flora [2].

Today a number of organisms can be listed in both hospitals and the community that thwart treatment because they are resistant to not one, but to many different antibiotics. Among these opportunistic pathogens are the enterococci, the coagulase-negative staphylococci, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* [3].

One strategy employed to overcome the resistance mechanism is the use of combination of drugs. Inhibitors of β -lactamase have been long known and they are administered with antibiotics as co-drugs [4]. However, combination therapy is also proving to be ineffective these days.

Over the past decade, there has been a resurgence of interest in the investigation of natural materials as a source of potential drug substance [5]. Few studies have found that the efficacy of antimicrobial agents can be improved by combining them with crude plant extracts against different pathogens [6]. There is also a possibility that herbs act as inhibitors of enzymes. Moreover, the plant extract can have synergistic effect with an antibiotic.

Previous study by us has indicated the effect of *Terminalia chebula* extract on *Pseudomonas* and *Acinetobacter* species [7]. However as per CLSI, the Checkerboard Assay carries more weight in establishing the synergy.

MATERIALS AND METHODS

Cultures

Isolate of *Acinetobacter baumannii* was collected from Brahmakumari hospital in Mumbai, India. The isolate was brought to

the research laboratory in transport media and checked for purity. The culture was maintained on Nutrient Agar slant containing 50 μ g/ml of Cefotaxime. Inocula were prepared by diluting overnight culture in saline. Fresh subcultures were used for each experiment.

Antimicrobial Susceptibility Testing (AST)

The isolate was subjected to antimicrobial susceptibility testing as per the Kirby Bauer disc diffusion method [8]. The medium used was Mueller-Hinton agar obtained from Hi-Media, Mumbai. The density of the culture was adjusted to 0.5 McFarland standard. The various antibiotics were used to determine the resistance of the test cultures. The antimicrobial agents tested were Cefotaxime (CTX 30), Ceftazidime (CAZ 30), Cefaclor (Cj 30), Cefuroxime (CXM 30) and Cefoxitin (CX 30). The culture was swabbed on Sterile Mueller-Hinton agar plates using sterile cotton swab. Using a sterile forcep, the discs of various antibiotics were placed aseptically on the plate. The plate was incubated overnight at 37°C for 24 hours. The zone sizes were interpreted as per the standard chart and the organisms were classified as sensitive, intermediate and resistant to the various antibiotics.

Collection and Authentication of *Terminalia chebula*

The dried plant part under study namely *Terminalia chebula* was provided by Konark Herbals and Healthcare, Mumbai. For the present study, the dried herb was examined for its authenticity by the Botany Department of Mithibai College, Mumbai.

Preparation of Hot Aqueous Extract of *Terminalia chebula*

Hot aqueous extract was prepared by boiling 10 g of *Terminalia chebula* fruit powder in 100 ml of distilled water for 30 mins and kept in a conical flask for 24 hours undisturbed [9]. The aqueous extract of *Terminalia chebula* thus prepared was then kept in the refrigerator for further use.

Antimicrobial Activity of Cefotaxime and *Terminalia chebula* by Disc Diffusion Method

For the Disc Diffusion method, the medium used was Mueller Hinton agar. The density of the culture was adjusted to 0.5 McFarland standard. With the help of sterile cotton swab, *Acinetobacter baumannii* was inoculated on the medium so as to obtain a lawn culture. Discs of Cefotaxime (30 μ g), discs containing 100 μ g/10 μ l of *Terminalia chebula* plant extract and discs of Cefotaxime (30 μ g) containing 100 μ g/10 μ l of *Terminalia chebula* plant extract were

used. Using a sterile forcep, these discs were placed on the surface of inoculated Mueller Hinton agar by pressing slightly. The plate was incubated at 37°C for 24 hours. At the end of the incubation period, the zone of inhibition formed was measured in mm.

Antimicrobial Activity of Cefotaxime and Terminalia chebula by Checkerboard Assay

The antibiotic Cefotaxime stock (10mg/ml) was used for the Checkerboard assay. It was stored at 2 to 8°C until use. 10% aqueous extract of Terminalia chebula was used and stored in refrigerator until use. The stock solutions of Cefotaxime used ranged from 0.004µg/ml to 128µg/ml. The stock solutions of aqueous extract of Terminalia chebula used ranged from 0.125% to 1%. Double dilutions of both the antibiotic as well as the aqueous extract of Terminalia chebula was used for the assay according to the recommendations of NCCLS immediately prior to testing. A total of 50 µl of sterile Mueller Hinton broth was distributed aseptically in all the sterile suspension tubes. The antibiotic Cefotaxime was serially diluted along the abscissa, while the aqueous extract of Terminalia chebula was diluted along the ordinate. An inoculum equal to 0.5 McFarland turbidity standard was prepared for each culture in sterile saline. Each suspension tube was inoculated with 100µl of the culture. All the tubes were incubated at 37°C for 24 hours. After incubation the growth was observed by streaking a loopful from each tube in sterile Mueller Hinton agar plates. The plates were incubated at 37°C for 24 hours. The plates were observed for growth of the test organism. The combination of the drugs in which the growth is completely inhibited was considered as effective MIC for the combination.

The ΣFICs were calculated as follows: ΣFIC=FIC A + FIC B, where FIC A is the MIC of drug A (Cefotaxime) in the combination/MIC of drug

A (Cefotaxime) alone, and FIC B is the MIC of drug B (T. chebula extract) in the combination/MIC of drug B (T. chebula extract) alone. The combination is considered synergistic when the ΣFIC is ≤0.5, indifferent when the ΣFIC is >0.5 to <2, and antagonistic when the ΣFIC is ≥2 [10].

RESULTS AND DISCUSSION

Isolate of Acinetobacter baumannii obtained from Brahmakumari hospital was subjected to AST to get the antibiogram. The antibiotics tested were Cefotaxime (CTX 30), Ceftazidime (CAZ 30), Cefaclor (Cj 30), Cefuroxime (CXM 30) and Cefoxitin (CX 30). Acinetobacter baumannii did not give any zone of inhibition to Cefotaxime, Ceftazidime, Cefaclor, Cefuroxime and Cefoxitin. Since Acinetobacter baumannii did not give the zone of inhibition at all to Cefotaxime, they were considered ESBL [11].

Researchers have reported substantial increase in the rates of antibiotic resistance of A. baumannii infections during the past decades. Recent microbiological surveillance have reported rates of multi-drug resistance in A. baumannii of approximately 30%. However, geographical differences in resistance patterns are evident [12]. Clinicians are faced with infections of P. aeruginosa and Acinetobacter species that are resistant to all or almost all β-lactam antibiotics, aminoglycosides, and quinolones [13].

In Disc Diffusion Method, Cefotaxime did not inhibit A. baumannii at all. Aqueous extract of Terminalia chebula inhibited A. baumannii giving a zone of inhibition of 8.6mm. However Cefotaxime and the aqueous extract of Terminalia chebula inhibited A. baumannii giving the zone of 16.3mm (Table 1). Thus synergistic interaction was observed between Cefotaxime and the aqueous extract of Terminalia chebula on A. baumannii by Disc Diffusion Method.

Table 1: Disc Diffusion Method

Name of the culture	Zone of inhibition (mm)		
	Cefotaxime	T. chebula extract	Cefotaxime + T. chebula extract
Acinetobacter baumannii	NI	8.6	16.3

Key: NI: No Inhibition

Checkerboard Assay was performed to study the synergistic activity between the antibiotic Cefotaxime and the aqueous extract of Terminalia chebula on Acinetobacter baumannii. Cefotaxime in combination with the aqueous extract of Terminalia chebula exhibited synergistic antibacterial action on Acinetobacter baumannii. FIC A was

0.0625 whereas FIC B was 0.5. The FIC value was 0.5625 for Acinetobacter baumannii thus indicating synergistic interaction between Cefotaxime and the aqueous extract of Terminalia chebula (Fig 1). Inhibition of bacterial growth was recorded at lower concentrations compared to the individually tested values.

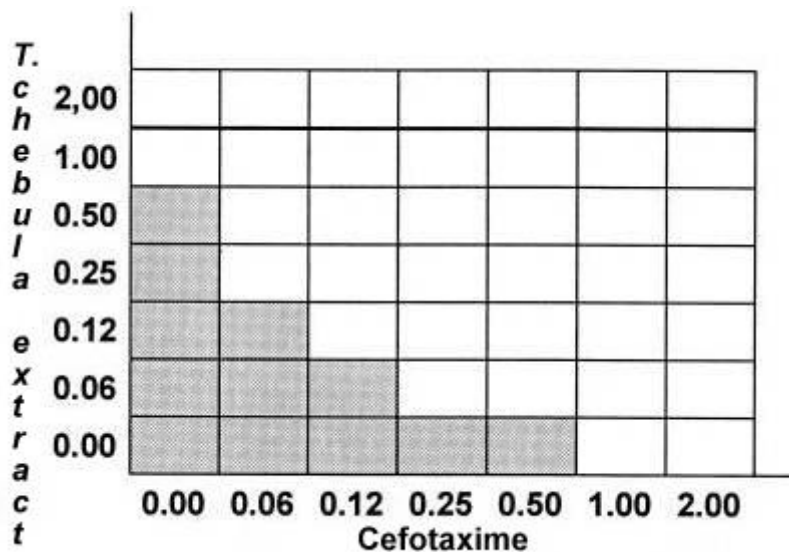


Fig. 1: The checkerboard method showing the synergy of a two-drug combination.

Gram-negative bacteria have an outer phospholipid membrane with structural lipopolysaccharide components, which make their cell wall impermeable to antimicrobial agents. Tannins present in the extract of *Terminalia chebula* may be working synergistically with Cefotaxime and making the cell wall of Gram-negative bacteria permeable due to which lysis of the cells occurs.

Different rates of synergy have been reported with various antibiotic combinations using checkerboard technique. These differences might be attributed to the different features of the strains. Although there is a standard procedure for checkerboard synergy testing it fails to exhibit standard results owing to different methods of interpretation of the results. Thus, there is a need to standardise the interpretation method for checkerboard synergy testing [14].

The ability of tannin compounds to cause the bacterial cells to disintegrate probably results from their interference with the bacterial cell wall; thereby inhibiting the microbial growth [15-16].

CONCLUSION

The resistance pattern of *Acinetobacter baumannii* was studied. The isolate was found to be an ESBL producer. Disc Diffusion Method and Checkerboard Assay showed synergistic interaction between Cefotaxime and the aqueous extract of *Terminalia chebula* against *Acinetobacter baumannii*. Thus, this study indicates that the combination of medicinal plant extracts and known antibiotics offers significant potential for the development of novel antimicrobial therapies and treatment of several diseases caused by microorganisms. Hence, research should be focused towards this direction to identify more medicinal plants which exhibit synergistic behaviour. Not only do more studies need to be conducted on the components of phytochemicals that show potential, but the mechanisms involved need to be understood and safety aspects of such combinations need to be studied too. A next step towards use in the clinic would then be *in vivo* studies to predict what will happen in the human situation.

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