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Synergistic Effect of *Terminalia chebula* and Antibiotics against Multidrug-resistant Uropathogenic *Escherichia coli*

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ABSTRACT

The present study evaluated the *in vitro* antibacterial efficacy of *Terminalia chebula* fruit extract with selected antibiotics: amoxicillin (penicillin antibiotics), ceftazidime (third generation of cephalosporins), ciprofloxacin (fluoroquinolones), gentamicin (aminoglycoside) and trimethoprim (sulfonamide group) against 8 clinical isolates of multidrug-resistant uropathogenic *Escherichia coli.* The effects of these drugs were examined by two *in vitro* methods (checkerboard titration and time-kill kinetics). On checkerboard titration, gentamicin and trimethoprim exerted 'synergy' with plant extract against the test isolates by 87 and 75%, respectively whereas amoxicillin, ceftazidime and ciprofloxacin showed an 'additive effect' with plant extract. No antagonistic effect was observed. With the time-killing method, the ¼X MIC of gentamicin or trimethoprim in combination with ¼X MIC of plant extract also showed synergistic activity. TLC-bioautography of plant extract revealed that phenols are the major active phytocompounds. These promising findings show a significant *in vitro* synergistic effect of plant extract with gentamicin and trimethoprim against uropathogenic *E. coli* that could be considered as a valuable support in combination therapy in the management of urinary tract infections and may contribute to the development of new and safe agents from plant origin.

Keywords: checkerboard titration, combination effect, plant extract, phytochemical analysis, resistant microbes, time-kill method
Abbreviations: AM, amoxicillin; **CZ**, ceftazidime; **Cip**, ciprofloxacin; **C**, chloramphenicol; **DMSO**, dimethylsulphoxide; **ER**, erythromycin; **FIC**, fractional inhibitory concentration; **GM**, gentamicin; **MHA**, Mueller Hinton Agar; **MHB**, Mueller Hinton Broth; **MIC**, minimal inhibitory concentration; **T**, tetracycline; **TMP**, trimethoprim

INTRODUCTION

Antibiotics are strong medicines and save lives but with the increasing use of antibiotics in the clinic, multidrug-resistant strains become frequently encountered, making treatment with a single agent more and more difficult to cure infections. Thus, it is extremely important to find new antimicrobial agents or new ways that are effective for the treatment of infectious diseases caused by drug-resistant bacteria (Taylor *et al.* 2002). Drug synergism between known antimicrobial agents and bioactive plant extracts is a novel concept. It could be beneficial (synergistic or additive) or deleterious (antagonistic or toxic effect). Research on synergism of plant extracts with antibiotics is very limited and a few studies have found that efficacy of antimicrobial agents can be improved by combining them with crude plant extracts against different pathogens (Ahmad and Aqil 2007; Horiuchi *et al.* 2007). *Terminalia chebula* (Retz.) is a medicinal plant, called the "King of Medicine" in Tibet and is always listed first in the list of Ayurvedic Materia Medica due to its extraordinary power of healing. Different parts of this plant have been reported to have different medicinal properties including antimicrobial activity (Sato *et al.* 1997; Malckzadeh *et al.* 2001; Rani *et al*. 2004; Kim *et al.* 2006; Carounanidy *et al.* 2007; Parekh and Chanda 2008; Kannan *et al.* 2009; Deepak *et al.* 2010). Studies conducted in our laboratory revealed that *T. chebula* fruit extract possessed strong antibacterial activity against multidrug-resistant uropathogenic *E. coli* (Bag *et al.* 2009). In the present investigation an attempt has been made to study the combination effect of *T. chebula* fruit extract with some selected antibiotics against multidrug-resistant uropathogenic *E. coli* with a view to elucidate its possible synergistic activity with antibiotics, if any.

MATERIALS AND METHODS

Collection, identification and processing of plant material

Fresh ripe fruits of *Terminalia chebula* Retz. were collected from local herbalist and were identified and authenticated by a botanist, Prof. S. Chanda, Agricultural and Ecological Research Unit, Indian Statistical Institute, Kolkata, India. Voucher specimen was stored in the Agricultural and Ecological Research Unit, Indian Statistical Institute, Kolkata, India. Shade dried seedless fruits of *T. chebula* were milled to fine powder. The powder was kept in an air-tight container to avoid contamination.

Extraction of plant material

25 g *T. chebula* fruit powder was kept in 70% ethanol for consecutive 3 days at room temperature and filtered through Whatman filter paper No. 1. The filtrate was centrifuged at 3000 rpm for 15 min and evaporated to dryness (yield = 18.3 g) and stored at 4°C in an air-tight jar until further use. For experimental purposes, extract was reconstituted in 5% dimethylsulfoxide (DMSO) to a final concentration of 100 mg/ml.

Microbial assays

1. Bacterial isolates and selected antibiotics

A total of 8 multidrug-resistant *E. coli* isolates were collected from urine specimens submitted to Department of Microbiology, Institute of Postgraduate Medical Education and Research, Kolkata,

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India. Their identity was confirmed using cultural, morphological and biochemical tests. The isolates were maintained on nutrient agar slants at 4°C. The following selected antibiotics: amoxicillin (AM) (penicillin antibiotics), ceftazidime (CZ) (third generation of cephalosporins), ciprofloxacin (Cip) (fluoroquinolones), gentamicin (GM) (aminoglycoside) and trimethoprim (TMP) (sulfonamide group) powder were procured from Sigma Chemicals, St. Louis, USA and used for combination study. To test antibiotic resistance of test isolates, following antibiotic discs: AM (30 µg/disc), CZ (30 µg/disc), chloramphenicol (C) (30 µg/disc), Cip (5 µg/disc), erythromycin (ER) (15 µg/disc), GM (10 µg/disc), tetracycline (T) (30 µg/disc) and TMP (5 µg/disc) were procured from Oxoid, UK.

2. Antibiotic resistance of test isolates

Antibiotic sensitivity of test isolates was determined by standard disc diffusion method against a number of standard antibiotics (Bauer *et al.* 1966). The isolates that showed resistance against at least three of antibiotics tested were considered as multidrug-resistant and selected for antibacterial assay. Internal quality assurance was ensured using reference *E. coli* strain (ATCC 8739).

3. Determination of minimal inhibitory concentrations (MICs)

The minimal inhibitory concentrations (MICs) of *T. chebula* fruit extract and antibiotics were determined in Mueller-Hinton broth with microdilution method (CLSI 2005). Briefly, overnight bacterial cultures were used to prepare 0.5 McFarland standard suspensions which were further adjusted to 5×10^5 CFU/ml. Serial dilutions of antibiotics and plant extract were prepared. The range of antibiotics dilution were AM (2-20 mg/L), CZ (2.5-10 mg/L), Cip (2-16 mg/L), GM (1-4 mg/L) and TMP (0.1-0.5 mg/L) and for plant extract, it was 97-1560 mg/L. Tubes containing antimicrobial agents and inoculum were incubated for 24 h at 37°C. The least concentration of antimicrobial agents showing no visible growth was taken as the MIC.

For calculation of MIC_{50} and MIC_{90}, after the MIC had been determined, a known quantity (0.1 ml) of inoculum from each tube containing broth was subcultured to solid agar plates. The antimicrobial agent that has carried with inoculum was removed by diffusion into agar and the effect was negated by spreading the inoculum over a large area. The number of colonies that grow on subculture after overnight incubation was then counted and compared to the number of CFU/ml in the original inoculum. MIC at which 90% and 50% of an isolate tested was inhibited, was referred to as MIC_{90} and MIC_{50}, respectively (Visalli *et al.* 1998).

4. Checkerboard titration

This method utilized an inoculum of approximately 5×10^5 CFU/ml on Mueller-Hinton Broth (MHB) with the plant extract and the antibiotics in combination ranging from 1/8 × MIC to 4 × MIC. The fractional inhibitory concentration (FIC) was derived from the lowest concentration of antibiotic and extract combination putting no visible growth of the test organisms on the MHB tubes after incubation for 24 h at 37°C. FIC indices were calculated using the formula: FIC_{index} = (MIC of antibiotic in combination/MIC of antibiotic alone) + (MIC of plant extract in combination/MIC of plant extract alone). The results were interpreted according to $FIC_{indices}$ as follows. 'synergy' (FICI ≤ 0.5), 'additive' (FICI > 0.5–4) and 'antagonism' (FICI > 4). All the experiments were independently repeated thrice and the data were expressed as arithmetic average (Leclercq *et al.* 1991).

5. Time-kill method

Bacterial killing effect was carried out on isolate no. 3 against which plant extract showed maximum synergistic activity in combination with GM and TMP by checkerboard method. The combination of ¼X MIC was applied: 390 mg/L plant extract, 1 mg/L GM and 0.125 mg/L TMP. An over-night grown bacterial culture was diluted to approximately 5×10^5 CFU/ml in fresh MHB and incubated at 37°C. The sample removed at 0, 3, 6 and 24 h were diluted and plated on MHA in order to determine viable cell counts. Synergy was defined as a >100-fold or $2\log_{10}$ decrease in colony count at 24 h by the combination compared with that by the most active single agent (Leclercq *et al.* 1991).

6. Phytochemical analysis of plant extract by thin layer chromatography

Major phytocompounds in the crude extract of plant material was detected by standard colour tests and thin layer chromatography. 50 µg of plant extract was spotted at 2.5 cm from the base of preparative E-Merck chromatographic Silica Gel F_{254} TLC plates (thickness 0.25 mm; 3 × 8 cm). Only one solvent system (acetone: ethanol; 1: 1) was used and run in duplicate. One set was used as the reference chromatogram and the other set was used for bioautography. The reference TLC plates were then developed using *p*-anisaldehyde-sulphuric acid reagent which were then heated at 110°C for 5-10 min (Harborne 1973; Wagner and Bladt 1996; Stafford *et al.* 2005).

7. TLC – bioautography

One set of bioautography plates, developed as described above, was placed in sterile petridishes. Inoculum of *E. coli* containing 5×10^5 CFU/ml for every 10 ml of melted nutrient agar was then distributed over the TLC plates. After the solidification of the medium, the TLC plate was incubated for 24 h at 37°C. Subsequently, bioautograms developed were sprayed with 0.2 mg/ml *p*-iodonitrotetrazolium violet. Clear zones on bioautograms indicate inhibition of growth after incubating for 1 hr at 37°C (Slusarenko *et al.* 1989; Eloff 1998; Holetz *et al.* 2002).

Statistical analysis

Data were statistically analyzed by analysis of variance (ANOVA) *F*-test and multiple comparison procedure using Scheffe's method (Woolson 1987).

RESULTS AND DISCUSSION

According to MIC_{90} values the most active antibiotic was TMP (MIC_{90}: 0.5 mg/L), followed by GM (4 mg/L), CZ (5 mg/L), Cip (8 mg/L) and AM (13.5 mg/L) (**Table 1**).

GM and TMP are synergistic when combined with plant extract against the majority of *E. coli* isolates tested. Plant extract-gentamicin combination showed synergy against 87% of test isolates whereas plant extract-trimethoprim showed 75%. Combinations of AM, CZ and Cip showed 'additive' effect with plant extract against majority of test isolates (**Table 2**).

Fig. 1 shows strong synergic activity of both plant extract-gentamicin and plant extract-trimethoprim combinations. In both cases >100-fold decrease in colony count were observed after 24 h.

In order to check whether there was a statistically significant difference in inhibitory action by the antibiotics and plant extract alone and in combinations at 24 h, Scheffe's test for multiple comparison for all antimicrobial agents with control group was applied. The estimated *F*-value at 5% level was higher than the tabulated figure indicating a statistically significant difference in inhibitory action achieved by certain combinations of plant extract and antibiotics at 24 h (**Fig. 1**).

Table 1 MIC ranges, MIC_{50} and MIC_{90} values of plant extract and antibiotics against uropathogenic *Escherichia coli*

Antimicrobial agents	MIC (mg/L)		
	MIC ranges	MIC_{50}	MIC_{90}
PE	97 - 1560	195	390
AM	2 - 20	4.5	13.5
Cip	2 - 16	6	8
CZ	2.5 - 10	2.5	5
GM	1 - 4	2	4
TMP	0.1 - 0.5	0.25	0.5

PE : Plant extract; AM : Amoxicillin; Cip : Ciprofloxacin; CZ : Ceftazidime; GM : Gentamicin; TMP : Trimethoprim

Table 2 Fractional inhibitory concentration (FIC) values for the combinations between the plant extract and antibiotics.

Strain No.	PE + GM		PE + TMP		PE + Cip		PE + AM		PE + CZ	
	FIC_{index}	Activity	FIC_{index}	Activity	FIC_{index}	Activity	FIC_{index}	Activity	FIC_{index}	Activity
1	0.37	S	1.00	ADD	2.13	ADD	1.50	ADD	1.13	ADD
2	0.43	S	0.36	S	1.38	ADD	2.73	ADD	4.63	AG
3	0.37	S	0.32	S	1.62	ADD	2.50	ADD	1.50	ADD
4	0.43	S	0.40	S	2.50	ADD	1.43	ADD	5.63	AG
5	0.50	S	0.40	S	2.13	ADD	4.70	AG	2.50	ADD
6	1.25	ADD	1.22	ADD	4.37	AG	2.73	ADD	1.50	ADD
7	0.43	S	0.50	S	1.63	ADD	1.50	ADD	1.63	ADD
8	0.50	S	0.32	S	1.38	ADD	4.70	AG	4.50	AG

S : Synergy; ADD : Additive; AG : Antagonism; PE : Plant extract; AM : Amoxicillin; Cip : Ciprofloxacin; CZ : Ceftazidime; GM : Gentamicin; TMP : Trimethoprim

Table 3 Phytochemical analysis and TLC-bioautography of *T. chebula* fruits.

Plant Constituents	*T. chebula* fruit	Inhibition
Alkaloids	-	Not detected
Phenols	+++	*
Flavonoids	+	#
Glycosides	-	Not detected
Terpenoids	+	No inhibition
Steroids	-	Not detected
Saponins	-	Not detected

(-) : negative; (+) : low concentration; (++) : medium concentration; (+++) : high concentration; * well-defined zone of inhibition; # inhibition zone less visible

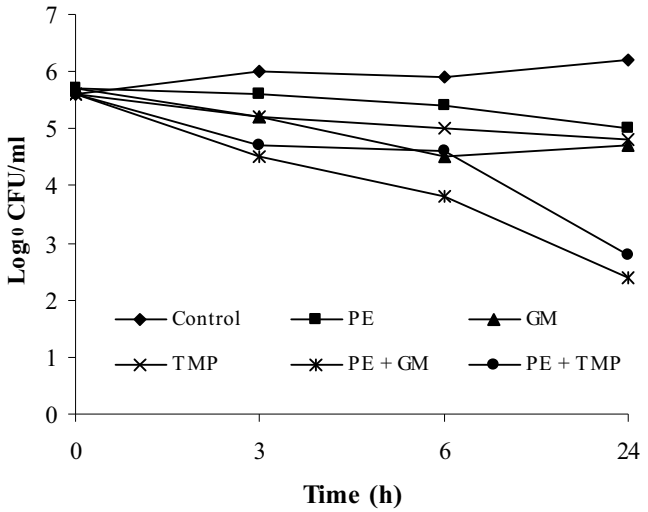

Fig. 1 Time-kill curves showing combination effect of plant extract with gentamicin and trimethoprim against multidrug-resistant uropathogenic *Escherichia coli* isolate. Groups PE, GM, TMP, PE plus GM and PE plus TMP are significantly ($P < 0.05$) different when compared with control group. Also pairs of groups between PE or GM or TMP vs. PE plus GM and PE plus TMP are significantly ($P < 0.05$) different at 24 h. But pairs of groups PE vs. GM; PE vs. TMP and GM vs. TMP are not significantly different.

Phytochemical analysis revealed that *T. chebula* fruits contain high concentration of phenols and low concentrations of flavonoids and terpenoids. TLC-bioautography indicated phenols as active phytocompounds (**Table 3**).

Urinary tract infections (UTIs) pose serious health problem affecting millions of people each year. *Escherichia coli* causes about 80% of the urinary tract infections in adults (Kunin 1987). Despite the recent introduction into clinical practice of highly potent newer antibiotics such as the newer aminoglycosides, fluoroquinolones and the third generation cephalosporins, *E. coli* infections pose a major therapeutic problem for clinicians world-wide. Synergism is of particular importance in cases in which the infecting organisms are relatively resistant to all available antimicrobial agents or in which the enhancement of drug activity is needed (Moellering 1995).

The results obtained in the present combination study which showed either synergy or additivity but no evidence of antagonistic effects are very encouraging (**Table 2**). The plant extract has the importance to control the test isolates under very low concentration of test antibiotics (GM and TMP) (**Fig. 1**) and thus minimizing the possible toxic effects. Furthermore, synergistic activity of the plant extract enables the use of the respective antibiotic when it is no longer effective by itself during therapeutic treatment. Our observations are in agreement with the findings of other workers where synergistic activity of different plant extracts with antibiotics against bacterial isolates have been reported (Zartoshti *et al.* 2007; Odunbaku *et al.* 2008).

Phytochemical analysis by colour tests and thin layer chromatography of plant material revealed the presence of high content of phenols and low content of flavonoids and terpenoids. Phenols showed strong antibacterial activity against the test isolates under TLC-bioautography (**Table 3**).

The possible mechanism behind the synergy is not clear right now. It may be due to the combined effect of antibiotics and the antimicrobial principles of *T. chebula* fruit which may have different mechanisms of action or may be inhibiting two different steps in the same biosynthetic pathway of the organism resulting in an overall synergy at certain combinations.

CONCLUSIONS

Results of our foregoing findings revealed that *T. chebula* fruit extract possessed strong antibacterial (synergistic) activity with antibiotics against the test isolates and reinforce the importance of ethnomedical approach as a potential source of bioactive compounds for the management of urinary tract infections caused by *E. coli*. Further studies on bioassay-guided fractionation both on the plant extract and phenolic composition as well as *in vivo* experiments should be performed to uncover the real therapeutic potential of the plant material. This report may serve as a footstep on this aspect.

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