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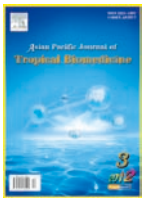

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# *In vitro* antimicrobial potential of *Terminalia chebula* fruit extracts against multidrug-resistant uropathogens

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ABSTRACT

**Objective:** *Terminalia chebula* Retz. (combretaceae) is called the "King of Medicine" in Tibet and is always listed at the top of the list of "Ayurvedic Materia Medica" because of its extraordinary power of healing. The present study was carried out to evaluate the possible *in vitro* antibacterial potential of different solvent extracts of *T. chebula* fruit against multidrug-resistant uropathogens. **Methods:** A total of 52 multidrug-resistant uropathogenic bacteria were used in this study. Successive extractions of *T. chebula* fruits were performed with solvents of different polarities. Agar well diffusion and microbroth dilution assay methods were used for antibacterial susceptibility testing. Kill-kinetics study was done to know the rate and extent of bacterial killing. Qualitative phytochemical screening was done to know the major phytoconstituents present in the plant material. Acute oral toxicity study in mice was performed to evaluate the toxic potential of the plant material, if any. **Results:** The ethanol extract of *T. chebula* fruits demonstrated a strong antimicrobial activity against all the test isolates and found to be most effective over others. Kill-kinetics study showed dose and time dependent antibacterial activity of ethanol extract. Phytochemical analysis revealed the presence of high concentration of phenolics and low concentration of flavonoids and terpenoids. In acute oral toxicity study, no gross behavioral changes were observed in mice at recommended dosage level and 24 h $LD_{50}$ of ethanol extract was found to be >4 g/kg, p.o. in mice. **Conclusions:** The results provide justification for the use of *Terminalia chebula* fruit in folk medicine to treat various infectious diseases and could be useful for the development of alternative/ complementary medicine for multidrug-resistant uropathogens.

## 1. Introduction

Despite tremendous progress in medical fraternity, infectious diseases caused by pathogenic microorganisms are still a major threat to public health worldwide[1]. One of the more alarming recent trends in infectious diseases has been the increasing frequency of antimicrobial resistance among microbial pathogens causing nosocomial and community-acquired infections. Numerous classes of antimicrobial agents have now become less effective as a result of the effective pressure of antimicrobial usage[2]. This resistance problem provides an impetus for further attempts to search for new antimicrobial agents from other sources to combat infections and overcome the problems of resistance and side effects of the currently available antimicrobials. Crude extracts of medicinal plants stand out as veritable sources of potential resistance modifying agents and the Indian biosphere promises to be a potential source of such compounds owing to its rich plant species diversity.

Among the infectious diseases, urinary tract infections (UTIs) are the second most common type of infections in the body accounting for morbidity and mortality in all the human populations from neonate to the geriatric age group. A high level of antibiotic resistance is very significant in uropathogenic bacteria especially *Escherichia coli*, the main aetiological agent of UTIs. It has developed resistance to conventional antibiotics including extended spectrum cephalosporins, fluoroquinolones and carbapenem[3, 4]. Other commonly found bacteria which causes urinary tract infections includes *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and others[5].

*Terminalia chebula* Retz. (Fam. Combretaceae), is a medicinal plant with diverse beneficial effects on human health because of its extraordinary power of healing[6-8]. Although numerous studies have shown antimicrobial activity of *T. chebula* fruit extracts against a number of microorganisms[9-12], but systematic and methodical investigations on antimicrobial potential of *T. chebula* fruit extracts against multidrug-resistant uropathogens

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seems to be dubious. The present investigation has therefore been designed to study the *in vitro* antibacterial potential of *T. chebula* fruit extracts against multidrug-resistant uropathogens with a view to develop an effective and practically non-toxic drug-resistance modifying antimicrobial agent from plant origin for the treatment of urinary tract infections caused by these drug-resistant bacterial pathogens and also to overcome the problems of toxic side effects of conventional antibiotics.

## 2. Materials and Methods

### 2.1. Collection, identification and processing of plant material

The fresh matured fruits of *T. chebula* were collected from local market (Bowbazar, Kolkata, India) and were identified and authenticated by a Botanist, Prof. Sunanda Chanda, Agricultural and Ecological Research Unit, Indian Statistical Institute, Kolkata. A voucher specimen (No. AERU/TC01/10) was deposited at the Dept. of Agricultural and Ecological Research Unit, Indian Statistical Institute, Kolkata. The fruits were washed thoroughly in tap water, dried and seeds were separated. The pericarp of fruits were then milled to fine powder.

### 2.2. Preparation of crude extracts of T. chebula fruits

Aqueous, ethanol and acetone extracts of *T. chebula* fruit powder were prepared by immersing 25g *T. chebula* fruit powder in each of the three conical flasks stoppered with rubber cork containing 150 ml of distilled water, 70% aqueous ethanol and acetone respectively with occasional shaking at room temperature and kept for 24 h and then filtered off using sterile filter paper (Whatman No. 1). The process was repeated twice using the remaining residues. The pooled filtrates were centrifuged at 3000 rpm for 15 min and concentrated under reduced pressure using a rotary evaporator (yield - aqueous extract : 22.69% ; ethanol extract : 30.25% and acetone extract : 28.2%).

All the dried extracts were stored at 4℃ in air-tight jars until further use. For experimental purposes extracts were reconstituted in 0.5% dimethylsulphoxide (DMSO) to a final concentration of 100 mg/ml.

### 2.3. Test microorganisms and antibiotic resistance of test isolates

52 multidrug-resistant clinical bacterial isolates: 21-*Escherichia coli* (amoxicillin-, trimethoprim- gentamicin-, *ceftazidine*- resistant and ciprofloxacin-, aztreonam - sensitive) , 16 - *Klebsiella pneumoniae* (ampicillin-, trimethoprim-, gentamicin - resistant; aztreonam-, imipenem-, ciprofloxacin - sensitive) , 9 - *Pseudomonas aeruginosa* (sulphamethoxazole-, gentamicin-, novobiocin - resistant; ciprofloxacin-, imipenem - sensitive) and 6 -*Staphylococcus aureus* (oxacillin-, gentamicin-, cefoxitin - resistant; erythromycin-, ciprofloxacin-, vancomycin - sensitive) collected from urine samples of urinary tract infected outdoor patients were kindly provided by the Department of Microbiology, Institute of Post Graduate Medical Education and Research, Kolkata, India. Internal quality assurance was ensured using reference *E. coli* (ATCC 8739) and *S. aureus* (ATCC 6538P) strains procured from National Chemical Laboratory, Pune, India. The isolates were maintained on nutrient agar slants at 4℃.

### 2.4. Preparation of inoculums

The inoculum size of the test isolates was standardized according to the National Committee for Clinical Laboratory Standards guidelines[13]. The bacterial isolates were inoculated in Mueller Hinton Broth (Hi-media, Mumbai, India) and incubated at 37℃ in a shaker water bath for 3 - 6 h until the culture attained a turbidity of 0.5 McFarland Unit. The final inoculum size was adjusted to $5\times 10^5$ CFU/ml.

### 2.5. Antimicrobial assay

#### 2.5.1. Determination of Inhibition Zone Diameter (IZD)

Susceptibility tests were performed by a modified agar well diffusion method[14]. One ml of standard suspension of each bacterial isolate was spread evenly on Mueller-Hinton Agar (Himedia, Mumbai, India) plates using a sterile glass rod spreader and the plates were allowed to dry at room temperature. Subsequently six mm diameter wells were bored on the surface of different plates into which 100 μL reconstituted solution of extract was added. After holding the plates at room temperature for 2 h to allow diffusion of extract into the agar, they were incubated at 37℃ for 24 h. Inhibition Zone Diameter (IZD) was measured to the nearest millimeter (mm). Ciprofloxacin (1 μg/ml) (Himedia, Mumbai, India) and gentamicin (8 μg/ml) (Himedia, Mumbai, India) were used as experimental positive controls and 0.5% DMSO as negative control. The tests were performed in triplicate for each microorganism used.

#### 2.5.2. Determination of minimal inhibitory concentration (MIC)

Ethanol extract having found most effective against the test isolates in agar-well diffusion method was selected for MIC determination. For this purpose, microdilution susceptibility test was performed in flat-bottom 96-well microtitre plates containing Mueller Hinton Broth medium (90 μL) in each well. The active extract was diluted two-fold serially ranging from 0.097 - 12.50 mg/ml. 100 μL of diluted solutions were given in each well containing broth (90 μL). 10 μL of working inoculum suspension ($5\times 10^5$ CFU/ml) was added to the wells. A number of wells were reserved in each plate for control of sterility (no inoculum added), inoculum viability (no sample solution added) and DMSO inhibitory effect. The plates were then incubated for 24h at 37℃. After 24h of incubation, 10 μL of Alamar Blue (Biosource Int., USA) was added in each well and further incubated for 4h for a colour change from blue to pink. A blue colour in the well was interpreted as no growth, and a pink indicated growth. Visual MICs were determined as the lowest concentration of the drug that prevented the colour change from blue to pink[15].

#### 2.5.3. Determination of $MIC_{50}$

For $MIC_{50}$ determination, the following formula of geometric means was used:

$$MIC_{50} = (M<50) + \frac{(n-x) \times [(M>50)-(M<50)]}{Y}$$

Where M < 50 is the MIC of highest cumulative percentage below 50%, M > 50 is the MIC of lowest cumulative percentage above 50%; n is 50% of the number of organisms tested, x is the number of organisms in the group at M < 50 and y is the number of organisms in the group at M > 50[16].

On the basis of strong antibacterial activity, the most active ethanol extract was further investigated for kill-kinetics study, qualitative phytochemical analysis and acute oral toxicity study.

### 2.5.4. Kill-Kinetics study

The rate and extent of bacterial killing by ethanol extract against the most susceptible *E. coli* isolate was determined by kill-kinetics assay method. Growing cultures ($5 \times 10^5$ CFU/ml) of test *E. coli* isolate was added to Mueller Hinton Broth (MHB) (Hi-Media, Mumbai, India) medium and were exposed to 0.5, 1, 2 and 4 times of MIC of ethanol extract of *T. chebula* fruits. Drug free inoculated medium was also plated as a growth control. Samples were removed for colony counts at 0, 2, 4, 6, 8 and 24 h. Viable counts were determined by the serial dilution method. Plates were incubated at 37℃ for 24 h. Plate counts were made after 24h of incubation and only plates containing between 30-300 counts for each series of dilution were counted. The experiment was repeated in triplicate and the means of the readings were computed and recorded. Colony count ($Log_{10}$ CFU/ml) was plotted against time. Antimicrobial agent was considered bactericidal at the lowest concentration that reduced the original inoculum size by > 3 $log_{10}$ CFU/ml (99.9%) and bacteriostatic, if the inoculum size was reduced by 0 - 3 $log_{10}$ CFU/ml[17]. Antibiotic carryover was prevented by serial dilution techniques.

### 2.6. Qualitative phytochemical analysis

Qualitative phytochemical analysis of the ethanol extract was performed as follows: Alkaloids with Dragendroff reagent; flavonoids using sodium hydroxide and dilute HCl; phenolic compounds with ferric chloride solution; saponins with ability to produce stable foam; anthraquinones by Borntrager test; glycosides with Keller-Kiliani test; terpenoids by Salkowski test ; steroids with Libermann-Burchard reagent and reducing sugars with Fehling's solution test. These phytoconstituents were identified by characteristic colour changes using standard procedures[18, 19].

### 2.7. Acute oral toxicity study in mice

The acute oral toxicity study of ethanol extract was carried out using albino mice of either sex weighing 20 - 25g each. They were maintained on standardized diet (Hindustan Lever, Mumbai, India) and water *ad libitum*. The animals were housed in cages at 23 ± 2℃ with 12h/12h light : dark cycle and were allowed to acclimatized to the laboratory conditions for one week. For experimental purposes, animals were kept fasting overnight but allowed free access to water. The animals were randomly divided into seven groups consisting of ten animals in each group, Group I served as control animals and Groups II, III, IV, V, VI and VII as drug treated animals. Group I animals received normal saline (2 ml/kg, p.o.) while groups II, III, IV, V, VI and VII received the ethanol extract at the concentration 100, 250, 500, 1000, 2000 and 4000 mg/kg, p.o. respectively. The animals were observed continuously for first 2h for gross behavioural changes or death, if any, and intermittently for the next 6 hrs and then at 24h after drug administration. The behavioural parameters like convulsions, hyper activity, sedation, grooming, loss of righting reflex and increased respiration were observed[20]. 24h $LD_{50}$ of ethanol extract against mice was calculated on the basis of percent mortality after 24h of ethanol extract administration.

### 2.6. Statistical analysis

Statistical analysis was performed using SPSS software : Version 18.0. The results were presented as the mean ± S.D. Two-way analysis of variance (ANOVA) followed by Tukey's test was applied for statistical analysis with the level of significance set at $P < 0.05$.

## 3. Results

The results of the antibacterial activity of the extracts of *T. chebula* fruits assessed by agar well diffusion method are shown in Table 1. All the tested extracts demonstrated varying degrees of strain specific antibacterial activity against all the test isolates. The inhibition zone diameter against drug-resistant clinical isolates ranged from 8.67 ± 0.92 mm to 24.51 ± 1.28 mm. Ethanol extract was found to be most effective (IZD : 10.67 ± 1.11 mm to 24.51 ± 1.28 mm) followed by acetone extract (IZD : 8.67 ± 0.92 mm to 17.33 ± 1.33 mm) and aqueous extract (IZD : 8.85 ± 0.95 mm to 10.48 ± 1.21 mm). Zone inhibition values of tested extracts against the type strains were ranged from 11.00 ± 1.01 mm to 28.33 ± 0.62 mm. Ciprofloxacin showed inhibition zone ranged from 18.00 ± 0.70 mm to 25.00 ± 1.09 mm against drug-resistant clinical isolates and 26.66 ± 0.92 mm to 28.33 ± 1.21 mm against standard type strains. Gentamicin showed no promising antibacterial activity against the drug-resistant test clinical isolates (IZD: 4.11 ± 0.78 mm to 7.90 ± 0.83 mm) but found sensitive against standard type strains (IZD: 23.66 ± 0.75 mm to 24.33 ± 1.14). The $MIC_{50}$ values assessed by microbroth dilution method confirmed these strain specific antibacterial activity of the plant extracts against the test isolates as shown in Table 2.

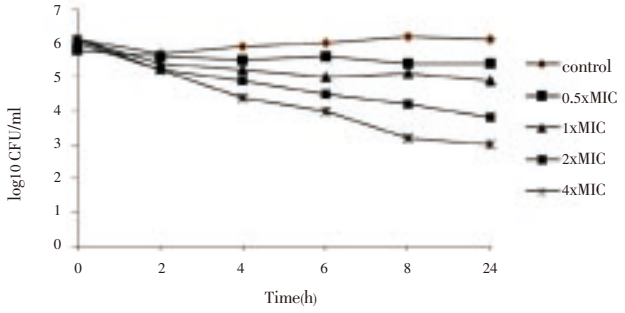


**Figure 1.** Dose and time dependent antibacterial activity (kill-kinetics) of ethanol extract of *Terminalia chebula* fruits against multi drug-resistant uropathogenic *Escherichia coli*

Kill-kinetics study revealed that ethanol extract at 4×MIC dosage level reduced the inoculum size of most susceptible drug-resistant *E. coli* isolate by > 3$log_{10}$CFU/ml at 24h (Fig

**Table 1.**
Inhibition zone diameter of different extracts of *Terminalia chebula* fruits against multidrug resistant uropathogens

| Microorganisms | Zone of inhibition (mm) | | | | | |
|---|---|---|---|---|---|---|
| | Aqueous | *Ethanol | Acetone | Ciprofloxacin | Gentamicin | DMSO |
| *E. coli*(MDR l.c.i.)(n = 21) | 10.48+1.21 | 24.51+1.28 | 17.33+1.33 | 25.00+1.09 | 4.11+0.78 | – |
| *P. aeruginosa* (MDR l.c.i.)(n = 9) | 8.85+0.95 | 10.67+1.11 | 8.67+0.92 | 18.00+0.70 | 7.90+0.83 | – |
| *K. pneumoniae* (MDR l.c.i.)(n = 16) | 9.27+0.98 | 17.5+1.07 | 12.77+1.03 | 24.00+1.03 | 7.74+0.71 | – |
| *S. aureus* (MDR l.c.i.)(n = 6) | 9.00+1.03 | 13.72+0.67 | 10.06+1.05 | 23.00+0.89 | 5.17+0.78 | – |
| *E. coli*(ATCC 8739) | 12.33+0.78 | 28.33+0.62 | 20.00+1.01 | 26.66+0.92 | 23.66+0.75 | – |
| *S. aureus* (ATCC 6538P) | 11.00+1.01 | 22.66+0.92 | 14.66+1.03 | 28.33+1.21 | 24.33+1.14 | – |

Results are mean ± SD of triplicate experiments.
*$P$<0.05, when compared with aqueous and acetone extracts against all the test isolates.

**Table 2.**
MIC ranges and $MIC_{50}$ values of extracts of *Terminalia chebula* fruits against multidrug resistant uropathogens

| Microorganisms | Aqueous extract | | Ethanol extract | | Acetone extract | |
|---|---|---|---|---|---|---|
| | MIC ranges(mg/ml) | $MIC_{50}$(mg/ml) | MIC ranges(mg/ml) | $MIC_{50}$(mg/ml) | MIC ranges(mg/ml) | $MIC_{50}$(mg/ml) |
| *E. coli* (MDR l.c.i.)(n = 21) | 0.39–3.12 | 0.866 | 0.097–1.560 | 0.200 | 0.194–3.120 | 0.261 |
| *K. pneumoniae* (MDR l.c.i.)(n = 16) | 0.78–6.25 | 0.877 | 0.194–6.250 | 0.269 | 0.39–6.25 | 0.465 |
| *P. aeruginosa* (MDR l.c.i.)(n = 9) | 6.25–12.50 | 3.902 | 0.39–12.500 | 1.716 | 3.12–12.50 | 3.433 |
| *S. aureus* (MDR l.c.i.)(n = 6) | 1.56–6.26 | 1.657 | 0.194–6.250 | 0.975 | 0.78–6.25 | 0.975 |

**Table 3.**
Phytochemical analysis of ethanol extract of *Terminalia chebula* fruits

| Phytoconstituents | Tests used | Ethanol extract |
|---|---|---|
| Alkaloids | Drangendorff test | – |
| Flavonoids | Sodium hydroxide test | + |
| Phenolics | Ferric chloride test | +++ |
| Saponins | Foam test | – |
| Anthraquinones | Borntrager test | – |
| Glycosides | Keller–Kiliani test | – |
| Terpenoids | Salkowski test | + |
| Steroids | Libermann– Burchard test | – |
| Reducing sugar | Fehling's test | – |

(+): Low concentration; (++) moderate concentration; (+++): High concentration; (–) : Absent

**Table 4.**
The acute oral toxicity study of ethanol extract of *Terminalia chebula* fruits in mice

| Group | Dose (mg/kg, p.o.) | No. of animals taken | No. of animals died | % mortality (24 h) | $LD_{50}$ |
|---|---|---|---|---|---|
| I | Control (saline) | 10 | 0 | 0 | >4g/kg, p.o. |
| II | 100 | 10 | 0 | 0 | |
| III | 250 | 10 | 0 | 0 | |
| IV | 500 | 10 | 0 | 0 | |
| V | 1000 | 10 | 0 | 0 | |
| VI | 2000 | 10 | 1 | 10 | |
| VII | 4000 | 10 | 2 | 20 | |

1) which indicated its bactericidal activity. Phytochemical analysis revealed that ethanol extract exhibited high concentration of phenolics and low concentration of flavonoids and terpenoids (Table 3).

In acute oral toxicity study, no gross behavioural changes were observed in mice at recommended dosage level and 24h $LD_{50}$ was found to be >4 g/kg, p.o. in mice (Table 4)

## 4. Discussion

With the increase in resistance by microorganisms to the currently used antibiotics and the high cost of production of synthetic compounds there is a need to seek alternative antimicrobials from other sources effective against pathogens resistant to current antibiotics[21]. Medicinal plants could be one of those alternatives because most of them are safe, cost less and affect a wide range of antibiotic resistant microorganisms. The rich chemical diversity in plants promises to be a potential source of antibiotic resistance modifying or modulating compounds and has yet to be adequately explored[22, 23].

From our foregoing findings on antimicrobial susceptibility testing of *T. chebula* fruit extracts against multidrug-resistant uropathogens, it was observed that all the extracts demonstrated varying degrees of strain specific antimicrobial activity against the test isolates. To check whether there

was any statistically significant difference in inhibition zone diameter achieved by different solvent extracts of *T. chebula* fruits against the test isolates, two-way analysis of variance followed by Tukey's test was applied. Two-way analysis of variance revealed significant variation (5% level) among the treatments as far as their antimicrobial activity was concerned. The follow-up Tukey's multiple comparison test showed the supremacy of ethanol extract over others. Mean inhibition zone diameter of ethanol extract against all the test isolates differed from both acetone and aqueous extracts by amount as great as minimum significance range at 5% level. Ethanol extract also showed bactericidal activity as evidenced from kill-kinetic study. The high content of phenolics in the ethanol extract of *T. chebula* fruits implied that phenolics may be responsible for this antibacterial activity. In acute oral toxicity study, no gross behavioural changes as well as toxic effects in mice were observed at recommended and higher dosage levels of ethanol extract which indicated that the test material can be considered as safe[24].

Thus our findings support the traditional use of *Terminalia chebula* fruits against infections and indicated its resistance modifying potential against multidrug-resistant uropathogens. This may help in developing an effective alternative antimicrobial agent from plant origin in near future. Further studies are needed to uncover the therapeutic relevance of this plant material. This promising report may serve as a footstep on this aspect.

## Conflict of interest statement

We declare that we have no conflict of interest.

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