

The Kill Kinetics of Phenolics of Chebolic Myrobalan (Fruit of *Terminalia chebula* Retz.) against Methicillin-Resistant *Staphylococcus aureus* and Trimethoprim-Sulphamethoxazole-Resistant Uropathogenic *Escherichia coli*

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ABSTRACT

In this study, phenolics of chebolic myrobalan (CM) (fruit of *Terminalia chebula* Retz.), which was previously determined to have strong antibacterial activity, were tested for the rate of killing bacteria in given time (kill kinetics). Assays were conducted against methicillin-resistant *Staphylococcus aureus* (MRSA) and trimethoprim-sulphamethoxazole (SXT/TMP)-resistant uropathogenic *Escherichia coli*. Inoculated strains were tested against serial dilutions at time intervals of 0, 2, 4, 6, 8 and 24 h with different concentrations of phenolics based on their MIC values against the tested strains. Results obtained showed that phenolics at dosage levels of 6.25 mg/ml (2X MIC) and 12.5 mg/ml (4X MIC) strongly inhibited the bacterial growth of MRSA. On the other hand, the growth of SXT/TMP-resistant uropathogenic *E. coli* was strongly inhibited by phenolics from 1.56 mg/ml (1X MIC) to 6.25 mg/ml (4X MIC) for 6 h. After that regrowth of the tested strain (*E. coli*) occurred. These findings revealed that phenolics have pharmacodynamic properties against the tested strains and reinforce the importance of an ethnomedical approach as a potential source of bioactive compounds for the treatment of multi-drug resistant key bacterial pathogens.

Keywords: multi-drug resistant pathogens, *Terminalia chebula* fruits, time-kill curves, total phenolics

Abbreviations: CM, chebolic myrobalan; MBC, minimal bactericidal concentration; MIC, minimal inhibitory concentration; MRSA, methicillin-resistant *Staphylococcus aureus*; SXT/TMP, trimethoprim-sulphamethoxazole

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a resistant variant of common bacterium *S. aureus* which is responsible for difficult-to-treat infections in humans. MRSA is resistant to a large group of antibiotics called the β -lactams, including aminoglycosides, the third and fourth generations of cephalosporins and monolactams (Livumore 1995). Among the wide array of antibiotics, β -lactams are the most varied and widely used agents accounting for over 50% of all systematic antibiotics in use (Bronson and Barrett 2001). On the other hand, the clinical management of urinary tract infections is complicated by the increasing incidence of infections caused by the strains of *Escherichia coli* that are resistant to commonly used antimicrobial agents (Zhanel *et al.* 2000). In recent years, in the United States, there has been a notable increase in the isolation of uropathogenic *E. coli* strains resistant to trimethoprim-sulphamethoxazole (SXT/TMP), representing as multi-drug-resistant pathogen (Sahm *et al.* 2001; Karlowsky *et al.* 2002). Antimicrobial resistance among key microbial pathogens continues to grow at an alarming rate worldwide (Archibald *et al.* 1997; Pfaller *et al.* 1998). The increased prevalence of antibiotic resistant bacteria emerging from the extensive use of antibiotics render the current antimicrobial agents insufficient to control the bacterial infections and have become a global concern. This resistance problem demands that a renewed effort be made to seek antimicrobial agents from other sources effective against pathogenic microorganisms resistant to current antibiotics and one of the possible strategies towards this objective is the rational localization of bioactive phytochemicals (Cordell 2000;

Rios and Recio 2005). Besides, presently the emergence of immuno-compromised cases and new strains of disease causing bacteria require that antibiotics are closely monitored (Kirby and Craig 1981; Kunin 1981). For example, a system that shows the rate and extent of bacterial killing (kill kinetics) provides more accurate description of antimicrobial activity than does the MIC (Craig and Vogelmann 1987; Vogelmann and Craig 1986; Zhanel *et al.* 1991). The strong antibacterial activity of extracts and phenolics of chebolic myrobalan (CM) (fruit of *Terminalia chebula* Retz.) against MRSA and SXT/TMP-resistant uropathogenic *E. coli* has already been reported by us (Chattopadhyay *et al.* 2008; Bag *et al.* 2009). Time-kill curves are pharmacodynamic examples of bactericidal activity expressed as the rate of killing by a fixed concentration of antimicrobial and are one of the most reliable methods of determining tolerance (NCCLS 1992). In the present investigation an attempt has been made to study the time-kill kinetics of phenolics of CM against both Gram-positive and -negative multi-drug resistant key pathogens (MRSA- and SXT/TMP-resistant uropathogenic *E. coli*) with a view to elucidate the rate and extent of bacterial killing.

MATERIALS AND METHODS

Collection of plant materials

Fresh ripe fruits of Chebolic myrobalan (*Terminalia chebula* Retz.) was collected from a local herbalist and identified and authenticated by a botanist, Prof. S. Chanda, Agricultural and Ecological Research Unit, Indian Statistical Institute, Kolkata, India.

Extraction and estimation of total phenolics

Shade dried seedless fruits of CM were milled to fine powder. The total phenolics were extracted using the protocol suggested by Price *et al.* (1980). Briefly, 5 g of powdered CM fruits was homogenized thoroughly in 200 ml of acetone using a mortar and pestle, transferred to a stoppered flask and kept overnight in a shaker. The supernatant was collected and the residue was extracted twice with 10 ml acetone. The collected extracts were pooled and filtered through Whatman No. 1 filter paper and the filtrate was centrifuged at $3000 \times g$ for 10 min. The supernatant was collected and evaporated to dryness and stored at -80°C until use.

Total phenolics were estimated by Folin Ciocalteu reagent (McDonald *et al.* 2001). A dilute extract (0.5 ml of 1: 10 g/ml) or gallic acid (standard phenolic compound) was mixed with Folin Ciocalteu reagent (5 ml, 1: 10 diluted with distilled water) and aqueous Na_2CO_3 (4 ml, 1 M). The mixtures were allowed to stand for 15 min and the total phenolics were determined by colorimetry at 765 nm. The standard curve was plotted using 0, 50, 100, 150, 200, 250 mg/L solutions of gallic acid in methanol: water (50: 50 v/v). Total phenolics were expressed in terms of gallic acid equivalent (mg/g of dry mass), which is a common reference standard.

Formulation of extract

The extract was diluted in 5% dimethylsulphoxide and adjusted to a final concentration of 100 mg/ml.

Microorganisms

For microbial analysis, freshly collected clinical isolates (i) MRSA- and (ii) SXT/TMP-resistant uropathogenic *E. coli* were used. These clinical isolates were procured from the Department of Microbiology, Institute of Postgraduate Medical Education and Research, Kolkata, India. All the tested strains were maintained in nutrient agar slants at 4°C .

Determination of minimal inhibitory concentration (MIC)

The minimal inhibitory concentrations (MICs) of the phenolics of CM against all the test strains were determined by macrobroth dilution assay method (NCCLS 1993). Two-fold serial dilutions of phenolics (0.1 to 100 mg/ml) were prepared in tubes with Mueller Hinton Broth (MHB) (Hi-media, Mumbai, India) as diluent. Each dilution was seeded with $20 \mu\text{l}$ of tested microorganisms to the standard concentration (5×10^5 cfu/ml). Two-fold serial dilution of Gentamicin (Nicholas, India) (0.215–512 $\mu\text{g/ml}$) was used as experimental positive control. The tubes were incubated at 37°C for 24 h. The least concentration of the phenolics or standard drug showing no visible growth was taken as the MIC.

Determination of minimal bactericidal concentration (MBC)

Minimal bactericidal concentrations (MBCs) were determined by aspirating 0.01 ml of the culture medium from each tube (in the macrobroth MIC assay) showing no apparent growth and subculturing it on fresh Mueller Hinton Agar (MHA) plates. The later was incubated at 37°C for 24 h. The MBC was read as the least concentration of the phenolics showing no visible growth on MHA subculture. Using the values of MIC and MBC, the $\text{MIC}_{\text{index}}$ (MBC/MIC) was computed against each tested strain.

Time-Kill assays

All isolates were tested by a standard time-kill methodology. Growing cultures (10^5 – 10^6 cfu/ml) of each strain were added to Mueller Hinton Broth (MHB) (Hi-Media, Mumbai, India) medium and were exposed to 0.5, 1, 2 and 4X the MIC of phenolics of CM. 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°C 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 procedure was repeated in triplicate for each organism and the means (X) of the readings computed and recorded. Log_{10} cfu/ml was plotted against time for each strain. Antimicrobial agent was considered bactericidal at the lowest concentration that reduced the original inoculum by $> 3 \text{ log}_{10}$ cfu/ml (99.9%) and bacteriostatic, if the inoculum was reduced by 0.3 log_{10} cfu/ml (NCCLS 1992). Antibiotic carryover was prevented by serial dilution techniques.

RESULTS

Table 1 shows the results of MIC, MBC and $\text{MIC}_{\text{index}}$ values of phenolics of CM against the test strains. The MIC values for phenolics against MRSA- and SXT/TMP-resistant uropathogenic *E. coli* were 3.12 and 1.56 mg/ml, respectively whereas MBC values were 6.25 and 1.56 mg/ml, respectively. All the strains were resistant to Gentamicin. $\text{MIC}_{\text{index}}$ values against MRSA- and SXT/TMP-resistant uropathogenic *E. coli* were 2 and 1, respectively.

Fig. 1 shows that *S. aureus* did not respond up to 3.12 mg/ml for 24 h, but rather increased almost at the same rate as the control. But at 6.25 and 12.5 mg/ml, the inoculum was reduced to 1×10^3 and 1×10^2 cfu/ml, respectively for 24 h.

E. coli, on the other hand (Fig. 2), showed no noticeable change in inoculum size at 0.78 mg/ml for 24 h. But at 1.56, 3.12 and 6.25 mg/ml, the inoculum was reduced to 3×10^4 , 1×10^3 and 1×10^2 cfu/ml, respectively for 6 h. But beyond 6 h, regrowth of the inoculum occurred and reached near to control levels after 24 h.

DISCUSSION

The principle constituents of CM are gallic acid, ellagic acid, chebulagic acid, chebulinic acid and corilagin which are phenolic in nature and have strong antibacterial activity against a variety of bacterial species (Mehta *et al.* 1993; Bruneton 1995; Chevallier 1996; Sato *et al.* 1997; Cowan 1999). In our study, the kill kinetics of phenolics of CM

Table 1 Minimal inhibitory concentration (MIC), minimal bactericidal concentration (MBC) and $\text{MIC}_{\text{index}}$ of phenolics of Chebulic myrobalan against MRSA and SXT/TMP resistant uropathogenic *Escherichia coli*

Treatment	Microorganisms	MIC (mg/ml)	MBC (mg/ml)	$\text{MIC}_{\text{index}}$
Phenolics	<i>S. aureus</i> (LCI)	3.12	6.25	2
	<i>E. coli</i> (LCI)	1.56	1.56	1
Gentamicin	<i>S. aureus</i> (LCI)	R	R	NA
	<i>E. coli</i> (LCI)	R	R	NA

R: resistant; NA: not applicable

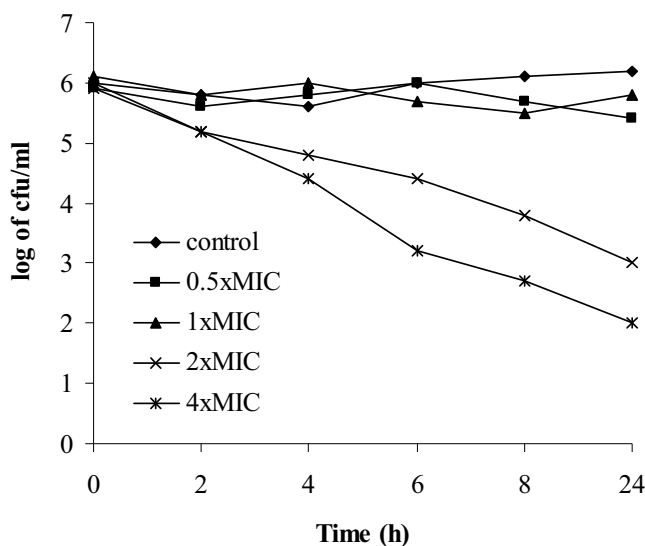


Fig. 1 Activity of varying concentrations of Chebulic myrobalan phenolics against methicillin-resistant *Staphylococcus aureus* (MRSA).

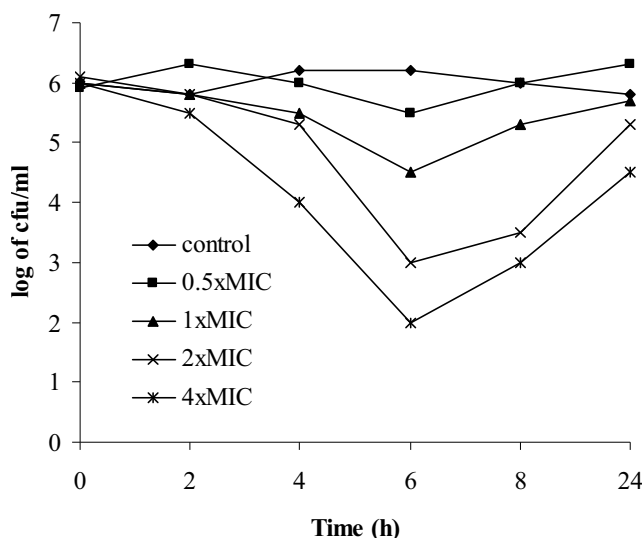


Fig. 2 Activity of varying concentrations of Chebolic myrobalan phenolics against trimethoprim-sulphamethoxazole (SXT/TMP)-resistant uropathogenic *Escherichia coli*.

revealed that phenolics have pharmacodynamic properties against both multi-drug resistant Gram-positive (MRSA) and Gram-negative (SXT/TMP-resistant uropathogenic *E. coli*) bacterial pathogens.

In Gram-positive MRSA (Fig. 1), phenolics of CM failed to inhibit growth up to 3.12 mg/ml. But at 6.25 and 12.5 mg/ml, the MRSA inoculum was gradually reduced and reached 1×10^3 and 1×10^2 cfu/ml, respectively in 24 h. The killing was both dose- and time-dependent and this is a more rational basis for determining optimal dosage for antimicrobial treatment regimens (Chalkley and Koornhof 1985).

On the other hand, Gram-negative *E. coli* did not respond at sub-inhibitory concentration (0.5X MIC) and remained almost static for 24 h. But at dosage levels from 1.56 to 6.25 mg/ml, phenolics possessed strong inhibitory action against the tested *E. coli* strains for 6 h then regrowth of the tested strains occurred and reached near to control at 24 h (Fig. 2).

The regrowth of *E. coli* beyond 6 h is not clear right now. It may be due to genetic factors or due to cell membrane permeability as observed in case of some microorganisms that are generally survive the action of antimicrobial agents and usually revert to parental forms when the cell wall inhibiting agent is removed (Woolfrey et al. 1990).

Thus, phenolics of CM may be used as chemotherapeutic agents to control both Gram-positive and Gram-negative multi-drug resistant key bacterial pathogens. Further studies are necessary to elucidate how these *in vitro* and pharmacodynamic data translate into therapeutic regimens. This report may serve as a footstep in this aspect.

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