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Full Length Research Paper

Evaluation of antibacterial properties of Chebulic myrobalan (fruit of *Terminalia chebula* Retz.) extracts against methicillin resistant *Staphylococcus aureus* and trimethoprim-sulphamethoxazole resistant uropathogenic *Escherichia coli*

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The aim of this study was to evaluate the possible antibacterial potential of Chebulic myrobalan extracts (cold aqueous, hot aqueous and ethanol) against multi-drug resistant bacterial pathogens. For this purpose both clinical isolates (methicillin resistant *Staphylococcus aureus* and trimethoprim-sulphamethoxazole resistant uropathogenic *Escherichia coli*) and standard type control strains were tested in this study. Antibacterial potency of the extracts was tested by standard growth inhibitory assay methods. All the tested extracts showed to varying degrees of strain specific antibacterial potential against tested strains of which ethanol extract showed superior activity against *E. coli* and hot aqueous extract against *S. aureus*. Cold aqueous extract exhibited the least antibacterial activity against all the tested strains. These promising findings suggest to antibacterial activity of the plant material exhibited bioactive compounds against multi-drug resistant bacterial pathogens and serving them as an alternative antimicrobial agent against diseases caused by these organisms.

Key word: Chebulic myrobalan, MRSA, *Escherichia coli*, antibacterial activity.

INTRODUCTION

In recent years, multiple drug resistance has been developed due to indiscriminate use of existing antimicrobial drugs in the treatment of infectious diseases (Westh et al., 2004). This resistance problem needs a re-newed effort, resulting in researching effective antibacterial agents against pathogenic microorganisms resistant to current antibiotics (Soulsby, 2005). Besides, though conventional antibiotics are strong medicine and save lives, they cause more harmful effects than good ones when they are not used in right way (Neu, 1992). Therefore, to improvement of alternative antimicrobial agent in agents in order to treat microbial infections is also a requirement compared to other sources and one of the possible strategies towards this objective is the rational localization of the bioactive phytochemicals (Cordell, 2000). According to World Health Organization (WHO), about 80% of the world population rely chiefly on the plant based traditional medicine especially for their primary healthcare needs and there has been a worldwide move towards the use of traditional medicines due to concern over the more invasive, expensive and potentially toxic main stream practices (WHO, 2002). Traditional healing systems around the world that utilize from herbal remedies are an important resource for the discovery of new antimicrobials (Cowan, 1999). Plant medicines are used on a worldwide scale to prevent and treat infectious diseases. They are of great demand both in the

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developed as well as developing countries for the primary health care needs due to their wide biological and medicinal activities, higher safety margin and lesser costs (Soulsby, 2005). Plants are rich in a wide variety of secondary metabolites such as tannins, alkaloids, terpenoids and flavonoids having been found *in vitro* since they have antimicrobial properties and may serve as an alternative, effective, cheap and safe antimicrobial for the treatment of microbial infections (Cowan, 1999).

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). MRSA, a strain of *Staphylococcus aureus* that is resistant to a large group of antibiotics called the β-lactams, including aminoglycosides, the third and fourth generations of cephalosporin and monolactams (Livumore, 1995). In recent years, in the United States, there has been a notable increase in the isolation of uropathogenic *Escherichia coli* strains resistant to trimethoprim-sulphamethoxazole (SXT/TMP), representing as multi-drug resistant pathogen (Sahm et al., 2001; Karlowsky et al., 2002), and these strains are generally marker strains in antimicrobial activity studies.

Terminalia chebula is called the 'King of Medicine' in Tibet and is always listed at the top of the list in Ayurvedic Materia Medica due to its extraordinary power of healing. The dried ripe fruits of Chebulic myrobalan has traditionally been used in the treatment of asthma, sore throat, vomiting, hiccough, diarrhoea, bleeding piles, gout, heart and bladder diseases (Kirtikar and Basu, 1935). It has antioxidant, free radical scavenging and anticarcinogenic properties (Cheng et al., 2003; Saleem et al., 2002). Antibacterial activity of *T. chebula* fruit extracts against several bacterial strains has been reported by us and also by other workers (Chattopadhyay et al., 2008; Malckzadeh et al. 2001; Kim et al. 2006). It is effective in inhibiting the urease activity of *Helicobactor pyroli,* an ubiquitous bacterium implicated in the development of gastritis, ulcers and stomach cancers (Malckzadeh et al., 2001). Diffusate of *T. chebula* showed an inhibitory effect against strain X-100 of the bacterium *Xanthomonas campestris pv. citri* indicating its usefulness for the management of citrus canker disease (Afzalakhtar et al., 1997). It has also growth inhibitory action against *Salmonella typhi* (Rani et al., 2004) and intestinal bacteria (Kim et al., 2006). An aqueous extract of *T. chebula* fruits exhibits antifungal activity against a number of dermatophytes and yeasts (Dutta et al., 1998). It protects epithelial cells against influenza A virus, supporting its traditional use for aiding in recovery from acute respiratory infections (Badmaev and Nowakowski, 2000). But systematic scientific investigations on antibacterial potency of Chebulic myrobalan against MRSA and SXT/TMP resistant uropathogenic *E. coli* have not been studied so far. In the present investigation we studied the possible antibacterial potential of *T. chebula* fruit extracts against both Gram-positive and Gram-negative multi-drug resistant bacterial pathogens (MRSA and SXT/TMP resistant uropathogenic *E. coli*) to elucidate possible alternative antimicrobial agent from *T. chebula* fruits.

MATERIALS AND METHODS

Chemical analysis

Collection of plant materials: The fresh matured fruits of *T. chebula* were collected from local herbalist and were identified and authenticated by a pharmacognosy expert.

Extract preparation: The seedless ripe fruits of *T. chebula* were dried in shadow and then milled to fine powder. This powdered *T. chebula* fruits was extracted using different solvents.

Cold aqueous extract: 25 g of *T. chebula* fruit powder was soaked into 150 ml cold water in a conical flask stoppered with rubber cork and left undisturbed for 24 h, then filtered off using sterile filter paper (Whatman No. 1) into a clear conical flask. The filtrate was centrifuged at 3000 rpm for 15 min and evaporated to dryness in a water bath below 60°C (yield = 8.174 g).

Hot aqueous extract: Hot aqueous extract was prepared by boiling 25 g of *T. chebula* fruit powder in 150 ml of distilled water for 30 min and kept in a conical flask for 24 h undisturbedly. The other steps were the same as followed in case of cold aqueous extract (yield = 2.569 g).

Ethanol extract: To prepare ethanol extract, 25 g powder of *T. chebula* fruits was kept in 70% ethanol for consecutive 3 days at room temperature and filtered. The filtrate was centrifuged at 3000 rpm for 15 min and evaporated to dryness in a water bath (below 60°C) (yield = 18.3 g).

All the three extracts were stored at 4°C in air-tight jars until further use.

Formulation of extracts: Ethanol extract was reconstituted in 5% Dimethylsulfoxide (DMSO) and aqueous extract (cold and hot) in sterile distilled water to a final concentration of 100 mg/ml.

Microbial analysis

Microorganisms

For microbial analysis two sensitive (type strains) and two multi-drug resistant (clinical isolates) bacterial stains were used. Type strains (i) *S. aureus* (ATCC 6538P) and (ii) *E. coli* (ATCC 8739) were procured from National Chemical Laboratory, Pune, India. Pure and clinical isolates (i) MRSA and (ii) SXT/TMP resistant uropathogenic *E. coli* were procured from Department of Bacteriology and Serology, Calcutta School of Tropical Medicine, Kolkata. All the tested strains were maintained in nutrient agar slants at 4°C.

Susceptibility tests

Inoculum preparation

Susceptibility tests were performed by a modified agar well diffusion method (Okunji et al., 1990; Okeke et al., 2001). The inoculum size of the test strains were standardized according to the National Committee for Clinical Laboratory Standards guidelines (NCCLS, 1993). The bacterial strains were inoculated in Mueller Hinton Broth (Hi-media, Mumbai, India) and incubated at 37°C 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 5×10^5 cfu/ml.

Table 1. Antibacterial potential of extracts of Chebulic myrobalan (fruits of *T. chebula* Retz.) against *S. aureus* (ATCC), *S. aureus* (LCI), *E. coli* (ATCC) and *E. coli* (LCI) in modified agar well diffusion method

Extracts	Microorganisms			
	***S. aureus* (ATCC)**	***S. aureus* (LCI)**	***E. coli* (ATCC)**	***E. coli* (LCI)**
	Inhibitory Zone Diameter (IZD) (mm)			
Cold Aqueous	14[a]	12[a]	14[a]	13[a]
Hot Aqueous	20[a]	19[a]	17[a]	15[a]
Ethanol	16[a]	15[a]	20[a]	21[a]
Gentamicin	24[a]	5[b]	22[a]	4[b]
DMSO	-	-	-	-

Values are average of triplicate experiments.
(-): absence of inhibition.
[a]Sensitive, [b]Resistant

Determination of inhibitory zone diameter (IZD)

One ml of standard suspension of each bacterial strain was spread evenly on Mueller-Hinton Agar (Hi-media, 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 in the agar and 100 µl volumes of 100 mg/ml of each reconstituted extract was pipetted into wells. After holding the plates at room temperature for 2 h to allow diffusion of extract into the agar, they were incubated at 37°C for 24 h. Inhibition Zone Diameter (IZD) was measured to the nearest millimeter (mm). Gentamicin (Nicholson) (10 µg/ml) was used as experimental positive control and 5% DMSO as negative control. The tests were performed in triplicate for each microorganism used and the final results were expressed as the arithmetic average of triplicate experiments.

Determination of minimal inhibitory concentration (MIC)

The minimal inhibitory concentrations (MICs) of the extracts of Chebulic myrobalan against all the test strains were determined by macro broth dilution assay method (NCCLS, 1993). Two-fold serial dilutions of all the extracts (0.1 to 100 mg/ml) were prepared in tubes with Mueller Hinton Broth (Hi-media, Mumbai, India) as diluent. Each dilution was seeded with 20 µl of test micro-organisms to the standard concentration (5 x 10^5 cfu/ml). Two-fold serial dilution of gentamicin (0.125 – 512 µg/ml) was used as experimental positive control. The tubes were incubated at 37°C for 24 h. The least concentration of the extract or standard drug showing no visible growth was taken as the MIC.

Determination of minimal bactericidal concentration (MBC)

Minimal bactericidal concentration (MBC) determination was determined by aspirating 0.01 ml of the culture medium from each tube (in the macro broth MIC assay) showing no apparent growth and sub-culturing it on fresh MHA. The later was incubated at 37°C for 24 h. The MBC was read as the least concentration of the extract showing no visible growth on MHA subculture. Using the values of MIC and MBC, the MIC_{index} (MBC/MIC) of each extract was computed against each test strain.

RESULTS

Table 1 shows the results of antibacterial potential of different extracts of Chebulic myrobalan. All the tested extracts showed varying degrees of strain specific inhibitory action (for *S. aureus* strains IZD ranged from 12 – 20 mm and for *E. coli* strains it was 13 – 21 mm). Hot aqueous extract was found to be more potent against the *S. aureus* strains (IZD ranged from 19 – 20 mm) whereas ethanol extract was found to be more potent against *E. coli* strains (IZD ranged from 20 – 21 mm). Gentamicin (positive control) showed strong antibacterial activity against type strains (IZD ranged from 22 – 24 mm) but very weak activity was observed against clinical isolates (IZD ranged from 4 – 5 mm) showing its resistance towards the clinical isolates tested. DMSO (negative control) showed no inhibitory action against any of the test strains.

Table 2 shows the results of MIC, MBC and MIC_{index} values of extracts of Chebulic myrobalan against the test strains. The MIC values for cold aqueous, hot aqueous and ethanol extracts against the test strains were ranged from 6.25 - 25.00 mg/ml; 3.12 - 12.50 mg/ml and 3.12 - 12.50 mg/ml respectively. MBC values for cold and hot aqueous extracts were ranged from 12.50 – 50.00 mg/ml and 3.12 – 25.00 mg/ml respectively. Whereas this value for ethanol extract was ranged from 3.12 - 25.00 mg/ml. MIC and MBC values of gentamicin against the test strains were from 2 - 4 µg/ml. MIC_{index} values against the test strains were ranged from 1 - 2.

DISCUSSION

Infectious diseases are the world's leading cause of premature deaths. In recent years, drug resistance to human pathogenic bacteria has commonly been reported from all over the world (Piddock and Wise, 1989). Even though pharmaceutical companies produced a number of new antibacterial drugs, resistance to these drugs by bacteria increased and became a global concern. MRSA and multi-drug resistant as well as SXT/TMP resistant uropathogenic *E. coli* have been recognized as the major causes of infections in humans. Therefore, the importance of identifying new effective antimicrobial agents

Table 2. Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and MIC_{index} of Chebulic myrobalan extracts against test bacterial strains.

Extracts	Microorganisms	MIC (mg/ml)	MBC (mg/ml)	MIC_{index}
Cold Aqueous	*S. aureus* (ATCC)	6.25	12.50	2
	S. aureus (LCI)	12.50	25.00	2
	E. coli (ATCC)	12.50	25.50	2
	E. coli (LCI)	25.00	50.00	2
Hot Aqueous	*S. aureus* (ATCC)	3.12	3.12	1
	S. aureus (LCI)	3.12	3.12	1
	E. coli (ATCC)	6.25	12.50	2
	E. coli (LCI)	12.50	25.00	2
Ethanol	*S. aureus* (ATCC)	6.25	12.50	2
	S. aureus (LCI)	12.50	25.00	2
	E. coli (ATCC)	3.12	3.12	1
	E. coli (LCI)	3.12	3.12	1
Gentamicin	*S. aureus* (ATCC)	2.00*	2.00*	1
	S. aureus (LCI)	R	R	NA
	E. coli (ATCC)	4.00*	4.00*	1
	E. coli (LCI)	R	R	NA

*µg/ml; R: Resistant; NA-: Not Applicable.

cannot be overemphasized. The use of plant extracts or phytochemicals with known antimicrobial properties can be of great significance of therapeutic treatments.

Results of our foregoing findings reveal that all the tested extracts of Chebulic myrobalan exhibited growth inhibitory activity against the bacterial strains evaluated. The susceptibility of both the reference standard control strains and clinical isolates towards the extracts were more or less the same (Table 1 and 2). It was also observed that hot aqueous extract was more potent against MRSA compared to other tested extracts (Table 2). But in case of SXT/TMP resistant uropathogenic *E. coli*, ethanol extract had superior activity, which was over others (Table 2). The reason for these findings is not clear right now. It may associate with the greater solubility of bioactive compounds responsible for growth inhibition of specific bacterial test strains in respective solvents. The result further revealed that antibacterial potency of the bioactive compounds was not affected when extracted in boiling water indicating that the plant material contains thermo stable bioactive compounds. It is also important to note that susceptibility of the pathogens was varied to solvent extract and aqueous extract. This indicates the involvement of more than one active principles of biological significance (Ming et al., 2005). The present finding is hence highly encouraging in recognizing a plant of interesting antibacterial activity.

Sato et al. (1997) have reported that 50% ethanol extract of fruiting bodies of *T. chebula* Retz. exhibits antibacterial activity against MRSA and the compounds responsible for this activity are gallic acid and its ethyl ester. Kim et al. (2006) have studied the effect of *T. chebula* fruits on six intestinal bacteria and ethanedioic acid isolated from fruit of *T. chebula* had moderate inhibitory activity whereas elagic acid exerted a potent inhibitory activity against intestinal *E. coli*. But in our study it was observed that hot aqueous extract resulted in higher antibacterial potential against MRSA than ethanol extract, indicating greater solubility of the bioactive compounds in boiling water rather than ethanol. In our previous work we have also observed that Chebulic myrobalan fruit powder contains high concentration of total phenolics, moderate concentration of flavonoids and minimum concentration of carotenoids. The high phenolics content in Chebulic myrobalan powder can be associated with this activity. But flavonoids and carotenoids may also have an important role (Chattopadhyay et al., 2008).

Thus Chebulic myrobalan has strong antibacterial activity against both the MRSA and SXT/TMP resistant uropathogenic *E. coli* and the compounds responsible for this activity was thermo stable, suggesting the importance of ethno medical approach as a potential source of bioactive compounds. Chebulic myrobalan can be helpful against diseases caused by these microorganisms and further studies both on the extract and/or its chemical constituents are needed to pinpoint of the findings. This report may serve as a footstep on this aspect.

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