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ORIGINAL RESEARCH

Allyl isothiocyanate from crucifers potentiates β-lactam activity against methicillin-resistant *Staphylococcus aureus*

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Abstract

Introduction: Increasing prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) worldwide with limited therapeutic options is a growing public health concern. Isothiocyanates (ITCs) from crucifers have been shown to possess antibacterial actions against MRSA by antagonizing its resistance mechanisms. Allyl isothiocyanates (AITC) is the predominant isothiocyanates (ITC) of commonly consumed cruciferous vegetables such as brussels, mustard, cabbage, cauliflower and kale.

Objectives: The aim of this study is to evaluate the potentiation effect of AITC on ampicillin and cefixime against clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) using broth microdilution method and checkerboard methods.

Results: AITC showed a promising antibacterial activity against a panel of clinical isolates of MRSA when used in alone. However, β-lactam antibiotics like ampicillin and cefixime had little or no activity against MRSA confirming their multi-drug resistance. When AITC combined with ampicillin and cefixime, MIC was reduced by ≥ 4 fold as compared to their monotherapy, evidencing a synergistic effect of AITC, as defined by a FICI of ≤0.5.

Conclusions: AITC showed promising synergistic and potentiation effect on ampicillin and cefixime at sub-MIC level against multi-drug resistant MRSA. This modulatory effect of AITC on β-lactam antibiotics could be useful as a synergistic therapeutic pair in combating MRSA infection in a hospital or community settings.

Keywords: Allyl isothiocyanate; Crucifers potentiates; β-lactam activity; Methicillin-resistant *Staphylococcus aureus*

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Introduction

Staphylococcus aureus can display an exceptional adaptive evolution in the antibiotic era, as it has demonstrated a unique ability to quickly respond to each new antibiotic with the development of a resistance mechanism [1]. Methicillin-resistant *Staphylococcus aureus* (MRSA) is a strain of *Staphylococcus aureus* that has developed resistance to most of the β-lactam antibiotics through the process of natural selection. MRSA infections are common among people who have weak immune systems and are in hospitals, nursing homes, and other health care centers. In medical facilities, MRSA

causes life-threatening bloodstream infections, pneumonia and surgical site infections [2].

MRSA develop resistance through several mechanisms such as enzymatic inactivation of antibiotics, decreased affinity of antibiotics targets, trapping of antibiotics and altered genetic makeup acquired through horizontal gene transfer, spontaneous mutations and positive selections [3, 4]. Finding antibiotic co-adjuvant capable of inhibiting bacterial resistance mechanisms and potentiating the existing antibiotics would be a valuable solution to combat community and healthcare-associated infection.

Plant secondary metabolites form the first-line defence mechanism for plants against their pathogenic invaders [5]. They have effective and broad-spectrum antibacterial properties against both Gram positive and Gram negative bacteria [6, 7]. Phytochemicals are known to modulate resistance mechanisms in bacteria [8] and that property can be utilized to potentiate the activity of β-lactam antibiotics for increased efficacy against resistant strains. Allyl isothiocyanates (AITC) is the predominant isothiocyanate (ITC) of commonly consumed cruciferous vegetables such as brussels, mustard, cabbage, cauliflower and kale [9]. AITC has been shown to possess wide-spectrum antimicrobial activity and inhibits bacteria at all growth stages [10]. In this study, the antimicrobial activities of AITC against methicillin-resistant *Staphylococcus aureus* isolated in a clinic were assessed using broth microdilution method and checkerboard methods for synergistic effect of its combination with ampicillin and cefixime.

Materials and methods

Materials

All antibiotics, AITC and other fine biochemicals were obtained from Sigma-Aldrich, US. Stock antibiotic solutions were prepared and dilutions were made according to the clinical and laboratory standards institute (CLSI) protocols [10] or manufacturer's recommendations. AITC was dissolved in ethanol (99% molecular grade, Sigma Aldrich). Ampicillin and cefixime were dissolved in sterile distilled water. The stock solution concentration for all antibiotics and AITC was 10 mg/ml and stored at -20°C for subsequent use for up to 6 weeks.

Preparation of bacterial strains

Six isolates of methicillin-resistant *Staphylococcus aureus* isolated from Mahavir Hospitals, Hyderabad and standard strains of methicillin-sensitive *S. aureus* (MSSA – ATCC 29213) and methicillin-resistant *S. aureus* (MRSA – ATCC 43300) were used in this study. Antibiotic susceptibility was determined in testing the inhibition zones (inoculums 0.5 McFarland suspension, 1.5 x 108 CFU/ml) and MIC/MBC (inoculums 5 x 105 CFU/ml) was measured by following the methods described in the National Committee for Clinical Laboratory Standards (NCCLS, 2000) [11]. Resistance to methicillin is determined by mecA gene, which encodes the low-affinity penicillin-binding protein PBP2A [12]. The mecA gene does not allow the ring-like structure of β-lactam antibiotics to attack the enzymes that help form the cell wall of the bacterium (transpeptidases), and hence the bacteria is allowed to replicate as normal. All the isolates were tested positive for mecA gene which was detected using polymerase chain reaction (PCR) method as described [13].

Minimum inhibitory concentration/minimum bactericidal concentration (MIC/MBC) assay

The antibacterial activities of AITC, ampillicin and cefixime against clinical isolates MRSA and reference strains were determined via broth dilution method [14, 15]. AITC and β-lactam antibiotics were tested in the range of 4.0 – 4000 µg/mL for measuring their MIC and MBC alone and in combination. The titer plates were inoculated with bacteria having 0.5 Macfarland turbidity and incubated aerobically at 37°C for 24 h. The minimum inhibitory concentration (MIC) was recorded as the lowest concentration of test samples resulting in the complete inhibition of visible growth. For clinical strains, MIC50s and MIC90s, defined as MICs at which, 50% and 90%, respectively of the isolates were inhibited, were determined. The minimum bactericidal concentration (MBC) was determined based on the lowest concentration of the extracts required to kill 99.9% of bacteria from the initial inoculum as determined by plating on agar.

Checkerboard dilution test

The synergistic combinations were investigated in the preliminary checkerboard method performed using the MRSA, MSSA, and clinical isolate strains via MIC determination [16]. The starting concentration

of the AITC and antibiotics for the checkerboard assay was 16x MIC, which was determined earlier. The fractional inhibitory concentration index (FICI) is the sum of the FICs of each of the drugs, which were defined as the MIC of each drug when used in combination divided by the MIC of each drug when used alone. The FIC index was calculated as follows:

$$FIC = \frac{\text{MIC of AITC in combination}}{\text{MIC of AITC alone}} + \frac{\text{MIC of Antibiotics in combination}}{\text{MIC of Antibiotics alone}}$$

FIC indices (FICI) were interpreted as follows:
≤0.5: Synergy; >0.5 – ≤1.0: Additive; >1.0 – ≤2.0: Indifference; >2.0: Antagonism

All experiments were performed at least three times with biological triplicates and technical replicates after which the means were determined.

Results

AITC exhibited significant inhibitory activities against clinical isolates MRSA and reference strains, MRSA ATCC 43300 and MSSA ATCC 29213. As depicted in Table 1, AITC displayed varying degrees of activity against clinical isolated MRSA 1 – 6 with MIC in the range of 16 – 250 µg/mL and MBC in the range of 125 – ≥ 500 µg/mL. The MICs/MBCs for ampicillin and cefixime were determined to be in the range of 500 – >4000 µg/mL against MRSA 1 – 6 isolates. For the isolates No 3 and 5, β-lactam antibiotics were totally ineffective as they were not showing any inhibition even at their highest range of 4000 µg/mL. The range of MIC50 and MIC90 of AITC were in the range of 8– 32µg/mL and 16 - 125µg/mL respectively.

The combination of ampicillin and cefixime with AITC resulted in the significant reduction of MIC/MBC for all clinical isolates and reference strains as presented as Table 2a and 2b. MIC of ampicillin and cefixime were decreased by ≥ 4 fold as compared to their monotherapy, evidencing a synergistic effect of AITC, as defined by a FICI of ≤0.5.

Table 1: Antibacterial activity of AITC and β-lactam antibiotics on clinical isolates and reference strain of MRSA and MSSA.

Sample	*AITC (µg/mL)*			*Ampillicin*	*Cefixime*
	MIC_{50}	*MIC_{90}*	*MIC/MBC*	*MIC/MBC (µg/mL)*	
MSSA (ATCC 29213)	4	16	16/32	0.032/1.25	0.016/0.64
MRSA (ATCC 43300	16	64	64/250	1000/4000	500/2000
MRSA 1	16	64	64/125	1000/>4000	1000/4000
MRSA 2	32	125	125/250	2000/>4000	2000/4000
MRSA 3	32	125	125/500	>4000	>4000
MRSA 4	32	125	125/500	500/2000	125/500
MRSA 5	64	250	250/1000	>4000	>4000
MRSA 6	32	125	125/500	1000/>4000	1000/>4000

Discussion

Combining antibiotic therapy with dietary phytochemicals is an attractive approach to enhance the efficacy of antibiotics in preventing the spread of multi-drug resistant strains and in minimizing community and healthcare-based infections. Combination of conventional antibiotics with plant phytochemicals is the latest alternative for the treatment of resistant bacteria like MRSA. Khan et al. [17] have demonstrated the potentiating effect of piperine on ciprofloxacin activity against *S. aureus* strains. Similarly, Soe et al. [7] found that ethyl gallate augment the antibacterial activity of tetracycline when used in combinations and eventually could overcome the resistance in MRSA. These studies illustrate the potentiating effect of phytochemicals on the mechanism of antibiotics.

In this study, AITC showed synergy with ampicillin and cefixime and able to reduce the MIC/MBC of

Table 2a: Synergistic effects of AITC with ampicillin on clinical isolates of MRSA.

Sample	*Agent*	*MIC (µg/mL)*		*FIC*	*FICI*	*Outcome*
		Alone	*Combination*			
MRSA 1	AITC	64	8	0.125	0.375	Synergistic
	Ampicillin	1000	250	0.250		
MRSA 2	AITC	125	8	0.064	0.125	Synergistic
	Ampicillin	2000	125	0.064		
MRSA 3	AITC	125	16	0.125	0.189	Synergistic
	Ampicillin	>4000	250	0.064		
MRSA 4	AITC	125	8	0.064	0.189	Synergistic
	Ampicillin	500	64	0.125		
MRSA 5	AITC	250	125	0.50	0.625	Additive
	Ampicillin	>4000	500	0.125		
MRSA 6	AITC	125	16	0.125	0.189	Synergistic
	Ampicillin	1000	64	0.064		

Table 2b: Synergistic effects of AITC with cefixime on clinical isolates of MRSA.

Sample	*Agent*	*MIC (µg/mL)*		*FIC*	*FICI*	*Outcome*
		Alone	*Combination*			
MRSA 1	AITC	64	8	0.125	0.375	Synergistic
	Cefixime	1000	250	0.250		
MRSA 2	AITC	125	8	0.064	0.125	Synergistic
	Cefixime	2000	125	0.064		
MRSA 3	AITC	125	16	0.125	0.189	Synergistic
	Cefixime	>4000	250	0.064		
MRSA 4	AITC	125	8	0.064	0.096	Synergistic
	Cefixime	125	4	0.032		
MRSA 5	AITC	250	125	0.50	0.625	Additive
	Cefixime	>4000	500	0.125		
MRSA 6	AITC	125	16	0.125	0.189	Synergistic
	Cefixime	1000	64	0.064		

antibiotics up to 4 – 8 folds. Significant difference in MICs between combinational and monotherapy clearly suggest the potentiating effect of AITC on antibiotics which otherwise had high growth and out of range MIC/MBC (>4000 µg/mL) when used alone.

AITC has been shown to exhibit broad-spectrum antimicrobial activity and possess multi-targeted mechanism in reducing the probability of developing resistance by microbes [10]. AITC showed positive interaction (mostly synergistic and/or additive with none showing antagonism) with ampicillin and cefixime, suggesting its potential application as resistance modulator in combating drug resistance MRSA in combination with β-lactam antibiotics. AITC is known to modulate membrane potential and increase the permeability of bacterial cell wall owing to its lipophilic property [10], which perturbation

coupled with the transpeptidase action of β-lactam would have led to enhanced antimicrobial effect.

Combinational therapy with synergistic antibacterial drugs has been identified as a rational approach to tackle concerns of MRSA resistance, owing to reduction in drug dosage and decline of resistance with negligible side effects. Combining phytochemicals and antibiotics at sub-MIC levels would be more suitable and realistic in a clinical setting which would eventually bring down the side effects caused by each of these antibacterial drugs. For clinical applications, the total toxicity level of phytochemicals must be taken into account, including the pharmacokinetics and pharmacodynamics models of drugs. The acute toxicity level of AITC was found to be LD50 > 112mg/kg (obtained from the manufacturer – Sigma-Aldrich USA), which is much higher than the MIC/MBC obtained for AITC alone as well as in combination.

Conclusions

AITC showed promising synergistic and potentiation effect on ampicillin and cefixime at sub-MIC level against multi-drug resistant MRSA. This modulatory effect of AITC on β-lactam antibiotics could be useful as a synergistic therapeutic pair to combat MRSA infection. Since AITC is a phytochemical found in many common indigenous vegetables, it could be taken up as a pilot project to study its efficacy along with β-lactam antibiotics in a hospital or community settings.

Conflict of interest

The authors declare no conflict of interest.

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