INTRODUCTION

During the past decade, *Acinetobacter* species have emerged as an opportunistic pathogen. They are rapidly evolving toward multidrug resistance and are often involved in various nosocomial infections that can be severe in intensive care units, burn units, and surgical wards [1]. Multidrug-resistant strains of *Acinetobacter* generally cause treatment failure as therapeutic options are limited for infections caused by them [2].

Skin or tissue infections, skin structure, and deep-seated soft tissue infections are general terms used for infections of the entire skin layer, including the subcutaneous and muscle tissue layers and their respective fascia structures. The beta-lactam antibiotics are the most commonly used drug for the treatment of various skin and soft tissue infections (SSTIs). The resistance against beta-lactam is swiftly increasing in *Acinetobacter* strains [3]. A variety of molecular mechanisms for resistance to broad-spectrum beta-lactams have been reported in *Acinetobacter*, such as mutations of penicillin-binding proteins and alterations of membrane permeability, but the most common mechanism is attributed to the presence of beta-lactamases encoded by either chromosomes or plasmids [4].

Extended-spectrum beta-lactamas (ESBLs) are typically plasmid-mediated clavulanate-susceptible enzymes that hydrolyze penicillins, expanded-spectrum cephalosporins, monobactams, and are commonly inhibited by beta-lactamase inhibitors such as clavulanic acid, sulbactam, and tazobactam [5]. Ambler Class C (AmbC) class beta-lactamases are cephalosporinases that are poorly inhibited by clavulanic acid. They can be differentiated from ESBLs by their ability to hydrolyze cephamycins as well as other extended-spectrum cephalosporins [6]. Metallo-beta-lactamas (MBLs) are a type of carbapenemase, which are characterized by the ability to hydrolyze carbapenems and are inhibited by ethylenediaminetetraacetic acid (EDTA), chelators of Zn++. They lack inhibition by the commercially available beta-lactam inhibitors; clavulanic acid, sulbactam, and tazobactam [7]. Often coexistence of multiple beta-lactamase enzymes in a single isolate is observed, which further complicates treatment options [8].

There is a lack of development of new synthetic antimicrobials against Gram-negative organisms, hence attention is increasingly focused on natural compounds either as stand-alone or adjunctive therapy [9]. Plants produce a wide variety of secondary metabolites, which are used either directly as precursors or as compounds in the pharmaceutical industry [10].

Garlic (*Allium sativum*) has been found to exhibit a wide spectrum of antibacterial activity against Gram-negative and Gram-positive bacteria, including species of *Escherichia*, *Salmonella*, *Staphylococcus*, *Streptococcus*, *Klebsiella*, *Proteus*, *Bacillus*, and *Clostridium* [11]. Cavallito and Bailey were the first to demonstrate that the antibacterial action of garlic is mainly due to allin [12]. Allin is generated by the enzyme alliinase when garlic is crushed. Garlic contains at least 33 sulfur compounds such as allin, allinol, ajoene, allyl propyl, diallyl trisulfide, s-allyl cysteine, S-allylmethycysteine, and others, which are responsible for its antibacterial activity [13].

Allin interacts with important thiol-containing enzymes such as cysteine protease and alcohol dehydrogenase, as well as the thiorodoxin reductases, which are critical for maintaining the correct redox state within microorganisms. It is unlikely that bacteria would develop resistance to allin because they would require modifying these vital enzymes, which are necessary for the survival of the microbes. Hence, garlic may prove to be a potent antimicrobial agent [14].
Very few studies have been documented on the antibacterial activity of aqueous garlic extract (AGE) against beta-lactamase producers. No study has reported the effect of AGE against beta-lactamase-producing Acinetobacter strains from SSTIs. Thus, this study aims to investigate the antibacterial activity of AGE on beta-lactamase-producing Acinetobacter strains isolated from SSTIs and also to study its combined activity along with ceftaxime.

**METHODS**

The present study was conducted in our tertiary care hospital and was approved by the Local Ethics Committee of the institution.

**Bacterial strains**

A total of 41 non-duplicate Acinetobacter strains isolated from SSTIs of patients from our tertiary care hospital were selected for the study. The samples were processed and were identified by standard laboratory methods [15].

**Antimicrobial susceptibility test (AST)**

The antimicrobial susceptibility was determined by Kirby-Bauer disk diffusion method in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines using commercially available antimicrobial discs (HiMedia, Mumbai, India) [16]. The following antibiotics were used - ampicillin (10 μg), amoxicillin (10 μg), ciprofloxacin (5 μg), gentamicin (10 μg), amoxiclav (30 μg), cefazidime (30 μg), cefoxitin (30 μg), imipenem (10 μg), meropenem (10 μg), piperacillin (10 μg), and piperacillin-tazobactum (100/10).

All the isolates which were resistant to ceftazidime, as per the CLSI-susceptible breakpoints, were further screened for beta-lactamase production by the confirmatory tests.

**Phenotypic confirmatory disc diffusion test (PCDDT)**

The phenotypic disc diffusion test was performed as per the CLSI guidelines. The current CLSI guideline does not describe any method for the detection of isolates producing AmpC beta-lactamases. The antibiotic discs were purchased from HiMedia Laboratories, Mumbai, India.

Ceftazidime (30 μg) - ceftazidime/clavulanic acid (30/10) was used for ESBL detection. If there was ≥5 mm increase in the inhibition zone diameter of ceftazidime/clavulanic acid versus ceftazidime alone, the isolate was considered an ESBL producer [16]. Imipenem/EDTA (10/750 μg) disc was used for the detection of MBL producers. An increase of ≥7 mm in the inhibition zone diameter of imipenem/EDTA versus imipenem alone indicated MBL production [16]. Cefoxitin (30 μg) - cefoxitin/cloxacillin (30/200) discs was used for AmpC detection. An increase of ≥4 mm in the inhibition zone diameter of cefoxitin/cloxacillin acid versus cefoxitin alone indicated AmpC production [17].

**E-test**

Detection by phenotypic testing may be misleading, especially when there is a coexistence of multiple beta-lactamase enzymes. They mask each other, which results in misreporting and failure in the clinical treatment of patients. For this reason, E-strips which differ from each other, which results in misreporting and failure in the clinical setting. The isolates were reported and confirmed as ESBL and AmpC beta-lactamase producer as per the application sheet supplied by the manufacturer. These strips are to be used along with pure ESBL detection strips (EM079-HiMedia, Mumbai, India) to avoid false-positive results [16-18].

The phenotypic MBL detection strip (EM078- HiMedia, Mumbai, India) is coated with a mixture of imipenem +EDTA and imipenem on a single strip in a concentration gradient manner. The upper half has imipenem +EDTA with highest concentration tapering downward, whereas the lower half is similarly coated with imipenem in a concentration gradient in the reverse direction. The isolates were reported and confirmed as MBL producer when the ratio of the value obtained for imipenem and the value of imipenem+EDTA was > 16 [16,19].

A standard reference strain of Escherichia coli ATCC 25922, susceptible to all antimicrobial drugs tested, and positive control strain Klebsiella pneumoniae ATCC 700603 were used as a quality control for AST, PCDDT and the E-test, as per the CLSI guidelines. These phenotypically confirmed ESBL, AmpC, and MBL producers were further utilized in the study as test strains.

**Preparation of AGE**

The garlic cloves were peeled, cut into pieces, and 20 g was put in a juice extractor to squeeze out the raw garlic extract. This extract was regarded as 100% extract and used for further studies. To arrive at various concentrations, the extract was diluted with sterilized distilled water. To estimate the dry weight of the extract, 10 ml of the extract was placed in a pre-weighted glass dish and re-weighed, followed by drying in an oven at 60°C. The sample was found to contain 5 g (equivalent dry weight) of garlic extract in 10 ml which was equivalent to 500 mg/ml and was used as a stock AGE [20]. This extract was then stored at 4°C for further use.

**High-performance liquid chromatography analysis (HPLC)**

The HPLC system consisted of a stainless steel column (0.25 m long and 4 mm in internal diameter, packed with silanised octadeckylsilica gel) combined with a stainless steel pre-column (20 mm long and 4 mm in internal diameter packed with silanised octadeckylsilica gel). The elution was carried out at a flow rate of 0.80 ml/min using mobile phase made up of a mixture of 40 volumes of a 1% v/v solution of anhydrous formic acid and 60 volumes of methanol. About 20.0 mg of butyl parahydroxybenzoate was dissolved in 100.0 ml of a mixture of equal volumes of methanol and water and was used as an internal standard. Detection was performed at a wavelength of 254 nm. About 0.800 g of garlic powder was homogenized in 20.0 ml of water, and 10 μl was injected into the HPLC (Natural Remedies, Bangalore, India). The percentage of allicin in the sample was calculated using the following formula:

$$\frac{S1 \times m2 + 22.75}{S2 \times mL} = \frac{S1}{S2}$$

S1=Area of the peak corresponding to allicin,
S2=Area of the peak corresponding to butyl parahydroxybenzoate in the chromatogram obtained with the test solution,
m1=Mass of the drug in grams,
m2=Mass of butyl parahydroxybenzoate in grams in 100.0 ml of the internal standard solution.

1 mg of butyl parahydroxybenzoate corresponds to 8.65 mg of allicin.

**Antibacterial activity of AGE**

The evaluation of antibacterial activity of AGE was conducted by the disc diffusion method using Mueller-Hinton agar as described by the CLSI [16]. The turbidity of the culture was adjusted to 0.5 McFarland standards. Sterile paper discs (6 mm, HiMedia, Mumbai, Maharashtra, India) were impregnated with 20 μl of the 500 mg/ml AGE and placed on the inoculated agar for the positive control, a disc of imipenem (10 μg), and for negative control, a disc impregnated with distilled water was placed on the inoculated Mueller-Hinton agar. The plate was incubated at 37°C for 24 hr. The experiment was performed in triplicate.
Determination of minimum inhibitory concentration (MIC)
The MIC of AGE and cefotaxime was determined by agar dilution method [21]. For MIC of AGE, dilutions were prepared by mixing AGE with sterile Mueller-Hinton agar to get final concentrations ranging between 0.25-4% (1.25-20 mg/ml). For MIC of cefotaxime, dilutions were prepared by mixing cefotaxime with sterile Mueller-Hinton Agar to get the final concentrations ranging from 25 to 500 μg/ml. A plate of Mueller-Hinton agar without AGE served as a control. These plates were seeded with bacterial suspensions and were incubated at 37°C for 24 h. The MIC was recorded as the lowest concentration of AGE and cefotaxime, at which visible bacterial growth was completely inhibited.

Determination of synergistic activity by checkerboard assay using Agar dilution method
Checkerboard assay was used to establish the presence of synergistic interaction between cefotaxime (stock - 10 mg/ml) and AGE (10 %). The agar plates were prepared by mixing AGE in the range of 0.1-2% (0.5-10 mg/ml) along with 10-200 μg/ml of cefotaxime. Visible growth after 24 hr of incubation at 37°C was checked, and the MIC in combination with cefotaxime and AGE was determined for all Acinetobacter strains. The results obtained were used to calculate fractional inhibitory concentration (FIC) indices. The FIC index (∑FIC) was calculated as follows:

\[ \sum \text{FIC} = \text{FIC}_A + \text{FIC}_B \]

\[ \text{FIC}_A = \frac{\text{MIC of cefotaxime in combination}}{\text{MIC of cefotaxime alone}} \]

\[ \text{FIC}_B = \frac{\text{MIC of AGE in combination}}{\text{MIC of AGE alone}} \]

A minimum FIC index of ≤0.5 indicates synergy, while a FIC index >2 indicates antagonism. If the minimum FIC index was >0.5 and ≤1, the effect of the combination was classified as additive. If the minimum FIC index was >1 and ≤2, the effect of the combination was classified as indifference [21].

RESULTS
The Acinetobacter strains isolated from SSTIs showed the highest rate of resistance against ciprofloxacin and ampicillin, 80% each (33/41). The lowest rate of resistance was exhibited against amikacin (17%). Among all the isolates, 63.4% (26/41) strains were resistant to ceftazidime whereas 21.95% (9/41) strains were resistant to carabapemems.

Resistance to a third-generation cephalosporin, ceftazidime, was indicative of beta-lactamase production, which was further confirmed by PCDDT and E-test. Nearly, 51.2% of the (21/41) strains were confirmed to be beta-lactamase producers, and these were used as test organisms further in the study. Among all the test strains, 73.1% (3/41) strains were detected to be pure AmpC producers. There were no pure ESBL producers, but 29.3% (12/41) of the strains were co-producers of ESBL and AmpC, i.e., they were multiple beta-lactamase producers. Out of the 9 strains resistant to imipenem, 6 strains were MBL producers. Thus, 15% of the isolates were MBL producers (6/41). Out of the 12 co-producers of ESBL and AmpC, 3 were reported to be sensitive to piperacillin-tazobactam combination, in vitro. All beta-lactamase producers were also found to be multi-drug-resistant, i.e., they were resistant to 5 or more than 3 groups of antibiotics.

The estimation of active ingredient allicin was carried out by HPLC analysis and was found to be 0.20, expressed as percentage w/w. Antibacterial activity of AGE was carried out by the disc diffusion method, and the average zone of inhibition ranged from 18 to 31 mm with a mean of 25.19±5.06 mm against the test strains (Fig. 1).

The MIC of AGE and cefotaxime was determined by the agar dilution method. The MIC of AGE was found to be in the range of 0.5-2% (2.5-10 mg/ml) with a mean of 0.86% (4.28±2.25 mg/ml) as shown in Table 1. The MIC of cefotaxime was found to be in the range of 100-400 μg/ml with an average of 252.38 μg/ml (Table 2).

Table 1 depicts the number and percentage of Acinetobacter strains inhibited by various minimal concentrations of AGE by agar dilution method (n=21).
Table 2 depicts the number and percentage of Acinetobacter strains inhibited by various concentrations of cefotaxime by agar dilution method (n=21).
Checkerboard assay was used to determine the combined activity of various concentrations of cefotaxime and AGE. FIC index was used to determine the type of association between cefotaxime and AGE. Based on the FIC index, 11 test strains showed synergism, 5 strains exhibited additive and indifference effect each. None of the strains showed an antagonistic effect between AGE and cefotaxime (Table 3).
Table 3 depicts the number of the Acinetobacter strains inhibited by combining the concentrations of AGE and cefotaxime (n=21). Synergistic interaction between AGE and cefotaxime was seen in 52.38% of the isolates, and none of the isolates exhibited antagonism.

DISCUSSION
Multidrug-resistant Acinetobacter strains have been identified to be among the most difficult antimicrobial-resistant Gram-negative bacilli to control and treat. The study of the pattern of bacterial resistance is important for epidemiological and clinical purposes. In the current study, amikacin was the most effective antibiotic for Acinetobacter isolates, followed by imipenem. The resistant rate in Acinetobacter strains toward ceftazidime and imipenem was in accordance with the study carried out by Parandekar and Peerapur at a hospital in Pune [22].

**Table 1: MIC of AGE against Acinetobacter strains producing beta-lactamase**

<table>
<thead>
<tr>
<th>Concentration of AGE (mg/ml)</th>
<th>1.25</th>
<th>2.5</th>
<th>5</th>
<th>10</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of strains inhibited</td>
<td>-</td>
<td>10</td>
<td>9</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>Percentage of strains inhibited</td>
<td>47.61</td>
<td>42.86</td>
<td>9.52</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2: MIC of cefotaxime against Acinetobacter strains producing beta-lactamase**

<table>
<thead>
<tr>
<th>Concentration of cefotaxime (µg/ml)</th>
<th>25</th>
<th>50</th>
<th>100</th>
<th>200</th>
<th>300</th>
<th>400</th>
<th>500</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of strains inhibited</td>
<td>-</td>
<td></td>
<td>7</td>
<td>4</td>
<td>2</td>
<td>8</td>
<td>-</td>
</tr>
<tr>
<td>Percentage of strains inhibited</td>
<td>-</td>
<td></td>
<td>33.3</td>
<td>19</td>
<td>9.5</td>
<td>38.0</td>
<td>-</td>
</tr>
</tbody>
</table>

MIC: Minimum inhibitory concentration, AGE: Aqueous garlic extract
Table 3: Combined effect of cefotaxime and AGE Acinetobacter strains producing beta-lactamase

<table>
<thead>
<tr>
<th>Type of beta-lactamase</th>
<th>ESBL</th>
<th>AmpC</th>
<th>ESBL+AmpC</th>
<th>MBL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of strains exhibiting synergy (FIC&lt;0.5)</td>
<td>-</td>
<td>-</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>Number of strains exhibiting additive (0.5&lt;FIC&lt;1)</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Number of strains exhibiting indifference (1&lt;FIC&lt;2)</td>
<td>-</td>
<td>3</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>Number of strains exhibiting antagonism (FIC&gt;2)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

AGE: Aqueous garlic extract, ESBL: Extended-spectrum beta-lactamases, AmpC: Ambler Class C, MBL: Multiple beta-lactamase, FIC: Fractional inhibitory concentration

The synergistic association between garlic and cefotaxime demonstrates the potential application of natural product and antibiotic combinations as an efficient, novel therapeutic tool for antibiotic-resistant bacterial infections.

CONCLUSION

Thus, it can be said that natural products have the ability to curb the growing menace of antibiotic-resistant bacteria. The use of different natural compounds as antibacterial agents is an interesting strategy for discovering bioactive products that could help in designing therapeutic tools. The augmented action of antibiotics along with natural substances may have positive synergistic effects toward specific, drug-resistant microorganisms, which are difficult to eradicate, particularly in hospital settings.

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