Fresh Garlic Extract has a Synergistic Effect with Antibiotics on ESBLs Producing E. coli Urinary Isolates

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Author’s contribution

The sole author designed, analysed, interpreted and prepared the manuscript.

ABSTRACT

Aim: Extended spectrum beta-lactamases (ESBLs)-producing bacteria often exhibit a multidrug-resistant phenotype limiting the therapeutic options available to the clinician. This study was conducted to investigate the antibacterial susceptibility pattern of ESBL producing E. coli isolates and evaluation of the antibacterial activity of Garlic extract against it.

Study Design: The study was carried out using antibiotics powder and Garlic extract.

Place and Duration of Study: At the microbiology lab, Faculty of Medicine, Minia University between January 2017 to August 2018.

Methods: The study was carried on 55 ESBL producing E. coli isolates isolated from patients with urinary tract infections (UTI). Screening and confirmation for ESBL was done according to clinical and laboratory standard institute guidelines (CLSI) guidelines. The antibacterial sensitivity to a panel of antibiotics and Garlic was performed by tube dilution method.

Results: The results of antibacterial susceptibility testing of ESBLs-producing E. coli isolates in our study showed a higher degree of resistance to the tested antibiotics. In the present study, Garlic showed an inhibitory effect on ESBLs- producing E. coli with concentrations ranging from 10-50.

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Garlic extract produce a synergistic effect with all tested antibiotics against all tested ESBL producing E. coli isolates. A Significant decrease in ESBLs genes expression (SHV, TEM and CTX-m) was reported after treatment with Garlic extract (P value: <0.001).

**Conclusion:** The combinations of antibiotics and Garlic may be more useful than individual agents, so we recommended that further researches should be undertaken to evaluate the combination of Garlic with antibiotics towards ESBL producing E. coli.

**Keywords:** E. coli; ESBLs; garlic; MIC; susceptibility; UTI.

### 1. INTRODUCTION

Antibiotic resistant mutants producing extended spectrum beta-lactamases (ESBLs) have emerged among Gram negative bacilli, predominantly Escherichia coli (E. coli) [1].

ESBLs are plasmid-mediated enzymes that hydrolyze penicillins, extended-spectrum cephalosporins, monobactams, and are inhibited by beta-lactamase inhibitors such as clavulanic acid, sulbactam, and tazobactam [2].

ESBLs producing bacteria continue to be associated with higher rates of mortality, morbidity, and health care costs. ESBLs arise because of mutations in the TEM, SHV, CTX-M and OXA genes, which are commonly found in the Enterobacteriaceae family [3].

ESBLs-producing bacteria often exhibit a multidrug-resistant phenotype, including resistance to aminoglycosides and fluoroquinolones, further limiting the therapeutic options available to the clinician [4]. Infectious Diseases Society of America listed ESBL-producing E. coli as one of the drug-resistant microbes to which new therapies are urgently needed [5].

*Allium sativum* commonly known as Garlic belongs to the Amaryllidaceae family. The inhibitory and lethal activity of Garlic extract against many pathogenic fungi and bacteria has been investigated by several researchers [6,7].

Garlic 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* [8,9,10]. Garlic contains at least 33 sulfur compounds such as alliin, allicin, ajoene, allyl propyl, diallytrisulfide, s-allyl cysteine, S-allylmercaptocysteine, and others, which are responsible for its antibacterial activity [11].

This study was conducted to investigate the antibacterial susceptibility pattern of ESBLs-producing clinical E. coli isolates and evaluation of the antibacterial activity of Garlic extract against ESBL producing E. coli isolates.

### 2. MATERIALS AND METHODS

The study was carried out at the Microbiology and Immunology department, Faculty of Medicine, Minia University, in the period from January 2017 to August 2018. The study was carried out on 55 ESBLs-producing E. coli isolates out of 130 E. coli isolates isolated from patients with urinary tract infections.

#### 2.1 Phenotypic Detection of ESBLs Production

ESBLs detection was performed by initial screening test and phenotypic confirmatory test as recommended by CLSI guidelines.

**2.1.1 Screening by Minimal Inhibitory Concentration (MIC) test**

Minimum inhibitory concentration of the isolates was determined by broth dilution method for ceftazidime and ceftriaxone. The values of concentration range of antibiotics tested were as followed: 0.25 µg/mL to 128 µg/ml. According to NCCLS guidelines, isolates with MIC ≥2 µg/mL for ceftazidime or ceftriaxone were recorded as potential ESBLs producers [12].

**2.1.2 Confirmation by combination disc method**

A phenotype confirmation test of ESBLs production by combination disc method was done to all the screened ESBLs positive isolates according to NCCLS recommendations. The bacterial suspension was first adjusted to 0.5 McFarland standards then it was spread on Muller–Hinton agar using a sterilized cotton swab. After incubation at room temperature for 15 minutes, the antibacterial discs (ceftriaxone 30
μg and ceftazidime 30 μg) and combination discs (ceftaxzone 30 μg with clavulanate 10 μg and ceftazidime 30 μg with clavulanate 10 μg) were placed on each plate. After 18 hours incubation at 37°C, each plate was examined. The diameters of the inhibition zone were measured. According to NCCLS guidelines, an organism was interpreted as ESBLs producer if there was an increase of ≥5 mm of the inhibition zone of the Combination disc when compared with the corresponding cephalosporin disc [12].

2.2 Antibacterial Susceptibility Testing

The antibiotic sensitivity test to a panel of antibiotics was performed by tube dilution method using antibiotic powders (Oxoid, UK). Two-fold serial dilutions of antibiotics were prepared. The MIC range varied with different drugs. All MIC ranges were followed according to the NCCLS guidelines [12].

2.3 Preparation of Garlic (A.sativum) Extract

The Garlic cloves were peeled, cut into pieces, and 100 g was mixed in 50 mL sterile distilled water. The mixture was crushed finely using a juicer. The resulting paste was centrifuged for 20 minutes and the supernatant was then sterilized by the 0.22 μm filter (Thermo Fisher Scientific, Germany). The final concentration of Garlic in aqueous solution was determined to be 50% (w/v) by subtracting the weight of the precipitate from the weight of the original Garlic bulbs. The Garlic extract was stored at -20°C until used. To achieve various concentrations, the extract was diluted with sterilized distilled water [13].

2.4 ESBLs E. coli Genes Expression

Semi-quantitative RT-PCR was performed to study the effect of garlic on the mRNA expression of the ESBLs E. coli genes (SHV, TEM and CTX-M) after RNA isolation and complementary DNA (cDNA) synthesis. Total RNA was extracted from overnight cultures. Cells were then cultured for 8 h at 37°C with shaking; with or without garlic (30 mg/ml; mean MIC). Total RNA extraction was performed according to manufacture instructions of QIAamp RNA Minikit (Qiagen, Valencia).

Total RNA was then quantified using a spectrophotometer (Genova, UK).

cDNAs were synthesized using cDNA Synthesis kit (Thermo Fisher Scientific, Inc) in a thermal cycler at 42°C for 60 min followed by 70°C for 5 min using random hexamer primer according to the manufacturer’s instructions. qPCR was done using Maxima SYBR Green qPCR kit (Thermo Fisher Scientific, Inc) in an ABI 7500 instrument (Applied Biosystems, USA). Each PCR reaction was performed in a 25-μ volume containing 50 ng of cDNA (2 μl), SYBR Green master mix (12.5 μl), 0.3 μM each primer (1 μl), 50x reference dye low (0.5 μl) and PCR-grade water (up to 25 μl). The reaction condition was 95°C for 10 minutes as initial denaturation, followed by 40 cycles of: 95°C for 15 seconds denaturation, annealing 60°C for 30 seconds, and extension 72°C for 30 seconds.

We analyzed PCR results with relative quantification to E. coli 16S rDNA gene as a reference gene. Gene specific primers used were listed in Table 1. We calculated the fold changes of mRNA levels using the comparative cycle threshold (ΔΔCt) method as follows: ΔCt = mean value Ct (cDNA of interest) - mean value Ct (reference), ΔΔCt = ΔCt test sample – Average Δ CT control sample. The fold change in gene expression normalized to reference gene and relative to the control sample, the negative value of this subtraction (-ΔΔCt) becomes the exponent of 2 (R = 2-ΔΔCt). Then the relative expression levels of cDNA were confirmed by using free data analysis tools.

2.5 Statistical Analysis

All statistical analyses were performed using the SPSS program for Windows (version 20 statistical software; Texas instruments, IL, USA). The Pearson’s chi-square test was used to determine the significance. A two-tailed p-value of < 0.05 was considered statistically significant.

3. RESULTS

3.1 ESBLs Detection

About 76% (99/130) of E. coli were suspicious of ESBLs production by screening test. The suspicious isolates of ESBLs production were further confirmed by combination disc method. Only 55/130 (42.3%) E. coli were confirmed as ESBLs producer.

3.2 Antibacterial Susceptibility Pattern of ESBLs- E. coli Isolates

The percentage of antibacterial resistance of ESBLs-producing E. coli isolates to different antibiotics is shown in Fig. 1.
All isolates were sensitive to imipenem. Co-resistance was detected to non-beta lactam antibiotics (Cotrimoxazole, ciprofloxacin and nitrofurantoin).

### 3.3 Antibacterial Activity of Garlic Extract

Antibacterial activity of different concentrations of Garlic extract for all ESBLs- *E. coli* isolates show that the lowest concentration that inhibit the growth of all tested organisms were at 40 mg/ml concentration and the activity was linear with increasing the concentration as shown in Fig. 2.

Mean MIC was 30 mg/ml. MIC$_{50}$ was 25 mg/ml, MIC$_{90}$ was 37.5 mg/ml and 100% of the isolates inhibited at MIC 40 mg/ml.

Table 2 shows the mean MIC of different antibiotics when tested alone and in combination with Garlic extract. A significant decrease in the mean MIC was detected for all tested antibiotics when combined with Garlic. There is no antagonistic effect was reported for Garlic when combined with antibiotics, instead a synergistic effect was detected for all tested antibiotics when combined with Garlic against all tested isolates.

Fractional inhibitory concentration (FIC) for all tested antibiotics and Garlic showing value less than 0.5 showing synergy as shown in Fig. 3.

The combined effect is analyzed by using measurements of the MIC to calculate the FIC Index according to the formulas:

\[
\text{FIC Index} = \text{FICA} + \text{FICB}
\]

Where,

\[
\text{FICA} = \frac{\text{MIC}_A}{\text{MIC}_{\text{combined}}} \quad \text{FICB} = \frac{\text{MIC}_B}{\text{MIC}_{\text{combined}}}
\]

### Table 1. Primers used for PCR reaction

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHV</td>
<td>3'-CGCCTGTGTATTATCTCCCT-5'</td>
<td>Bali et al. 2010</td>
</tr>
<tr>
<td></td>
<td>5'-CGAGTAGTCCACCAGATCCT-3'</td>
<td></td>
</tr>
<tr>
<td>TEM</td>
<td>3'-TTTCGTGTCGCCCTATTCC-5'</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5'-ATCGTTTGCAGAAGTTTGG-3'</td>
<td></td>
</tr>
<tr>
<td>CTX-M</td>
<td>3'-CGCTGTGGTATAGGAGTG-5'</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5'-GGCTGGGTGAAGTAGTGAC-3'</td>
<td></td>
</tr>
<tr>
<td>16S rDNA (reference gene)</td>
<td>3'-AGAGTTTGATCCTGCTTCAG-5'</td>
<td>Magray et al. 2011</td>
</tr>
<tr>
<td></td>
<td>5'-TTTGGGCTGCTAGAT-3'</td>
<td></td>
</tr>
</tbody>
</table>

Gen Bank no for SHV, TEM and CTX-M were EF125011, AB282997 and DQ303459 respectively

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**Fig. 1. Antibacterial resistance pattern of ESBLs- *E. coli* isolates**
Fig. 2. Antibacterial activity of different concentrations of garlic extract against ESBL *E. coli* isolates

Table 2. Comparison between mean MIC of tested antibiotics alone and in combination with garlic extract against ESBLs- *E. coli*

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Mean MIC Antibiotic Alone (µg/ml)</th>
<th>Mean MIC Garlic Alone (mg/ml)</th>
<th>Mean MIC Antibiotic / Garlic (µg/ml) (mg/ml)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>1024</td>
<td>64/12</td>
<td></td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>512</td>
<td>32/10</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>Cotrimoxazole</td>
<td>32/1216</td>
<td>8/152/10</td>
<td>0.004</td>
<td></td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>128</td>
<td>8/9</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>512</td>
<td>16/8</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>32</td>
<td>2/7</td>
<td>0.004</td>
<td></td>
</tr>
<tr>
<td>Nitrofurantoin</td>
<td>256</td>
<td>32/5</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>Amox-clav</td>
<td>32/16</td>
<td>8/8/5</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>Amikacin</td>
<td>16</td>
<td>2/5</td>
<td>0.006</td>
<td></td>
</tr>
<tr>
<td>Imipenem</td>
<td>0.5</td>
<td>0.125/3</td>
<td>0.04</td>
<td></td>
</tr>
</tbody>
</table>

*P value <0.05 was significant. The mean MIC for all tested antibiotics was significantly decreased when combined with garlic than when tested alone.*

Fig. 3. FIC of all tested antibiotics and garlic against ESBLs producing *E. coli*. showing value less than 0.5 showing synergy
Table 3. Fold changes in ESBLs genes expression with and without garlic extract

<table>
<thead>
<tr>
<th>ESBLs genes</th>
<th>Fold change without Garlic (mean±SD)</th>
<th>Fold change with Garlic (mean±SD)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHV</td>
<td>253.7±68.2</td>
<td>21.3±10.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TEM</td>
<td>178.9±50.0</td>
<td>12.7±3.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CTX-M</td>
<td>85.6±23.6</td>
<td>34.8±7.9</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

SD (standard deviation), fold changes decrease was significant, P value was <0.05

The MICA +B value is the MIC of compound A in the presence of compound B, and vice versa for MICB+A. FIC Index results are interpreted as synergistic if FIC Index <0.5, additive if 0.5 >FIC Index <4, or antagonistic if FIC Index >4.

3.4 ESBLs E. coli Genes Expression

A comparison between ESBLs genes (SHV, TEM and CTX-M) expression with and without Garlic extract was done. A Significant decrease in ESBLs genes expression was reported after treatment with Garlic extract as shown in Table 3.

4. DISCUSSION

In recent years, the problem of increasing resistance to antibiotics has threatened the entire world. Resistance to extended spectrum β-lactams among Gram negative bacteria is increasingly associated with ESBLs [14].

In our study the occurrence of ESBLs producers in urinary isolates of E. coli was found to be 42.3%, this high prevalence rates agree with that reported in other studies, Al-Agamy et al. [15], Thabit et al. [16], Khater and Sherif [17] and Elsayed et al. [18] in Egypt reported that the prevalence of ESBLs producers in urinary isolates of E. coli was 60.9%, 53%, 53.3% and 36% respectively. Also many studies in different countries reported a high prevalence rate of ESBLs producers in urinary isolates of E. coli [1,19,20,21] contrary to the relatively low prevalence rates of ESBLs producers in urinary isolates of E. coli in Europe: 5.8-18.2% [22], India: 20.4% [23], UK from 4.6 to 6.6% [24], USA: 27.4% [25], Taiwan: 33.3% [26].

This may be due to the fact that the prevalence of ESBLs among clinical isolates varies greatly worldwide and are rapidly changing over time.

The results of antibacterials susceptibility testing of ESBLs- E. coli isolates in our study showed a higher degree of resistance to ampicillin (98%), cefalothin (97%), ceftazidime (56.9%) and ceftriaxone (59.4%), this is in agreement with other studies [18,21,27].

The overall resistance to various antibiotics was as followed: amikacin (11.9%), nitrofurantoin (39.2%), ciprofloxacin (55%) and cotrimoxazole (80.6 %).

Although clavulamic acid is an inhibitory compound which is expected to hinder the activity of ESBLs, 23.5% of ESBLs- E. coli isolates in our study were resistant to Amox-Clav, so the usefulness of β-lactam/β-lactamase inhibitors have been decreased. It is worth noting that the considerable reduction in the activity of the commonly used drugs in treatment of UTI, this is because ESBLs-producing organisms show cross-resistance with non β-lactam antibiotics. ESBLs, plasmid-mediated enzymes are transferable between bacterial species and are also capable of incorporating genetic material coding for resistance to other antibiotics.

A higher percentage of resistance among ESBLs- E. coli to non-beta lactam antibiotics was reported in different studies that have shown that ESBLs-producing organisms was an alarming trend of associated resistance to other classes of antibacterial agents [18,21,23,28].

All our isolates were sensitive to Imipenem. Although carbapenems are widely regarded as the drugs of choice for treatment of infection caused by ESBLs-producing organisms, production of β-lactamases capable of hydrolyzing carbapenems has been reported in Enterobacteriaceae mostly in Enterobacter and Serratia [29]. In E. coli, the first report of carbapenem resistance appeared in 1999 [30].

If Garlic is to be considered as an alternative medicine, various studies would have to be carried out to determine the antibacterial activity and side effects. Garlic appears to satisfy all the criteria for antibacterial agents, being cheap and safe. Since there is increasing resistance to most of antibacterial agents, search for new antibacterials is very important in recent times. Because Garlic is known to act synergistically
with antibiotics, and resistance has not been reported, more preclinical and clinical studies should be done to assess the use of an antibiotic/Garlic combination for bacteria that are difficult to eradicate.

In the present study, Garlic showed an inhibitory effect on ESBLs- *E. coli* with concentrations ranging from 10-40 mg/ml and a mean of 30 mg/ml, comparable results reported by Wali and Awad, [31] where the range of Garlic MIC was: 6.2 to 100 mg/ml and a mean of 37 towards their ESBLs- *E. coli* isolates.

Also Iwalokun et al. [32] reported a mean Garlic MIC of 20 mg/ml towards multi-drug resistant *E. coli*. In addition, Abubakar, [33] reported Garlic MIC values of 50 and 100 mg/ml towards a standard laboratory *E. coli* strain and a nosocomial *E. coli* isolate, respectively; However, lower results have been demonstrated for commensal and pathogenic *E. coli* isolates with Garlic MIC ranging between 3 and 12 mg/ml in a study carried by Ross et al. [34]. Shayan et al. [35] reported that The MIC of Garlic ranged from 2.5 mg/ml to 10 mg/ml with a mean of 5 against AmpC and ESBL producing *E. coli* in their study in Iran.

The disparity of antibacterial potency for Garlic observed among studies might be attributed to the geographical variation which affects the intensity and range of antibacterial effects of Garlic.

There was a significant decline in the mean MIC values for each of antibiotics tested (ampicillin, cephalothin, cotrimoxazole, ceftriaxone, ceftazidine, ciprofloxacin, nitrofurantoin, amikacin and imipenem) and Garlic when combined against all tested ESBL- *E. coli* urinary isolates compared to their values when tested alone (P value <0.05).

Wali and Awad, [31] reported that there was a significant decline in the MIC values of nitrofurantoin when combined with Garlic for all tested ESBL producing *E. coli* isolates compared to their MICs alone. These findings further support the idea that Garlic combination with antibiotics holds promising effects. Synergism has been observed between Garlic and vancomycin against vancomycin resistant enterococci [36]. Also Garlic has also shown synergy with streptomycin against streptomycin resistant *E. coli* [37]. Li et al. [13] reported a synergistic effect of Garlic with certain antibiotics against Methicillin resistant *S. aureus* (MRSA) and Pseudomonas.

Also we studied the effect of Garlic extract on ESBLs genes (SHV, TEM and CTX-M) expression. A Significant decrease in all ESBLs genes expression was reported after treatment with Garlic extract.

5. CONCLUSIONS

Garlic extract produce a synergistic effect with all tested antibiotics against all tested ESBL producing *E. coli* isolates. The combinations of antibiotics and Garlic may be more useful than individual agents. Various studies would have to be carried out to determine the antibacterial activity and side effects of Garlic extract in vitro and in vivo. Garlic appears to satisfy all the criteria for antibacterial agents, being cheap and safe. Since there is increasing resistance to most of antibacterial agents, search for new antibacterial is very important in recent times.

CONSENT

Written informed consents were obtained from all patients.

ETHICAL APPROVAL

The study was approved by the Ethical Committee of Minia University, Faculty of Medicine.

COMPETING INTERESTS

Author has declared that no competing interests exist.

REFERENCES


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Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sdiarticle3.com/review-history/47146