ANTIMICROBIAL ACTIVITY OF GARLIC AND CEFOTAXIME AGAINST EXTENDED SPECTRUM β-LACTAMASE (ESBL) - PRODUCING ESCHERICHIA COLI AND ITS ASSOCIATION WITH THE MOLECULAR PROFILE OF β-LACTAMASE GENES

by

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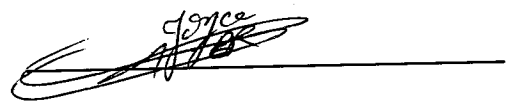

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Abstract

Since the first rise of extended spectrum beta lactamases (ESBL), ESBL-producing organisms have posed a remarkable threat to hospitalized patients due to their activity against cephalosporins, restricting available treatment option. This rapid emergence of ESBLs has been confronted by limited data regarding both phenotypic and genotypic profiles. This lack of information requires more exploration. The aim of this study was, in the first place, to investigate the effect of garlic (*Allium sativum*) ethanolic plant extract against ESBL- producing *Escherichia coli* and its effect in combination with cefotaxime, and secondly, to characterize the genotypic properties of ESBL-producing *E. coli* and correlate it with the efficacy of the combinations of cefotaxime and garlic. The methods used included the agar-well diffusion method to determine the inhibition zone with the double disk synergy test, the microdilution method to determine minimum inhibitory concentration (MIC), the checkerboard technique to quantitatively determine the ameliorative effect, and the polymerase chain reaction method to detect genotypically ESBL genes. The results showed that 30 μg cefotaxime had inhibition zones ranging between 7 and 22mm against ESBL-producing *E. coli* indicating that all isolates as resistant to cefotaxime. Using the checkerboard method, all the combinations of ethanolic garlic extract and CTX showed indifference except for some combinations that demonstrated an antagonistic activity with ΣFIC>4. PCR analyses showed that the most common ESBL gene belonged to the CTX-M group. All isolates of *E. coli,* except isolate 2, contain this allele according to the following proportions: 36.36% harbored CTX-M gene only, 36.36% harbored CTX-M and OXA genes, and 9.09% harbored CTX-M and TEM. TEM was found in 18.18% of the isolates. No correlation between the phenotype and the genotype of the bacteria was

found. A highly resistant isolate of ESBL-producing E. coli (isolate 2) to garlic ethanolic extract was discovered. Further research is needed in order to understand the mechanism of this resistance.,

Keywords: Ethanolic garlic extract, Cefotaxime, FIC, ESBL-producing *E. coli*, PCR, CTX-M, TEM, SHV, OXA.

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1. Introduction

Enterobacteriaceae is a family of small Gram negative, non-sporing straight rods (Hayek, Willis, 1984). Many species of this family inhabit the intestinal flora and are among the most common human pathogens (Nordmann et. Al, 2005). This family is a source of community- and hospital-acquired infections. Lately, the evolution of antimicrobial resistance by Enterobacteriaceae was considered as a serious problem in the treatment of bacterial infections (Ventola, 2015). The emergence of *Escherichia coli* isolates with multiple antibiotic- resistant phenotypes is one example of resistance development in Enterobacteriaceae. *E. coli*, is a major nosocomial pathogen that causes intra-abdominal infection, urinary tract infection, and primary bacteremia (Kang et. Al, 2004). *E. coli* has been susceptible to cephalosporins (oxymino-β-lactam antibiotics) for many years. However, their excessive use resulted in the development of extended-spectrum beta-lactamase (ESBL)-producing *E. coli* strains that are resistant to this group of antibiotics and resulting in therapeutic challenges. ESBLs are plasmid-mediated enzymes evolving through point mutations of key amino acids in TEM and SHV enzymes (Paterson, Bonomo, 2005). Two new families of enzymes known as the CTX-M and OXA-type enzymes emerged later. Although ESBLs vary in their ß-lactam substrate affinity and enzyme kinetics, they confer resistance to cephalosporins which are the principal antibiotic families of which the β-lactam ring is part of the core structure (Munita & Arias, 2016). These enzymes have the ability to hydrolyze cephalosporins and are inhibited by clavulanic acid (Queenan & Bush, 2007). Hence, there is an urgent need for finding alternatives to enhance the activity of cephalosporins to combat ESBL- producing *E. coli.* A recent study indicates the good efficacy of combining cefotaxime, a third-generation cephalosporin, and Garlic (*Allium satvium L.*)

against ESBL- producing *E. coli* (Halawi, 2017). However, this work was limited to one strain and ESBL genotype that was not characterized. For clinical purposes, it is of paramount importance to test the efficacy of a specific antibiotic or a combination of antibiotics against a specific ESBL genotypes (Turnidge & Paterson, 2007).

Therefore, the aim of this study was, firstly, to investigate the possible ameliorative effect of cefotaxime when combined with garlic against 11 isolates of ESBL producing *E. coli*. Secondly, to study, at a molecular level, the influence of ESBL plasmid carriage on the host bacterium expansion of resistance by characterizing the genotypic properties of ESBL-producing *Escherichia coli* and correlates it with the efficacy of cefotaxime and garlic alone and in combination.

2. Literature Review

This literature review focuses on extended spectrum beta-lactamases (ESBL)-producing *Escherichia coli,* its mode of action, and the rise of antibiotic resistance. It highlights its causes and the mechanisms of bacterial resistance that relate ESBL-producing *E. coli* to cefotaxime. In addition, this review draws some guidelines for combating antimicrobial resistance. Then, it puts special emphasis on ethanolic garlic extract as an antibacterial agent. Finally, it shows synergistic, additive or indifference, and antagonistic effects of the interaction between plant extracts and antibiotics.

2.1 Escherichia coli

Escherichia coli (E. coli) is a Gram-negative rod in the Enterobacteriaceae family. It is the predominant facultative anaerobe of the human colonic flora. This gram-negative bacterium is comprised of both nontoxic commensal strains and different pathogenic forms with the capacity causing intestinal and extraintestinal diseases (Dobrindt, 2005). The commensal strains typically colonize the infant gastrointestinal tract within hours of life, and, thereafter, E. coli and the host derive mutual benefit (Nataro, Kaper, 1998). In fact, *Escherichia coli* is a remarkable and diverse organism. The pathogenic form is capable of causing a range of diseases, from gastroenteritis to extraintestinal infections of the urinary tract, bloodstream and central nervous system (Croxen, et. Al, 2013).

2.2 The rise of antibiotic resistance

The rapid emergence of resistant bacteria is taking place globally, threatening the efficacy of antibiotics and resulting in a public health threat. (D'Agata, et al., 2008). Even though many considerable interventions are planned to prevent the emergence and diffusion of antimicrobial-resistant bacteria, the rates continue extending promptly. This increase in antibiotic resistance was generated by the chaotic overuse of antibiotics in

human health, food industry and agriculture (Alanis, 2005; Nimmo & Turnidge, 2015), as well as the lack of new drug evolution by pharmaceutical industries due to reduced economic considerations and challenging requirements.

2.3 Mechanisms of bacterial resistance

The molecular mechanism by which bacteria acquire resistance to antibiotics are diverse and complex (Alanis, 2005), which could be one of the reasons behind the failure of combating resistance. Gram-negative organism develop resistance by five main methods. Resistance can occur when bacteria carry genes coding for enzymes such as beta-lactamases which hydrolyze and inactivate beta-lactam antibiotics. Resistance also can manifest by mutations in the genes for binding sites for antibiotics which change its specific target or its function. In addition, alteration of the membrane porins leading to reduced permeability results in bacterial resistance. As well, bacteria can reveal efflux pumps that transport antibiotics out of the cell, thus developing resistance. Bacteria can also alternate pathways to by-pass paths inhibited by antibiotics (Mcdermott, et al., 2003).

β-lactam antibiotics are a widespread class of antibiotics consisting of agents that contain a β-lactam ring as the chemical base in their molecular structure (Worthington and Melander, 2013). This chemical structure that forms the β-lactam ring includes a three-carbon, one-nitrogen cyclic amine structure. This ring is vital for antimicrobial activity (Sherris, 2004). The antibacterial activity of these antibiotics is due to the side chain variability that consist of a variable group, the beta-lactam ring, attached to the core structure by a peptide bond (Thenmozhi, 2014).

2.4 Extended Spectrum Beta-Lactamases

Extended spectrum beta-lactamases are enzymes capable of conferring bacterial resistance by hydrolyzing the beta-lactam ring of Beta-lactam antibiotics rendering the organism resistant. They were first described in Escherichia coli isolates before the release of the first β-lactam drug, penicillin (Abraham & Chain, 1940). These lactamases develop resistance to the penicillins, third-generation cephalosporins, and aztreonam (but not the cephamycins or carbapenems) and which are inhibited by β-lactamase inhibitors such as clavulanic acid (Paterson & Bonomo, 2005). These enzymes can be divided into many groups based on their amino acid similarities or functional characteristics. The first β-lactamase categorization is the Ambler scheme, Classes A to D based on molecular structure, and the second is the Bush-Jacoby-Medeiros scheme based on functional similarities, Groups 1-4. Ambler class A, C and D are serine- β-lactamases and class B are metallo- β-lactamases. Bush scheme groups consist of three groups: group one is cephalosporinases which are not inhibited by clavulanic acid, the larger group two are broad spectrum enzymes which are generally inhibited by clavulanic acid and the group three are metallo-β-lactamases (Thenmozhi, 2014). ESBLs are Class A β-lactamases and may be defined as plasmid-mediated enzymes. Moreover, several families of β-lactamase exist. Of these, the most common are the SHV, TEM, OXA, and CTX-M types.

2.4.1.1 TEM β-lactamases

TEM-1 β lactamase was the first β-lactamase discovered in Europe, during the early 1960s (Datta et al., 1965), and was obtained from an *Escherichia coli* isolate from a Greek patient from Athens named Temoneira (Datta et al., 1965). In fact, TEM is known to be the most common β-lactamase encountered in Gram-negative bacteria,

leading to approximately 90% of ampicillin resistance in *E. coli.* TEM is considered as well responsible for ampicillin and penicillin resistance identified, in large numbers, in *H.influenzae* and *N.gonorrhoeae*. More than 150 TEM type have been discovered, all of them been TEM-1 and TEM-2 derivatives by point mutations.

2.4.1.2 SHV β-lactamases

Another type of β-lactamases is the SHV (sulfydryl variable) enzymes. SHV was first described in *Klebsiella pneumoniae*, having a similar overall structure as TEM and known to share about 68% of its amino acids with TEM-1. As it is most commonly found in K. pneumoniae, SHV β-lactamase is responsible for up to 20% of the plasmid mediated ampicillin resistance in this species (Paterson & Bonomo, 2005). The SHV-type ESBL was the most common enzyme detected in *E. coli* isolates found in some retail raw meat samples in Spain (Rodríguez-Baño, 2009). To date, more than 50 SHV derivatives are discovered, all derived from SHV-1 or SHV-2 (Gupta, 2007).

2.4.1.3 CTX-M β-lactamases

CTX-M enzymes are one of the β-lactamase family, first isolated in Munich. They were named CTX-M for their great hydrolytic activity against cefotaxime than other β-lactam substrates as ceftriaxone, cefepime or ceftazidime (Gupta, 2007). These CTX-M enzymes do not arise by mutation as other types, but instead, represent examples of plasmid acquisition of β-lactamase genes normally found on the chromosome of a rarely pathogenic commensal organisms named Kluyvera species. These enzymes show only 40% identity or even less with TEM and SHV type ESBLs (Paterson & Bonomo, 2005). So far, more than 80 CTX-M enzymes are currently known, and are divided into 5 clusters based on the amino acid sequence of each one: CTX-M-1, CTX-M-2, CTX-M-9 and CTX-M-25 (Gupta, 2007).

2.4.1.4 OXA β-lactamases

OXA β-lactamases are a type of β-lactamases recognized as a less common type in this family, and named so because of their oxacillin-hydrolizing capabilities. They do not hydrolyze extended-spectrum cephalosporins, but instead hydrolyze oxacillin and related anti-staphylococcal Penicillins. Some exceptions exist including OXA-1, and OXA-13 to OXA-19 (Toleman et al., 2003). The difference between OXA enzymes and the TEM and SHV enzymes lies in the classification of OXA enzymes, in that they belong to molecular class D and functional group 2d. The OXA type ESBLs have been found mainly in *Pseudomonas aeruginosa* rather than *Escherichia coli* and *Klebsiella pneumoniae*, and other Enterobacteriaceae where all other ESBLs are almost found.

2.5 Combating antimicrobial resistance

One of the major healthcare problems in the 21st century are the infections caused by bacteria that show resistance to commonly used antibiotics. These infections are more severe, need more complex and expensive diagnosis and treatments. In fact, with the widespread use of antibiotics in a random way, more and more bacteria that was previously susceptible to common antimicrobials have been reported to have developed resistance to different antibiotics (Alanis, 2005). These bacteria, that once leaded to significant nosocomial infections resulting in major morbidity and mortality rates in hospitalized patients, have become after acquiring resistance more severe, spreading to the community and causing severe illnesses in previously healthy and non-vulnerable patients.

The American Dental Association (ADA) Council on Scientific Affairs (2004) have five guidelines that should be considered when prescribing antibiotics in order to reduce or limit the spread of antimicrobial resistance. First, usage of antibiotics should

be appropriate and under right dosing schedules. Second, in simple infections, narrow-spectrum antibiotics should be used, and broad-spectrum antibiotics should be preserved for complex infections. Third, obtaining knowledge about the side effects of an antibiotic before prescribing it avoids many misuses of these drugs. Forth, unnecessary use of antibiotics in treating viral infections should be avoided. And lastly, patients should be educated about the proper use of the drugs and stress the importance of completing the full course of therapy (Musoke, Revathi, 2000).

In order to succeed in the resistance prevention measures and to recommend best empiric therapies, Isaacs and Andresen (2013) and the WHO (2001) proposed increasing financing of antibiotic resistant research programs and improving surveillance of antibiotic resistance. Certainly, it is fundamental that the scientific community, besides developing new antibacterial drugs should engage in combining different antibiotics in order to combat bacterial resistance (Maheshwari, 2007; WHO, 2001).

2.6 Use of garlic as antibacterial agent

As vigorous antibiotics are losing their thump against microorganisms, scientists are searching for new antimicrobial agents from natural sources to overcome the problem of antibiotic resistance. Many phytochemicals have drawn much attention for their antibacterial activity. This study will focus on garlic as a potent antimicrobial agent.

2.6.1.1 Garlic

Garlic, *Allium sativum,* is a member of Liliaceae family that is used worldwide as a vegetable, spice, and medicinal plant (Lewis & Elvin-Lewis, 2003). Its widespread pharmacological effects have been very significant over the years. *A.sativum* have many

biological activities including antibacterial (Abdulzahra & Mohammed, 2014; Harris et al., 2001), antifungal, antioxidant, anticancer, antiviral (Harris et al., 2001), antiseptic (Abdulzahra & Mohammed, 2014), antiatherosclerosis (Campbell et al., 2001), cholesterol lowering (Yeh & Liu, 2001), anti-asthmatic, and expectorant activities (Zare et al., 2008). Garlic consists of 33 sulfur compounds at least that are responsible for its odor and medicinal effects including an antimicrobial effect (Gebreyohannes & Gebreyohannes, 2013). Allicin is the main active ingredient of garlic. After cutting and crushing garlic, Allin (S-allyl-L-cysteine sulfoxides), which is one of the major sulfur-containing compounds present in intact garlic (Stoll & Seebeck, 1948), reacts enzymatically with allinase leading to the formation of a low-molecular-weight organosulfur compound which is the allicin. this main active ingredient is responsible for garlic's antimicrobial activity (Amagase, 2006; Barone & Tansey, 1977). Though, allicin is very unstable and decomposes rapidly (Brodnitz et al., 1971). Other active ingredients include diallyl trisulfide, ajoene, allyl alcohol, diallyl disulfides and other organosulfides (Harris et al., 2001).

2.6.1.2 Antibacterial activity of garlic

The first scientist that have described the antibacterial effect of garlic extracts was Louis Pasteur (Lupoae et al., 2013). Remarkably, to date, bacteria has not developed a resistance to garlic (Admin, 2014), which is attributed to garlic's wide spectrum activity against gram positive bacteria including: *Staphylococcus aureus, Staphylococcus epidermidis, Bacillus. subtilis,* MRSA and multidrug resistant: *Bacillus* spp. and *S.aureus* (Arora & Kaur, 1999; Durairaj et al., 2009; Elsom et al., 2000; Karuppiah & Rajaram, 2012; Srinivasan et al., 2001) and against gram negative bacteria including: *Pseudomonas aeruginosa, Salmonella typhi, Helicobacter pylori, Klebsiella*

pneumonia, Proteus mirabilis, E. coli, ESBL-producing *E. coli*, and multidrug resistant: *Proteus* spp., *E. coli, P. aeruginosa, K. pneumonia, Salmonella* mgulani, *Aeromonas caviae, Aeromonas hydrophila* and *Enterobacter* spp (Akkiraju et al., 2015; Arora & Kaur, 1999; Durairaj et al., 2009; Elsom et al., 2000; Karuppiah & Rajaram, 2012; Srinivasan et al., 2001).

Allicin, the active ingredient of garlic, possesses an inhibitory effect against various bacteria including *S. aureus, B. subtilis, P. mirabilis, Salmonella typhi, Vibrio cholera, Shigella dysenteriae* and *Streptococcus viridans* (Cottrell, 2004). However, allicin is highly unstable and, when exposed to heat, its biological activity is rapidly destroyed (Kumar & Sharma, 2009). Autoclaving garlic extract or heating the extract at 100°C for 20 min results in complete loss of its activity (Sato et al., 1990; Shashikanth et al., 1981). Yet, at -20°C, the antibacterial activity of garlic extract is maintained for ninety days (Durairaj et al., 2009; Harris et al., 2001).

Moreover, many studies have demonstrated that the antibacterial activity of several antibiotics is enhanced by garlic extracts and allicin against resistant bacteria, a process known as synergism. Here some examples of synergistic activities between garlic and antibiotics: Garlic ethanol extract in combination with nitrofurantoin shows a synergistic activity against ESBL-producing *E. coli* (Wali & Awad, 2014). Moreover, fresh garlic extract and ampicillin displays synergy against ampicillin resistant *S. aureus* (Pillai et al., 2013). Synergism appears also in fresh garlic extract or allicin when combined with vancomycin against vancomycin-resistant enterococci (Jonkers et al., 1999). Allicin and β-lactam antibiotics cefazolin and oxacillin are also a synergistic combination against *S. aureus* and *S. epidermidis* clinical isolates (Cai et al., 2007). One

more synergistic activity is noticed between allicin and cefoperazone against resistant *P. aeruginosa* (Cai et al., 2007).

2.6.2 Mechanism of antibacterial activity

The thiosulfinate allicin is the main attributor to the prime antibacterial activity of garlic which has many targets in the bacterial cell. Allicin works by inhibition of many processes inside these cells:

1- Inhibition of sulfydryl enzymes including enzymes for primary metabolism like succinic dehydrogenase, hexokinase, alcohol dehydrogenase and triosphosphate dehydrogenase (Wills, 1956) by inactivating the thiol group (Rabinkov et al., 1998; Roos et al., 2013). This activity is due to the sulfhydryl modifying activity of Allicin (Wills, 1956)

2- Inhibition of some enzymes that do not contain the thiol group (Borlinghaus et al., 2014).

3- Inhibition of RNA synthesis inside the bacterial cell (Hindi, 2013).

4- Inhibition of DNA and protein synthesis leading to the inhibition of the growth and development of the bacteria (Durairaj et al., 2009; Hindi, 2013).

5- Interference of Allicin with lipid synthesis leading to a malformation of the phospholipid bilayer of the bacterial cell wall.Therefore, the bacteria cannot grow in the presence of garlic extract or allicin (Durairaj et al., 2009; Hindi, 2013).

Besides Allicin, there is other organosulfur compounds that are responsible for the antibacterial activity of garlic but to a lesser extent (Borlinghaus et al., 2014). The increase in the number of sulfur atoms in these compounds causes an increase in the antimicrobial activity. Many morphological changes occur after exposure to these

organosulfur compounds including a loss of cell wall, cell membrane and intracellular matrix structural integrity resulting in their breakage, condensation of cellular material and leakage of cytoplasmic components and membrane fragments (Lu et al., 2011). With these wide destinations that garlic extracts and allicin can access inside the cell, it is very difficult for bacteria to develop resistance. This acute activity makes garlic suitable to be used in fighting hospital-based pathogens (Abubakar, 2009), since it has been reported that the development of resistance to beta-lactam antibiotics was 1000-fold easier than the development of resistance to allicin.

2.7 Interaction of plant extracts and antibiotics

Combinations of plant extracts with antibiotics lead to different modes of interactions. These interactions are classified mainly as synergism, additive or indifference, and antagonism (Pillai et al., 2005).

Synergism is a positive interaction where the combined effect of the drug and plant extract is significantly greater than the expected result, based on their individual effects when the agents are used separately (Pillai et al., 2005).

The additive effect results when the effect of combination is the sum of the separate effects of the agents being tested if they do not interact with one another (Pillai et al., 2005); however, indifference occurs when the combined effect of agents that do not interact with one another is the effect of the more active agent alone (King & Krogstad, 1983; King et al., 1981).

Antagonism is a negative interaction where the combined effect of the agents is significantly less than their independent effects when tested separately (Pillai et al., 2005).

3. Materials and Methods

3.1 Media

Mannitol Salt Agar (MSA; Conda/Pronadisa, Madrid, Spain), Nutrient Agar (NA; HiMedia, Mumbai, India), MacConkey (Mc; HiMedia, Mumbai, India) agar, Muller Hinton agar (MHA; Conda/Pronadisa, Madrid, Spain), Nutrient broth (NB; HiMedia, Mumbai, India) and Muller Hinton broth (MHB; Conda/Pronadisa, Madrid, Spain) were prepared according to the manufacturer's instructions.

3.2 Strains and isolates

ESBL-producing *E. coli (11 strains)* were streaked on MacConkey (Mc) agar. The mentioned bacteria were streaked using the four-quadrant technique to isolate single colonies. All the plates were incubated at 37°C for 24h.

Subcultures were made 24-48h before performing the respective experiment by taking a single colony from the agar plate and streaking it on its relevant new agar plate using the four-quadrant technique for the bacteria. The plates were incubated at 37°C for 24h.

3.3 Sample collection

Garlic *(Allium sativum)* was obtained from a local store in Lebanon.

3.4 Garlic and cefotaxime solutions preparations

Garlic bulbs were tested using ethanol extracts. Cefotaxime (Sanofi-aventis, Paris, France) was obtained as a powder. A stock solution of 10^5 µg/ml was prepared and stored at -20°C for further use.

3.4.1.1 Garlic bulbs ethanol extracts preparations

Raw garlic cloves were soaked in water for 1 day. Sixty grams of garlic cloves were washed with distilled water, cut into small pieces and crushed in an electric

blender with 30% ethanol for ethanol extract preparation. The crushed material was centrifuged (Z 323 K model, Hermle; Wehingen, Germany) for 15min at 5500 rpm. The supernatant was collected and filtered through a sintered glass filter No 1, then through a sintered glass filter No 3, then through Whatman No 1 filter paper, and finally through a qualitative filter paper with a size/diameter of 240mm. Stock solution of: 1.51 x 10^6µg/ml for garlic 30% ethanol extract was obtained. Finally, aliquots were transferred into sterile cryotubes (1.8mL) and stored at -80°C for further use.

3.5 Testing for the antibacterial activity

The antibacterial activity of garlic extracts and cefotaxime was investigated alone, and in paired combinations, against selected bacteria using the microdilution method and the checkerboard technique.

3.5.1.1 Antibacterial activity of cefotaxime (CTX) using the disk diffusion method

Cefotaxime was screened against ESBL-producing *E. coli* using the disk diffusion method. Inoculum preparation was as following: using a sterile loop, 2 to 3 normal appearing colonies were taken from each bacterial plate and suspended in 2 mL sterilized distilled water. The tubes were adjusted equivalent to 0.5 McFarland standard (approximately $1.5x10^8$ CFU/ml) and diluted with sterilized distilled water to $1.5x10^6$ CFU/ml. Within 15 min of preparing the inoculum, the surface of the MHA plates was flooded with 2 mL of the inoculated sterile water or nutrient broth ($1.5x10^6$ CFU/mL), shaken gently and left to stand for 2 or 3 minutes. Excess inoculum was removed using a sterile pipette. CTX (30µg) disks were added to its respective well. The plates were left to dry for 30 minutes and then were turned upside down and incubated at 37°C for 18 hours. Following the incubation period, the diameter of the inhibition zones was measured in mm.

3.5.1.2 Detection of ESBL production by Double disk synergy test

The double disk synergy test was designed to detect ESBL production in *Escherichia coli*. The test was performed on agar with a 30µg disk of cefotaxime and a disk of amoxicillin clavulanate (containing 10 µg of clavulanate) positioned at a distance of 30 mm. The test was considered as positive when a decreased susceptibility to cefotaxime was combined with a clear-cut enhancement of the inhibition zone of cefotaxime in front of the clavulanate-containing disk, often resulting in a characteristic shape-zone referred to as 'champagne-cork' or 'keyhole'.

3.5.1.3 Determining the minimum inhibitory concentration (MIC)

The MIC of garlic 30% ethanol extract (EG) and CTX was determined against ESBL-producing *E. coli* using microdilution method. One row of the 96-well sterile microtiter plate was used for each test with up to 10 different dilutions. 200 µL of MHB was dispersed into the first well and well 12 (sterility control well) of the microtiter plate and 100 µL of MHB was dispersed into wells 2 till 11. Based on the calculations done, 22.8µl of MHB was removed from the first well and 22.8µl of EG was added to have a starting concentration of 85500µg/ml for all the strains. For dispensing Cefotaxime (CTX), 65.53µL of MHB was removed from the first well and 65.53 µL of 10^5µg/mL CTX was added to have a starting concentration of 16384µg/ml against strain 1, 2, 7 and 8; 32,76 µL of 10^5µg/mL CTX added to have a starting concentration of 8192µg/ml against strain 5 and 10; 16.38 µL of 10^5µg/mL CTX added to have a starting concentration of 4096 µg/ml against strain 11; and 81,92 µL of 10^4µg/mL CTX added to have a starting concentration of 2048µg/ml against strain 3, 4 and 6 (table 1). Then, twofold serial dilution of EG or CTX was carried in which 100 µL was transferred from one well to another and the last 100 µL from the last column (column 10) was

discarded. Following twofold serial dilution, each column containing the EG or CTX solution and the growth control well (well 11) were inoculated with 100µL of the bacterial suspension starting from column 11 backward till column 1. This resulted in the final desired inoculum of 5 x10^5 CFU/ml. The microplates were covered with a plastic lid and incubated at 37°C from 16–20h. From one to four hours before the end of the incubation period, 50 µL of 0.01% solution of 2, 3, 5-Triphenyl Tetrazolium Chloride (TTC) may be added if turbidity is not observed visually. TTC is a colorless salt that changes to red in the presence of viable bacteria. Following the incubation period, the MIC panels were read as follows:

1. The positive control well was checked for turbidity. Turbidity should be present, indicating adequate growth of the bacteria.
2. The negative control well was checked for the absence of turbidity.
3. The MIC endpoint was read as the lowest concentration of antimicrobial agent that completely inhibited the growth of the organism as detected by the unaided eye or TTC (Coyle, 2005; Lessem, 2007; Simmons & Williams, 1967; Witkowska et al., 2013).

Table 1. Total volume of cefotaxime used in the minimum inhibitory concentration with the starting concentration of each isolate

Isolates	Total Volume (µL)	Starting concentration (µg/ml)
1	65.53	16384
2	65.53	16384
3	81.92	2048
4	81.92	2048
5	32.76	8192
6	81.92	2048
7	65.53	16384
8	65.53	16384
9	32.76	8192
10	32.76	8192
11	16.38	4096

3.5.1.4 Synergy testing of garlic 30% ethanol extract (EG) and cefotaxime (CTX) using the checkerboard technique

Synergistic activity was determined for EG and CTX against ESBL-producing *E. coli* using the checkerboard technique.

3.5.1.5 Details of the procedure followed

First, in panel B: 200µl of MHB was added from H1 → H12 and 100µl of MHB was added in all wells except row A. For each type of bacteria, a specific volume of MHB was removed and cefotaxime was added into each well of row H (H1 → H12). This was followed by twofold serial dilution from H to B where 100µL was removed from one row to the other using a multichannel pipette to have a final concentration of 8x MIC to MIC/8. The last 100µl was discarded from row B.

Second, in panel A: 100µL of MHB was added from A12→H12 and 50µl of MHB was added in all wells except column 1. For each type of bacteria, a specific volume of MHB was removed and EG was added into each well of column 12 (A12→H12). This was followed by twofold serial dilution from column 12 to 2 where 50µl was removed from one column to the other using a multichannel pipette to have a final concentration of 32 x MIC to MIC/32. The last 50µl was discarded from column 2. 150µl MH broth was added to A1 to serve as a negative control.

Third, after serially diluting panel A and panel B, 50µl from each well of panel B was transferred into the corresponding well of panel A using a multichannel pipette. This was followed by addition of 50µl of the bacterial inoculum to the wells containing the combination of extracts and antibiotic and 100µl of bacterial inoculum to the wells having one type of extract or antibiotic (A12→A2 & B1→H1) to have a total volume of 150µl and an inoculum of 5 x10^5 CFU/ml.

Finally, the microplates were covered with a plastic lid and incubated at 37°C for 18-24h. Note that 50 µL of a 0.01 % solution of 2, 3, 5-Triphenyl Tetrazolium Chloride (TTC) could be applied to the wells 1-4 hours before the end of the incubation.

3.5.1.6 Interpretation of the results:

The FIC_{index} was calculated based on the equation:

$$\sum FIC = FIC\ of\ EG + FIC\ of\ CTX = \frac{MIC\ of\ EG\ in\ combination}{MIC\ of\ EG\ alone} + \frac{MIC\ of\ CTX\ in\ combination}{MIC\ of\ CTX\ alone}$$

The results were classified as: Synergy for $\Sigma FIC \leq 0.5$, Indifference for $0.5 < \Sigma FIC \leq 4$, and Antagonism for $\Sigma FIC > 4$ (Ben-Ami et al., 2011; Daoud et al., 2013; Hsieh et al., 1993; Schwalbe et al., 2007).

3.6 Detection of ESBL type using PCR

Multiplex PCR Master Mix, with template DNA, and primer mix were thawed first. Bacterial DNA extraction was done by suspending one or two colonies of each test isolate grown overnight in 200 µl of distilled water in 2 ml Eppendorf tubes, followed by heating the solution at 95°C for 10 min. The tubes were then centrifuged for 10 minutes at maximum speed at 4°C. Then, a mixture of all primers was prepared following manufacturer's instruction, containing 10 µl of each primer. Mixing all the primers in one tube was done to avoid pipetting of individual primers for each experiment, to reduce pipetting time and to increase reproducibility of results. The presence of blaCTX-M, blaSHV, blaTEM, and blaOXA genes was tested using previously published primer sets and conditions. A reaction mix was also prepared, containing all the components required for multiplex PCR except the template DNA. Therefore, 11 PCR tubes were labeled containing 19 µl of the reaction mix with 1 µl DNA. The PCR reaction conditions involved a 15 min denaturation step at 95 °C, followed by 30 amplification cycles of 30 s at 94 °C, 90 s at 62 °C, and 60 s at 72 °C, with a final extension step of 10 min at 72 °C (Daoud et al., 2013). The primer sequences with the expected amplicon sizes of the target ESBL genes were: for blaSHV: F-CTTTATCGGCCCTCACTCAA, R-AGGTGCTCATCATGGGAAAG (327 bp); blaTEM: F-CGCCGCATACAC- TATTCTCAGAATGA, R-ACGCTCACCGGCTCCAGATTTAT (445 bp); blaCTX-M: F-ATGTGCAGYACCAGTAARGTKATGGC, R-TGGGTRAAR-TARGTSACCAGAAYCAGCGG (593 bp); blaOXA: F-ACACAATACATAT-CAACTTCGC, R-AGTGTGTTTAGAATGGTGATC (813 bp) (table 2). PCR products were visualized by gel electrophoresis. The gel was prepared by weighing 1 g of

agarose into an Erlenmeyer flask with 100 ml of 1× TAE buffer added. Then, the agarose/buffer mixture was melted by heating in a microwave for 1 min. At 30s intervals, the flask was removed to swirl the content for a total mixing. This step was repeated until the agarose was completely dissolved. 5 µl of Ethidium Bromide (EtBr) was added and the solution was left to cool. After placing the gel tray into the casting apparatus with an appropriate comb to create the wells, the molten agarose was poured into the gel mold and allowed to set at room temperature. Then, the comb was removed and the gel was placed in the gel box. In new tubes, 12 µl from each sample tube was mixed with 2 µl of Thermo Scientific loading dye. Each well on the gel was filled with its respective mixture. The gel was run at 130 V for 45 min. Amplicons were visualized using an ultraviolet transilluminator system for analysis. The gel had one well containing a DNA ladder (100 bp; Thermo Scientific) in order to be able to estimate the size of the DNA amplicons. A negative control was used containing all the mixture except the template DNA.

Table 2. Amplicons with primers sequence of each genes and their respective sizes.

Amplicon	Primer sequence (5' to 3')	Size (bp)
bla_{SHV}	CTTTATCGCCCCTCACTCAA AGGTGCTCATCATGGGAAAG	237
bla_{TEM}	CGCCGCATACACTATTCTCAGAATGA ACGCTCACCGGCTCCAGATTTAT	445
$bla_{CTX\text{-}M}$	ATGTGCAGYACCAGTAARGTKATGGC TGGGTRAARTARGTSACCAGAAYCAGCGG	593
bla_{OXA}	ACACAATACATATCAACTTCGC AGTGTGTTTAGAATGGTGATC	813

4. Results

The ultimate aim of this study was to investigate the possible ameliorative effect of cefotaxime when combined with garlic against the ESBL-producing *E. coli* and to study, at a molecular level, the influence of ESBL plasmid carriage on the host bacterium expansion of resistance by characterizing the genotypic properties of ESBL-producing *Escherichia coli* and correlates it with the efficacy of combinations of cefotaxime and garlic. Before determining the effect of the combination, the antibacterial activity of cefotaxime was investigated qualitatively by the agar well diffusion method and through the double disk synergy test. Then, the effect of the combination between cefotaxime and garlic was determined quantitatively by the checkerboard technique. Finally, ESBL isolates were subjected to multiplex PCR to screen for resistant genes and to estimate a possible correlation between the genotype and the MIC of the isolates.

4.1 Determination of ESBL- producing E. coli isolates

30μg Cefotaxime (CTX) was selected to qualitatively investigate its inhibitory effect against ESBL-producing *E. coli*. The results of the current study showed that 30μg cefotaxime had inhibition zones ranging approximately between 7mm and 22mm against all isolates of ESBLs (figure 1). No isolates were found susceptible for cefotaxime. For cefotaxime ≥26 is susceptible, 23-25 is intermediate and ≤22 is resistant. Therefore, they are considered resistant as shown in table 3.

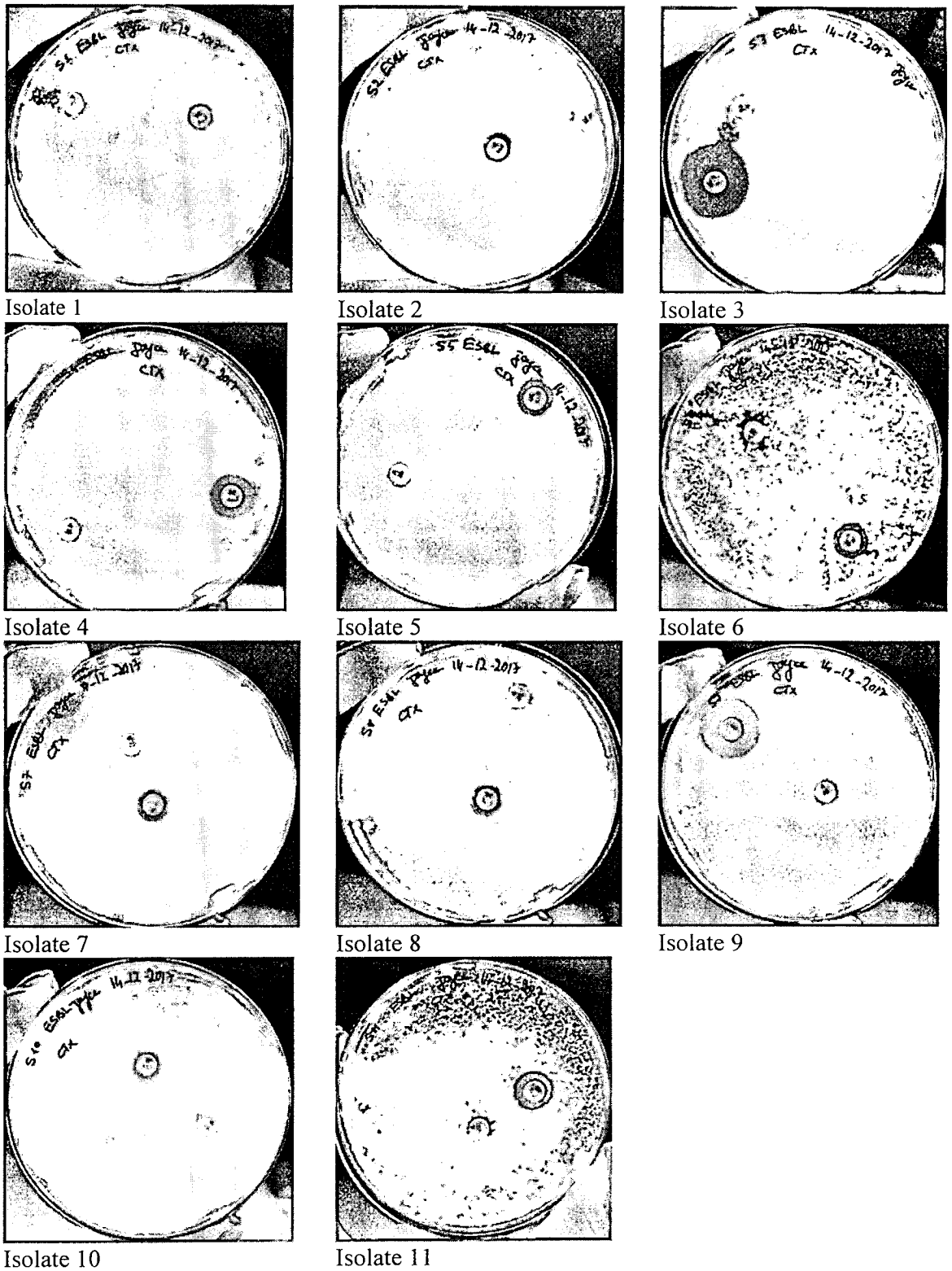

Figure 1. Inhibition zones obtained by 30µg cefotaxime (CTX) against ESBL-producing Escherichia coli (106 CFU/mL) isolates.

Table 3. Antibacterial activity of 30μg cefotaxime against ESBL-producing E. coli.

Isolates of ESBL-producing *E. coli*	Inhibition zone (mm)
1	7
2	8
3	22
4	13
5	10
6	10
7	10
8	10
9	18
10	13
11	13

4.2 Testing for interaction between garlic 30% ethanol extract (EG) and cefotaxime (CTX) using checkerboard technique

The minimum inhibitory concentration (MIC) results of garlic 30% ethanol extract (EG) and cefotaxime (CTX) against, ESBL-producing *E. coli* are presented in Table 4. All isolates showed an approximately similar MICs of garlic ranging between 2671.875 and 5343.75 μg/ml except for isolate 2 having a MIC of 42750 μg/ml. For cefotaxime MICs, isolates showed a wide range of MICs with isolate 2, isolate 9 and isolate 11 having the highest values (512 μg/ml), and isolate 3 having the lowest MIC (16 μg/ml).

Table 4. MIC of garlic 30% ethanol extract (EG) and MIC cefotaxime (CTX) against ESBL-producing E. coli with the fractional inhibitory concentration index (ΣFIC).

Isolate	MIC of EG	MIC of CTX	ΣFIC_{min}	ΣFIC_{max}	ΣFIC_{ave}
1	2671.8 – 5343.7	256	1.72	3.30	2.43
2	42750.0	512	1.06	2.50	1.72
3	5343.7	16	1.05	3.16	1.88
4	2671.8 – 5343.7	32 – 64	1.7	3.00	2.34
5	2671.8 – 5343.7	64 – 128	2.016	3.41	2.9
6	2671.8	32 – 64	1.35	3.33	3.41
7	5343.7	128 – 256	2.01	3.16	2.5
8	5343.7	128 – 256	1.016	2.50	2.39
9	5343.7	256 – 512	0.53	3.50	0.87
10	2671.8 – 5343.7	128 – 256	1.45	4.60	3.02
11	2671.8– 5343.7	256 – 512	1.04	2.50	1.81

After determining the MIC values, the interactive activity of EG and CTX was examined against ESBL-producing *E. coli* using the checkerboard technique. FIC index (ΣFIC) was calculated for each well along the growth-no-growth interface. FIC index was used to determine the type of association between cefotaxime and garlic extract. Based on the FIC index, 10 strains exhibited additive and indifference effect each. One isolate showed antagonism in specific wells with $\Sigma FIC \geq 4$ but having in average ΣFIC showing an indifference activity. None of the isolates showed a synergistic effect between garlic and cefotaxime.

4.3 Genetic molecular characterization of beta- lactamase genes

Multiplex PCR was performed in order to determine the type(s) of ESBLs present in each of the clinical isolates (*bla* CTX-M, *bla* SHV, *bla* TEM, and *bla* OXA), which are sequence-specific primers capable of distinguishing the genes coding for the main ESBL types were used in this experiment (Fang et al., 2008). Amplicons of different sizes produced by each reaction allowed a clear determination of which genes were present in a single reaction (figure 1).

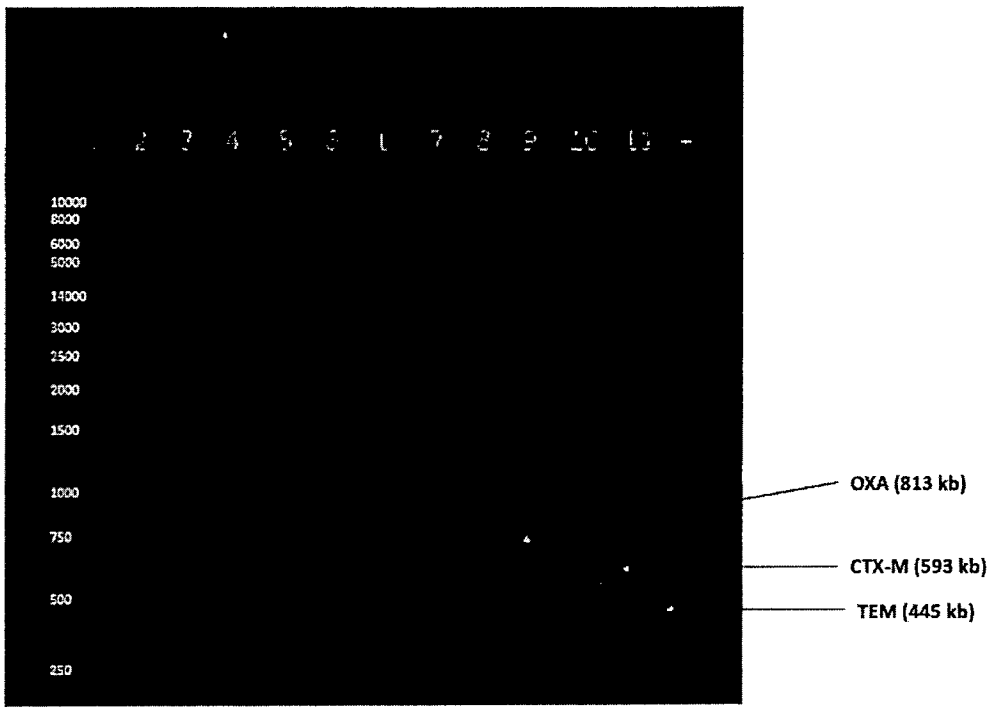

Figure 2. Amplified products to TEM, SHV, OXA, and CTX-M genes. 1% agarose gel analysis of PCR amplified fragments of blaTEM, blaOXA, blaSHV and blaCTX-M using specific primers. Lanes 1-11 are PCR products of ESBL genes from *E. coli* isolates. Ladder size 100 bp is shown in Lane L.

The results showed that out of 11 ESBL- producing *E. coli*, 5 isolates (45.5 %) harbored 2 β-lactamase genes and the 6 remaining isolates (54.5 %) harbored just a single β-lactamase gene. The most common ESBL gene belonged to the CTX-M group,

with all isolates of *E. coli* containing this allele except isolate 9 and 11. Isolates 1, 4, 6 and 10 harbored CTX-M only, while isolates 2, 3, 5 and 8 harbored CTX-M and OXA genes. Isolate 7 was the only strain harboring CTX-M and TEM genes together. TEM was found in isolate 9 and 11 (table 5 and 6).

Table 5. Molecular characterization of β-lactamase genes among ESBL-producing *E.coli* isolates (n=11).

bla genes	No. of isolates
CTX-M	4 (36.36%)
TEM	2 (18.18%)
CTX-M and OXA	4 (36.36%)
CTX-M and TEM	1 (9.09%)

Table 6. Presence (+) or absence (-) of *bla* genes using multiplex PCR genotyping results of ESBL-producing *E coli* isolates.

Isolate	CTX-M	CTX-M	CTX-M	CTX-M
1	+	-	-	-
2	+	-	+	-
3	+	-	+	-
4	+	-	-	-
5	+	-	+	-
6	+	-	-	-
7	+	+	-	-
8	+	-	+	-
9	-	+	-	-
10	+	-	-	-
11	-	+	-	-

The MICs of cefotaxime of the isolates with the most-prevalent ESBL genotypes bla_{CTX-M} were high to moderate ranging between 32 and 256 µg/ml and having the same MICs of garlic (isolates 1, 4, 6 and 10). Interestingly, some of the bla genotypes harboring CTX-M and OXA genes showed clinically significant differences in MIC levels for different isolates. In fact, despite the fact that isolates 2, 3, 5, and 8 harbored all CTX-M and OXA genes, MICs of CTX for each isolate differed, being respectively 512, 16, 128 and 256 µg/ml. on the other hand, isolate 2 had the highest MIC of garlic, 42750 µg/ml. Bla_{TEM} isolates (isolate 9 and 11) expressed high MICs of cefotaxime 512 µg/ml. Isolate 7 is the only isolate harboring CTX-M and TEM genes together (256 µg/ml) (table 7).

Table 7. Phenotypic and genotypic characteristics of ESBL-producing *E. coli* including zone of inhibition, MICs and presence of *bla* genes

Isolate	CTX-M	TEM	SHV	OXA	Zone Of Inhibition (mm)	MIC of CTX (µg/ml)	MIC of garlic (µg/ml)
1	+	-	-	-	7	256	5343.7
2	+	-	-	+	8	512	42750
3	+	-	-	+	22	16	5343.7
4	+	-	-	-	13	32	5343.7
5	+	-	-	+	10	128	5343.7
6	+	-	-	-	10	64	2671.8
7	+	+	-	-	10	128	5343.7
8	+	-	-	+	1	256	5343.7
9	-	+	-	-	18	256	5343.7
10	+	-	-	-	13	256	5343.7
11	-	+	-	-	13	512	5343.7

5. Discussion

This study was conducted to investigate the effect of garlic ethanolic extract and the antibiotic cefotaxime against a selected group of isolates of ESBL- producing *E. coli,* and to study, at a molecular level, the genotypic properties of these isolates.

The isolates tested showed variable sensitivities to cefotaxime and garlic extracts. All isolates were verified and confirmed to be ESBL positive strains by the disk diffusion method and by the Double Disk Synergy test. The isolates were resistant to cefotaxime with variable inhibition zones that are less than 22mm, the cut-off value for resistance (Hombach et al., 2012). Our results substantiate earlier findings where ESBL conferred resistance to cefotaxime by 99.2% according to Wani et al., 2009.

Regarding garlic, isolates showed variable MIC results to garlic ethanolic extract with the minimum inhibitory concentration method. Our results substantiate earlier findings, where garlic ethanolic extract had inhibitory activity against ESBL producing multidrug resistant bacteria including *E. coli* (Abdulzahra & Mohammed, 2014; Abubakar, 2009; Durairaj et al., 2009; Elsom et al., 2000; Gull et al., 2012; Kumar *et al.*, 2012; Shayan et al., 2014).

Checkerboard assay was used to determine the interaction of the combined activity of cefotaxime and ethanolic garlic extract by determining the FIC index. Antagonism was found in specific wells of the isolate 10 although the average FIC index showed an indifference activity. This finding merit to be more investigated in the future to study the mechanism of resistance of this isolate that showed an antagonistic activity with the combination of garlic and cefotaxime. All remaining strains exhibited additive or indifference activity in specific wells. No synergistic effect between garlic and cefotaxime was found. These results differ from those reported by Halawi, 2017

who conducted similar study on ESBL-producing *E. coli* and obtained many wells with synergistic effect.

In contrast to our study, many studies reported a synergistic activity when combining ethanolic garlic extracts and antibiotics against ESBLs. According to Wali & Awad (2014), a synergism between garlic ethanol extract and nitrofurantoin was present against ESBL-producing *E. coli* ($\Sigma FIC \leq 0.5$). Halawi, 2017 stated also a synergistic activity between ethanolic garlic extract and cefotaxime against ESBL *E. coli.* The difference between these findings and our results regarding synergy may be due to the difference in garlic's genotype, age and. concentrations of active incredients (Bokaeian & Bameri, 2013 and Iwalokun et al., 2004),

In this study, the molecular profile of the isolates for the presence of beta-lactamase genes was investigated. The results of the study confirmed the high prevalence of CTX-M genes alone or in combination with other genes (90.9%). All isolates of *E. coli* containing this gene except two isolates nine and eleven. The combination of CTX-M and OXA genes were also present in relatively high levels (36.36%). CTX-M and TEM genes together were present in 9.09% of the isolates. TEM was found in less frequency (18.18%).

The predominance of CTX-M is consistent with an earlier report that found CTX-M as the most widespread β-lactamase in Lebanon (Moubareck et al., 2005; Salem et al., 2013). In 2007, Seputiene et al. also showed the high prevalence of CTX-M among E. coli (96%) and K. pneumoniae (71%) isolates. A study conducted in Portugal in 2007 showed that CTX-M strains were prevalent among urinary tract infections (76%), where 181 unduplicated E. coli strains were isolated in nine different hospitals and studied genotypically (Mendonça *et. Al,* 2007). Another study conducted

in Turkey on 2013 showed that CTX-M was the most prevalent beta-lactamase among ESBL-producing E. coli isolates (83.18%) (Copur Cicek *et. Al,* 2013).

Other studies from different geographic locations showed variable results regarding the prevalence of beta lactamase genes. Bajpai et. Al, 2017 reported that TEM gene predominated the SHV and CTX-M genes responsible for ESBL production. Until the year 2000, TEM was the most prevalent ESBL gene in the Indian bacterial population but was replaced by CTX-M in the following decade (Shahid *et. Al,* 2011). This difference between the results of the studies indicated that the prevalence and type of ESBL genes may vary from one geographical region to another.

Regarding the MICs and the molecular profile of the beta lactamase genotypes, the behavior of the isolates having the same profile is different. Beta lactamase genotypes harboring a combination of CTX-M and OXA genes have been shown to have noticeable differences in MIC levels among isolates. Also, 4 isolates harbored CTX-M gene in their profile, yet they had different MICs. This was also applicable for isolates harboring TEM genes. Therefore, there is no correlation between the molecular profile of beta lactamase genes and the degree of resistance to cefotaxime and garlic. This is in agreement with a previous study conducted by Harris, 2014 that stated the non-necessary correlation between the genotype and the phenotype of bacteria.

In fact, this absence of correlation between phenotype and genotype may be due extra genes conferring this variance in resistance that needed to be studied in the future, or even due to some mutations in the currently studied genes. Our work was focused on the four beta-lactamase genes OXA, CTX-M, TEM and SHV because they are among the most prevalent genes present in Lebanon (Moubareck et al., 2005; Salem et al., 2013). It is of great importance for these beta lactamase genes to be sequenced in further

studies because DNA sequencing can characterize the plasmids in more details for determining if the resistance is correlated with these genes or there are extra genes that needed to be sequenced and studied in the future. Regarding sequencing, it is worth mentioning that CTX-M-15 in particular should be studied and sequenced in the future since it was found to be the most widespread β-lactamase in Lebanon (Salem et al., 2013).

Among the 11 isolates tested, isolate 2 showed the highest MIC for garlic ethanolic extract, 42750 μg/ml, and at the same time the highest MIC for cefotaxime, 512 μg/ml. This is an important finding that merits further investigation in order to understand the mechanism of this resistance. To our knowledge, this is the first report of isolating an ESBL-producing *E. coli* that is highly resistant to ethanolic garlic extract. This may pose a threat to the clinical usefulness of using garlic as an antibacterial agent against ESBL-Producing E. coli. This source of resistance may be due to extra genes conferring this high resistance. Garlic extract have different mode of actions against bacteria. In fact, the active ingredient in garlic, allicin, to which the antibacterial activity of garlic is widely attributed, works by inhibiting RNA synthesis as a primary target and acts by partially inhibiting DNA and protein synthesis (Eja et al., 2007). Moreover, organosulfur and phenolic compounds have been reported to be involved in the antimicrobial activity of garlic (Griffiths et al., 2002; Jombo et al., 2011). In view of the different mode of action of garlic as an antibacterial agent that affect multiple genes (polygenic) in bacteria, it is less likely that resistance will develop due to a reduced selection pressure. Future work will emphasize the molecular characterization of isolate two to better characterize this source of resistance.

6. Conclusion

The increasing spread of ESBL-producing organisms remains a big threat affecting the clinical outcome of patients. There is an urgent need for new antibiotics, and enhancements in the antibiotic programs, and regulations. *In vitro* studies showed that strains of ESBL-producing *E. coli* confer resistance to cefotaxime and that garlic ethanolic extract had antibacterial activity against these gram-negative bacteria resistant strains. The combination of garlic and cefotaxime showed overall an indifference effect against the tested strains. Further studies are needed to explore the antimicrobial effects of the active compounds of garlic against ESBL-producing *E. coli* as an alternative approach to bacterial resistance management. The highly resistant isolate of ESBL-producing E. coli to ethanolic garlic extract discovered in this study deserves further research in order to understand the mechanism of this resistance.

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