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Inhibition of ***Escherichia coli*** biofilm formation by natural extracts

By

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Dedication

To my loving parents

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Inhibition of *E. coli* biofilm formation by natural extracts

Mohammad Jaafar

ABSTRACT

Biofilms are defined as a buildup of microorganisms in a mostly polysaccharide environment, rendering bacterial growth immotile and sessile. Biofilm formation is well known to facilitate bacterial adherence to multiple surfaces that may include live tissue as well as inert matter. Once microorganisms initially attach themselves to such surfaces they synthesize a dome like extracellular polymer that results in the formation of a highly hydrated matrix. *Escherichia coli*, a known biofilm former and a cause of many infections, over time, developed resistance against many antibacterial agents, creating a major problem in treating patients with infection caused by this organism. Uropathogenic *E.coli* strains that were isolated from patients in LAUMC-RH were definitively identified, tested for the production of extended spectrum beta lactamases (ESBL). and genetically characterized by PFGE. The strains of *E. coli* selected (10 ESBLs producing and 10 non-ESBLs producing) were those already tested for their ability to produce biofilms. A number of natural products was tested for their effect on the ability of the test strains to form their biofilm. Two of the tested plant extracts were proved to influence biofilm formation of the tested strains: namely: *Salvia officinalis* (sage) and *Thymus vulgaris* (thyme). The major organic compounds that mainly make up these two plants were individually tested for their ability to inhibit the biofilm formation of the tested strains. Camphor from sage and terpinene from thyme demonstrated a clear effect to inhibit biofilm formation of the two groups of *E.coli* tested at different molar concentrations (0.025, 0.05, and 0.10) The use of combinations of these compounds did not demonstrate synergism in their ability to inhibit biofilm formation.

Keywords: Biofilms, *Escherichia coli*, *Salvia officinalis* and *Thymus vulgaris*, camphor, terpinene, ESBL.

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List of Abbreviations

PFGE: Pulsed field gel electrophoresis

ESBL: Extended spectrum beta-lactamases

N-ESBL: Non- Extended spectrum beta-lactamases

XbaI : Recommended restriction enzyme

E.coli : *Escherichia coli*

G: garlic ethanolic extract

O: onion ethanolic extract

P: parsley ethanolic extract

M: mint ethanolic extract

Cl: clover leaf ethanolic extract

Ba: basil ethanolic extract

Po: pomegranate ethanolic extract

R: rosemary ethanolic extract

S: sage ethanolic extract

T: thyme ethanolic extract

Ty: thymol ethanolic solution

Cy: cymene ethanolic solution

Te: terpinene ethanolic solution

Ca: camphor ethanolic solution

Th: thujone ethanolic solution

Pi: pinene ethanolic solution

CHAPTER I

Introduction

1.1 *Escherichia coli*

1.1.1 Introduction

The main goal once dealing with infectious diseases is to eliminate the main cause, the microbial organisms behind such conditions (Hentzer & Givskov. 2003.). One possible approach is by total and utter elimination of the colonies, through the administration of antimicrobials (Lewis, 2001.).

The first antibiotic was discovered by Alexander Fleming back in 1928. He was able then to isolate and concentrate an active antimicrobial substance from *Penicillium* which he then named penicillin (Clardy et al, 2009.). This radical sighting led to an increase in research and development in the field of antibiotic production building up the prospect it is in today (Clardy et al, 2009.); where a number of major antibiotics exist and work on inhibiting bacterial growth effectively, with the least side effects possible Hentzer & Givskov, 2003.).

The continuous exposure of microrganisms to antibiotics introduced and increased their ability to adapt, evolve, and resist such agents, through many techniques and pathways (Hancock, 1998.). Mainly their aptitude to degrade antibacterial compounds by the use of enzymes that target the active site of such compounds similar to beta-lactamase; or by the facility of reducing the permeability of antibiotics by the production and formation of biofilms (Hancock, 1998.).

The increase in mutation frequency and bacterial resistance against antibiotics shifted the scope of research into an era of post-antibiotic treatments, where the focus now depends on the capacity to reduce bacterial virulence and disrupt infection formation through the application of antipathogenic compounds, composites that can simultaneously able to reduce virulence and clear the host immune system (Hancock, 1998., Hentzer & Givskov, 2003.).

Plants and natural components form the scaffold of such complexes, since they were always thought to have various medicinal roles, and distinctive healing abilities and potential, long before the discovery of microbes and antibiotics (Heinrich et al, 2004); for this microbiologist, botanists, and physicians have shifted their focus onto

plant essential oils and flavonoids in an attempt to resolve this widely spread threat (Ncube et al, 2008.)

1.1.2 E.coli

Escherichia coli is, a gram negative, rod-shaped bacterium usually found in the intestine of warm blooded animals like humans and represent a highly diverse group of microbes. While the majority of *E.coli* strains are harmless and line the normal flora, some strains are highly pathogenic and may lead to serious and sometimes deadly conditions such as urinary tract infections, diarrhea, respiratory illness and pneumonia. Pathotypes, disease-causing variant, is the mode of pathogenic *E.coli* categorization and several pathotypes were associated with diarrhea such as Enterotoxigenic *E.coli* (ETEC), Shiga toxin-producing *E. coli* (STEC), and Enteroaggregative *E*. *coli* (EAEC). Moreover, and due to the increased exposure to antibiotics, *E.coli* strains began forming and acquiring resistance against effective groups of antibiotics (center of disease control and prevention, 2012).

1.1.3 Extended spectrum beta lactamases

Peptidoglycans are an essential component of the cell wall of both gram positive and gram negative bacteria. It's formed of alternating units of N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) lycan chains with tetrapeptide side chains

and polypeptide cross bridges. The final stages of peptidoglycan synhesis, in gram-negative bacteria, which occur at the level of the periplasmic membrane comprises a specific set of proteins, penicillin binding protein (PBPs), involved in conserving the firmness of the peptidoglycan and the sustainability of the bacterial cell. (Pinheiro, 2013)

PBPs are considered to be the main targets of β-lactam antibiotics, an antibiotic encompassing a β-lactam ring, and include penicillins, cephamycins, cephalosporins, carbapenems and monobactams. Beta-lactamses work on inhibiting the activity of PBPs via an acyl-enzyme complex formation at the level of the proteins active site, specifically at the serine residue, thus inflicting bacterial cell lysis and death, through loss of selectivity elongation and lesions of the peptidoglycan. (Jacobs et al, 1997).

With time and throughout the progression of evolution, bacteria began to develop various modes of resistance against beta-lactams, one of which was the expression and biosynthesis of lytic enzymes, beta-lactamases, which dominated gram negative microbes, and that work on disrupting the beta lactam ring of the antibiotic. (Sainsbury, 2011)

Beta lactamases were found to have two different modes of action. The first was dependent on the presence of a zinc metal ion in the active site, and was found to be less common, while the other was a serine-ester mechanism following the noncovalent

binding of the enzyme to the target antibiotic, and that is with the help of a glutamate residue situated on a peptide strand in the gamma-loop of the enzyme. (Nicholas et al, 2003.).

Today beta lactamases are being classified based on their structure, via the Ambler classification, and by their given function, via the Bush-Jacoby-Medeiros classification. Ambler divided the enzymes into four distinct groups entitled A, B, C, and D based on their peptides motifs and structure. Class B was considered to represent metallo beta lactamses, thus the enzymes requiring one or two zinc ions linked to a histinde or to a cysteine in order to be able to react and hydrolyse most of the beta-lactam antibiotic including carbapenems; classes A, C, and D sustained the serine-ester mode of hydrolysis.(Bush, 1989., Bush & Jacoby 1995, 2010)

As for Bush-Jacoby-Medeiros classification, it updated Ambler grouping by assembling enzymes within the same group according to functional similarities, such as susceptibility and specificity. This updated mode of arrangement substantially improved initial diagnosis and time of treatment by physcians including group 1 and group2(Bush, 1989., Bush and Fischer, 2011.)

One way of combating beta-lactam resistant species, such as *E.coli* and *Klebsiellsa pneumoniae*, was the introduction of the third generation cephalosporins in the early 1980s, which in addition to presenting potential in fighting the resistant

bacteria also reduced the nephrotoxic effects when compared to other comparable antibacterials, like aminoglycosides and polymyxins. (Paterson and Bonomo, 2005).

This prospect did not last long for by the mid-1980s reports of plasmid coded beta-lactamases which attained the capacity of lysing third generation cephalosporin started surfacing, and was found in a number of bacterial species, including *E.coli*. (Pinheiro, 2013)

These enzymes, known as extended spectrum beta lactamases (ESBL), were found to be notorious for their acquired ability to hydrolyze and subdue the influence of first, second, and third generation cephalosporin and aztreonam, but not carbapenems and cephamycins. The main inhibitor of these was acknowledged to be clavulanic acid (Majiduddin et al, 2002).

More than two-hundred different forms of ESBLs have been segregated and categorized from the time they were discovered, some of which situated in hospital communities while others stand widespread in the community, as will be discussed shortly. (Majiduddin et al, 2005)

Following the classification scheme mentioned previously it was noted that the most prevalent ESBL enzymes, do belong to group 2be following the Bush-Jacoby-Medeiros classification, which coordinates to group A according to Ambler's cataloging. Suggesting that they present an extended spectrum of activity, hence, as well as the ability of hydrolyzing ampicillin, penicillin, and to a slighter extent cephalothin,

but lack the ablity to hydrolyze extended-spectrum aztreonam and cephalosporins to any effective grade .(Bush et al, 1995)

CTX-M variants, the more prevalent form of enzymes found in South America, and Eastern Europe, as well as India and China in more recent years. Its nomenclature refers to the high lytic action of beta-lactamases, mainly for cefotaxime. Microorganisms expressing CTX-M beta-lactamases typically have high minimal inhibitory concentrations (MICs) against cefepime, cefotaxime, and Tazobactam, ranging from around 64 μg/ml for cefotaxime to 256 μg/ml for cephalosporin, whereas these were susceptible to ceftazidime with an MIC of 8μg/ml. Aztreonam MICs are mostly flexible.(Baraniak et al, 2002., Poirel, 2002.). More than 40 different enzymes are found in the CTX enzyme family that differ from each other by some amino acid sequences where multiple SNPs (single nucleotide polymorphisms) have been perceived when members of this family are associated. These have been divided into distinct clusters, counting CTX-M-1, CTX-M-2, structurally related to the naturally produced b-lactamase of *Kluyvera ascorbata* (20 of 52), 8 associated to beta-lactamase of *Kluyvera georgian,(*21of 52) CTX-M-9 linked with enzymes from *Kluyvera* species isolated from Guyana (23of 52).

CTX-M-encoding genes are located on plasmids with sizes ranging from 7 kb to 160 kb. These plasmids were found also to convey genes encoding resistance for

numerous additional antibiotics, including aminoglycosides, sulfonamides chloramphenicol, trimethoprim, and tetracycline. Those plasmids are normally transmissible by conjugation with relatively high frequency of transfer, sometimes reaching 1:100 per donor cell, thus illuminating the ease of transmission and transfer(Sturenburg et al, 2004).

The CTX-M enzyme is around 291 amino acid residues spanning a mass of 28 kDa with amino acid positions 240 and 167 look to be tangled in the evolution of CTX-M enzymes; for instance, CTX-M-15, and CTX-M-27 originate from CTX-M-3, and CTX-M-9, respectively, by a Gly240Asp substitution, conferring an increased catalytic activity against ceftazidime. (Tzouvelekis et al, 2000)

Another group of ESBLs firmly related to *E.coli* is TEM, named after Temoneira, the name of the greek patient from which it was first isolated. (Paterson and Bonomo, 2005)

More than a hundred TEM beta-lactamase have been characterized since first reported, most of them well known to be ESBLs, with a number showing reduced susceptibility to beta-lactamase inhibitors, like tazobactams. At first, enzymes conferring the previously stated qualities lacked or showed reduced capacity to hydrolyse third generation cephalosprins and were not considerd as ESBLs. However with time, mutants showing those abilities started evolving and were named complex

mutants of TEM (CMT-1 to -4) (Sirot et al, 1997., Fiett et al, 2000., Neuwirth et al, 2001., Poirel et al, 2004.).

Most of the TEM variants were shown to evolve from either TEM-1 or TEM-2 by the induction of point mutation spanning many residues of the enzymes thus effecting their overall function such as arg244 that was replaced by a short side chain amino acid, like serine or histidine, the enzyme– substrate interface was weakened by reduction of substrate affinity. (Livermore, 1995.)

It should be noted though, that the serine residue at position 268 of the TEM structure is well known to be the reactive amino acid residue and any perturbation or blockage at its level by mutation, or at nearby amino acids may and will have severe side effect on the scope of affinity and activity of the enzyme. (Livermore, 1995.)

Other categories of ESBLs, which have been poorly linked with ESBL producing *E.coli* are numerous. One is SHV (sulfhydryl variable) that was recovered in clinical isolates more frequently than any other ESBLs (Jacoby, 1997.). It was complete due to the possibility that p-chloromercuribenzoate may hinder its activity, and was first reported in *Klebsiella ozaenae* isolates from Germany. (Sykes and Bush, 1982., Tzouvelekis and Bonomo, 1999.). It is now responsible for more than 20 percent of plasmid-mediated ampicillin resistance in *Klebsiella pneumoniae* (Ishii et al, 2005.).

Only few derivatives of SHV were categorized, such as SHV-1, 11, 3 and 14. They all differ from one another by point mutation directed toward the key amino acid

and nucleotides at the level of the blaSHV gene existing on transferable plasmids (Jacoby and Sutton, 1991.).The majority of SHV variant at present, are characterized with having serine 238 substitutions to glycine, playing a major part in the enzyme scope of activity against cefotaxime (Huletsky et al, 1993.).

Most of the SHV variants do present an ESBL phenotype, except for SHV variant 10; as this odd phenotype was related to a substitution mutation at serine 140 to glycine which seemed to predominate the previously stated key SNP at amino acid position 238. (Bradford et al, 1995., El Harrif-Heraud et al, 1997., Naas et al,1999.).

Another growing group of ESBLs is OXA, belonging to group 2d. Group D according to Ambler shares a lot of fundamental characteristics with group 2be, since both are serine hydrolase enzymes with the main difference being that group D does not display susceptibility against tazobactam and is not always inhibited by clavulanic acid. (Naas et al, 1999., Paterson and Bonomo, 2005., Pinheiro, 2013)

On the other hand OXA ESBLs were mainly isolated from *P. aeruginosa* unlike other classes of extended spectrum beta-lactamses that were primarily found in *E.coli* and *K..pneumoniae* species. OXA variants just like any other group of enzymes differ from one another by the substitution of one or two amino acids in particular, the Gly157Asp substitution essential for resistance against ceftazidime (Danel et al, 1995., Danel et al, 1999.)

While TEM and SHV do form the majority of ESBLs, other subgroups of beta-lactamses did prevail and were widespread across various countries spanning many organisms other than *E.coli* and *K. pneumoniae* like *Acinetobacter baumannii* and *Salmonella enterica* serovar Typhimurium (Vahaboglu et.al, 1995., Vahaboglu et.al, 1998.). The PER beta-lactamases is another group mainly widespread in Turkey with a significant ceftazidime resistance. (Vahaboglu et.al, 1997.). Another beta-lactamase subgroup is VEB which was first isolated in Vietman from *E.coli* but was later on found in *P. aeruginosa* in a patient from Thailand (Naas et.al, 1999.)

As previously noted, not all beta-lactamase derivatives are in fact ESBLs , early on, this group of beta-lactamses was not inhibited by clavulanic acid so it was ruled out as non-ESBL beta lactamses. With the dominance of nucleotide sequencing some of the beta-lactamases previously ruled out as non ESBL showed high sequence similarity to ESBLs. Those enzymes mainly belonged to the TEM group and for that they were considered as inhibitor resistant TEMs and included, IRT, found in *P. mirabilis*, *Citrobacter freundii* , *K. pneumoniae*, as well *as E. coli* (Bonomo et al, 1997., Chaibi et.al, 1999.).

The origins of this rather extraordinary phenomenon were point mutation at specific amino acids, far diverse from those responsible for regular ESBL phenotypes,

some of these are Arg-275, Asn-276, Arg-244, and Met-69 (Belaaouaj et al, 1993., Henquell et al, 1995.). Moreover, laboratory constructed mutants that contained two alterations were not able to present both phenotypes as only one predominated, eliminating the possibility of this occurrence in real life any time soon (Stapleton et al, 1999.)

1.1.4 ESBL expressing E.coli in health care associated infections

A standardized identification method for ESBLs approved by the national committee for clinical laboratory standards, NCCLS, is by testing antibiotic susceptibility in either disk diffusion susceptibility tests or dilution tests using cefpodoxime, since it helped in detecting ESBLs with higher efficiency and accuracy than other cephalosporins such as cefotaxime, and ceftazidime. (Emery et al, 1997., CLSI, 2012., Moland et al, 1998.) .

However it was later shown that the previously established procedure was not accurate as cefpodoxime caused an increasing number of false positives (Tenover et al, 1999.).

To compensate for the inaccuracy attained by cefodoxime susceptibility testing other modes of detection were suggested, like disk diffusion assay using oxyimino-beta-

lactam antibiotics placed 30 mm apart from each other and the Epsilometer test ESBL with ceftazidime. However, none of these approaches single-handedly displayed high precision in detection of ESBLs, and it was noted by Tenover et.al (1999.) that one possible effective process of detecting potential beta-lactamase producers is through the usage of several beta-lactam antibiotics.

On the other hand, molecular identification and classification of the target genes indeed provided the ability to accurately categorize the isolate under study. The easiest and less laborious intensive classification procedure to date is the polymerase chain reaction, PCR, using taq polymerase and specifically designed primers that work on targeting, amplifying, and binding to both the 5' and 3' ends of the DNA strand under investigation, which can then easily be viewed after electrophoresis. The only disadvantage of this approach is its inability to differentiate between derivatives. For this, one step further was to be made, and it was by cleaning up and sequencing the previously amplified sequences using multiple sequencing procedures, like Sanger sequencing. (Paterson et al, 2005.)

Recent reports on community colonization and infection associated with *E.coli* producing extended spectrum beta lactamases were linked to urinary tract infection that

are wide spread across all continents. Such cases showed significant resistance against UTI first-line treatment regiemns including ciprofloxacin, ceftriaxone, and gentamicin (Paterson et al, 2005.)

1.2 Bacterial Biofilms

Compared with higher order life forms, microbes were once assumed to exist singly, an argument that soon began to fail with the prevalence of evidence and scientific data proving the ability of bacteria to undergo multiplex behaviors and differentiation protocols, most importantly their ability to colonize, adhere and form biofilms on and in a given surface and/or area (O'toole et al, 2000.).

Biofilms are a buildup of microorganisms in a polysaccharide environment (O'toole et al, 2000.) altering their mode of growth from a planktonic to a sessile state, shielding the given organisms from harsh neighboring settings (Costertron et al, 1999.), a phenomenon that was first noted by Claude Zobell on marine bacteria (Zobell, 1943.) The Costerton group acknowledged these findings and worked on exploring the microbial species that retained this beneficial capability including many gram positive as well as gram negative microorganism (Costertron et al, 1999.). *Escherichia coli, Staphylococcus aureus, Staphylococcus epidermidis, Enterococcus faecalis, klebsiella*

pneumonia, *Proteus mirabilis* and *Pseudomonas aeruginosa* which are found capable of forming biofilms (Khoury et al, 1992.).

Nowadays it is recognized that apart from their ability to settle on live matter, bacteria in biofilms can essentially form on a number of surfaces, even on dead inert tissue. In addition it was established that biofilms can form on medical devices such as urinary catheters, central venous catheters, and prosthetic heart valves. Countless chronic infections like cystic fibrosis, prostatis, and periodontitis were indeed an outcome of this distinctive quality (Donlan and Costerton, 2002).

When microbial pathogenesis was first categorized, pure cultures were necessary in order to correlate a disease with a particular microbe. This statement is gratified in some cases when it comes to biofilm related infections, particularly with nosocomial infections such as UTIs (urinary tract infections). However in most cases biofilms embrace numerous bacterial species, and occupy different environments from soil to marine (Adal and Farr, 1996., Archibald and Gaynes, 1997., Dickinson and Bisno, 1993.).

As the bacterial cells adhere, imperative phases of development arise, synthesizing and building up extracellular polymers, converting the bacterial approach of growth form a planktonic into a sessile state (O'toole et al, 2000.).

Initially, free floating, mobile bacterial cells move along a surface and adhere to it, establishing a reversible monolayer of cells and paving the way to the next phase of growth and maturation. This is when the microorganism attachment to the surface becomes irreversible and establishes maximum thickness of the outermost shell of the biofilm. This is produced through a process of water loss as well as extracellular polysaccharide production and build-up (Donlan and Costerton, 2002.). The final step of biofilm formation is dispersion and includes the release of microbial cells from the biofilm, hence reestablishing their planktonic state. This last stage of development could be manifested through one of two procedures, either by erosion, referring to continuous detachment of small portions of the biofilm at various time points or by sloughing where the majority of the biofilm is dispersed at once (Caldwell et al, 1992.).

In order for initial attachment and bacterial surface adhesion to take place, the microorganism should be able to signal the activation of given molecular pathways (Ribet & Cossart, 2015.).

Bacterial adhesion was initially thought of as a mean for bacterial-host colonization and fortification from destruction by shielding the given microbe from numerous classes of innate defense secretion such as mucosal secretions, as well as, peristalsis. Adhesins which are adhesion molecules, are the first bacterial cell

components to make contact with the host that can lead to a series of events, most importantly the secretion of polysaccharide scaffold molecules needed for biofilm establishement (Khoury et al, 2009.).

Fimbrae were the main adhesins expressed in gram negative microorganism. They are known as hair-like protrusions found on bacterial surfaces (Pizarro-Cerdá & Cossart, 2006., Khoury et al, 2009.). The base of these structures attaches to the outer bacterial membrane while the tip contains binding factors that sustain adherence (Pizarro-Cerdá & Cossart, 2006., Khoury et al, 2009.).

This structure presents multiple well defined assemblies. For example some uropathogenic strains of *Escherichia coli* (UPEC) infecting the urinary tract and kidney epithelium express pyelonephritis-associated (P) pili, characterized by PapG adhesion factor presented at its tip and works on binding to kidney epithelial glycosphingolipids (Roberts et al, 1994.). While other UPEC strains require type I pili on their outer membrane that bind with high specificity to D-mannosylated receptors found on the surface of the host's cells, such as the bladder's uroplankins (Lillington et al,2014.).
The production and assembly of both the type 1 and P-pili are considered to be standard for pilus formation and bacterial adhesions in host-microbe interactions. Assembled via the chaperone/usher pathway, pili formation begins with the release of structurally unfinished pilin subunits bound to periplasmic chaperone proteins such as FimC for type 1 pili, and PapD for P pili, consisting of two immunoglobulin-like domains stabilized by

a salt bridge, via the SecA/Y pathway all the way through the cytoplasmic membrane. SecA, an ATPase, is a cell membrane associated protein subunit essential for the movement of protein substrates across the translocon channel found in Type II secretory pathway, a system responsible for secreting proteins through the cell membrane mainly in gram-negative bacteria (Kline et al, 2009., du Plessis et al, 2011.).

Translocon channels are proteins complexes responsible for polypeptides and proteins translocation across cytoplasmic membranes (Johnson and van Waes, 1999.). The bacterial hetero-trimeric protein complex SecYEG translocon, consists of three subunits SecE, SecG, and SecY. The alpha-subunit, SecY consists of two halves, linked extracellularly through a loop between their trans-membrane segments. The alpha subunit can open laterally at the front permitting the passage of target proteins. The gamma-subunit, SecE, wraps dorsally around the two halves of SecY. SecG (beta-subunit) which lies on the side of the alpha-subunit and makes only few contacts with it is considered to be inessential. *(*Van den Berg et al, 2004.).

The transported chaperone-subunit complexes are then directed towards outer membrane ushers, such as FimD for type 1 pili, and PapC for P pili, the chaperone-pilin complex then disassemble themselves and the subunits are added to the bases of the growing pilus according to their affinity for each other and to the usher , for example in P pili, chaperone-adhesin, PapD-PapG binds first to empty PapC sites(Dodson, et al, 1993., Lee, et al, 2004, 2007., Saulino et al, 1998.)

Type IV pili is another important class, found in many gram negative microorganisms such as enteropathogenic *E.coli* as well as some gram positive bacteria like *Clostridium perfringens*, as it enables twitching motility of the microorganism expressing it (Melville and Craig, 2013). This form of mobility is maintained by the ability to retract through the bacterial cell wall while the tip is still attached to the target cell (Mattick, 2002.). Type IV pili are polymeric structures of processed pilin, a protein synthetized in the bacterial cytoplasm and proteolytically altered across the inner bacterial membrane before being assembled and expressed on the outer membrane (Melville and Craig, 2013.).

The progression of assembly for the type IV pili is in fact very different from types 1 and p-pili where the pre-pilin proteins are shuffled to the inner-membrane where they undergo a form of post-translational modification at the level of the amino-terminus, where the leader sequence of that terminus is cleaved via the pre-pilin peptidase. Afterwards the mature pilin peptides are released in the inner-membrane where the pilus assembly takes place utilizing ATPase enzymes. The newly formed pili are then secreted to the surface of the cell, where they reside, with the aid of a secretin pore located at the outer-membrane (Carbonnelle et al, 2005., Wolfgang et al, 2000.).

A number of commensal and pathogenic enteric gram negative microorganism present thin sticky aggregative fimbriae capable of adhering to various surfaces, curli. These adhesive amyloids are formed in a two-step process in which the major protein subunit, CsgA, is released towards the inner membrane under the influence of type 2 secretion systems and then with the presence of a multimeric pore, CsgG. Once found at the outer-membrane, the major subunit is susceptible to conformational modification when in-contact with related surface bound subunit, CsgB, finally forming insoluble curli. It should be noted that apart from being expressed in commensal bacteria it was found that a number of clinical urosepsis *E.coli* were in fact capable of expressing and presenting adhesive amyloids at human physiological temperatures signifying a virulence effect (Bian et al, 2000.).

In concordance with the structures and mechanisms discussed earlier, some bacterial species and strains are able to express and inject host cells with specific receptors that they could bind to, known as exogenous receptors. Both the entero-pathogenic and entero-hemorragic strains of *E. coli*, EPEC and EHEC respectively, are capable of undertaking this task by expressing the Tir receptor, which binds to bacterial cell surface protein intimin, that they than inject and deliver to the host cell via their type 3 secretion system (T3SS), which is a needle like structure that crosses the bacterial cell wall as well as the host's cell plasma membrane (Kenny et al, 1997.). The injection

of the Tir into the host's cell leads to a series of remodeling steps at the level of the actin cytoskeleton by the recruitment of cytoskeleton regulators such Wiskott-Aldrich syndrome protein (N-WASP) and the actin-related protein 2/3 (Arp2/3) (Lai et al, 2013.). This remodeling of the host membrane creates a pedestal under the attached pathogen sustaining the qualifying "attaching and effacing" concept (Crepin et al, 2010.).

Flagellar mediated motility is another way for establishing initial contact. Many bacterial species that have the ability to express flagella are well known to be biofilm formers, examples include motile *E.coli* strains. This was concluded after a study done by Genevaux and colleagues, (1996) who screened a group of mutant *E.coli* strains for biofilm formation capabilities and found that around half of the strains that weren't able to form biofilms were defective in flagellar-mediated motility, while the other half had a defective type I pili especially the *E.coli* K12 strain .

Sigma factor 28, encoded by RNA polymerase flagellum gene *rpoF,* sustains flagellar biosynthesis of various bacteria including: *Bacillus subtilis*, *Legionella pneumophila*, *Salmonella* Typhimurium, and *Escherichia coli*. This is usually done by directing RNA polymerase to the consensus sequence TAAAGTTX GCCGATAAC, found in several genes linked to production of flagella (Studholme and Buck, 2000.).

All the previously mentioned modes pathways for adherence and attachment, provide the means necessary for cell to surface and cell to cell interactions assisting the

development of a rudimentary sessile, monolayer, state surrounded with little exopolymeric substances, for the bacterial cells expressing it. And albeit bacterial attachment and adhesion are crucial steps and a start for biofilm development, if not quickly retained this system will be held off, and will deteriorate swiftly. At this level the bacteria that are found in the colonies under sessile conditions can revert back to a planktonic state with the help of pilus and flagellar driven mobility (Korber et al, 2003.). For this a transition from weak to permanent interactions, bonding has to take place at the level of the newly adherent bacterial communities during maturation, and it's mostly achieved by the production, secretion, and presence of extracellular polymers (EPS) in the area surrounding the attached colonies. EPS are biosynthetic polymers that may include polysaccharides, proteins, phospholipids as well as nucleic acids (Wingender et al, 1999.).

The composition of the expressed and synthesized EPS can extremely differ from one microorganism to another (Wingender et al, 1999.). For example colanic acid is the major compound in the extracellular polymers produced by multiple *E.coli* strains, which is required for the synthesis of the biofilm architecture (O'toole et al, 2000.). On the other hand alginate is the major EPS produce by *P. aeruginosa* (DeVault et al, 1989.).

However the expressed EPS alone is not capable of sustaining the outgrowth of the now forming biofilm microcolonies, the newly adherent cells undergo binary fission

leading to the spreading of the daughter cells upward and outwards increasing the covered area's surface and width (Heydorn et al, 2000.). Another approach for surface area expansion is by the recruitment of planktonic cells from the fluid state of the biofilm by sessile cells. Both of these approaches may not take place at the same time, and if they do, their contribution to the formation and maturation of biofilm will not be the same, it is all based on the physical and chemical properties of the surface upon which the cells are adhering (Tolker-Nielson et al, 2000.).

In several cases biofilms may need up to ten days to reach structural maturity and stability (Heydorn et al, 2000.). At this stage, with the help of the released EPS an architectural change and development starts taking place creating a protective surrounding. This includes increasing the thickness of the biofilm, establishing hydrated channels for essential nutrient delivery and intercellular communication (Costertron et al, 1999.), conserving the survival of the colonies at the core of the biofilm. This maturation will eventually lead to the development of a profound characteristic of biofilms: antibiotic resistance.

The morphological changes that take place during maturation affect the colonizing bacterial cells, as manifested by an alteration in physiological processes to accommodate the conditions of a particular niche, effecting gene and protein expression, where the expressed proteins from bacteria under sessile conditions showed a significant difference at the level of expression and the nature, function, of the proteins themselves

when compared with microbial communities under planktonic settings. For example more than 800 proteins and 70 genes had different and/or greater levels of expression in sessile *Pseudomonas aeruginosa* (Ascon-Cabrera et al, 1995., Dewanti and Wong, 1995., Fletcher, 1996.). Most importantly the alginate, *algC*, one of the genes involved in EPS synthesis in *Pseudomonas aeruginosa*, which showed an increase in expression of around five folds in sessile organisms once compared with planktonic ones.(Davies, et al, 1993., Davies and Geesey, 1995.). In addition to the circumstance that in order for alginate to be synthesized sigma factors AlgT/AlgU need be to expressed, and genetic analysis have revealed that those same factors needed for alginate production and release play a major role in rendering the microorganism under study, *Pseudomonas aeruginosa*, immotile by down-regulating a key biosynthetic gene in flagellar expression, hence aiding the microbe in settling in the biofilm promoting its maturation. (Garrett et al, 1999.)

As for *E.coli* strains, transcription of around 38% of genes were affected within biofilms. Some expressed an increase, such as the *proU* operon that encodes for production of glycine betaine transport system, the OmpC porin, letting the organism to recover from the harmful effects of hyperosmotic shock. The same occurred in the nickel high-affinity transport system encoded by *nikA* . On the other hand some genes were down regulated just like *fliC* , which codes for flagellin synthesis, which

implement the preservation of biofilm maturation as well as the survival of the bacterial cell. (Prigent-Combaret et al, 1999.).

Another transcription modification that takes place in biofilm forming microbes during maturation, especially in *E.coli*, happens to be the production of sigma factors. Sigma factors are known to be proteins needed for RNA synthesis initiation as they enable RNA polymerase binding to specific gene promoters. Sigma factors vary depending on the gene coding for them (Gruber and Gross, 2003.). In colonies of mature biofilms colonies two main sigma factors are effected *rpoH* and *rpoS*. During the developmental phase an increase in sigma factor *sigma-38*, encoded by *rpoS*, the main regulator of stress-response and stationary phase genes expression, was noted especially for the biosynthesis of alginate regulated by *sigma-38*. This increase in expression was not maintained in mature biofilms; in effect a significant down-regulation was prominent (Gruber and Gross, 2003.). On the other hand *rpoH*, the heat shock sigma factor, enables the cell

to survive higher temperatures, was found to be upregulated in mature biofilms, hitting to the idea that specific genes are activated to allow biofilm formation while others will attain the microbe survival within the mass(Gruber and Gross, 2003.).

Those alterations of proteins and their level of expression, which are now considered as an answer to stressful environmental settings, will help microbial colonies to progress into a fully differentiated irreversible biofilm since they alter the expression

of genes encoding proteins involved in translation, metabolism, and gene regulation (O'toole et al, 2000.). At this point bacterial micro colonies situated within biofilms develop and organize themselves into communities with sustainable heterogeneity, where most biofilms are formed of mixed colonies (Costertron et al, 1999.). The hydrophilic channels responsible for nutritional management may also allow the exchange and transfer of genetic material by horizontal gene transfer, such as exchange plasmids by conjugation which can contain resistance factors, thus increasing the pathogenicity of the organism, and the probability of antibiotic resistant microorganisms (Wuertz et al, 2004.).

Detachment of microbial communities from biofilms is another step in biofilm development and as previously stated can be accomplished in two different procedures, either by erosion, referring to continuous detachment of small portions of the biofilm at various time points; or through sloughing where the majority of the biofilm is dispersed at once. Studies done on the mode of detachment of biofilms structures from medical devices showed that an increase of shear, for example increasing the flow rate, could cause bacterial cell erosion, while sloughing can occur in case of a change in substrate concentration (Characklis, 1990., Characklis, et al, 1990.). This was further acknowledged by Sauer et al, 2002 who showed that this process of aggregate sloughing can occur when bacterial colonies enter starvation mode, consequently allowing the microbes to search for a recovered, nutrient rich habitations. The physiological

mechanisms backing up this phase is not really known yet, but some studies have suggested that a decrease in EPS production may be one of the causes of bacterial dispersion from biofilms (Stoodley, et al, 2001.).

The proteins produced at this stage of development are no more considered to be related to the sessile state that the bacteria was experiencing, they revert back to the conditions that were predominant during planktonic status.

Biofilms established the trait of counteracting antibiotics, through different morphological qualities. The increased production of extracellular polymers, which maximizes the thickness of the biofilm structure, ultimately results in lowering the penetrative abilities of antibiotics. Another factor that will contribute in neutralizing antibiotics is the fact that the majority of biofilms are produced by many species creating an extremely unique phenotypic state shielding the biofilm. Currently antibiotics are capable of rapidly eliminating the majority of bacterial colonies, but not all, whereas the surviving resilient minority persists despite continuous exposure to antibiotics (Stewart & Costerton, 2001.).

Also, since biofilms are rather considered to be more of a closed setting for bacterial colonies the slow buildup of metabolic waste products helps in establishing a PH difference that is well greater than 1 between the bulk and the interior of the biofilm counteracting the work of many types of antibiotics, for instance aminoglycosides. This

lower PH condition will also help the micro colonies to enter a non-growth phase, protecting them from further elimination (Stewart & Costerton, 2001.).

Another proposed mode of resistance comes from the fact that during their presence in biofilms, bacteria slow down their growth significantly due to the stressful conditions, such as nutrient and oxygen starvation, by activating sigma factors, like *rpoS* in *E.coli*. subsequently decreasing the penetration of antibiotics hence limiting their activity to a bare minimum. Evans et al (1990) found that the slowest growing *Escherichia coli* cells in biofilms are in fact the most resistant to cetrimide.

In all cases, the antimicrobial agents are most likely not capable of attacking or eliminating the core of the biofilm and can only induce an effect on cells and colonies floating around or the edges and surfaces as a result of the highly isolated organization adapted throughout. This explains why many patients suffering from symptoms related to biofilm forming microorganisms, do in fact show recurring symptoms a short period of time after stopping antibiotic therapy. In most cases, the only solution for biofilm caused bacterial infection is the surgical removal of the sessile population from the body of the patient.

Nowadays, biofilms are showing intense resistance, many preventative techniques are taking place, but by far the most promising mode of interference is

inhibiting initial attachment by changing the characteristic of the host surface along with eradicating the initially adherent microorganism (Donlan & Costerton, 2002.).

1.3 whole genome fingerprinting and pulsed field gel electrophoresis

1.3.1 Conventional approaches and gel electrophoresis

Dealing with and preventing the spread of bacterial infections is always key in order to encompass a given situation (Goering, 2010.). With the purpose of governing the circumstances and consequences of bacterial infection spread and recurrence, microbiologist had an urge to appropriately recognize and classify the microorganism in question (Goering, 2010.).

Conventional approaches of strain typing, for epidemiological analysis involved bacteriophage typing and serotyping (Tenover et al, 1995.). Serotyping epidemiological classification relies mainly on cell surface antigens that are presented by cells and microbes, a serotype or serovar expresses a group of antigens that are distinct to it (Baron, 1996.. Dictionary, 2007.) . For example *Salmonella* has been found to have more than 2600 serotypes including *Salmonella enterica* serovar Typhimurium (Ryan et

al, 2004.). Serotyping is performed primarily through serological methods that deals with antiserum and target specific antigens presented on bacterial surfaces (Baron, 1996., Dictionary, 2007.).

Phage typing depends on the concept that only a given class of bacterial isolates of a given strain and or species are in fact susceptible to bacteriophages, viruses that target bacteria; accordingly scientists were able to categorize and classify isolates and it is mostly considered as a phenotypic and not genotypic mode of strain classification (Baggesen et al, 2010.).

While both methods proved to be useful, and are still used in a number of applications mainly in *Salmonella* classification, they did show signs of inefficacy and other methods of classification needed to be functional to encompass a wider array of bacterial specimens (Arbeit, 1996.).

For this various new molecular procedures have replaced the conventional approaches in the last few years, such as polymerase chain reaction (PCR) based protocols, and DNA fingerprinting via pulse field gel electrophoresis, which still presents itself as being the best and most suitable methodology (Arbeit, 1996. Maslow, 1993., Swaminathan, 1993).

1.3.2 Pulsed field gel electrophoresis techniques, procedures, and result analysis

The model of pulsed field gel electrophoresis was first introduced in 1984 by Schwartz and Cantor and it enabled the separation of DNA molecules of up to ten mega base pair in size, and achieved a much higher resolving power and resolution than that of conventional gel electrophoresis that is only capable of splitting up to twenty kilo base pair fragments (Anand, 1986.).

This exceptional increase in resolving power arises from exposing the restricted DNA fragments to alternating perpendicular electric fields, counting on the idea that depicting distinct DNA fragments to different electrical fields will affect their rate of migration in agarose gel and eventually lead to their separation hence increasing resolving power (Anand, 1986.).

For this, and to be able to properly visualize large restricted DNA fragments separately many electrophoretic methodologies and instrumentations have been applied throughout the course of protocol optimization; the important practice deviations were the variation of gel run time and electrodes switch time (Goering, 2010.).

Many instrumental tactics have been used for pulsed-field gel electrophoresis, most notably field inversion gel electrophoresis (FIGE) and the more commonly used contour-clamped homogeneous electric-field gel electrophoresis (CHEF) (Chu, et al, 1986.).

With contour-clamped homogeneous electric-field gel electrophoresis DNA separation is accomplish through an electric field applied by 24 electrodes uniformly distributed at an angle of 120 degree ; yet its run may be effected negatively by modification in gel thickness and running buffer PH out of the range of the required parameters(Birren et al, 2012., Struelens et al, 2001.) .

On the other hand field inversion gel electrophoresis does not necessitate a specific electrophoresis chamber, all what it needs is an alternating electric current at 180 degree angle rather than 120 degree like CHEF, and it is consider to be considerably faster but with lower discriminatory power (Turmel et al, 1990.).

The main purpose behind strain typing and pulsed field gel electrophoresis is to study the genetic relatedness of isolates collected in a given area, hence determining if the epidemiologically related specimen present an extent of genetic coherence (Kreiswirth, 1993.).

The DNA restriction pattern generated after a gel run, of enzyme restricted DNA samples, need to be properly interrupted into useful epidemiological information in order to classify the studied isolates and to determine whether or not an outbreak took place (Haley, 1995.).

Indistinguishable isolates, are specimens that show the exact same pattern of restriction without any change or modification, and this only shows that the studied isolates are the same strain; and represent the outbreak strain in ideal situations (Miranda et al, 1991., Olsen et al, 1994.). But that is not the case constantly since uncontrollable genetic modifications may take place such as deletions, insertions, and single nucleotide polymorphism (SNPs) changing the pattern of some of the bands, adding or deleting a few (Sader et al, 1993.).

If two bands present a change in analysis and pattern of distribution, correlating with the addition or deletion of one restriction site henceforth one genetic modification, the isolates are presumed of being closely related; a marginal fluctuation in pattern, and restriction sites, out of that scope and up to a difference of four to six bands, hence two independent genetic modifications, the compared specimens are still considered to be possibly related (Miranda et al, 1991., Olsen et al, 1994., Tenover et al, 1994.).

Any alteration out of that range the strains are ruled of being unrelated, and largely three independent genetic alterations in restriction site number and pattern of restriction will lead to the assumption of genetically unrelated isolates indicating the presence of different bacterial strains (Tenover et al, 1995.).

Pulsed-field gel electrophoresis presents itself as the golden method for epidemiological examination due to its highly stable, reproducible restriction pattern and ability for it to be applied against a large range of bacterial specimens with minute change in run parameters and choice of restriction enzyme; however it displays breaking limitations especially when it comes to inter-labortory reproducibility as well as low discriminatory power between unrelated specimens. Due to this molecular examination nowadays is shifting towards whole genome sequencing (center of disease control and prevention, 2017.).

1.4 Natural extracts of plants

1.4.1 Ethno botany

Traditional medicine and the use of medicinal plants and herbs played an important role in old ages, since it helped in treating various cases and medical conditions. With the prevalence of antibiotic resistance bacterial species, this traditional approach needed to be reinitiated to sustain a non-invasive method of recuperation (Hernández et al, 2003.).

1.4.2. Natural extracts

1.4.2.1Petroselinum cripsum

Petroselinum cripsum (parsley) is an aromatic biennial herb, characterized by having unequal flat leaves, and yellowish green crescent flowers. It has shown to retain a diuretic, carminative action due to the active ingredients in its fruit, namely flavone and apigenin-7- apioglucoside, myristicin (Husain, 1992., Yoganarasimhan, 2000.).

Furthermore a study done by Devi et al (2010) did show that in addition to the previously mentioned benefits of *Petroselinum cripsum*, it also exhibits powerful antifungal, antimicrobial properties presented by its methanol extract against a wide range of gram negative bacteria *Escherichia coli Pseudomonas aureginosa* and

Salmonella typhi , gram positive bacteria *Bacillus subtilis* ,*Staphylococcus aureus* as well as fungal growth of *Candida albicans* which showed dose dependent susceptibility.

1.4.2.2 Mentha spicata

Mentha spicata (spearmint) natively found in the Mediterranean region has displayed a potential in pharmaceutical and medicinal applications, which dates back to medieval times. It was used for ulcerative colitis, bronchitis, liver malfunctions in addition to physiological complications (Baytop, 1999., Brown, 1995., Cowan, 1999.)

Menthol the major component of the essential oils of *Mentha spicata*, menthol was also capable of reducing bacterial growth and presenting a significant antimicrobial activity against a wide range of gram negative as well as gram positive microorganims such as *Escherichia coli* and *Staphylococcus aureus* (İşcan et al, 2002.). Low doses of menthol, ranging from 0.07 to 2.5 mg/ml, were able to eradicate and inhibit bacterial growth of twenty one different bacterial species using the disk diffusion method (İşcan et al, 2002.).

1.4.2.3 Ocimum basilicum

Ocimum basilicum (basil) essential oils, are mostly found in the aerial parts of the plant, exhibited good antioxidant activity due to the presence of linalool, the major constituent, to bergamotene and cadinene; that reduced the risk of chronic diseases such as cardiovascular diseases and some types of cancer, like colon cancer (Choi et al, 2007., Majhenič et al, 2007., Hussain et al, 2008.).

The antimicrobial activity of basil essential oils was tested against a wide range of gram negative as well as gram positive microorganisms like *Escherichia coli* and *Staphylococcus aureus* respectively (Hussain et al, 2008.). All isolates exhibited to a certain extent susceptibility to the oils, but what was most significant in addition to the fact that the oils presented a much more potent effect against gram positive than gram negative bacteria, is that the outcome varied seasonally (Hussain et al, 2008.). Essential oils obtained during winter and autumn exhibited higher effect once compared with those obtained in spring or summer. One possible explanation to this phenomenon was that linalool and other components were present in greater percentages and are more oxygenated during winter and autumn than in summer and spring (Hussain et al, 2008.).

1.4.2.4 Rosmarinus officinalis

Rosmarinus officinalis (rosemary) is mostly found in Turkey and the Mediterranean region previous studies showed that methanolic extracts of rosemary did not present a noticeable inhibitory effect against many bacterial isolates (Celiktas et al, 2007.). Nevertheless, a substantial influence was seen against *Proteus vulgaris*, and *Staphylococcus epidermidis* with a minimal inhibitory concentration as low as 10mg/ml. These results were linked to the effect generated by 1,8-cineole and alpha-pinene the major components of rosemary (Celiktas et al, 2007.).

1.4.2.5 Trifolium alexandrinum

Trifolium alexandrinum (clover leaf) was used in traditional medicine in many areas of the world (Khan et al, 2012.). *Trifolium alexandrinum* is characterized by having hollow stems and oblong leaflets comprising glycosides, flavonoids, and fatty acids, In fact various species and sub-groups *Trifolium* have been shown to have antiseptic in addition to analgesic properties and has been related to possible treatments for rheumatic ache (Sabudak et al, 2009.).

These primeval properties of clover leaf initiated the quest to test for its anti-bacterial action. *Trifolium alexandrinum* found all over the United States, Europe, and Asia, was able to hinder and prevent the development of some human pathogenic isolates (Khan et al, 2012., Sabudak et al, 2009.).

1.4.2.6 Punica granatum

Punica granatum (Pomegranate) is one of the oldest fruits know to humanity and has been recognized in many cultures, such as the Greek and Babylonian; its seed oils contain mainly punicic acid and ellagic acid as well as a minority of fatty acids and sterols (Jurenka, 2008., Mahdihassan, 1984.).

Puninic acid, is directly associated with many of the biological properties and health benefits of pomegranate, since it demonstrates anticarcenogenic properties against several types of cancer, in addition to antidiabetic activity (Aruna et al, 2016).

A few years back, pomegranate metahnolic seed extract was found to be able to inhibit the growth of highly pathogenic bacterial isolates, primarily the gram positive *Bacillus cereus* and gram negative *Klebsiella pneumoniae* with a zone of inhibition

ranging between 25 and 20 millimeters respectively on disk diffusion assays (Dahham et al, 2010.).

1.4.8 Allium cepa and *Allium sativum*

Allium cepa (onion) and *Allium sativum* (garlic), are watery crops that have been known and used for ages; water percentage ranges between sixty to ninety percent of the total mass of any of those two crops (Rivlin, 2001.).

In fact the most noteworthy constituents, from a medical perspective, in both *A. cepa* and *A. sativum* were presumed to be the sulfur containing compounds (Benkeblia, 2004., Lawson, 1998.). Moreover it has been reported that sulfur is more abundant in garlic than in onion, encompassing a threefold increase in garlic when compared with onions elements (Benkeblia, 2004., Lawson, 1998.).

These sulfur containing compounds have extremely effective nutritional values in addition to validated antifungal and antimicrobial properties against a large scope of gram-negative and gram-positive microorganisms specifically towards *S.aureus* specimens (Naidu, 2000., Benkeblia et al, 2004.).

1.4.2.8 Salvia officinalis

Salvia officinalis (sage), the native Mediterranean evergreen subshrub, is considered of high importance in the medical field; it has been shown to have high antioxidant activity due to the presence of flavone glycosides as well as rosmaric acid derivatives (Lu et al, 2001.).

Recent studies have shown that *S. officinalis* does present within it a potential antimicrobial ability. In fact a study by Horiuchi et al in 2007, stressed on the high antimicrobial activity of lipids present, such as oleanolic and ursolic acids against serious, life threatening, bacterial isolates like vancomycin resistant enterococci (VRE) (Horiuchi et al, 2007.).

Moreover, in a related study, the methylene chloride extract of *S. officinalis* was capable of reducing the growth of many gram negative bacterial isolates including *Salmonella* Enteritidis and *Escherichia coli*. This effect was correlated with the high percentage of camphor and alpha thujone, both of which convey high antimicrobial activity, in the organic composite solution of sage (Veličković et al, 2003.).

1.4.2.9 Thymus vulgaris

Thymus vulgaris's (thyme) essential oils were long used as food and beverage preservatives as they seemed to have a bacteriostatic effect on both gram negative and gram positive bacterial species. Marino and his colleagues (1999) worked on extracting the organic constituents of thyme during various growth stages and were able to conclude that at all times and in all stages, especially in mature leafs, *T. vulgaris's* essential oils were capable of either partially or completely inhibiting bacterial growth and colonization. They also found that thyme oils, which include alpha and beta pinene and gamma terpinene, were most effective on gram negative isolates such as *Proteus mirabilis* and *Salmonella* Typhimurium as well as *E. coli* O157:H7 which was considered as the most virulent strain of *E.coli* (Marino et al, 1999.)

CHAPTER II

Materials and methods

2.1 Processing of bacterial isolates

The bacterial isolates used in the study are clinical isolates provided by the Clinical Microbiology Laboratory of the Lebanese American University Medical Center- Rizk Hospital (LAUMC- RH). The isolates used were 20 samples of each of *Escherichia coli.*

2.2Detection of extended spectrum beta lactamases

2.2.1 Identification of *Escherichia coli.*

All studied isolates have been properly identified according to conventional biochemical techniques such as API 20e as well as grams staining.

2.2.2 Antimicrobial susceptibility testing

The diffusion assay was performed as recommended by the Clinical Laboratory Standards Institute (CLSI) guidelines (Clinical Laboratory Standard Institute, 2012.). The following antibiotic discs were used: ceftazidime,

aztreonam, cefotaxime, and ceftriaxone. Inoculum with a turbidity of 0.5 McFarland standards was prepared from colonies on agar plates. A sterile cotton swab was dipped into the inoculum suspension. The swab was rotated several times and pressed firmly against the inside wall of the tube above the fluid level and inoculated on the dried surface of a Mueller-Hinton agar (MHA) plate by streaking the swab over it. With a sterile forceps ceftazidime, cefotaxime, ceftriaxone, and aztreonam disks were placed on the MHA plate and the plate was incubated at 35°C for 18–24 hours (Kumar et al, 2014.).

The antibiotic discs were removed and gently pressed by a sterile forceps to ensure complete contact with the agar surface (forceps is flamed and dipped in ethanol after moving to other disc) Amoxicillin-clavulanic acid disc was placed towards the center of the plate. 15 mm out from the edge of that disc to the left Ceftazidime disc was put, 15 mm to the right Ceftriaxone disc is injected, 15mm downwards Aztreonam discs was positioned respectively, and Cefoxitin disc was put in any available space remaining on the plate.

2.3 Pulsed-field gel electrophoresis fingerprinting

The genetic affiliation of the strains, were analyzed via pulsed-field gel electrophoresis (PFGE) fingerprinting on the 20 isolates using XbaI restriction enzyme (Thermo Fisher Scientific, MA, USA), 1% SeaKem agarose gel and lambda ladder 170-3635 (Bio-Rad Laboratories, Inc, CA, USA) according to the standard C.D.C protocol (Centers for Disease Control and Prevention, 2013). Electrophoresis was performed on Bio-Rad Laboratories CHEF DR-III system (Bio-Rad Laboratories, Inc, CA, USA) under the conditions set for O157 *E. coli* strains (Centers for Disease Control and Prevention, 2013). Gels were stained with ethidium bromide, and imaged on Chemidoc XRS (Bio-Rad Laboratories, Inc, CA, USA), the gel were then analyzed and dendograms were generated via BioNumerics servers 7.6.2.

2.3.1 Making plugs

TE Buffer preparation:

- 2 ml of 0.5 M EDTA, pH 8.0
- 10 ml of 1 M Tris, pH 8.0
- Diluted to 1000 ml with sterile water

1% SeaKem Gold agarose plugs preparation:

- Weigh 0.50 g of SeaKem Gold agarose
- Add 50.0 ml TE Buffer

- microwave for 30 seconds intervals until agarose is completely dissolved

Cell Suspension Buffer preparation:

- 200 ml of 0.5 M EDTA, pH 8.0
- 100 ml of 1 M Tris, pH 8.0
- Diluted to 1000 ml with sterile water

2 ml of Cell Suspension Buffer were transferred to small labeled,according to samples, tubes, with a sterile cotton swab that has been moistened with sterile Cell Suspension Buffer some of the growth from agar plate was removed and suspended in the buffer containing tube. The concentrations of cell suspensions were then adjusted by sectrophometry at 610 nm to reach an optical density of 0.8 to 1.0.

2.3.2 Casting Plugs

400 µl adjusted cell suspensions were transferred to labeled 1.5-ml microcentrifuge tubes, 20 µl of Proteinase K (20 mg/ml stock) and were then added to each tube and mixed gently with pipet tip, 400 µl melted 1% SeaKem Gold agarose is then added and the mixture was pipetted gently. 200 µl of the solution was immediately dispensed in disposable sterile plug molds.

2.3.3 Lysis of Cells in Agarose Plugs

Cell Lysis Buffer preparation:

- 100 ml of 10 % Sarcosyl (N-Lauroylsarcosine, Sodium salt)
- 100 ml of 0.5 M EDTA, pH 8.0
- 50 ml of 1 M Tris, pH 8.0

Lysis buffer (5 ml) wasa transferred to designated conicales onto which 25 µl Proteinase K stock solutions (20 mg/ml) was added. The plugs were then added to the conicales separately after trimming the excess agar off of them. The plugs were then incubated in a 42°C shaker incubator overnight for 8-12 hours with constant and vigorous agitation (130-150 rpm).

2.3.4 Washing of Agarose Plugs after Cell Lysis

The tubes were removed from the shaker incubator, the lysis buffer was then carefully poured out, 10-15 ml of sterile water that has been preheated to 42°C is then added to each tube which is then shaken (130-150 rpm) in a 42°C incubator for 10-15 minutes. This step was repeated twice, water from the plugs was then poured and the plugs were then washed with 10-15 ml of preheated, at 42°C, TE buffer and left to shake in the incubator (130-150 rpm) for 10-15 minutes. This step was repeated three times

2.3.5 Restriction Digestion of DNA in Agarose Plugs

A small slice of the plug was digested with the restriction enzyme (Xba I).

The restriction enzyme master mix for 15 plugs has been prepared accordingly:

- Sterile water 2595 µl
- 10X Restriction Buffer 300 µl
- BSA (10mg/ml) 30 µl
- XbaI (10U/ µl) 75 µl

Master mix (200 micro-liter) was then poured in culture numbered 1.5ml microcentrifuge tubes and left at 40°C in the water bath for 4-5 hours.

2.3.6 Casting an Agarose Gel

Preparation of 10X TBE buffer:

- 121.1g Tris base
- 61.8 g of boric acid
- 7.4 g EDTA
- Dissolved in 1 liter sterile water and PH adjusted to 8.3.

Tris-Borate EDTA Buffer (TBE) (2.2 liters of 0.5X) needed for both the gel and electrophoresis running buffer was prepared from the stock 10X TBE buffer.

1% SeaKem Gold (SKG) Agarose in 0.5X TBE preparation:

- 1.5 g agarose is mixed with 150 ml 0.5X TBE for 21cm-wide gel form (15 wells).
- Microwave for 60 seconds; mix gently and repeat for 15 second intervals until agarose is completely dissolved.
- Restricted plug slices are then removed from water bath. plug slices are carefully removed from tubes and loaded on the bottom of the comb teeth
- Agarose is poured into the gel form and bubbles or debris are removed.

2.3.7 Electrophoresis Conditions for *Escherichia coli* O157:H7

- Initial switch time: 2.2 s
- Final switch time: 54.2 s
- Voltage: 6 V
- Included Angle: 120°
- Run time: 18-19 hours

2.3.8 Analysis of patterns

The PFGE patterns were analysed using BioNumerics (Applied Maths, Belgium

2.4 Detection of biofilm formation

2.4.1 Preparation of bacterial isolates

From fresh agar plates, each of the test organisms was used to inoculate a 10 ml trypticase soy broth (TSB) tube with 1% glucose. The inoculated TSB tubes were left in the incubator at 37°C for 24 h after which, the culture tubes were diluted 100 times with fresh media.

2.4.2 Detection of biofilm formation:

The biofilm forming bacterial strains were further screened via the tissue culture plate method, where the organisms were isolated from fresh agar plates and inoculated in 10 ml of trypticase soy broth with 1% glucose. The broth mixtures were then left in the incubator at 37 degrees Celsius for 24 hours. The cultures was then diluted 100 times with fresh media. Single wells of sterile 96 well flat-bottom tissue culture plates were filled with 200 micro-liters of the diluted culture and incubated at 35 degrees Celsius for 24 hours. Afterwards the content of the wells was gently removed by repeated soft tapping, and the wells were washed with P.B.S (phosphate buffer saline, PH of 7.2) several times. The wells were then stained by crystal violet (0.1%), but the biofilm formed by the bacterial colonies were first fixed by 0.2 % sodium acetate solution.

Excess stain was removed by deionized water and left to dry. The optical density of stained adherent biofilm was obtained by a microplate autoreader at 570 nm wavelength.

2.4.3 Effect of the natural extracts on biofilm formation

The ability of biofilm formation by the isolates and its possible inhibition by the ethanol solution of natural extracts, a method very slightly modified from that used by Mathur et al. (2006) was performed. The ethanol solution was added to the wells being tested in 96 well flat-bottom tissue culture plates and the plates were left to dry in the incubator under aseptic conditions. Upon drying, 200 µl of sterile TSB were added to the wells of the plates with 10 µl of the diluted cultures (previous section) and incubated at 35°C for 24 h. The contents of the wells were then gently discarded by repeated soft tapping, after which the wells were washed with phosphate buffered saline (PBS, pH of 7.2) several times. Then, 0.2% sodium acetate was added to fix any biofilms that may have formed and a 0.1% solution of crystal violet was finally added to stain the biofilms, when present. Excess stain was then removed with deionized water and the plates were left to dry. The optical densities were later determined by using a microplate auto-reader at 570 nm wavelength. The reported optical densities in the study were averaged for each extract.

It should be noted that in order to be able to visualize the effect of natural extract against biofilms the negative control readings in all microtiter plates were eliminated via subtraction upon analysis.

2.5 Preparation of Natural extracts

2.5.1 Natural products used in the study

The natural extracts were picked out from local supermarkets (picked up in the fall season).

Scientific name	products
Petroselinum cripsum	Parsley
Mentha sepicata,	Mint
Ocimum basilicum,	Basil
Rosmarinus officinalis,	Rosemary
Trifolium alexandrinum,	Clover
Punica granatum	Pomegranate
Allium cepa	Onion
Allium sativum	Garlic
Salvia officinalis	Sage
Thymus vulgari	Thyme

Table1. Natural products used in the study.

2.5.2 Preparation of natural product ethanol extracts:

Various natural compounds have been selected based on reports of their possible antimicrobial effect such as clover, sage, rosemary, basil, pomegranate, bay leaf etc. These products (200 grams of each) were placed in an 80% ethanol solutions and left in the orbital shaker for a week at room temperature (25-28 degrees Celsius). The

extracts were then filtered and stored in dark and low temperature (8-10 degrees Celsius); for further testing.

2.5.3 Source of organic compounds

The three major chemical composites of each one of the extracts were purchased from (Sigma-Aldrich) in both powder and liquid state.

2.5.4 Preparation of ethanol solutions of specific organic compounds:A stock alpha-thujone, camphor, therpinene, pinene, thymol, cymene solutions were provided by Sigma-Aldrich, from which they were diluted with 80% ethanol into solutions with distinctive molar concentrations (0.1, 0.05, 0.025, 0.0125 M).

CHAPTER III

Results

3.1 Detection of extended spectrum beta lactamases

The *E.coli* strains were tested for their susceptibility to beta-lactam antibacterials, as per the standard procedure in section 2.0, the results are shown in tables 1 and 2 below.

Antibiotic bacterial isolate	ceftazidime,	aztreonam	cefotaxime	ceftriaxone	Calvulanic acid
10052	-	-	-	+	-
9457	+	-	-	-	-
9127	-	+	-	-	-
8869	-	+	-	-	-
1711	-	-	-	-	-
1723	-	-	-	-	-
1676	-	-	-	-	-
1068	+	-	-	-	-

720	-	-	-	-	-
411	-	-	-	+	-

Table2. Antibacterial susceptibility testing of the ESBL expressing *E.coli* isolates, the numbers the designated study number, -: resitant, +: susceptible

antibiotic / bacterial isolate	ceftazidime,	aztreonam	cefotaxime	ceftriaxone	Calvulanic acid
241	+	+	+	+	+
8881	+	±	+	+	+
658	+	+	+	+	+
518	+	+	+	+	+
527	+	±	+	+	+
504	+	+	+	+	+
1389	+	+	+	+	+
12950	+	+	+	±	+
12738	+	+	+	+	±
12945	+	±	+	+	+

Table3. Antibacterial susceptibility testing of the Non-ESBL *E.coli* isolates, the numbers the designated study number, ±: intermediately susceptible, +: susceptible.

The Antibiotic susceptipility testing helped in the identification of the ESBL and non-ESBL *E.coli* isolates to be used in the study.

3.2 DNA fingerprinting of the *E.coli* isolates

The procedure recommended by the Centers for Disease Control and Prevention (C.D.C) was applied for the molecular and epidemiological analysis of the studied *E.coli* strains (method 2.2). The gel patterns are demonstrated below in Figures 1, and 2.

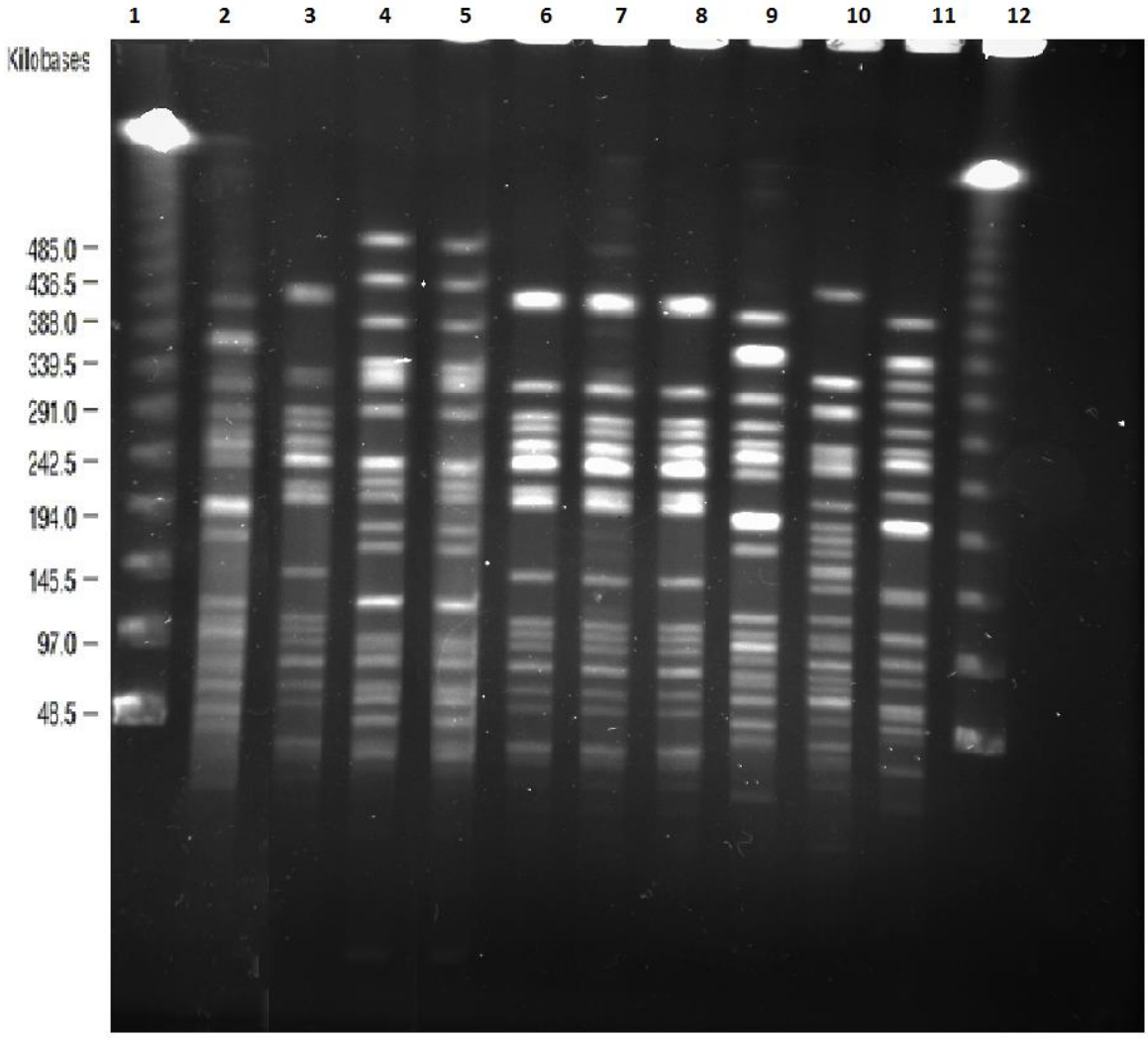

Figure 1. Pulse field gel electrophoresis of *Xba1* digested DNA from ESBL-*E.coli* isolates. Lane 1 and 12: lambda ladder, lane 2: isolate No 10052, lane 3: isolate No 9457, lane 4: isolate No 9127, lane 5: isolate No 8869, lane 6: isolate No 1711, lane 7:

isolate No 1723, lane 8: isolate No 1676, lane 9: isolate No 1068, lane 10: isolate No 720, lane 11: isolate No 411.

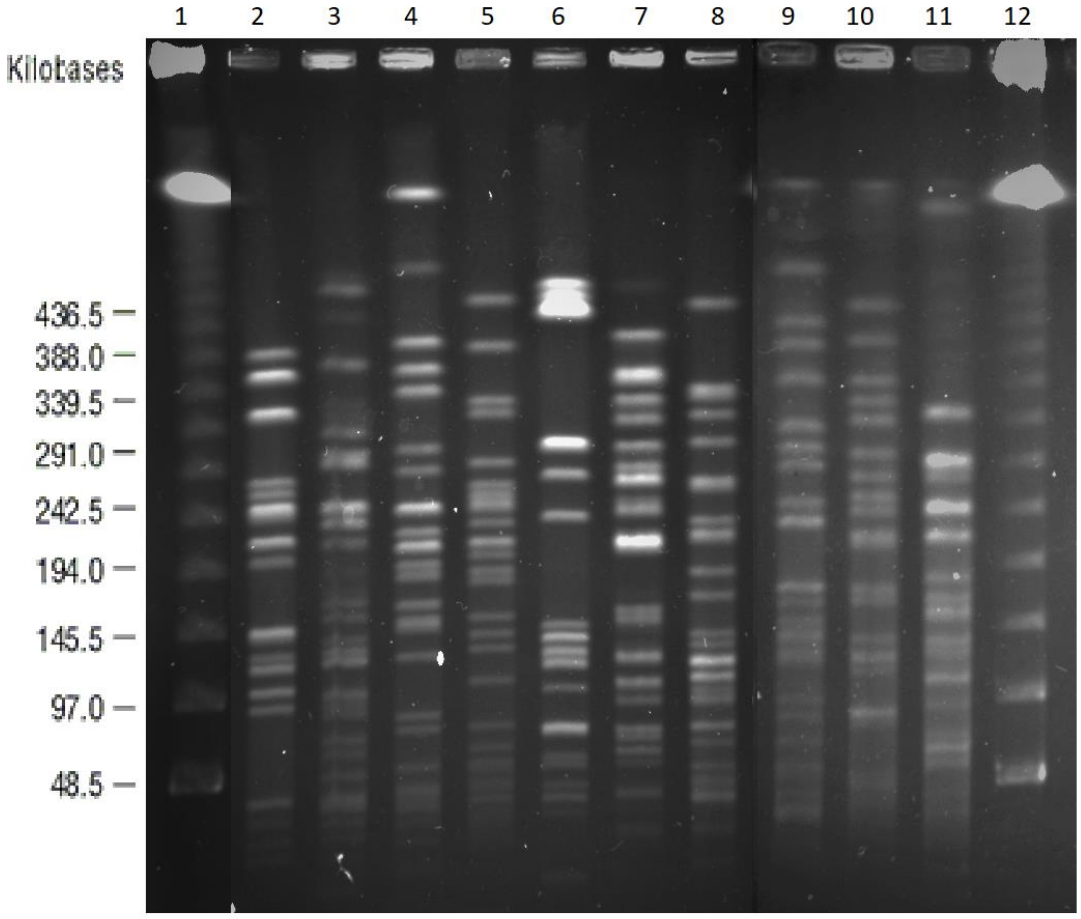

Figure 2. Pulse field gel electrophoresis of *Xba1* digested DNA from Non-ESBL-*E.coli* isolates. Lane 1 and 12: lambda ladder, lane 2: isolate No 241, lane 3: isolate No 8881, lane 4: isolate No 658, lane 5: isolate No 518, lane6: isolate No 527, lane 7: isolate No 504, lane 8: isolate No 1389, lane 9: isolate No 12950, lane 10: isolate No 12738, lane 11: isolate No 12945.

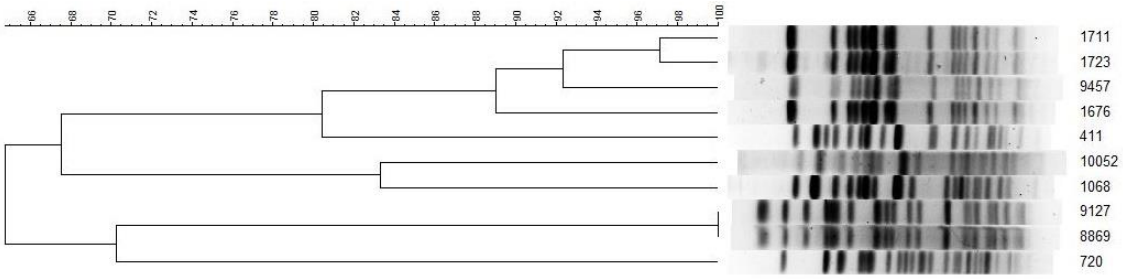

Figure 3. Dendogram of the ESBL *E.coli* strains in this study.

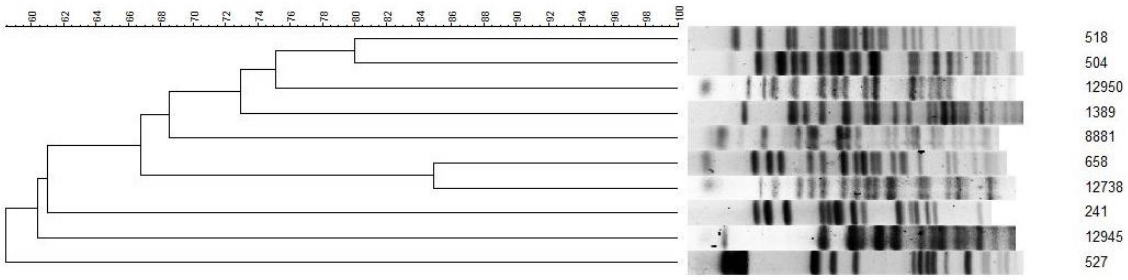

Figure 4. Dendogram of the Non-ESBL *E.coli* strains in this study.

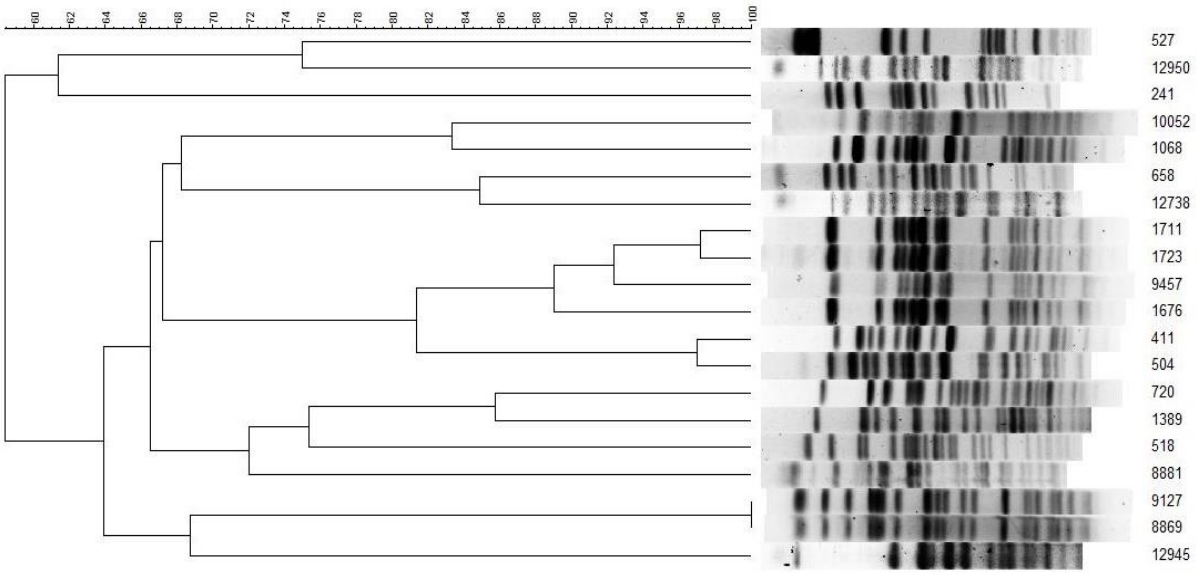

Figure 5. Dendogram of the *E.coli* strains in this study.

The data presented in Figure 3 shows that while most of the ESBL *E.coli* isolates were found to be of high relatedness, with the lowest percentage of relatedness between the studied groups was at 62%, the majority of the strains had a common ancestor and a selected some were ruled as identical. It is presumed that they all were derived from the pressures of the hospital environment.

This relatedness, however, was not seen in the finger prints of the non-ESBL strains, Figure 4, shows that the percent relatedness between these strains did not exceed 82% signifying a distant association between them and a geographical scattering of the isolates, indicating that probably they did not originate from the hospital environment.

The percent relatedness of all studied specimens was found to be relatively low, as shown in Figure 5, since it ranged between 60 and 70 %. Overall the ESBL and non-ESBL expressing *E.coli* were considered to be distantly related, stressing the presumption of being geographically scattered.

3.3 Screening for biofilm formation

All studied specimens were chosen to be biofilm formers, following the confirmatory protocol, stated in methods 2.4, reported separately for ESBL, and non-ESBL, *E.coli* as shown in Tables 4 and 5 and in Figures 6 and 7.

Isolates	**ESBL 10052**	**ESBL 9457**	**ESBL 9127**	**ESBL 8869**	**ESBL 1711**	**Control**
Average readings	0.44402613	0.48907975	0.54953475	0.43366175	0.4011205	0.33358
Control elimination	0.11044164	0.155495268	0.215950268	0.100077268	0.06753602	0
Isolates	**ESBL 1723**	**ESBL 1676**	**ESBL 1068**	**ESBL 720**	**ESBL 411**	**Control**
Average readings	0.585094	0.44491125	0.461237038	0.42729725	0.42604025	0.33358
Control elimination	0.25150952	0.111326768	0.127652555	0.093712768	0.09245577	0

Table4. Average optical density (O.D.) readings at 570nm wavelength of different ESBL isolates tested in this study. ESBL: extended spectrum beta lactamases *E. coli.*

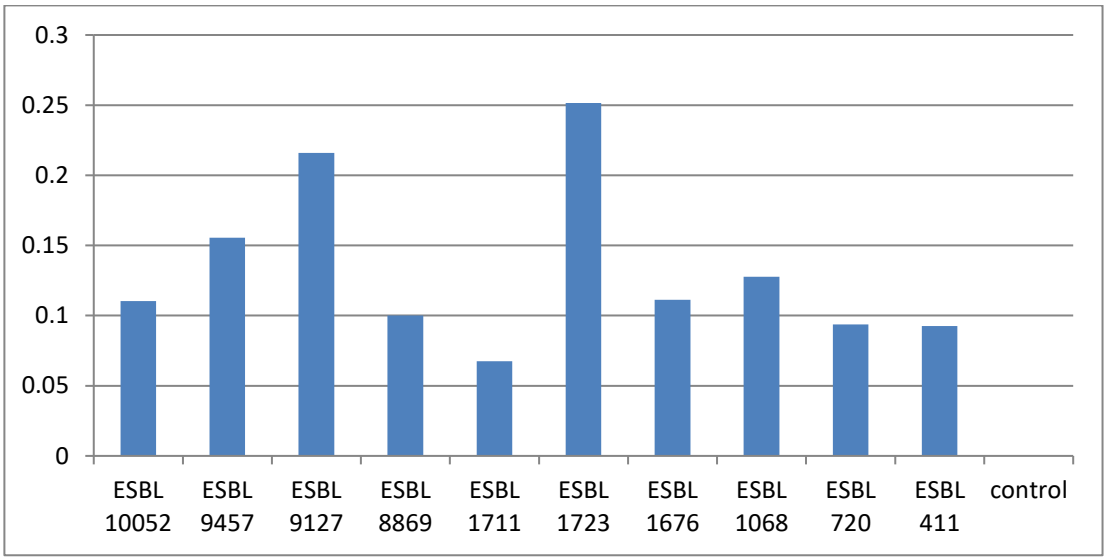

Figure 6. Graphic representation of the data in table 4. X axis: samples tested for biofilm formation/inhibition; Y axis: optical density (O.D.) readings at 570 nm wavelength. ESBL: extended spectrum beta lactamases *E. coli.*

Isolates	**N-ESBL 241**	**N-ESBL 8881**	**N-ESBL 658**	**N-ESBL 518**	**N-ESBL 527**	**Control**
Average readings	0.440776	0.494222	0.449093	0.405909	0.510059	0.373008
Control elimination	0.067767	0.121214	0.076084	0.0329	0.13705	0
Isolates	**N-ESBL 504**	**N-ESBL 1389**	**N-ESBL 13009**	**N-ESBL 12950**	**N-ESBL 12738**	**Control**
Average readings	0.403619	0.583221	0.496903	0.418334	0.492259	0.373008
Control elimination	0.030611	0.210213	0.123894	0.045325	0.11925	0

Table 5. Average optical density (O.D.) readings at 570nm wavelength of different ESBL isolates tested in this study. N-ESBL: Non-extended spectrum beta lactamases presenting *E. coli.*

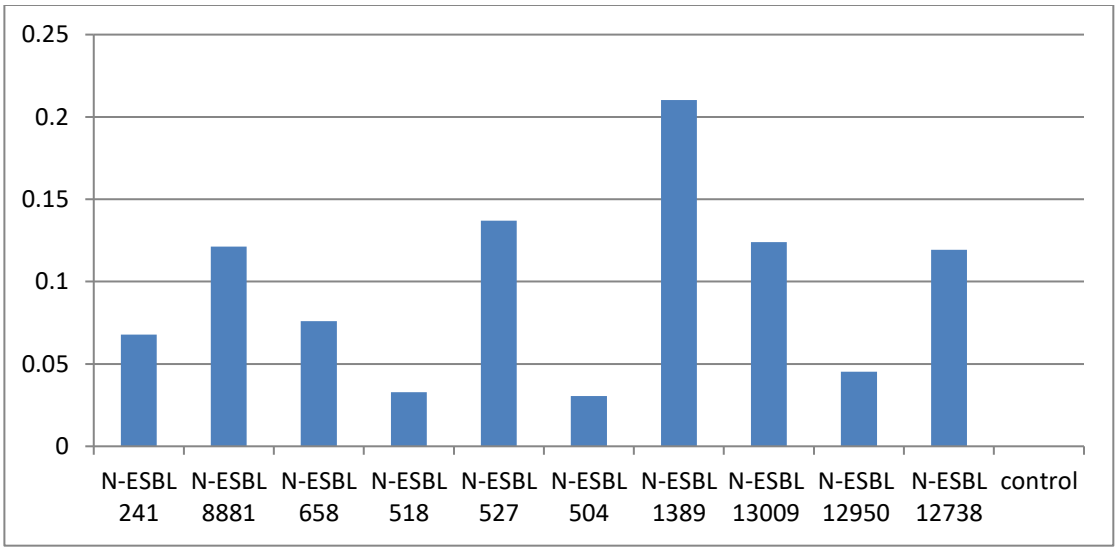

Figure 7. Graphic representation of the data in table 5. X axis: samples tested for biofilm formation/inhibition; Y axis: optical density (O.D.) readings at 570 nm wavelength. N-ESBL: Non-extended spectrum beta lactamases producing *E. coli.*

These results obtained show that the 20 chosen isolates definitely identified as *E.coli* were capable of forming biofilms.

3.4 Antibiofilm effect of Natural extracts

All the natural extracts tested in this study (Table 1) were reported to have a significant antimicrobial effect; they were picked out from local supermarkets that sell organic products.

These natural extracts were put to the test against the chosen biofilm forming (*E.coli*) isolates to test for their potential to prevent biofilm formation

A pilot study using 10 *E.coli* isolates, 5 of which were ESBL producers was conducted at first in order to test for their ability to inhibit biofilm formation. The results are shown in Tables (6 and 7) and Figures (8 and 9).

Pilot extracts	**G**	**G + B**	**O**	**O + B**	**B**	**Control**
Average readings	0.81136	0.898524	1.20906	0.86371	0.49001	0.36854
Control elimination	0.44281	0.52998	0.84052	0.49516	0.12147	0
Pilot extracts	**P**	**P + B**	**M**	**M + B**	**B**	**Control**
Average readings	3.33546	1.146738	1.33347	0.94278	0.49001	0.36854
Control elimination	2.96692	0.778194	0.96492	0.57423	0.12147	0

Table 6. Average optical density (O.D.) readings at 570nm wavelength of different natural extract with chosen bacterial isolates under examination in this study. B: bacterial biofilm readings, G: garlic ethanolic extract, O: onion ethanolic extract, P: parsley ethanolic extract, M: mint ethanolic extract.

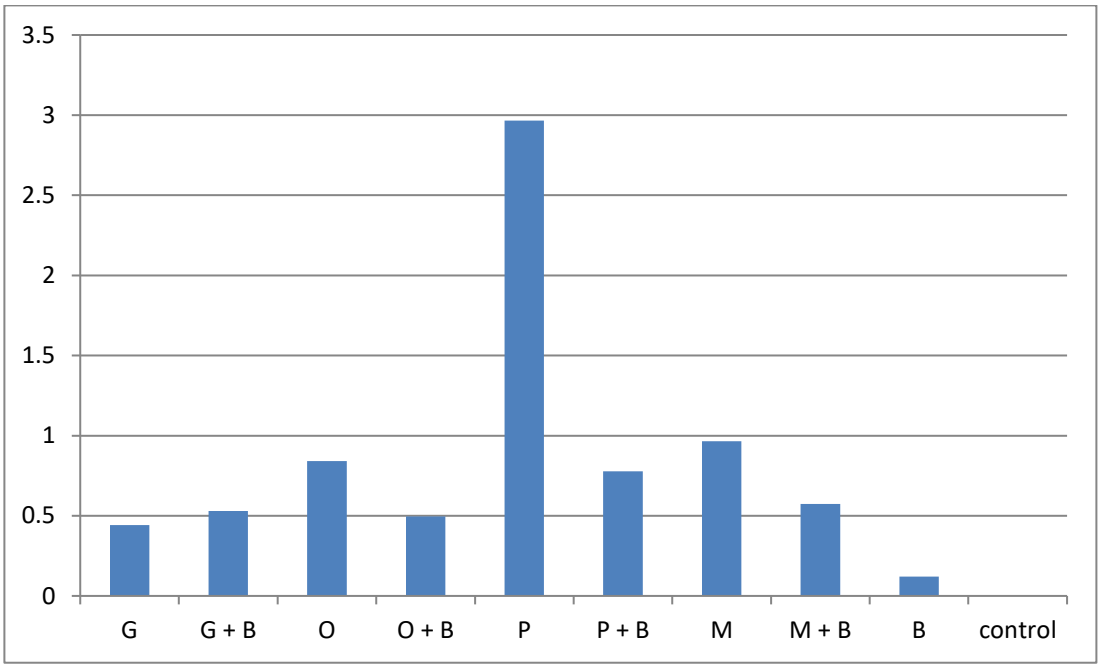

Figure 8. Graphic representation of the data in table 6. X axis: samples tested for biofilm formation/inhibition; Y axis: optical density (O.D.) readings at 570 nm wavelength. B: bacterial biofilm readings, G: garlic ethanolic extract, O: onion ethanolic extract, P: parsley ethanolic extract, M: mint ethanolic extract.

Pilot extracts	**Cl**	**Cl + B**	**Ba**	**Ba + B**	**Po**	**Po + B**	**B**	**Control**
Average readings	2.932072083	3.76045	1.43479	1.5475	0.42341	0.56877	0.4261	0.31252
Control elimination	2.619554125	3.44793	1.12227	1.23499	0.11089	0.25625	0.11358	0
Pilot extracts	**R**	**R + B**	**S**	**S + B**	**T**	**T + B**	**B**	**Control**
Average readings	1.180551833	1.95935	2.82569	2.02612	2.21396	1.13258	0.4261	0.31252
Control elimination	0.868033875	1.64684	2.51317	1.7136	1.90144	0.82006	0.11358	0

Table 7. Average optical density (O.D.) readings at 570nm wavelength of different natural extract against chosen bacterial isolates under examination in this study. B: bacterial biofilm readings, Cl: clover leaf ethanolic extract, Ba: basil ethanolic extract, Po: pomegranate ethanolic extract, R: rosemary ethanolic extract, S: sage ethanolic extract, T: thyme ethanolic extract.

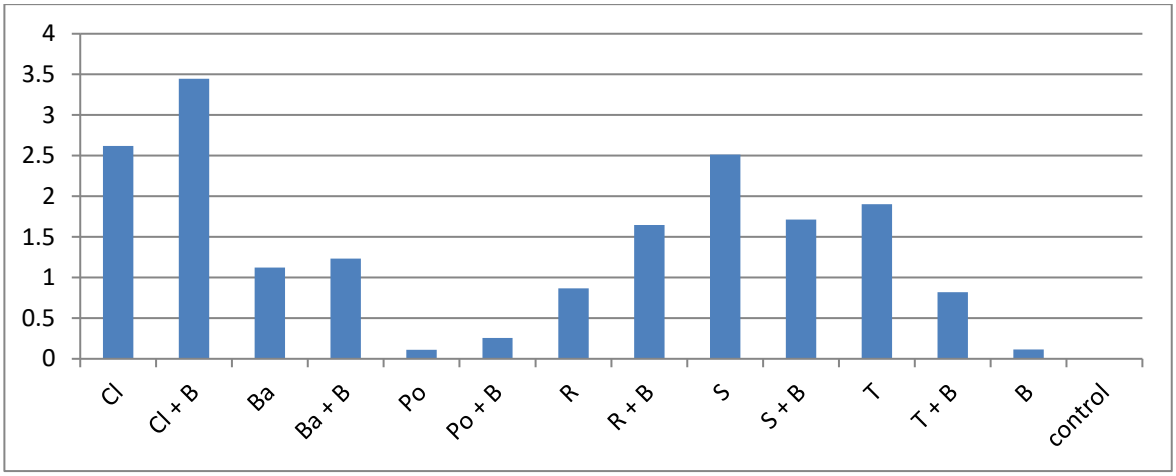

Figure 9. Graphic representation of the data in table 7. X axis: samples tested for biofilm formation/inhibition; Y axis: optical density (O.D.) readings at 570 nm wavelength. . B: bacterial biofilm readings, Cl: clover leaf ethanolic extract, Ba: basil ethanolic extract, Po: pomegranate ethanolic extract, R: rosemary ethanolic extract, S: sage ethanolic extract, T: thyme ethanolic extract.

This pilot study revealed that only *Allium cepa* (onion) , *Salvia officinalis* (sage), and *Thymus vulgari* (thyme) showed an ability to inhibit biofilm formation and were then tested using all the isolates selected for the study.

Extract	S	S + BE	T	T + BE	O	O + BE	BE	Control
Average readings	2.18943	1.68665	1.90764	1.30199	1.02367	0.92834	0.41389	0.33358
Control elimination	1.86214	1.35936	1.58035	0.9747	0.69638	0.60105	0.0866	0.00

Table 8. Average optical density (O.D.) readings at 570nm wavelength of different natural extract against ESBL bacterial isolates under examination in this study. BE: ESBL bacterial biofilm readings, S: sage ethanolic extract, T: thyme ethanolic extract, O: onion ethanolic extract.

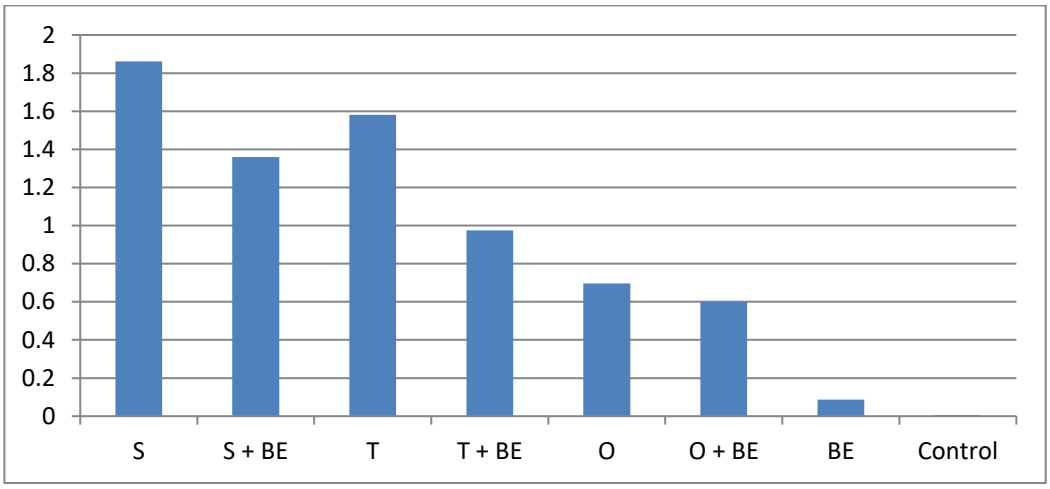

Figure 10. Graphic representation of the data in table 8. X axis: samples tested for biofilm formation/inhibition; Y axis: optical density (O.D.) readings at 570 nm wavelength. . BE: ESBL bacterial biofilm readings, S: sage ethanolic extract, T: thyme ethanolic extract, O: onion ethanolic extract.

Extract	S	S + BNE	T	T + BNE	O	O + BNE	BNE	Control
Average readings	2.540615	1.339525	1.759205	1.763863	1.257443	1.410661	0.472975	0.373008
Control elimination	2.167607	0.966516	1.386197	1.390854	0.884434	1.037653	0.099966	0

Table 9. Average optical density (O.D.) readings at 570nm wavelength of different natural extract against non-ESBL bacterial isolates under examination in this study. BNE: non-ESBL bacterial biofilm readings, S: sage ethanolic extract, T: thyme ethanolic extract, O: onion ethanolic extract.

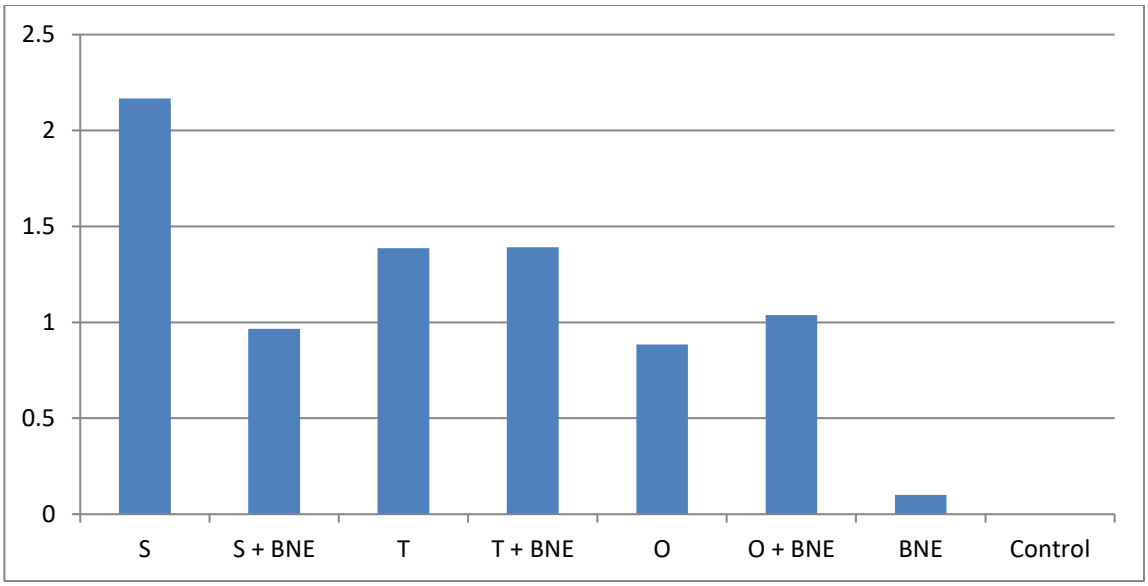

Figure 11. Graphic representation of the data in table 9. X axis: samples tested for biofilm formation/inhibition; Y axis: optical density (O.D.) readings at 570 nm wavelength. . BNE: non- ESBL bacterial biofilm readings, S: sage ethanolic extract, T: thyme ethanolic extract, O: onion ethanolic extract.

The full analysis of *Allium cepa* (onion) , *Salvia officinalis* (sage), and *Thymus vulgari* (thyme) effect on inhibiting biofilm formation against all isolates lead to the elimination of onion from further analysis due to the fluctuating results Figures 10,11 that were detected. The antibifilm effect of sage and thyme was consistent and the study was continued using these two products.

3.5 Antibiofilm effect of major acting organic compounds

The major organic constituents of sage and thyme that presented a substantial antibiofilm effect where put to the test at a specific molar concentration in order to, isolate the active compounds.The same procedure used for the detection of the antibiofilm effect of the natural products was used (section 2). The results for the 3 major compounds composing sage and thyme shown on Tables 10, 11 and Figures 12 and 13.

Organic compounds	**Ty + BE**	**Ty**	**Cy + BE**	**Cy**	**BE**	**Control**
Average readings	0.75857	0.60776	0.43391	0.39164	0.43371	0.33565
Control elimination	0.42292	0.27211	0.09826	0.05599	0.09806	0
Organic compounds	**Te + BE**	**Te**	**Ca + BE**	**Ca**	**BE**	**Control**
Average readings	0.37106	0.33028	0.40687	0.32878	0.43371	0.33565
Control elimination	0.03541	-0.0054	0.07122	-0.0069	0.09806	0
Organic compounds	**Th + BE**	**Th**	**Pi + BE**	**Pi**	**BE**	**Control**
Average readings	0.38776	0.24869	0.41158	0.37095	0.43371	0.33565
Control elimination	0.05211	-0.087	0.07593	0.0353	0.09806	0

Table 10. Average optical density (O.D.) readings at 570nm wavelength of different Organic compounds at 0.1 molar concentration against ESBL bacterial isolates under examination in this study. BE: ESBL bacterial biofilm readings, Ty: thymol ethanolic

solution, Cy: cymene ethanolic solution, Te: terpinene ethanolic solution, Ca: camphor ethanolic solution, Th: thujone ethanolic solution, Pi: pinene ethanolic solution.

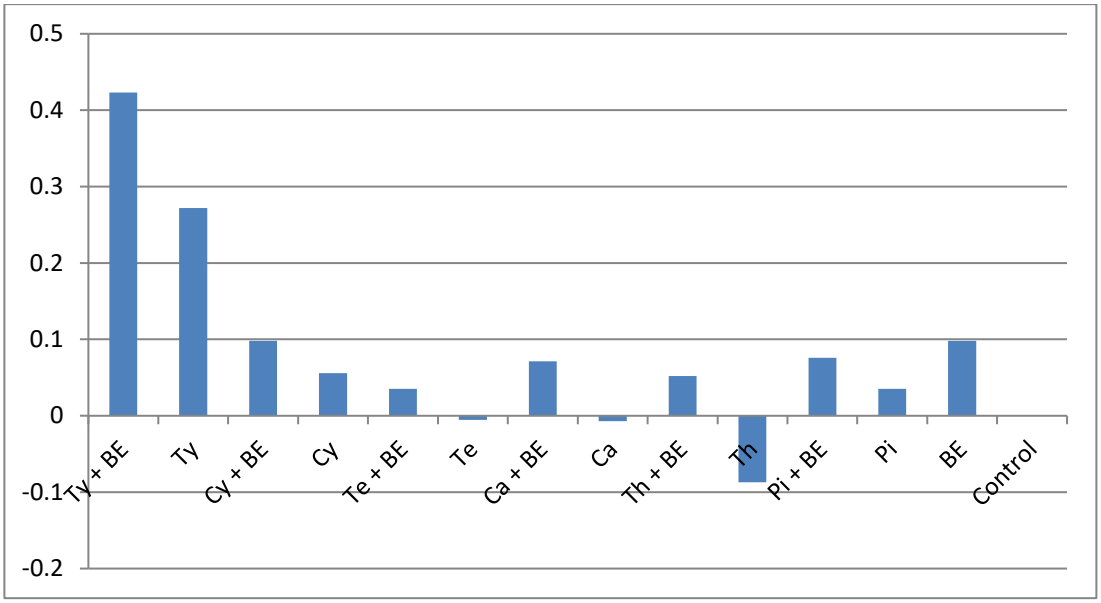

Figure 12. Graphic representation of the data in table 10. X axis: samples tested for biofilm formation/inhibition; Y axis: optical density (O.D.) readings at 570 nm wavelength. BE: ESBL bacterial biofilm readings, Ty: thymol ethanolic solution, Cy: cymene ethanolic solution, Te: terpinene ethanolic solution, Ca: camphor ethanolic solution, Th: thujone ethanolic solution, Pi: pinene ethanolic solution.

Organic compounds	**Te + BNE**	**Te**	**Ca + BNE**	**Ca**	**BNE**	**Control**
Average readings	0.38298	0.32898	0.34214	0.31001	0.43278	0.35037
Control elimination	0.03262	-0.0214	-0.0082	-0.0404	0.08242	0
Organic compounds	**Cy + BNE**	**Cy**	**Pi + BNE**	**Pi**	**BNE**	**Control**
Average readings	0.4618	0.30687	0.46768	0.29572	0.43278	0.35037
Control elimination	0.11143	-0.0435	0.11732	-0.0546	0.08242	0
Organic compounds	**Ty + BNE**	**Ty**	**Th + BNE**	**Th**	**BNE**	**Control**
Average readings	0.53444	0.33317	0.44007	0.30379	0.43278	0.35037
Control elimination	0.18408	-0.0172	0.0897	-0.0466	0.08242	0

Table 11 . Average optical density (O.D.) readings at 570nm wavelength of different Organic compounds at 0.1 molar concentration against non-ESBL bacterial isolates under examination in this study. BNE: non-ESBL bacterial biofilm readings, Ty: thymol ethanolic solution, Cy: cymene ethanolic solution, Te: terpinene ethanolic solution, Ca: camphor ethanolic solution, Th: thujone ethanolic solution, Pi: pinene ethanolic solution.

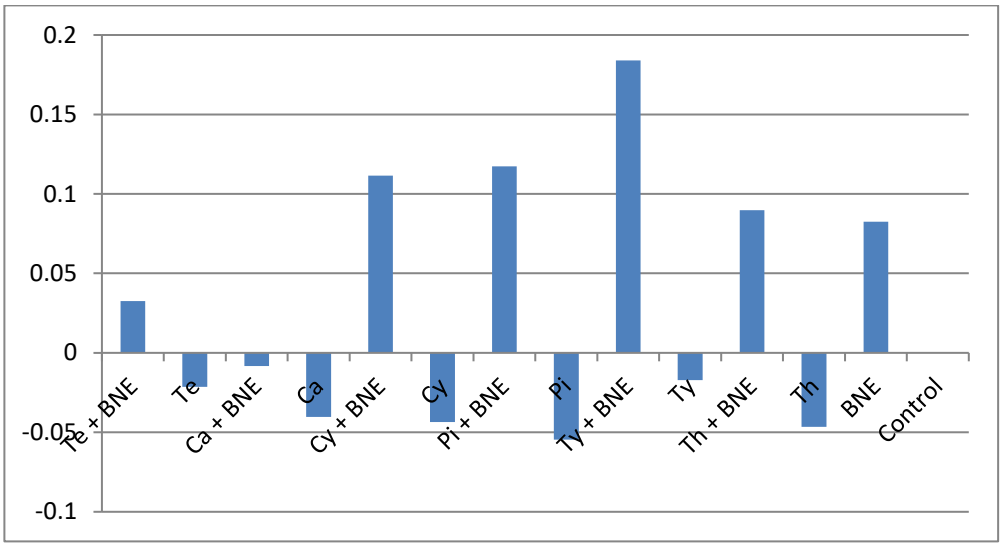

Figure 13. Graphic representation of the data in table 11. X axis: samples tested for biofilm formation/inhibition; Y axis: optical density (O.D.) readings at 570 nm wavelength. BNE: ESBL bacterial biofilm readings, Ty: thymol ethanolic solution, Cy: cymene ethanolic solution, Te: terpinene ethanolic solution, Ca: camphor ethanolic solution, Th: thujone ethanolic solution, Pi: pinene ethanolic solution

The effect of the major organic compounds of *Salvia officinalis* (sage), α-Thujone, Camphor, and α-Pinene, against biofilm formation in ESBL and non-ESBL presenting *E.coli* revealed that Camphor was capable to inhibit biofilm formation at 0.1 molar (Tables 10, 11, Figures 12 and 13).

Moreover the effect of the main organic compounds of *Thymus vulgaris* (thyme), Thymol, gamma-terpinene, and para-cymene, against biofilm formation in ESBL and

non-ESBL presenting *E.coli* revealed that gamma-terpinene was capable to inhibit biofilm formation at 0.1 molar Tables 10, 11 and Figures 12 and 13.

The results of these experiments showed that biofilm inhibitory potential of organic compiounds against biofilm forming ESBL- *E.coli* specimens (Table 10 and figure 9). However, contradictory results were perceived when the compounds were tested against non-ESBL producing *E.coli* (Table 10 and Figure 10).

3.6 Synergistic effect of the effective organic compounds

The major organic compounds where mixed together in various concentrations, to study the potential of a synergic effect that would increase the biofilm inhibitory effect. The concentrations prepared where then put to the test against all the isolates in this study. The organic compounds were tested against two biofilm forming bacterial isolates (isolates 1723, and 1389) in a checkerboard approach, where multiple concentrations of camphor were mixed with a range of gamma-terpinene concentrated solution as seen in Table 12, and Figure 14. Out of the group three mixtures showed promising results, highlighted in bold in Table 12, and they corresponded to 0.0125M camphor mixed with 0.1M, 0.05M, and 0.025 M gamma-terpinene respectively.

Mixtures	**0.1t/0.1c**	**0.1t/0.05c**	**0.1t/0.025c**	**0.1t/0.0125c**
Average readings	0.42312	0.40218	0.34602	0.27093
Control elimination	0.12419	0.10326	0.0471	-0.028
Percent effect	-23.062	-2.3165	53.3319	**127.742**
Mixtures	**0.05t/0.1c**	**0.05t/0.95c**	**0.05t/0.025c**	**0.05t/0.0125c**
Average readings	0.35034	0.39951	0.37383	0.31604
Control elimination	0.05142	0.10059	0.07491	0.01712
Percent effect	49.0478	0.32972	25.7743	**83.04**
Mixtures	**0.025t/0.1c**	**0.025t/0.05c**	**0.025t/0.025c**	**0.025t/0.0125c**
Average readings	0.43199	0.36392	0.36819	0.29393
Control elimination	0.13306	0.065	0.06927	-0.005
Percent effect	-31.85	35.5931	31.3626	**104.95**
Mixtures	**0.0125t/0.1c**	**0.0125t/0.05c**	**0.0125t/0.025c**	**0.0125t/0.0125c**
Average readings	0.43947	0.36976	0.41735	0.40267
Control elimination	0.14054	0.07084	0.11843	0.10374
Percent effect	-39.264	29.8048	-17.349	-2.7978

bacteria	**control**	**mix**
0.39984	0.29892	0.30797
0.10092	0	0.00904
0	100	not applicable

Table 12. Average optical density (O.D.) readings at 570nm wavelength of checker board plate of different camphor concentrations against various terpinene concentrations. C: camphor, T: terpinene, mix: reading of 0.1 molar camphor and 0.1 molar terpinene alone, 0.1, 0.05, 0.025, 0.0125: multiple molar concentrations.

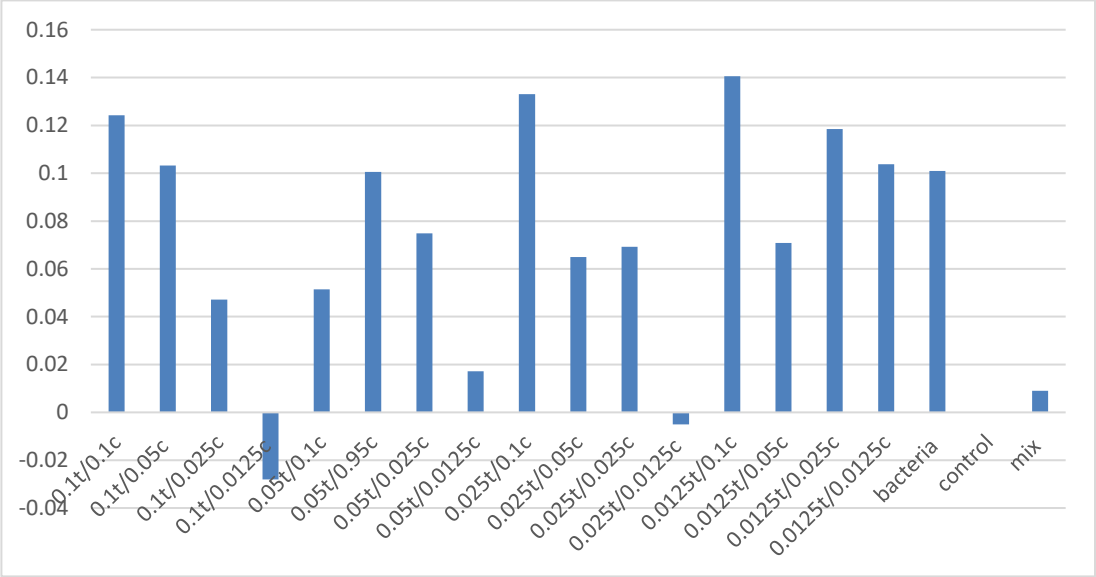

Figure 14. Graphic representation of the data in table 12. X axis: samples tested for biofilm formation/inhibition; Y axis: optical density (O.D.) readings at 570 nm wavelength. C: camphor, T: terpinene, mix: reading of 0.1 molar camphor and 0.1 molar terpinene alone, 0.1, 0.05, 0.025, 0.0125: multiple molar concentrations.

The promising mixtures, after checkerboard analysis pilot study, were then put to the test against all bacterial isolates included in this study, shown in Tables 14 to 20; throughout the different mixtures and solutions some antibiofilm effect was noted, but no synergism was recorded.

Mixtures	ESBLs + 0.1t+0.0125c	ESBLs	0.1t+0.0125c	control
Average readings	0.32495	0.37642	0.28115	0.27606
Control elimination	0.0489	0.10037	0.0051	0
Percent effect		51.2825		

Table 13. Average optical density (O.D.) readings at 570nm wavelength of checker board plate of ESBL isolate with camphor and terpinene concentrations. C: camphor, T: terpinene, 0.1, 0.0125: molar concentrations.

Mixtures	ESBLs + 0.05t +0.0125c	ESBLs	0.05t +0.0125c	control
Average readings	0.339916	0.376422	0.28913	0.276057
Control elimination	0.063859	0.100365	0.013074	0
Percent effect		36.37304		

Table14. Average optical density (O.D.) readings at 570nm wavelength of checker board plate of ESBL isolate with camphor and terpinene concentrations. C: camphor, T: terpinene, 0.05, 0.0125: molar concentrations.

Mixtures	ESBLs + 0.025t +0.0125c	ESBLs	0.025t +0.0125c	control
Average readings	0.354792	0.376422	0.291101	0.276057
Control elimination	0.078736	0.100365	0.015044	0
Percent effect		21.5507		

Table 15. Average optical density (O.D.) readings at 570nm wavelength of checker board plate of ESBL isolate with camphor and terpinene concentrations. C: camphor, T: terpinene, 0.025, 0.0125: molar concentrations.

Mixtures	Non-ESBLs + 0.1t+0.0125c	non-ESBLs	0.1t+0.0125c	control
Average readings	0.282837	0.345099	0.281154	0.276057
Control elimination	0.006781	0.069042	0.005098	0
Percent effect		**90.17879**		

Table16. Average optical density (O.D.) readings at 570nm wavelength of checker board plate of non-ESBL isolate with camphor and terpinene concentrations. C: camphor, T: terpinene, 0.1, 0.0125: molar concentrations.

Mixtures	Non-ESBLs + 0.05t +0.0125c	non-ESBLs	0.05t +0.0125c	control
Average readings	0.306529	0.345099	0.28913	0.276057
Control elimination	0.030473	0.069042	0.013074	0
Percent effect		**55.86356**		

Table17. Average optical density (O.D.) readings at 570nm wavelength of checker board plate of non-ESBL isolate with camphor and terpinene concentrations. C: camphor, T: terpinene, 0.05, 0.0125: molar concentrations.

Mixtures	Non-ESBLs + 0.025t +0.0125c	non-ESBLs	0.025t +0.0125c	control
Average readings	0.293942	0.345099	0.291101	0.276057
Control elimination	0.017885	0.069042	0.015044	0
Percent effect		**74.09545**		

Table18. Average optical density (O.D.) readings at 570nm wavelength of checker board plate of non-ESBL isolate with camphor and terpinene concentrations. C: camphor, T: terpinene, 0.025, 0.0125: molar concentration

Wells content	Non-ESBLs + 0.1t	0.1t	Non-ESBLs + 0.05t	0.05t	Non-ESBLs + 0.025t	0.025t	Non-ESBLs	control
Average readings	0.53298	0.42898	0.5154	0.35103	0.52399	0.38192	0.580294	0.427389
Control elimination	0.10559	0.00158	0.088011	-0.07636	0.096605	-0.04547	0.152905	0
Percent effect	**30.9415**		**42.4410**		**36.8206**			

Table19. Average optical density (O.D.) readings at 570nm wavelength of checker board plate of all non-ESBL isolates with different terpinene concentrations. T: terpinene, 0.1, 0.05, 0.025, 0.0125: molar concentrations.

wells content	ESBLs+0.1t	0.1t	ESBLs+0.05t	0.05t
Average readings	0.46687	0.330285	0.431274	0.351034
Control elimination	0.039481	-0.0971	0.003885	-0.07636
percent effect	**42.90984**		**94.38223**	
wells content	**ESBLs+0.025t**	**0.025t**	**ESBLs**	**control**
Average readings	0.423881	0.381919	0.496544	0.427389
Control elimination	-0.00351	-0.04547	0.069155	0
percent effect	**105.0721**			

Table20. Average optical density (O.D.) readings at 570nm wavelength of checker board plate of all ESBL isolates with different terpinene concentrations. T: terpinene, 0.1, 0.05, 0.025: molar concentrations.

Organic compounds	BNE + 0.1c	BNE + 0.0125c	BNE	C	control
Average readings	0.34214	0.40973	0.40844	0.31001	0.35037
Control elimination	-0.0082	0.05936	0.05807	-0.0404	0
Percent effect	**114.172**	**-2.2211**			

Table21. Average optical density (O.D.) readings at 570nm wavelength of checker board plate of all Non-ESBL isolates with different camphor concentrations. C: camphor, 0.1, 0.0125: molar concentrations.

Organic compounds	BE + 0.1c	BE + 0.0125c	BE	C	control
Average readings	0.40687	0.422409	0.43371	0.31001	0.35037
Control elimination	0.0565	0.072039	0.08334	-0.04036	0
Percent effect	**32.20542**	**13.5599**			

Table22. Average optical density (O.D.) readings at 570nm wavelength of checker board plate of all ESBL isolates with different camphor concentrations. C: camphor, 0.1, 0.0125: molar concentrations.

CHAPTER IV

Discussion

With the increasing prevalence of antibiotic resistant bacterial strains and the fear of outbreaks caused by multidrug resistant bacteria, the search for effective compounds that can inhibit the formation of biofilms, a step performed by most bacterial groups prior to initiating an infection, has been the focus of research scientists. With this goal in mind, this study was planned to try, to use natural extracts from different plants ,(listed in Table 1). In an attempt to identify a compound which can help in controlling infections caused by an organism that is very common in clinical specimens, *Escherichia coli*.

The generated dendogram for ESBL E.coli (Figures 3) indicates that the studied strains originated from either the same or closely geographical related environment; one possible environment was the hospital environment, however this needs more investigation with a bigger number of strains.

Where non-ESBL strains 504, 1389 and ESBL strains 411 and 720 were found to be highly related respectively (95 and 89 %) which indicates that at one point the

newly attained Non-ESBL strains were able to acquire resistance against beta-lactams quickly and with minimal genetic variations if the surrounding environment attained stress factors and excess exposure to factors requiring such changes.

Inhibition of biofilm formation can be manifested by hindering the initial reversible bacterial attachment to surfaces. This is usually done by either limiting the number of attaching bacteria or downregulating gene expression and the pathways responsible for production and release of excess exopolyscharides needed for the biofilm formation. Earlier studies showed that coating surfaces with certain chemical compounds can aid in stopping biofilm development and maturation, the reason why it was decided to use the same proceudure of biofilm inhibition implemented earlier in this study.

Several studies showed the positive outcome of this technique using different bacterial species. Antoci et al (2008) applied this protocol against *Staphylococcus epidermidis*, a Gram-positive organism prevalent in orthopaedic infections, by coating titanium alloy surfaces with vacomyocin in an attempt to prevent bacterial colonization

and were able to obstruct biofilm formation while retaining the antibiotic sensitivity and specificity of the organism.

Bazzi et al (2013), also applied this procedure by coating microtiter plates with micafungin, an antifungal agent known to hinder 1,3-β-D-glucan biosynthesis in *Candida albicans*, and were capable not only to inhibit biofilm formation but also to decrease the expression levels of *algC* and *pelC*, *ndvB*, genes encoding for alginate, pellicles, and cell wall 1,3-β-D-glucan respectively; all of which are also responsible for the progression formation of biofilms in *Pseudomonas aeruginosa* .

As a first step all the tested and selected natural extracts were put to the test for their potential biofilm inhibition ability ; against multiple biofilm forming bacterial *E.coli* strains to test for their potential antibiofilm effect; the detection protocol was similar to the one applied in biofilm detection (method 2.4.2), with only adding the ethanolic extracts on the wells to see their effect on the readings.

A pilot study using 10 *E.coli* isolates, 5 of which were ESBLs was conducted at first in order to test for the biofilm inhibitory potential of the different natural compound against biofilm formation.

Tables 6 and 7 as well as Figures 8 and 9 shows that more than one natural product were indeed capable of reducing biofilm formation, namely: onion, sage, and thyme. Extracts from other products did not show a clear negative or positive effect; probably due to the high chromatographic index in some of the compounds like parsley, mint, and clover leaf; that masked the inhibitory potential of these extracts in the study (Tables 6 and 7, and Figures 8 and 9). Yet again extratcs from other products did not show any sign of biofilm inhibition such as garlic, basil, rosemary, and pomegranate (Tables 6 and 7, and Figures 8 and 9).

The results of the pilot study showed that out of the tested products three natural extracts were able to inhibit biofilm formation, and those were those of onions, thyme, and sage (Tables 6 and 7, Figures 8 and 9).

The potentially effective extracts were once more put to the test against all biofilm forming isolates in this study as shown in Tables 8, and 9 and Figures 10, and 11. The results showed that on one side sage and thyme ethanolic extratcs were clearly able to inhibit biofilm formation effectively and significantly (Tables 8, and 9 and Figures 10, and 11); while onion ethanolic extract was not able to inhibit biofilm formation on non-ESBL producing *E.coli* (Table 9 and Figure 11) and thus was excluded from further study.

This fluctuating outcome in the case of onion ethanolic extract may have been due to a number of reasons. One plausible explanation is that, as a result of the greatly concentrated ethanolic extract the inhibitory complexes obtained may have been hindered sterically leading to the conflicting effects discussed previously. Another possible reason may have been since the strains that did not exhibit extended spectrum beta-lactamases were able to resist the inhibitory effect of onions against biofilm formation; the increased gene expression of beta–lactamases may have hindered or down regulated genes responsible for the irreversible settling of the biofilm.

As this study showed that the prospective natural products that required more investigations were sage and thyme, the literature was reviewed to detect the organic compounds making these products. Previous work, using gas chromatography and mass spectrometry, defined the exact organic constituents of these two plants (Sparkman et al, 2011., Porte et al, 2013., Borugă et al, 2014.) as shown in Tables 23 and 24 for sage and thyme respectively.

Compounds	**%**
α-Thujone	40.90
Camphor	26.12

α-Pinene	5.85
β-Thujone	5.62

Table23. Major chemical constituents of the essential oil from leaves of *Salvia officinalis* (Porte et al, 2013.).

Compounds	**%**
Thymol	47.59
gamma-Terpinene	30.90
para-Cymene	8.41

Table24. Major chemical composition of *Thymus vulgaris*(Borugă et al, 2014.)

The obtained information about the inhibition of biofilm formation potential of each one of the tested plants lead to the following step, to test the ability of the organic compounds in each to inhibit the ability of the tested strains to form biofilms; the initial concentration of 0.1 molar solution in 80% ethanol of the selected chemicals tested, was set in order to minimize the possibility of chemical compound emulsification against the walls of the microtiter plates, which may lead to incorrect readings.

The results showed that only two chemical compounds of those tested for bothe products were found to inhibit biofilm formation of both sets of strains (ESBL and non-ESBL producing *E.coli*), and these were Camphor and Terpinene (Tables 10, 11 and Figures 12, 13).

It should be noted that during the course of testing for the effect of the major organic compounds many compounds registered readings slightly lower than the negative control (empty well stained), less than 0.1 difference, and that probably was due to the high viscosity of the chemical compounds that may have hindered the well staining by the used stain crystal violet.

Camphor oil is known to have several health and medicinal benefits; the waxy, highly aromatic ten carbon containing organic compound was found to have anti-inflammatory, analgesic, and antiseptic properties and is widely in far-east Asia (Chang et al, 2006., Mann et al, 1994.).

Likewise gamma-terpinene, the isomeric hydrocarbon of terpenes is highly valued in the medical field, as it is considered as a very powerful antioxidant and has a notable bactericidal effect against a range of microorganisms; it was found to be present in a number of plants from thyme to citrus (NCBI, 2017.).

As noted earlier no synergism was recorded in the case of using camphor and gamma-terpinene, but solution mixtures were able to inhibit biofilms with higher efficacy than individual organic compounds in the case of non-ESBLs as seen in Tables 16, 17, 18, 19, and 21.

This variability in the results between ESBLs and non-ESBLs was probably due to the idea that overexpression of beta-lactamase related genes has hindered, masked or down regulated other genes responsible for biofilm irreversible settling. The overall presented results hint to the posibility that camphor and terpinene worked on downregulating and inhibiting pathways responsible for biofilm formation and development. These results howerver also suggest that both terpinene and camphor may work on targeting separate regulatory pathways responsible for biofilm formation. This hypothesis needs to be tested in future studies by isolating, determining and examining expression levels of genes known to be involved in *E.coli* biofilm formation through qRTPCR.

In 2009 a patent was granted on the use and production of ursolic and asiatic acid that have showed ability to modulate the expression level of *cysB* gene in *E.coli*; the transcriptional regulator *cysB* is known to play a major role in biofilm production and development since it helps in exopolysscharide synthesis and release (Eldridge, 2009.), hence showing the ability of natural organic compounds to reduce and inhibit

biofilm formation by downregulating pathways needed for the settlement and formation of biofilms.

This study conformed the idea that biofilm formation can be inhibited with the use of organic compounds found in common natural products. This and future work will help in limiting infections that initially depend on biofilm formation on body tissues. This approach may not solve the developing problem of increae in resistant bacterial strains, but will definitely decrease the risk of infections, if used properly in and out of the hospital environment.

References

Adal, K. A., & Farr, B. M. (1996). Central venous catheter-related infections: a review. *Nutrition*, *12*(3), 208-213.

Anand, R. (1986). Pulsed field gel electrophoresis: a technique for fractionating large DNA molecules. *Trends in Genetics*, *2*, 278-283.

Antoci, V., Adams, C. S., Parvizi, J., Davidson, H. M., Composto, R. J., Freeman, T. A., Hickok, N. J. (2008). The inhibition of staphylococcus epidermidis biofilm formation by vancomycin-modified titanium alloy and implications for the treatment of periprosthetic infection. *Biomaterials*, *29*(35), 4684–4690.

Arbeit, R. D. (1996). Laboratory procedures for the epidemiologic analysis of microorganisms. *Manual of clinical microbiology*.

Archibald, L. K., & Gaynes, R. P. (1997). Hospital-acquired infections in the United States: the importance of interhospital comparisons. *Infectious disease clinics of North America*, *11*(2), 245-255.

Aruna, P., Venkataramanamma, D., Singh, A. K., & Singh, R. P. (2016). Health benefits of punicic acid: a review. *Comprehensive Reviews in Food Science and Food Safety*, *15*(1), 16-27.

Ascón-Cabrera, M. A., Ascón-Reyes, D. B., & Lebeault, J. M. (1995). Degradation activity of adhered and suspended *Pseudomonas* cells cultured on 2, 4, 6-trichlorophenol, measured by indirect conductimetry. *Journal of Applied Microbiology*, *79*(6), 617-624.

Baggesen, D. L., Sorensen, G., Nielsen, E. M., & Wegener, H. C. (2010). Phage typing of *Salmonella* Typhimurium-is it still a useful tool for surveillance and outbreak investigation. *Euro Surveill*, *15*(4), 19471.

Baraniak, A., J. Fiett, W. Hryniewicz, P. Nordmann, and M. Gniadkowski. 2002. Ceftazidime-hydrolysing CTX-M-15 extended-spectrum beta-lactamase (ESBL) in Poland. J. Antimicrob. Chemother. 50:393–396.

Baron, S. (1996). *Epidemiology--Medical Microbiology*. University of Texas Medical Branch at Galveston.

Baytop, T. (1999). Therapy with medicinal plants in Turkey (Past and Present).

Bazzi, W., Sabra, A., Zahreddine, L., Khairallah, M. T., Baroud, M., Hadi, U., & Matar, G. M. (2013). The inhibitory effect of micafungin on biofilm formation by Pseudomonas aeruginosa. *Biofouling*, *29*(8), 909-915.

Belaaouaj, A., C. Lapoumeroulie, M. M. Canic ¸a, G. Vedel, P. Ne ´vot, R. Krishnamoorthy, and G. Paul. 1994. Nucleotide sequences of the genes coding for the TEM-like -lactamases IRT-1 and IRT-2 (formerly called TRI-1 and TRI-2). FEMS Microbiol. Lett. 120:75–80.

Benkeblia, N. (2004). Antimicrobial activity of essential oil extracts of various onions (*Allium cepa*) and garlic (*Allium sativum*). *LWT-Food Science and Technology*, *37*(2), 263-268.

Bian, Z., Brauner, A., Li, Y., & Normark, S. (2000). Expression of and Cytokine Activation by Escherichia coli Curli Fibers in Human Sepsis. *The Journal of infectious diseases*, *181*(2), 602-612.

Birren, B., & Lai, E. (2012). *Pulsed field gel electrophoresis: a practical guide*. Academic Press.

Bonomo, R.A., S.A.Rudin,and D.M. Shlaes.(1997).Tazobactamisapotent inactivator of selected inhibitor-resistant class A beta-lactamases. FEMS Microbiol. Lett. 148:59–62.

Borugă, O., Jianu, C., Mişcă, C., Goleţ, I., Gruia, A. T., & Horhat, F. G. (2014). *Thymus vulgaris* essential oil: chemical composition and antimicrobial activity. *Journal of medicine and life*, *7*(Spec Iss 3), 56.

Bradford, P. A., C. Urban, A. Jaiswal, N. Mariano, B. A. Rasmussen, S. J. Projan, J. J. Rahal, and K. Bush. 1995. SHV-7, a novel cefotaxime-hydrolyzing -lactamase, identified in *Escherichia coli* isolates from hospitalized nursing home patients. Antimicrob. Agents Chemother. 39:899–905

Brown, D. (1995). *Encyclopedia of Herbs and their Uses. Dorling Kindersley London*. ISBNO 7513-020-31.

Bush, K. & Fisher, J. F. (2011). Epidemiological Expansion, Structural Studies, and Clinical Challenges of New βlactamases from Gram-Negative Bacteria. Annual Review of Microbiology, 65: 455-478.

Bush, K. & Jacoby, G. A. (2010). Updated Functional Classification of β-lactamases. Antimicrobial Agents and Chemotherapy, 54(3): 969-976.

Bush, K. (1989a). Characterization of β-lactamases. Agents Chemotherapy, 33:259-263.

Bush, K., Jacoby, G. A. & Medeiros, A. A. (1995). A Functional Classification Scheme for β-lactamases and its Correlation with Molecular Structure. Antimicrobial Agents and Chemotherapy, 39(6): 1211-1233.

Bush. K. (1989b). Classification of β-lactamases - Groups 1, 2a, 2b and 2b'. Antimicrobial Agents and Chemotherapy, 33(3): 264-270. Caldwell, D. E., Korber, D. R., & Lawrence, J. R. (1992). Confocal laser microscopy and digital image analysis in microbial ecology. In *Advances in microbial ecology* (pp. 1-67). Springer US.

Carbonnelle, E., Hélaine, S., Prouvensier, L., Nassif, X., & Pelicic, V. (2005). Type IV pilus biogenesis in Neisseria meningitidis: PilW is involved in a step occurring after pilus assembly, essential for fibre stability and function. *Molecular microbiology*, *55*(1), 54-64.

Celiktas, O. Y., Kocabas, E. H., Bedir, E., Sukan, F. V., Ozek, T., & Baser, K. H. C. (2007). Antimicrobial activities of methanol extracts and essential oils of Rosmarinus officinalis, depending on location and seasonal variations. *Food Chemistry*, *100*(2), 553-559.

Centers for Disease Control and Prevention. (2013). Standard operating procedure for PulseNet PFGE of Escherichia coli O157: H7, *Escherichia coli* non-O157 (STEC), *Salmonella* serotypes, *Shigella sonnei* and *Shigella flexneri. Centers for Disease Control and Prevention, Atlanta*.

Chaibi, E. B., D. Sirot, G. Paul, and R. Labia. (1999). Inhibitor-resistant TEM--lactamases: phenotypic, genetic and biochemical characteristics. J. Antimicrob. Chemother. 43:447–458.

Chang, C. P., Leung, T. K., Lin, S. M., & Hsu, C. C. (2006). Release properties on gelatin-gum arabic microcapsules containing camphor oil with added polystyrene. *Colloids and Surfaces B: Biointerfaces*, *50*(2), 136-140.

Characklis, W. G. 1990. Biofilm processes, p. 195–232. In W. G. Characklis and K. C. Marshall (ed.), Biofilms. John Wiley & Sons, New York, N.Y.

Characklis, W. G., G. A. McFeters, and K. C. Marshall. 1990.Physiological ecology in biofilm systems, p. 341–394. In W. G. Characklis and K. C. Marshall (ed.), Biofilms. John Wiley and Sons, New York, N.Y.

Choi, Y., Jeong, H. S., & Lee, J. (2007). Antioxidant activity of methanolic extracts from some grains consumed in Korea. *Food chemistry*, *103*(1), 130-138.

Chu, G., Vollrath, D., & Davis, R. W. (1986). Separation of large DNA molecules by contour-clamped homogeneous electric fields. *Science*, *234*, 1582-1586.

Clardy, J., Fischbach, M. A., & Currie, C. R. (2009). The natural history of antibiotics. *Current biology*, *19*(11), R437-R441.

Clinical Laboratory Standard Institute . Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Second Informational Supplement. Vol. 32. Clinical Laboratory Standard Institute; Wayne, Pennsylvania, USA: (2012). pp. 70–71.

Costerton, J. W., Stewart, P. S., & Greenberg, E. P. (1999). Bacterial biofilms: a common cause of persistent infections. *Science*, *284*(5418), 1318-1322.

Cowan, M. M. (1999). Plant products as antimicrobial agents. *Clinical microbiology reviews*, *12*(4), 564-582.

Crepin, V. F., Girard, F., Schüller, S., Phillips, A. D., Mousnier, A., & Frankel, G. (2010). Dissecting the role of the Tir: Nck and Tir: IRTKS/IRSp53 signalling pathways in vivo. *Molecular microbiology*, *75*(2), 308-323.

Dahham, S. S., Ali, M. N., Tabassum, H., & Khan, M. (2010). Studies on antibacterial and antifungal activity of pomegranate (Punica granatum L.). *Am. Eurasian J. Agric. Environ. Sci*, *9*(3), 273-281.

Danel, F., L. M. C. Hall, B. Duke, D. Gur, and D. M. Livermore. 1999. OXA-17, a further extended-spectrum variant of OXA-10 -lactamase, isolated from Pseudomonas aeruginosa. Antimicrob. Agents Chemother. 43:1362–1366.

Danel, F., L. M. C. Hall, D. Gur, and D. M. Livermore. 1995. OXA-14, another extended-spectrum variant of OXA-10 (PSE-2) -lactamase from Pseudomonas aeruginosa. Antimicrob. Agents Chemother. 39:1881–1884.

Davies, D. G., & Geesey, G. G. (1995). Regulation of the alginate biosynthesis gene algC in Pseudomonas aeruginosa during biofilm development in continuous culture. *Applied and environmental microbiology*, *61*(3), 860-867.

Davies, D. G., Chakrabarty, A. M., & Geesey, G. G. (1993). Exopolysaccharide production in biofilms: substratum activation of alginate gene expression by Pseudomonas aeruginosa. *Applied and environmental microbiology*, *59*(4), 1181-1186.

DeVault, J. D., Berry, A., Misra, T. K., Darzins, A., & Chakrabarty, A. M. (1989). Environmental sensory signals and microbial pathogenesis: Pseudomonas aeruginosa infection in cystic fibrosis. *Nature Biotechnology*, *7*(4), 352-357.

Devi, P., Meera, R., Chithambaranathan, N., Kameswari, B., & Badmanaban, R. (2010). Diuretic and antimicrobial activity of methanolic extract of Petroselinum Cripsum leaves. *International Journal of Pharmtech Research*, *2*(1), 228-231.

Dewanti, R., & Wong, A. C. (1995). Influence of culture conditions on biofilm formation by Escherichia coli O157: H7. *International journal of food microbiology*, *26*(2), 147-164.

Dickinson, G. M., & Bisno, A. L. (1993). Infections associated with prosthetic devices: clinical considerations. *The International journal of artificial organs*, *16*(11), 749-754.

Dictionary, A. H. (2007). The American Heritage Medical Dictionary.

Dodson, K. W., Jacob-Dubuisson, F. R. A. N. I. O. I. S. E., Striker, R. T., & Hultgren, S. J. (1993). Outer-membrane PapC molecular usher discriminately recognizes periplasmic chaperone-pilus subunit complexes. *Proceedings of the National Academy of Sciences*, *90*(8), 3670-3674.

Donlan, R. M., & Costerton, J. W. (2002). Biofilms: survival mechanisms of clinically relevant microorganisms. *Clinical microbiology reviews*, *15*(2), 167-193.

du Plessis DJF; Nouwen N; Driessen AJM (2011). "The Sec translocase". Biochim. Biophys. Acta. 1808 (3): 851–865. PMID 20801097. doi:10.1016/j.bbamem.2010.08.016), Kline, K. A., Dodson, K. W., Caparon, M. G., & Hultgren, S. J. (2010). A tale of two pili: assembly and function of pili in bacteria. *Trends in microbiology*, *18*(5), 224-232.

El Harrif-Heraud, Z., C. Arpin, S. Benliman, and C. Quentin. 1997. Molecular epidemiology of a nosocomial outbreak due to SHV-4 producing strains of Citrobacter diversus. J. Clin. Microbiol. 35:2561–2567.

Eldridge, G. R. (2009). *U.S. Patent No. 7,604,978*. Washington, DC: U.S. Patent and Trademark Office.

Emery, C.L., and L.A .Weymouth.(1997).Detectionandclinicalsignificance of extended-spectrum beta-lactamases in a tertiary-care medical center. J. Clin. Microbiol. 35:2061–2067.

Evans, D. J., Allison, D. G., Brown, M. R. W., & Gilbert, P. (1990). Effect of growth-rate on resistance of gram-negative biofilms to cetrimide. *Journal of Antimicrobial Chemotherapy*, *26*(4), 473-478.

F.C. Tenover, P.Raney, P.P. Williams, K. L. Brittan, C. D. Steward, S. K. Fridkin, R. P. Gaynes, and J. E. McGowan, Jr., 40th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 1606, 2000

Fiett, J., A. Palucha, B. Miaczynska, M. Stankiewicz, H. Przondo-Mordarska, W. Hryniewicz, and M. Gniadkowski. 2000. A novel complex mutant beta-lactamase, TEM-68, identified in a *Klebsiella pneumoniae* isolate from an outbreak of extended-spectrum beta-lactamase-producing klebsiellae. Antimicrob. Agents Chemother. 44:1499–1505.

FLETCHER, M. (1996). DIVERSITY OF SURFACES AND ADHESION STRATEGIES. *Bacterial adhesion: molecular and ecological diversity*, *19*, 1.

Garrett, E. S., Perlegas, D., & Wozniak, D. J. (1999). Negative control of flagellum synthesis in Pseudomonas aeruginosa is modulated by the alternative sigma factor AlgT (AlgU). *Journal of bacteriology*, *181*(23), 7401-7404.

Genevaux, P., Muller, S., & Bauda, P. (1996). A rapid screening procedure to identify mini-Tn10 insertion mutants of *Escherichia coli* K-12 with altered adhesion properties. *FEMS microbiology letters*, *142*(1), 27-30.

Goering, R. V. (2010). Pulsed field gel electrophoresis: a review of application and interpretation in the molecular epidemiology of infectious disease. *Infection, Genetics and Evolution*, *10*(7), 866-875.

Goering, R. V. (2010). Pulsed field gel electrophoresis: a review of application and interpretation in the molecular epidemiology of infectious disease. *Infection, Genetics and Evolution*, *10*(7), 866-875.

Gruber, T. M., & Gross, C. A. (2003). Multiple sigma subunits and the partitioning of bacterial transcription space. *Annual Reviews in Microbiology*, *57*(1), 441-466.

Haley, R. W., Cushion, N. B., Tenover, F. C., Bannerman, T. L., Dryer, D., Ross, J., ... & Siegel, J. D. (1995). Eradication of endemic methicillin-resistant Staphylococcus aureus infections from a neonatal intensive care unit. *Journal of Infectious Diseases*, *171*(3), 614-624.

Heinrich, M., Barnes, J., Gibbons, S., & Williamson, E. M. (2004). Fundamentals of Pharmacognosy and Phytotherapy. pub Churchill Livingstone an imprint of Elsevier Science Limited. *Dr Duke's Phytochemical and Ethnobotanical Databases http://www. ars-grin. gov/duke*.

Henquell, C., C. Chanal, D. Sirot, R. Labia, and J. Sirot. 1995. Molecular characterization of nine different types of mutants among 107 inhibitorresistant TEM -lactamases from clinical isolates of Escherichia coli. Antimicrob. Agents Chemother. 39:427–430.

Hentzer, M., & Givskov, M. (2003). Pharmacological inhibition of quorum sensing for the treatment of chronic bacterial infections. *The Journal of clinical investigation*, *112*(9), 1300-1307.

Hernández, T., Canales, M., Avila, J. G., Duran, A., Caballero, J., De Vivar, A. R., & Lira, R. (2003). Ethnobotany and antibacterial activity of some plants used in traditional medicine of Zapotitlán de las Salinas, Puebla (México). *Journal of ethnopharmacology*, *88*(2-3), 181-188.

Heydorn, A., Nielsen, A. T., Hentzer, M., Sternberg, C., Givskov, M., Ersbøll, B. K., & Molin, S. (2000). Quantification of biofilm structures by the novel computer program COMSTAT. *Microbiology*, *146*(10), 2395-2407.

Horiuchi, K., Shiota, S., Hatano, T., Yoshida, T., Kuroda, T., & Tsuchiya, T. (2007). Antimicrobial activity of oleanolic acid from Salvia officinalis and related compounds on vancomycin-resistant enterococci (VRE). *Biological and Pharmaceutical Bulletin*, *30*(6), 1147-1149.

Huletsky, A., J. R. Knox, and R. C. Levesque. 1993. Role of Ser-238 and Lys-240 in the hydrolysis of 3rd-generation cephalosporins by SHV-type beta-lactamases probed by site-directed mutagenesis and 3-dimensional modeling. J. Biol. Chem. 268:3690–3697.

Husain, A. (1992). Dictionary of Indian medicinal plants.

Hussain, A. I., Anwar, F., Sherazi, S. T. H., & Przybylski, R. (2008). Chemical composition, antioxidant and antimicrobial activities of basil (Ocimum basilicum) essential oils depends on seasonal variations. *Food chemistry*, *108*(3), 986-995.

İşcan, G., Kirimer, N., Kürkcüoğlu, M., Başer, H. C., & DEMIrci, F. (2002). Antimicrobial screening of Mentha piperita essential oils. *Journal of agricultural and food chemistry*, *50*(14), 3943-3946.

Ishii, Y., S. Kimura, J. Alba, K. Shiroto, M. Otsuka, N. Hashizume, K. Tamura, and K. Yamaguchi. (2005). Extended-spectrum -lactamase-producing shiga toxin gene (stx1)-positive *Escherichia coli* O26:H11: a new concern. J. Clin. Microbiol. 43:1072–1075.

Jacobs, C., Frère, J-M. & Normark, S. (1997). Cytosolic Intermediates for Cell Wall Biosynthesis and Degradation Control Inducible β-lactam Resistance in Gram-Negative Bacteria. Cell, 88: 823-832.

Jacoby, G. A. 1997. Extended-spectrum beta-lactamases and other enzymes providing resistance to oxyimino-beta-lactams. Infect. Dis. Clin. N. Am. 11:875–887.

Jacoby, G. A., and L. Sutton. 1991. Properties of plasmids responsible for production of extended-spectrum -lactamases. Antimicrob. Agents Chemother. 35:164–169.

Johnson, A. E., & van Waes, M. A. (1999). The translocon: a dynamic gateway at the ER membrane. *Annual review of cell and developmental biology*, *15*(1), 799-842.

Jurenka, J. (2008). MT (ASCP). *Alternative Medicine Review*.

Kenny, B., DeVinney, R., Stein, M., Reinscheid, D. J., Frey, E. A., & Finlay, B. B. (1997). Enteropathogenic E. coli (EPEC) transfers its receptor for intimate adherence into mammalian cells. *Cell*, *91*(4), 511-520.

Khan, A. V., Ahmed, Q. U., Shukla, I., & Khan, A. A. (2012). Antibacterial activity of leaves extracts of Trifolium alexandrinum Linn. against pathogenic bacteria causing tropical diseases. *Asian Pacific journal of tropical biomedicine*, *2*(3), 189-194.

Khoury, A. E., Lam, K., Ellis, B., & Costerton, J. W. (1992). Prevention and control of bacterial infections associated with medical devices. *ASAIO journal*, *38*(3), M174-M178.

Kline, K. A., Fälker, S., Dahlberg, S., Normark, S., & Henriques-Normark, B. (2009). Bacterial adhesins in host-microbe interactions. *Cell host & microbe*, *5*(6), 580-592.

Korber, D. R., Lawrence, J. R., & Lappin-Scott, H. M. (2003). Growth of Microorganisms on. *Microbial biofilms*, *5*, 15.

Kreiswirth, B., Kornblum, J., Arbeit, R. D., Eisner, W., Maslow, J. N., McGeer, A., ... & Novick, R. P. (1993). Evidence for a clonal origin of methicillin resistance in *Staphylococcus aureus. SCIENCE-NEW YORK THEN WASHINGTON-*, *259*, 227-227.

Kumar, D., Singh, A. K., Ali, M. R., & Chander, Y. (2014). Antimicrobial Susceptibility Profile of Extended Spectrum β-Lactamase (ESBL) Producing *Escherichia coli* from Various Clinical Samples. *Infectious Diseases*, *7*, 1–8.

Lai, Y., Rosenshine, I., Leong, J. M., & Frankel, G. (2013). Intimate host attachment: enteropathogenic and enterohaemorrhagic Escherichia coli. *Cellular microbiology*, *15*(11), 1796-1808.

Lawson, L. D. (1998). Garlic: a review of its medicinal effects and indicated active compounds.

Lee, Y. M., DiGiuseppe, P. A., Silhavy, T. J., & Hultgren, S. J. (2004). P pilus assembly motif necessary for activation of the CpxRA pathway by PapE in Escherichia coli. *Journal of bacteriology*, *186*(13), 4326-4337.

Lee, Y. M., Dodson, K. W., & Hultgren, S. J. (2007). Adaptor function of PapF depends on donor strand exchange in P-pilus biogenesis of Escherichia coli. *Journal of bacteriology*, *189*(14), 5276-5283.

Lillington, J., Geibel, S., & Waksman, G. (2014). Biogenesis and adhesion of type 1 and P pili. *Biochimica et Biophysica Acta (BBA)-General Subjects*, *1840*(9), 2783-2793.

Livermore, D. M. 1995. Beta -Lactamases in laboratory and clinical resistance. Clin. Microbiol. Rev. 8:557–584.

Lu, Y., & Foo, L. Y. (2001). Antioxidant activities of polyphenols from sage (Salvia officinalis). *Food chemistry*, *75*(2), 197-202.

Mahdihassan, S. (1984). Outline of the beginnings of alchemy and its antecedents. *The American journal of Chinese medicine*, *12*(01n04), 32-42.

Majhenič, L., Škerget, M., & Knez, Ž. (2007). Antioxidant and antimicrobial activity of guarana seed extracts. *Food chemistry*, *104*(3), 1258-1268.

Majiduddin, F. K., Materon, I. C. & Palzkill, T. G. (2002). Molecular Analysis of Beta-lactamase Structure and Function. International Journal of Medical Microbiology, 292: 127-137

Mann, J., Davidson, R. S., Hobbs, J. B., Banthorpe, D. V., & Harborne, J. B. (1994). *Natural products: their chemistry and biological significance*. Longman Scientific & Technical.

Marino, M., Bersani, C., & Comi, G. (1999). Antimicrobial activity of the essential oils of *Thymus vulgaris* L. measured using a bioimpedometric method. *Journal of Food Protection*, *62*(9), 1017-1023.

Maslow, J. N. (1993). Application of pulsed-field gel electrohoresis to molecular epidemiology. *Diagnostic molecular microbiology: principles and applications*.

Mathur. T., Singhal, S., Khan, S., Upadhyay, D.J., Fatma, T., Rattan, A. (2006). Detection of biofilm formation among the clinical isolates of staphylococci: an evaluation of three different screening methods. Indian J. Med. Microbiol. 24(1):25.

Mattick, J. S. (2002). Type IV pili and twitching motility. *Annual Reviews in Microbiology*, *56*(1), 289-314.

Melville, S., & Craig, L. (2013). Type IV pili in Gram-positive bacteria. *Microbiology and molecular biology reviews*, *77*(3), 323-341.

Miranda, A. G., Singh, K. V., & Murray, B. E. (1991). DNA fingerprinting of Enterococcus faecium by pulsed-field gel electrophoresis may be a useful epidemiologic tool. *Journal of clinical microbiology*, *29*(12), 2752-2757.

Moland, E. S., C. C. Sanders, and K. S. Thomson. 1998. Can results obtainedwithcommerciallyavailableMicroScanmicrodilutionpanelsserve as an indicator of beta-lactamase production among *Escherichia coli* and *Klebsiella* isolates with hidden resistance to expanded-spectrum cephalosporins and aztreonam? J. Clin. Microbiol. 36:2575–2579.

Naas, T., L. Philippon, L. Poirel, E. Ronco, and P. Nordman. 1999. An SHV-derived extended-spectrum beta-lactamase in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. 43:1281–1284.

Naas, T., W. Sougakoff, A. Casetta, and P. Nordman. 1998. Molecular characterization of OXA-20, a novel class D -lactamase, and its integron from *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. 42:2074–2083.

Naidu, A. S. (Ed.). (2000). *Natural food antimicrobial systems*. CRC press.

National Center for Biotechnology Information. PubChem Compound Database. 2017.

Ncube, N.S., Afolayan, A.J., Okoh, A.I. (2008). Assessment techniques of antimicrobial properties of natural compounds of plant origin: current methods and future trends. Afr. J. Biotechnol. 7(12):1797-1806.

Neuwirth, C., S. Madec, E. Siebor, A. Pechinot, J. M. Duez, M. Pruneaux, M. Fouchereau-Peron, A. Kazmierczak, and R. Labia. 2001. TEM-89 betalactamase produced by a *Proteus mirabilis* clinical isolate: new complex mutant (CMT 3) with mutations in both TEM-59 (IRT-17) and TEM-3. Antimicrob. Agents Chemother. 45:3591–3594.

Nicholas, R. A., Krings, S., Tomberg, J., Nicola, G. & Davies, C. (2003). Crystal Structure of Wild-Type PenicillinBinding Protein 5 from Escherichia coli. Journal of Biological Chemistry, 278(52): 52826-52833.

Olsen, J. E., M. N. Skov, E. J. Threlfall, and D. J. Brown. 1994. Clonal lines of Salmonella enterica serotype enteritidis documented by IS200-, ribo-, pulsed-field gel electrophoresis and RFLP typing. J. Med. Microbiol. 40:15– 22.

O'Toole, G., Kaplan, H. B., & Kolter, R. (2000). Biofilm formation as microbial development. *Annual Reviews in Microbiology, 54*(1), 49-79.

Paterson, D. L., & Bonomo, R. A. (2005). Extended-spectrum β-lactamases: a clinical update. *Clinical microbiology reviews*, *18*(4), 657-686.

Pinheiro, H. M. H. (2013). *Xenology of beta-lactamases: association of its genetic sources and putative pleiotropism* (Doctoral dissertation).

Pizarro-Cerdá, J., & Cossart, P. (2006). Bacterial adhesion and entry into host cells. *Cell, 124*(4), 715-727.

Poirel, L., H. Mammeri, and P. Nordmann. 2004. TEM-121, a novel complex mutant of TEM-type beta-lactamase from Enterobacter aerogenes. Antimicrob. Agents Chemother. 48:4528–4531.

Poirel, L., M. Gniadkowski, and P. Nordmann. 2002. Biochemical analysis of the ceftazidime-hydrolysing extended-spectrum beta-lactamase CTXM-15 and of its structurally related beta-lactamase CTX-M-3. J. Antimicrob. Chemother. 50:1031–1034.

Porte, A., Godoy, R. L. O., & Maia-Porte, L. H. (2013). Chemical composition of sage (Salvia officinalis L.) essential oil from the Rio de Janeiro State (Brazil). *Revista Brasileira de Plantas Medicinais*, *15*(3), 438-441.

Pratt, L. A., & Kolter, R. (1998). Genetic analysis of Escherichia coli biofilm formation: roles of flagella, motility, chemotaxis and type I pili. *Molecular microbiology*, *30*(2), 285-293.

Prigent-Combaret, C., Vidal, O., Dorel, C., & Lejeune, P. (1999). Abiotic surface sensing and biofilm-dependent regulation of gene expression in Escherichia coli. *Journal of bacteriology*, *181*(19), 5993-6002.

Ribet, D., & Cossart, P. (2015). How bacterial pathogens colonize their hosts and invade deeper tissues. *Microbes and Infection*, *17*(3), 173-183.

Rivlin, R. S. (2001). Historical perspective on the use of garlic. *The Journal of nutrition*, *131*(3), 951S-954S.

Roberts, J. A., Marklund, B. I., Ilver, D., Haslam, D., Kaack, M. B., Baskin, G., ... & Normark, S. (1994). The Gal (alpha 1-4) Gal-specific tip adhesin of Escherichia

coli P-fimbriae is needed for pyelonephritis to occur in the normal urinary tract. *Proceedings of the National Academy of Sciences*, *91*(25), 11889-11893.

Ryan, K. J., & Ray, C. G. (2004). Medical microbiology. *McGraw Hill*, *4*, 370.

Sabudak, T., & Guler, N. (2009). Trifolium L.–a review on its phytochemical and pharmacological profile. *Phytotherapy research*, *23*(3), 439-446.

Sader, H. S., Pignatari, A. C., Leme, I. L., Burattini, M. N., Tancresi, R., Hollis, R. J., & Jones, R. N. (1993). Epidemiologic typing of multiply drug-resistant *Pseudomonas aeruginosa* isolated from an outbreak in an intensive care unit. *Diagnostic microbiology and infectious disease*, *17*(1), 13-18.

Sainsbury, S., Bird, L., Rao, V., Shepherd, S. M., Stuart, D. I., Hunter, W. N., Owens, R. J. & Ren, J. (2011). Crystal Structures of Penicillin-Binding Protein 3 from Pseudomonas aeruginosa: Comparison of Native and AntibioticBound Forms. Journal of Molecular Biology, 405(1-3): 173-184.

Sauer, K., Camper, A. K., Ehrlich, G. D., Costerton, J. W., & Davies, D. G. (2002). Pseudomonas aeruginosa displays multiple phenotypes during development as a biofilm. *Journal of bacteriology*, *184*(4), 1140-1154.

Saulino, E. T., Thanassi, D. G., Pinkner, J. S., & Hultgren, S. J. (1998). Ramifications of kinetic partitioning on usher-mediated pilus biogenesis. *The EMBO Journal*, *17*(8), 2177-2185.

Sirot, D., C. Recule, E. B. Chaibi, L. Bret, J. Croize, C. Chanal-Claris, R. Labia, and J. Sirot. (1997).AcomplexmutantofTEM-1beta-lactamasewith mutations encountered in both IRT-4 and extended-spectrum TEM-15, produced by an Escherichia coli clinical isolate. Antimicrob. Agents Chemother. 41:1322–1325.

Sparkman, O. D., Penton, Z., & Kitson, F. G. (2011). *Gas chromatography and mass spectrometry: a practical guide*. Academic Press.

Stapleton, P., K. P. Shannon, and G. L. French. 1999. Construction and characterization of mutants of the TEM-1 -lactamase containing amino acid substitutions associated with both extended-spectrum resistance and resistance to beta-lactamase inhibitors. Antimicrob. Agents Chemother. 43: 1881–1887.

Stewart, P. S., & Costerton, J. W. (2001). Antibiotic resistance of bacteria in biofilms. *The lancet*, *358*(9276), 135-138.

Stoodley, P., Wilson, S., Hall-Stoodley, L., Boyle, J. D., Lappin-Scott, H. M., & Costerton, J. W. (2001). Growth and detachment of cell clusters from mature mixed-species biofilms. *Applied and environmental microbiology*, *67*(12), 5608-5613.

Struelens, M. J., De Ryck, R., & Deplano, A. (2001). Analysis of Microbial Genomic Macrorestriction Patterns by Pulsed-Field Gel Electrophoresis (PFGE) Typing-7.

Studholme, D. J., & Buck, M. (2000). The alternative sigma factor σ28 of the extreme thermophile Aquifex aeolicus restores motility to an Escherichia coli fliA mutant. *FEMS microbiology letters*, *191*(1), 103-107.

Sturenburg, E., A. Kuhn, D. Mack, and R. Laufs. 2004. A novel extendedspectrum beta-lactamase CTX-M-23 with a P167T substitution in the active-site omega loop associated with ceftazidime resistance. J. Antimicrob. Chemother. 54:406–409.

Swaminathan, B., & Matar, G. M. (1993). Molecular typing methods. *Diagnostic molecular microbiology: principles and applications. American Society for Microbiology, Washington, DC*, 26-50.

Sykes, R. B., and K. Bush. 1982. Physiology, biochemistry and inactivation of beta-lactamases, p. 155–207. In R. B. Morin and M. Gorman (ed.), The chemistry and biology of beta-lactam antibiotics, vol. 3. Academic Press, London, England.

Tenover, F. C., Arbeit, R. D., Goering, R. V., Mickelsen, P. A., Murray, B. E., Persing, D. H., & Swaminathan, B. (1995). Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *Journal of clinical microbiology*, *33*(9), 2233.

Tenover, F. C., Arbeit, R., Archer, G., Biddle, J., Byrne, S., Goering, R., & Hollis, R. (1994). Comparison of traditional and molecular methods of typing isolates of *Staphylococcus aureus*. *Journal of clinical microbiology*, *32*(2), 407-415.

Tenover, F. C., J. M. Mohammed, T. Gorton, and Z. F. Dembek. 1999. Detection and reporting of organisms producing extended-spectrum -lactamases: survey of laboratories in Connecticut. J. Clin. Microbiol. 37:4065– 4070.

Tolker-Nielson T, Brinch UC, Ragas PC, Andersen JB, Jacobsen CS, Molin S. 2000. Development and dynamics of *Pseudomonas* sp. biofilms. J. Bacteriol. 182:6482–89.

Turmel, C., Brassard, E., Forsyth, R., Hood, K., Slater, G. W., & Noolandi, J. (1990). High-resolution zero integrated field electrophoresis of DNA. *Current communications in cell & molecular biology*.

Tzouvelekis, L. S., and R. A. Bonomo. 1999. SHV-type beta-lactamases. Curr. Pharm. Des. 5:847–864.

Tzouvelekis, L. S., E. Tzelepi, P. T. Tassios, and N. J. Legakis. 2000. CTX-M-type beta-lactamases: an emerging group of extended-spectrum enzymes. Int. J. Antimicrob. Agents 14:137–142.

Vahaboglu, H., L. M. C. Hall, L. Mulazimoglu, S. Dodanli, I. Yildirim, and D. M. Livermore. 1995. Resistance to extended-spectrum cephalosporins, caused by PER-1 -lactamase, in Salmonella typhimurium from Istanbul, Turkey. J. Med. Microbiol. 43:294–299.

Vahaboglu, H., R. O ¨ztu ¨rk, G. Aygu ¨n, F. Coskunkan, A. Yaman, A. Kaygusuz, H. Leblebicioglu, I. Balik, K. Aydin, and M. Otkun. 1997. Widespread detection of PER-1-type extended-spectrum -lactamases among nosocomialAcinetobacterandPseudomonasaeruginosaisolatesinTurkey:a nationwide multicenter study. Antimicrob. Agents Chemother. 41:2265– 2269.

Vahaboglu, H., S. Dodanli, C. Eroglu, R. O ¨ztu ¨rk, G. Soyletir, I. Yildirim, and V. Avkan. 1996. Characterization of multiple-antibiotic-resistant Salmonella typhimurium strains: molecular epidemiology of PER-1-producing isolates and evidence for nosocomial plasmid exchange by a clone. J. Clin. Microbiol. 34:2942–2946.

Van den Berg, B., Clemons Jr, W. M., Collinson, I., & Modis, Y. (2004). X-ray structure of a protein-conducting channel. *Nature*, *427*(6969), 36.

Veličković, D. T., Ranđelović, N. V., Ristić, M. S., Veličković, A. S., & Šmelcerović, A. A. (2003). Chemical constituents and antimicrobial activity of the ethanol extracts obtained from the flower, leaf and stem of Salvia officinalis L. *Journal of the Serbian Chemical Society*, *68*(1), 17-24.

Wingender, J., Neu, T. R., & Flemming, H. C. (1999). What are bacterial extracellular polymeric substances?. In *Microbial extracellular polymeric substances* (pp. 1-19). Springer Berlin Heidelberg.

Wolfgang, M., Van Putten, J. P., Hayes, S. F., Dorward, D., & Koomey, M. (2000). Components and dynamics of fiber formation define a ubiquitous biogenesis pathway for bacterial pili. *The EMBO journal*, *19*(23), 6408-6418.

Wuertz, S., Okabe, S., & Hausner, M. (2004). Microbial communities and their interactions in biofilm systems: an overview. *Water Science and Technology*, *49*(11-12), 327-336.

Yoganarasimhan, S. N. (2000). Medicinal plants of India. Volume 2. Tamil Nadu. *Bangalore: The Author i*.

Zobell, C. E. (1943). The effect of solid surfaces upon bacterial activity. *Journal of* bacteriology, *46*(1), 39.