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Eugenia caryophyllata Thunberg, a Spice Significant in Therapy of Bacterial Infections Highly Resistant to Antibiotics

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ABSTRACT

Background The antibiotic resistance of Gram-negative bacteria ESBL isolated from UTI infections of hospitalized patients has become a major problem for medical practice in the last decade. With the advent of new drugs, the bacteria have developed new virulence factors. The use of spices herbs could successfully replace the antibacterial activity in many critical circumstances. This study illustrates the antibacterial activity of the hydroethanolic extract of *Eugenia caryophyllata* against a lot of ESBL Gram-negative bacteria MDR isolated from nosocomial infections. From a lot of 80 Gram-negative bacteria isolated, that were tested, 70% of strains were resistant to the extended spectrum β-lactams (cephalosporins), 45% were resistant to carbapenems and 100% were resistant to amoxicillin/clavulanic acid. Phenotypic analysis showed the presence of proteases and pore-forming enzymes. Genetic analysis indicated the presence of ESBL strains type (52% $bla_{\text{CTX-M,}}$ 32% bla_{TEM} and 3% $bla_{\text{OXA-48}}$ and bla_{KPC} type). TLC analysis of ethanol extract of *E. caryophyllata* showed the presence of following antibacterial potential compounds: gallic acid, quercetin and kaempferol. The total content of polyphenols was 264.44 mg acid galic/g, and antioxidant activity was 4160.87 mg acid ascorbic/g. MIC measured via single-wavelength spectrophotometry at 620 nm of ethanol extracts of *E. caryophyllata* against MDR strains, was between 7.8 and 62.5 □ g / mL. The results demonstrate the high potential of the antibacterial activity of *E. caryophyllata* compared with usual antibiotics. The antibacterial activity can be attributed to the high content of polyphenols and a very high antioxidant activity.

INTRODUCTION

Infections remain one leading cause of death in the world, which amplifies the global crisis that characterizes the health of the global population. The incidence of infectious processes caused by resistant bacteria is growing steadily and is today one of the major health risks. For almost every existing antibiotic, bacteria have developed a resistance factor that protects them. For each resistance factor, pharmaceutical companies have created a stronger antibiotic. Antibiotic resistance is due to a variety of less known biochemical and physiological processes that change continuously. Antimicrobial agents can not cover all these mechanisms and the development of antibiotic resistance is relentless [1]. Antibiotic resistance genes for all classes of antibiotics showed a numerical increase since 1940, especially those which code for resistance to the tetracycline and β-lactam classes of compounds [2]. ESBLs (Extended Spectrum Beta-Lactamases) [EC3.5.2.6] are enzymes that hydrolyze oximino group of beta-lactam ring to inactivate compounds such as penicillins, oximinomethyl cephalosporins (ceftazidime, cefotaxime, ceftriaxone) and wide spectrum monobactams. They are generally derivatives of TEM (attacking all anti-gram-negative-bacterium penicillins except temocillin) and SHV (sulfhydryl variable, indicating that p-chloromercuribenzoate, which binds sulfhydryl groups, inhibits hydrolysis of cephaloridine but not of benzylpenicillin) type enzymes. ESBL-producing Enterobacteriaceae strains are responsible for most outbreaks of infection [3]. Since 1994 mutant strains producing TEM and SHV type enzymes have emerged, replaced with the CTX-M type assigned to *K. pneumoniae* and *E. coli* strains, especially in hospitals [4]. Many ESBL-producing strains show resistance to aminoglycosides by co-transfer of a plasmid factor. Also, there is an association between increased production of ESBLs and fluoroquinolone resistance. Although the ESBLs are inhibited *in vitro* by some inhibitors such as clavulanic acid, the activity of inhibiting agents is influenced by the bacterial inoculum, the dose of administration and the particular ESBL type. Carbapenems (imipenem/cilastatin, meropenem, ertapenem and doripenem) are used in therapy of infections caused by ESBL-producing strains. However, use of carbapenems led to the emergence of new resistant strains to carbapenems, such as some strains of *Pseudomonas spp*. [3] or *Acinetobacter spp*. The therapeutic options available for the treatment of bacterial infections are limited by their drug resistance conferred by ESBL, with frequent co-resistance observed for various classes of antibiotics, cephamycins, fluoroquinolones, aminoglycosides, tetracyclines, and trimethoprim /sulfamethoxazole. Relevant clinical data regarding the treatment efficacy of infections

associated with ESBLs are limited. Although some cephalosporins may have *in vitro* activity, often clinical outcomes associated to them are suboptimal. Thus, according to the recommendations of CLSI (Clinical and Laboratory Standards Institute), ESBL-producing strains of *Escherichia coli*, *Klebsiella pneumoniae, Proteus mirabilis* and *Klebsiella oxytoca* are reported as resistant to penicillins, cephalosporins and monobactams, whatever the results of susceptibility *in vitro* [5]. Use of natural products as drugs has been described throughout history. Traditional medicine practitioners used remedies such as oils, tinctures, potions, many of these bioactive natural products being still unidentified. The medicinal properties of bioactive compounds from plants were discovered following direct experiments over the centuries, through studies that experimenters often performed on themselves, by tasting minute or larger amounts of remedy and carefully describing subsequent symptoms and signs, or by registering premature deaths in search of food available to treat various diseases [6]. Using spices in world history proved to be beneficial not only to the preservation of aromatic properties and preservation of foods but also for their antibacterial properties [7]. In India, and generally speaking, in the Orient, medicinal herbs, especially spices, are used to treat chronic diseases on a much wider scale compared to the West. Whole foods are used as functional foods rather than as supplements [8]. *Eugenia caryophyllata* Thunberg 1788 (syn. *Syzygium aromaticum* Linn.) belonging to the *Myrtaceae* family, known as cloves, is an aromatic tree, native to tropical area. About 75 genera with 3000 *Eugenia* species are currently known [9]. In traditional Asian and Australian medicine buds of *E. caryophyllata* are used in various diseases, such as asthma, gastrointestinal infections, headache [10]. Activity against Gram positive and Gram negative bacteria of the essential oil of cloves extract has been reported in many studies [11, 12, 13]. Therefore, within the present study, we aimed an analysis of active antimicrobial compounds in cloves and a thorough characterization of their activity on several bacterial strains resistant to multiple antibiotics. To highlight the antibacterial activity of the extract of buds of *E. caryophyllata* in the case of MDR (multidrug resistant) strains, we pursued two main objectives: 1. identifying the factors of phenotypic and genotypic resistance of strains taken from nosocomial infections, and 2. obtaining extract of cloves, testing and analyzing its major components with activity against MDR strains.

MATERIALS AND METHODS

A total of 80 strains highly resistant to antibiotics were isolated from several infections from the patients hospitalized at "Theodor Burghele" Hospital, Bucharest. Identification of bacterial strains and antibiotic resistance profile were performed using a compact automated VITEK®2 system (BioMérieux Inc, Durham, NC) according to the manufacturer's instructions, in the hospital laboratory. A summary of experimental protocols is presented in Table 1.

Screening for phenotypic and genotypic virulence factors of strains

Order to identify soluble virulence factors, the strains were seeded on mediums with specific substrate and the results were read directly on the plate according to the protocol of table 1. Identification of the genes encoding for resistance to beta-lactam antibiotics was achieved by PCR (Polymerase Chain Reaction). DNA extraction was carried out using a rapid alkaline lysis method and thermal shock [14]. To evidence β-lactamase-producing genes we used amplifying primers for bla_{TEM} and $bla_{\text{CTX-M}}$ genes, and for carbapenemases-producing genes, we used amplifying primers for $bla_{\text{OXA-23}}$, $bla_{\text{OXA-48}}$, bla_{VIM}, bla_{NDM}, bla_{KPC} and bla_{IMP}, as described in the literature. PCR amplicon sizes are also listed in Table 1. Dosage for gene amplification reaction of interest and amplification reactions program were carried out as shown in the same table. The amplification products of each PCR were verified by electrophoresis in 1.5% agarose gel, staining with ethidium bromide (10 μg/mL), and identified by their typical dimensions to the specific molecular weight markers.

Obtaining hydroethanolic extract of *E. caryophyllata*, antibacterial testing and analysis of major compounds

Buds of *E. caryophyllata* were purchased from an Arab spice grocery. For obtaining the hydroethanolic extract, we transformed 300 g of buds of *E. caryophyllata* into dust using a grinder. Over the powder, we poured 700 mL solution obtained from 200 mL of distilled water and 500 mL ethanol. The solution thus obtained was kept in an amber glass container at a temperature of 4°C with stirring every day. After ten days the solution was placed in a rotary evaporator for 10-15 minutes after which the supernatant was removed using a Whatman no. 1 filter. Finally, we obtained a stock solution of which serial dilutions were prepared. For the calculation of MIC (Minimum Inhibitory Concentration), we used sterile sets of disposable 96-well flat bottom plastic plates, containing 12 rows, with a capacity of

approximately 300 µL/well. For columns 2-12, 100 µL of nutrient broth was distributed, and for the first column 180 µL per well were distributed. Of the stock solution obtained from extract of cloves, we distributed 20 µL per well in the first column, mixed it with 180 µL of medium, then we took in pipette 100 µL of mixes and we pipetted into next column, repeating the same operation up to the tenth column, then threw the 100 µL mixes. These were the decimal dilutions. In the last two columns, extract of clove was not pipetted. After this stage, 20 µL per well of bacterial suspension adjusted to 0.5 McFarland units were distributed in columns 1-11, the last column (12) being negative control. The plates were placed in an incubator at 37°C for 24 hours. MIC was read spectrophotometrically as the absorbance at 620 nm.

Quantitative analysis of the antibacterial compounds of hydroethanolic extract of cloves by spectrophotometry

For the measurement of antioxidant activity, we used ABTS (2, 2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) assay, a method based on electron transfer reactions. The capacity to scavenge free radicals of hydroethanolic extract of cloves was evaluated by measuring the absorbance of the sample treated with radical cation $ABTS^{\bullet+}$. Calculation of the total content of phenolic compounds, phenolic acids and flavonoids, resulting from the secondary metabolism of plants has been performed according to the protocol summarized in Table 1. The absorbance measurements were performed using a T80+ UV/VIS Spectrophotometer (PG-Instruments). Each experiment has been performed in triplicate.

Qualitative analysis of antibacterial compounds of hydroethanolic extract of cloves by TLC (Thin Layer Chromatography)

The standard compounds with antibacterial activity (ferulic acid, gallic acid, chlorogenic acid, quercetin, rutin, kaempferol) were purchased from the Bucharest Chemical Company. We have used a semiautomatic applicator (Linom 5 - CAMagic, Muttenz, Switzerland). The spraying was achieved using a plate spray device (Merck). Reading of the plates was performed using a device for TLC imaging (Digistore 2 - CAMagic), and the images were stored as JPEG files, without compression, to avoid losing image quality. The advantage of this method is that the detection by natural fluorescence or fluorescence quenching does not modify or destroy the compounds. A systematized protocol is shown in Table 1.

Table 1. Experimental protocols used in characterizing antimicrobial effects of *E. cariophyllata* ethanol extracts

Culture media used for testing the phenotypic virulence of the strains			
Virulence factors	Medium used	Sterilization	Macroscopic observation
Lipase	[pepsic pepton (10 g) + NaCl (5 g) + $CaC1_2$ (0.1 g) + agar (25 g) + distilled water (1000 mL)]+ 1% TWEEN 80	120°C (20 min)	translucent halo
DN-ase	DNA agar (42 g) + distilled water (1000 mL)	121°C (15 min)	pink halo
Hemolysin	simple agar (7000 mL) + sheep blood (5-7 m L)	37°C (24 h)	transparent halo
Caseinase	nutrient agar (500 mL) + nonfat milk 0, I % fat (250 mL)	121°C (20 min)	off-white precipitate
Gelatinase	nutrient agar + gelatine	121°C (20 min)	halo
Lecithinase	nutrient agar (700 mL) + egg yolk emulsion (35 mL)	121°C (20 min) before adding yolk emulsion	halo and/or opacifying
Amylase	nutritive agar (700 mL) + (7 g starch + 70 ml distilled water)	121°C (20 min) 115ºC (30 min)	yellow ring in blue medium colored with Lugol
Esterase	nutritive agar (700 mL) +1% ($FeC_6H_5O_7$)	121ºC (20 min)	black precipitateof esculetol
Primers used for amplification			
Gene	Primers sequence (5’ to 3’, as synthesized)	Expected amplicon size	Reference
$bla_{OXA\text{-}48}$	OXA-48 F: GCG TGG TTA AGG ATG AAC AC OXA-48 R: CAT CAA GTT CAA CCC AAC CG	438 bp	[15]
$bla_{OXA\text{-}23}$	OXA-23 F: GAT CGG ATT GGA GAA CCA GA OXA-23 R: ATT TCT GAC CGC ATT TCC AT	501 bp	[16]
bla_{TEM}	TEM F: ATA AAA TTC TTG AAG ACG AAA TEM R: GTC AGT TAC CAA TGC TTA ATC	1080 bp	[17]

bla_{CTX-M}	CTX-M F: CGC TGT TGT TAG GAA GTG TG CTX-M R: GGC TGG GTG AAG TAA GTG AC	730 bp	[18]
bla_{NDM}	NDM F: GGT TTG GCG ATC TGG TTT TC NDM R: CGG AAT GGC TCA TCA CGA TC	621 bp	[19]
bla_{IMP}	IMP F: GGA ATA GAG TGG CTT AAY TCT C IMP R: CCA AAC YAC TAS GTT ATC T	232 bp	[15]
bla_{VIM}	VIM F: CAG ATT GCC GAT GGT GTT TGG VIM R: AGG TGG GCC ATT CAG CCA GA	523 bp	[20]
bla_{KPC}	KPC F: CGT CTA GTT CTG CTG TCT TG KPC R: CTT GTC ATC CTT GTT AGG CG	798 bp	[15]

Concentrations of PCR reaction components

Primers	$MgCl_2$	dNTP	DNA-TaqPol	Buffer	DNA	Final volume
0.2 µM	2 mM	0.2 µM	2.5 µL	1X	10X	25 µL

Program amplification reactions

Step	Temperature							Duration
Initial denaturation	95°C							15 min
Denaturation 30 cycles	94°C							30 s
Primer annealing 30 cycles	bla_{OXA} 57°C	bla_{TEM} 50°C	bla_{CTX-M} 58°C	bla_{NDM} 52°C	bla_{IMP} 55°C	bla_{VIM} 55°C	bla_{XPC} 55°C	30 s
Elongation 30 cycles	72°C							1 min
Final elongation	72°C							10 min

Measurement of antioxidant activity

Reagent	Quantity of extract	Absorbance	Calibration
$ABTS^{\bullet+}$ (8 mM ABTS +3 mM $K_2S_2O_8$)	20 µL	734 nm (20 min)	Ascorbic acid (50-250 µg/mL)

Measurement of total contents of phenolic compounds

Compounds	Reagents			
Total phenols	Folin-Ciocalteu's reagent 1.5 mL (0.2 M/L) +Na_2CO_3 1.2 mL (0.7 M/L)	0.3 mL	760 nm	Gallic acid (0-100 mg/mL)
Total	$AlCl_3$ 0.4 mL (25 g/L) +	0.5 mL	430 nm	Rutin

flavonoids	CH_3COONa 0.5 mL (100 g/L) + aqua distillata			(0-120 mg/mL)

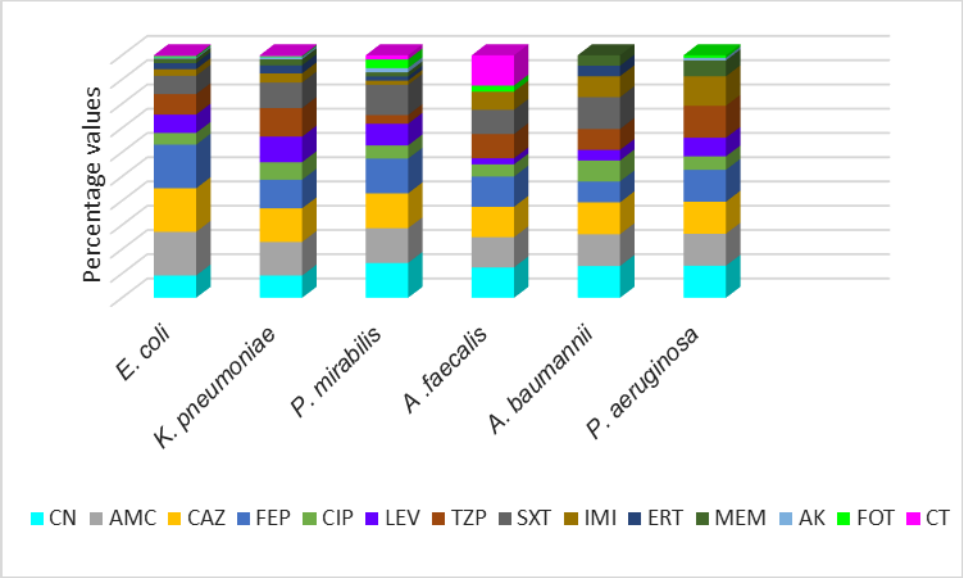
Qualitative analysis of antibacterial compounds of hydroethanolic extract of cloves by TLC

Stationary phase	Mobile phase	Development	R_f	Detection		
				Visible light	UV_{254}	UV_{365}
Silica gel (SiO_2) 60F254 HPTLC (80 nL/s) 8 rows on the Al plate (20x10 cm) prewashed with methanol for 3 min at 100°C	toluene: acetone: formic acid 9:9:2 (v/v/v)	100°C for 3 min spraying with NP	$Rf = \frac{\text{distance the cente of the spot move}}{\text{distance the solve front moved}}$	For natural colored compounds	NP / PEG green back-ground	NP / PEG black back-ground

RESULTS AND DISCUSSION

Analysis of the antibiotic resistance spectrum (figure 1) revealed the existence of acquired phenotypes. The strains of *Escherichia coli* are natively susceptible to ampicillin, while resistance to this antibiotic is phenotype acquired. The analyzed strains showed susceptibility to cefoxitin (second generation of cephalosporin), except for two strains of *Klebsiella pneumoniae*. This phenotype suggests the presence of ESBLs. All strains (except for *Pseudomonas aeruginosa* strains) showed a high resistance to trimethoprim-sulfamethoxazole. The drug has a broad spectrum and is generally prescribed by family doctors; it is relatively inexpensive compared to other drugs. Nevertheless, secondary effects are not rare, most of them being skin rash and gastrointestinal diseases. Recently complications due to treatment such as hyperkalemia were reported [21]. Multidrug resistance in *Enterobacteriaceae* and especially in *Klebsiella pneumoniae* is a growing problem and can lead to dangerous limitations of treatment options. The resistance to carbapenems is caused mainly by carbapenemase production. In this study, we found that all strains of *K. pneumoniae* feature the class A of carbapenemases type, which explains resistance to penicillins, cephalosporins and carbapenems. Phenotypic analysis spectrum of antibiotic resistance of all studied strains suggested the presence of ESBL enzymes type.

However, a higher tolerance of all strains to colistin and fosfomycin was observed. One strain of *E. coli* and one of *K. pneumoniae* showed resistance to colistin. The resistance to fosfomycin was observed in two strains of *E. coli*. In the last two decades, the paucity of novel antibiotics to treat drug-resistant infections, especially those caused by Gram-negative pathogens, has led to the reconsideration of some old antibiotics (fosfomycin and colistin) as a therapeutic option. The polymyxin group of polypeptide antibiotics, discovered in the 1940s, contains some of the first antibiotics with significant activity against Gram-negative bacteria. The emergence of colistin-resistant *K. pneumoniae* has been described following widespread use of colistin [22].



Abbreviations: CN – gentamicin; AMC – amoxicillin-clavulanic acid; CAZ – ceftazidime; FEP – cefepime; CIP- ciprofloxacin; LEV – levofloxacin; TZP – piperacillin-tazobactam; SXT – sulfamethoxazole; IPM – imipenem; ERT – ertapenem; MEM – meropenem; AK – amikacin; FOT – fosfomycin; CT– colistin.

Figure 1. Antibiotic resistances of Gram-negative bacteria

The spectrum of soluble virulence factors, characteristic of each strain analyzed has revealed the presence of pore-forming enzymes (lipase, lecithinase and hemolysin)and proteases (caseinase, amylase, gelatinase, esculinase and DN-ase) in strains analyzed (fig. 2). Extracellular enzymes have a role in the degradation of tissue components, facilitating cell invasion and dissemination.

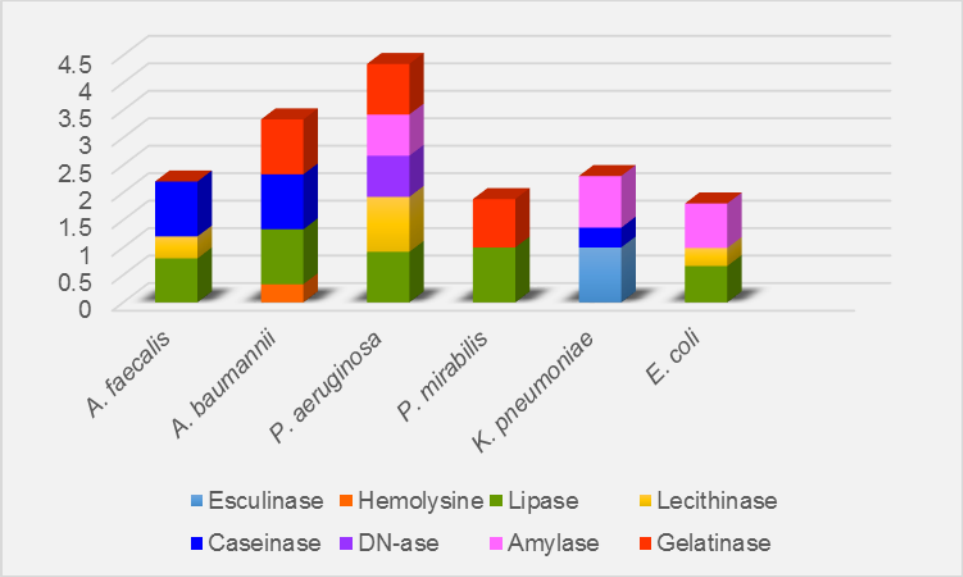


Figure 2. Spectrum of soluble virulence factors in strains isolated from nosocomial infections

The results of the phenotypic factors resistance analysis to β-lactam antibiotics has led to the next step, molecular screening for major β-lactamase encoding genes described in the literature as frequently encountered. The presence of resistance to penicillin and cephalosporin was considered relevant for screening bla_{TEM} genes, TEM β-lactamases encoders and $bla_{CTX\text{-}M}$ genes, encoding CTX-M-type β-lactamases. To highlight the carbapenemase enzymes presence in *K. pneumoniae*, we considered appropriate to screen for gene encoders for OXA-48, OXA-23, VIM, KPC and NDM carbapenemases. The multiplex PCR assay described in this paper offers the advantage that it can be used for rapid screening of large numbers of clinical strain isolates with respect to genes for β-lactamases and carbapenemases types (Fig. 3-5). Usually, Gram-negative bacteria possess a naturally occurring chromosomally mediated β-lactamase probably due to the selective pressure exerted by β-lactam producing organisms found in the environment. In this paper, the bla_{TEM} ESBL genotype was detected in ten strains of *E. coli*, two strains of *K. pneumoniae*, two strains of *Alcaligenes faecalis*, one of *Proteus mirabilis* and one strain of *Acinetobacter baumannii*. The PCR results showed that three isolates (*K. pneumoniae*$_{14I}$, *K. pneumoniae*$_{15I}$, *K. pneumoniae*$_{19I}$) contained both the CTX-M ESBLs and the OXA-48 carbapenemases. PCR analysis for the $bla_{CTX\text{-}M}$ family identified twenty one strains of *E. coli*, fifteen strains of *K. pneumoniae* and a strain of *Proteus mirabilis*. The finding of bla_{TEM} genotype in two *A. faecalis* and one *Proteus mirabilis* strain was unexpected. This genotype, generally

characteristic for *Enterobacteriaceae*, encountered also in other group families, may be due to the abuse of antibiotics. The CTX-M and TEM-type ESBL enzymes have emerged in the late 1980s, a few years after the introduction of cefotaxime as a treatment for bacterial infections [23]. Only a strain of *K. pneumoniae* showed the presence of KPC carbapenemases [Fig. 5]. This KPC carbapenemase is an alarm signal, as the first of this type lactamase reported in Romania. KPC enzymes confer resistance to all β-lactam antibiotics, including penicillin, cephalosporins, carbapenems and monobactams [24]. The multiplex PCR for bla_{VIM}, bla_{IMP}, bla_{NDM} and bla_{OXA-23}, with subsequent amplicon detection by reverse hybridization did not indicate their presence in the investigated strains collection [Figure 3]. The other undetected amplicons are nonspecific, although failure to detect them does not exclude β-lactamase activity.

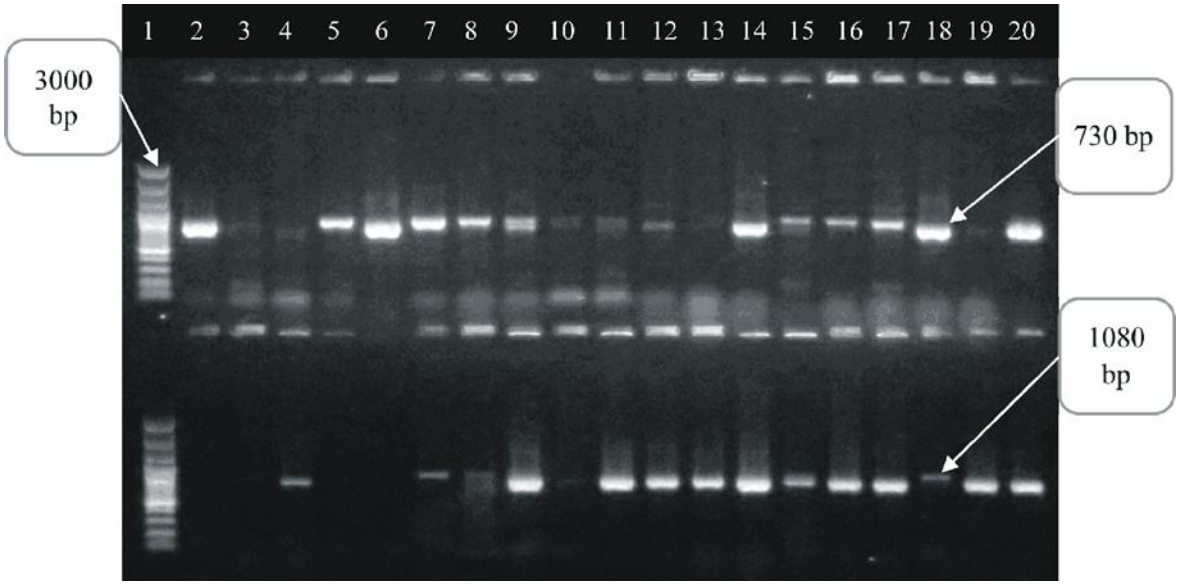


Figure 3. Agarose gel electrophoresis by multiplex PCR amplification for simultaneous detection of $bla_{TEM\text{-}like}$ and $bla_{CTX\text{-}M\text{-}like}$ genes; numbered lanes correspond to the following samples: top row(1-20): molecular weight markers Gene Ruler 3000bp (Fermentas) (MGM), *E.coli*$_7$, *E.coli*$_{29}$, *E.coli*$_{2R}$, *E.coli*$_{13}$, *E.coli*$_{3R}$, *E.coli*$_{22}$, *E.coli*$_{19}$, *A. faecalis*$_4$, *A. faecalis*$_{7R}$, *A. faecalis*$_2$, *E.coli*$_{28}$, *E.coli*$_{1022}$, *E.coli*$_{36}$, *E.coli*$_{12}$, *E.coli*$_{1018}$, *E.coli*$_{10101}$, *E.coli*$_{11}$, *E.coli*$_{33}$, *E.coli*$_{17R}$; bottom row: (1-20): (MGM), *E.coli*$_{24I}$, *E.coli*$_{26}$, *E.coli*$_5$, *E.coli*$_{1138}$, *E.coli*$_{13R}$, *E.coli*$_{32}$, *E.coli*$_{102}$, *E.coli*$_{0R}$, *A. baumanni*$_{22R}$, *E.coli*$_{16R}$, *K. pneumoniae*$_{6R}$, *K. pneumoniae*$_{3R}$, *E.coli*$_{112937}$ *K. pneumoniae*$_{2R}$ *K. pneumoniae*$_{15R}$, *K. pneumoniae*$_{20}$, *A. faecalis*$_{41}$, *K. pneumoniae*$_{19I}$, *K. pneumoniae*$_{12R}$

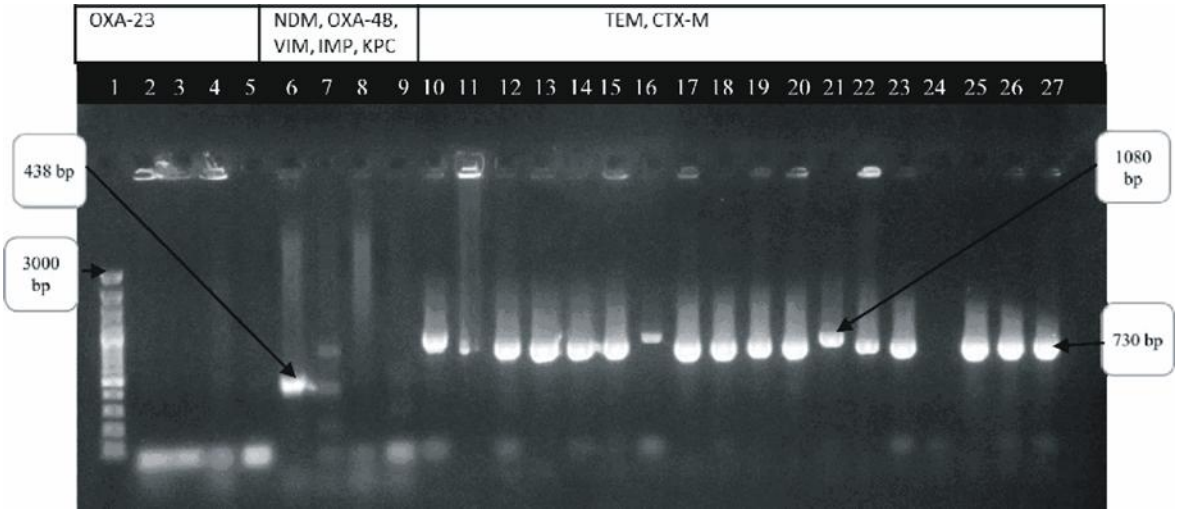


Figure 4. Agarose gel electrophoresis by multiplex PCR amplification for simultaneous detection of *bla*$_{\text{OXA-23}}$, *bla*$_{\text{NDM-like}}$, *bla*$_{\text{OXA-48-like}}$, *bla*$_{\text{VIM-like}}$, *bla*$_{\text{IMP-like}}$, *bla*$_{\text{KPC-like}}$, *bla*$_{\text{TEM-like}}$ and *bla*$_{\text{CTX-M-like}}$ genes; numbered lanes correspond to the following samples: (MGM), *E.coli*$_{22R}$, *K. pneumoniae*$_{1}$, *A. baumannii*$_{5R}$, *A. baumannii*$_{45R}$, *K. pneumoniae*$_{34}$, *E.coli*$_{41R}$, *K. pneumoniae*$_{34}$, *E.coli*$_{41R}$, *E.coli*$_{46}$, *K. pneumoniae*$_{38}$, *P. mirabilis*$_{32}$, *E.coli*$_{43}$, *E. coli*$_{39}$, *E. coli*$_{40}$, *P. mirabilis*$_{31}$, *K. pneumoniae*$_{34}$, *E. coli*$_{42}$, *E. coli*$_{36}$, *K. pneumoniae*$_{30}$, *A. baumannii*$_{45R}$, *E. coli*$_{37}$, *E. coli*$_{44}$, (CTR), *E. coli*$_{41R}$, *K. pneumoniae*$_{28}$, *E. coli*$_{33}$.

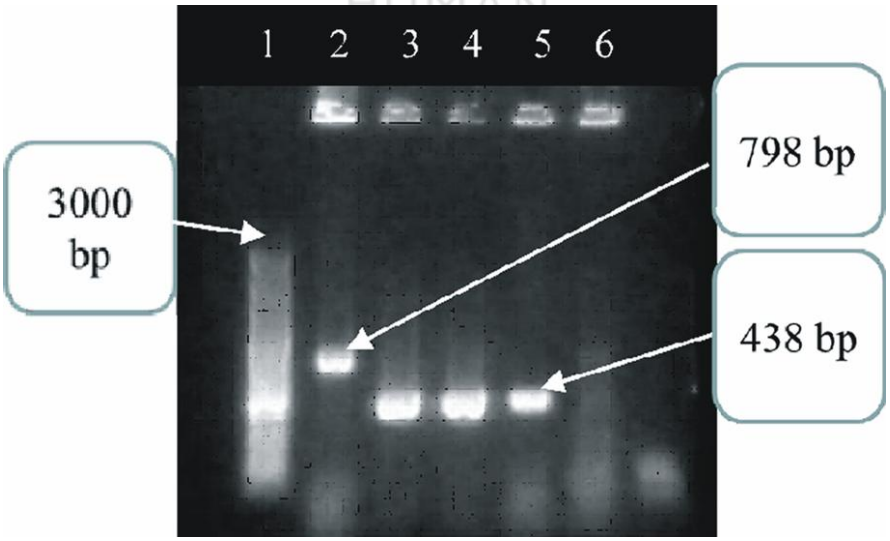


Figure 5. Simultaneous detection of *bla*$_{\text{KPC-like}}$ and *bla*$_{\text{OXA-48-like}}$ genes: (MGM), *K. pneumoniae*$_{6I}$, *K. pneumoniae*$_{14I}$, *K. pneumoniae*$_{15I}$, *K. pneumoniae*$_{19I}$, *K. pneumoniae*$_{0}$.

As it can be observed in figure 6, *K. pneumoniae* strains have been producing of the several types of β-lactamases.

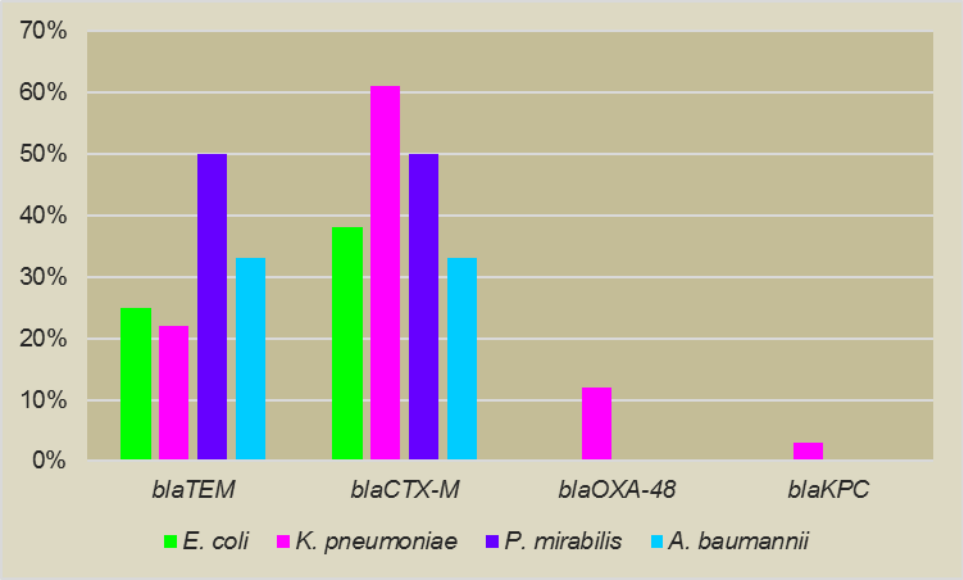


Figure 6. The distribution of β-lactamases in strains isolates of nosocomial infections

Chemical analysis of the hydroethanolic extract of E. caryophyllata

The method of separation and identification of compounds by TLC consists of partitioning compounds of the mixture between an adsorbent (the stationary phase, silica gel) and a solvent (the mobile phase) which flows through the adsorbent. The stationary phase is very "polar". The mobile phase is relatively nonpolar and is capable of interacting with analytes by stronger London forces, as well as by dipole-dipole and H-bonds. In figure 6 we show the chromatographic fingerprints of isolated compounds observed at different wavelengths by spraying with reagents, or directly in visible light.

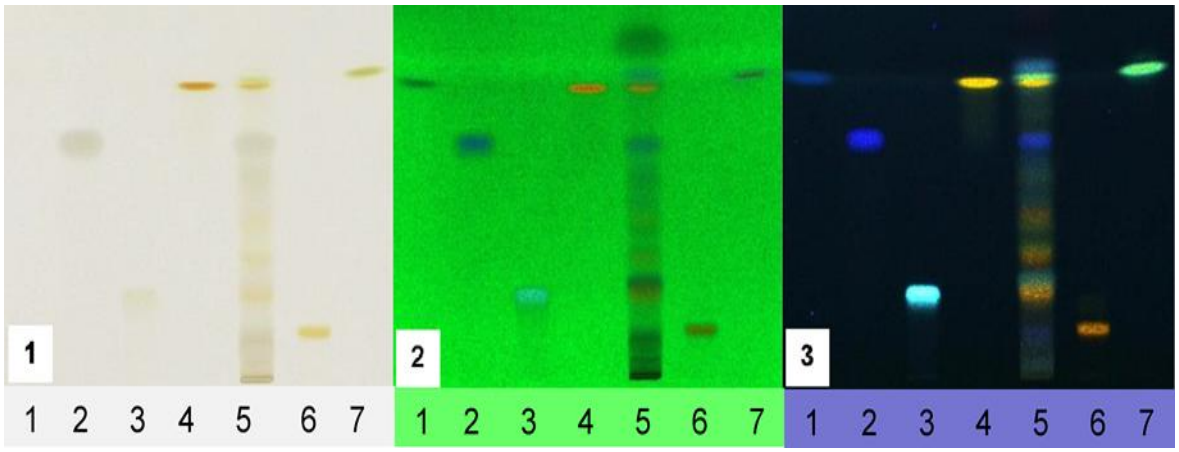


Figure 7. Identification and separation of the antibacterial compounds of hydroethanolic extract of *E. Caryophyllata* using the TLC assay method: (1) light

detection, (2) detection at 254 nm detection at 400 nm and (3) detection at 400 nm. The sequencing of standard plates and extract compounds: 1- ferulic acid, 2- gallic acid, 3- chlorogenic acid, 4- quercetin, 5- plant extract, 6- rutin, 7- kaempferol. The best resolution was obtained at 400 nm, where we identified the compounds constituents: ferulic acid, gallic acid, quercetin and kaempferol.

The Rf values in the order of migration of the compounds on the plate were 0.89, 0.75, 0.38, 0.87, 0.29 and 0.90.

The specificity and complexity of the interactions at molecular level between the components of the bacterial cell and plant extract compounds are largely elucidated. Ferulic acid (FA) is a constituent commonly found in plants that arise from the metabolism of aromatic amino acids L-phenylalanine and L-tyrosine, as key entities [25, 26]. FA exhibits a wide range of biomedical effects including antioxidant, antiallergic, hepatoprotective, anticarcinogenic, anti-inflammatory, antimicrobial, antiviral [27]. The structural characteristics of FA might give potential antioxidant characteristics. Due to phenolic ring and unsaturated side chain, it can easily establish resonance free radicals giving them the steady state by pooling a hydrogen atom with formation of phenoxy radicals. Additional phenoxy radical stabilization can be expanded in unsaturated side chain. Phenoxy radicals may not initiate a chain reaction propagation, collision between them thus achieving a condensation to give curcumin dimer. The presence of the phenolic hydroxyl group confers the additional ability to capture free radicals and forming of o-quinone [26]. Recent research has shown that GA (3,4,5-trihydroxybenzoic acid) is entirely or principally synthesized by dehydrogenation of DHS (dehydroshikimate). DHS is a key enzyme in the shikimate pathway. Previously, DHS has proved to catalyze the NADPH-dependent reductase of 3-DHS to GA and to shikimic acid [28]. The shikimate pathway (specific for plants and microorganisms), links the carbohydrate metabolism to biosynthesis of aromatic compounds, phenylalanine, tyrosine and tryptophan [29]. Flavonol quercetin is an important flavonoid which represents with its glycosylated forms 60-70% of flavonoid intake. The most important effect of quercetin is scavenging of oxygen-derived free radicals and chelating transition metal ions [30,31]. Quercetin (3,30,40,5,7-pentahydroxy flavone) resonates with free radicals and by donating a proton they become energetically stabilized. The resulting unpaired electron is delocalized by resonance, resulting in low energy quercetin radical. Quercetin antioxidant potential is given by *o*-dihydroxy B ring, 4-oxo group in conjugation with 2,3-alkene, and 3- and 5-hydroxyl

groups. Quercetin glycosylation is achieved by the hydroxyl groups. The most common quercetin glycosides have a glucose group at position 3, quercetin (-3-O-β-glucoside) [31]. Kaempferol (3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4*H*-chromen-4-one), like all other flavonoids studied for therapy, can be applied to various diseases due to binding specificity and lack of toxicity. The only difference of molecular structure between quercetin and kaempferol is the existence of the 3'-hydroxyl, which in kaempferol is missing. This group confers to quercetin additional properties in interactions with proteins. In comparison with drugs which bind weakly and non-specifically to proteins, flavonoids feature highly specific and strong binding [32].

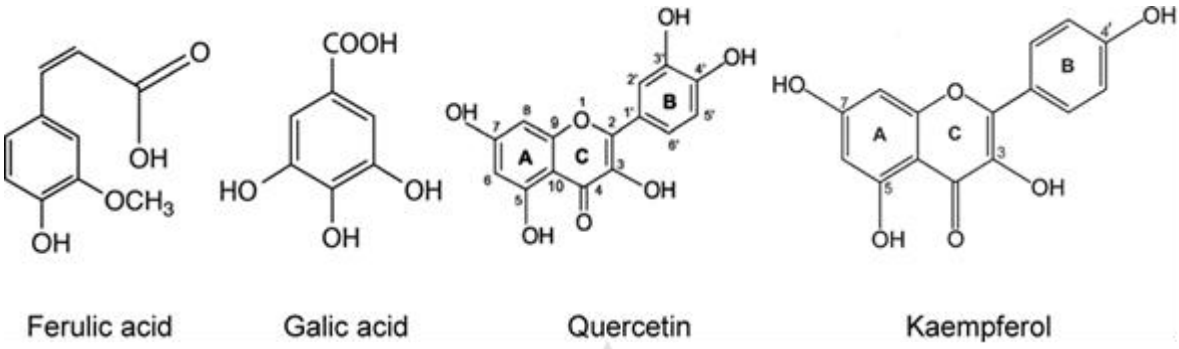
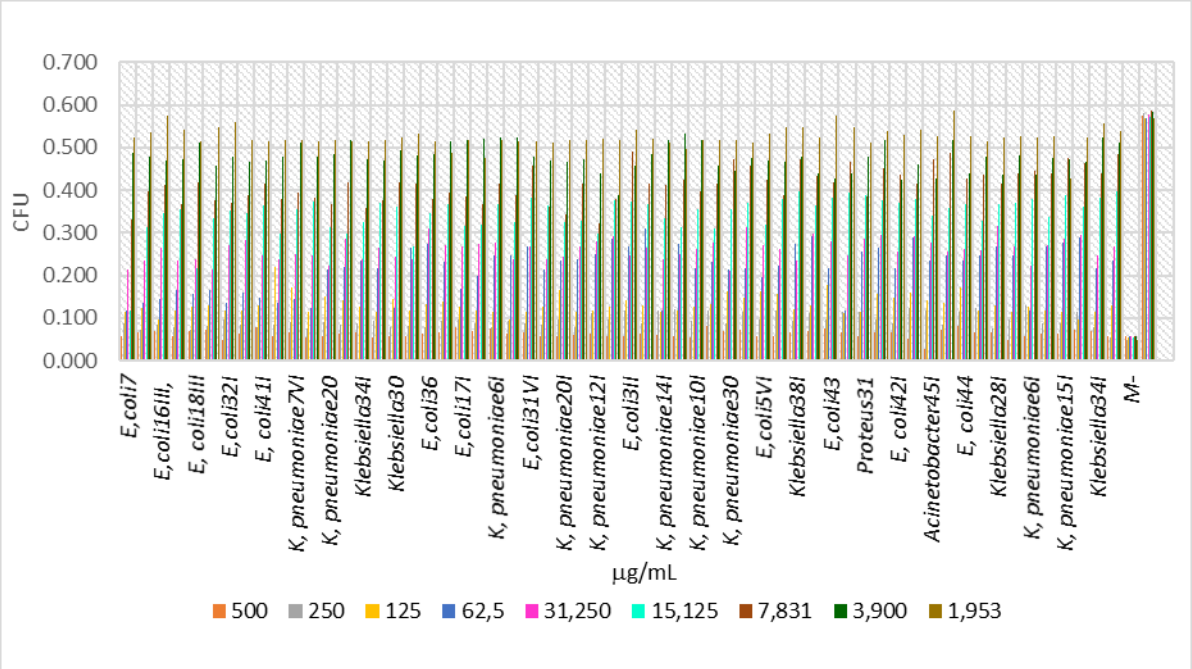


Figure 8. Molecular structure of compounds isolated from *E. caryophyllata.*

Total phenolic content calculated it was 264.44 mg gallic acid/g herbs, flavonoid content it was 0.33 mg rutin/g extract and antioxidant capacity expressed in terms of ascorbic acid equivalents it was a very remarkable corresponds to 4160.87 mg/g extract.

Quantitative evaluation of the antibacterial activity of hydroethanolic extracts of E. caryophyllata

The antimicrobial activity of plant extracts is the first demonstrated pharmacological property for these. The medicinal plants have a great potential to produce new drugs of great benefit to mankind. Higher plants are capable of synthesizing unlimited numbers of highly complex and unusual chemical substances [33]. The specificity and complexity of the molecular interactions between cellular components and potential antibacterial compounds from extracts of plants are not elucidated. The calculation of MIC via dilution method in 96-well plates with the liquid medium yielded values between 7.8 and 62.5 µg/ mL for hydroethanolic extracts of *E. caryophyllata.* Figure 7 shows the values of MIC read at an absorbance of 620 nm for Gram-negative MDR bacteria in which they were detected presence of the carbapenemases and ESBL.



Abbreviations: M^+ =positive control, M^- =negative control; CFU= Colony Forming Units.

Figure 9. The MIC values of hydroethanolic extracts of *E. caryophyllata* against MDR bacteria via single-wavelength spectrophotometry at 620 nm.

The high content of phenols resulting from semiquantitative analysis might suggest that these compounds had the highest antibacterial activity. β-lactamases are the main factor of resistance of Gram-negative bacteria to β-lactam antibiotics. The prevalence of these enzymes in *Enterobacteriaceae*, particularly, has been reported in numerous articles [34]. Despite a large number of antibiotics and increased incidence of isolates resistant to penicillins, β-lactam antibiotics are the most used. β–lactamases have evolved as a protective mechanism against β-lactam antibiotics produced by fungus and bacteria in the environment, having an assigned role in peptidoglycan assembly [35].

CONCLUSION

The high resistance to antibiotics of Gram-negative strains isolated from nosocomial infections has been attributed to the presence of ESBL and carbapenemases and as well of virulence soluble factors (proteases and pore-forming enzymes). MIC of cloves extract had low values, between 7.8 and 62.5 µg/mL, fact that expresses the antibacterial qualities of cloves extract. Powerful antibacterial activity of the hydroethanolic extract of *E. caryophyllata* is due to the synergistic action of chemical compounds, resulting from secondary metabolism identified and separated by TLC: ferulic acid, gallic acid, quercetin

and kaempferol. Also, powerful antioxidant activity, 4160.87 (equivalents in mg ascorbic acid/g extract) is associated with antibacterial activity. Obtaining drugs based on synthetic compounds derived from secondary metabolism of *E. caryophyllata*, or using crude extracts in bacterial infections could be a success in the medical practice, by stopping evolution of the infection or the possibility of developing new virulence genes.

AUTHOR DISCLOSURE STATEMENT

The authors declare no conflicting interests related to the present work.

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