Eugenia caryophyllata Thunberg - A MIRACULOUS HERB

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Abstract. *Eugenia caryophyllata* Thunberg 1788 belongs to the family Myrtaceae, is an aromatic tree, originally from Indonesia used as a condiment. Its antibacterial, antifungal and anthelmintic properties are known from ancient times. Despite the development of the pharmaceutical industry, currently at least 30 000 people die every year in Europe due to infections caused by antibiotic-resistant microorganisms: *Staphylococcus aureus* resistant to methicillin (MRSA), *Staphylococcus aureus* vancomycin-resistant (VRSA), *Entercoccus spp.* vancomycin-resistant (VRE), *Streptococcus pneumoniae* penicillin-resistant (PRSP), Enterobacteriaceae (*Escherichia coli, Klebsiella pneumoniae*) resistant to the third generation cephalosporins, Enterobacteriaceae (*K. pneumoniae*) carbapenems-resistant and non-enteric bacteria (*Pseudomonas aeruginosa*) resistant to carbapenems. For almost every existing antibiotic, bacteria have developed a resistance factor that protects them from the action. For each resistance factor, pharmaceutical companies have developed a stronger antibiotic - until today. In the battle between bacteria and antibiotics the balance of victory begins to tilt toward these microorganisms. In these circumstances, the return to traditional medicine seems to be the solution. In order to determine the antibacterial activity of the compounds of *Eugenia caryophyllata* buds extract against bacteria isolated from nosocomial infections MDR (multiple drug resistant), we used the disc-diffusion method and inoculation of 96-well plate BHI. Screening of genes coding β- lactam antibiotics resistance was performed by PCR. The active compounds of the ethanol extracts and essential oil of *Eugenia caryophyllata* were determined by HPTLC, respectively by GC-MS. Gram positive and Gram negative strains isolated from nosocomial infections showed genotypic resistance characteristics to lactam antibiotics. Hydroalcoholic extracts and essential oil of *E. caryophyllata* were active against all MDR bacteria. Eugenol is the main component of the extract of *E. caryophyllata*. In conclusion, the extracts of *E. caryophyllata* can successfully replace antibiotics whose action against MDR bacteria proved to be ineffective.

Keywords: *Eugenia caryophyllata*, eugenol, MDR bacteria.

Rezumat. *Eugenia cariophyllata* Thunberg - o plantă miraculoasă. *Eugenia caryophyllata* Thunberg 1788, face parte din familie Myrtaceae, este un arbore aromatic, original din Indonezia folosit ca şi condiment. Proprietăţiile sale antibacteriene, antifungice şi antihelmitice sunt cunoscute din antichitate. În ciuda dezvoltării industriei farmaceutice, în prezent, cel puţin 30 000 de oameni mor anual în Europa datorită infecţiilor cauzate de microorganisme rezistente la antibiotice: *Staphylococcus aureus* rezistent la meticilină (MRSA), *Staphylococcus aureus* rezistent la vancomicină (VRSA), *Entercoccus spp*. rezistente la vancomicină (VRE), *Streptococcus pneumoniae* rezistent la penicilină (PRSP), enterobacterii (*Escherichia coli, Klebsiella pneumoniae*) rezistente la cefalosporine de generaţia a treia, enterobacterii (*K. pneumoniae*) rezistente la carbapeneme, şi non-enterici (*Pseudomonas aeruginosa*) rezistente la carbapeneme. Pentru aproape fiecare antibiotic existent în prezent, bacteriile au dezvoltat un factor de rezistenţă care le protejează de acţiunea sa. Pentru fiecare factor de rezistenţă, companiile farmaceutice au dezvoltat un antibiotic mai puternic – până astăzi. În lupta dintre bacterii şi antibiotice, balanţa victoriei începe să se încline către aceste microorganisme. În aceste condiţii, întoarcerea la medicina tradiţională pare a fi soluţia. Pentru a determina activitatea antibacteriană a compuşilor din extract de muguri de *Eugenia caryophyllata* împotriva bacteriilor MDR izolate din infecţii nosocomiale, am folosit metoda disc-difuzimetrică adaptată şi însămânţare în plăci cu 96 godeuri cu mediu BHI. Screening-ul genelor codificatoare ale rezistenţei la antibioticele β- lactamice s-a realizat prin metoda PCR. Compuşii activi din extractele etanolice şi ulei esenţial din *Eugenia caryophyllata* au fost determinaţi prin HPTLC respetiv, GC. Tulpinile Gram pozitive şi Gram negative izolate din infecţii nosocomiale au prezentat caracteristicile genotipice de rezistenţă la antibioticele lactamice. Extractele hidroalcoolice şi uleiul volatil din *E. caryophyllata* au avut activitate împotriva tuturor bacteriilor MDR. Eugenolul este componentul principal al extractului din *E. caryophyllata*. În concluzie, extractele din *E. caryophyllata* pot înlocui cu succes antibioticele a căror acţiune împotriva unor bacterii MDR s-a dovedit a fi ineficientă.

Cuvinte cheie: *Eugenia caryophyllata*, eugenol, bacterii MDR.

INTRODUCTION

The healing with medicinal plants is as old as mankind itself. The relationship between man and aromatic herbs dates back to the distant past, as it results from different sources: written documents, preserved monuments, and even the original drugs from plants (PETROVSKA, 2012). *Eugenia caryophyllata* Thunb, with EUCA15 symbol is a spice widely praised for its antibacterial and antioxidant properties, used for centuries as a food preservative. Currently, many articles in PubMed render the antibacterial, antifungal, antiviral and anticarcinogenic activity of the extracts of *E. caryophyllata* buds (clove). *E. caryophyllata* is an evergreen tree with a height of 10-20 m (TAJUDDIN et al., 2004). It is part of the Myrtaceae, being indigenous in India, Indonesia, Zanzibar, Mauritius and Sri Lanka. Buds of this tree are currently marketed worldwide. The production of flower buds begins 4 years after planting, being collected in the maturation phase before flowering (CORTÉS-ROJAS, 2014).

The classes of compounds with antimicrobial activity obtained from the extracts of *E. caryophyllata* are secondary metabolites derived from fundamental processes of photosynthesis, glycolysis and the Krebs cycle. Secondary metabolites in general are not essential for growth, development and reproduction of an organism and are either the result of the body's adaptation to external environmental factors or defense mechanisms against predator bodies, helping the survival of the organism. The changes of the biosynthetic pathways may be due to natural causes (for example, viruses or environmental changes), or unnatural causes (for example, chemical pollutants), in an effort to adapt to survive (DIAS, 2012). Plant extracts have multiple action target in the microbial cell, which is an advantage of their use as antimicrobials, with synergistic or

concerted activity of components - their antimicrobial activity, and not only as a result of the fact that they are multicomponent systems. This pharmacologic synergistic or additive effect may be beneficial by eliminating the side effects associated with the predominance of one of the xenobiotic compounds in the body.

This role of several chemicals to act synergistically or the additive myth originated in plant secondary compounds involved in the defense for the survival of the species. For example, role-products in the defense of a mixture of chemicals that have additive or synergistic effects on multiple target sites would not only ensure efficacy against a wide variety of herbivores or pathogens, but would also reduce the chances of these bodies to develop and adapt their reactions. Although secondary compounds may have a variety of functions in plants, it is likely that the functions can have an effect on the human body with medicinal potential. For example, cytotoxicity secondary compounds involved in plant defense against microbial pathogens could be useful as antimicrobial agents in humans (BRISKIN, 2000).

Ethanolic extracts and essential oil of clove have been shown to be an important source of phenolic compounds such as flavonoids, hydroxybenzoic acid, hydroxycinnamic acid and hydroxyphenyl propene. Eugenol is the main bioactive compound that was reported by different authors as having the largest activity (TAJUDDIN et al. 2004, JOSHI et al., 2010. BHOWMIK et al., 2010). Tannins were reported in large quantities: phenolic acids (caffeic acid, ferulic, ellagic and salicylic acid), gallic acid can be found in higher concentration. Flavonoids such as kaempferol, quercetin and its derivatives (glycosylated) have also lower concentrations. Another important compound in the essential oil of clove found in concentrations of up to 2.1% is α-humulen. Other volatile compounds present in low concentrations in the essential oil of cloves are β-pinene, limonene, farnesol, benzaldehyde, 2-heptanone, and ethyl hexanoate (CORTÉS-ROJAS, 2014).

MATERIAL AND METHODS

1. Preparation of the extract and analysis of the compounds

The buds of *Eugenia caryophyllata* were purchased in store. The ethanolic extract was prepared by macerating the clove powder in 95% ethyl alcohol for 24 hours (1: 4 w / v), after which it was introduced into a rotary evaporator for 10-15 minutes and then the supernatant was purified by Whatman filter paper no. 41. The extract thus obtained was stored in an amber glass container at 4° C. The analysis of the compounds of the ethanolic extracts was made using HPTLC **(**High Performance Thin-Layer Chromatography method). Chemicals and reagents use: 2,2' azinobis (3- ethylbenzothiazoline- 6 sulfonic acid) diammonium salt (ABTS), ascorbic acid (vitamin C), gallic acid, Folin–Ciocalteu's reagent, and sodium persulfate ($Na_2S_2O_8$) were purchased from Merck (Darmstadt, Germany). Aluminium chloride ($AlCl_3$), sodium acetate (CH_3COONa), sodium carbonate (Na_2CO_3) and methanol were obtained from Chimopar (Bucharest, Romania). All chemicals and solvents were analytical grade. The absorbance measurements were performed using a T80+ UV/VIS Spectrophotometer (PG-Instruments). Each experiment has been performed in triplicate. For antioxidant activity of extracts we performed the following steps: the cationic radical $ABTS^+$ was obtained from the reaction of 7 mmol/L ABTS diammonium salt solution with 2.45 mmol/L $Na_2S_2O_8$ solution mixed in 1:1 (v/v) ratio, incubated for 24 h at room temperature in the dark. Then, 0.5 mL of appropriately diluted extract were added to 3 mL $ABTS^{+\bullet}$ solution diluted to achieve an absorbance less than 0.800, and the absorbance was measured at 734 nm, after 20 min. Calibration was performed using vitamin C as standard, in the concentration range of 50–250 μg/ mL, following the same procedure.

The calibration curve was used to calculate antioxidant activity. For total phenolic content (TPC) determination, 1.5 mL of Folin–Ciocalteu reagent (0.2 mol/L) was added to 0.3 mL of plant extract appropriately diluted with distilled water. The reaction mixture was allowed to react 5 min and then, 1.2 mL of 0.7 mol/L Na_2CO_3 was added. The results were read using an absorbance spectrophotometry at 760 nm. A calibration curve was obtained using 0–100 mg gallic acid/mL and was used to calculate the total phenolic content of the extracts. The total flavonoids content of extracts was determined by treating 0.5 mL of extract appropriately diluted with distilled water with 0.4 mL of 25 g/L $AlCl_3$ solution, 0.5 mL of 100 g/L CH_3COONa solution and 4 mL distilled water. After 15 min, the absorbance of the mixture was measured at 430 nm and the flavonoid content express in mg rutin/g of plant, was calculated using the calibration curve obtained in the 0–120 mg/mL concentration range. The volatile oil of cloves was obtained by hydro distillation in a Clevenger-Neo for 4 h. The volatile oil was dried with Na_2SO_4 and stored in a dark glass bottle at 4° C. Oil samples were diluted in dichloromethane (1/200) for the analysis of the chemical compounds. To identify the compounds of the clove oil extract we used a GC-MS. The used gas chromatograph was Fisons Instruments GC 8000 coupled with mass spectrometer ionization quadruple analyzer impact, pattern MD 800. Ionization energy was 70 eV. Capillary column used was a fused silica column with 5% phenyl poly (dimethylsiloxane) (SLB-5ms, 30m x 0,32mm id, film thickness of stationary phase 0.25μm). The operating conditions were: split splitless injector (injection mode - split flow dividing ratio 1/30) to 280°C, ion source temperature 200°C and 280°C interface; initial column temperature was 40° C scheduled as follows: 3 min at 40° C, 4° C / min up to 280°C, isothermal conditions (at 280°C) for 20 min; the flow rate of the carrier gas (helium) was 2 ml / min; injected sample volume was 1 ml. Data acquisition was performed with the software MassLab in Table 30-600 amu, with a scan rate of 1 scan / s. Identification of the detected compounds was based on comparing their mass spectra with those in databases (NIST, WILEY). Relative concentration of the compounds was calculated using the values chromatographic peak areas under the curves, without applying correction factors.

2. Analysis of resistance factors MDR strains from nosocomial infections

Strains isolated from urogenital infections were from patients hospitalized at Theodor Burghele Hospital Bucharest. Phenotypic resistance to antibiotics was determined by disk diffusion method. For the analysis of virulence factors we used

phenotypic tests that allow the detection of six extracellular virulence factors: hemolysis, caseinase, gelatinase and pore-forming toxins: DN-ase, lipase and lecitinase. These extracellular proteins known as the invasins have a role in the degradation of tissue and invasion. Highlighting these soluble factors of virulence was achieved by growing strains on culture media and studying the specific detection of each type of virulence factor. β-lactamases are the most important enzymes that confer antibacterial resistance. To identify genes β-lactam antibiotic resistance we used PCR method. Preparation of bacterial DNA to screen for gene was made by the method of bacterial lysis. The strains were seeded on an agar plate and kept at 37 ° C for 24 hours. In the PCR tubes there were added 20 µl NaOH (0.05 M) and SDS (0.25%), which made suspensions of 1-5 colonies. The tubes bacterial suspensions were kept for 15 minutes at 95°C, after which there were added 180 ml extraction of TE solution (10 mM Tris-HCl, pH 8, 0.1 mM EDTA), followed by centrifugation at 13,000 rpm / min for 3 minutes. The supernatant was recovered, and then stored in a refrigerator. The verification of the product obtained was carried out by electrophoresis of 0.8% agarose gel stained with ethidium bromide 3.5µg / ml. Following observation of bacterial phenotypes it was checked the existence of several genes commonly associated with the production of β-lactamases: countertop, $bla_{\text{CTX-M}}$, bla_{NDM}, bla_{OXA}, bla_{IMP}. For the amplification of this gene, we used the primer sequences described in the literature.

Table 1. Sequences of the primers used in the PCR reaction.

Gene	Primers sequence (5' to 3', as synthesized)	Expected amplicon size (bp)	Reference
$bla_{\text{OXA-48}}$	OXA-48 F: GCG TGG TTA AGG ATG AAC AC OXA-48 R: CAT CAA GTT CAA CCC AAC CG	438 bp	Poirel et.al.,2004
bla_{TEM}	TEM F: ATA AAA TTC TTG AAG ACG AAA TEM R: GTC AGT TAC CAA TGC TTA ATC	1080 bp	Eftekhar et. al.,2005
$bla_{\text{CTX-M}}$	CTX-M F: CGC TGT TGT TAG GAA GTG TG CTX-M R: GGC TGG GTG AAG TAA GTG AC	730 bp	Schlesinger et. al., 2005
bla_{NDM}	NDM F: GGT TTG GCG ATC TGG TTT TC NDM R: CGG AAT GGC TCA TCA CGA TC	621 bp	Nordmann et. al., 2011
bla_{VIM}	VIM F: CAG ATT GCC GAT GGT GTT TGG VIM R: AGG TGG GCC ATT CAG CCA GA	523 bp	Docquier et. al., 2003
bla_{IMP}	IMP F: GGA ATA GAG TGG CTT AAT TCT IMP R: GTG ATG CGT CYC CAA YTT CAC	361 bp	Docquier et. al., 2003

3. Determination of antimicrobial activity of extracts of clove

The quantitative determination of antimicrobial activity and establishing the minimum inhibitory concentration (MIC) was made by the method of serial microdilution in a liquid medium BHI (Hearth Infusion Broth) in 96well plates / Ependorf tubes of 1.5ml (CHIFIRIUC et. Al., 2011). MCI was established macroscopically, as the last concentration at which no growth of the microbial environment was observed and that the appearance of turbidity are read spectrophotometrically to the absorbance at 620 nm. For the qualitative testing of the antimicrobial activity, there were prepared microbial suspension adjusted to 1.5x108 CFU / mL of the 0.5 McFarland standard, 15-18 hour cultures grown on solid medium (Sabouraud). Antimicrobial activity was determined by disc diffusion method adapted to standardized control antimicrobial activity of antibiotics, CLSI, 2009. The microbial suspension, adjusted to 0.5 McFarland standard in the cloth was seeded in the cloth agar Petri dishes which pipetted 10µL of the stock solution obtained of the plant extract. The plates are incubated for 16-18 hours at 35 ± 2° C, with the lid down. Reading the results was done by measuring the diameters of the zones of inhibition comparatively. The influence on the ability adhesion to the inert substrate was quantified after the protocol quantitative analysis of the effect of antimicrobial, evaluating the biomass, after fixation with methanol and staining with crystal violet.

The optical density was determined spectrophotometrically and the biological material resuspended at 490 nm (NORUZI et. al., 2010). For determining the inhibitory activity of clove extracts against soluble virulence factors, the strains were grown in increasing concentrations of stock solutions subinhibitory essential oil: DMSO (CMI / 2) and the positive control (untreated strain) were used for seeding each medium with each specific culture for detecting soluble virulence factor (hemolysins caseinase, lipase, lecithinase, gelatinase and DN-ase).

RESULTS AND DISCUSSION

Table 2. From the analysis of the ethanol extract of cloves, HPTLC method has shown the following results:

Ethanolic extract	Antioxidant activity (mg vitamin C / g of herbs)	Total polyphenol content (mg gallic acid / g herbs)	The content of flavonoids (mg rutin / g herbs)
Eugenia caryophyllata	4.16087	264.44	0.33

It is a very remarkable antioxidant, so a gram of ethanol extract clove corresponds to 4160.87 g of vitamin C. It is also important to note the high concentration of polyphenols (264.44 mg gallic acid / g herbs). However, the concentration of flavonoids is almost insignificant. The concentration of polyphenolic compounds in herbs is coupled with their antioxidant activity. Antioxidant properties are induced by redox activity and mechanisms of action such as: free-radical scavenging activity, transition-metal-chelating activity and/or singlet-oxygen-quencing capacity. Polyphenols have an important role in the stabilization of lipid peroxidation and inhibit various types of oxidative

enzymes. The relationship between total antioxidant activity and the phenolic content of a large number of spices has not been investigated sufficiently. Many researchers have argued that the phenolic compounds in spices were responsible for their antioxidant activity, but few could correlate these relations on the basis of statistical data (SHAN et al., 2005; DE ROSA & CRUTCHLEY, 2002).

The oil obtained by hydrodistillation of cloves was intense yellow with characteristic aromatic and persistent odour. The average yield clove oil extraction was 8.40%, the density was 1.055 (g/cm3) and the index of fraction was 1.535. In clove oil there were identified five compounds representing approximately 95.51% of the total. In diagram 1 are shown the major component is eugenol, present in an amount of 87%, followed by caryophyllene (4.87%), acetyl eugenol (2.11%), β–pinene (1.021%) and hexadecanoic acid, octadecyl ester (0.51%).

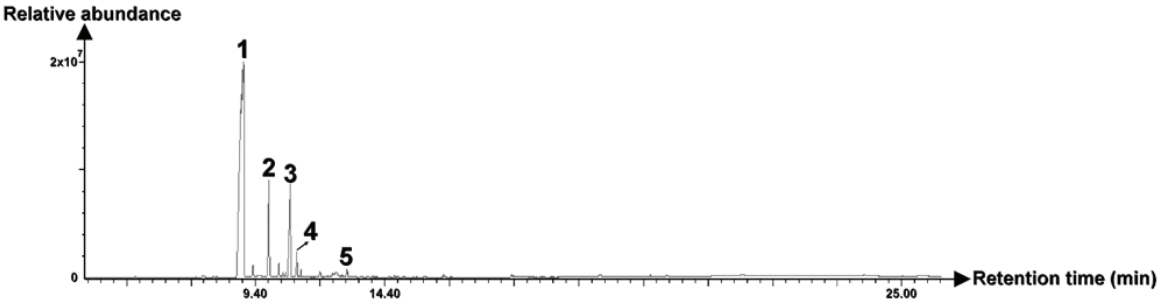


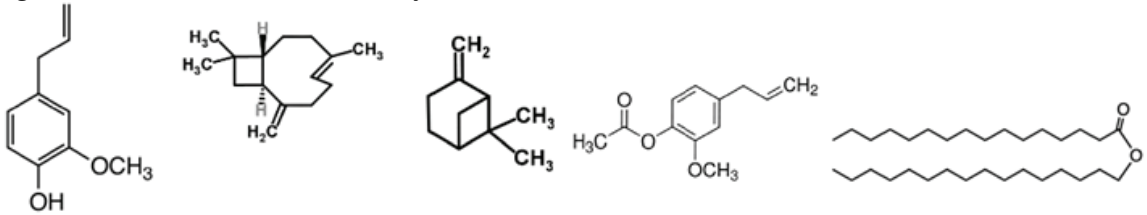
Diagram 1. Chromatogram of the essential oil of clove: the abscissa is the last retention time (minutes) and the ordinate, the relative abundance. Compounds are listed in order of elution: 1= eugenol, 2= caryophyllene, 3= acetyl eugenol, 4= β–pinene, 5= hexadecanoic acid, octadecyl ester.

Eugenol or phenol, 2-methoxy-3-(2-propenyl) has the molecular formula $C_{10}H_{12}O_3$ and a molecular weight of 164 201 Da. Eugenol is an aromatic compound, the ether-oxide group with methylenedioxy group attached to the benzene nucleus in para-position to the allyl chain. Eugenol increased the permeability of the membrane; the deformation of macromolecules in the membrane was verified by spectroscopy (DEVI et al., 2011). The compound is a very promising candidate for versatile applications; eugenol possesses significant antioxidant and has also been used as a penetration enhancer (PRAMOD et al. 2010). Eugenol is also known for his property against microorganisms forming biofilms. Adherence of the microorganisms to host cells and tissues is the first event required for initial colonization or establishment of infection. Moreover, the microbial surface contact can trigger various cellular behaviours, including biofilm formation. Biofilms can be defined as irreversibly surface-attached communities of cells (sessile cells) embedded in a self-produced exopolymeric matrix, displaying a distinctive phenotype compared to their free-floating (planktonic cells) counterparts. Sessile cells are less susceptible to medicaments. Biofilms are thereby difficult to eradicate (DE PAULA et. al., 2014, CHIFIRIUC et. al., 2011). Eugenol is polar due to the acidic hidroxyl (OH) group, but acetyl-eugenol is not polar. As a result, they can be separated by extraction from a 5% aqueous NaOH solution. Acetyl-eugenol will dissolve in the organic CH_2Cl_2 layer, while eugenol remains in the aqueous base layer as a phenoxide.

Caryophyllene is a bicyclic sesquiterpene, usually found as a mixture of isocaryophyllene (cis double bond isomer) and α- humulen (obsolete name: α-caryophyllene), an open-ring isomer. Caryophyllene is considered to be a rare structure in nature, a cyclobutane ring (GERTSCH et al., 2008) with molecular formula $C_{15}H_{24}$. CALLEJA et al., 2014, reported the antimicrobial activity of caryophyllene. β-pinene, a monoterpene, is one of the two isomers of pinene; the other one is α-pinene. It is soluble in alcohol but not in water. It smells of green wood. It is also known as 6,6-dimethyl-2-methylenebicyclo [3.1.1] heptane. Bactericidal and bacteriostatic activity of β-pinene has been reported in many articles. β-pinene has a direct effect on the respiratory system of fungi by inhibiting the efflux pump protons, K^+ and H^+ that are found in the mitochondrial membrane, since the loss of respiratory control and the disintegration of organelles followed by an inhibition of breathing at higher concentrations of terpenes . β– pinene has a preferential affinity for mitochondria (URIBE et al., 1985).

Acetyl-eugenol or phenol, 2-methoxy-4- (2-propenyl) - acetate has a molecular formula $C_{12}H_{14}O_3$.

Hexadecanoic acid, octadecyl ester or cetyl palmitate is the ester derived from palmitic acid and cetyl alcohol. Palmitic acid, in IUPAC nomenclature, is the most common fatty acid (saturated) found in animals, plants and microorganisms. Hexadecanoic acid, octadecyl ester has a molecular formula $C_{34}H_{68}O_2$.



Eugenol β- caryophyllene β- pinene Acetyl-eugenol Cetyl palmitate

Following the comments of phenotypic resistance to antibiotics by disc-diffusion method, 70% of strains were resistant to extended spectrum β-lactams (ceftazidime, cefotaxime, aztreonam), 54% were resistant to clavulanic acid, 41% were resistant to carbapenems (meropenem, imipenem) and 39% were resistant to oxacillin.

The results of the analysis of soluble factors revealed the presence of exotoxins and pore-forming toxins. 7 strains showed simultaneous four soluble virulence factors, 9 strains presented three virulence factors simultaneously and 12 showed two virulence factors simultaneously. Of the 82 studied strains, following phenotypic tests for evidence of virulence factors, 56 strains showed proteases by testing simple agar medium with the addition of milk. The test was considered positive when observed around colonies yellowish-white precipitate of calcium paracaseinase. In tis paper 20 strains of all studied strains showed gelatinase on average egg yolk medium with added lecithin seed purified. In the case of positive reaction around the bacterial colony emerged crop or a halo and / or opacified area. Clarification result from the release of more soluble lecithin of lipid complex (presence of lecitinase). 18 strains showed lipase on agar medium with the addition of TWEEN 80 (sorbitol monooleate). The presence of TWEEN-lipase crystals oleate led to the emergence Ca^{2+} insoluble (crystal formed between fatty acids and the release of Ca^{2+} ions). Of the total studied strains, 17 strains showed hemolysins on agar medium with addition of 5% sheep blood or rabbit blood (highlighting the presence of Kanagawa hemolysins). Viewing the occurrence of areas of haemolysis around the colonies, as a transparent halo, the clear characteristic was β-haemolytic strains (which achieve a complete haemolysis; the haemoglobin released from red blood cells by the action of α-hemolysins generates a green halo, emphasizing partial degradation). Of the total strains, 16 strains showed DN-aze on agar medium with DNA with toluidine blue. DNA hydrolysis was revealed by the appearance of a halo around the spot pink culture, environment while the rest remained blue coloured. Of the 82 studied strains, 21 showed gelatinase nutrient agar medium with gelatine. After precipitation around the colonies a halo appeared due to the hydrolysis of gelatine.

For screening the activity of oil clove against soluble virulence factors, the strains were seeded in BHI broth medium with the addition of the clove oil extract diluted in DMSO (1: 2) as the minimum inhibitory. Thus, the obtained strains were passaged in specific medium of virulence factors. The inhibitory effect of the extracts of clove against virulence factors has been reported in a ratio of 100%. According to the assessment, the phenotypical resistance factor β-lactam antibiotics, there have been selected primers for molecular screening of the major coding for the β-lactamase gene described in the literature as being frequent (PATERSON & BONOMO, 2005, LIVERMORE, 1995). For β- extended spectrum lactamases in the case of bacteria resistant to penicillins and cephalosporins there have been used $bla_{\text{CTX-M}}$ and bla_{TEM} genes encoding for β-lactamases CTX-M. and TEM. For metal-β- lactamase we used $bla_{\text{VIM,}}$ bla_{KPC} and $bla_{\text{OXA-23}}$ genes coding for VIM, KPC and OXA-23 carbapenemases (BUSH & JACOBY, 2010). Figure 1 shows the negative image of ethidium bromide-stained agarose gel of the multiplex PCR assay of $bla_{\text{TEM-like}}$ and $bla_{\text{CTX-M-like}}$ for strains investigated.

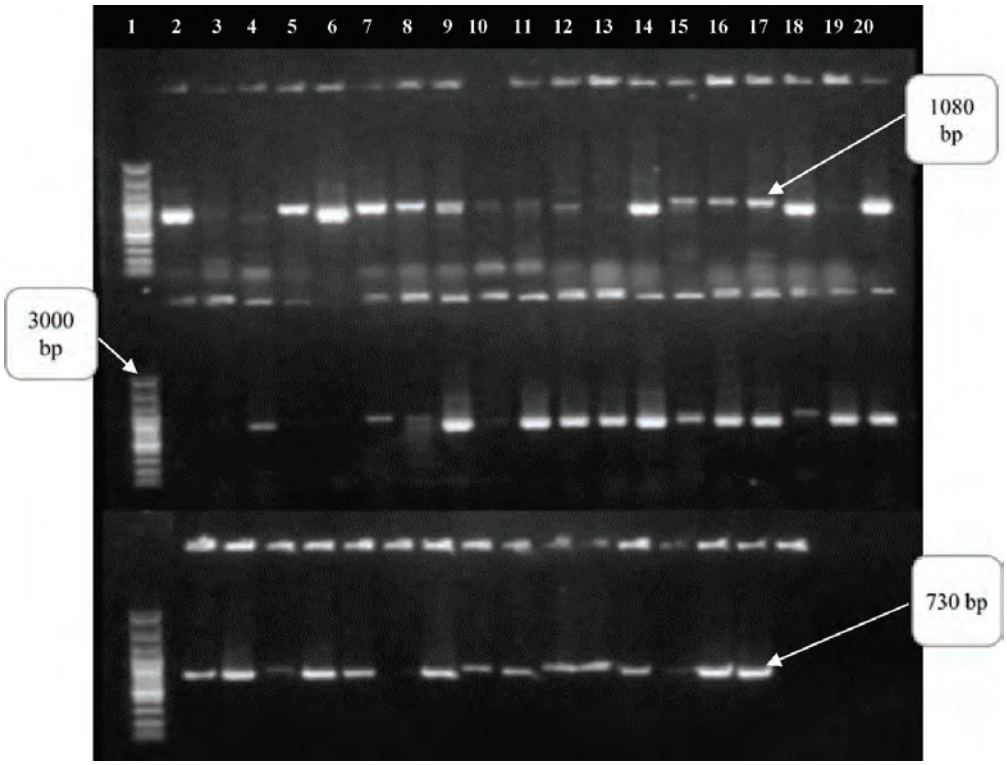


Figure 1 Agarose gel electrophoresis by multiplex PCR amplification for simultaneous detection of $bla_{\text{TEM-like}}$ and $bla_{\text{CTX-M-like}}$ genes, numbered lanes correspond to the following samples: top row(1-20): molecular weight markers Gene Ruler 3000bp (Fermentas) (MGM), *E.coli*$_{7}$, *E.coli*$_{29}$, *E.coli*$_{2R}$, *E.coli*$_{13}$, *E.coli*$_{3R}$, *E.coli*$_{22}$, *E.coli*$_{19}$, *E. faecalis*$_{4}$, *E. faecalis*$_{7R}$, *E. 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*$_{17R}$; middle 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*$_{20}$, *E. faecalis*$_{41}$, *K. pneumoniae*$_{19I}$, *K. pneumoniae*$_{12R}$; bottom row: *K. pneumoniae*$_{9III}$, *E.coli*$_{81}$, *K. pneumoniae*$_{26I}$, *Proteus miserabilis*$_{1I}$, *K. pneumoniae*$_{14I}$, *K. pneumoniae*$_{31I}$, *E.coli*$_{1138}$, *K. pneumoniae*$_{113740}$, *K. pneumoniae*$_{10I}$, *E.coli*$_{5III}$, *K. pneumoniae*$_{KI}$, *K. pneumoniae*$_{K2}$, *K. pneumoniae*$_{K3}$, *E.coli*$_{E1}$, *E.coli*$_{E2}$, *E.coli*$_{E3}$; (**B**): simultaneous detection of $bla_{\text{NDM-like}}$ and $bla_{\text{OXA-48-like}}$ genes: (MGM), *K. pneumoniae*$_{6I}$, *K. pneumoniae*$_{14I}$, *K. pneumoniae*$_{15I}$, *K. pneumoniae*$_{19I}$, *K. pneumoniae*$_{0}$.

Amplicon approx. 1080 bp corresponding bla_{TEM} gene confirming the presence of β-lactamases TEM can be observed in the wells 5, 7, 8, 9, 15, 16, 17 (in the top row), in the wells 7, 8, 18 (in the middle row) and the wells 4, 9, 11, 12 in the lower row (representing 26% of the 54 strains). Amplicons approx. 754 bp corresponding bla_{CTX-M} gene confirming the presence of β-lactamases CTX-M can be observed in the wells 2, 6, 14, 18, 20 (in the top row), in the wells 4, 9, 11, 12, 13, 14, 15, 16, 17, 19, 20 (in the middle row) and in the wells 2, 3, 5, 6, 8, 10, 13, 14, 15, 16 (in bottom row). Molecular analysis showed that the 26% of strains showed the presence of β-lactamases TEM-type compared with 48% of the strains showed β-lactamase CTX-M type. The multiplex PCR method did not reveal the presence of $bla_{VIM,}$ bla_{IMP} bla_{NDM} and bla_{OXA-48} genes coding for VIM, IMP, NDM and OXA-48 carbapenemases. The other amplicons are not detected although not ruling out nonspecific β-lactamase. The existence of these types of β-lactamases confirms lactam antibiotics resistance to pathogens.

Qualitative and quantitative evaluation of the antimicrobial activity of clove extracts

The qualitative testing by disc diffusion method Kirby-Bauer, adapter (CHIFIRIUC et al, 2011) emphasized that hydroalcoholic extracts and essential oil of *E. caryophyllata* shoots had antimicrobial activity for all bacterial strains from the collection achieved, quantified by the appearance of a zone of growth inhibition around the spot stock solution deposited on the seeded agar medium as can be seen in the example from Figure 2.

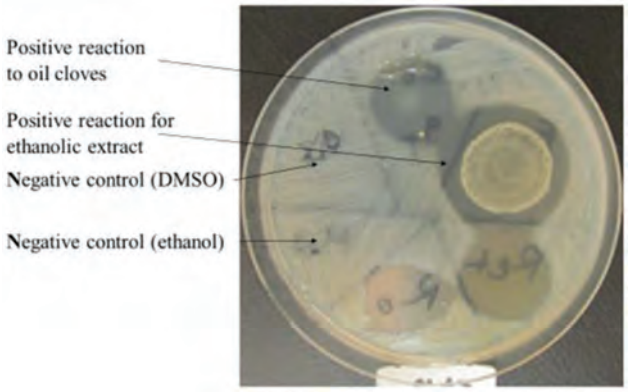


Figure 2. Qualitative testing of susceptibility to volatile oil and ethanol extract of *E. caryophyllata* strains studied, for example *Pseudomonas. aeruginosa*$_{1III.}$ (original).

Qualitative evaluation is an estimate, because it is not known the extent of absorption of the compound by the medium. Quantitative determination of antimicrobial activity was performed by the technique of successive binary microdilution in a liquid medium, which is a method of determining the minimum inhibitory concentration (MIC). The assay was performed according to the method described by CHIFIRIUC et al., 2011. Out of stock solutions of the compounds to be analysed there are performed micro binary successive dilutions in liquid BHI medium, distributed in 96-well plates. No dilutions were made in the wells treated for positive control (culture medium) and negative control (the control microbial culture).

MIC values are between 7.8 and 62.5 µL/ mL for ethanolic extracts and essential oils of *E. caryophyllata*. In Table 2, there are shown the MIC values for Gram negative strains from the collection of study. Considering some of the compounds resulting from the secondary metabolism of plants, also have a protective role, for example against phytopathogens, which are generally Gram-negative bacteria, we can make a correlation between these phytopathogenic bacteria and pathogenic bacteria in humans. Thus, we could explain the action of active compounds from plants against a broad spectrum of pathogens.

Table 2. The minimal inhibitory concentrations (MICs) for the essential oil and ethanolic extracts of *E. caryophyllata* against Gram negative bacteria.

	MIC ethanolic extract (µl/ml)		MIC essential oil (µl/ml)	
Strain	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}
Escherichia coli	15,625	62,5	7,8	31,25
Klebsiella pneumoniae	62,5	125	7,8	31,25
Proteus mirabilis	31,25	62,5	31,25	7,8
Alcaligenes faecalis	31,25	62,5	15,625	31,25
Acinetobacter baumannii	15,625	62,5	7,8	15,625
Pseudomonas aeruginosa	31,25	62,5	7,8	31,25

CONCLUSIONS

Ethanolic extracts and the essential oil of clove can be used successfully for severe nosocomial infections, due in particular to MDR Gram negative bacteria. Eugenol (the main component of *E. caryophillata*) and the concerted action of the compounds resulting from secondary metabolism of *E. caryophillata* had through several targets inhibitory action against Gram negative bacteria. The importance of isolating synthetic compounds in the extracts of cloves can be materialized by obtaining of new drugs for treatment of infections caused by MDR bacteria. Promoting the use of plant extracts is the main aim to eradicate microorganisms whose virulence is due to the abuse of drugs. The use of spices in daily consumption, especially cloves, for a long-term therapy, will result in increased body immunity against pathogens.

ACKNOWLEDGEMENS

We express our gratitude to Professor Coordinator, Mihăescu Grigore, Mrs. Carmen Maria Chifiriuc director of the Research Institute of the Faculty of Biology, Mrs. Dr. Camelia Tudor, head of the laboratory of Theodor Burghele Hospital Bucharest.

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Received: March 27, 2015
Accepted: May 12, 2015