Eugenia caryophyllata THUNBERG, A SPICE SIGNIFICANT IN THE PREVENTION AND THERAPY OF BACTERIAL INFECTIONS HIGHLY RESISTANT TO ANTIBIOTICS

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Abstract. The antibiotic resistance of Gram negative bacteria isolated from kidney infections of hospitalized patients has become a major problem for medical practice in the last decade. The use of spices herbs could successfully replace the antibacterial activity of antibiotics, in many critical circumstances. This study illustrates the antibacterial activity of the hydroethanolic extract of *Eugenia caryophyllata* Thunberg, against a lot of Gram negative bacteria isolated from nosocomial infections with high resistance to all antibiotics tested. Hydroethanolic extract of clove (in a ratio of 3: 2: 5) was tested in vitro from a lot of MDR Gram negative bacteria isolated from renal infections. Identification of bacterial strains and antibiotic resistance profile were performed using a compact automated VITEK®2 system. CMI was made by decimal dilutions method and it was quantified by spectrophotometry. Qualitative analysis of compounds of clove extract was made by TLC. TLC analysis of *E. caryophyllata* ethanol extract showed the presence of the following antibacterial potential compounds: gallic acid, quercetin and kaempferol. The total content of polyphenols (equivalent gallic acid) was 264.44 and antioxidant activity (equivalent in ascorbic acid content) was 4160.87. 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 µg / 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.

Keywords: *E. caryophyllata*, MDR Gram negative bacteria, antibacterial testing.

Rezumat. *Eugenia cariophyllata* Thunberg, o plantă aromatică importantă în prevenirea și terapia infecțiilor bacteriene cu rezistență multiplă la antibiotice. Rezistența la antibiotice a bacteriilor Gram negative izolate din infecțiile tractului urinar al pacienților spitalizați, reprezintă o problemă medicală majoră cu care se confruntă știința medicală în ultimul deceniu. Folosirea unor plante aromatice ar putea înlocui cu succes activitatea antibacteriană a unor antibiotice, în multe situații critice. Acest studiu ilustrează activitatea antibacteriană a extractului hidroetanolic de *Eugenia caryophyllata* Thunberg, asupra bacteriilor Gram negative izolate din infecții nosocomiale cu rezistență multiplă la antibioticele testate. Extractul hidroetanolic de cuișoare (în raport de 3: 2: 5) a fost testat *in vitro* pentru bacteriile Gram negative MDR izolate din infecțiile tractului urinar. Identificarea tulpinilor bacteriene și a profilului de rezistență la antibiotice au fost realizate cu ajutorul unui sistem automatizat VITEK®2 compact. CMI (Concentrația Minimă Inhibitorie) a fost realizată prin metoda diluțiilor zecimale seriale și a fost cuantificată prin spectrofotometrie. Analiza calitativă a compușilor extractului din cuișoare a fost făcută prin metoda TLC (cromatografie pe strat subțire). Analiza TLC a extractului hidroetanolic de *E. caryophyllata* a pus în evidență prezența următorilor compuși potențiali antibacterieni: acid galic, quercetin și kaempferol. CMI măsurată prin spectrofotometrie la lungimea de undă de 620 nm a extractului de *E. caryophyllata* asupra tulpinilor MDR, a avut valori cuprinse între 7,8 și 62,5 µg / ml. Rezultatele au demonstrat un înalt potențial antibacterian a *E. Caryophyllata* comparativ cu antibioticele uzuale. Activitatea antibacteriană poate fi atribuită unui înalt conținut de polifenoli și a unei activități antioxidante foarte ridicate.

Cuvinte cheie: *Eugenia caryophyllata*, bacterii Gram negative MDR, test antibacterian.

INTRODUCTION

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. Antibiotic resistance is due to a variety of less known biochemical and physiological processes that change continuously. Antimicrobial agents cannot cover all these mechanisms and the development of antibiotic resistance is relentless (DAVIES & DAVIES, 2010). Using spices in world history proved to be beneficial not only for the preservation of aromatic properties and preservation of foods, but also for their antibacterial properties (SETHI et al., 2012). *Eugenia caryophyllata* Thunberg 1788 known as cloves, is an aromatic tree, native to tropical area. In traditional Asian and Australian medicine, buds of *E. caryophyllata* are used invarious diseases, such as asthma, gastrointestinal infections, headache (SINGH et al., 2012). Activity against pathogenic bacteria of the essential oil of cloves extract has been reported in many studies (ALI-MOHAMMED & BAHAA, 2014; LUANGNARUMITCHAI et al., 2007).

MATERIAL AND METHODS

The 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.

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 (200 mL of distilled water and 500 mL ethanol). The solution thus obtained was kept in an amber glass container at 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. 41 filter. Finally, we obtained 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, 100µL 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 the 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 (ROMAN et al., 2015). MCI was established macroscopically, as the last concentration at which no growth of the microbial environment was observed, and the appearance of turbidity was read spectrophotometrically at the 620 nm.

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

The standard compounds with antibacterial activity (ferulic acid, gallic acid, chlorigenic acid, quercetin, rutin, kaempferol) were purchased from Bucharest Chemical Company. We 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. Qualitative analysis of antibacterial compounds of *E. caryophyllata* hydroethanolic extract.

Qualitative analysis of antibacterial compounds of hydroethanolic extract of cloves by TLC						
Stationary phase	Mobile phase	Development	Rf	Detection		
				Visible light	UV254	UV365
Silicagel (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	$Rf = \frac{\text{distance the center of the spot moved}}{\text{distance the solvent front moved}}$	for natural colored compounds	NP / PEG green back-ground	NP / PEG black back-ground

RESULTS AND DISCUSSION

The analysis of the antibiotic resistance spectrum (fig. 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 cefoxitim (second generation of cephalosporin), except for two strains of *Klebsiella pneumoniae*. This phenotype suggests the presence of ESBLs resistance phenotype. 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 (MARK, 1997). Multidrug resistance in *Enterobacteriaceae* and especially in *K.-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 addition, β-lactam antibiotics, cephalosporins, monobactams, and carbapenems resistance of Gram negative bacteria has been described in many researches. In this study, we found that all strains of *K. pneumoniae* feature the class A of carbapenemases type, which explains the 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 *Algaligenes faecalis* 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 emergence of colistin resistant *K. pneumoniae* has been described following widespread use of colistin (YAHAV et al., 2012).

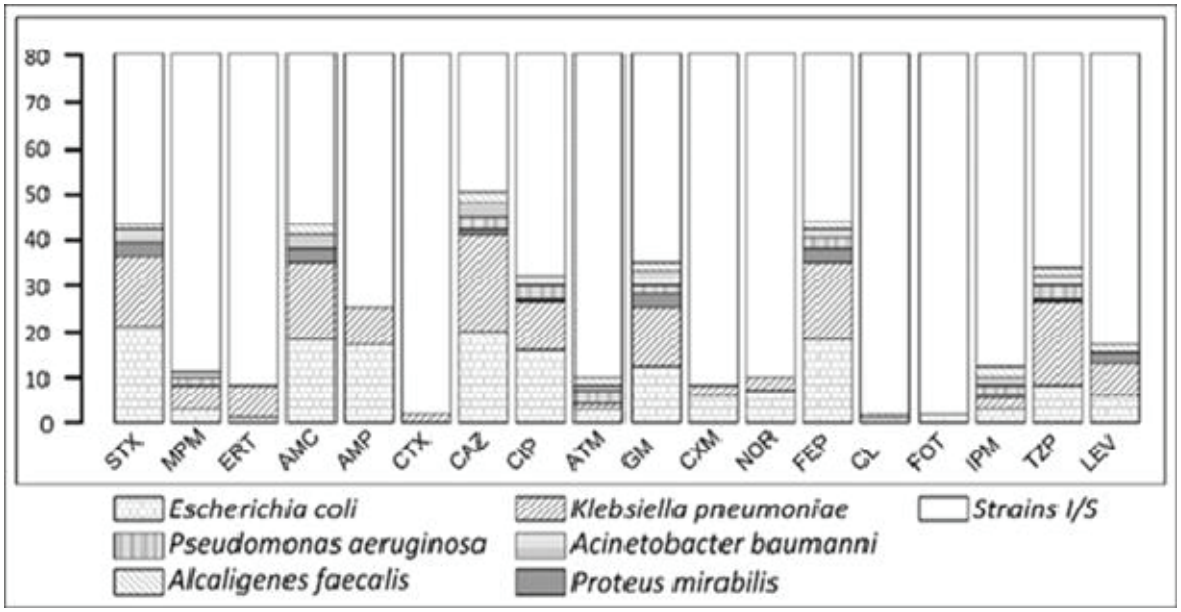

Figure 1. Antibiotic resistances of Gram negative bacteria. (Abbreviations: STX= sulfametoxazol/trimetoprim; MPM= meropenem; ERT= ertopenem; AMC= amoxicilin/clavulanic acid; AMP=ampicillin; CTX= ceftriaxone; CAZ= ceftazidime: CIP=ciprofloxacin; ATM= azteronam; GM=gentamicin; CXM=cefuroxime; NOR=norfloxacin; FEP=cefepime; CL=colistin; FOT=fosfomycin; IPM=imipenem; TZP= piperacillin/ tazobactam; LEV=levofloxacin)

Chemical analysis of the hydroethanolic extract of *E. caryophyllata*

The method of separation and identification of compounds by TLC consists in 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 Fig. 2, we show the chromatographic fingerprints of isolated compounds observed at different wavelengths by spraying with reagents, or directly in visible light.

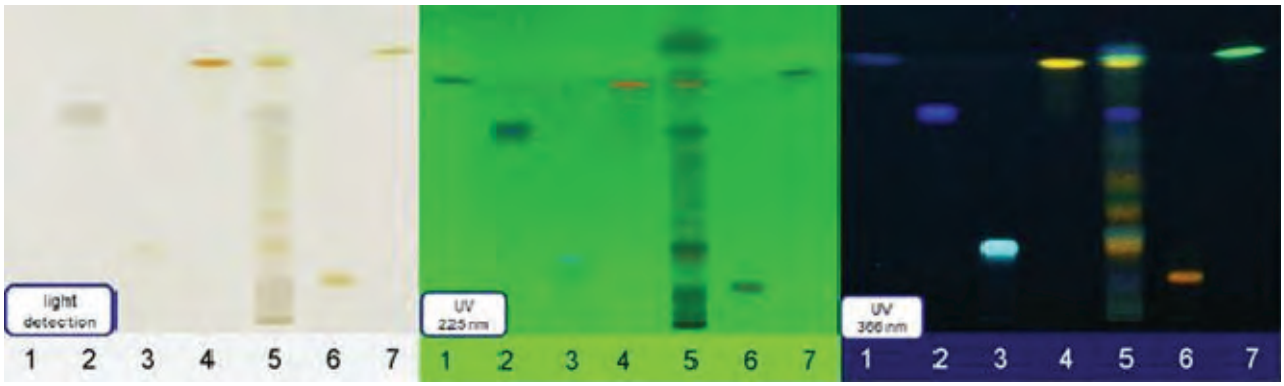

Figure 2. Identification and separation of the antibacterial compounds of hydroethanolic extract of clove using the TLC assay method: 1-ferulic acid, 2- gallic acid, 3- chlorogenic acid, 4- quercetin, 5- plant extract, 6- rutin, 7- kaempferol (original).

Analyzing the chromatographic fingerprints in hydro ethanol extract of *E. caryophyllata* there were identified four synthesis compounds: ferulic acid, Gallic acid, quercetin and kaempferol. Ferulic acid (FA) is a constituent commonly found in plants that arises from the metabolism of aromatic amino acids L-phenylalanine and L-tyrosine, as key entities (KUMAR & PRUTH HTTP, 2014; SRINIVASAN et al., 20017). FA exhibits a wide range of biomedical effects including antioxidant, antiallergic, hepatoprotective, anticarcinogenic, anti-inflammatory, antimicrobial, antiviral (KUMAR & PRUTHHTTP, 2014; BORGES et al., 2013). Gallic acid (GA) is a result of secondary metabolites in plants, especially *Gallo* and *Ellagitannins*. The most important effect of quercetin is scavenging oxygen-derived free radicals and chelating transition metal ions (NIJVELDT et al., 2001; BENTZ, 2009). Quercetin (3,3o,4o,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 (BENTZ, 2009). Kaempferol (3,5,7-

trihydroxy-2-(4-hydroxyphenyl)-4*H*-chromen-4-one), like all other flavonoids studied for therapy, can be applied in 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 (LIU et al., 2008).

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 (FARNSWORTH, 1988). The specificity and complexity of the molecular interactions between cellular components and potential antibacterial compounds from extracts of plants are not elucidated. The qualitative evaluation of antibacterial activity of plant extracts on solid medium is an estimate because the extent of absorption of the compound by the medium is unknown. 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*. Table 2 shows the values of MIC read at an absorbance of 620 nm for Gram negative bacteria, which showed resistance to all tested antibiotics.

Table 2. MIC of ethanol extracts of *E. caryophyllata* against bacterial strains resistant to all tested antibiotics measured via single-wavelength spectrophotometry at 620 nm.

Extract of cloves	**CFU (observed at 620 nm)**								
	*Ps a*1 1III	*Ps a*20III	*Al f*25I	*E c*01	*A b*5I	*Ps a*24II	*Ps a*27II	*Ps a*31III	*Ps a*1 III
500µg/mL	0.058	0.052	0.054	0.049	0.107	0.049	0.058	0.047	0.051
250µg/mL	0.098	0.064	0.063	0.051	0.086	0.050	0.067	0.049	0.059
125µg/mL	0.075	0.081	0.076	0.056	0.076	0.051	0.121	0.051	0.063
62.5µg/mL	0.078	0.084	0.087	0.058	0.083	0.068	0.149	0.089	0.096
31.25µg/mL	0.098	0.107	0.099	0.078	0.095	0.097	0.198	0.129	0.131
15.625µg/mL	0.168	0.178	0.207	0.097	0.164	0.174	0.209	0.183	0.159
7.831µg/mL	0.121	0.108	0.078	0.259	0.271	0.169	0.328	0.478	0.149
3.90µg/mL	0.253	0.476	0.498	0.287	0.296	0.375	0.427	0.309	0.375
1.953µg/mL	0.392	0.497	0.652	0.318	0.379	0.399	0.357	0.391	0.401
0.976µg/mL	0.409	0.597	0.514	0.375	0.487	0.439	0.524	0.549	0.523
M+	0.599	0.579	0.609	0.578	0.586	0.594	0.593	0.592	0.592
M-	0.048	0.046	0.046	0.048	0.047	0.045	0.048	0.045	0.048

Legend: *Ps a = Pseudomonas aeruginosa, Al f = Alcaligenes faecalis, Ec= Escherichia coli, Pm= Proteus mirabilis, Ab= Acinetobacter baumannii*, M+ = positive control, M- = negative control.

The high content of phenols resulting from semiquantitative analysis might suggest that these compounds had the highest antibacterial activity. This result led to the next stage of testing of the phenolic compounds isolated from the cloves extract against bacteria of its own collection. MIC values of phenolic compounds were very close to those obtained from hydroethanolic extracts of cloves. The MIC values of phenolic compounds are shown in Table 3.

Table 3. MIC values of phenolic compounds against bacterial strains resistant to all tested antibiotics measured via single-wavelength spectrophotometry at 620 nm.

Concentration of phenols	**CFU (observed at 620 nm)**								
	*Ps a*1 1III	*Ps a*20III	*Al f*25I	*E c*01	*A b*5I	*Ps a*24II	*Ps a*27II	*Ps a*31III	*Ps a*1 III
500µg/mL	0.051	0.047	0.052	0.046	0.049	0.048	0.051	0.055	0.048
250µg/mL	0.068	0.058	0.057	0.050	0.059	0.052	0.054	0.049	0.051
125µg/mL	0.073	0.075	0.069	0.053	0.073	0.049	0.088	0.051	0.057
62.5µg/mL	0.084	0.079	0.071	0.061	0.078	0.057	0.091	0.071	0.087
31.25µg/mL	0.089	0.086	0.083	0.072	0.086	0.089	0.173	0.091	0.089
15.625µg/mL	0.093	0.124	0.088	0.078	0.121	0.093	0.197	0.175	0.105
7.831µg/mL	0.117	0.131	0.097	0.189	0.233	0.137	0.316	0.421	0.113
3.90µg/mL	0.238	0.375	0.249	0.267	0.291	0.324	0.418	0.316	0.298
1.953µg/mL	0.387	0.426	0.527	0.307	0.376	0.399	0.325	0.378	0.377
0.976µg/mL	0.486	0.525	0.597	0.331	0.438	0.549	0.574	0.549	0.498
M+	0.581	0.577	0.684	0.548	0.579	0.588	0.587	0.573	0.516
M-	0.043	0.045	0.044	0.044	0.045	0.046	0.043	0.045	0.047

The ratio of the similarities between MIC values of the extract and the MIC values of phenols was between 0.839 and 0.939%. The biggest similarity was for *P. aeruginosa*$_{3III}$ (0.939%) and *P. aeruginosa*$_{24II}$ (0.930%) and the smallest similarity for *P. aeruginosa*$_{1III}$ (0.839%) and *A. faecalis*$_{25I}$ (0.855%). This result suggests to us that the antibacterial effect of the extract can be attributed to the high content of phenols.

CONCLUSIONS

The antimicrobial activity of ethanol extract of clove is closely related to the chemical structures of the components presented in the extract. Thus, the high antioxidant activity and the large polyphenol content were the main factors in the eradication of bacteria highly resistant to antibiotics. 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. The usefulness of medicinal plants in long term therapy is not only in stopping the abuse of antibiotics, but also in increasing immunity given that bacterial infections seem to take unprecedented dimensions.

ACKNOWLEDGEMENS

I bring gratitude to Professor Coordinator, Mihăescu Grigore, Professor Carmen Mariana Chifiriuc director of the Research Institute of the Faculty of Biology, Dr. Camelia Tudor, head of the laboratory at the Hospital Theodor Burghele Bucharest.

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Received: March 12, 2017
Accepted: May 11, 2017