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Flow Cytometry and Liquid Broth Microdilution Assay Evaluation of the Synergistic Activity of Eugenol with Current Antibiotics Against Eskape Clinical Strains

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Abstract

Considering the increase in antibiotic-resistant (AR) bacterial strains and the lack of new antibiotics, alternative/complementary strategies need to be founded to solve the infections due to AR pathogens. A possible solution may be to combine existing antibiotics with phytochemicals to enhance their efficacy. The purpose of this study was to assess the synergy between eugenol and a set of antibiotics currently used for the treatment of infections produced by Gram-positive and Gram-negative strains.

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Introduction

The antibiotics resistance problem in microbial strains is presumed as a method for continuity [1,2]. lately, the rapid growth of resistant strains is occurring worldwide, endangering the potency of antibiotics, which have transformed medicine and saved millions of lives [1]. *P. aeruginosa* is one of the most common bacteria isolated from chronic wounds is an opportunistic pathogen with innate resistance to several classes of antibiotics because of the low permeability of its outer membrane, the essential expression of various efflux pumps, and the production of antibiotic-inactivating enzymes (e.g., cephalosporinases) [3]. Methicillin-resistant *S. aureus* (MRSA) is a common problem in hospital settings and in the community. The most common cause of hospital-acquired infections is represented by hospital-acquired MRSA (HA-MRSA), and CA-MRSA (MRSA acquired in the community) [4,5].

There is a continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action because there has been a threatening increase in the incidence of new and re-emerging infectious diseases. Another big problem is the development of antibiotics resistance in current clinical use. Higher plants produce hundreds to thousands of diverse chemical compounds with different biological activities. The antimicrobial compounds produced by plants are active against pathogenic microorganisms. It is expected that plant extracts showing target sites other than those used by antibiotics will be active against drug-resistant microbial pathogens [6].

Thus, herbal treatment would promise a greater viable solution for the effective treatment of diseases caused by bacteria [7,8]. In vitro and in vivo studies have revealed that essential oils mixtures, oleoresin, flavonoids, alkaloids, phenol, and phenolic compounds, tannin, xanthone and xanthone derivatives, diterpene acid, phenylpropanoid glycosides, acteoside, and the bisnaphthquione derivatives have antimicrobial and anti-inflammatory activities [9]. The World Health Organization (WHO) estimates that 80 % of the world's population presently uses herbal medicine for some aspect of primary health care. Our aims for this study are: To assess the anti-pathogenic activity of the extracts from commercial oils and to search and propose a mechanism of action for the tested extracts.

Materials and methods

Microbial strains. several 27 clinical resistant isolates of *Pseudomonas aeruginosa* (10), (6 tracheal secretion, 3 surgical secretion, 1 nasal exudate), (10) *Escherichia coli* (4 urine, 4 anal swab, 2 wound secretion), and (10) *Staphylococcus aureus* (5 wound secretion, 2 tracheal secretion, 2 venous hemoculture, 1 skin). Strains were used Collected in the Bacteriology Laboratory of Al-Hussein teaching hospital, Identified by the Vitek2 automatic system.

Antibiotic susceptibility testing. The antibioresistance profile - obtained by disc diffusion method performed by Kirby-Bauer standard disk diffusion method The antibiotic disks used were Amykacin (15 µg), Ticarcillin-Clavulanate (75/10 µg), Aztreonam (Colistin (30 µg), Ciprofloxacin (5 µg), Ofloxacin (5 µg), Gentamicin (10 µg), Norfloxacin, Imipenem (10 µg), Cefotaxime, Ceftazidime (30 µg), Ticarcillin (75 µg), Piperacillin-Tazobactam (30 µg), Piperacillin-tazobactam, and Piperacillin (100 µg).

The antibiotic disks used for *S. aureus* were Oxacillin (30

μg), Tetracycline (30 μg), Amikacin (30 μg), Trimethoprim-Sulfamethoxazole (1.25/23.75 μg), Clindamycin (2 μg), Erythromycin (15 μg), Ciprofloxacin (5 μg), Gentamicin (10 μg), Rifampin (5 μg), *Penicillin* (10 μg), Chloramphenicol (30 μg) and Teicoplanin (30 μg). The antibiotic disks used for *E. coli* were Ampicillin (10 μg), Tetracycline (30 μg), Ciprofloxacin (5 μg), Trimethoprim-Sulfamethoxazole (1.25/23.75 μg), Norfloxacin (10 μg), Gentamicin (10 μg), Amikacin (30 μg), Kanamycin (30 μg), Chloramphenicol (30 μg), Cefepime (30 μg), Aztreonam (30 μg), Imipenem (10 μg), Amoxicillin-Clavulanate (20/10 μg).

At first, the bacteria were cultured into tryptic soy agar (TSA) and incubated at 37°C for 24 hours. After 24 hours, microbial suspensions were prepared, with a density equivalent to the turbidity of the 0.5 McFarland standard and plated with sterile swabs on Muller Hinton (MH) agar. The antibiotic disks were placed on the plate and incubated at 37°C for 24 hours. Following incubation, the diameters of the growth inhibition zone were measured.

Screening of antimicrobial synergistic effect of combinations eugenol-antibiotics. The quantitative analysis of the synergy between antibiotics and eugenol was performed by the serial microdilution technique, allowing to establish and compare the minimum inhibitory concentrations (MIC) obtained for eugenol and each antibiotic, separately and in the association. The evaluation of the possible mechanism of action of the extract was determined by flow cytometry (FC), using MIC and MIC/2 concentrations.

Flow cytometric (FCM) assay for the detection of eugenol antimicrobial action mechanisms. The tested microbial strains treated with eugenol at 1/2 MIC were analyzed by FCM, using the acid nucleic intercalating dyes: propidium iodide (PI) and ethidium bromide (EB). The dyes do not penetrate and accumulate inside viable cells, therefore the fluorescence (median fluorescence intensity (MFI) measured in channel 2 (ethidium bromide) and respectively in channel 3 (propidium iodide) is low. If the microbial cell wall is affected (loss of integrity/ affected efflux pump activity), the dyes enter the cell and bind to nucleic acid, resulting in increased fluorescence. Samples were stained at room temperature with 10 μL/ml for PI and 5 μg/mL EB, in the dark, for 10 minutes. The fluorescence measurements were performed using the FACSCalibur flow cytometer (BD, Sparks, USA) equipped with an argon laser with an excitation wavelength of 488 nm. For each sample, a total of 10,000-30,000 events were acquired. CellQuest Pro software was used for statistical analysis. Microbial populations were gated based on the FSC/SSC characteristics.

Results

In the present study, data indicate that both Gram-positive and Gram-negative bacteria were affected by the plant extracts tested. However, Gram-positive strains were more sensitive when treated with eugenol. This finding is agreeable with previous studies on a range of herbs and spices and maybe mainly related to differences in the cell wall structure and the outer membrane arrangement between Gram-positive and Gram-negative bacteria. Gram-negative bacteria possess a hydrophilic outer membrane rich in lipopolysaccharide molecules, this structure which serves as a penetration barrier towards macromolecules [10,11].

The resistance profiles were all *S. aureus* strains MRSA (methicillin-resistant *S. aureus*), most *P. aeruginosa* strains were resistant to cefotaxime and Ticarcillin-Clavulanate, and all strains from *E. coli* strains were resistant to Ampicillin and Tetracycline. Among the 10 strains of *P. aeruginosa*, most of them showed an increased susceptibility by the synergistic activity of eugenol with Aztreonam (ATM), Ticarcillin-clavulanate (TIM), and Ciprofloxacin (CIP), only two strains manifesting the same susceptibility pattern as in control test. Most of *E. coli* isolates had synergism with ATM and piperacillin. Regarding the strains of *S. aureus*, their susceptibility was increased by the synergistic activity of eugenol with Oxacillin (OX), penicillin, gentamycin, and CIP in most strains, excepting two strains which hadn't synergistic effect with OX.

The possible mechanisms of action revealed by FC were: permeabilization of the cellular wall (outer membrane of Gram-negative bacteria) and of cellular membrane and inhibition of efflux pumps too. Several reports discussed the antimicrobial features of fundamental oils and their components have been completed by many specialists. Although some oils have been explained in many studies previously, essentials learning of a large portion of the mixes and their system of activity is yet deficient [12]. This knowledge especially necessary to assessment the volatile oils activity on various pathogens, however they work together with different antimicrobial agents, and their interaction with food matrix parts. Furthermore, the resistance to different antimicrobial agents by different microorganisms (bacteria, fungi, viruses, parasites, etc.) is a massive threat in the medical domain for treating the diseases caused by these resistance microorganisms. For this reason, raising an acute need for alternative/ complementary strategies to solve the infections due to these resistant microorganism strains. To beat these problems, nano-encapsulation of essential oils and exploiting the synergies between essential oils, constituents of essential oils and antibiotics at the side of essential oils are counseled as a solution to the present issue. Nevertheless, few is understood regarding the interactions which allow to additive, synergistic, or antagonistic activities [12]. A possible solution may be to combine existing antibiotics with phytochemicals to enhance the efficacy of antibiotics [13]. A synergistic effect tested for eugenol with conventional clinically used antibiotics, and the results showed a synergism for eugenol combined with antibiotics against isolates of *P. aeruginosa, E. coli and S. aureus*. We used different antibiotics were completely resistant to tested strains. Two strains of *P. aeruginosa* weren't revealed synergism with most of tested antibiotics, while there was a synergy activity in the rest of *P. aeruginosa* isolates especially with Aztreonam (ATM), Ticarcillin-clavulanate (TIM), and Ciprofloxacin (CIP). Most of *E. coli* isolates had synergism with Aztreonam (ATM) and Piperacillin (PRL), regarding *S. aureus*. Flow cytometry is a rapid mechanism for the detailed analysis of microorganisms by multiparametric measurement at a rate of several thousand individual cells per second. This technique provides quantitative data on the size or granularity of cells by means of light dispersion signals and by fluorescence signals, quantitative information regarding the expression of surface antigens or intracellular components such as nucleic acids, proteins or lipids on the potential of the membrane and ion fluxes [14]. The possible mechanisms of the antimicrobial action of eugenol were previously reported to be the membrane damage potential and efflux pumps inhibitor (EPI) activity [15]. In our study, microbial cells exposed to eugenol at ½ x MIC exhibited an increased fluorescence after staining with PI and EB, respectively. The MFI of the treated cells was significantly higher than the MFI of the controls (two-fold or higher values than of viable control cells) suggesting an increase in the fraction of microbial cells with permeabilized cell envelope, at ½ x MIC. The PI and EB dyes were able to enter the permeabilized cells, bind nucleic acids resulting in an enhanced fluorescence of the cells. Therefore, the FCM results confirm that one of the mechanisms

by which the eugenol exerts its antimicrobial activity is represented by inducing damages to the microbial cell wall. This mechanism was detected in all tested microbial isolates. The researchers Engel J, (2007) and Martins M, et al. (2013) suggested a relation between the intensity of fluorescence (FI) of the cells labeled with ethidium bromide EB and the influx ratio net of EB/EB extracellular ejection via the ratio of efflux pump activity [16,17].

Concerning the Gram-negative bacteria, *P. aeruginosa* isolates were least sensitive to the action of eugenol. The analysis of action mechanisms by using flow cytometry assay showed that at sub-inhibitory concentrations, the plant EOs caused damage to the *P. aeruginosa* cytoplasmic membrane (in five to seven out of the ten *P. aeruginosa* isolates) allowing the PI molecules to penetrate the cells, and thus an increase of MFI values detected for this dye. Also, eugenol treatments affected the efflux pump activity, although this mode of action was less frequent detected (in 1-4 out of 10 isolates), of the tested EO, eugenol predominantly affected the efflux pumps activity of *E. coli* strains with little effect on the integrity of the cytoplasmic membrane. Whereas, eugenol determined bacterial cell depolarization at both MIC and ½ MIC in one strain of *P. aeruginosa* strains and *E. coli*. Eugenol didn't produce the depolarization of *S. aureus* Gram-positive bacterial cells.

Conclusions

Our results showed the potential use of Syzygium aromaticum essential oil (eugenol) as an adjuvant of antibiotic therapy, acting by decreasing the effective antibiotic concentrations, thus increasing their efficiency in the treatment of many diseases caused by drug-resistant bacterial pathogens.

A synergistic effect between the essential oil of Syzygium aromaticum (eugenol) with conventional clinically used antibiotics has been demonstrated, the combinations being active against some strains of *P. aeruginosa, E. coli,* and *S. aureus.*

Flow cytometry has proven to be a quick and reliable analysis for sensitivity testing and determination of how essential oils work; giving very useful results that could support the development of new antimicrobial agents.

The cytometric analysis revealed that eugenol produced bacterial cell depolarization at MIC and ½ MIC, suggesting that the cytoplasmic membrane is a target of the tested essential oil.

The results of this experiment are significant and can be a motivation to continue the research on essential oils and other potential natural antimicrobials, in order to find active agents/ combinations on free and biofilm embedded cells too, without selective pressure on bacteria and connective effect of antibioresistance amplification.

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