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Rosmarinus officinalis essential oil as antibiotic potentiator against *Staphylococcus aureus*

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ABSTRACT

Essential oil extracted by hydrodistillation from *Rosmarinus officinalis* was characterized by means GC-MS. *R. officinalis* contained α-pinene, β-pinene, eucalyptol, camphor and caryophyllene, respectively, as the major compounds. An adapted difussion method was used in order to assess the potentiator effect of the essential oil and the analytical standard (eucalyptol) on the antibiotic susceptibility of *S. aureus* strains to some of the currently used antibiotics, i.e. tetracycline, oxacillin, erythromycin, penicillin, cefoxitin, doxycycline as well as suphametoxazole. Five *S. aureus* strains were tested, four of clinical origin (two wound secretions and two blood cultures) and one reference strain, i.e. *S. aureus* ATCC 25923. The *R. officinalis* essential oil and its main constituent, eucalyptol, exhibited a strong, strain specific influence on the antibiotic susceptibility of the tested strains.

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Keywords: *S. aureus*, *R. officinalis, potentiator of antibiotics, eucalyptol*

1. INTRODUCTION

The antibiotic era in clinical medicine, launched more than 70 years ago with the introduction of sulfonamides and the stunning success of penicillin which led to a drastic increase in survival from bacterial infections, clearly changed the world. However, as early as 1942, René Dubos predicted that bacterial resistance should be expected. “Rather than counter bacterial resistance with even more potent weapons”, he argued that we should, “seek instead more peaceful coexistence with pathogens”. When Alexander Fleming received the Nobel Prize in 1945, he noted, “it is not difficult to make microbes resistant to penicillin”. Now the discovery of new antibacterial strategies represents a real scientific challenge. The high expectations following the genomic breakthrough revealing numerous potential bacterial drug targets were followed by disillusionment and a poor success rate. The success rate of obtaining new chemical leads from these screens was only 7%, which is substantially lower than other therapeutic areas. There are several reasons for the difficulties, including the fact that most antibacterial targets are hard to inhibit enzymes, the toxicity of some antibacterial compounds on mammalian targets and spectrum challenges of antibacterials development, especially when it comes to Gram-negative bacteria. More innovative approaches are

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needed. Some areas currently being investigated include returning to natural product screening, exploring novel chemical space (e.g., boron chemistry) and development of antibiotic potentiators [1]. Initial studies on the mechanisms of action of essential oils components are related to the use of microorganisms in remediation biotechnologies. Microbial transformation of cyclic hydrocarbons is of ecological and biotechnologcal importance, in wastewater treatment or waste gas, in bioremediation and biocatalysis. In these processes, cyclic hydrocarbons, such as terpenes, aromatic substances and cycloalkanes, are present as pollutants or raw materials for biochemical transformation reactions [2]. Terpenoids are phytochemical compounds consisting of isoprene units, components of the essential oils, present in the environment due to the biological synthesis. In plants, the physiological role of the essential oils is poorly understood. It seems that they play important roles in self-defense against bacterial and fungal infections and in pollination, as well as in intraspecific communication. Because some constituents can be solubilized by glycosylation and stored in tissues, was proposed the hypothesis that essential oils could play a reserve substances role [3,4,5]. Industrial applications of terpenoids include pharmaceutical, food, perfume, chemical (solvents) industries, etc. Cyclic hydrocarbons are converted sometimes by microorganisms to complete mineralization. Researches have elucidated the metabolic pathways of the cyclic hydrocarbons transformation in general and terpenoids in particular, some literature data addressing their genetic aspects. The terpenoids transformation is closely correlated with the cytotoxic effect manifested by these compounds on microorganisms, with an impressive number of studies in the literature on the subject. However, the cytotoxicity mechanisms are relative poorly understood, especially for essential oils, which are complex mixtures of terpenoids [2]. Overall, the antimicrobial result involving biocides is a sum of actions with different targets in the microbial cells, leading to the microbiostatic or the microbicidal effect [6]. The antimicrobial effect of the essential oils is concerted, rather than being an amount of antimicrobial activity of the pure constituent compounds [5]. To date no essential oils extracts have been approved for the systemic use in infectious diseases, probably due to the lack of specificity of action or to the difficulty of obtaining pure substances from the complex mixtures [7]. A first aspect of those compounds cytotoxicity is their lipophilic character, which would favor hydrophobic interaction with the external membrane/cell coatings (cell wall, S layer). The lipophilic character is defined as a sum of the physico-chmical properties, eg the molecular surface area, molecular volume, and polarity. The estimation of the antimicrobial potential could use the octanol/water coefficient, a measure of differential solubility of the compound between these two solvents, forecasting the distribution of the tested compound within the cell. Bioavailability is essential the embedded antimicrobial substances mechanism of action, so their solubility in water is of a particular importance. In order to ensure bioavailability one can use co-solvents or emulsifiers, lipophilic molecules that facilitate solubilization of essential oils (such as dimethylformamide, dimethyl sulfoxide, ethanol, Tween 80) [2]. The effect of the essential oils constituents upon outer membrane/cell coatings can be used to potentiate the antimicrobial effect of commonly used antibiotics. The synergism between major classes of antibiotics and sesquiterpenoids as farnesol and nerolidol have been demonstrated. In contrast to inhibition of efflux pumps, the terpenoids increased permeability of cell envelopes, as evidenced by flow cytometric measurement of the intracellular accumulation of the ethidium bromide. A recent study shows a significant increase in MRSA (metycilin-resistant *Staphylococcus aureus*) susceptibility to the β-lactam antibiotics, particularly by inhibiting lipid C55 recycling, a transport molecule involved in the cell wall synthesis. The virulence attenuation, as opposed to the direct microbicidal effect, seems to be a

strategy in the current research studies. The anti-pathogenic molecules inhibit the production of toxins in the microbial cells, or affect the ability of microorganisms to adapt to the environmental conditions provided by the host, thus giving a competitive advantage to the host immune system that can accomplish the infecting microorganisms clearance. The advantage of this approach lies in preserving the normal microbiota of the host, which will not be affected by the anti-pathogenic molecules, and the lack of the selective pressure that leads to the selection of the resistant and multidrug-resistant microbial strains [7]. Structure-based drug design for discovery of novel antibacterial drugs to circumvent some of the bottlenecks in the search for these antibiotics is the identification of new inhibitors of both classical and novel bacterial target proteins was predicted to increase the success rate for discovery of antibacterial drugs in the near future. In this respect, it may be possible to design molecules that simultaneously inhibit two or more functional sites in a target enzyme, which could minimize the potential for the development of resistance [1]. Having a setup stage with the multidrug-resistance issue and keeping in mind the need of novel antibacterial strategies, our aim was to assess the anti-pathogenic activity of the *Rosmarinus officinalis* essential oil and of the eucaliptol analytical standard.

2. EXPERIMENTAL SECTION

2.1. Extraction and characterization of essential oils. *Rosmarinus officinalis* plant material was purchased from a local supplier and subjected to essential oil extraction. A Neo Clevenger type apparatus according to European Pharmacopoeia 6 was used performing two microwave assisted extractions from 225g plant material [8]. Finally DMSO was added to form a stock solution (1:1 with essential oil v/v) and it was kept in a cool place until was used. Chemical composition was settled by GC-MS analysis. Gas chromatographic analysis was performed using an Agilent 6890 Series GS System gas chromatograph Detection was carried out with a 5973 mass-selective single quadrupole detector (Agilent technologies). The mass spectrometer was calibrated before use with perfluorotributylamine (PFTBA) as a calibration standard. GC-MS parameters were described in a previous study [9].

2.2. Microbial strains. Five *S. aureus* strains were tested, four of clinical origin (two wound secretions and two blood cultures) and one reference strain, i.e. *S. aureus* ATCC 25923. Isolates were identified by using an automatic Vitek II system.

2.3. Quantitative assay of the antimicrobial activity. MIC (Minimal Inhibitory Concentration) values for the essential oil and the main tested constituent were determined by two-fold microdilution technique, in 96 multi-well plates, starting from 100µL/mL to 12.5 µL/mL, for each tested microbial strain. Simultaneously, there were achieved serial dilutions for DMSO in the same volume, in order to obtain the negative control. Each well was inoculated with 10 µL of 0.5 Mc Farland adjusted microbial suspension. Negative (MH broth) and positive controls (MH broth and microbial inoculum) were used. The plates were incubated for 24 h at 37°C, and MICs were read as the lowest concentration of the essential oil which inhibited the microbial growth.

2.4. Soluble enzymatic virulence factors. Bacterial cultures grown for 18 hours in nutrient broth with and without tested solutions as well as with the addition of DMSO were evaluated for seven enzymatic virulence factors [17] (pore forming toxins: lecithinase, lipase, hemolysins; exoenzymes: gelatinase, amylase, caseinase, DN-ase; siderophores-like activity). The bacterial cultures were spotted on the available media containing specific substrate for enzymes activity detection.

2.5. The antibiotic potentiator effect of the essential oil and eucalyptol. An adapted diffusion method was used in order to assess the potentiator effect of the essential oil and of the analytical

standard on the antibiotic susceptibility of the tested *S. aureus* strains to some of the currently used antibiotics, chosen according to CLSI reccomandations i.e. tetracycline, oxacillin, erythromycin, penicillin, cefoxitin, doxicycline, suphametoxazole. Standardized antibiotic discs have been placed on the Mueller Hinton agarised medium in Petri dishes, previously seeded with a bacterial inoculum adjusted to the 0.5 McFarland standard. Stock solutions of the *R. officinalis* essential oil and eucalyptol were spoted on the antibiotic discs. The plates were incubated 24h at 37°C, and the differences between inhibition zones diameters were quantiffied.

3. RESULTS SECTION

The results obtained by GC–MS analysis of the essential oils *R. officinalis* is presented in table 1. *Rosmarinus officinalis* essential oil proved to be reach in α-pinene, β-pinene, eucalyptol, camphor and caryophyllene. Differences in oil composition of *R. officinalis* have already been reported [10].The major components, alpha-pinene, borneol, camphene, camphor and bornyl-acetate, were also reported to be present in Sardinian *R. officinalis* L.oil [11].

Table 1: The main compounds and their percentage in *Rosmarinus officinalis* essential oil

Peak	R.T.	Pct Total	compounds
1	4.871	20.574	α-Pinene
2	5.251	3.823	Camphene
3	6.024	8.497	β-Pinene
4	7.457	3.259	o-Cymol
5	7.625	37.334	Eucalyptol
6	10.961	10.974	Camphor
7	15.114	2.45	Bornyl acetate
8	18.607	9.097	Caryophyllene

All the tested microbial strains had the same MIC value for the *Rosmarinus officinalis* essential oil and for the eucalyptol analytical standard, 25µL/mL, demostrating that eucalyptol is one of the major antimicrobial compounds of this essential oil. No significant influence was observed in the soluble virulence factors production, except for the stimulation of the siderophores-like molecules production in three strains. Differences between inhibition zones diametres obtained for the antibiotic control and for the tested solutions are ploted in figure 1a-e. The chosen antibiotics covered few important targets of microbial cell, i.e. the cell wall, protein and folic acid synthesis.

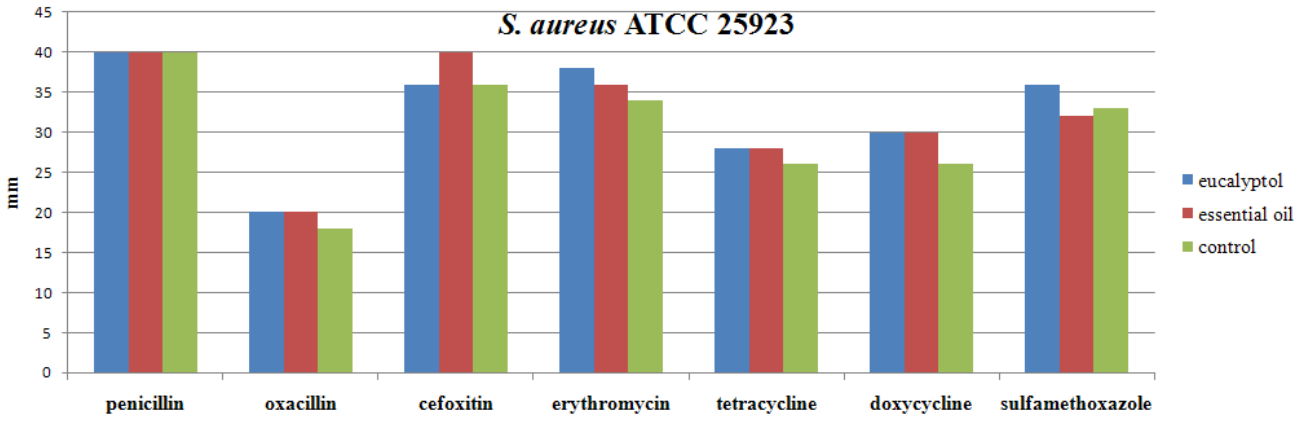

Figure 1: The graphic representation of zone inhibition diameters obtained for different antibiotics in the presence of the essential oil and eucalyptol

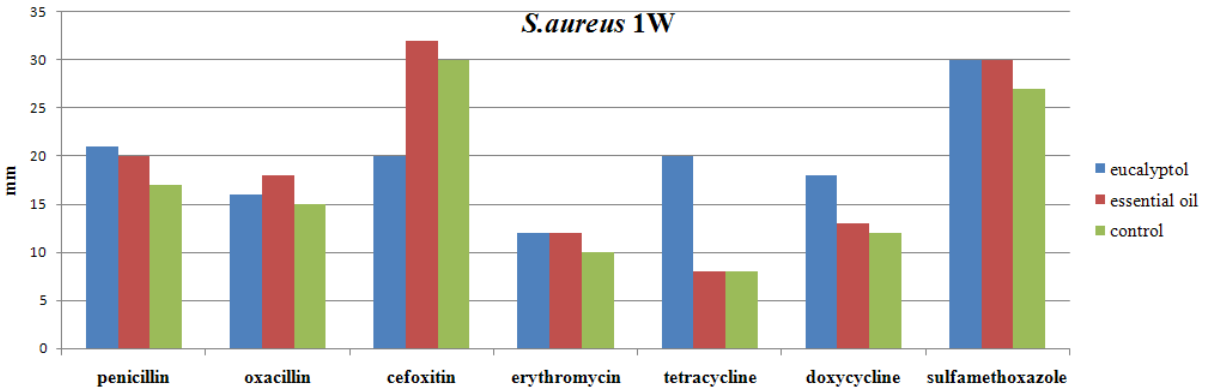

Figure 2: The graphic representation of zone inhibition diameters obtained for different antibiotics in the presence of the essential oil and eucalyptol

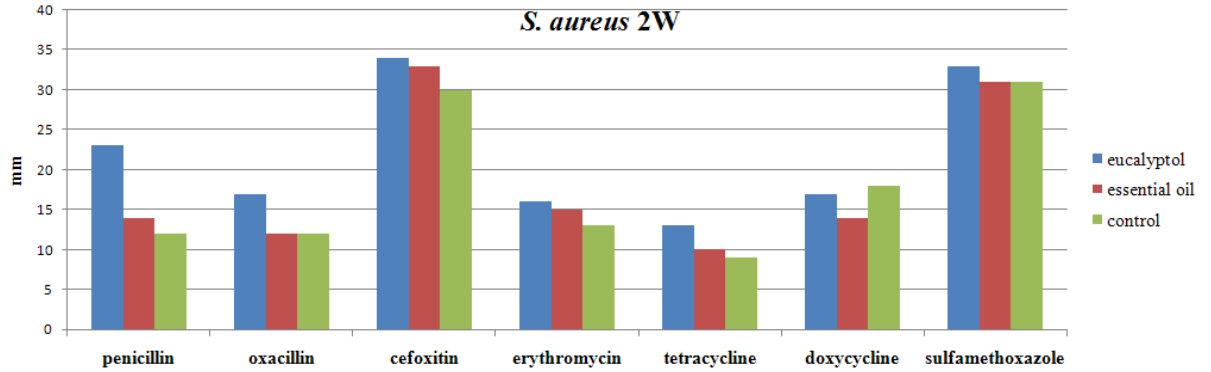

Figure3: The graphic representation of zone inhibition diameters obtained for different antibiotics in the presence of the essential oil and eucalyptol

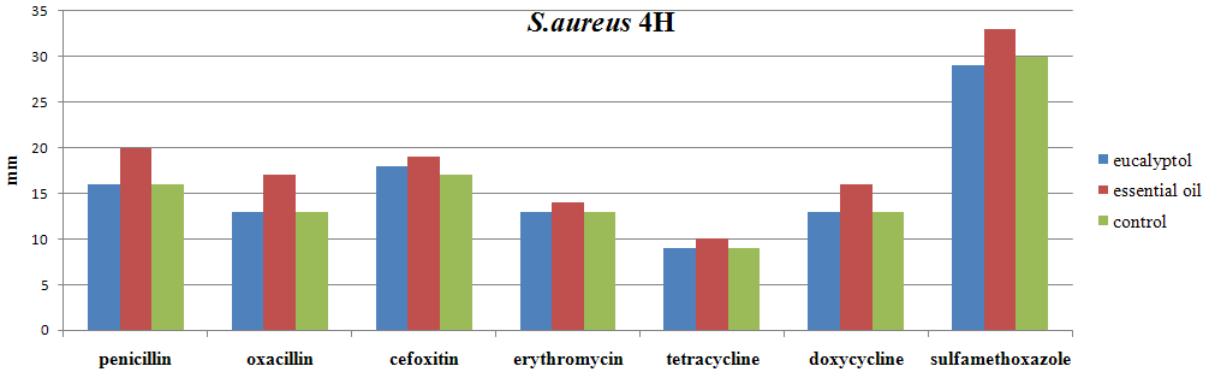

Figure 4: The graphic representation of zone inhibition diameters obtained for different antibiotics in the presence of the essential oil and eucalyptol

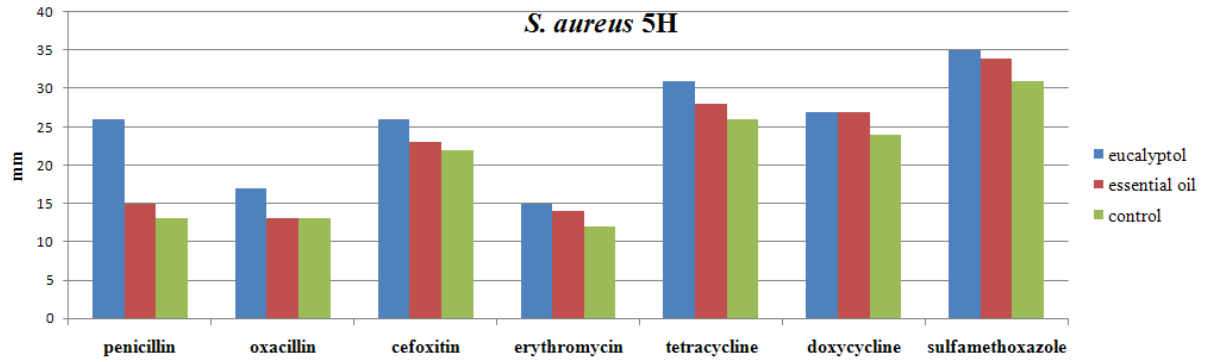

Figure 5: The graphic representation of zone inhibition diameters obtained for different antibiotics in the presence of the essential oil and eucalyptol

The activity of the *R. officinalis* and eucalyptol stock solutions as antibiotic potentiator proved to be strain apecific, the most sensitive strain being *S. aureus* ATCC 25923, whose susceptibility was influenced by both working variants. *S. aureus* 1W and *S. aureus* 4H in the presence of the essential oil have converted from resistant to sensitive to oxacillin, *S. aureus* 2W and *S. aureus* 5H to penicillin and *S. aureus* 5H to cefoxitin, in the presence of eucalyptol.

4. CONCLUSIONS

The intensity of the antimicrobial effect was similar for the *R. officinalis* essential oil and eucalyptol analytical standard, as revealed by similar MIC values and induced no significant changes in the virulence profile of the tested strains. In exchange, both the essential oil and to a greater extent, the eucalyptol standard increased their susceptibility to antibiotics of *S. aureus* strains.

5. ACKNOWLEDGEMENT

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