

# Enhancement of *Escherichia coli* and *Staphylococcus aureus* Antibiotic Susceptibility Using Sesquiterpenoids

Manuel Simões<sup>1,2,3,\*</sup>, Sílvia Rocha<sup>4</sup>, Manuel A. Coimbra<sup>4</sup>, and Maria J. Vieira<sup>1</sup>

<sup>1</sup>IBB-Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, University of Minho, Campus de Gualtar, Braga, Portugal; <sup>2</sup>CITAB-Departamento de Fitotecnia e Engenharia Rural, Universidade de Trás-os-Montes e Alto Douro (UTAD), Edifício Ciências Agrárias, Apartado 1013, 5001-801 Vila Real, Portugal; <sup>3</sup>LEPAE, Department of Chemical Engineering, Faculty of Engineering, University of Porto, Rua Dr. Roberto Frias, s/n, 4200-465 Porto, Portugal; <sup>4</sup>Department of Chemistry, University of Aveiro, Aveiro, Portugal

**Abstract:** The present work examines the potential of sesquiterpenoids to sensitize *Escherichia coli* and *Staphylococcus aureus*, and modulate their susceptibility to the standard antibiotics ciprofloxacin, erythromycin, gentamicin and vancomycin. It was tested samples of three sesquiterpenoids: guaiazulene, nerolidol (racemic mixture of the *cis* and *trans* isomers) and germacrene D enriched natural extract. Experiments were conducted aiming to assess the antimicrobial effects of the antibiotic-sesquiterpenoid combination on bacterial growth inhibition, by the disc diffusion assay and the minimum inhibitory concentration (MIC) assessment, the bactericidal effects, the post-antibiotic effect (PAE) and the effect on membrane permeability. The data related with the antimicrobial activity evidenced, through the disc diffusion assay, an antibiotic *S. aureus* antimicrobial activity enhancement by sesquiterpenoids presence. The MIC value for *E. coli* decreased significantly by sesquiterpenoids combination with ciprofloxacin, erythromycin and gentamicin, and for *S. aureus*, with all four selected antibiotics. This combination also increased the PAE, with the exception of guaiazulene, which seemed to quench antibiotic antimicrobial action. A moderate correlation between antimicrobial action and impairment of cell membrane function was detected for germacrene D enriched extract, and nerolidol, as single treatments and in combination with antibiotic, while a poor correlation was obtained for guaiazulene.

This study provides basis for the evaluation of sesquiterpenoids as alternative or possible synergistic compounds for current antimicrobial chemotherapeutics, showing the practical utility of natural derived products to increase the susceptibility of *E. coli* and *S. aureus*.

**Key Words:** Antibacterial drug screening, antibiotics, antimicrobial action, mechanisms of action, natural products, post-antibiotic effect, resistance, sesquiterpenoids.

## INTRODUCTION

To survive in the environment, bacteria must respond to several stresses that lead to non-ideal growth conditions. As an additional stress, they may be exposed to a wide range of antimicrobial agents, such as antibiotics, that can act as a selective pressure for the development of resistant microorganisms [1]. As a result of antibiotic use and misuse, increasing incidence of resistance to antimicrobials is a growing concern of the medical, food and sanitation areas [2-6]. Reduced susceptibility of microorganisms to antimicrobial agents may be acquired through mutation, by plasmid or transposon acquisition, or by the microorganisms intrinsic properties conferring reduced susceptibility to antimicrobial agents [3,7-10]. To counter the increasing emergence of resistant microorganisms, substantial resources have been invested in the research of new antimicrobial compounds, mainly of microbial and plant origin [11-13].

A wide variety of essential oils derived from plants are known to have antimicrobial properties which, in many cases, are due to the presence of active terpene constituents

[12]. These oils are secondary metabolites highly enriched of compounds based on an isoprene (C<sub>5</sub> chain) structure [14]. Sesquiterpenoids (C<sub>15</sub> chain), formed by the assembly of three isoprenoid units synthesized from acetate units, share their origins with fatty acids, and are known to have antimicrobial activity [11,14,15].

Terpenoids and other essential oils constituents occur widely in nature contributing to characteristic plants and products flavours and aromas [11]. Their mechanism of antibacterial action is yet not fully understood, but it is speculated to involve membrane disruption through lipophilic compounds [11,14, 16]. According to a previous study [16], this action results in membrane expansion, increase of membrane fluidity and permeability, disturbance of membrane embedded proteins, inhibition of respiration, and alteration of ion transport processes. This action can contribute to the decrease of microbial resistance and avoid the spread of resistant strains [6,17].

In this study, we report the antimicrobial and sensitizing actions of three selected samples of sesquiterpenoids, presenting different structural features (Fig. 1), against two clinically significant microorganisms, *E. coli* and *S. aureus*. These actions were assessed through the combination of antibiotics and sesquiterpenoids on bacterial growth inhibitory

Manuel Simões, LEPAE, Department of Chemical Engineering, Faculty of Engineering, University of Porto, Rua Dr. Roberto Frias, s/n, 4200-465 Porto, Portugal; Tel: 00 351 253 604404; Fax: 00 351 253 678986; E-mail: mjvsimoes@deb.uminho.pt

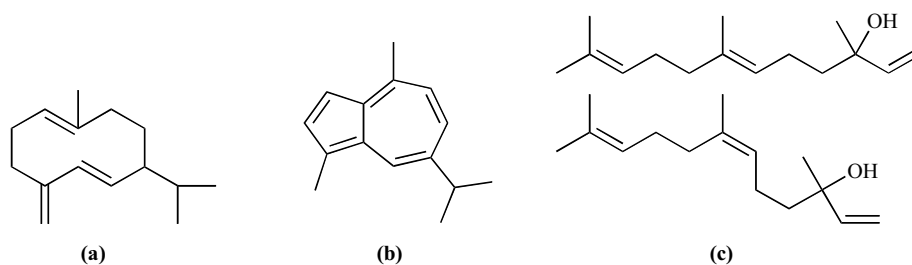


Fig. (1). Chemical structure of germacrene D (a), guaiazulene (b), and *trans*- and *cis*-nerolidol.

and bactericidal effects, post-antibiotic effect, and effect on the membrane permeability.

## MATERIALS AND METHODS

### Bacterial Strains and Culture Conditions

The bacterial strains used were *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923). Mueller-Hinton broth (Merck, Portugal) was used to culture both bacteria, at 37 °C.

### Sesquiterpenoids and Antibiotics

Three samples of sesquiterpenoids were used: guaiazulene- GU (> 98 %, TCI Europe, Zwijndrecht, Belgium), nerolidol- NL (> 98 %, racemic mixture of the *cis* and *trans* isomers, Aldrich, Madrid, Spain) and germacrene D- GE (40 %, natural extract enriched in germacrene D, this extract also contains other sesquiterpenoids,  $\alpha$ -farnesene (*ca.* 10 %) is the second most abundant, R. C. Treatt & CO, LTD, Suffolk, United Kingdom). These samples were tested at 20 mM for every experiment. Ciprofloxacin (CIP), erythromycin (ERY), gentamicin (GEN) and vancomycin (VAN) were obtained from Sigma (Portugal) and paper discs, containing known quantities of the same antibiotics, were obtained from Quilaban (Portugal).

### Antibiotic Disk Assay

Cells from an overnight growth culture (log phase cultures) were suspended in 0.5 mL of phosphate (KH<sub>2</sub>PO<sub>4</sub>; Na<sub>2</sub>HPO<sub>4</sub>) buffer (2 mM, pH 7) and mixed with 5 mL (0.7% w/v) of Mueller-Hinton agar (Oxoid, England), tempered at 45 °C. Sesquiterpenoids dissolved in ethanol (0.5 % v/v) were added to the cell/Mueller-Hinton broth mixture yielding final concentrations of 20 mM. The final cell concentration was approximately 10<sup>8</sup> CFU/mL. The vortexed (Heidolph, model Reax top – 50 % of maximum power input) mixtures were poured over hardened Mueller-Hinton agar (1.5 % w/v) plates using a sterilized cotton swab and allowed to set. Antibiotic discs containing CIP (5  $\mu$ g/disc), ERY (15  $\mu$ g/disc), GEN (10  $\mu$ g/disc) and VAN (30  $\mu$ g/disc) were placed on the surface of plates containing sesquiterpenoids and bacteria. After a 37 °C, 24 h incubation period, zones of growth inhibition were measured, according to the CSLI/NCCLS standard [18]. The experiments were repeated at three different occasions for every scenario tested.

### Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) was determined as the lowest concentration of antibiotic where no growth was detected by the standard CSLI/NCCLS broth

microdilution method [19]. A constant concentration of 20 mM of each sesquiterpenoid, with a varying antibiotic concentration, was used for MIC determination. The experiments were repeated at three different occasions for every condition tested.

### Post-antibiotic Effect - Determination of Lag of Regrowth

Post-antibiotic effect (PAE) experiments were carried out at 37 °C through the determination of the lag of regrowth. Log phase cultures of approximately 10<sup>8</sup> CFU/mL were mixed in glass tubes, in a total phosphate buffer volume of 2 mL, mixed with the different sesquiterpenoids at the desired concentration or with a sesquiterpenoid free control. A 1/5 MIC concentration of CIP was applied to bacteria in order to allow a lag evaluation, not possible when using a MIC. After incubation at 37 °C and 120 rpm, for 30 min, the action of sesquiterpenoids, CIP, and the combination of sesquiterpenoids with antibiotic was terminated by a dilution procedure. Aliquots of 100  $\mu$ L were removed from each culture and diluted in 900  $\mu$ L pre-warmed (37 °C) 2 mM phosphate buffer. After mixing and incubation for 2 - 3 min, 200  $\mu$ L of each suspension was mixed with 1800  $\mu$ L pre-warmed Mueller-Hinton broth and incubated at 37 °C. The resulting regrowth curve was constructed by viable counts at the time of antimicrobial inactivation (dilution to sub-inhibitory concentrations) and at appropriate intervals thereafter (up to 8 h). Aliquots were removed from all cultures, serially diluted in 2 mM phosphate buffer, and plated as 10  $\mu$ L spots in triplicate on Mueller-Hinton agar plates (undiluted suspensions were plated with 100  $\mu$ L). After incubation for 18 - 24 h at 37 °C, CFU were counted. Lag of regrowth duration was calculated by means of the equation lag (time) = T - C, where T is the time required for the CFU count in the test culture to increase by 1log<sub>10</sub>, immediately after the dilution procedure, and C is the time required for the CFU count in an untreated culture to increase by 1log<sub>10</sub> above the count observed after chemical removal. A significant lag of regrowth was defined as a lag  $\geq$  0.5 h [20]. The experiments were repeated at three different occasions for every condition tested.

### Time-kill Curves

Time-kill curves were performed with CIP at 1/5 MIC and/or sesquiterpenoids (20 mM) in Mueller-Hinton broth at 37 °C, in a total volume of 3 mL. An inoculum of 1  $\times$  10<sup>8</sup> CFU/mL of *S. aureus* and *E. coli*, in the log phase of growth, was used. Samples were taken at 0, 30, 60, 90 and 120 min after inoculum addition. The number of bacteria in the samples was determined by making serial dilutions in 2 mM

phosphate buffer. Thirty  $\mu\text{L}$  of each dilution were plated on Mueller-Hinton agar plates and incubated overnight, at 37 °C. Colonies were counted after 18-24 h incubation period. Each curve was determined in triplicate.

### Assessment of Membrane Integrity - Propidium Iodide Uptake

The Live/Dead<sup>®</sup> BacLight<sup>™</sup> kit (Molecular Probes, L-7012, Leiden, Netherlands) assesses membrane integrity by selective stain exclusion [21]. The BacLight<sup>™</sup> kit is composed of two nucleic acid-binding stains: SYTO 9<sup>™</sup> and propidium iodide (PI). SYTO 9<sup>™</sup> penetrates all bacterial membranes and stains the cells green, while PI only penetrates cells with damaged membranes, and the combination of the two stains produces red fluorescing cells. After bacterial exposure to the antimicrobial treatment (single application of CIP, sesquiterpenoids, and a combination of CIP/sesquiterpenoids) for a period of 30 min, the various bacterial suspensions ( $1 \times 10^8$  CFU/mL) were diluted 1:10 and 300  $\mu\text{L}$  of each diluted suspension were filtered through a Nucleopore<sup>®</sup> (Whatman) black polycarbonate membrane (pore size 0.22  $\mu\text{m}$ ) and stained with 250  $\mu\text{L}$  diluted (1:250) SYTO 9 and 250  $\mu\text{L}$  diluted (1:250) PI for 15 min in the dark at  $37 \pm 1$  °C, according to the manufactures protocol. To observe the stained bacteria, a Zeiss (AXIOSKOP) microscope fitted with fluorescence illumination was used with a 100 $\times$  oil immersion fluorescence objective. The optical filter combination for optimal viewing of stained preparations consisted of a 480 to 500 nm excitation filter in combination with a 485 nm emission filter. Several microphotographs of the stained bacterial samples were obtained using a microscope camera (AxioCam HRC, Carl Zeiss), and a program path (AxioVision, Carl Zeiss Vision) involving image acquisition and processing. A program path (Sigma Scan Pro 5), for object measurement and data output, was used to obtain the total cells number (both stains) and the number of PI stained cells. The total cells number and the number of PI stained cells, on each polycarbonate membrane, was estimated from counts of a minimum of 20 fields of view. The range of cell counts for each field was between 50-200 cells/field. The experiments were repeated at three different occasions for every condition tested.

### Statistical Analysis

The data were analysed using the statistical program

SPSS version 14.0 (Statistical Package for the Social Sciences). The mean and standard deviation within samples were calculated for all cases. Paired *t*-test analyses were performed for data assuming a normal distribution. The other data were statistically analyzed by the nonparametric Wilcoxon test. Statistical calculations were based on confidence level equal or higher than 95% ( $P < 0.05$  was considered statistically significant).

## RESULTS

### Antibiotic Disc Diffusion Assay – Sesquiterpenoids Action

As measured by antibiotic disc diffusion assay (Table 1), an increase in antimicrobial activity, promoted by the combination of antibiotics and sesquiterpenoids, was only found for *S. aureus*. Furthermore, it was only found for particular combinations: CIP/GU; CIP/NL; ERY/GE; ERY/GU; GEN/GU; GEN/NL; VAN/GE; VAN/GU; VAN/NL. A remarked *S. aureus* growth inhibition was promoted by the combination of CIP with NL, and VAN with all three sesquiterpenoids ( $P < 0.05$ ). Experiments with *E. coli* showed no significant growth inhibition enhancement by sesquiterpenoids, when comparing with single exposure to antibiotics ( $P > 0.05$ ).

### Minimum Inhibitory Concentration

A growth inhibitory effect was found with the presence of antibiotics against both bacteria (Table 2). The combination of sesquiterpenoids and antibiotics decreased the MIC of CIP, ERY and GEN against *E. coli*, and decreased the VAN antimicrobial effects. In fact, VAN is known to inhibit cell wall synthesis, being ineffective against Gram-negative bacteria. For *S. aureus*, except for VAN with GE, the combination of antibiotics with the tested sesquiterpenoids decreased the MIC value of those antibiotics.

### PAE after Exposure to Sesquiterpenoids and a sub-MIC of CIP

An important feature involved in antibiotics activity is the post-antibiotic effect (PAE) or lag of bacterial regrowth, following a short bacterial exposure to an antimicrobial agent. Such data may contribute to the choice of the most suitable antimicrobial agent, as an extended lag will induce an antibacterial effect longer than expected from the contact time. CIP was selected for PAE (Table 3), time-kill (Fig. 2)

**Table 1. Antimicrobial Activity Against *E. coli* and *S. aureus* Observed by Disc Diffusion Assay. The Means  $\pm$  SD for at Least Three Replicates are Illustrated. GE - Germacrene D; GU - Guaiazulene; NL - Nerolidol**

	Diameter of Zone of Inhibition (mm)							
	<i>E. coli</i>				<i>S. aureus</i>			
	Control	GE	GU	NL	Control	GE	GU	NL
Ciprofloxacin (CIP)	36.0 $\pm$ 0.0	29.0 $\pm$ 1.4	31.5 $\pm$ 1.0	31.5 $\pm$ 1.0	24.0 $\pm$ 0.0	22.5 $\pm$ 2.1	33.5 $\pm$ 1.0	45.0 $\pm$ 4.2
Erythromycin (ERY)	11.0 $\pm$ 1.4	9.50 $\pm$ 1.0	8.00 $\pm$ 0.0	12.0 $\pm$ 0.0	11.0 $\pm$ 0.0	14.5 $\pm$ 1.0	15.0 $\pm$ 4.2	11.5 $\pm$ 1.0
Gentamicin (GEN)	22.5 $\pm$ 3.5	18.5 $\pm$ 1.0	19.0 $\pm$ 0.0	20.0 $\pm$ 0.0	21.5 $\pm$ 1.0	18.5 $\pm$ 1.0	27.0 $\pm$ 2.8	28.0 $\pm$ 1.4
Vancomycin (VAN)	1.00 $\pm$ 0.5	2.50 $\pm$ 1.0	3.00 $\pm$ 0.0	1.50 $\pm$ 1.0	5.00 $\pm$ 1.0	18.5 $\pm$ 1.0	11.0 $\pm$ 0.0	9.50 $\pm$ 3.5

**Table 2.** Susceptibility of *E. coli* and *S. aureus* to Antibiotics by the Microdilution Method. GE - Germacrene D; GU - Guai-azulene; NL - Nerolidol

	MIC ( $\mu\text{g/mL}$ )							
	<i>E. coli</i>				<i>S. aureus</i>			
	Control	GE	GU	NL	Control	GE	GU	NL
Ciprofloxacin (CIP)	0.01	0.007<MIC<0.01	0.007<MIC<0.01	0.007<MIC<0.01	0.3	0.22<MIC<0.3	0.15	0.15
Erythromycin (ERY)	60	3<MIC<4.5	30<MIC<45	30	0.24	0.12	0.12	0.12
Gentamicin (GEN)	6	0.3<MIC<0.5	0.3<MIC<0.5	0.4	0.45	0.22<MIC<0.3	0.2	0.2
Vancomycin (VAN)	280	> 280	> 28	> 280	0.35	> 0.35	0.26<MIC<0.3 5	0.175

and PI (Fig. 3) studies due to its effects on cellular susceptibility observed by the MIC values (Table 2), in the absence and in combination with sesquiterpenoids. In all situations, a decrease in the MIC value was registered when both bacteria were exposed to both CIP and sesquiterpenoids.

Single application of GE and NL increased the *E. coli* lag of regrowth, *i.e.*, the time to repair sub-lethal injuries, while GU had no effect (Table 3). Concerning *S. aureus*, GE and GU did not promote a lag of regrowth, nevertheless, the application of 20 mM NL originated one higher than 7 h. In fact, this lag (> 7 h) was actually significantly higher than the one promoted by CIP (0.15 h) alone. The combined treatment increased the lag of regrowth of both bacteria, in all situations, except for *S. aureus* when exposed to CIP and GU. Such exposure had a reverse effect, inducing the growth ability of *S. aureus* (PAE = -1.3 h).

#### Sub-MIC CIP/Sesquiterpenoids Post-exposure Bacterial Counts and Membrane Permeability

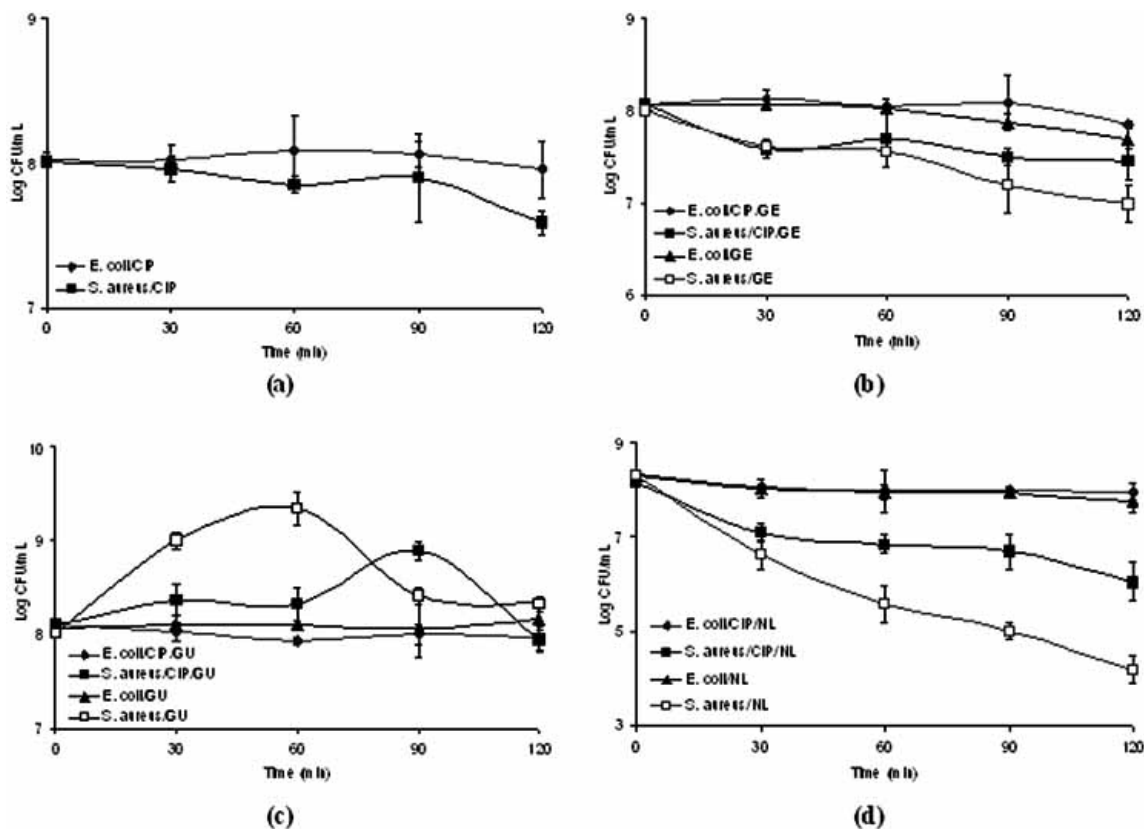
The combinatory effects of sub-inhibitory CIP (1/5 MIC)

and sesquiterpenoids concentrations were also assessed by means of time-kill assays (Fig. 2). Control tests were conducted by assessment of bacterial culturability along time, in chemicals absence (results not shown), showing that the number of CFU remained unchanged during the 2 h ( $P > 0.05$ ). Exposure to CIP decreased, along time, the number of cultivable *S. aureus* corresponding to a 0.5 log CFU/mL ( $P < 0.05$ ). The CIP antimicrobial effect was not detected for *E. coli* ( $P > 0.05$ ). GE decreased the number of CFU, a phenomenon more significant for *S. aureus* ( $P < 0.05$ ) than for *E. coli* ( $P > 0.05$ ). However, in combination with CIP such effect was attenuated for both bacteria (Fig. 2b).

Fig. (2c) shows that 20 mM of GU was not efficient in decreasing total counts of both bacteria ( $P > 0.05$ ). For *S. aureus*, the presence of GU even increased the cellular growth potential, as the number of cells increased along time (higher than 1 log CFU), in comparison to chemical-free experiments. This effect was more pronounced at the 60 (*S. aureus* exposed to GU) and 90 min (*S. aureus* exposed to CIP and GU) sampling times.

**Table 3.** Duration of lag of regrowth of *E. coli* and *S. aureus* due to exposure to the single and combination of ciprofloxacin (CIP) and sesquiterpenoids (GE - Germacrene D; GU - Guai-azulene; NL - Nerolidol)

	Duration of Lag of Regrowth (h)	
	<i>E. coli</i>	<i>S. aureus</i>
CIP	1.75	0.15
GE	0.33	-0.2
GU	0	-0.6
NL	0.5	> 7
CIP/GE	2.7	1.9
CIP/GU	2.0	-1.3
CIP/NL	2.5	> 7



**Fig. (2).** Antimicrobial effect of 1/5 of the MIC value of ciprofloxacin (a) with 20 mM of germacrene D (b), guaiazulene (c) and nerolidol (d) against *E. coli* and *S. aureus* at 37 °C in neutral phosphate buffer. The means  $\pm$  SD for at least three replicates are illustrated.

NL moderately decreased the culturability of *E. coli*. This effect was also observed when in combination with CIP ( $P > 0.05$ ). NL decreased significantly the culturability of *S. aureus* (Fig. 2d). However, in combination with CIP, NL antimicrobial effect on *S. aureus* was reduced by 2 log CFU ( $P < 0.05$ ).

Due to the sesquiterpenoids inherent lipophilicity properties, they show an affinity to interact with biological membranes, where their accumulation may cause substantial impacts at structural and functional levels [11]. In order to clarify this issue, sesquiterpenoids and/or CIP effect was ascertained through PI uptake determination (Fig. 3). The PI inability to penetrate cells with intact outer membranes was confirmed by the low level of uptake observed in bacteria not exposed to either CIP or sesquiterpenoids (results not shown). According to Fig. (3a), CIP was able to damage the outer layers of *E. coli* and *S. aureus*. Two hours of cell exposure to antibiotic, about 70% of the total population was PI stained. This effect was time dependent for both species ( $P < 0.05$ ), being the ratio of *E. coli* stained cells higher for lower exposure times, in comparison to *S. aureus*. In fact, the proportion of *E. coli* and *S. aureus* stained cells was not equivalent ( $P < 0.05$ ), except for prolonged exposure times (2 h).

GE seems to act on the outer layers of *S. aureus* and *E. coli*, as proposed by Fig. (3b). Its interaction with *S. aureus* was prompt, as 15 % of the bacterial population was stained with PI, immediately after contact with the chemical. The number of PI stained cells was invariable for the several sampling times ( $P > 0.05$ ). Concerning *E. coli*, GE had a

more marked effect on its cellular outer layers than on *S. aureus*, this effect being strongly time dependent ( $P < 0.05$ ). For an exposure time of 2 h, the number of PI stained cells was more than 75 % of the total population. The combination of GE with antibiotic increased the number of *S. aureus* PI stained cells, in comparison with CIP absence ( $P < 0.05$ ). Once more, the number of PI stained cells remained constant ( $P > 0.05$ ). An *E. coli* conflicting antimicrobial effect was found by applying a CIP/GE combination ( $P < 0.05$ ), in comparison to antibiotic absence.

Fig (3c) shows that GU at 20 mM did not promote *S. aureus* outer layers disturbance, for any sampling time, promoting only a modest damage on the *E. coli* population, for sampling times of 60, 90 and 120 min, being the proportion of *E. coli* stained cells always smaller than 20 %. The CIP/GU action against *E. coli* resulted in comparable results with CIP absence ( $P > 0.05$ ). On the contrary, a CIP/GU combination promoted an extreme effect against *S. aureus*, for sampling times over 60 min, as the entire population presented outer layer damages. For sampling times of 0 and 30 min no cells were PI stained.

The results of NL applied independently, or in combination with CIP, (Fig. 3d) show how it strongly interacts with *S. aureus* outer layers as, at 30 min of sampling time, all the population was PI stained. The presence of CIP decreased the number of PI stained cells for all sampling times, a statistically significant event ( $P < 0.05$ ). NL also damaged *E. coli* outer layers, a phenomenon not time-dependent ( $P > 0.05$ ). The presence of CIP increased the percentage of *E. coli*

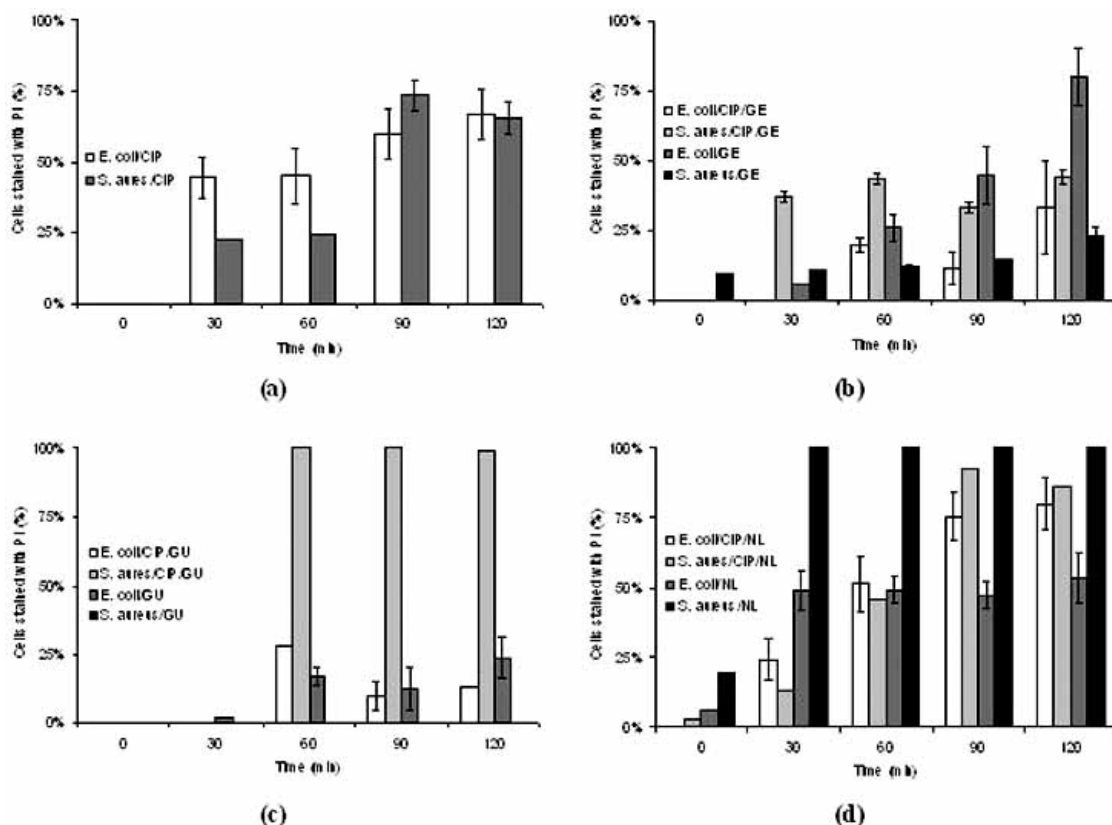


Fig. (3). Permeability of ciprofloxacin (1/5 MIC) treated *E. coli* and *S. aureus* (a) to propidium iodide (PI) when in combination with germacrene D (GE) (b), guaiazulene (GU) (c) and nerolidol (NL) (d). The means  $\pm$  SD for at least three replicates are illustrated.

stained cells which, furthermore, increased with time ( $P < 0.05$ ).

The decrease on the total bacterial counts number was not directly correlated with the PI uptake results (Fig. 4), being found for all cases a correlation factor smaller than 0.85. The highest correlation obtained was for NL ( $R^2 = 0.839$ ), when applied to *S. aureus*. Poor correlation factors (below 0.5) were found for GU, when applied to both bacteria, and for the combination of CIP/GU against *S. aureus* (Fig. 4). GU seems to prevent CIP from being uptaken by cells.

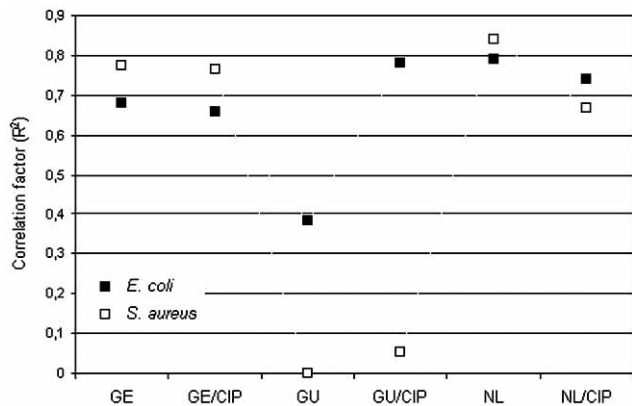


Fig. (4). Correlation factor between loss of culturability and cellular permeability to PI for *E. coli* and *S. aureus*.

### DISCUSSION

In a chemotherapeutic context, the aim of any antibacterial treatment is to maximize the positive effects (such as microbial inactivation, killing) during the period in which active levels of chemical are present, and to minimize the negative effects (such as microbial regrowth, induction of resistant phenotypes) when in presence of sub-inhibitory chemical concentrations [20]. Due to the increased bacterial resistance to conventional antibiotics, new strategies need to be developed. In this context, plants secondary metabolites demonstrate potential to act as antimicrobials, or synergists for other compounds [11,14]. It is known that terpenoids by themselves may exert some antimicrobial activity. Previous studies [11,15,16,22,23] already described the antimicrobial potential of these plant secondary metabolites.

In this study, the antimicrobial efficacy of four antibiotics representing drug families of major clinical importance, aminoglycoside (GEN), fluoroquinolone (CIP), glycopeptides (VAN) and macrolide (ERY) were tested, alone or in combination with three samples of sesquiterpenoids (Fig. 1), against *E. coli* and *S. aureus*. The GU is composed by two unsaturated ring structures which confer rigidity and hydrophobicity to its molecular structure; and NL is composed by a linear structure containing one hydroxyl group that confers molecular flexibility and polarity to one part of the backbone chain. The GE molecular structure is composed by a 10 carbon ring containing two double bonds that confers hydrophobicity and semi-rigidity to the molecule. It is important to

point out that the GE enriched extract also contains other sesquiterpenoids, namely the  $\alpha$ -farnesene that is composed by a linear structure containing one hydroxyl group that confers molecular flexibility and polarity to one part of the backbone chain (Fig. 1). The effects observed with this natural extract may not be explained only by the presence of GE. This study reinforces the positive effects of the combination of some natural derived compounds with standard antimicrobial agents to improve the efficacy of antibacterial treatments.

Antimicrobial activity testing by disc diffusion assay, revealed the increase of activity for the several antibiotics applied to inhibit *S. aureus* growth, in the presence of GU and NL (except for ERY), and also for GE when in the presence of ERY and VAN. *E. coli* was insusceptible to the presence of sesquiterpenoids, as assessed by disc diffusion assay. This result is not corroborated by MIC assays, as the presence of sesquiterpenoids always decreased the MIC value of CIP, ERY and GEN, when applied to *E. coli*. This reinforces the advantageous antimicrobial effect of sesquiterpenoids combination with antibiotics, an effect related with the increase of antibiotic bacterial sensitivity. A similar effect was also described by Brehm-Stechter and Johnson [11], for NL. The combination of sesquiterpenoids with VAN revealed an undesirable antimicrobial effect against *E. coli*. Such a phenomenon is probably related with chemical interactions, which lead to an increase in the *E. coli* growth potential. In fact, VAN is known to inhibit the cell wall synthesis of Gram-positive bacteria, being inactive against Gram-negative [6]. *E. coli* was, with the exception of CIP (MIC for *S. aureus* > MIC *E. coli*), generally less susceptible to the antibiotics comparatively to *S. aureus*, as its outer membrane constitutes a stronger barrier to antibiotic entry [3]. In Gram-negative bacteria, an outer membrane and a set of multi-drug resistance pumps are quite effective barriers for antimicrobial compounds [14,24]. Gram-positive bacteria possess a permeable cell wall that usually does not restrict the penetration of antimicrobials [25]. Furthermore, the processes involved in the antimicrobial action comprise transportation of the antimicrobial agent to the surface of the cell, adsorption, diffusion, penetration and interaction at the target site. These processes are not instantaneous, and the time they take to fully occur, and the correspondent killing time, may differ within antimicrobial agents. The differences also depend on its mode of action, as well as on the chemical constitution and the antimicrobials chemical-physical properties [26].

PAE studies constitute important data providers for efficient chemotherapeutic strategies implementation. Such studies showed that GE extract and NL increased moderately the *E. coli* lag of regrowth, while only NL significantly increased the *S. aureus* lag. PAE values are important as pharmacodynamic predictors [20], allowing the assessment of an antimicrobial agent impact on a target microorganism. Appropriate antibiotic dose is the key to infection-causing bacteria eradication, and an important factor in the emergence and proliferation of antibiotic-resistant strains. A combined treatment CIP/sesquiterpenoid, lead to a prolonged lag of regrowth, for both bacteria and all situations, except in the presence of GU for *S. aureus*. In this particular situation, the potential of regrowth increased significantly (1.3 h).

Time-kill assays demonstrate a significant antibacterial effect of GE and NL against *S. aureus*, together with a modest effect against *E. coli*. Increased uptake of the nucleic acid stain PI, to which cell membrane is normally impermeable, was observed, revealing cell permeabilization. The PI results demonstrated marked interactions with bacterial outer layers, by a single application of CIP (both bacteria), GE (*E. coli*), GU (*S. aureus*) and NL (both bacteria). However, Fig. (4) shows that bacterial viability (Fig. 2) was not only associated with cellular permeabilization (Fig. 3). This result leads to speculate that other cellular targets, critical for cell survival, can be behind the mechanism of action of the distinct sesquiterpenoids. On a previous study [11], NL was found to cause the rapid and non-specific uptake of exogenous compounds, such as antibiotics, by both Gram-negative and -positive bacteria. CIP exerts its antimicrobial action by interfering with DNA synthesis. Its mode of action depends upon the blockage of bacterial DNA replication, by binding itself to a DNA gyrase, thereby causing double-stranded breaks in the bacterial chromosome [27]. Fluoroquinolones uptake occurs by simple diffusion [28], and a sesquiterpenoids damaged bacterial outer layer allows an increased antibiotic antimicrobial action. However, the sesquiterpenoids molecular properties can account for only part of the antimicrobial properties, probably acting as a multi-target agent, enabling the prediction and observation of specific sesquiterpenoid/microorganisms interactions.

The overall results show that GE extract and NL are promising chemicals for antimicrobial treatments, enhancing antimicrobial activity and pharmacodynamic parameters of conventional antibiotics. As sesquiterpenoids are lipophilic chemicals, sensitizing bacterial outer layers [11,16], they can also act by avoiding active efflux mechanism of resistance. Furthermore, these substances seem to be adjuvant agents for fighting multi-drug resistant microorganisms.

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#### REFERENCES

- [1] Russell, A.D. *Lancet Infect. Dis.*, **2003**, *3*, 794-803.
- [2] Gilbert, P.; McBain, A.J.; Bloomfield, S.F. *J. Antimicrob. Chemother.*, **2002**, *50*, 137-139.
- [3] McDonnell, G.; Russell, A.D. *Clin. Microbiol. Rev.*, **1999**, *12*, 147-179.
- [4] Simões, M.; Simões, L.C.; Cleto, S.; Machado, I.; Pereira, M.O.; Vieira, M.J. *J. Basic Microbiol.*, **2007**, *47*, 230-242.
- [5] Snardy, E.L.; Kelly, L.A.; Davison, H.C.; Teale, C.J.; Wooldridge, M. *J. Antimicrob. Chemother.*, **2004**, *53*, 906-917.
- [6] Tenover, F.C. *Am. J. Infect. Control*, **2006**, *34*, S3-S10.
- [7] Gilbert, P.; Allison, D.G.; McBain, A.J. *J. Appl. Microbiol.*, **2002**, *92*, 98S-110S.
- [8] Gilbert, P.; McBain A.J. *Clin. Microbiol. Rev.*, **2003**, *16*, 189-208.
- [9] Levin, B.R. *Science*, **2004**, *305*, 1578-1579.
- [10] White, D.G.; McDermott P.F. *Curr. Opin. Microbiol.*, **2001**, *4*, 313-317.
- [11] Brehm-Stechter, B.F.; Johnson E.A. *Antimicrob. Agents Chemother.*, **2003**, *47*, 3357-3360.
- [12] Cox, S.D.; Mann, C.M.; Bell, H.C.; Gustafson, J.E.; Warmington, J.R.; Wyllie S.G. *J. Appl. Microbiol.*, **2000**, *88*, 170-175.

- [13] Røssland, E.; Langsrud, T.; Granum, E.P.; Sørhaug T. *Int. J. Food Microbiol.*, **2005**, *98*, 193-200.
- [14] Cowan M.M. *Clin. Microbiol. Rev.*, **1999**, *12*, 564-582.
- [15] Griffin, S.G.; Wyllie, S.G.; Markham, J.L.; Leach D.N. *Flavour Frag. J.*, **1999**, *14*, 322-332.
- [16] Trombetta, D.; Castelli, F.; Sarpietro, M.G.; Venuti, V.; Cristani, M.; Daniele, C.; Saija, A.; Mazzanti, G.; Bisignano G. *Antimicrob. Agents Chemother.*, **2005**, *49*, 2472-2478.
- [17] Vatopoulos, A.C.; Kalapothaki, V.; Legakis N.J. *Emerg. Infect. Dis.*, **1999**, *5*, 471-476.
- [18] Clinical and Laboratory Standards Institute/NCCLS. *Performance Standards for Antimicrobial Disc Susceptibility Tests*; Approved Standard-Eighth Edition. CLSI/NCCLS document M2-A8. West Valley Road, Pennsylvania, USA, **2006**.
- [19] Clinical and Laboratory Standards Institute/NCCLS. *Performance Standards for Antimicrobial Susceptibility Testing*; Fifteenth Informational Supplement. CLSI/NCCLS document M100-S15. West Valley Road, Pennsylvania, USA, **2006**.
- [20] Fursted, K.; Hjort, A.; Knudsen L. *J. Antimicrob. Chemother.*, **1997**, *40*, 221-226.
- [21] Simões, M.; Pereira, M.O.; Vieira M.J. *Biofouling*, **2005**, *21*, 9-17.
- [22] Carson, C.F.; Mee, B.J.; Riley T.V. *Antimicrob. Agents Chemother.*, **2002**, *46*, 1914-1920.
- [23] Luna-Herrera, J.; Costa, M.C.; González, H.G.; Rodrigues, A.I.; Catilho P.C. *J. Antimicrob. Chemother.*, **2007**, *59*, 548-552.
- [24] Tegos, G.; Stermitz, F.R.; Lomovskaya, O.; Lewis K. *Antimicrob. Agents Chemother.*, **2002**, *46*, 3133-3141.
- [25] Lambert P.A. *J. Appl. Microbiol.*, **2002**, *92*, 46S-54S.
- [26] Paulus W. *Microbiocides for the Protection of Materials – a Handbook*. Chapman & Hall, **1993**.
- [27] Bazile, S.; Moreau, N.J.; Bouzard, D.; Essiz M. *Antimicrob. Agents Chemother.*, **1992**, *36*, 2622-2627.
- [28] Zeller, V.; Janoir, C.; Kitzis, M.-D.; Gutmann, L.; Moreau N.J. *Antimicrob. Agents Chemother.*, **1997**, *41*, 1973-1978.

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