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Proceeding Paper

Phytochemicals as Adjuvants of Topical Antibiotics to Treat Biofilm Related *Staphylococcus aureus* Wound Infections †

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Abstract: Diabetic foot ulcers (DFUs) are a complex secondary complication of diabetes *mellitus*. Infection progression occurs in more than half of the DFUs with *Staphylococcus aureus* being the most prevalent microorganism. We propose the use of topical antibiotics (mupirocin and gentamicin) in combination with natural adjuvants, particularly chalcone and farnesol. After the determination of the minimum inhibitory and bactericidal concentrations (MIC and MBC) against a clinical S. aureus isolate from diabetic foot wound (MJMC109), it was evaluated their potentiation effect on the antibiotics through the disc diffusion method. The combined effect of both phytochemicals and antibiotics were evaluated on the potential to eradicate a pre-formed *S. aureus* biofilm. The results showed a significant culturability reduction with both combinations. In conclusion, this study reveals the great potential for the topical application of different phytochemicals as adjuvants of mupirocin to combat multidrug resistant wound infections.

Keywords: antibiotic adjuvants; combination; diabetic foot ulcers; phytochemicals; *Staphylococcus aureus*; topical antibiotics

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1. Introduction

Diabetes *mellitus* is a global fast-growing severe endocrine disease characterized by an increase in blood glucose levels. Among diabetes complications, foot ulcers are at higher risk to occur. As many as 30% of people with diabetes will develop a diabetic foot ulcer at least once in their lifetime [1]. Infections may occur in half of DFUs, increasing the risk of lower limb amputations (LLAs) [2]. When it occur, LLAs are in 80% of the cases preceded by biofilm formation at the wound site [3]. *Staphylococcus aureus* are between the most prevalent pathogenic bacteria found at DFUs, specially with those related with biofilm formation [4,5]. Biofilms have an important role in the treatment failure of DFUs and so, new approaches to treat these infections, particularly those caused by multidrug resistant (MDR) bacteria are required.

Plant secondary metabolites (phytochemicals) are an underexploited diverse group of compounds responsible for promising therapeutic effects. Their richness of structural

diversity and different modes of action brings hope to face the resistance phenomena. Usually, phytochemicals possess weaker antimicrobial effect when compared to antibiotics, but this characteristic may be a remarkable one if synergistic combinations are found [6]. In the present study, chalcone and farnesol were selected to be tested as adjuvants of topical antibiotics mupirocin and gentamicin in the treatment of *S. aureus* biofilm related DFUs infection. The minimum inhibitory and bactericidal concentration (MIC and MBC) of both phytochemicals and antibiotics were assessed, as well as their combined effect with antibiotics against a clinical *S. aureus* isolate from diabetic foot ulcer. The combined effect of both phytochemicals/antibiotics was evaluated in pre-formed biofilms.

2. Materials and Methods

2.1. Preparation of the Phytochemicals and Antibiotics

Chalcone (Sigma-Aldrich, St. Louis, MO, USA), farnesol (Sigma-Aldrich, St. Louis, MO, USA), mupirocin (AppliChem, GmbH, Darmstadt, Germany) and gentamicin (AppliChem, GmbH, Darmstadt, Germany) were purchased as pure compounds. Stock solutions of phytochemicals and mupirocin were prepared in dimethyl sulfoxide (DMSO, 100%), while gentamicin was prepared in distilled water. For phytochemical-based molecules, serial dilutions from 1000 mg/L to 6.25 mg/L were prepared, when needed. With respect to antibiotics, the concentrations range varies from 1024 mg/L to 0.0625 mg/L. The percentage of DMSO never exceeded 10% (*v*/*v*) of the final volume. For the disc diffusion method, the mass of antibiotics on the disc used was selected according to Clinical and Laboratory Standards Institute (CLSI) guidelines (mupirocin: 200 μg/disc and gentamicin: 10 μg/disc). All tests were performed at least three times with three replicates.

2.2. Bacterial Strains

The *S. aureus* strain selected for this study is a methicillin-susceptible clinical isolate from foot wounds (MJMC109). This clinical isolate belongs to the MJMC collection and was isolated from diabetic foot ulcer exudates of a patient hospitalized in the Hospital Centre of Trás-os-Montes and Alto Douro (CHTMAD), located in the north of Portugal (Vila Real). The study was granted approval by the Ethics Committee of CHTMAD, according to a protocol established in 2004.

2.3. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The antibacterial effect of chalcone, farnesol, mupirocin and gentamicin against *S. aureus* was evaluated through MIC and MBC determination according to Baptista et al. [7].

2.4. Antibiotic/Phytochemical Dual Combination: Disc Diffusion Method

The study of the dual combination of phytochemicals and antibiotics was performed by a modification of the disc diffusion assay, according to Abreu et al. [8]. In this method, the natural compounds were added to MHB agar (at 0.1 × MIC). The results were expressed as the mean of inhibition zone diameters (IZD, mm).

Classification

The effect of dual combinations of antibiotics and phytochemicals can be classified according to Abreu et al. [8]:

- Potentiation (+++): (IZDa+p–IZDa) ≥ 6 mm;
- Additive (++): 6 mm > (IZDa+p–IZDa) ≥ 4 mm;
- Indifferent (+): 4 mm > (IZDa+p–IZDa) > −6 mm;
- Negative (–): (IZDa+p–IZDa) ≤ −6 mm,

where IZD_a corresponds to antibiotic, and IZD_P represents phytochemicals.

2.5. Effect of Phytochemical and/or Antibiotic on Pre-formed Biofilms

The effect of both phytochemicals and antibiotics on biofilm removal, metabolic activity and culturability was performed according to Baptista et al. [7]. To do so, a 24-h old *S. aureus* biofilm was exposed to a specific phytochemical (at 10× MIC) and antibiotic (at MIC) and also to the combination of both. For this evaluation a 96-well flat clear bottomed PS microtiter plates was filled with 200 μL of cells suspension (~1 × 10^8 CFU/mL) and left incubating at 37 °C for 24 h. After the incubation period, the content of each well was discarded and washed once with sterile NaCl (8.5 g/L). Then, 20 μL of the specific phytochemical and antibiotic was introduced or 10 μL of the each in case of combination. Bacterial suspensions with DMSO and without compounds were used as controls. After 24 h of contact, the microtiter plates were analyzed in terms of biomass quantification by crystal violet staining (CV; Merck, Darmstadt, Germany), metabolic activity by alamar blue (AB; Merck, Darmstadt, Germany) staining and culturability through colony forming units (CFUs), according to Baptista et al. [7]. All tests were performed in triplicate with six replicates. The results were presented in terms of percentage of biofilm mass removal and biofilm metabolic activity reduction according to Equation (1):

$$\%\ \text{BR or \%MAR} = \frac{[OD\ or\ FI]_{control} - [OD\ or\ FI]_{phytochemical/antibiotic}}{[OD\ or\ FI]_{control}} \quad (1)$$

In the following equation %BR is the percentage of biofilm removal, while %MAR is the percentage of biofilm inactivation. The $OD_{control}$ is the OD (λ = 570 nm) for biofilms exposed to DMSO at the respective concentration or fluorescence intensity ($FI_{control}$) in case of alamar blue staining procedure. $OD_{phytochemical/antibiotic}$ is the OD (λ = 570 nm) for biofilms exposed to the specific phytochemical/antibiotic or to the combination of both, while once again $FI_{phytochemical/antibiotic}$ refers to the fluorescence intensity. The number of colony forming units (CFUs) was counted and expressed in logarithmic CFU/mL.

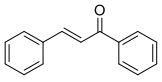
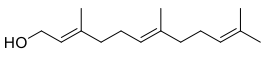
2.6. Statistical Analysis

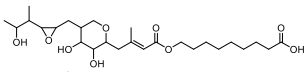
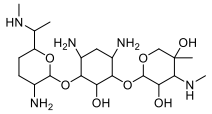
Statistical analysis was performed using GraphPad Prism software version 9.1.1 (GraphPad Software Inc., San Diego, CA, United States). One-way ANOVA and multiple comparisons were used to test the significance based on a confidence level of ≥95% ($p < 0.05$, statistically significant). All experiments were performed in triplicate with at least three replicates for each condition tested.

3. Results and Discussion

In this study, the phytochemicals chalcone and farnesol and the antibiotics mupirocin and gentamicin were first evaluated for their inhibitory (MIC) and bactericidal (MBC) activities against a clinical isolate of methicillin susceptible *S. aureus* (MSSA) from diabetic foot wounds (MJMC109). As shown in Table 1, between the two phytochemicals tested, farnesol was the molecule with the highest antibacterial activity, presenting the lowest MIC and MBC values.

Table 1. Chemical structure, MIC and MBC (mg/L) values for the selected phytochemicals and antibiotics against MJMC109, a *S. aureus* clinical isolate.

	Class	Compound	Chemical Structure	MJMC109	
				MIC (mg/L)	MBC (mg/L)
Phytochemicals	Phenolics	Chalcone		200	>1000
	Sesquiterpenoid constituents of essential oils	Farnesol		25	800

Antibiotics	Carboxylic acid	Mupirocin		0.5	4
	Aminoglycoside	Gentamicin		1	16

The effect of all the selected molecules on the activity of the antibiotics mupirocin and gentamicin was assessed through the disc diffusion method. The results showed in Figure 1 demonstrate that chalcone possesses an additive effect on both antibiotics, while farnesol increases the effect of gentamicin and has an indifferent behaviour when combined with mupirocin.

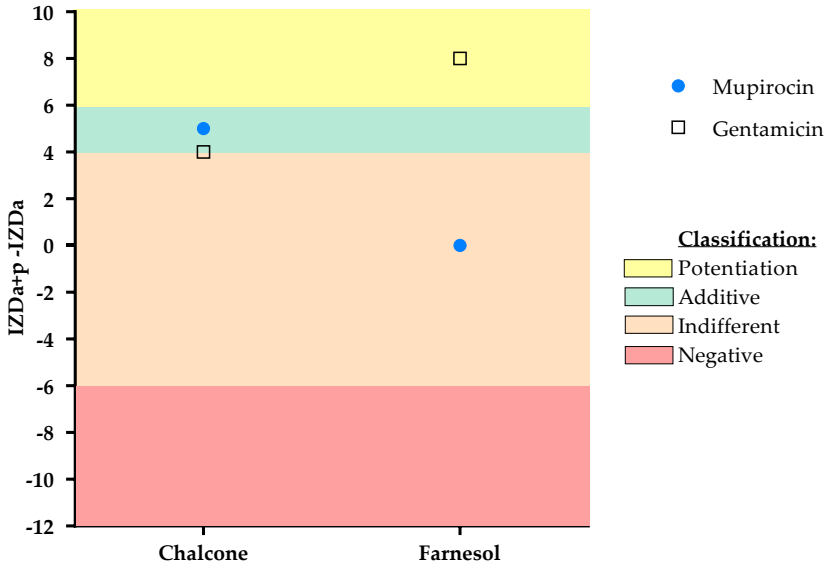
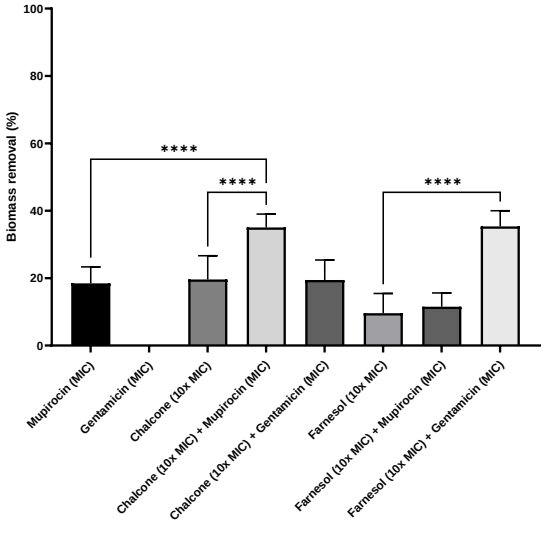


Figure 1. IZD values (mm) and respective classification according to the combined application of the selected phytochemicals and antibiotics against MJMC109 *S. aureus* strain. The effect of dual combinations of antibiotics and phytochemicals was classified as potentiation when IZD_{a+p}–$IZD_a \geq 6$ mm (yellow zone), additive when 6 mm > IZD_{a+p}–$IZD_a \geq 4$ mm (green zone), indifferent when 4 mm > IZD_{a+p}–$IZD_a > -6$ mm (light pink zone) and negative when IZD_{a+p}–$IZD_a \leq -6$ mm (dark pink zone), where IZD corresponds to the inhibition zone diameter, a = antibiotic and p = phytochemical.

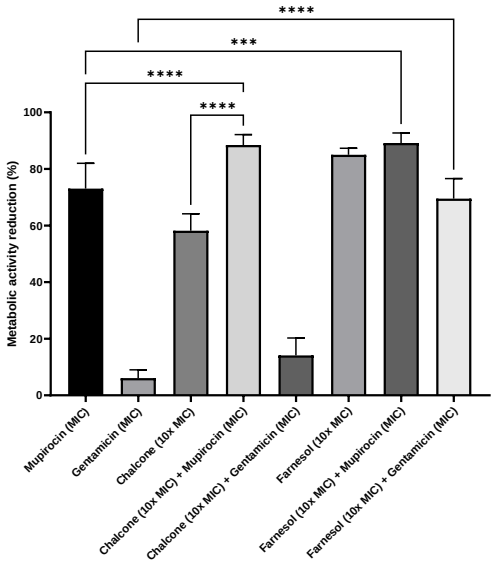
The effect of the phytochemicals, antibiotics and combinations were evaluated in terms of biomass removal, metabolic activity reduction and culturability (Figure 2). The results demonstrated that, practically in all aspects evaluated, the phytochemicals tested presented a better anti-biofilm activity compared to the antibiotics alone. Chalcone combined with mupirocin was able to increase significantly the biomass removal and inactivation of the *S. aureus* biofilm compared to the phytochemical or antibiotic alone. The combination of farnesol with both mupirocin and gentamicin was able to reduce in 4-log the biofilm cell culturability. The effect is statistically different from the result obtained for the phytochemical alone, which *per se* is already substantial ($p < 0.05$). This result is in accordance with the literature, since this sesquiterpenoid was found to control and reduce the formation of single and multiple biofilms of *Candida albicans* (ATCC 10231) and *Streptococcus mutants* (ATCC 25175) [9]. In that study farnesol was able to significantly reduce the viable cells and the dense structure of the biofilms, by reducing the protein content. Also, some studies indicate the synergism of farnesol with different classes of antibiotics. For instance, Castelo-Branco et al. [10] showed a synergistic effect with amoxicillin, ceftazidime, doxycicline, sulfamethoxazole/trimethoprim against *Burkholderia pseudomallei*'s biofilms, while Pammi et al. [11] indicated a synergisitic effect between this

phytochemical and vancomycin and nafcillin against biofilms of *S. epidermidis* (ATCC 55 133 and 1457).

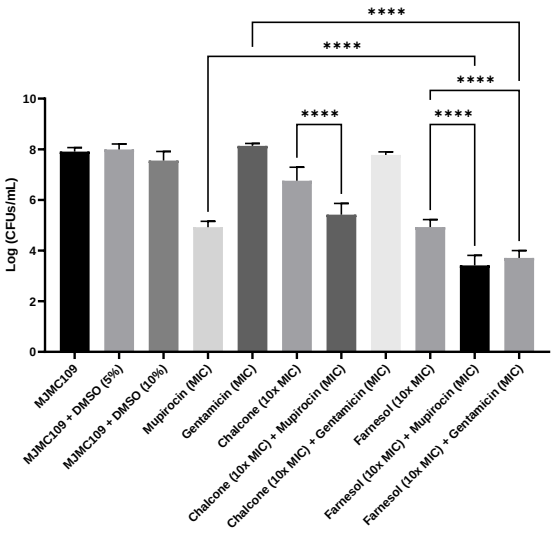
Apart from the promise demonstrated in the use phytochemical-based molecules as antibiotic adjuvants, there is little knowledge on the molecular basis of these synergistic interactions to better understand their combined mechanism of action. The same happens to the possible cytotoxic effect of the molecules alone or in combination. Despite this, information about the phytochemicals possible mechanism of action, especially their chemical class, has already been reported in the literature [12–14]. Even though, their mecanism of action is somehow reported much remains to be understood. Actually, the mechanism of action of each component of the mixture is not necessarily the same of each component alone, nor the sum of each one. The same happens with the possible cytotoxic effect of the molecules alone or in combination. Although some concerns might appear around the topical application of this phytochemical/antibiotics formulations and resistance appearance, the inclusion of phytochemicals as resistance-modifying agents may overcome this challenge and bring back to life antibiotics that are no longer in use.



(**a**)



(**b**)



(**c**)

Figure 2. Effect of phytochemicals and/or antibiotics on a 24-h old *S. aureus* biofilm in terms of biomass removal (**a**), metabolic activity reduction (**b**) and culturability (**c**). Bars with * are statistically different from each other (*** $p < 0.001$; **** $p < 0.0001$).

4. Conclusions

Antibiotic resistance is a serious public health threat that calls for a concerted global action. New treatment strategies to combat these life-threatening infections, especially those caused by *S. aureus*, are urgently required. Our findings demonstrate that phytochemicals, a clearly underexploited resource, possess promising characteristics as antibiotic adjuvants and especially as antibiotic resistance-modifying agents. We also emphasize the great potential of a more routine usage of topical antimicrobials in the treatment of DFU's infections.

Author Contributions: All authors have read and agreed to the published version of the manuscript. D.O., A.B. and M.S. designed the study and the manuscript; D.O. was responsible for data acquisition, analysis, interpretation of data and manuscript drafting; A.B., M.S. and F.B. made critical revisions to the article. M.J.S. provided and helped in the selection of the clinical isolates used in the study. All authors have read and agreed to the published version of the manuscript.

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