ECA 2021

sciforum

Conference Proceedings Paper

Antimicrobial activity of phytochemical-antibiotic combinations against pathogenic bacteria †

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† Presented at the The 1st International Electronic Conference on Antibiotics—The Equal Power of Antibiotics And Antimicrobial Resistance, 08-17 May 2021.

Abstract: The treatment of biofilms has been increasingly troubled due to the rising of antibiotic resistance in pathogenic bacteria, making the use of antibiotics alone ineffective for treating biofilm-associated infections. Natural products, particularly phytochemicals, have been thoroughly studied as a means to circumvent the emergence of resistant pathogenic bacteria due to their multiple modes of action. Thus, the present study investigated the antimicrobial potential of selected phytochemicals alone and in combination with standard antibiotics (gentamicin, mupirocin and fusidic acid) against *Staphylococcus aureus*, including a multidrug-resistant strain, and *Escherichia coli*. Among the selected phytochemicals, citronellol presented the highest antimicrobial activity against *S. aureus* and cis-6-nonen-1-ol displayed the highest antimicrobial activity against the multidrug-resistant *S. aureus* and *E. coli*. In addition, bacterial cells were found to be eradicated at lower doses of selected phytochemicals and antibiotics when combined. This study highlights the promising phytochemical-antibiotic combinatorial approach for dealing with biofilm-associated infections.

Keywords: biofilms; antimicrobial; antibiotics; phytochemicals.

1. Introduction

Biofilm development is a crucial virulence factor in the pathogenesis of several medically important bacteria, including *Staphylococcus aureus* and *Escherichia coli* [1,2]. Indeed, biofilms account for up to 80% of all microbial infections in humans, underpinning major health and economic burdens [3]. Moreover, increased antibiotic resistance in pathogenic bacteria is a general trait related to biofilms, which makes the use of antibiotics alone ineffective for treating biofilm-associated infections [3]. Thus, novel strategies to target pathogenic bacteria should be designed outside the constricted antibiotics box.

Natural products, particularly phytochemicals (molecules from the secondary metabolism of plants), have proven to be outstanding broad-spectrum antimicrobial compounds, in parallel with other unique characteristics such as anti-inflammatory, antioxidant, anticancer and regenerative activities, making them perfect candidates for these much-needed novel antimicrobials [4–6]. Furthermore, phytochemicals commonly act through different mechanisms of action than conventional antibiotics, which can be of great relevance to prevent the emergence of resistant pathogenic bacteria [4,5]. Such natural products may not necessarily have strong antimicrobial activities themselves but may synergize with classical antibiotics.

Therefore, the combinatorial approach of phytochemicals with the already available antibiotics could be a different paradigm to control biofilm-associated infections, dealing simultaneously with the microbial resistance and toxicity, since lower concentrations of both compounds can be used. Herein, we investigate the *in vitro* antimicrobial potential of selected phytochemicals alone and in combination with standard antibiotics, namely gentamicin, mupirocin and fusidic acid, against *Staphylococcus aureus* (Gram-positive bacteria), including a multidrug-resistant strain, and *Escherichia coli* (Gram-negative bacteria).

2. Materials and Methods

2.1. Bacteria and Culture Conditions

The collection stains *S. aureus* CECT 976 and *E. coli* CECT 434, and a methicillin-resistant *S. aureus* (MRSA) XU212, which was kindly provided by Simon Gibbons (University College London, UCL), were used in all experiments. The bacteria were preserved at −80 °C in Mueller-Hinton (MH) broth containing 30 % (*v*/*v*) glycerol. The bacterial cultures were grown overnight in MH broth at 37 °C under 160 rpm of agitation before all the experiments.

2.2. Phytochemicals and Antibiotics

The antibiotics gentamicin (GEN) and mupirocin (MUP) were purchased from Panreac. The antibiotic fusidic acid (FUS) and the phytochemicals cis-6-nonen-1-ol (CIS), citronellic acid (CA) and 3-7-dimethyl-1-octanol (3,7DOC) were obtained from Sigma-Aldrich. The phytochemical citronellol (CITRO) was obtained from Acros Organics. All the phytochemicals and MUP were dissolved in dimethyl sulfoxide (DMSO). The GEN and FUS were dissolved in sterile distilled water. Stock antibiotic solutions were prepared and dilutions were performed according to the CLSI protocols, with concentrations ranging from 0.0625 to 1024 μg/mL. Each phytochemical was tested at various concentrations in the range of 0.0625-8192 μg/mL.

2.3. Determination of Minimum Bactericidal Concentration (MBC)

The MBC of each antibiotic and phytochemical was determined by the broth microdilution method. After overnight incubation, bacterial cultures were adjusted to a cell density of approximately 1×10^6 cells/mL in MH broth. Then, 180 μL of the adjusted bacterial suspension were added to a sterile 96-well microtiter plates, along with 20 μL of 2-fold dilutions of the compounds to test, and the plates were incubated at 37 °C for 1 h and 160 rpm. Subsequently, the suspensions were subjected to a process of antimicrobial neutralization (15 min contact time) by the following solution: lecithin (3 g/L), tween 80 (30 g/L), sodium thiosulphate (5 g/L), α-histidine (1 g/L), and saponin (30 g/L) in phosphate buffer 0.25 M at 1%. Afterwards, 10 μL of each suspension was dropped on plate count agar (PCA) plates and incubated at 37 °C for 24 h. The plates were then analyzed and the MBC of each compound corresponded to the lowest concentration causing no bacterial growth on solid medium. Three independent experiments were performed for each compound.

2.4. Determination of Fractional Bactericidal Concentration (FBC) Index

The FBC index was established to understand the effect between combinations of phytochemicals and antibiotics under investigation. For that, this was assessed by checkboard broth microdilution method in 96-well microtiter plates via MBC determination. Bacterial suspensions (~1 × 10^6 cells/mL) were added to each well, along with the antibiotics and phytochemicals (in a total volume of 200 μL) in different concentrations, so that each well contains the same amount of the antibiotic, which is being 2-fold diluted along the x axis (rows), and the same amount of the phytochemical being 2-fold diluted on the y axis (columns). The antimicrobial solution did not exceed 10% (*v*/*v*) of the well. The range of the tested concentrations of each compound was from MBC/16 to 2 × MBC. The MBC of each combination was then determined as previously described in Section 2.3.

At least three replicates and two independent assays were performed for each combination. The FBC index for all combinations was determined using the following equation [7]:

FBC index = $FBC_A + FBC_B = (MBC_{A \text{ in the presence of } B}/MBC_{A \text{ alone}}) + (MBC_{B \text{ in the presence of } A}/MBC_{B \text{ alone}})$

where the nature of interaction of the two compounds was described by the value of FBC index and interpreted as follows: ≤ 0.5 synergy; > 0.5 and ≤ 1 additive; > 1 and < 2 indifference; and ≥ 2 antagonism [7].

3. Results and Discussion

This study investigated the antimicrobial activity of selected phytochemicals alone and their combinations with GEN, MUP and FUS against *S. aureus* CECT 976, MRSA XU212 and *E. coli* CECT 434. It is well-known that variations in the structure of phytochemicals can result in differences in their antimicrobial effects [8]. The presence of functional groups, such as hydroxyl (OH), oxygenated and double bond, can affect the antimicrobial activity on multiple levels, having an essential role in the polarity, solubility, hydrogen bonding capacity and pKa of the compounds [8]. Therefore, an attempt to correlate the antimicrobial activity of the compounds tested in this study with their chemical structure was made (Figure 1).

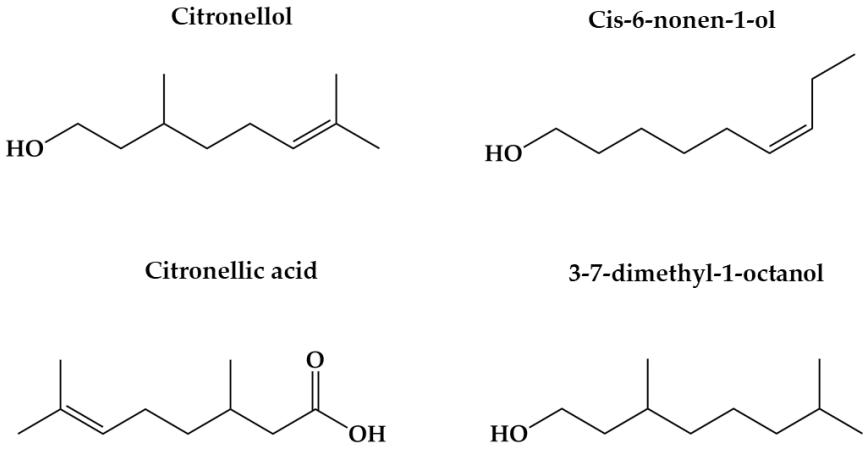

Figure 1. Chemical structure of selected phytochemicals tested in this study. The structures were created using ChemDraw software.

3.1. Bactericidal Activity of Phytochemicals and Antibiotics

According to the results shown in Table 1, GEN was the most effective antibiotic against both *S. aureus* and *E. coli*, with MBC values of 8 μg/mL and 64 μg/mL, respectively. The lower antimicrobial activity of GEN against *E. coli* could be related with the more complex cell wall of Gram-negative bacteria. It is constituted by a thin peptidoglycan layer adjacent to cytoplasmatic membrane, and an outer membrane composed by phospholipids and lipopolysaccharides, which serves as a selective barrier inhibiting the penetration of antimicrobials, such as antibiotics [9]. GEN is an aminoglycoside antibiotic that bind to the 16S rRNA component of the 30S ribosomal subunit, disturbing the mRNA and, thus, leading to the formation of truncated or nonfunctional proteins [10]. Interestingly, MUP was the only antibiotic presenting bactericidal activity against MRSA (MBC of 64 μg/mL), besides its activity against *S. aureus* (MBC of 32 μg/mL). In fact, this antibiotic has been demonstrated to be highly active against staphylococci, including MRSA strains [11]. MUP exerts its antimicrobial action by inhibiting bacterial RNA and protein synthesis through binding to bacterial isoleucyl-tRNA synthetase, ultimately leading to bacterial death [11]. The antibiotic FUS also exhibited bactericidal activity against *S. aureus* (MBC of 128 μg/mL), yet at higher doses compared to GEN and MUP.

Table 1. MBC (μg/mL) of selected phytochemicals and standard antibiotics against pathogenic bacteria.

	***S. aureus* CECT 976**	**MRSA XU212**	***E. coli* CECT 434**

Antibiotics	GEN	8	NA	64
	MUP	32	64	NA
	FUS	128	NA	NA
Phytochemicals	CITRO	512	NA	2048
	CIS	1024	2048	1024
	CA	2048	NA	4096
	3,7DOC	NA	NA	NA

NA: no activity (MBC > 1024 μg/mL for antibiotics; MBC > 8192 μg/mL for phytochemicals).

Among the selected phytochemicals, CITRO presented the highest bactericidal activity against *S. aureus* (MBC of 512 μg/mL) and CIS exhibited the highest bactericidal effect against both MRSA and *E. coli* (MBC ranging from 1024 to 2048 μg/mL). Compared to CA and 3,7DOC, the chemical structures of CITRO and CIS are characterized by the C=C double bond and OH group (Figure 1), which could be responsible for the higher antimicrobial activity of these compounds. The OH groups are thought to interact with the cell membrane of bacteria, causing the destabilization of the cytoplasmic membrane and reducing the pH gradients across the membrane, which eventually leads to the leakage of cellular components and cell death [8]. Furthermore, it has been demonstrated that the number of double bonds are also significant in relation to antimicrobial effectiveness, in which the increasing number of double bonds may enhance the antimicrobial effect [12].
These findings support the idea that the compounds GEN, MUP, CITRO and CIS presented considerably more effective antimicrobial activity against pathogenic bacteria and deserve further investigation regarding their potential for synergistic effects when combined.

3.2. Combinatorial Activity between Phytochemicals and Antibiotics

Combinations of antimicrobial agents can provide many benefits, such as increased antimicrobial activity and reduced toxicity effects of the combined compounds. Accordingly, in order to check the combinatorial effects between selected phytochemicals and standard antibiotics against pathogenic bacteria, the FBC index was adopted and the data are shown in Table 2.

Table 2. FIC index of the different combinations between selected phytochemicals and standard antibiotics against pathogenic bacteria.

		***S. aureus* CECT 976**	**MRSA XU212**	***E. coli* CECT 976**
CITRO	GEN	1.125 (I)	NA	0.188 (S)
	MUP	0.562 (A)	*	–
CIS	GEN	0.75 (A)	–	0.25 (S)
	MUP	0.75 (A)	0.562 (A)	–

NA: no activity; S: synergy; A: additive; I: indifference; − no significant bactericidal effect when combining the compounds (compared with the compound alone at MBC); * the concentration of MUP was reduced from 64 μg/mL (MUP alone) to 32 μg/mL in the presence of CITRO (NA alone against MRSA).

The bactericidal potential of GEN against *E. coli* was significantly enhanced by its combination with both CITRO and CIS, with a clearly synergistic activity between the compounds (FBC index of 0.188 for the combination CITRO-GEN and 0.25 for the combination CIS-GEN). It has been proposed that phytochemicals and antibiotics mat act synergistically mostly by multi-target effect in which compounds target different bacterial sites, by pharmacokinetic or physicochemical effects (e.g., enhancement of solubility or bioavailability), or by targeting a specific resistance mechanism of the bacteria [13]. Noteworthy is the fact that when the bacterial cells were treated with these antibiotic-phytochemical combinations, the bactericidal effect was observed at significantly lower concentrations. Indeed, the effective doses of GEN were reduced by 8-fold in *E.coli*. This outcome is particularly interesting since, due to the distinctive structure of Gram-negative bacteria, they are often more resistant than Gram-positive bacteria [9]. In fact, the majority of the World Health Organization (WHO) list of priority pathogens is composed of Gram-negative bacteria, which have

been associated with substantial morbidity and mortality worldwide [14]. In addition, the combination of CIS with GEN caused an additive effect against *S. aureus*, whereas the interaction between CITRO and GEN was indifferent against this bacterial strain.

Regarding the effects of combining the phytochemicals CITRO and CIS with MUP, FBC index values revealed that these combinations act additively against both *S. aureus* and MRSA. Indeed, the most interesting effect was observed for the combination CITRO-MUP against MRSA, in which the concentration of MUP needed to have bactericidal activity against this bacterial pathogen was reduced from 64 μg/mL (MUP alone) to 32 μg/mL in the presence of CITRO. Herein, it is important to highlight that the CITRO alone did not demonstrate bactericidal activity against MRSA, showing the potential of these natural products as drug modulating or modifying agents when combined with antibiotics. As a result, the use of phytochemicals in association with antibiotics could be an effective tool for the management of multidrug-resistant bacteria.

4. Conclusions

This work showed the antimicrobial efficacy of selected phytochemicals to be used as an alternative to and/or in combination with standard antibiotics against pathogenic bacteria. Besides the possibility of reducing the toxicity of the compounds when used in combination, the side effects occurred by phytochemicals are considerably less as they are derived from plants. Experimental investigations are under development to further assess the interaction between phytochemicals and antibiotics against bacterial biofilms.

Author Contributions: M.R. and M.B.S. conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft. M.S. conceived and designed the experiments, analyzed the data, contributed reagents/materials/analysis tools, authored or reviewed drafts of the paper, approved the final draft. All authors have read and agreed to the published version of the manuscript.

Funding: This research was financially supported by: Base Funding - UIDB/00511/2020 funded by national funds through the FCT/MCTES (PIDDAC); Project Biocide_for_Biofilm - PTDC/BII-BTI/30219/2017 - POCI-01-0145-FEDER-030219, ABFISH – PTDC/ASP-PES/28397/2017-POCI-01-0145-FEDER-028397 and ALGAVALOR - POCI-01-0247-FEDER-035234, funded by FEDER funds through COMPETE2020–Programa Operacional Competitividade e Internacionalização (POCI) and by national funds (PIDDAC) through FCT/MCTES.

Conflicts of Interest: The authors declare no conflict of interest.

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