

***Antimicrobial and antibiofilm effect of
phytochemicals as a potential treatment for
nosocomial infections***

Master's Dissertation

by

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“Our greatest weakness lies in giving up. The most certain way to succeed is always
to try just one more time.”

Thomas A. Edison

Thesis outputs

This work already resulted in the following publications:

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Resumo

O tratamento de infeções bacterianas tem sido progressivamente perturbado devido à crescente resistência a antibióticos. Os biofilmes agravam esta luta contra as infeções porque fornecem um ambiente protetor às células microbianas. Muitos agentes antimicrobianos de primeira linha tornaram-se ineficazes no tratamento de infeções causadas por biofilmes, levando à procura de novas terapias capazes de ajudar nesta tarefa. Na verdade, os produtos naturais, como os fitoquímicos, provaram já ser agentes antibacterianos de largo espectro, eficazes, seguros e baratos.

Neste estudo, foi avaliada a bioatividade de cinco fitoquímicos: 7-hidroxicumarina, hidrato de naringina, teofilina, álcool perílico e ácido hidrocínâmico contra duas estirpes da *Spanish Type Culture Collection* (CECT): *Escherichia coli* CECT 434 e *Staphylococcus aureus* CECT 976. Como a combinação de fitoquímicos com antibióticos tem mostrado ser uma alternativa eficaz aos tratamentos convencionais, foi avaliado o seu perfil de resistência a nove antibióticos/combinações de antibióticos tendo sido selecionados o cloranfenicol e a amoxicilina. No geral, *S. aureus* revelou um bom perfil de suscetibilidade aos antibióticos enquanto *E. coli* parece estar perto de adquirir resistência ao cloranfenicol e à amoxicilina.

Foram também determinados os valores de concentração mínima inibitória (CMI) e concentração mínima bactericida (CMB), o que permitiu limitar os ensaios posteriores a quatro compostos de interesse: cloranfenicol, amoxicilina, álcool perílico e ácido hidrocínâmico. Quatro compostos apresentaram CMIs detetáveis: cloranfenicol (4 µg/mL para *S. aureus* e 16 µg/mL para *E. coli*), amoxicilina (0,08 µg/mL para *S. aureus* e 8 µg/mL para *E. coli*), álcool perílico (1024 µg/mL para *S. aureus* e 256 µg/mL para *E. coli*) e ácido hidrocínâmico (2048 µg/mL para ambas as bactérias). Três compostos tiveram CMBs detetáveis: cloranfenicol (64 µg/mL para ambas as bactérias), amoxicilina (2 µg/mL para *S. aureus* e 8 µg/mL para *E. coli*) e álcool perílico (2048 µg/mL para *S. aureus* e 512 µg/mL para *E. coli*). Todos os fitoquímicos e os antibióticos selecionados foram analisados com base na *rule of five* de Lipinski e concluiu-se que apenas o hidrato de naringina não pode ser considerado uma molécula "drug-like".

Posteriormente, foram realizados ensaios com as células em estado sésil, tendo ambas as bactérias sido classificadas como fracas produtoras de biofilmes. Seguiu-se a exposição dos biofilmes aos compostos selecionados por 24 h, tendo-se avaliado a sua inativação metabólica, remoção da biomassa e culturabilidade celular. No que diz respeito à inativação da atividade metabólica, sete tratamentos consistindo em concentrações distintas apresentaram eficácia moderada, catorze resultaram numa eficácia elevada e três apresentaram uma eficácia excelente. Em termos de redução da biomassa, quinze tratamentos apresentaram eficácia baixa e nove resultaram numa eficácia moderada. Notavelmente, o ácido hidrocínâmico não foi capaz de causar qualquer redução da biomassa.

No que diz respeito à culturabilidade das células dos biofilmes, foi obtida uma ausência total de culturabilidade celular tanto para os biofilmes de *S. aureus* como para os biofilmes de *E. coli* tratados com álcool perílico e ácido hidrocínâmico para 5×CMI e 10×CMI. De facto, os dois fitoquímicos revelaram os maiores valores de eficácia no que diz respeito à inativação metabólica e à culturabilidade celular. Além disso, os compostos testados tiveram um maior efeito nos biofilmes de *S. aureus* do que nos biofilmes de *E. coli*, exceto a amoxicilina.

Numa segunda fase, foi investigada a bioatividade de combinações duplas e triplas, com foco em quatro compostos: álcool perílico, ácido hidrocínâmico, cloranfenicol e amoxicilina, e uma estirpe microbiana: *E. coli* CECT 434. Primeiramente, foi avaliada a toxicidade e eficácia de um neutralizador universal, concluindo-se que este não é tóxico para *E. coli* e é eficaz na neutralização dos compostos selecionados. Depois, as combinações foram avaliadas por *checkerboard assay* (pela classificação *Fractional Inhibitory Concentration (FIC) Index (FICI)* e pelo *software Combenefit*) e por *time-kill assay* (através do $\log_{10}$ (CFU/mL) em função do tempo de incubação). Por último, foi avaliada a atividade das combinações sobre os biofilmes.

Para o ensaio *checkerboard* obteve-se sinergismo para 37,5% das combinações, efeito aditivo para 12,5% e indiferença para 50,0%. A análise com o *Combenefit* mostrou que as combinações da amoxicilina com cada fitoquímico selecionado resultaram em sinergismo para várias concentrações, sendo este mais intenso para uma concentração de amoxicilina igual a 2 µg/mL. As combinações do cloranfenicol com cada fitoquímico selecionado revelaram sinergismo principalmente para concentrações mais baixas do antibiótico e foi observada uma zona de antagonismo muito ligeiro para concentrações intermédias ou mais elevadas do cloranfenicol. O ensaio *time-kill* revelou que 16,7% das combinações resultaram em sinergismo, 16,7% num efeito aditivo e 66,7% em indiferença. A concordância entre os dois ensaios foi de 87,5%, tendo ambos demonstrado que deve ser dado um foco particular às combinações cloranfenicol/álcool perílico, cloranfenicol/ácido hidrocínâmico, amoxicilina/ácido hidrocínâmico e amoxicilina/metronidazol, enquanto as combinações triplas não foram particularmente eficazes quando comparadas com as combinações duplas. Os ensaios com biofilmes demonstraram que, em relação à inativação metabólica, todas as combinações resultaram numa eficácia elevada e em termos de redução da biomassa todas as combinações revelaram uma eficácia moderada. Considerando a culturabilidade das células sésseis de *E. coli*, foi determinado sinergismo para 20,0% das combinações, efeito aditivo para 60,0% e indiferença para 20,0%. A combinação mais eficaz foi cloranfenicol/álcool perílico/ácido hidrocínâmico e a menos ativa foi álcool perílico/ácido hidrocínâmico.

Assim, este estudo revelou o potencial dos compostos naturais avaliados e das terapias combinatórias no controlo de infeções associadas aos cuidados de saúde.

Palavras-chave: Biofilmes; Resistência Microbiana; Fitoquímicos; Antibióticos; Combinações.

Abstract

The treatment of bacterial infections has been progressively troubled due to the increasing resistance to antibiotics. Biofilms aggravate this fight against infections because they provide a protective environment to microbial cells. Many first-line antimicrobial agents became ineffective in treating infections caused by biofilm, instigating the search for new therapies able to help with this task. In fact, natural products such as phytochemicals, have already proven to be broad-spectrum, effective, safe and inexpensive antibacterial agents.

In this study, the bioactivity of five phytochemicals was evaluated: 7-hydroxycoumarin, naringin hydrate, theophylline, perillyl alcohol and hydrocinnamic acid against two strains from the Spanish Type Culture Collection (CECT): *Escherichia coli* CECT 434 and *Staphylococcus aureus* CECT 976. Since combinations of phytochemicals with antibiotics has been shown to be an effective alternative to conventional treatments, their resistance profile to nine antibiotics/combinations of antibiotics was evaluated being selected chloramphenicol and amoxicillin. Overall, *S. aureus* showed a good antibiotic susceptibility profile while *E. coli* seems to be close to acquiring resistance to chloramphenicol and amoxicillin.

Also, the values of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined, which allowed the subsequent assays to be limited to four compounds of interest: chloramphenicol, amoxicillin, perillyl alcohol and hydrocinnamic acid. Four compounds showed detectable MICs: chloramphenicol (4 µg/mL for *S. aureus* and 16 µg/mL for *E. coli*), amoxicillin (0.08 µg/mL for *S. aureus* and 8 µg/mL for *E. coli*), perillyl alcohol (1024 µg/mL for *S. aureus* and 256 µg/mL for *E. coli*) and hydrocinnamic acid (2048 µg/mL for both bacteria). Three compounds had detectable MBCs: chloramphenicol (64 µg/mL for both bacteria), amoxicillin (2 µg/mL for *S. aureus* and 8 µg/mL for *E. coli*) and perillyl alcohol (2048 µg/mL for *S. aureus* and 512 µg/mL for *E. coli*). All phytochemicals and selected antibiotics were analysed based on the Lipinski's rule of five, and it was concluded that only naringin hydrate can not be considered a "drug-like" molecule.

Subsequently, tests were performed with sessile cells being both bacteria classified as weak biofilm producers. It was followed by exposure of biofilms to selected compounds for 24 h being evaluated their metabolic inactivation, biomass reduction and cellular culturability. Regarding metabolic activity inactivation, seven treatments consisting of distinct concentrations showed moderate efficacy, fourteen resulted in high efficacy and three displayed excellent efficacy. In terms of biomass reduction, fifteen treatments showed low efficacy and nine resulted in moderate efficacy. Remarkably, hydrocinnamic acid was not able to cause any biomass reduction. Regarding the culturability of biofilms cells, total absence of cellular culturability was obtained both for *S. aureus* biofilms and *E. coli* biofilms treated with perillyl alcohol and hydrocinnamic acid at 5×MIC and 10×MIC. In fact, the two phytochemicals

revealed the highest efficacy values regarding metabolic inactivation and cellular culturability. Furthermore, the compounds tested had a greater effect on *S. aureus* biofilms than on *E. coli* biofilms, except amoxicillin.

In a second stage, the bioactivity of dual and triple combinations was investigated, focusing on four compounds: perillyl alcohol, hydrocinnamic acid, chloramphenicol and amoxicillin, and one microbial strain: *E. coli* CECT 434. In first place, the toxicity and efficacy of a universal neutralizer was evaluated, concluding that it is not toxic to *E. coli* and is effective in neutralizing the selected compounds. Then, combinations were assessed through checkerboard assay (by Fractional Inhibitory Concentration (FIC) Index (FICI) classification and software Combeneft) and through time-kill assay (by $\log_{10}$ (CFU/mL) as a function of incubation time). Lastly, the activity of combinations on biofilms was evaluated.

For checkerboard assay, synergism was obtained for 37.5% of combinations, additivity for 12.5% and indifference for 50.0%. The analysis with Combeneft showed that combinations of amoxicillin with each selected phytochemical resulted in synergism for various concentrations being more intense for a concentration of amoxicillin equal to 2 $\mu\text{g/mL}$. Combinations of chloramphenicol with each selected phytochemical revealed synergism mainly at lower concentrations of antibiotic and a very slight antagonism zone was observed at intermediate or higher concentrations of chloramphenicol. Time-kill assay revealed that 16.7% of combinations resulted in synergism, 16.7% in additivity and 66.7% in indifference. The agreement between the two assays was 87.5% and both demonstrated that a particular focus should be given to combinations chloramphenicol/perillyl alcohol, chloramphenicol/hydrocinnamic acid, amoxicillin/hydrocinnamic acid and amoxicillin/metronidazole while triple combinations were not particularly effective when compared to dual combinations. The assays with biofilms demonstrated that, regarding metabolic inactivation, all combinations resulted in high efficacy and in terms of biomass reduction all combinations revealed moderate efficacy. Considering the culturability of sessile cells of *E. coli*, synergism was determined for 20.0% of combinations, additivity for 60.0% and indifference for 20.0%. The most effective combination was chloramphenicol/perillyl alcohol/hydrocinnamic acid and the least active was perillyl alcohol/hydrocinnamic acid.

Thereby, this study revealed the potential of natural compounds evaluated and combinatorial therapies for the control of healthcare-associated infections.

Keywords: Biofilms; Microbial Resistance; Phytochemicals; Antibiotics; Combinations.

Statement

It is declared, under oath, that this study is original and all non-original contributions have been duly referenced with identification of the source.

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Abbreviations and units list

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AUC – Area Under The Curve
agg – Aggregation Substances
AHL – Acyl-Homoserine Lactones
AI – Autoinducers
AMR – Antimicrobial Resistance
CAPD – Citric Acid:1,2-Propanediol
CECT – Spanish Type Culture Collection
CFU – Colony-Forming Units
CLSI – Clinical & Laboratory Standards Institute
CTAB – Cetyltrimethylammonium Bromide
CV – Crystal Violet
DMSO – Dimethyl Sulfoxide
EC50 – Half Maximal Effective Concentration
eDNA – Environmental DNA
EPS – Extracellular Polymeric Substances
Esp – Enterococcal Surface Protein
EUCAST – European Committee on Antimicrobial Susceptibility Testing
FIC - Fractional Inhibitory Concentration
FICI - Fractional Inhibitory Concentration Index
gelE – Gelatinase
HPLC – High-Performance Liquid Chromatography
IZD – Inhibition Zone Diameter
LA – Lactic Acid
MBC – Minimum Bactericidal Concentration
MDR – Multidrug-Resistant
MHA – Mueller-Hinton Agar
MHB – Mueller-Hinton Broth
MIC – Minimum Inhibitory Concentration
MRSA – Methicillin-Resistant *S.aureus*
Log *P* – Octanol-Water Partition Coefficient
MW – Molecular Weight
NADES – deep eutectic solvents
n-OHND donors – Number of Hydrogen Bond Donors
n-ON acceptors – Number of Hydrogen Bond Acceptors
OD – Optical Density
OD_c – Optical Density of the Control
OM – Outer Membrane
p – Statistical Significance Level
PBS – Phosphate Buffered Saline
PVL – Panton-Valentine Leukocidin
QS – Quorum Sensing
SPSS – Statistical Package for the Social Sciences
TNase – Thermostable Nuclease
WHO – World Health Organization
XXGIH – Choline Chloride:Glucose
XXGly – Choline Chloride:Glycerol
XXPD – Choline Chloride:Propanediol
ZDB – Zone Diameter Breakpoint

Units list

h – hour
v/v – volume per volume
°C – degrees Celsius
µg – microgram
mL – milliliter
mg – milligram
µL – microliter
rpm – rotations per
minute
nm – nanometer
min – minute
mm – millimeter

Chapter 1 – Work outline

1.1. Background

Since its discovery, antibiotics have been essential for the treatment and control of previously fatal infections (Allen et al. 2019; Micoli et al. 2021). Nevertheless, the improper use and over-prescription of antibiotics, the poor hygiene and sanitation habits, the lack of infection control and the absence of new antibiotics led to a rapid spread of resistant microbial strains (Barbieri et al. 2017; Allen et al. 2019; Liu et al. 2021).

Antimicrobial resistance (AMR) is a natural result of the adaptation of infectious pathogens to antibiotics used in diverse fields. Moreover, the economic burden associated with these multidrug-resistant (MDR) bacteria is greatly significant (Monte et al. 2014; Prestinaci et al. 2015). Since antibiotic resistance is a multifactorial issue, the World Health Organization (WHO) recommends coordinated actions, from preventing the spread of infections to promoting the research and development of new treatment strategies (Ventola 2015; Barbieri et al. 2017). In addition, microorganisms that live in communities, the so-called biofilms, are responsible for serious and dangerous infections and combating them is difficult or requires high doses of antibiotics (Llor and Bjerrum 2014; Frieri et al. 2017). It is well known that the acquisition of antibiotic resistance by bacteria is facilitated when biofilms are developed (Kostakioti et al. 2013; de Carvalho 2018; Yin et al. 2019). In fact, bacteria in biofilms can be up to one thousand times more tolerant to antibiotics than free-floating microorganisms, which complicates the treatment options (Potera 2010; Venkatesan et al. 2015; Sharma et al. 2019).

Plants have been explored as a source of interesting compounds, most of which with efficacy against a variety of organisms, including fungi, yeasts, bacteria, insects, nematodes, viruses and other plants (Monte et al. 2014; Wintola and Afolayan 2015). Phytochemicals are non-nutritive secondary metabolites from plants with biological activity (Huang et al. 2016; Jimenez-Garcia et al. 2018). Different parts of plants from several species are rich in different classes of phytochemicals, which may be a source of effective, inexpensive and safe antimicrobial agents (Chouhan et al. 2017; Aung et al. 2020). They are able to inhibit peptidoglycan synthesis, destroy the structure of microbial membranes, change the hydrophobicity of bacterial membranes and interfere with quorum sensing (QS) (Nazzaro et al. 2013; Monte et al. 2014). Phytochemicals are promising antimicrobial agents due to their cost-effectiveness, eco-friendliness, large structural diversity and capacity of reducing AMR (Giaouris and Simões 2018; Sakarikou et al. 2020).

1.2. Main objectives

The main objective of this study was to perform a screening of the bioactivity of five phytochemicals: 7-hydroxycoumarin, naringin hydrate, theophylline, perillyl alcohol and hydrocinnamic acid, against *Escherichia coli* CECT 434 and *Staphylococcus aureus* CECT 976. These microorganisms were chosen because of their clinical relevance resulting from widespread and worrying antibiotic resistance (Simões et al. 2008). Moreover, the antibiotics chloramphenicol and amoxicillin were selected based on the resistance profiles of *E. coli* CECT 434 and *S. aureus* CECT 976, after testing nine antibiotics/combinations of antibiotics. Then, the values of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined, and four compounds of interest were selected: chloramphenicol, amoxicillin, perillyl alcohol and hydrocinnamic acid. After, all phytochemicals and selected antibiotics were analysed based on Lipinski's rule of five. Towards biofilms assays, in first place, bacteria were classified according to their ability to form biofilms. Secondly, the antimicrobial effect of chloramphenicol, amoxicillin, perillyl alcohol and hydrocinnamic acid, against 24 h old biofilms, was investigated. Biofilms were exposed to the selected compounds for 24 h. Afterwards, the culturability of biofilm's cells (measured by colony-forming units (CFU) counts), biofilm's metabolic inactivation (measured by fluorescence values) and biomass reduction (measured by optical density (OD) at 570 nm values) were determined.

In a second phase of this work, the bioactivity of dual and triple combinations was evaluated. For this purpose, based on the previous results, only four compounds were selected: perillyl alcohol, hydrocinnamic acid, chloramphenicol and amoxicillin as well as one microbial strain: *E. coli* CECT 434. First, the toxicity and efficacy of a universal neutralizer were evaluated. Then, all possible dual and triple combinations were investigated, excluding chloramphenicol/amoxicillin. In the case of planktonic cells, dual and triple combinations were evaluated by checkerboard and time-kill assays. Considering checkerboard assay, the results were evaluated by Fractional Inhibitory Concentration (FIC) Index (FICI) classification and by the software Combenefit. Concerning time-kill assay, the results were evaluated by $\log_{10}$ (CFU/mL) as a function of incubation time. With regard to biofilms, the same tests were conducted as for single compounds. In all assays, the combination amoxicillin/metronidazole was used as a positive control and compared to other combinations involving amoxicillin.

1.3. Thesis organization

This master thesis was organized in five chapters, as shown in **Figure 1**.

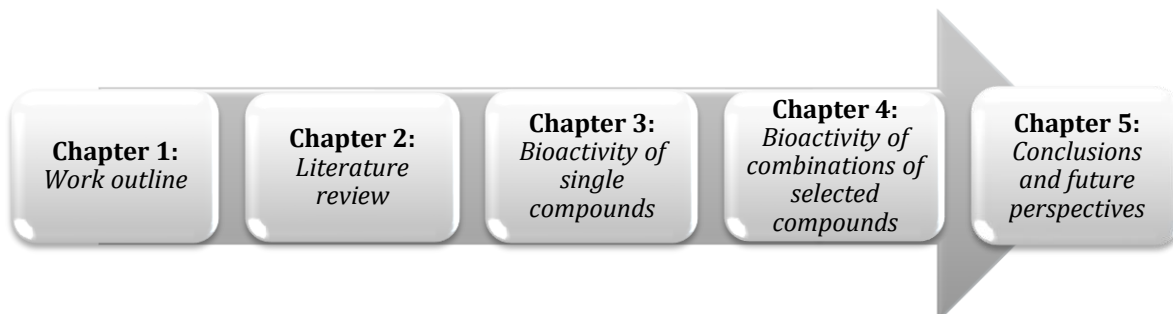


Figure 1. Organization of this master thesis by chapters.

In **chapter 1**, the background, the motivations and the major objectives were discussed.

In **chapter 2**, a literature review about the topics covered was presented. First, the bacterial resistance to traditional antibiotics and the main problems associated were reported. Then, the relevant concepts related to the most critical microorganisms, due to their multidrug resistance, were highlighted. Afterwards, the complex issue of infections caused by biofilms and some concepts about biofilm formation were addressed. Phytochemicals were introduced, focusing on their sources, properties, mechanism of action, bioactivity, therapeutic effects, different classes and some studies reporting their antibiofilm activity. Finally, studies associated with targeting bacterial infections with phytochemicals and new perspectives for their used in combinatorial therapies as well as biomimetics solutions were discussed.

In **chapter 3**, the antibacterial activity of single compounds against planktonic cells and biofilms of *E. coli* CECT 434 and *S. aureus* CECT 976 was investigated. Based on the results for the resistance profiles together with MICs and MBCs values, four compounds were selected: chloramphenicol, amoxicillin, perillyl alcohol and hydrocinnamic acid. Then, all phytochemicals and selected antibiotics were analysed based on Lipinski's rule of five. Afterwards, bacteria biofilms were classified, and the compounds selected were evaluated for biofilm control purposes. The culturability of biofilm's cells was determined by CFU counts, biofilm's metabolic inactivation was assessed by alamar blue staining procedure and biomass reduction was evaluated by crystal violet (CV) staining method.

In **chapter 4**, dual and triple combinations were studied. For this purpose, four compounds: perillyl alcohol, hydrocinnamic acid, chloramphenicol and amoxicillin as well as one bacterial strain: *E. coli* CECT 434, were selected. Combinations (all possible excluding chloramphenicol/amoxicillin) were assessed by checkerboard and time-kill assays. Then, the antibiofilm activity of combinations was assessed.

In **chapter 5**, the main conclusions and some indications for future work were presented.

Chapter 2 – Literature review

2.1. Bacterial resistance

Bacterial resistance is a natural result of the adaptation of infectious pathogens to antibiotics used in diverse situations, including medicine, food, crop production, but also disinfectants for farms, hospitals and houses. Moreover, the economic burden associated with these MDR bacteria is highly significant (Monte et al. 2014; Prestinaci et al. 2015). Bacterial resistance mechanisms are specific strategies of microbial evolution being inevitable (Simões et al. 2009; Upmanyu and Malviya 2020). For this reason, it is not surprising that new drug resistance mechanisms are still emerging (Barbieri et al. 2017; Sekyere and Asante 2018; Diggle and Whiteley 2020). Some phenomena can explain the resistance of microorganisms to antibiotics: (i) cells can present an intrinsic and natural resistance to drugs being “genetically programmed” to resist to antibiotics (Beceiro et al. 2013; Holmes et al. 2016); (ii) microorganisms may suffer some genetic modifications, as mutations, that make them not susceptible to antibiotics (Beceiro et al. 2013; Fair and Tor 2014; Meredith et al. 2015); (iii) cells may become resistant to antimicrobial agents through the horizontal acquisition of genes from other microorganisms that confer resistance to certain antibiotics. This is a very extensive phenomena and increases the probability of survival under the selective pressure caused by antibiotics presence (Beceiro et al. 2013; Meredith et al. 2015; Bello-López et al. 2019). Moreover, bringing a new antibiotic to the market may take up to ten years and it is very economically expensive and time consuming (Power 2006; Spellberg 2014; Barbieri et al. 2017). Furthermore, it is well known that the acquisition of resistance by microorganisms to antibiotics is facilitated when they are present in biofilms because these structures provide protection against temperature and pH fluctuations, ultraviolet radiation exposure, changes in salinity, depletion of water, oxygen and nutrients and antimicrobial compounds (Bridier et al. 2011; Kostakioti et al. 2013; de Carvalho 2018; Yin et al. 2019). Regarding biofilm recalcitrance, there are two types of factors responsible for that: innate resistance factors and induced resistance factors. While innate resistance factors are activated during the biofilm development and are integral part of biofilm structure and physiology, the induced resistance factors are triggered by the antibiotic itself (Anderson and O’Toole 2008). Innate resistance pathways include poor antibiotic penetration, nutrient limitation and slow growth, adaptive stress responses and formation of persister cells. Biofilm recalcitrance is usually a combination of both innate and induced mechanisms (Anderson and O’Toole 2008; Lebeaux et al. 2014).

2.1.1. Critical multidrug-resistant pathogens

According to the global priority list of pathogens published in 2017 by the WHO (2017), certain microorganisms are particularly critical for human health, including some Gram-negative bacteria such as Enterobacteriaceae, *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. Moreover, this list divides microorganisms into different priority categories (critical, high and medium) being *E. coli* in the critical priority class and *S. aureus* in the high priority class (World Health Organization 2017). According to the same institution, ESKAPE pathogens, which are a group of six highly virulent, pathogenic and resistant microorganisms, should also be a subject of concern. This group include *Enterococcus faecium*, *S. aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp. (World Health Organization 2017; Skariyachan and Garka 2018). These Gram-positive and Gram-negative bacteria can evade or "escape" commonly used antibiotics because of their high multidrug resistance being also responsible for increasing the expression of AMR worldwide (Mulani et al. 2019; Schultz et al. 2020). Therefore, it is important to be aware of the main virulence factors associated with some of the most concerning bacteria, which are shown in **Table 1**.

Table 1. Main virulence factors of some critical bacteria.

<i>Species</i>	<i>Main virulence factors</i>	<i>References</i>
<i>S. aureus</i>	Membrane-damaging toxins; shock syndrome toxin; epidermolytic toxin; staphylococcal enterotoxins; pyrogenic exotoxin; exoenzymes; Panton-Valentine Leukocidin (PVL) toxin; thermostable nuclease (TNase); adhesins; phenol-soluble modulins; surface proteins; capsular polysaccharides; invasins; surface inhibitors.	(Dinges et al. 2000; Balasubramanian et al. 2017; Fisher et al. 2018; Tam and Torres 2019; Etter et al. 2020)
<i>E. coli</i>	Adhesins; toxins; iron acquisition factors; lipopolysaccharides; polysaccharide capsules; invasins.	(Kaper et al. 2004; Sarowska et al. 2019; Shah et al. 2019)
<i>P. aeruginosa</i>	Pyocyanin; pyochelin; other pigments; elastase; phospholipase C; protease A; exotoxins and cytotoxins; flagella and pili; QS regulatory system proteins.	(Cox 1986; Britigan et al. 1992; Alonso et al. 2020)
<i>E. faecium</i>	Proteins and carbohydrates; enzymes; enterococcal surface protein (Esp); cytolysin; cell wall adhesins efaAfm; gelatinase (gelE); aggregation substances (agg).	(Huycke et al. 1998; Mascini et al. 2006; Ike 2017)
<i>A. baumannii</i>	Porins; capsular polysaccharides; phospholipases; lipopolysaccharides; outer membrane (OM) vesicles; metal acquisition systems; protein secretion systems.	(Lee et al. 2017; Aliramezani et al. 2019; Sarshar et al. 2021)

2.2. Biofilm-related infections

According to WHO, antibiotic resistance is a major issue with severe consequences for public health and a great economic burden (Levy and Marshall 2004). In addition, it compromises the prevention and treatment of a large range of previously treatable infections, especially the ones caused by biofilms (Jindal et al. 2015; Micoli et al. 2021). Biofilms are well-structured and high cell density communities attached to a certain biotic or abiotic surface. Here, cells are embedded in a matrix composed by polysaccharides, proteins and extracellular DNA produced by the cells themselves (Donlan 2002; Høiby et al. 2010, 2011; Limoli et al. 2015; Karygianni et al. 2020). Surprisingly, biofilms can play an important positive role in many ecosystems, namely breaking down sewage and decontaminating polluted sites, although having many well known negative effects already discussed (Hunter 2008; Muhammad et al. 2020). One of the most important characteristics of biofilms is their capacity to ensure a greater protection to bacterial cells and survive in the presence of high concentrations of antibiotics, known as “recalcitrance”, which leads to treatment failure and infection recurrence (Lebeaux et al. 2014; Aggarwal et al. 2015; Giulia et al. 2021). In fact, it is estimated that bacteria in biofilms are up to one thousand times more resistant to antibiotics than free-floating bacteria, which severely complicates treatment options. Consequently, by living in a community, microorganisms are responsible for serious and dangerous infections and combating them usually requires high doses of antibiotics (Llor and Bjerrum 2014; Frieri et al. 2017). Therefore, even in unfavorable and harsh environments, biofilms can promote the survival of several microorganisms, including pathogens, and facilitate their diffusion as well as the colonization of new niches (Kostakioti et al. 2013; Wu et al. 2015; Roy et al. 2018; Del Pozo 2018; Muhammad et al. 2020). Biofilms are also associated with device-related infections, which can be responsible for malfunctions of devices and the introduction of microorganisms in the human body (Del Pozo 2018; Khatoon et al. 2018). In fact, biofilms are responsible for up to 80% of human infections such as cystic fibrosis, endocarditis, osteomyelitis and sepsis. The resistance of biofilms to most available antibiotics continues increasing (Jamal et al. 2018; Banerjee et al. 2020). Given the relevance of infections caused by biofilms, it is possible to conclude that the development of biofilms is intrinsically related to antibiotic resistance (Miquel et al. 2016; Batoni et al. 2021).

2.2.1. Biofilm formation and specific mechanisms

A biofilm occurs when planktonic cells adhere to biotic or abiotic surfaces and initiate the development of sessile microcolonies, surrounded by an extracellular matrix produced by the cells (Otto 2009). The structures originated by biofilms are morphologically and physiologically

distinct from those developed by free-living bacteria (Davies et al. 1998). Biofilms allow planktonic cells to live, although temporarily, in a microbial consortium. This cellular community provides cells with protection against severe environmental conditions and antimicrobial agents (Arunasri and Mohan 2019). In addition, biofilms consist of a reserve of essential resources for microbial survival, namely nutrients, and allow the exchange of genetic material between cells, which is important for their adaptation and evolution (Hobley et al. 2015). In fact, the stages of development of biofilms are similar for different microorganisms. Nevertheless, the molecules associated with this process such as nucleic acids, proteins, exopolysaccharides and adhesive fibers are specific for a particular species or strain (Limoli et al. 2015). Furthermore, the structure/architecture of the biofilm, the kinetics of biofilm formation stages, the interactions between cells and between cells and the surface as well as the regulatory factors may differ from microorganism to microorganism (Floyd et al. 2017).

In fact, biofilm formation has been recognized as a multistage process (Francolini and Piozzi 2016). In other words, it is believed that the biofilm development process occurs through several stages, as schematized in **Figure 2**.

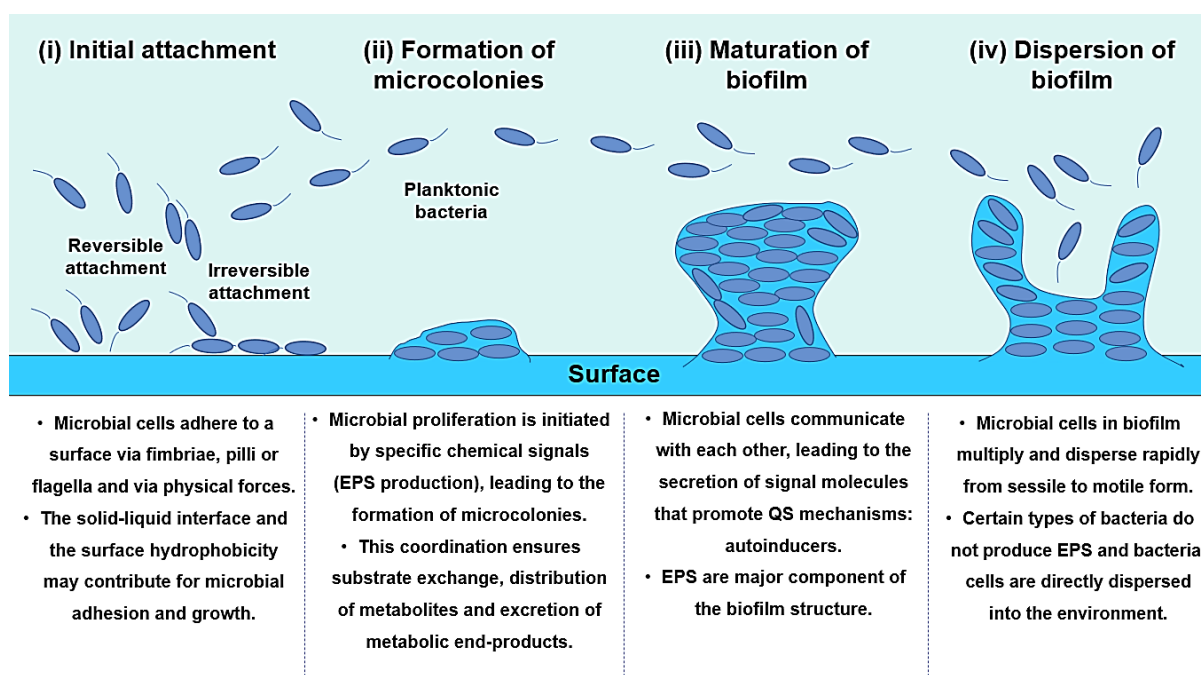


Figure 2. Representation of biofilm formation stages: initial attachment; formation of microcolonies; maturation of biofilm and dispersion of biofilm (based on Jamal et al. 2018; López and Soto 2020).

Briefly, those are (i) initial attachment: microbial cells adhere to a surface through their appendages, namely pili and flagella, but can also get attached through other physical forces such as van der Waal's forces, electrostatic interactions, among others; (ii) formation of microcolonies: the process of microbial cell proliferation and division begins, which is initiated by specific chemical signals, through EPS; (iii) maturation of the biofilm: the secretion of signal

molecule called autoinducers (AI), which promotes the QS phenomena responsible for cell-to-cell communication, is important during this stage of biofilm development because the required microbial cell density has to be achieved and (iv) dispersion of biofilm: microbial cells in the biofilm multiply and disperse rapidly to transform from sessile to motile form (Jamal et al. 2018). Consequently, biofilm development requires a particular kind of molecular signaling, QS mechanisms as well as the expression of different genes, when compared to those of the planktonic cells (De Kievit et al. 2001; Lebeaux et al. 2014). Regarding surface properties, the attachment is facilitated on rough, hydrophobic and coated surfaces (Rowthu and Hoffmann 2019). However, the attachment is also affected by the flow velocity, the water temperature and the nutrient concentration (Donlan 2002).

For the successful development and establishment of a biofilm, specific mechanisms and the respective virulence factors are crucial (Phillips and Schultz 2012). Virulence factors are molecules produced by bacteria, which are needed to enable bacteria to attack eukaryotic hosts and cause infections (Sharma et al. 2017). They are usually encoded on mobile and integrative genetic elements, namely plasmids, bacteriophages, conjugative transposons, integrative and conjugative elements and pathogenicity islands (Boyd 2012; Lami 2019). Virulence factors allow the pathogens to evade the host immune response and facilitate the establishment and long-term survival of biofilms in tissues (Phillips and Schultz 2012; Zhao and Ma 2015). Besides virulence factors, there are also several mechanisms that are extremely important for their survival and proliferation in host tissues when bacteria live in biofilm communities (Vestby et al. 2020). Thereby, it is important to understand these mechanisms and known the respective virulence factors. The main bacterial mechanisms responsible for the establishment of biofilms are QS, adhesion, motility and EPS production, characterized in **Table 2**.

Table 2. Biofilm mechanisms for cell protection and advantage under stressing conditions.

<i>Biofilm mechanisms and their main characteristics</i>	<i>References</i>
QS • QS allows bacteria to communicate and modulate the expression of genes involved in bacterial survival, virulence and pathogenicity. • The production and release of AIs allow bacteria to detect changes in population density and the interaction of AIs with specific transcriptional regulators leads to population-dependent changes in gene expression. • QS systems in Gram-negative bacteria are related to LuxI/LuxR-Type QS System. LuxI-type proteins are synthase proteins that catalyze the synthesis of acyl-homoserine lactones (AHL) while LuxR type proteins are transcriptional activator proteins that binds to AHL, triggering transcriptional modifications. • In Gram-positive bacteria, peptides are secreted and detected by two-component systems but can also be actively transported via ABC transporters to bind a cytoplasmic regulator.	(Miller and Bassler 2001; Frederix and Downie 2011; Rutherford and Bassler 2012; Goulden et al. 2013; Floyd et al. 2017; AL-Mamun et al. 2018; Clark et al. 2019; Harriott 2019; Pena et al. 2019; Smith et al. 2020)
Adhesion • Adhesion is the first step for biofilm development, and it is controlled by a complex set of chemical and physical interactions. • The first adhesion process of a planktonic microorganism is reversible, and it is dictated by several physicochemical interactions being mostly associated with van der Waals forces and electrical double layer forces (DLVO theory). • Adhesion depends on the net sum of attractive and repulsive forces between the surfaces and the microorganism. • The second stage implies a stronger and irreversible binding of the microorganism to the surface through bacterial adhesins.	(Garrett et al. 2008; Almaguer-Flores 2013; Abraham et al. 2015; Stones and Krachler 2016; Berne et al. 2018)
Motility • Motility is one of the most dynamic bacterial phenomena, contributing to virulence through adhesion and biofilm formation. • Six distinct categories of motility have been identified: swimming, swarming, gliding, twitching, sliding and darting. • Swimming and swarming are dependent on flagella. • Twitching and some ways of gliding requires type IV pili. • Sliding/spreading are passive translocation types of motility. • Information about darting is still scarce.	(Eberl et al. 1999; Harshey 2003; Xu et al. 2014; Monte et al. 2014; Tomada et al. 2016; Pollitt and Diggle 2017)
EPS production • EPS are organic polymers, namely exopolysaccharides, proteins, lipids and DNA, crucial for the interaction between bacteria and the environment. • The extracellular matrix, composed by water and EPS, is essential for the establishment and maintenance of the structure and properties of biofilms. • The matrix stability is guaranteed by non-covalent binding between EPS involving weak physicochemical forces. • The EPS network confers cohesion and viscoelasticity to the biofilm's structure.	(Costerton et al. 1999; Flemming and Wingender 2010; Sheng et al. 2010; Jiao et al. 2010; Di Martino 2018; Gao et al. 2019)

2.3. Phytochemicals as natural antimicrobial agents

In order to find novel antimicrobial agents with different mechanisms, plants have been explored as a source of interesting compounds, most of which with efficiency against several types of organisms, including fungi, yeasts, bacteria, insects, nematodes, viruses, among others (Cowan 1999; Monte et al. 2014; Wintola and Afolayan 2015). Medicinal plants are the most abundant biological resources in traditional and modern medicine, nutraceuticals, food

supplements, folk medicines, pharmaceutical intermediates and chemical components of synthetic medicines (Pan et al. 2013; Desai et al. 2015; Saranraj et al. 2016).

Phytochemicals are non-nutritive and natural compounds derived from plants with biological activity, consisting of secondary metabolites with a significant structural and functional diversity (Liu 2013; Diep et al. 2015; Huang et al. 2016; Jimenez-Garcia et al. 2018). They are responsible for the color, flavor, aroma and texture of plants (Barbieri et al. 2017). Different parts of plants from several species such as leaves, roots, barks, stem bark and flowers are rich in different classes of phytochemicals, which are a source of effective, cheap and safe antimicrobial agents (Barbieri et al. 2017; Chouhan et al. 2017; Aung et al. 2020). In addition, it is estimated that more than five thousand phytochemicals are present in fruits, vegetables, whole grains, beans, seeds, and nuts (Liu 2003; Chandra et al. 2012; Huang et al. 2016; Jimenez-Garcia et al. 2018). Phytochemicals, whether used alone and/or in combination, have great therapeutic potential for preventing and treating several diseases (Liu 2013). Phytochemicals with nutritional and health-care properties in food are of great significance for human health (Jimenez-Garcia et al. 2018). In fact, they are able to avoid and assist in the combat of a large variety of diseases such as cancer (Forni et al. 2019), coronary heart disease (Kibe et al. 2017), diabetes (Zhang et al. 2015), hypertension (Kibe et al. 2017), inflammation (Forni et al. 2019), microbial, viral and parasitic infections (Thakur et al. 2020), psychosis (Guan et al. 2021), spastic diseases (Forni et al. 2019), ulcers (Gupta et al. 2021), osteoporosis (Sharan et al. 2009) and neurological diseases (Howes and Simmonds 2014). Among the phytochemicals considered to have potential health benefits, there are some major categories such as phenolic compounds (flavonoids and phenolic acids), alkaloids, organosulfur compounds, phytoestrogens, terpenoids, carotenoids and phytosterols (Bayir et al. 2019; Andrade et al. 2020; Singh et al. 2020; Thakur et al. 2020).

Furthermore, recent studies reported some interesting and relevant antibiofilm activities of phytochemicals, which are compiled in **Table 3** (Afonso et al. 2022). To complement this information, **Table A1** in **Appendix A** contains some examples, a structural description and the main benefits/activities for the different classes of phytochemicals.

Table 3. Recent studies providing some examples of antibiofilm activity of phytochemicals (based on Afonso et al. 2022).

<i>Class</i>	<i>Phytochemical</i>	<i>Pathogen</i>	<i>Mechanism</i>	<i>Dose; Effect</i>	<i>References</i>
Carotenoids	Lutein	<i>P. aeruginosa</i> PAO1	Not determined.	20 µg/mL; 61% biofilm inhibition.	(Sampathkumar et al. 2019)
Phenolic compounds (flavonoids and phenolic acids)	Quercetin	<i>P. aeruginosa</i> PAO1	Transcriptional changes associated with QS and expression levels of <i>lasI</i> , <i>lasR</i> , <i>rhlI</i> and <i>rhlR</i> significantly reduced.	250 µg/mL; 43 to 78% biofilm inhibition.	(Vipin et al. 2019)
		<i>P. aeruginosa</i> PAO1		16 µg/mL; 51% biofilm inhibition.	(Ouyang et al. 2016)
	Caffeic acid	Methicillin-Resistant <i>S. aureus</i> (MRSA)	Interference with bacterial motility, modification of physical and chemical properties of cell surfaces, which alter the adhesion potential, interference with QS, and inhibition of biofilm formation.	24% biofilm reduction.	(Kot et al. 2018)
	p-coumaric acid			28% biofilm reduction.	
	Trans-cinnamaldehyde			42% biofilm reduction and 79% inactivation.	
	Ferulic acid	<i>E. coli</i> CETC 434		2500 µg/mL; 13% biofilm prevention and 38% biofilm reduction.	(Borges et al. 2015)
		<i>P. aeruginosa</i> ATCC 10145		500 µg/mL; 81% biofilm prevention and 42% biofilm reduction.	
		<i>S. aureus</i> CETC 976		5000 µg/mL; 6% biofilm prevention.	
	Gallic acid	<i>E. coli</i> CETC 434		5000 µg/mL; 64% biofilm prevention and 29% biofilm reduction.	
		<i>P. aeruginosa</i> ATCC 10145		500 µg/mL; 84% biofilm prevention and 48% biofilm reduction.	
		<i>S. aureus</i> CETC 976		500 µg/mL; 70% biofilm prevention.	
Alkaloids	Indole-3-carbinol	<i>E. coli</i> CETC 434		800 µg/mL; 51% biofilm reduction and 72% biofilm inactivation.	(Borges et al. 2012)
		<i>S. aureus</i> CETC 976		200 µg/mL; 10% biofilm reduction and 40% biofilm inactivation.	
	Saponin	<i>E. coli</i> CETC 434		3200 µg/mL; 34% biofilm reduction and 75% biofilm inactivation.	
		<i>S. aureus</i> CETC 976		3200 µg/mL; 48% biofilm inactivation.	
		<i>S. aureus</i> CETC 976		3200 µg/mL; 48% biofilm inactivation.	
Organosulfur compounds	Allyl isothiocyanate	<i>E. coli</i> CETC 434		1000 µg/mL; 87% biofilm prevention.	(Borges et al. 2016)
		<i>P. aeruginosa</i> ATCC 10145		1000 µg/mL; 99% biofilm prevention.	
		<i>S. aureus</i> CETC 976		1000 µg/mL; 96% biofilm prevention.	
	2-phenylethyl isothiocyanate	<i>E. coli</i> CETC 434		1000 µg/mL; 100% biofilm prevention.	
		<i>P. aeruginosa</i> ATCC 10145		1000 µg/mL; 93% biofilm prevention.	
		<i>S. aureus</i> CETC 976		1000 µg/mL; 90% biofilm prevention.	

2.3.1. Targeting bacterial infections with phytochemicals

2.3.1.1. *Effects of phytochemicals in QS mechanisms*

The study of natural molecules with the ability to interfere with QS and understanding their effects may be an important way to fight bacterial resistance and biofilm establishment (Lade et al. 2014; Borges et al. 2014a). Studies show the action of some phytochemicals on QS and the possibility to retard or avoid biofilm development.

The synthetic halogenated furanone, a secondary metabolite derived from furanone present in the Australian macroalgae *Dilsea pulchra* has shown an enormous potential in affecting the bacterial signaling QS as well as the motility. Based on the structural similarity between *D. pulchra* furanone and AHLs molecules, it was hypothesized that this furanone may be responsible for interfering with the interaction of putative regulatory protein and AHL molecule through its competitive binding to the receptor. Moreover, furanones can also inhibit surface aggregation, when in an ecologically significant concentration (Roy et al. 2018). Hentzer et al. (2002) suggested that *rhl* system, which is a QS mechanism in *P. aeruginosa*, is a target to furanone. It can penetrate the biofilm matrix of *P. aeruginosa*, interfering with some genes related to QS and biofilm maturity (Asfour 2018; Roy et al. 2018).

Polyphenols compounds such as epigallocatechin gallate, tannic acid, ellagic acid, gallic acid and ferulic acid are also promising molecules for inhibiting biofilm development. Studies related the antibiofilm activity of these polyphenols to some interference in QS mechanisms. Huber et al. (2003) demonstrated that some polyphenolic compounds such as gallic acid, epigallocatechin gallate, (+)-catechin and tannic acid were capable to interfere with QS mechanism of *E. coli* and *Pseudomonas putida* through the blockade of AHL system. Quercetin as well as other flavonoids have also shown to be quite effective in affecting the QS mechanisms of several microorganisms such as *P. aeruginosa*, *S. aureus*, *E. coli*, *Enterococcus faecalis* and *Streptococcus mutans* (Quave et al. 2012).

Curcumin, from the rhizome of *Curcuma longa*, have shown a strong biofilm inhibitory activity due to the regulation of the expression of genes involved in QS and associated to the production of alginate and exopolysaccharides. Curcumin can also inhibit the swimming and swarming motilities and increase the biofilm susceptibility to antibiotics (Packiavathy et al. 2013, 2014; Borges et al. 2016). A study performed by Kali et al. (2016) demonstrated that curcumin increased the activity of ciprofloxacin against biofilms of Gram-positive bacteria. Moreover, this compound was also capable to potentiate the effect of amikacin, gentamicin and cefepime against Gram-negative biofilms. These results revealed the potential of using curcumin in a combination therapy with antibiotics. In addition, emodin induced the proteolysis of *E. coli* QS signal receptor TraR and enhanced the activity of ampicillin against *P. aeruginosa*

(Kali et al. 2016). This compound has also been associated with the downregulation of the *cidA* gene, which is involved in cell lysis and environmental DNA (eDNA) release (Borges et al. 2016; Yan et al. 2017; Zhang et al. 2020).

In addition, there are more examples of studies that equally proved the promising results of phytochemicals in QS inhibition, that is, with quorum quenching ability. It was demonstrated by Niu et al. (2006) that *Cinnamomum cassia* extract, containing cinnamaldehyde and eugenol, significantly inhibited QS of *E. coli* and *Vibrio harveyi*. Moreover, clove, cinnamon, peppermint and lavender also exhibited anti-quorum sensing effect against *Chromobacterium violaceum* and *P. aeruginosa*, according to Khan et al. (2009). Iberin, which is an isothiocyanate, and sulfoxide, interfered with the QS mechanism of *P. aeruginosa* and induced apoptosis. Organosulfur compounds from garlic such as allicin and ajoene, were able to inhibit QS of *P. aeruginosa* and *E. coli* (Borges et al. 2013a). In another study developed by Vikram et al. (2010), flavonoids were evaluated for their capacity to interfere with QS of *Vibrio harveyi* and inhibit the development of *E. coli* O157:H7 and *V. harveyi* biofilms. The results showed that kaempferol, naringenin, quercetin and apigenin were able to function as nonspecific inhibitors of autoinducer-mediated cell-cell signaling processes. Furthermore, these molecules inhibited the formation of *V. harveyi* and *E. coli* O157:H7 biofilms (Vikram et al. 2010).

2.3.1.2. Effects of phytochemicals on motility

It has been shown that many natural compounds, extracts and pure products have the ability to interfere with the bacterial motility of several microorganisms, which may be reflected on the reduction of biofilm formation (Vattem et al. 2007; Liaqat et al. 2018).

According to Packiavathy et al. (2012), swimming and swarming motilities of *P. aeruginosa*, *Proteus mirabilis* and *Serratia marcescens* were inhibited by methanolic extracts of *Cuminum cyminum*, consisting essentially of methyl eugenol. This is a compound with a well-studied antibiofilm activity (Packiavathy et al. 2012). In addition, cinnamaldehyde and eugenol from *C. cassia* were able to compromise the development of *E. coli* biofilms by interfering with their swimming motility in a study developed by Niu and Gilbert (2004). Gallic and ferulic acids showed potential to inhibit the motility and adhesion of four pathogenic bacteria, including *E. coli*, *P. aeruginosa*, *S. aureus* and *Listeria monocytogenes*. Identical results were obtained for allyl isothiocyanates isothiocyanate and 2-phenylethyl isothiocyanate (Borges et al. 2012). Ferulic and salicylic acids activities were reported as inhibitory of swimming motility of *Bacillus cereus* and *Pseudomonas fluorescens* (Lemos et al. 2014).

In another study developed by Borges et al. (2014b) the effect of allyl isothiocyanates isothiocyanate and 2-phenylethyl isothiocyanate on planktonic cell susceptibility, bacterial motility and adhesion and biofilms of *E. coli*, *P. aeruginosa*, *S. aureus* and *Listeria*

monocytogenes was evaluated. Allyl isothiocyanates caused complete inhibition of swimming motility of *P. aeruginosa* and total inhibition of swarming motility of *E. coli*. 2-Phenylethyl isothiocyanate caused complete inhibition of swimming motility of *E. coli*, *P. aeruginosa* and *L. monocytogenes* and swarming motility of *E. coli* and *P. aeruginosa*. Moreover, the spreading motility of *S. aureus* was totally inhibited by 2-phenylethyl isothiocyanate. Allyl isothiocyanates isothiocyanate and 2-phenylethyl isothiocyanate presented a preventive effect on biofilm formation, which may be related to the effects on the inhibition of bacterial motility. Moreover, allyl isothiocyanates isothiocyanate and 2-phenylethyl isothiocyanate reduced the mass of biofilms formed by the Gram-negative bacteria (Borges et al. 2014b).

2.3.1.3. Effects of phytochemicals on adhesion

Since bacterial adhesion to a surface is an initial prerequisite for biofilm formation, it is essential to identify natural compounds able to interfere with these phenomena, which may constitute an important target when developing strategies for controlling pathogenicity (Kostakioti et al. 2013; Verderosa et al. 2019).

Within this context, phenolic compounds have been shown to exhibit important roles in preventing bacterial adhesion, including the adhesion of *Campylobacter jejuni* (Klančnik et al. 2021). In a study performed by Pogačar et al. (2016), thyme ethanolic extract, thyme post-hydrodistillation residue and olive leaf extract were evaluated for their phytochemical composition using High-Performance Liquid Chromatography (HPLC) with photodiode array, and the antimicrobial activity against *C. jejuni*. This analysis showed that the main components present in thyme extracts were flavone glucuronides and rosmarinic acid derivatives while in olive leaf extract, verbascoside, luteolin 7-O-glucoside and oleuroside were the major compounds. Thyme ethanolic extract and thyme post-hydrodistillation residue were able to decrease *C. jejuni* adhesion to an abiotic surface by up to 35%, at a concentration range between 50-200 µg/mL, and up to 30%, at a concentration range between 0.2-12.5 µg/mL. Thyme post-hydrodistillation residue was more effective than thyme ethanolic extract and displayed a meaningful inhibition of *C. jejuni* adhesion (higher than 30%), reaching up to 40%. When considering a concentration range of olive leaf extract between 3.125-12.5 µg/mL, biofilm formation was affected, and the adhesion inhibition reached 10% to 23%. In addition, the anti-adhesion effect of *C. jejuni* to cell cultures was also assessed and it was observed that *C. jejuni* adhesion towards pig small intestine epithelial cell line, PSI cl1 cells, was remarkably decreased up to 30% in the presence of thyme ethanolic extract, thyme post-hydrodistillation residue and olive leaf extract at a concentration range between 0.78-200 µg/mL (Pogačar et al. 2016).

Cranberry species are recognized for its high levels of phytochemicals, including phenolic acids, flavonoids and ellagic acid (Walton 2014). Furthermore, cranberry extracts had inhibitory effects on tissue adhesion with regard to Gram-negative bacteria, including the ones of the genera *Proteus*, *Klebsiella*, *Enterobacter* and *Pseudomonas* (Walton 2014).

Toivanen *et al.* (2010) evaluated the anti-adhesion activity of different molecular size fractions of wild cranberry *Vaccinium oxycoccos* polyphenols against *Streptococcus pneumoniae* and *Streptococcus agalactiae*. It was found that *S. pneumoniae* cells bound mostly to the low-molecular size fraction of bilberry and cranberry juices while *S. agalactiae* attached most actively to medium- and high- molecular size fraction of berry and juice of *V. oxycoccos*. After a phytochemical analysis of the medium- and high-molecular size fractions, it was concluded they contain polyphenol macromolecular complexes, including proanthocyanidins and polyhydroxy flavonoids (Toivanen *et al.* 2010).

Moreover, according to Borges *et al.* (2013a), there are some other phytochemicals with very impressive anti-adhesion activity, namely cinnamaldehyde and eugenol from *C. cassia*, which interfered with adhesion and biofilm formation of *E. coli*. Polyanacardic acid, polysalicylic acid, catechin, epigallocatechin and tannic acid also exhibited anti-adhesion activity and inhibited the formation of *S. mutans* and *P. aeruginosa* biofilms (Borges *et al.* 2013a). In another work of Lee *et al.* (2011), the potential effects of phloretin, which can be found for instance in apples, were investigated. Phloretin reduced the attachment of *E. coli* O157:H7 to human colon epithelial cells and decreased the formation of *E. coli* O157:H7 biofilms. It was also demonstrated that phloretin was able to repress genes associated with toxicity, *hlyE* and *stx(2)*; the autoinducer-2 importer genes, *IsrACDBF*; curli genes *csgA* and *csgB*; and also, prophage genes of *E. coli* O157:H7 biofilms (Lee *et al.* 2011).

2.3.1.4. Effects of phytochemicals on the EPS production

It is fundamental to understand the role of natural compounds capable of inhibit the production of EPS in terms of controlling and fighting biofilms (Mishra *et al.* 2020).

Borges *et al.* (2013a) showed that methyl eugenol interfered with EPS production. In agreement, Packiavathy *et al.* (2012) found the methanolic extract of *C. cyminum*, at a concentration of 2 mg/mL, was able to inhibit the production of violacein by *C. violaceum*. In addition, it was demonstrated that the *C. cyminum* extract highly interfered with EPS production and flagellar movement by affecting the physiological functions regulated by AHL and the formation of biofilms. At a sub-MIC level, it promoted damage to the biofilm structure and strongly inhibited the biofilm formation of *P. aeruginosa* PAO1, *P. mirabilis* and *S. marcescens*. The QS inhibitory effects shown by this plant were assigned to methyl eugenol, considering the results of molecular docking analysis (Packiavathy *et al.* 2012). Abraham *et al.*

(2011) assessed the inhibition of EPS production by methanolic fraction of the dried fruits of *Caesalpinia spinosa*. It was observed that the extract of *C. spinosa* interfered with EPS production, swimming and swarming motility, and biofilm formation in *E. coli*, *P. mirabilis*, *S. marcescens* and *P. aeruginosa* PAO1. At a concentration of 2 mg/mL, this extract significantly restricted the EPS production to 58, 46, 66 and 67% and limited biofilm formation to 79, 75, 73 and 70%, in *S. marcescens*, *P. aeruginosa* PAO1, *E. coli* and *P. mirabilis*, respectively. The extract tested revealed a strong QS inhibition effect, in a concentration dependent way, however without interfering with the microbial growth (Abraham et al. 2011).

(-)-Epigallocatechin gallate is a well known compound that has several antimicrobial mechanisms of action, including the ability to decrease EPS production (Borges et al. 2013a). Epigallocatechin gallate is the major component of tea catechins and displayed an inhibitory effect, EPS degradation capacity and a destructive action against *E. coli* biofilms (Maeyama et al. 2005). Taking into account the epigallocatechin gallate effects, Maeyama et al. (2005) developed a new bactericidal surface based on a catechin polymer. The surfaces, containing epigallocatechin gallate, were prepared through photopolymerization of liquid biodegradable polyesters and the rate of releasing was potentiated by increasing the rate of surface-erosion of the polymers. Polymers exhibiting a higher releasing ability showed a lower biofilm development. Moreover, epigallocatechin gallate induced a biofilm-destructing effect such as EPS degradation, damage of bacterial membrane and cell detachment (Maeyama et al. 2005). Lihua et al. (2013) investigated the activity of allicin against *P. aeruginosa* biofilms, specifically their effect on the production of virulence factors controlling the QS mechanism and EPS production. The authors found that the EPS production increased over time but decreased with the concentration of allicin used in the treatment (Lihua et al. 2013).

2.3.2. New perspectives for combinatorial use of natural products

Interestingly, novel antimicrobial strategies based on combinations of phytochemicals with other natural compounds have been described in the literature (Khare et al. 2021). Biomimetics is a revolutionary design concept inspired by nature, its constituents and processes, in order to address and solve complex human issues, in this case, related to infection control (Thanigaiarasu 2020). In the context of biomimetics, combinations of natural compounds, namely those involving phytochemicals (in an attempt to approach the complex mixtures naturally produced by plants) and natural molecules able to mimic the action of antibiotics can be highlighted (AlSheikh et al. 2020). This is the case of phytochemicals, biosurfactants and antimicrobial peptides, all of them with an eminent antimicrobial and antibiofilm activity (Mishra et al. 2020). The solvent used for the extraction of natural compounds and how it can potentiate the antimicrobial properties of the extracted can also be

a good example of biomimetics, when natural extraction solvents are used (Hamidi et al. 2022). A few studies drawing attention to the potential of biomimetics are presented and discussed.

In a recent work developed by Leclercq *et al.* (2020), the authors developed two self-assembled and highly stable emulsions consisting of natural oils with known biological properties, carvacrol and terpinen-4-ol, in combination with the oligosaccharide β -cyclodextrin (a cyclic oligosaccharide compound composed by seven units of glucose). It was demonstrated that the emulsions were able to enhance the antibacterial, antifungal and antibiofilm effect of miconazoylium bromide, a compound for which bioactivity has already been demonstrated. The emulsions based on biocidal/phytochemical were effective against MRSA, *E. coli* and *C. albicans* being carvacrol the most active oil. In fact, the bacterial death for all carvacrol-based emulsions was close to 100%, acting as very effective biofilm-eradicating agent. Moreover, this emulsion was two times more active against *C. albicans* and MRSA than the commercial cream, containing only miconazole nitrate. Also, carvacrol-based emulsions showed a high activity against *E. coli* being this result a significant improved compared to the commercial cream that is completely ineffective against *E. coli*. Finally, this formula provided a synergistic response against *C. albicans* and an additive effect against bacteria (Leclercq et al. 2020).

In another study performed by Sharma *et al.* (2014), it was intended to assess the synergistic effect of curcumin when combined with cinnamaldehyde, eugenol and ellagic acid, against *Staphylococcus epidermidis*. The results showed that the combination of curcumin with cinnamaldehyde provided a synergetic effect against *S. epidermidis* (FICI = 0.5) while the combinations of curcumin and both ellagic acid and eugenol had an additive outcome (FICI = 0.75). Time–kill assay showed that the effect of curcumin and cinnamaldehyde at $0.5 \times \text{MIC}$ was negligible. However, their combination at $1/2 \times \text{MIC}$ and $2 \times \text{MIC}$ (individual MICs values) significantly reduced the bacterial growth (over $2 \log_{10}$ reduction), resulting in synergism. The combination of the two phytochemicals against *S. epidermidis* biofilms also resulted in synergism, but in this case the FICI value was equal to 0.248. (Sharma et al. 2014).

Sharma *et al.* (2020) intended to evaluate the combinations of Bacteriocin-GAM217 (a bacterial extracellular peptide produced by bacteria with antibacterial activity) with two phytochemicals, namely curcumin or cinnamaldehyde. FICI values were in the range between 0.19 and 0.5 so both combinations were classified as synergistic, for *S. epidermidis* and *E. coli*. The combination of Bacteriocin-GAM217 with cinnamaldehyde was able to synergistically inhibit and disrupted >80% of established biofilms of *S. epidermidis* and *E. coli*. The combination of bacteriocin and curcumin had a synergistic effect regarding the inhibition of biofilm formation for *S. epidermidis* and *E. coli* and an additive effect against established biofilm of both microorganisms, with a 4-16-fold reduction in the concentration required to inhibit biofilm formation when compared with the individual MIC. A reduction of 4-8-fold was obtained

for antibiotic-resistant strains (*E. coli* 220, *Enterobacter intermedium* 60, *Citrobacter diversus* 497, *P. aeruginosa* 85 and *K. pneumonia* 507)(Sharma et al. 2020).

A study performed by Malheiro et al. (2019) evaluated the capability of selected phytochemicals and derivatives, including cinnamic acid, cinnamaldehyde, cinnamyl alcohol, allyl cinnamate, methyl trans-cinnamate, cinnamamide, hydrocinnamic acid, alpha-methylhydrocinnamic acid, alpha-methylcinnamic acid, alpha-fluorocinnamic acid, to enhance the activity of two commonly used biocides, lactic acid (LA) and cetyltrimethylammonium bromide (CTAB), against *E. coli* and *S. aureus*. FICI values lower or equal to 1 were obtained for both bacteria, except for cinnamyl alcohol and cinnamamide that did not potentiate the activity of any biocide. Moreover, the combination of LA with allyl cinnamate revealed the higher and stronger activity against *E. coli* biofilms being responsible for a $\log_{10}$ (CFU/cm²) reduction of 2.98 ± 0.76 . The combinations of CTAB were successful for most phytochemicals/derivatives, standing out the combination of CTAB with allyl cinnamate that displayed a maximum effect against sessile *E. coli* corresponding to a $\log_{10}$ (CFU/cm²) reduction equal to 2.20 ± 0.07 , and the combination of CTAB with alpha-methylcinnamic acid with a maximum activity against *S. aureus* biofilms corresponding to a $\log_{10}$ (CFU/cm²) reduction equal to 1.68 ± 0.30 (Malheiro et al. 2019).

In a study developed by Grozdanova et al. (2020), the antimicrobial, genotoxic and cytotoxicity potential of the two extracts, *Sideritis scardica* and *Plantago major*, were investigated. However, these extracts were obtained from plants not with traditional solvents, but with four natural deep eutectic solvents (NADES): Choline chloride:Glycerol (XXGly)(molar ratio 1:2), Citric acid:1,2-Propanediol (CAPD)(molar ratio 1:4), Choline chloride:Glucose plus 30% of Water (XXGIH)(molar ratio 5:2) and Choline chloride:1,2-Propanediol (XXPD)(molar ratio 1:3). NADES are a new generation of solvents consisting of mixtures of one or more primary metabolites from plants. Regarding the extraction performance, it was higher for XXGIH (6.3 ± 0.2 for *S. scardica* and 6.99 ± 0.5 for *P. major*) but showed no antimicrobial activity. Considering MICs and MBCs values, the best antimicrobial effect was obtained for CAPD with *S. scardica* against *S. pyogenes*, *E. coli*, *S. aureus* and *C. albicans* (between 0.098 and 0.39 $\mu\text{g/mL}$). CAPD with *P. major* was active against *S. aureus*, *E. coli* and *C. albicans* (between 1.99 and 3.98 $\mu\text{g/mL}$). Low genotoxicity and cytotoxicity were observed for all four combinations of NADES and extracts with antimicrobial activity (Grozdanova et al. 2020).

Thus, after careful analysis of the studies presented in this section, it was possible to verify and understand the importance of biomimetics in the context of infection control, with a particular focus on the combinations of natural compounds. In fact, all these research works highlight the high potential of the combinations involving phytochemicals and their derivatives in the formulation of solutions for antimicrobial and antibiofilm applications. Therefore, this is a field that should be further explored and receive more attention and efforts from researchers.

Chapter 3 – Bioactivity of single compounds

3.1. Introduction

Pathogenic and virulent microorganisms have been enhancing their ability to survive after being exposed to drugs and antimicrobial agents such as antibiotics. Consequently, several antibiotics became ineffective mainly due to their extensive and inappropriate use (Abreu et al. 2013b). *S. aureus* and *E. coli* are two relevant human pathogens responsible for healthcare-associated infections being two of the most widespread species of Gram-positive and Gram-negative bacteria, respectively (Bannerman et al. 2004; Beenken et al. 2004).

The methods for determining MIC and MBC values are commonly applied to assess the antimicrobial activity of agents against planktonic bacteria (Sykes and Rankin 2013). MIC can be defined as the minimum concentration of a certain drug able to inhibit the growth of a microorganism after a fixed incubation period (commonly 18 h to 24 h). MBC is the minimum concentration of an agent necessary to kill 99.9% of the bacterial population after a defined time, commonly 18 h or 24 h (Parvekar et al. 2020). In fact, there are some *in vitro* factors that can affect MIC and MBC, namely the composition of the medium, the inoculum, the incubation conditions, the host factors and the presence of resistant bacterial subpopulations within the inoculum (Loose et al. 2020).

Cells can generate biofilms when in contact with surfaces, experiencing a diversified spectrum of interactions between themselves and the environment (Donlan 2002). The cells that are part of a biofilm have some particularities, namely the ability to produce EPS constituting the matrix of the biofilm, lower growth rates and the capacity to express specific genes different from those that are active in planktonic cells (Donlan 2002).

It is known that around 80% of chronic and recurrent infections affecting the human body are caused by bacterial biofilm. Moreover, microbial cells that are part of biofilms are ten to one thousand times more resistance to antibiotics than planktonic cells (Sharma et al. 2019). According to Anderson and O'Toole (2008), the four main causes for antibiotic resistance in bacterial biofilms are the poor antibiotic penetration in biofilms, the nutrient limitation and slow growth, the adaptive stress responses and the formation of persister cells. This leads to antibiotics not being able to reach the innermost regions of biofilms, either being inactivated or sequestered (Sabnis et al. 2018). For example, enzymes such as β -lactamases and chloramphenicol acetyltransferases are able to destroy or inactivate antibiotics molecules when they penetrate biofilms (Lewis 2001; Bottery et al. 2021). Another issue that should be considered with regard to biofilm antibiotic resistance is the fact that there are many antibiotics whose action is dependent on microbial growth (Stewart 2002). For instance, β -lactam antibiotics disrupt the cross-linking of peptidoglycans during the cell wall synthesis, which can

only happen during bacterial growth (Whitehouse et al. 2018). Cells in the inner layers of biofilms, having reduced access to nutrients that have difficulty to penetrate these regions, may be metabolically inactive or dormant, so they are expected to be more resistant to antibiotics (Dincer 2020). The induction of the general stress response by cells and their ability to acquire resistance are also important for recalcitrance (Anderson and O'Toole 2008).

Hence, it is essential to treat infectious diseases and avoiding the resistance to antibiotics (Donlan and Costerton 2002). Phytochemicals are known for their capacity to harm planktonic cells and biofilms as well as enhance the activity of antibiotics, despite may not have antimicrobial properties alone (Gorlenko et al. 2020; Rieusset et al. 2020).

The aim of this chapter was to assess the bioactivity of five phytochemicals: 7-hydroxycoumarin, naringin hydrate, theophylline, perillyl alcohol and hydrocinnamic acid against planktonic cells and biofilms of *E. coli* CECT 434 and *S. aureus* CECT 976. Furthermore, their resistance profile to nine antibiotics/combinations of antibiotics was evaluated, allowing the selection of chloramphenicol and amoxicillin. Afterwards, MICs and MBCs values were assessed, and four compounds of interest, namely chloramphenicol, amoxicillin, perillyl alcohol and hydrocinnamic acid, were selected for further assays. All phytochemicals and the selected antibiotics were analysed based on Lipinski's rule of five. Then, the bacterial strains were classified according to their biofilm formation ability. The activity of chloramphenicol, amoxicillin, perillyl alcohol and hydrocinnamic acid against 24 h old biofilms of *E. coli* and *S. aureus* was evaluated. After exposure for 24 h, the culturability of biofilm's cells was determined by CFU counts, biofilm's metabolic inactivation (measured by fluorescence values) was assessed through alamar blue staining method and biomass reduction (measured by OD at 570 nm values) was evaluated by CV staining procedure.

3.2. Materials and methods

3.2.1. Bacterial strains and culture conditions

The bacterial strains tested, *S. aureus* CECT 976 and *E. coli* CECT 434, belong to the Spanish Type Culture Collection (CECT). *S. aureus* and *E. coli* are commonly nosocomial pathogens and some of the most worrying Gram-positive and Gram-negative bacteria, respectively. For these reasons, the development of new strategies and methods to fight them are of extreme importance and urgency (Tan et al. 2014). Moreover, they are considered reference strains because they don't have any resistance known (Borges et al. 2012). Both strains were stored in cryovials with glycerol 30% (v/v) at $(-80 \pm 2) ^\circ\text{C}$. For recovery, bacteria were cultured in Mueller-Hinton Agar (MHA)(Sigma-Aldrich, Merck KGaA, France) plates and

allowed to grow for 24 h at $(37 \pm 2) ^\circ\text{C}$ in a refrigerated incubator with an orbital shaker (IKA® KS 130 shaker).

3.2.2. Phytochemicals and antibiotics

Five phytochemicals (7-hydroxycoumarin, naringin hydrate, theophylline, perillyl alcohol and hydrocinnamic acid) and nine antibiotics/combinations of antibiotics (chloramphenicol, gentamicin, minocycline, ciprofloxacin, levofloxacin, ceftazidime, tetracycline, amoxicillin and sulfamethoxazole/trimethoprim) were assessed in this study. Stock solutions were prepared for all the compounds, with a certain concentration and using dimethyl sulphoxide (DMSO)(Avantor, VWR, France) as solvent. Compounds were weighed on an analytical balance (A&D Weighing, model GR-200). After their preparation, stock solutions were stored at $(-18 \pm 2) ^\circ\text{C}$, in a freezer (Balay, 3KVB5610). **Appendix B** contains information about the chemical, structural and molecular properties of these molecules.

Table 4 shows the disk content, the zone diameter breakpoint (ZDB) values and MICs breakpoint values established by the Clinical & Laboratory Standards Institute (CLSI) guidelines (2022) for susceptibility tests with *S. aureus*.

Table 4. Disk content and breakpoints for *S. aureus*.

	<i>Disk content</i> (μg)	<i>ZDB (mm)</i>			<i>MIC ($\mu\text{g/mL}$)</i>		
		S	I	R	S	I	R
<i>Gentamicin</i>	10	≥ 15	13-14	≤ 12	≤ 4	8	≥ 16
<i>Minocycline</i>	30	≥ 19	15-18	≤ 14	≤ 4	8	≥ 16
<i>Ciprofloxacin</i>	5	≥ 21	16-20	≤ 15	≤ 1	2	≥ 4
<i>Levofloxacin</i>	5	≥ 19	16-18	≤ 15	≤ 1	2	≥ 4
<i>Sulfamethoxazole/Trimethoprim</i>	1,25/23,75	≥ 16	11-15	≤ 10	$\leq 2/38$	-	$\geq 4/76$
<i>Chloramphenicol</i>	30	≥ 18	13-17	≤ 12	≤ 8	16	≥ 32
<i>Tetracycline</i>	30	≥ 19	15-18	≤ 14	≤ 4	8	≥ 16
<i>Amoxicillin</i>	10	≥ 29	-	≤ 28	≤ 0.12	-	≥ 0.25

S – Susceptible; I - Intermediate; R - Resistant

Ceftazidime was not tested against *S. aureus* because, according to the CLSI guidelines (2022), it is not used to combat infections caused by *S. aureus*, as it has little or no activity against most Gram-positive organisms. In fact, this antibiotic is inactive against staphylococcal

microorganisms because it presents a reduced binding to enzymes that function in staphylococcal cell wall synthesis (CLSI 2022).

Table 5 presents the disk content and breakpoints established by the CLSI guidelines (2022) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (2022) for susceptibility tests with *E. coli*.

Table 5. Disk content and breakpoints for *E. coli*.

	Disk content (μg)	ZDB (mm)			MIC ($\mu\text{g/mL}$)		
		S	I	R	S	I	R
Gentamicin	10	≥ 15	13-14	≤ 12	≤ 4	8	≥ 16
Minocycline	30	≥ 16	13-15	≤ 12	≤ 4	8	≥ 16
Ciprofloxacin	5	≥ 26	22-25	≤ 21	≤ 0.25	0.5	≥ 1
Levofloxacin	5	≥ 21	17-20	≤ 16	≤ 0.5	1	≥ 2
Sulfamethoxazole/ Trimethoprim	1,25/23,75	≥ 16	11-15	≤ 10	$\leq 2/38$	-	$\geq 4/76$
Ceftazidime	30	≥ 21	18-20	≤ 17	≤ 4	8	≥ 16
Chloramphenicol	30	≥ 18	13-17	≤ 12	≤ 8	16	≥ 32
Tetracycline	30	≥ 15	12-14	≤ 11	≤ 4	8	≥ 16
Amoxicillin	-	-	-	-	≤ 8	-	>8

S – Susceptible; I - Intermediate; R – Resistant

3.2.3. Antibiogram

Antibiogram was performed using the Kirby Bauer method, as described by Gerbig Jr. *et al.* (2013). Nine antibiotics/combinations of antibiotics were tested in this assay: levofloxacin, ceftazidime, minocycline, ciprofloxacin, gentamicin, chloramphenicol, tetracycline, amoxicillin and sulfamethoxazole/trimethoprim. Antibiotics were in the form of commercially available disks with 6 mm of diameter (Oxoid, UK) with a fixed concentration, according to CLSI guidelines (2022). First, the inoculum was prepared by growing *S. aureus* and *E. coli* overnight, in 50 mL of liquid culture medium, at $(37 \pm 2) ^\circ\text{C}$ and with agitation (150 rpm), using the same refrigerated incubator with an orbital shaker. According to CLSI, the medium of choice for the susceptibility testing should fulfil some requirements such as: (i) the composition of the medium should give sufficient growth for the strain tested, (ii) the medium must not contain material that could interfere with the test itself or react with any drug tested, and (iii) the media used in performing such tests should be specifically formulated. Therefore, Mueller-Hinton Broth (MHB)(Millipore, Merck KGaA, Germany) was used to prepare the inoculum and to performed

the microdilution method because it is recommended by CLSI guidelines, allows the growth of non-fastidious microorganisms, does not require supplementation and meets all CLSI requirements mentioned above (Darwish et al. 2008; Holland et al. 2009; CLSI 2018). For solvent change, approximately 30 mL of bacterial cultures were transferred to 50 mL Falcon tubes (Avantor, VWR, USA) and centrifuged (Eppendorf centrifuge 5810R) for 10 min at 4000 rpm. After centrifugation, the supernatant was removed, and the pellet was resuspended in approximately 30 mL of sterile NaCl (8.5 g/L NaCl)(Avantor, VWR, Belgium). These steps of centrifugation and resuspension of the pellet in sterile NaCl were repeated for a second time. The turbidity was then adjusted to 0.5 McFarland Standard, that is, OD_{620nm} of 0.040 ± 0.020 (1.5×10^8 CFU/mL), for *E. coli*, and OD_{600nm} of 0.132 ± 0.020 (1.5×10^8 CFU/mL), for *S. aureus*, in sterile NaCl. The bacterial culture was spread on MHA plates, using a sterile cotton swab. After setting for 15 min, the antibiotic disks were placed on the MHA plates already inoculated. Finally, the plates were incubated for 24 h at $(37 \pm 2) ^\circ C$, using the same refrigerated incubator. Each inhibition zone diameter (IZD) was measured, registered and evaluated according to CLSI (2022) guidelines.

3.2.4. MIC determination

MIC values were determined by the microdilution method according to CLSI guidelines (2018). First, the inoculum was prepared by growing *S. aureus* and *E. coli* overnight, in 50 mL of MHB, at $(37 \pm 2) ^\circ C$ and with agitation (150 rpm), using the same refrigerated incubator with an orbital shaker. Then, the concentrations tested were prepared by starting from the stock solution and making successive dilutions with DMSO. The concentrations tested for phytochemicals were between 16 $\mu g/mL$ and 2048 $\mu g/mL$ and in the case of antibiotics were in the range from 0.01 $\mu g/mL$ to 102.4 $\mu g/mL$. The final concentrations were achieved on the sterile 96-wells flat-bottomed polystyrene tissue culture microtiter plates (Avantor, VWR, USA).

Then, 10 μL of each dilution was pipetted into the wells of the same column of the microtiter plate. The OD of the inoculum was then adjusted: for *S. aureus* the OD_{600nm} should be equal to 0.132 ± 0.020 (approximately 1.5×10^8 CFU/mL) and for *E. coli* the OD_{620nm} should be equal to 0.040 ± 0.020 (approximately 1.5×10^8 CFU/mL), in sterile fresh MHB. For this purpose, a spectrophotometer (Thermo Electron Corporation, EP1000T) was used. Control wells contained only sterile fresh MHB without bacterial cells, cells with DMSO 5% (190 μL of cells and 10 μL of DMSO) and only cells (200 μL). Bacteria-free controls constituted by the drugs in sterile fresh MHB were also prepared. To the testing wells, 190 μL of inoculum was added. Afterwards, it was necessary to measure the absorbance of microtiter plates, at 0 h, using a microtiter plate reader (SPECTROstar® Nano, BMG LABTECH). The microtiter plates were then incubated for 24 h under the same growth conditions and using the same

refrigerated incubator with an orbital shaker. After 24 h, the absorbance was read (at 600 nm for *S. aureus* and 620 nm for *E. coli*), using the same equipment.

To interpret the results obtained, it was necessary to start by checking the control wells: in wells containing sterile fresh MHB, it should be noted that there was no growth; in wells containing inoculum only, microbial growth should be recorded and in wells with inoculum and DMSO, it should be noted that DMSO 5% did not inhibit microbial growth. In fact, the negative control with DMSO 5% caused no inhibition of bacterial growth. Then, the mean and standard deviation for the absorbance values were calculated. After comparing the means obtained at 0 h and 24 h, it was necessary to search for the concentration from which the values at 24 h were equal to or lower than those recorded at 0 h: MIC.

3.2.5. MBC determination

MBCs values were determined by drop plate method according to CLSI guidelines (1999). After the incubation of microtiter plates, 10 μ L were taken from three different wells of each column, gently scraping the bottom of the well with the tip of the micropipette (Eppendorf Research, Merck KGaA, France) to loosen the adhered biofilm, and plated in MHA. Then, the Petri dishes (Avantor, VWR, France) were incubated in the same refrigerated incubator, at $(37 \pm 2^\circ\text{C})$ for 24 h. The MBC was then determined visually by observing the lowest concentration from which colonies did not grow. In the case of bacteria alone and bacteria with DMSO, colony formation was always observed.

3.2.6. Biofilm bacteria classification

The biofilm formation assay was performed according to a modified microtiter plate method described by Stepanović *et al.* (2000). Briefly, 50 mL of *S. aureus* and *E. coli* cultures were grown overnight at $(37 \pm 2)^\circ\text{C}$ and 150 rpm, using the same refrigerated incubator with an orbital shaker, and 200 μ L of bacterial suspension ($\text{OD}_{620\text{nm}}$ of 0.040 ± 0.020 (1.5×10^8 CFU/mL), for *E. coli*, and $\text{OD}_{600\text{nm}}$ of 0.132 ± 0.020 (1.5×10^8 CFU/mL), for *S. aureus*, in sterile fresh MHB) was transferred into the wells of two sterile microtiter plate. They were incubated aerobically in the same refrigerated incubator with an orbital shaker at 150 rpm and $(37 \pm 2)^\circ\text{C}$, one for 24 h and another for 48 h. For the 48 h old biofilms the medium was carefully discarded and replaced by fresh one on a daily basis. After each biofilm development period, the content of the wells was removed, and each well was washed with 200 μ L of NaCl solution to discard non-adhered bacteria. The remaining attached bacteria were fixed with 250 μ L of ethanol 99% (Avantor, VWR, France) for 15 min. Then, after discarding the content of each well, the fixed bacteria were stained for 5 min with 200 μ L of CV 1% (Millipore, Merck KGaA,

Germany). After, the excess of stain was gently withdrawn. Finally, the dye bound to the adherent cells was solubilized with 200 μ L of glacial acetic acid 33% (v/v) (Thermo Fisher Scientific, Fisher Scientific GmbH, UK). The OD was then measured at 570 nm using the same microtiter plates reader. In addition, control wells contained sterile fresh MHB without bacterial (200 μ L) cells and cells only (200 μ L).

Bacteria were classified using the scheme of Stepanović *et al.* (2000) as follow - non-biofilm producer: $OD \leq OD_c$ (OD of the control); weak biofilm producer: $OD_c < OD \leq 2 \times OD_c$; moderate biofilm producer: $2 \times OD_c < OD \leq 4 \times OD_c$; strong biofilm producer: $OD > 4 \times OD_c$.

3.2.7. Evaluation of biofilm control for single compounds

3.2.7.1. *Biofilm formation*

This assay was performed according to Stepanović *et al.* (2000). Bacterial cells were grown overnight using 50 mL of sterile fresh MHB at $(37 \pm 2)^\circ\text{C}$ and 150 rpm, using the same refrigerated incubator with an orbital shaker. After the overnight growth, the cultures were adjusted to an $OD_{620\text{nm}}$ of 0.040 ± 0.020 (1.5×10^8 CFU/mL), for *E. coli*, and to an $OD_{600\text{nm}}$ of 0.132 ± 0.020 (1.5×10^8 CFU/mL), for *S. aureus*, with sterile fresh MHB. Sterile microtiter plates were filled with 200 μ L of bacterial suspension. In addition, control wells contained sterile fresh MHB without bacterial (200 μ L) cells and cells only (200 μ L). The microtiter plates were incubated for 24 h at $(37 \pm 2)^\circ\text{C}$ and agitated with 150 rpm, using the same refrigerated incubator with an orbital shaker.

3.2.7.2. *Biofilm eradication (biofilm control)*

This assay was performed according to Simões *et al.* (2010). After biofilm formation for 24 h, the content of each well was removed and washed with 200 μ L of sterile NaCl solution. Afterwards, 10 μ L of each compound at MIC, 5×MIC and 10×MIC was applied in each well and 190 μ L of sterile fresh MHB was also added. In control wells with sterile fresh MHB, it was added 200 μ L of sterile fresh MHB and in wells that contained only cells, it was added 200 μ L of sterile fresh MHB. For negative control, 10 μ L of DMSO was added to 190 μ L of sterile fresh MHB. Then, the microtiter plates were incubated for more 24 h at $(37 \pm 2)^\circ\text{C}$ and agitated with 150 rpm, using the same refrigerated incubator with an orbital shaker.

According to Lemos *et al.* (2014), biofilm metabolic activity inactivation and biomass reduction were classified as follow - low efficacy: $< 25\%$; moderate efficacy: $\geq 25\%$ to $< 60\%$; high efficacy: $\geq 60\%$ to $< 90\%$; and excellent efficacy: $\geq 90\%$ to $\leq 100\%$.

3.2.7.3. Biofilm mass quantification by crystal violet staining

This assay was performed according to Chaves *et al.* (2007). The content of each well was removed, and the wells were washed as explained in section 3.2.6. The remaining attached bacteria were fixed with 250 μ L of ethanol 99% for 15 min. Then, after discarding the content of each well, the fixed bacteria were stained for 5 min with 200 μ L of CV 1%. After, the excess of stain was gently withdrawn. Finally, the dye bound to the adherent cells was solubilized with 200 μ L of glacial acetic acid 33% (v/v). The OD was measured at 570 nm using the same microtiter plates reader. The percentage of biomass reduction was calculated according to **Equation (1)**, where OD control₅₇₀ is the OD of the control at 570 nm and OD compounds₅₇₀ is the OD of the tested compounds at 570 nm (Monte et al. 2014).

$$\% \text{ of Biomass Removal} = \frac{OD_{control_{570}} - OD_{compounds_{570}}}{OD_{control_{570}}} \times 100 \quad (1)$$

3.2.7.4. Biofilm metabolic activity quantification by alamar blue

This assay was performed according to Sarker *et al.* (2007). The content of each well was removed, and the wells were washed as explained in section 3.2.6. For the staining procedure, 190 μ L of sterile fresh MHB was added to the microtiter plates. To each well, 10 μ L of alamar blue indicator solution (0.4 mM)(Sigma-Aldrich, Merck KGaA, France) was added. Then, microtiter plates were incubated during 20 min in darkness at $(37 \pm 2)^\circ\text{C}$ and 150 rpm, using the same refrigerated incubator with an orbital shaker. Finally, fluorescence was measured at $\lambda_{excitation} = 570 \text{ nm}$ and $\lambda_{emission} = 590 \text{ nm}$ with another microtiter plate reader (FLUOstar® Omega, BMG LABTECH). The percentage of metabolic inactivation was calculated according to **Equation (2)**, where FLUO_{control} is the fluorescence intensity of the control, that is, the biofilms not treated while FLUO_{compounds} is the fluorescence intensity of the biofilms exposed to the compounds tested (Monte et al. 2014).

$$\% \text{ of Metabolic Inactivation} = \frac{FLUO_{control} - FLUO_{compounds}}{FLUO_{control}} \times 100 \quad (2)$$

3.2.7.5. Quantification of the biofilm culturable cells

This assay was performed following the drop plate method, according to Herigstad *et al.* (2001). The content of each well was removed, and the wells were washed as explained in section 3.2.6. Biofilms were scraped three times (during periods of 1 minute each) after the addition of 200 μ L of sterile NaCl in order to resuspend the attached sessile cells. The content

of each well was transferred to independent sterile labeled microcentrifuge tubes (Avantor, VWR, USA). The first tube contained 200 μL of each scrapping step (a total of 600 μL after the three scrapping steps) and 400 μL of sterile NaCl. In this assay, one control was included, consisting of untreated bacteria. Afterwards, ten-fold serial dilutions in sterile NaCl were performed, from 10^{-1} to 10^{-7} . Then, 10 μL of each dilution was plated on MHA plates and incubated at $(37 \pm 2)^\circ\text{C}$ for 24 h, using the same refrigerated incubator. Finally, CFU were visually counted for plates containing > 10 and < 100 colonies and expressed per square centimeter of microtiter plate well: $\log_{10} (\text{CFU}/\text{cm}^2)$, following the **Equation (3)** and **Equation (4)**, where n is the number of CFU, SV is the sample volume, 0.01 mL, WWV is the working volume of each well, 0.2 mL, and 1.53 is the area of each well, in cm^2 .

$$\frac{\text{CFU}}{\text{mL}} = \frac{n}{SV \times \text{Dilution}} \quad (3)$$

$$\frac{\text{CFU}}{\text{cm}^2} = \frac{\frac{\text{CFU}}{\text{mL}} \times WWV}{1.53} \quad (4)$$

The quantification of the biofilm culturable cells was also assess through the logarithmic reduction, $\log_{10}$ reduction, according to **Equation (5)**, where N_0 and N are the CFU/cm^2 for the untreated bacteria (control) and treated bacteria, respectively. One control was included, consisting of untreated bacterial culture.

$$\log_{10} \text{ reduction} = \log_{10}(N_0) - \log_{10}(N) \quad (5)$$

3.2.8. Statistical analysis

Data were analyzed using the statistical program SPSS (Statistical Package for the Social Sciences) version 28.0 (IBM Corp., Armonk, New York, USA). Bearing in mind that a small sample leads to uneven variation, a nonparametric test, more specifically the Wilcoxon statistical test, was used to assess the statistical significance value. For assays involving CFU counting, statistical analysis was performed using One-Way ANOVA test followed by Sidak's multiple comparisons test. The results were presented as mean $\pm$ standard deviation. Significance level for the differences was set at $p < 0.05$ and the calculations were based on confidence level $\geq 95\%$. At least two independent experiments, with at least two replicates, were performed for each tested condition.

3.3. Results and discussion

3.3.1. Antibiogram results and MICs and MBCs values

Antibiogram (for antibiotics) and the determination of MICs and MBCs values are commonly performed for assessing the antimicrobial activity of molecules against planktonic bacteria (Balouiri et al. 2016).

In this study, the susceptibility profile of the reference strains *E. coli* CECT 976 and *S. aureus* CECT 434, both without any resistance known, to nine antibiotics/combinations of antibiotics was tested.

Table 6 shows the classification of *S. aureus* and *E. coli* regarding their antibiotic resistance profile based on IZDs values and their comparison with CLSI (2022) and EUCAST (2022) breakpoints.

Table 6. Antibiotic resistance profile of *S. aureus* and *E. coli*. Values are the mean $\pm$ standard deviation for at least two independent experiments.

	<i>S. aureus</i>		<i>E. coli</i>	
	IZD (mm)	Classification	IZD (mm)	Classification
<i>Gentamicin</i>	21.5 $\pm$ 0.7	S	20.2 $\pm$ 0.4	S
<i>Minocycline</i>	32.4 $\pm$ 0.8	S	29.3 $\pm$ 1.0	S
<i>Ciprofloxacin</i>	35.5 $\pm$ 0.7	S	42.0 $\pm$ 0.0	S
<i>Levofloxacin</i>	34.9 $\pm$ 0.2	S	32.5 $\pm$ 0.7	S
<i>Sulfamethoxazole/Trimethoprim</i>	28.0 $\pm$ 0.0	S	26.4 $\pm$ 0.6	S
<i>Ceftazidime</i>	-	-	29.8 $\pm$ 0.3	S
<i>Chloramphenicol</i>	23.5 $\pm$ 0.7	S	16.3 $\pm$ 0.4	I
<i>Tetracycline</i>	30.0 $\pm$ 0.0	S	28.5 $\pm$ 0.7	S
<i>Amoxicillin</i>	38.2 $\pm$ 0.3	S	12.2 $\pm$ 0.2	-

S – Susceptible; I – Intermediate; R – Resistant

In this study, the evaluation of antimicrobial activity against planktonic cells was also assessed through the determination of MICs and MBCs values. MICs values were understood as the lowest concentration that visibly inhibited bacterial growth after 24 h. MBCs values were interpreted as the lowest concentration that could lead to 99.9% of bacterial death after 24 h (Borges et al. 2013b). MICs and MBCs values were both expressed with units of $\mu\text{g/mL}$. **Table 7** shows the results of MICs and MBCs for five phytochemicals and two antibiotics, against *S.*

aureus and *E. coli*. Chloramphenicol and amoxicillin were selected for further studies because they provided a low IZD for at least one of the tested microbial strains (*E. coli*).

Table 7. MICs and MBCs values. Values are the mean $\pm$ standard deviation for at least two independent experiments.

	<i>S. aureus</i>		<i>E. coli</i>	
	MIC ($\mu\text{g/mL}$)	MBC ($\mu\text{g/mL}$)	MIC ($\mu\text{g/mL}$)	MBC ($\mu\text{g/mL}$)
<i>Chloramphenicol</i>	4	64	16	64
<i>Amoxicillin</i>	0.08	2	8	8
<i>Naringin hydrate</i>	> 2048	> 2048	> 2048	> 2048
<i>Theophylline</i>	> 2048	> 2048	> 2048	> 2048
<i>7-Hydroxycoumarin</i>	> 2048	> 2048	> 2048	> 2048
<i>Perillyl alcohol</i>	1024	2048	256	512
<i>Hydrocinnamic acid</i>	2048	> 2048	2048	> 2048

For both bacteria, that four compounds displayed detectable MICs values (up to 2048 $\mu\text{g/mL}$): chloramphenicol with a MIC of 4 $\mu\text{g/mL}$ for *S. aureus* and 16 $\mu\text{g/mL}$ for *E. coli*; amoxicillin with a MIC of 0.08 $\mu\text{g/mL}$ for *S. aureus* and 8 $\mu\text{g/mL}$ for *E. coli*; perillyl alcohol with a MIC of 1024 $\mu\text{g/mL}$ for *S. aureus* and 256 $\mu\text{g/mL}$ for *E. coli* and hydrocinnamic acid with a MIC of 2048 $\mu\text{g/mL}$ for both bacteria. For the other compounds, MICs was not detected, in other words, it was higher than 2048 $\mu\text{g/mL}$. Regarding MBCs values, only three compounds exhibited detectable values (up to 2048 $\mu\text{g/mL}$): chloramphenicol, with a MBC value of 64 $\mu\text{g/mL}$ for both bacteria; amoxicillin with a MBC of 2 $\mu\text{g/mL}$ for *S. aureus* and 8 $\mu\text{g/mL}$ for *E. coli* and perillyl alcohol with a MBC value of 2048 $\mu\text{g/mL}$ for *S. aureus* and 512 $\mu\text{g/mL}$ for *E. coli*. All MICs were lower or equal to MBCs, as expected since the concentration of a given compound capable of inhibiting microbial growth can not be higher than the necessary concentration to cause bacterial death. With this study, it was possible to conclude that, from all the compounds, the ones providing the lowest MICs and MBCs values were both antibiotics. Considering the phytochemicals tested, the most effective compound against *S. aureus* and *E. coli* was perillyl alcohol, followed by hydrocinnamic acid.

It is also important to point out the data referring to MICs and MBCs contained in the literature are difficult to compare. These values are not constant for a given antimicrobial agent, which can be explained by the use of various methods for assessing antibacterial activity, different solvents, different origin and purity of the compounds tested, often isolated from various plant extracts and different bacterial strains (Adamczak et al. 2019). These results are also influenced by the amount of inoculum, the incubation time, the composition of the culture medium and environmental conditions such as temperature, pH and aeration. Only when all these parameters are standardized it is possible to accurately compare the antimicrobial

activity of a given agent against a certain strain or analyze the antibacterial activity of the same compound for different microorganisms (Ostrosky et al. 2008).

Regarding the molecules with detectable MICs values, and starting with chloramphenicol, the values found in the literature vary considerably. This is probably due to the fact that chloramphenicol is a bacteriostatic agent, which means that its mechanism of action stalls bacterial cellular activity without directly causing bacterial death, in this case, through inhibition of bacterial protein synthesis, which can lead to a greater variability in its antimicrobial activity (Bakheit et al. 2014). Some other studies also report higher MICs values for *E. coli* when compared to *S. aureus*, as observed in this work (Sueke et al. 2010; Kidsley et al. 2018; Wozniak and Grinholc 2018; Lorenzo 2019). In fact, a study carried out by Sueke et al. (2010) claimed a range between 0.200 and 256 µg/mL for *S. aureus*. In the case of *E. coli*, a study developed by Kidsley et al. (2018) obtained a MIC of 16 µg/mL. These values are in agreement with the ones obtained in this study. In fact, Gram-negative bacteria such as *E. coli* have an OM that make them less susceptible than Gram-positive bacteria such as *S. aureus*, although Gram-positive bacteria have a thicker peptidoglycan layer (Vaara 1992; Simões et al. 2008; Monte et al. 2014). Gram-negative bacteria have lipopolysaccharides in their OM, which constitute a barrier that hinders penetration. Also, the porins of the OM do not allow the passage of hydrophobic compounds (Bos and Tommassen 2004; Monte et al. 2014). However, the mechanism of action of the various compounds depends on the ease with which molecules cross the cell wall and the cell membrane of bacteria and on the site of action. In the case of chloramphenicol, its site of action is the cytoplasm (more precisely, the ribosomes). Consequently, the activity of chloramphenicol will also depend on the affinity of this antibiotic for the constituents of the cell wall and the cell membrane (Fair and Tor 2014; Kapoor et al. 2017). In the case of amoxicillin, it is a semisynthetic penicillin derivative with a bactericidal activity against susceptible bacteria. This antibiotic inhibits the cell wall biosynthesis, leading to the bacterial death, along the active multiplication stage (Castle 2007). Gram-negative bacteria are more resistant to antibiotics, namely chloramphenicol and amoxicillin, than Gram-positive ones (Breijyeh et al. 2020), as demonstrated in this study. In a study published by Girlich et al. (2005) a MIC for amoxicillin against *E. coli* of 8 µg/mL was determined and Yang et al. (2017a) obtained a MIC for amoxicillin against *S. aureus* equal to 0.125 µg/mL. Both values are in agreement with the values obtained in this study. Regarding perillyl alcohol, the exact antimicrobial mechanism of action is unknown, but it might be involved with the cell membrane lysis (Chastain et al. 1992). In fact, the results of the present study suggest this phytochemical had greater affinity for some cell wall element of *E. coli* when compared to *S. aureus*. In the case of hydrocinnamic acids, the literature suggests that most of the phenolic acids have a stronger antibacterial activity against Gram-positive bacteria than Gram-negative bacteria (Patra 2012; Merkl et al. 2018). Based on the present study and on the literature,

hydrocinnamic acid seems to be an exception (Shaala et al. 2020). Although no justification for this fact was found, it can be hypothesized that perhaps hydrocinnamic acid had a greater affinity for the cell wall and/or the cell membrane of *E. coli*. In addition, the antimicrobial mechanisms of phenolic acids are related to cell membrane damage, virulence factors limitation and biofilm formation inhibition (Miklasínska-Majdanik et al. 2018).

Regarding the compounds with a MIC greater than 2048 µg/mL, and focusing on naringin hydrate, studies as the ones conducted by Özçelik et al. (2011) and Basile et al. (1999) showed a greater activity of alkaloids against *E. coli* than *S. aureus*. A study developed by Efimova et al. (2020) suggested the primary target of action of alkaloids is the cell membrane being that the amphiphilic nature of alkaloids certainly facilitates their incorporation into the cell membranes, affecting the activity of diverse receptors, ion channels and enzymes (Efimova et al. 2020). Regarding theophylline, the literature seems to suggest there is not a significant difference between its activity on Gram-positive and Gram-negative bacteria (Abreu et al. 2014; Wambaugh et al. 2017). However, the concrete mechanism of toxicity of theophylline has not yet been described (Olson 2012). With respect to 7-hydroxycoumarin, a study developed by Yang et al. (2016) defended that hydroxycoumarins can destroy the cell membrane through the repression of flagellar genes *fliA* and *flhC* (Yang et al. 2016). Moreover, a study developed by Monte et al. (2014) showed a greater activity of 7-hydroxycoumarin against *S. aureus* than *E. coli* (Monte et al. 2014).

It is possible to find in literature references to several natural products from plants and extracts with MICs values above 1000 µg/mL (Monte et al. 2014). However, these values are considered to have little clinical relevance (Gibbons 2004). Molecules are considered as antimicrobial agents if they display a MIC in the range of 100-1000 µg/mL (Tegos et al. 2002; Borges et al. 2012). Accordingly, in this study, only chloramphenicol, amoxicillin and perillyl alcohol can be classified as antimicrobial agents. However, although not all the phytochemicals assessed in this study can be considered antimicrobial agents, all of them could show promising results when incorporated in combinations.

In brief, both MICs values and antibiogram results showed that the *E. Coli* strain evaluated is close to acquiring resistance to chloramphenicol (based on MIC and IZD) and amoxicillin (only based on MIC value), so it would be interesting to act in order to prevent this strain from developing a "clear" resistance to these antibiotics. In fact, resistance can happen easily and quickly, for example through mutations or changes in the environmental conditions (Martinez and Baquero 2000). When resistance is on the verge, it becomes urgent to ensure that it is not fully developed. To this end, combinations of antibiotics with other antimicrobial agents could be a good strategy (Annunziato 2019). When resistance has already been developed, it would not be reversed unless there is a structural modification in the antibiotic or

if it is used in combination with another drug (Jasovský et al. 2016). On the other hand, *S. aureus* remains susceptible to all the antibiotics tested.

3.3.2. Lipinski's rule of five analysis

“Drug-likeness” properties of the compounds tested were assessed according to the rule of five described by Lipinski (2004) and are presented in **Table 8**. The rule of five was initially developed in order to evaluate orally active compounds regarding four physicochemical parameter, namely molecular weight (MW), octanol-water partition coefficient ($\log P$), the number of hydrogen bond acceptors (n-ON acceptors) and the number of hydrogen bond donors (n-OH/NH donors). “Drug-like” molecules must present some properties, namely solubility in water and fat, because an orally administered drug has to pass the intestinal lining, be transported in blood (an aqueous solution) and penetrate the lipid-based cell membrane in order to reach the intracellular medium (Walters et al. 1999). When it is intended that a certain compound presents an acceptable water and lipidic solubility as well as intestinal permeability, the first parameter should be ≤ 500 (g/mol), the second ≤ 5 , the third ≤ 10 and the fourth ≤ 5 (Lipinski 2004). If a certain compound does not meet one or more rules, there is a high chance of oral activity issues (Lipinski 2004). Only naringin hydrate does not meet all these parameters, failing in three of them: MW with a value of 580.54 g/mol, the number of hydrogen bond acceptors which was equal to 14 and the number of hydrogen bond donors that was equal to 8. Nevertheless, this rule is limited as it can not predict the biological activity nor the toxicity of molecules (Lipinski 2004). Data was calculated with Molinspiration Cheminformatics software (Bratislava University, Slovensky Grob, Slovak Republic), available at www.molinspiration.com.

Table 8. Structural and molecular properties of the molecules evaluated, where MW is the molecular weight, Log *P* is the octanol-water partition coefficient, n-ON acceptors is the number of hydrogen bond acceptors and n-OHND donors is the number of hydrogen bond donors. These data was determined with Molinspiration Cheminformatics software.

<i>Name and molecular structure</i>	<i>Chemical structure</i>	<i>MW (g/mol)</i>	<i>Log P</i>	<i>n-ON acceptors</i>	<i>n-OHND donors</i>
Chloramphenicol $C_{11}H_{12}Cl_2N_2O_5$		323.13	0.73	7	3
Amoxicillin $C_{16}H_{19}N_3O_5S$		365.41	-1.35	8	5
Perillyl alcohol $C_{10}H_{16}O$		152.24	2.37	1	1
Hydrocinnamic acid $C_9H_{10}O_2$		150.18	1.88	2	1
7-Hydroxycoumarin $C_9H_6O_3$		162.14	1.51	3	1
Naringin hydrate $C_{27}H_{34}O_{15}$		580.54	-0.37	14	8
Theophylline $C_7H_8N_4O_2$		180.17	-0.01	6	1

3.3.3. Biofilm bacteria classification

It is widely known that biofilm formation is a relevant way for bacterial cells to ensure their survival, both in natural and artificial environments (Tanaka et al. 2017). This process has been increasingly accepted as a passive virulence factor, helping the establishment of pathogenic microorganisms (Schulze et al. 2021). For this reason, studying the ability of a given bacterial strain to form biofilms is considered very important (Molobela and Ilunga 2012). In this study, the ability of the strains tested to form biofilms was assessed by the values of OD at 570 nm as a measure of the biomass for 24 h and 48 h old biofilms (**Figure 3**).

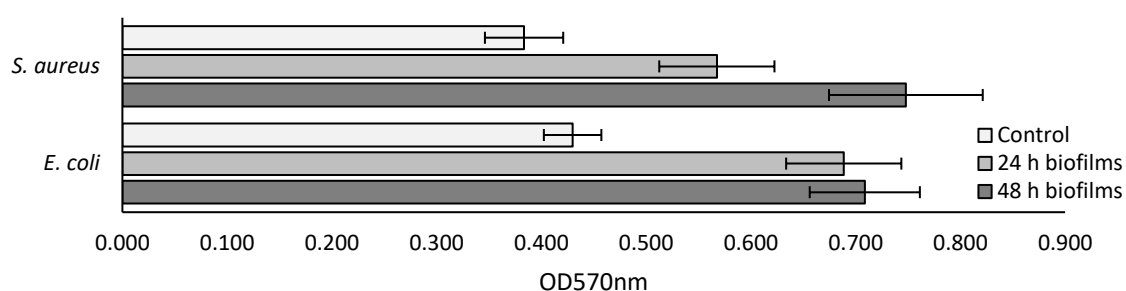


Figure 3. OD at 570 nm as a measure of 24 and 48 h old biofilms mass. Values are the mean $\pm$ standard deviation for at least two independent experiments.

The results showed that both strains were able to form biofilms, however they had not statistically distinct productivities ($p > 0.05$). Both bacteria were classified as weak biofilm producers, according to the classification of Stepanović et al. (2000) in **Table 9**, indicating that it may be less difficult to fight these bacterial structures (Lajhar et al. 2018). This classification was based on the difference between the values for the control (sterile fresh MHB) and for biofilms with 24 h and 48 h, with statistical significance difference ($p < 0.05$). The highest biomass amounts were observed at 48 h for both bacteria, with *S. aureus* producing the greatest biofilm mass. In fact, both bacteria have been previously classified as weak biofilm (Monte 2013).

Table 9. Biofilm bacteria classification (Stepanović et al. 2000).

Biofilm bacteria classification	OD values
<i>Non-biofilm producer</i>	$OD \leq OD_c$
<i>Weak biofilm producer</i>	$OD_c < OD \leq 2 \times OD_c$
<i>Moderate biofilm producer</i>	$2 \times OD_c < OD \leq 4 \times OD_c$
<i>Strong biofilm producer</i>	$OD > 4 \times OD_c$

3.3.4. Evaluation of biofilm control for single compounds

S. aureus and *E. coli* are capable of developing three-dimensional structures, called biofilms, responsible for the adhesion of cells to a surface (in the innermost layers of the biofilm) or to other cells (in the case of the outermost layers). Bacterial cells in biofilms are incorporated into the polymeric extracellular matrix, providing them a protective environment to external agents such as antibiotics (Pinto et al. 2019). Therefore, it becomes clear that the control and eradication of biofilms is a very complicated task (Sillankorva et al. 2004; Monte et al. 2014). This is mainly due to the inefficient penetration of molecules into the biofilm matrix (Anderson and O'Toole 2008; Srivastava and Bhargava 2016; Pinto et al. 2019), variations in the microenvironment of biofilms influencing the efficiency of antibacterial agents (del Pozo and Patel 2007) as well as varied mechanisms responsible for their resistance to diverse compounds (Pinto et al. 2019).

For these reasons, the effects of chloramphenicol, amoxicillin, perillyl alcohol and hydrocinnamic acid against *E. coli* and *S. aureus* biofilms, at concentrations equal to MIC, 5×MIC and 10×MIC, were evaluated. These four compounds were selected for biofilm control assays based on their IZDs (in the case of antibiotics) and MICs values (for antibiotics and phytochemicals). Biofilm formation was conducted for 24 h in sterile microtiter plates and then, the capacity of the tested compounds to reduce the biomass, to inactivate the metabolic activity of biofilms and to reduce their cellular culturability were studied.

Figure 4 shows the percentages of metabolic inactivation, for *S. aureus* and *E. coli* 24 h biofilms, after exposure to the tested compounds at MIC, 5×MIC and 10×MIC for 24 h.

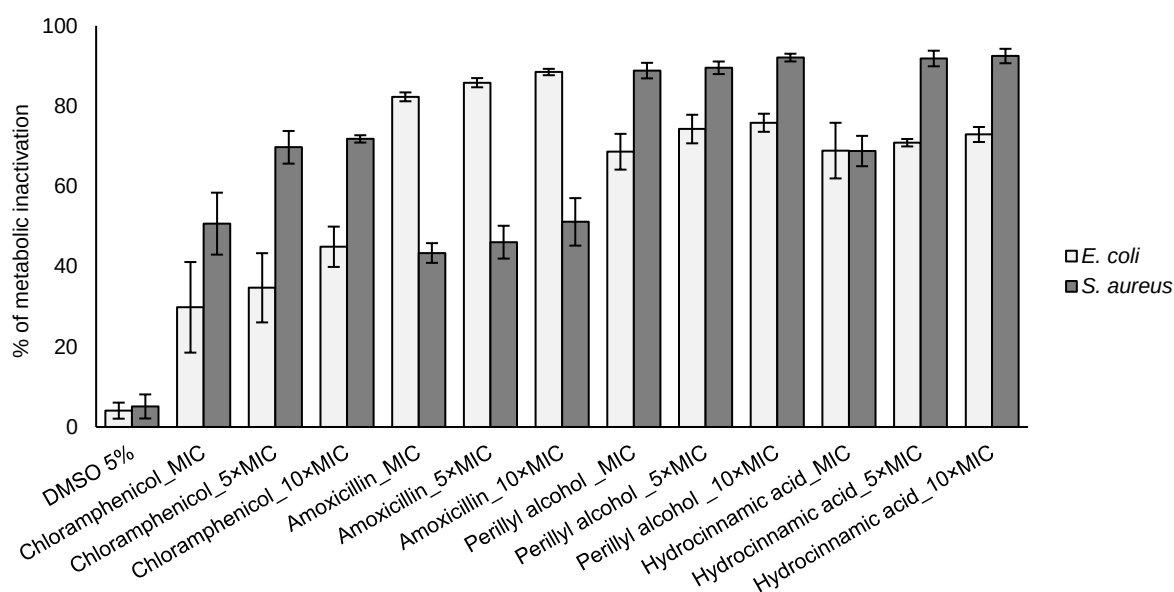


Figure 4. Percentages of metabolic inactivation, for *S. aureus* (black) and *E. coli* (white) 24 h biofilms, after exposure to the tested compounds at MIC, 5×MIC and 10×MIC for 24 h. Values are the mean ± standard deviation for at least two independent experiments.

Firstly, the results obtained for the two strains, considering the same compound, were statistically significant different ($p < 0.05$), with an exception for hydrocinnamic acid at MIC, where the percentage of metabolic activity inactivation were almost the same for both bacteria ($p > 0.05$). In general, the metabolic activity inactivation was higher for *S. aureus* than for *E. coli*, except for amoxicillin. Moreover, the negative control with DMSO 5% was not able to cause a remarkable reduction in metabolic activity both for *S. aureus* and *E. coli* ($p > 0.05$). The lowest percentage of metabolic inactivation was equal to 29.8% and was obtained for *E. coli* treated with chloramphenicol (for a concentration equal to MIC) while the highest value was equal to 92.5% and was obtained for *S. aureus* treated with hydrocinnamic acid (for a concentration equal to 10×MIC). The percentages obtained for treated and untreated biofilms were statistically significant different ($p < 0.05$). In general, there was a statistically significant difference when comparing different compounds for the same strain ($p < 0.05$). The only exception occurred for the phytochemicals, perillyl alcohol and hydrocinnamic whose activity against the same strain was not statistically significant different ($p > 0.05$). Surprisingly, perillyl alcohol and hydrocinnamic acid were the compounds that promoted the highest percentages of metabolic inactivation. Furthermore, it was observed that increasing the concentration of the compounds tested, the percentage of inactivation of the metabolic activity tends to increase, both for *S. aureus* and *E. coli*. It was possible to conclude that the metabolic inactivation was dependent on the compound and on its concentration. Although there was not total inactivation of the metabolic activity, very high percentages were obtained, especially for *S. aureus* and for higher concentrations of both phytochemicals. According to the classification of Lemos *et al.* (2014), seven treatments showed moderate efficacy, fourteen treatments resulted in high efficacy and three treatments displayed excellent efficacy.

Figure 5 presents the percentages of biomass reduction, for *S. aureus* and *E. coli* 24 h biofilms, after exposure to the tested compounds at MIC, 5×MIC and 10×MIC for 24 h.

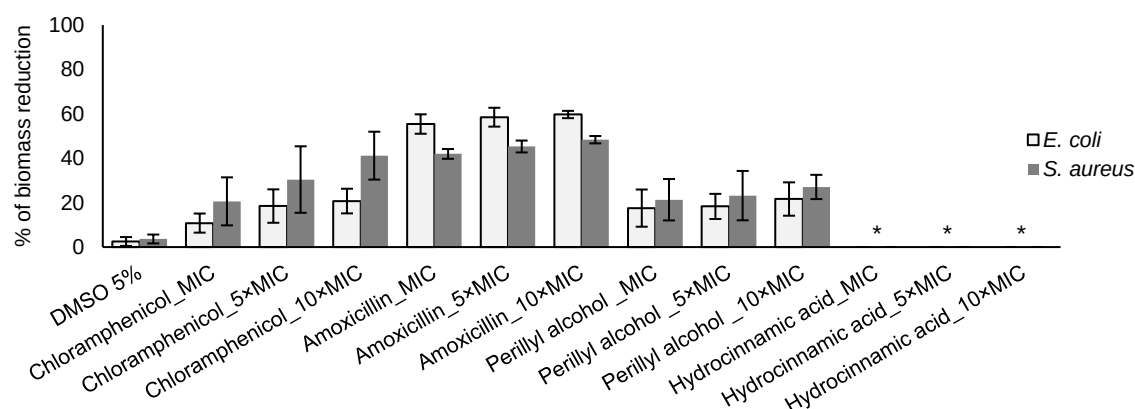


Figure 5. Percentages of biomass reduction, for *S. aureus* (black) and *E. coli* (white) 24 h biofilms, after exposure to the tested compounds at MIC, 5×MIC and 10×MIC for 24 h. (*) means that the result obtained was null. Values are the mean $\pm$ standard deviation for at least two independent experiments.

Once again, the results obtained for the two strains, considering the same compound, were statistically significant different ($p < 0.05$), and the biomass reduction was in general higher for *S. aureus* than for *E. coli*, except for amoxicillin. Moreover, the negative control with DMSO 5% was not able to cause a remarkable reduction in biomass reduction both for *S. aureus* and *E. coli* ($p > 0.05$). The lowest percentage of biomass reduction was equal to 10.8% and was obtained for *E. coli* treated with chloramphenicol (for a concentration equal to MIC) while the highest value was equal to 59.7% and was obtained for *E. coli* treated with amoxicillin (for a concentration equal to 10×MIC). It should also be noted that hydrocinnamic acid was not able to cause any reduction in biomass for any concentration or strain. The percentages obtained for treated and untreated biofilms were statistically significant different ($p < 0.05$). There was, one more time, a statistically significant difference when comparing different compounds against the same strain ($p < 0.05$), except for chloramphenicol and perillyl alcohol for all concentrations and both strains ($p > 0.05$). Furthermore, it was observed that increasing the concentration of the compounds tested, the percentage of biomass reduction tends to increase, both for *S. aureus* and *E. coli*. Therefore, it was possible to conclude that the biomass reduction was dependent on the compound and on its concentration. Moreover, there was not total biomass reduction. According to the classification proposed by Lemos *et al.* (2014), fifteen treatments showed low efficacy and nine treatments resulted in moderate efficacy.

Figure 6 presents the $\log_{10}$ (CFU/cm²) and **Figure 7** shows the $\log_{10}$ reduction, for *S. aureus* and *E. coli* 24h biofilms, after exposure to the tested compounds at MIC, 5×MIC and 10×MIC for 24 h.

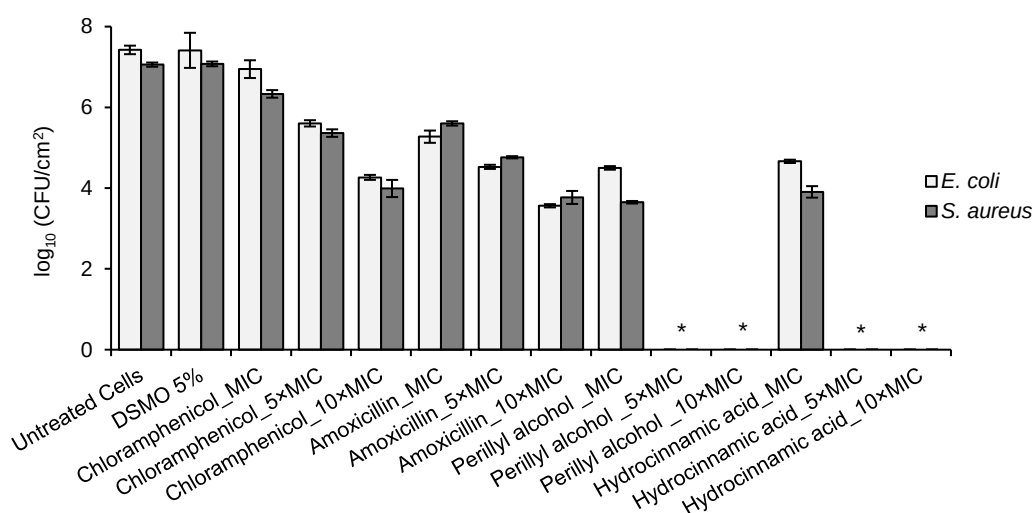


Figure 6. $\log_{10}$ (CFU/cm²), for *S. aureus* (black) and *E. coli* (white) 24 h biofilms, after exposure to the tested compounds at MIC, 5×MIC and 10×MIC for 24 h. (*) means that the result obtained was lower than the detection limit. Values are the mean $\pm$ standard deviation for at least two independent experiments.

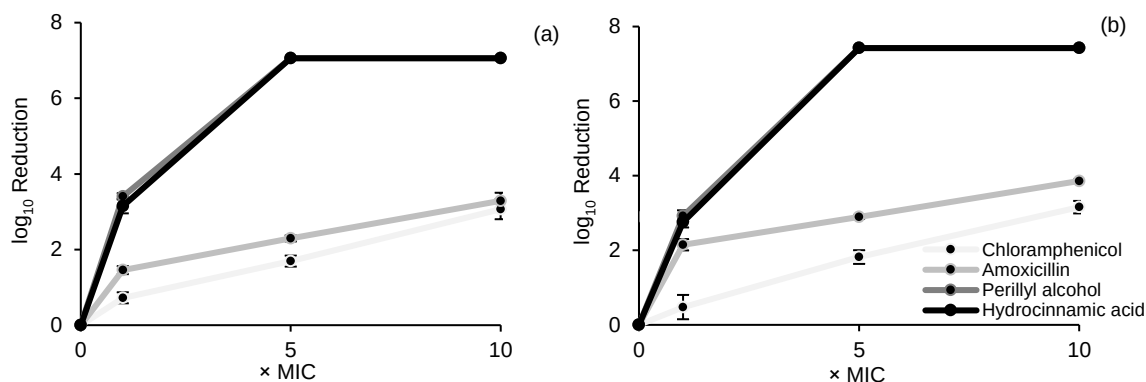


Figure 7. log₁₀ reduction, for *S. aureus* (a) and *E. coli* (b) 24 h biofilms, after exposure to the tested compounds at MIC, 5×MIC and 10×MIC for 24 h. Values are the mean ± standard deviation for at least two independent experiments.

Again, the results obtained for the two strains, considering the same compound, were statistically significant different ($p < 0.05$), and the compounds tested were more effective in reducing the culturability of biofilms of *S. aureus* than *E. coli*, except for amoxicillin. Moreover, the negative control with DMSO 5% was not able to cause a remarkable reduction in cellular culturability both for *S. aureus* and *E. coli* biofilms ($p > 0.05$). The lowest effect was obtained for *E. coli* treated with chloramphenicol (for a concentration equal to MIC), corresponding to a log₁₀ (CFU/cm²) of 6.95 and a log₁₀ reduction of 0.48 while the highest values, equivalent to a total absence of cellular culturability, was obtained both for *S. aureus* and *E. coli* biofilms treated with perillyl alcohol and hydrocinnamic acid (for a concentration equal to 5×MIC and 10×MIC). Moreover, the results obtained for treated and untreated biofilms were statistically significant different ($p < 0.05$), and the same scenario was verified when comparing different compounds against the same strain ($p < 0.05$), with the exception of both phytochemicals ($p > 0.05$). Perillyl alcohol and hydrocinnamic acid were the compounds that promoted better results. Furthermore, it was observed that increasing the concentration of the compounds tested, the log₁₀ (CFU/cm²) tends to decrease, and log₁₀ reduction tends to increase, both for *S. aureus* and *E. coli*. Therefore, it was possible to conclude that cellular culturability was dependent on the compound and on its concentration.

Overall, there was an excellent agreement in the results obtained for inactivation of metabolic activity and cellular culturability, which can be explained by the fact that a functional cellular metabolism is crucial for cellular viability (Rampersad 2012). The same does not seem to apply to biomass reduction.

In general, biofilm control assays revealed a greater effect of the compounds tested on *S. aureus* biofilms when compared to *E. coli*. In fact, according Simões *et al.* (2008), *E. coli* is less susceptible to antimicrobials agents than *S. aureus* because of its OM, which constitutes a barrier for the penetration of drugs. However, *E. coli* has shown to be more susceptible to amoxicillin than *S. aureus*. This can be explained considering that the concentrations tested

were established as a function of MIC (defined for cells in planktonic state and not for their sessile form). The value for amoxicillin against *E. coli* was two orders of magnitude higher than the MIC of the same antibiotic against *S. aureus*, which implied that the biofilms of both bacteria were exposed to very different concentrations of amoxicillin, influencing the antibiofilm activity obtained. The same reasoning can also justify that in certain cases the phytochemicals tested were more effective than both antibiotics. Abreu *et al.* (2016) reported similar results when testing antibiotics and phytochemicals against *S. aureus* biofilms.

Moreover, the bacterial resistance strategies valid for planktonic cells may not apply to biofilms, and vice versa, because of the differences in morphology and density of distinct biofilms (Furukawa *et al.* 2010; Monte *et al.* 2014), the differences in the chemical composition of their matrix (Meyer 2003; Abreu *et al.* 2013a) and the greater number of defense mechanisms of biofilms when compared to planktonic cells (Monte *et al.* 2014).

Chapter 4 – Bioactivity of combinations of selected compounds

4.1. Introduction

AMR is one of the most worrying issues faced by public health. In fact, this alarming situation is especially worthy of attention when microorganisms are resistant to several antimicrobial agents as well as when they create biofilms (Ribeiro et al. 2016). As a result, it leads to limited options for infections treatment, recurrent infections, treatment shortcoming and increased morbidity and death (Borges et al. 2016; Ayaz et al. 2019). Therefore, it is essential to discover new compounds to control and prevent the formation and development of infections (Ayaz et al. 2019). For this purpose, phytochemicals can be an attractive option since they have already shown to have a great therapeutic potential as antibacterial agents (Simões et al. 2009). These compounds can be used alone and/or in combination with other antibacterial products, acting as synergistic/potentiating agents of the antimicrobial activity. It is one possible strategy to prevent the occurrence of bacterial resistance (Sakharkar et al. 2009; AlSheikh et al. 2020).

An effective and common approach consists in combinatorial therapies, adding conventional antibiotics to phytochemicals, which usually enhances the efficacy of the treatment by generating a synergistic effect and allowing the reduction of antibiotic concentration (Jayaraman et al. 2013; Shuchi et al. 2016). When antimicrobial agents are combined, they can function as synergistic or potentiating agents of the antimicrobial activity. The combinatorial approach aims to combat the mechanisms of resistance and make the antibacterial drug still being effective, even against resistant microorganisms (Ayaz et al. 2019). In fact, combinations had already shown, in previous studies, highly promising and significant results as bacterial resistance modifying elements (Sakharkar et al. 2009).

This combinatorial approach aims to combat the mechanisms of resistance and make the antibacterial drug still being effective, even against resistant microorganisms. In fact, combinations have already shown, in previous studies, promising results as bacterial resistance modifying elements (Sakharkar et al. 2009; Ayaz et al. 2019). However, there should be more studies on the antimicrobial potential of combinations involving phytochemicals and antibiotics for the treatment of infections caused by planktonic cells and biofilms, due to their interest.

There are two main assays that are usually used for assessing the antimicrobial effect of combinations: checkerboard assay and time-kill assays (Magi et al. 2015). Checkerboard assay is based on the standard method for determining MIC. Briefly, it is intended to evaluate

the ability of a given microorganism to grow in the presence of different concentrations of drug combinations after an incubation period, generally 18 h to 24 h (Brennan-Krohn and Kirby 2019). Regarding the analysis of the results obtained, the antimicrobial activity of individual compounds is compared with the effect of combinations of compounds. This is achieved by calculating the FICI, which relates the MIC of the combinations with the MIC of the individual molecules (Lorian 2005). Time-kill assay is a method used to evaluate how an antimicrobial agent or combination can affect the culturability of a specific bacterial strain in a planktonic state being classified as bactericidal or bacteriostatic. Results are obtained after incubation in solid medium over time and by CFU counts (CLSI 1999).

Regarding MICs values and the data from the antibiogram, four compounds: perillyl alcohol, hydrocinnamic acid, chloramphenicol and amoxicillin, and one microbial strain: *E. coli* CECT 434, were selected for the subsequent assays contained in this chapter. First, the toxicity and efficacy of a universal neutralizer was evaluated. Then, all possible dual and triple combinations were assessed, with the exception of chloramphenicol/amoxicillin. Dual and triple combinations were assessed by checkerboard and time-kill assays for planktonic cells. In the case of checkerboard assay, the results were evaluated through FICI classification and the software Combenefit. Regarding time-kill assay, the results were analysed through $\log_{10}$ (CFU/mL) as a function of incubation time. After exposure for 24 h to dual and triple combinations, the culturability of biofilm's cells was evaluated by CFUs count, biofilm's metabolic inactivation (measured by fluorescence values) was assessed through alamar blue staining method and biomass reduction was determined by CV staining procedure. In all assays, the combination amoxicillin/metronidazole was used as a positive control and compared to other combinations involving amoxicillin.

4.2. Materials and methods

4.2.1. Bacterial strains, culture conditions and tested compounds

For the assays presented in this chapter, only *E. coli* CECT 434 were used being stored in cryovials with glycerol 30% (v/v) at $(-80 \pm 2) ^\circ\text{C}$. First, *E. coli* was cultured in MHA plates and allowed to grow for 24 h, using the same refrigerated incubator. Combinations of chloramphenicol, perillyl alcohol, hydrocinnamic acid and amoxicillin were assessed in this chapter. Preparation and conservation of the stock solutions were performed as described in section 3.2.2. These compounds were selected, once again, considering the results presented in the previous chapter. **Appendix B** contains the chemical, structural and molecular properties of the compounds studied in this chapter.

4.2.2. Toxicity and efficacy evaluation for a universal neutralizer

These assays were performed according to the European Standard (1997).

For the toxicity assessment, *E. coli* was grown overnight using 50 mL of sterile fresh MHB at $(37 \pm 2)^\circ\text{C}$ and 150 rpm, using the same refrigerated incubator with an orbital shaker. After the overnight growth, the culture OD was adjusted to 0.040 ± 0.020 (1.5×10^8 CFU/mL) at 620 nm, with sterile fresh MHB. Afterwards, 100 μL of bacteria suspension were added to 100 μL of sterile distilled water and 800 μL of a universal neutralizer: 30 g/L of polysorbate 80 (Avantor, VWR, France), 30 g/L of saponin (Avantor, VWR, Germany), 1 g/L of L-histidine (Sigma-Aldrich, Merck KGaA, Germany), 3 g/L of lecithin (Thermo Fisher Scientific, Alfa Aesar, Germany), 5 g/L of sodium thiosulphate (ACP Chemicals Inc., Labkem, Spain) in 0.0025 M phosphate buffer. The mixture was incubated for 10 min at room temperature and ten-fold serial dilutions were performed in sterile NaCl, from 10^{-1} to 10^{-7} . Next, 10 μL were plated on MHA plates, following the drop plate method, and the plates were incubated at $(37 \pm 2)^\circ\text{C}$ for 24 h in the same refrigerated incubator. A negative control was also performed, replacing the universal neutralizer with sterile NaCl. Finally, CFU were visually counted for plates containing > 10 and < 100 colonies.

For the efficacy assessment, *E. coli* was grown overnight using 50 mL of sterile fresh MHB at $(37 \pm 2)^\circ\text{C}$ and 150 rpm, using the same refrigerated incubator with an orbital shaker. Then, 800 μL of each compound (chloramphenicol, amoxicillin, perillyl alcohol and hydrocinnamic acid at $2 \times \text{MIC}$), prepared in DMSO 8%, was added to 100 μL of sterile distilled water and 100 μL of phosphate buffered saline (PBS): 8 g/L of NaCl, 0.2 g/L of KCl (Avantor, VWR, Belgium), 1.44 g/L of Na_2HPO_4 (Avantor, VWR, Belgium) and 0.24 g/L of KH_2PO_4 (Chem-Lab NV, Belgium) at pH 7.4. After incubation for 30 min at $(37 \pm 2)^\circ\text{C}$ in the same refrigerated incubator, 100 μL of this mixture was added to 800 μL universal neutralizer for 10 min at room temperature. Afterwards, the culture OD was adjusted to 0.040 ± 0.020 (1.5×10^8 CFU/mL) at 620 nm, with sterile fresh MHB, and 100 μL of bacteria suspension were added. The mixture was incubated for 30 min at $(37 \pm 2)^\circ\text{C}$ in the same refrigerated incubator and ten-fold serial dilutions were performed in sterile NaCl, from 10^{-1} to 10^{-7} . Next, 10 μL were plated on MHA plates, following the drop plate method, and the plates were incubated at $(37 \pm 2)^\circ\text{C}$ for 24 h in the same refrigerated incubator. Two negative controls were also performed, replacing the universal neutralizer with sterile NaCl and the compounds with DMSO. Finally, CFU were visually counted for plates containing > 10 and < 100 colonies.

4.2.3. Checkerboard microdilution assay

Checkerboard microdilution assay was performed according to CLSI guidelines (2018) and Stein *et al.* (2015) with some modifications. First, *E. coli* culture was grown overnight at $(37 \pm 2)^\circ\text{C}$ under agitation (150 rpm), using the same refrigerated incubator with an orbital shaker previously mentioned. In this assay, all the possible dual combinations involving chloramphenicol, amoxicillin, perillyl alcohol and hydrocinnamic acid (excluding chloramphenicol/amoxicillin) were evaluated. For dual combinations, concentrations equal to $1/32 \times \text{MIC}$, $1/16 \times \text{MIC}$, $1/8 \times \text{MIC}$, $1/4 \times \text{MIC}$, $1/2 \times \text{MIC}$, MIC and $2 \times \text{MIC}$ of individual compounds, prepared with DMSO, were combined. One compound was diluted along the x axis and another compound was diluted along the y axis. In this case, 5 μL of each compound in a certain concentration and 190 μL of inoculum were added (bacterial culture OD was adjusted in sterile fresh MHB as described in the section 3.2.3). For triple combinations, the following procedure was performed: based on the results of dual combinations, the two selected phytochemicals were diluted, one along the x axis and the other along the y axis. Then, one antibiotic was added to the combination of phytochemicals in each well, combining concentrations between $1/4 \times \text{MIC}$ and $2 \times \text{MIC}$ of individual compounds. The final volume in each well was always equal to 200 μL . In this case, 4 μL of each phytochemical, 2 μL of one antibiotic and 190 μL of inoculum (bacterial culture OD was adjusted in sterile fresh MHB as described in the section 3.2.3) were added to each well (in order to guarantee a percentage of DMSO equal to 5%). Two controls were added: 200 μL of sterile fresh MHB and 200 μL of bacterial culture. **Table A2** and **Table A3** of **Appendix C** represent the microtiter plates layout for dual and triple combinations, respectively. The microtiter plates were incubated for 24 h under the same growth conditions mentioned above and using the same refrigerated incubator with an orbital shaker. The absorbance of microtiter plates was read at 0 h and 24 h (after incubation), at 620 nm, using the aforementioned equipment. Other controls were made in another sterile microtiter plate, consisting of sterile fresh MHB (200 μL), bacterial culture alone (200 μL), bacterial culture (190 μL) with DMSO (10 μL) and sterile fresh MHB (195 μL) with each compound (5 μL at $2 \times \text{MIC}$). The combination amoxicillin/metronidazole was used as a positive control. All the results obtained for metronidazole are in **Table A4** of **Appendix D**.

4.2.4. Time-kill assay

Time-kill were performed based on the works of Tängdén *et al.* (2014), Ha and Ha (2010) and Zhou *et al.* (2020) with some modifications. Firstly, *E. coli* culture was grown overnight at $(37 \pm 2)^\circ\text{C}$ and with agitation (150 rpm), using the same refrigerated incubator with an orbital shaker, and bacterial culture OD was adjusted in sterile fresh MHB as described in the section

3.2.3. Concentrations equal to $1/2 \times \text{MIC}$, MIC and $2 \times \text{MIC}$ of individual compounds were tested, both for assessing the individual compounds and the combinations. The combinations of compounds evaluated in this assay were those mentioned in section 4.2.3. The combinations were performed in independent sterile labeled microcentrifuge tubes and inoculated with bacterial culture. Total volume was always equal to 1000 μL : 50 μL of single compounds or 50 μL of combinations (16.7 μL and 25 μL of each compound that constitutes the triple and the dual combinations, respectively) were added to 950 μL of bacterial culture. Untreated bacteria and bacterial culture with DMSO 5% were also evaluated. The tubes were incubated at $(37 \pm 2)^\circ\text{C}$ and 150 rpm for 24 h in the same refrigerated incubator. Samples were taken after 0, 1, 3, 6, 8 e 24 h of incubation. Then, neutralization was performed by the dilution-neutralization method, using the universal neutralizer described in section 4.2.2. Then, 100 μL of sample was mixed with 800 μL neutralizer and 100 μL of sterile distilled water. After 10 min at room temperature, ten-fold serial dilutions were prepared in sterile NaCl, from 10^{-1} to 10^{-7} , performed in a sterile microtiter plate. Next, 10 μL were plated on MHA plates, following the drop plate method, and the plates were incubated at $(37 \pm 2)^\circ\text{C}$ for 24 h in the same refrigerated incubator. Finally, CFU were visually counted for plates containing > 10 and < 100 colonies and expressed as $\log_{10}$ (CFU/mL), for the different incubation times, according to **Equation (3)**. The combination amoxicillin/metronidazole was used as a positive control and compared to other combinations involving amoxicillin.

4.2.5. Classification and evaluation of combinations

4.2.5.1. *Interpretation of checkerboard assay results – FICI*

There are four possible results when combining different compounds:

- **Synergism** refers to the combinations of two or more drugs that results in a greater effect than the sum of the effects for each drug alone (Bollenbach 2015).
- **Additivity** refers to the combinations of two or more drugs that results in an equal effect than the sum of the effects for each drug alone (Yang et al. 2017b).
- **Indifference** refers to the combinations of two or more drugs that results in an equal effect to this of the most active compound of the combination (Drugeon et al. 1987).
- **Antagonism** refers to the combinations of two or more drugs that results in a smaller effect than the sum of the effects for each drug alone (Drugeon et al. 1987).

According to Stein *et al.* (2015), $\text{FICI} = \text{FIC(A)} + \text{FIC(B)}$, where the fractional inhibitory concentration of A, FIC(A) , is the ratio between the MIC of A in combination and the MIC of A

alone ($MIC_A^{combination}/MIC_A^{alone}$), and $FIC(B)$ is the ratio between the MIC of B in combination and the MIC of B alone ($MIC_B^{combination}/MIC_B^{alone}$), as explained in **Equation (6)**. For triple combinations, $FICI = FIC(A) + FIC(B) + FIC(C)$, where $FIC(A)$ is the ratio between the MIC of A in combination and the MIC of A alone ($MIC_A^{combination}/MIC_A^{alone}$), $FIC(B)$ is the ratio between the MIC of B in combination and the MIC of B alone ($MIC_B^{combination}/MIC_B^{alone}$), and $FIC(C)$ is the ratio of the MIC of C in combination and the MIC of C alone ($MIC_C^{combination}/MIC_C^{alone}$), according to **Equation (7)**. Applying Stein *et al.* classification with some modifications, $FICI \leq 0.80$ corresponds to synergism, $0.80 < FICI \leq 1.00$ indicates additivity, $1.00 < FICI \leq 4.00$ is classified as indifference and $FICI > 4.00$ is considered antagonism (Stein et al. 2015).

$$FIC(A) + FIC(B) = (MIC_A^{combination}/MIC_A^{alone}) + (MIC_B^{combination}/MIC_B^{alone}) \quad (6)$$

$$FIC(A) + FIC(B) + FIC(C) = (MIC_A^{combination}/MIC_A^{alone}) + (MIC_B^{combination}/MIC_B^{alone}) + (MIC_C^{combination}/MIC_C^{alone}) \quad (7)$$

4.2.5.2. Interpretation of checkerboard assay results – Combeneft

Checkerboard assay results were also analysed with Combeneft (Cancer Research UK Cambridge Institute, Cambridge, United Kingdom). Combeneft is a free software to analyze and classify combinations according to their effects. The software uses the classic synergism models, namely Loewe, Bliss and HSA models, to process data (Di Veroli et al. 2016). In the present study, Bliss model was selected because it is appropriate to study drugs with independent responses, for example, when they have distinct modes of action (Tang et al. 2015). That is expected to be the case of combinations of antibiotics with phytochemicals. First, the software converts the dose-response surface into a matrix of percentages, correlating the determined concentrations to control values. Then, the software uses the results for the effects of single agents and creates a dose-response curve. The same considerations apply to combinations. Finally, the experimentally determined dose-response surface for the drug combination is compared to the theoretical model. This comparison provides the distribution of synergism in the concentration space (Di Veroli et al. 2016).

Combeneft provides the following graphical outputs: the single agent dose response; combination dose response; the model-generated reference combination dose response and the synergism distribution (Di Veroli et al. 2016). The single agent dose response and the synergism distribution were selected given their relevance to this study. Combeneft uses Bliss model to statistical determine synergism based on Bliss definition of drugs independence,

where the surviving fraction of cells following single-agent administration, this is, after exposure to drug A or drug B alone, can be denoted by S_A and S_B , respectively. The cellular surviving fraction of cells upon exposure to the combination is given by $S_A \times S_B$. The mortality caused by the combination of drugs, M_{AB} , is given by the **Equation (8)**, where M_A is the mortality caused by agent A and M_B is the mortality caused by drug B (Demidenko and Miller 2019).

$$M_A + M_B - M_A \times M_B = M_{AB} \quad (8)$$

4.2.5.3. Interpretation of time-kill assay results

A $\geq 2 \log_{10}$ (CFU/mL) decrease between the combination and its most active constituent was defined as synergism, for 24 h. Likewise, a $\log_{10}$ (CFU/mL) decrease between ≥ 1 and < 2 were defined as additivity and a $\geq 2 \log_{10}$ increase was defined as antagonism. Indifference was defined as any effect between the limits for additivity and antagonism (Zhou et al. 2020). Nevertheless, this classification was only applied to treatments for which the CFU counts were above the detection limit, constituting a modification to the classification of Zhou *et al.* (2020). It was intended not to misinterpret the cases for which the CFUs counts were below the detection limit and not to ignore unknown phenomena that may eventually occur in the interaction between compounds.

4.2.6. Evaluation of biofilm control for dual and triple combinations

To evaluate the dual and triple combinations against biofilms of *E. coli*, the assays described in sections 3.2.7.1 to 3.2.7.5. were performed again. Concentrations equal to MIC and 5×MIC of individual compounds were combined. The dual and triple combinations assessed were those mentioned in section 4.2.3. guaranteeing a percentage of DMSO equal to 5%. To compare the cellular culturability of biofilm cells obtained for individual compounds and for dual and triple combinations, the classification previously mentioned for time-kill assay in the section 4.2.5.3. was applied. The combination amoxicillin/metronidazole was used as a positive control and compared to other combinations involving amoxicillin.

4.2.7. Statistical analysis

Data were analyzed using the statistical program SPSS version 28.0 (IBM Corp., Armonk, New York, United States of America). Bearing in mind that a small sample leads to uneven variation, a nonparametric test, more specifically the Wilcoxon statistical test, was used

to assess the statistical significance value. For assays involving CFU counting, statistical analysis was performed using the One-Way ANOVA test followed by Sidak's multiple comparisons test. For time-kill curves, the area under the curve (AUC) was first calculated and then One-Way ANOVA test followed by Dunnett's multiple comparisons test was performed to compare the control with each treatment while One-Way ANOVA test followed by Sidak's multiple comparisons test was carried out for other multiple comparisons. The results were presented as mean $\pm$ standard deviation. Significance level for the differences was set at $p < 0.05$ and the calculations were based on confidence level $\geq 95\%$. At least two independent experiments, with at least two replicates, were performed for each tested condition.

4.3. Results and discussion

4.3.1. Toxicity and efficacy evaluation for a universal neutralizer

These assays were performed considering the microbial strain (*E. coli* CECT 434), the compounds (chloramphenicol, amoxicillin, metronidazole, perillyl alcohol and hydrocinnamic acid) and the maximum concentration ($2\times\text{MIC}$) evaluated in the tests of this chapter for which neutralization was required. In fact, complete neutralization is important to guarantee the accuracy of the results since antimicrobial activity is very often assessed by the cellular culturability over time (Russell et al. 1979; Sutton et al. 2002). In this sense, an effective neutralization is crucial to avoid an overestimation of the antimicrobial and antibiofilm activities (Sutton et al. 2002). In this study, the toxicity and efficacy of a universal neutralizer were evaluated, and the results are shown in **Figure 8**.

The neutralizer toxicity was assessed based on the comparison between the culturability of the population exposed to the neutralizer and the population not exposed to the neutralizer (the division of these two parameters defines the neutralizer toxicity ratio). The evaluation of the neutralizer efficacy was performed comparing the cellular culturability of the population exposed to the neutralizer plus antimicrobial agent and the population exposed only to the neutralizer (the division of these two parameters defines the neutralizer efficacy ratio)(Sutton et al. 2002). Following the criterion presented by Sutton *et al.* (2002), neutralization is acceptable when a neutralizer efficacy and a neutralizer toxicity ratios of ≥ 0.75 is obtained (Sutton et al. 2002).

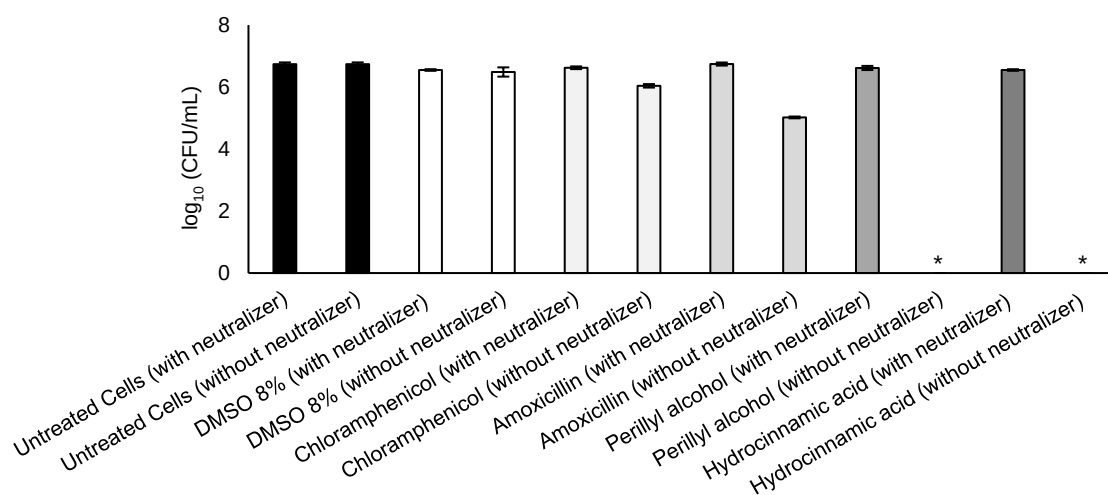


Figure 8. Toxicity and efficacy evaluation for a universal neutralizer. (*) means that the result obtained was lower than the detection limit. Values are the mean $\pm$ standard deviation for at least two independent experiments.

Table 10 presents the neutralizer efficacy and neutralizer toxicity ratios, according to Sutton *et al.* (2002).

Table 10. Neutralizer efficacy ratios and neutralizer toxicity ratios.

Neutralizer toxicity and neutralizer efficacy ratios	
Neutralizer toxicity ratio	1.00
Neutralizer efficacy ratio for DMSO 8%	0.98
Neutralizer efficacy ratio for chloramphenicol	0.98
Neutralizer efficacy ratio for amoxicillin	1.00
Neutralizer efficacy ratio for perillyl alcohol	0.98
Neutralizer efficacy ratio for hydrocinnamic acid	0.98

It was possible to observe that the neutralizer did not seem to present toxicity for *E. coli* since cell growth in the presence and absence of the neutralizer was equal ($p > 0.05$). It was also possible to conclude that DMSO 8% did not affect the cellular culturability of *E. coli*, whereas there was no statistically significant difference between untreated bacteria and bacteria exposed to DMSO 8% ($p > 0.05$). Moreover, bacterial population exposed to neutralizer plus DMSO 8% did not have its culturability affected ($p > 0.05$). The same scenario was observed for each cellular population exposed to the four compounds evaluated plus neutralizer, with no statistically significant difference between any of these population and the control (bacteria exposed to neutralizer) ($p > 0.05$). Therefore, the neutralizer was able of cease the activity of all the compounds, as it was expected based on previous results obtained regarding toxicity and efficacy obtained for this particular universal neutralizer (Fernandes *et al.* 2020; Oliveira *et al.* 2022). Moreover, when bacteria were exposed to the compounds without neutralizer, the cellular culturability observed was dependent on their effect, considering their characteristic action time after 30 min of exposure.

4.3.2. Checkerboard assay results

Over the years, phytochemicals have been exploited for their ability to increase the susceptibility of pathogens to several antimicrobial drugs and reduce their toxicity when used in combinations (Jayaraman et al. 2010). For the evaluation of two antimicrobial agents in combination, checkerboard is widely used, providing an excellent analysis of the bacterial growth inhibition. Furthermore, this technique can also be adapted to allow the study of combinations of a greater number of drugs. In fact, this is one of the most consolidated methods being described in numerous scientific publications for the assessment of combinations (Bellio et al. 2021). Here, six dual combinations: chloramphenicol/perillyl alcohol, chloramphenicol/hydrocinnamic acid, amoxicillin/perillyl alcohol, amoxicillin/hydrocinnamic acid, amoxicillin/metronidazole and perillyl alcohol/hydrocinnamic acid as well as two triple combinations: chloramphenicol/perillyl alcohol/hydrocinnamic acid and amoxicillin/perillyl alcohol/hydrocinnamic acid were assessed through checkerboard.

Table 11 shows the FICI values and the classification of combinations evaluated in this study, considering the interpretation proposed by Stein *et al.* (2015) with some modifications.

Table 11. FICI value and classification of dual and triple combinations. Values are the mean $\pm$ standard deviation for at least two independent experiments.

Combination	FICI	Classification
<i>Chloramphenicol/Perillyl alcohol</i>	0.56	Synergism
<i>Chloramphenicol/Hydrocinnamic acid</i>	1.00	Additivity
<i>Amoxicillin/Perillyl alcohol</i>	1.50	Indifference
<i>Amoxicillin/Hydrocinnamic acid</i>	0.75	Synergism
<i>Amoxicillin/Metronidazole</i>	0.63	Synergism
<i>Perillyl alcohol/Hydrocinnamic acid</i>	1.50	Indifference
<i>Chloramphenicol/ Perillyl alcohol/Hydrocinnamic acid</i>	1.75	Indifference
<i>Amoxicillin/Perillyl alcohol/Hydrocinnamic acid</i>	1.75	Indifference

The FICI values obtained in this study allowed classifying three combinations as synergistic: chloramphenicol/perillyl alcohol, amoxicillin/hydrocinnamic acid and amoxicillin/metronidazole; one as additive: chloramphenicol/hydrocinnamic acid; and four as indifferent: amoxicillin/perillyl alcohol, perillyl alcohol/hydrocinnamic acid, chloramphenicol/perillyl alcohol/hydrocinnamic acid and amoxicillin/perillyl alcohol/hydrocinnamic acid. Thereby, synergism was obtained for 37.5% of the combinations, additivity was verified for 12.5% of the cases studied and indifference was the outcome for 50.0% of the combinations. There was always inhibition of bacterial growth at concentrations equal to MIC and 2×MIC of the individual compounds.

The classification proposed by Stein *et al.* (2015) was used with some modifications. The original classification does not distinguish between indifference and additivity. In this study, it was decided to classify combinations that resulted in a FICI in the range between 0.80 and 1.00 as additivity, taking into account the classifications proposed by other authors, since a FICI of 1.00 is always equivalent to growth inhibition for the combination of sub-inhibitory concentrations for all the compounds combined (Zhou *et al.* 2020; Noel *et al.* 2022). In the case of triple combinations, FICI resulted from the sum of three individual FIC while for dual combinations it only resulted from the sum of two FIC values. Consequently, for the same type and magnitude of interaction between compounds, FICI for triple combinations will be higher than for dual combinations. In fact, triple combinations presented the highest FICI revealing that they were not particularly effective when compared to dual combinations.

Several studies reported the potentiation of antibiotics, namely chloramphenicol and amoxicillin, by plant extracts, when the combinations were tested against *E. coli* strains (Olajuyigbe and Afolayan 2012; Kon and Rai 2013; Sahra 2017; Seebaluck-Sandoram *et al.* 2018). Few studies described the outcome of combinations of these antibiotics with isolated phytochemicals, particularly against *E. coli*. However, the studies developed by Santos *et al.* (2015) and Rao *et al.* (2017) are good examples of how the interactions between amoxicillin and chloramphenicol with phytochemicals, respectively, can be interesting for the control of infections caused by *E. coli*, with synergism being reported. Similarly, this study showed that these combinations may have high potential, as 50.0% of the dual combinations between antibiotics and phytochemicals resulted in synergism. It is even less common to find studies in the literature referring to combinations of phytochemicals. Moreover, combinations of molecules with similar or the same mechanism of action are usually not particularly effective since molecules can "muddle" each other even if their combination doesn't result in antagonism (Rizvi *et al.* 2013). In this study, it seemed to happen for the combination perillyl alcohol/hydrocinnamic acid, but also for the combination amoxicillin/perillyl alcohol as well as for the triple combinations. Regarding the combination amoxicillin/metronidazole, it is known that metronidazole is often used as an adjuvant of amoxicillin, enhancing its effect against periodontal infections (Pavicić *et al.* 1991). Several studies developed in the dental medicine field claimed that the combination of metronidazole, targeting the bacterial DNA, and amoxicillin, targeting the bacterial cell wall, had a synergistic effect (Dabija-Wolter *et al.* 2018). This study proved that this combination may also have a high potential in combating other healthcare-associated infections such as those caused by *E. coli*.

The results obtained for Checkerboard assay were also analyzed using the software Combenefit. **Figure 9** presents the single agent dose response and the synergism distribution (matrix synergism plot and synergism mapped to D-R, based on Bliss model), for the dual combination amoxicillin/hydrocinnamic acid. The results for other combinations can be found

in **Figure A1**, **Figure A2**, **Figure A3**, **Figure A4** and **Figure A5** of **Appendix E**. The single agent dose response allows to evaluate how the activity or effect of a given molecule varies with the increase of its concentration, until a maximum action is reached. Furthermore, it also enables the determination of half maximal effective concentration (EC₅₀), which is the concentration of a drug that originates 50.0% of its maximal response (Meyer et al. 2019). Bliss model was used to assess the interactions between commercial antibiotics and phytochemicals, between amoxicillin and metronidazole and between perillyl alcohol and hydrocinnamic acid. The matrix synergism plot shows the synergism scores for each combination. Stars indicate the level of significance and only significant combinations are colored. The number of replicates is reported in the top left corner (in this case, three replicates were performed) (Di Veroli et al. 2016). The third-dimension graph (synergism mapped to D-R) shows the percentage of synergism for different concentrations and combinations, demonstrating a concentration dependent dual effect based on the percentage of synergism. In this graphical outputs, blue represents the synergistic combinations, green is used for indifference and red, orange and yellow indicates the combinations that resulted in antagonism. Each point represents the EC₅₀ for the different concentrations combined. Points that are above zero correspond to synergism and points that are below zero correspond to antagonism. Likewise, the points located at the plane or at zero synergism percentage correspond to additivity or zero percentage synergism (Di Veroli et al. 2016; Otto et al. 2019; Guerrero Pabón et al. 2021).

Regarding the results for single agent dose response, it was possible to obtain the EC₅₀ for all compounds studied. Therefore, EC₅₀ for chloramphenicol was (1.57 ± 0.04) µg/mL, for amoxicillin it was (3.67 ± 0.28) µg/mL, for perillyl alcohol it was (96.9 ± 2.1) µg/mL and for hydrocinnamic acid it was (1208 ± 1) µg/mL. For metronidazole, only one determination was made, 62.2 µg/mL. As it was expected, EC₅₀ was always lower than MIC for all compounds.

Looking at the matrix synergism plot and the synergism mapped to D-R, and starting by analyzing the outcomes obtained for chloramphenicol/perillyl alcohol (**Appendix E, Figure A1**), it was possible to verify that for lower concentrations of chloramphenicol, up to 2 µg/mL, and for particularly lower concentrations of perillyl alcohol, there was synergism. For a concentration of chloramphenicol of 4 µg/mL and concentrations of perillyl alcohol up to 64 µg/mL, there was a slight antagonism zone. For the highest concentrations, indifference occurs since growth inhibition was observed at these concentrations. The highest synergism score obtained was (19 ± 0) for 1 µg/mL of chloramphenicol and 8 µg/mL of perillyl alcohol and the lowest was (-15 ± 1) for 4 µg/mL of chloramphenicol and 16 µg/mL of perillyl alcohol.

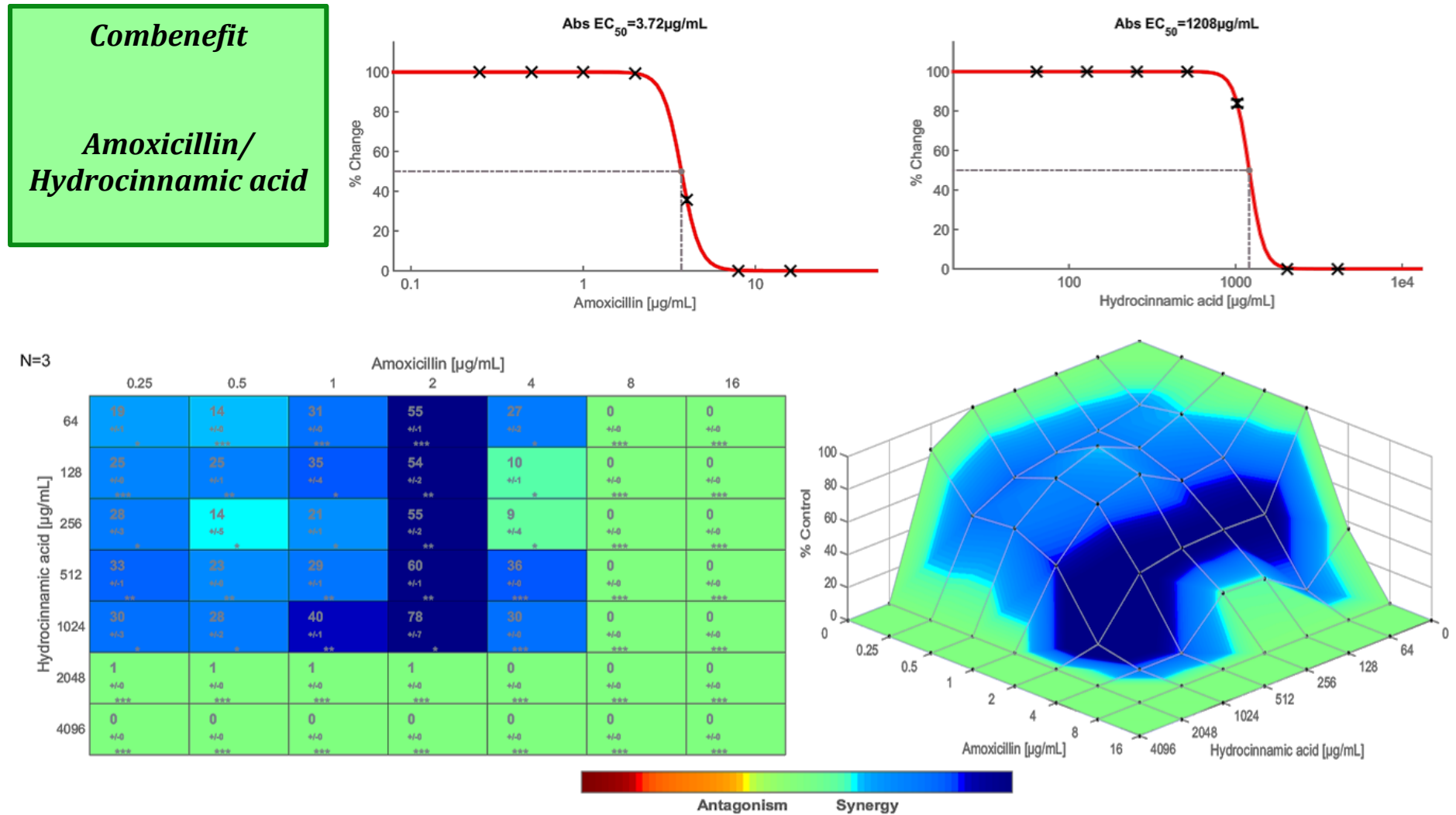


Figure 9. Single agent dose response (at the top), matrix synergism plot (at bottom left) and synergism mapped to D-R (at bottom right) for the combination amoxicillin/hydrocinnamic acid. Values are the mean $\pm$ standard deviation for at three independent experiments.

For chloramphenicol/hydrocinnamic acid (**Appendix E, Figure A2**), it was verified that for lower concentrations of chloramphenicol, especially for 0.5 µg/mL, and in particular for concentrations of hydrocinnamic acid between 64 µg/mL and 1024 µg/mL, there was synergism (mild for concentrations of chloramphenicol above 0.5 µg/mL). For a concentration of chloramphenicol of 2 µg/mL and 4 µg/mL and concentrations of hydrocinnamic acid between 128 µg/mL and 512 µg/mL, there was a zone of transition between indifference and antagonism. For the highest concentrations, indifference occurs since growth inhibition was observed at these concentrations. The highest synergism score obtained was (35 ± 0) for 0.5 µg/mL of chloramphenicol and 512 µg/mL of hydrocinnamic acid and the lowest was (-11 ± 0) for 4 µg/mL of chloramphenicol and 256 µg/mL of hydrocinnamic acid.

For amoxicillin/perillyl alcohol (**Appendix E, Figure A3**), it was verified that for a concentration of amoxicillin up to 4 µg/mL and a concentration of perillyl alcohol up to 32 µg/mL, there was a zone of synergism. It should also be noted that for a concentration of amoxicillin of 2 µg/mL and concentrations of perillyl alcohol between 8 µg/mL and 32 µg/mL, a more intense synergism was observed. For concentrations of amoxicillin up to 4 µg/mL and for concentrations of perillyl alcohol of 64 µg/mL and 128 µg/mL, there was a zone of transition between synergism and indifference. For the highest concentrations, indifference was observed since growth inhibition occurred at these concentrations. The highest synergism score obtained was (46 ± 5) for 2 µg/mL of amoxicillin and 8 µg/mL of perillyl alcohol and the lowest was (0 ± 0).

For amoxicillin/hydrocinnamic acid (**Figure 9**), it was possible to verify that for a concentration of amoxicillin up to 4 µg/mL and a concentration of hydrocinnamic acid up to 1024 µg/mL, there was a zone of synergism. It should also be noted that for a concentration of amoxicillin of 2 µg/mL and concentrations of hydrocinnamic acid between 64 µg/mL and 1024 µg/mL, a more intense synergism zone was observed. For the highest concentrations there was the occurrence of indifference since growth inhibition was observed at these concentrations. The highest synergism score obtained was (78 ± 7) for 2 µg/mL of amoxicillin and 1024 µg/mL of hydrocinnamic acid and the lowest was (0 ± 0).

For amoxicillin/metronidazole (**Appendix E, Figure A4**), it was verified that for a concentration of amoxicillin up to 2 µg/mL and a concentration of metronidazole of 32 µg/mL, there was a mild synergism zone. For concentrations of amoxicillin between 0.5 µg/mL and 2 µg/mL and for a concentration of metronidazole of 512 µg/mL, there was a strong antagonism zone. For the highest concentrations, indifference occurred since growth inhibition was observed at these concentrations. The highest synergism score obtained was (19 ± 4) for 0.25 µg/mL of amoxicillin and 32 µg/mL of metronidazole and the lowest was (-45 ± 3) for 1 µg/mL for amoxicillin and 512 µg/mL for metronidazole.

For perillyl alcohol/hydrocinnamic acid (**Appendix E, Figure A5**), it was possible to verify that for concentrations of perillyl alcohol between 8 µg/mL and 64 µg/mL and concentrations of hydrocinnamic acid between 64 µg/mL and 1024 µg/mL, there was synergism. For a concentration of perillyl alcohol of 128 µg/mL and concentrations of hydrocinnamic acid between 64 µg/mL and 512 µg/mL, there was a zone of slight antagonism. For a concentration of perillyl alcohol of 256 µg/mL and concentrations of hydrocinnamic acid between 64 µg/mL and 1024 µg/mL, there was a transition zone between synergism and indifference. For the highest concentrations, indifference occurred since growth inhibition was observed at these concentrations. The highest synergism score obtained was (31 ± 11) for 8 µg/mL of perillyl alcohol and 1024 µg/mL of hydrocinnamic acid and the lowest was (-14 ± 4) for 128 µg/mL of perillyl alcohol and 64 µg/mL of hydrocinnamic acid.

Overall, the highest synergism score obtained was (78 ± 7) for 2 µg/mL of amoxicillin and 1024 µg/mL of hydrocinnamic acid and the lowest synergism score was (-45 ± 3) for 1 µg/mL of amoxicillin and 512 µg/mL of metronidazole. Not considering the zones of indifference, combinations of amoxicillin with each phytochemical resulted in synergism for various concentrations, which was more intense for a concentration of amoxicillin equal to 2 µg/mL in both cases being these combinations especially interesting. Combinations of chloramphenicol with each phytochemical resulted in synergism mainly at lower concentrations of the antibiotic and a very slight antagonism zone was observed at intermediate or higher concentrations of chloramphenicol. Summing up, Combeneft shows the type and nature of interaction that occurs for each concentrations and FICI is calculated based only on the concentrations for which there is bacterial growth and those for which there is growth inhibition. While FICI determination takes into account the concentrations for which growth is inhibited, the synergism distribution mainly considers what type of interaction occurs until there is growth inhibition. Therefore, it is not possible to establish a clear analogy for these two different analysis (Otto et al. 2019). Based on these results, the potential of therapies based on combinations of antibiotics with phytochemicals in the inhibition of *E. coli* growth was also clear and notorious. Moreover, this assay has shown that a particular focus should be given to combinations chloramphenicol/perillyl alcohol, chloramphenicol/hydrocinnamic acid, amoxicillin/hydrocinnamic acid and amoxicillin/metronidazole while triple combinations were not particularly effective when compared to dual combinations.

4.3.3. Time-kill assay results

Time-kill assay has been widely used to evaluate the eventual bactericidal effect of single compounds and combinations. In fact, it is considered a highly relevant and reliable method for clinical purposes (Pankey et al. 2014). It allows the study of the pharmacodynamics,

providing the rate at which different concentrations of a compound kill a certain bacteria (Ferro et al. 2015). This assay is an excellent test for bactericidal effect assessment because it is defined as $\geq 3 \log_{10}$ (CFU/mL) decrease between starting and ending points of curves, which is equivalent to 99.9% killing of the initial inoculum (Pfaller et al. 2004). In this study, time-kill was performed to assess the extent to which the selected compounds (chloramphenicol, amoxicillin, perillyl alcohol and hydrocinnamic acid) and their dual and triple combinations were able to reduce cellular culturability of *E. coli*. **Figure 10**, **Figure 11** and **Figure 12** represent the time-kill curves for individual compounds, dual combinations and triples combinations, respectively. All the graphs also contain the curves for the controls *E. coli* and *E. coli* plus DMSO 5%. Data were presented in the form of $\log_{10}$ (CFU/mL).

For single compounds, the average $\log_{10}$ (CFU/mL) ranged between 0.00 (for treatments that completely reduced the cellular culturability to a null value) and 8.89 after 24 h for untreated *E. coli*. Among treated cells, the highest $\log_{10}$ (CFU/mL) value was 8.72 and was obtained for *E. coli* exposed to perillyl alcohol at $1/2 \times \text{MIC}$ for 24 h. For all compounds it was found that for concentrations equal to or greater than MBC, there was a total reduction in cellular culturability and $\log_{10}$ (CFU/mL) takes the value of 0.00. In the case of chloramphenicol, this never happened because its MBC (64 $\mu\text{g/mL}$) is greater than $2 \times \text{MIC}$ (32 $\mu\text{g/mL}$). For hydrocinnamic acid, MBC was not detectable, but the time-kill curves suggested that this value should be between MIC and $2 \times \text{MIC}$. In fact, based on MBCs values, biocidal effect was expected at least for amoxicillin and perillyl alcohol (Simep 1987).

For concentrations equal to $1/2 \times \text{MIC}$, there was a net growth of *E. coli* exposed to both phytochemicals after 24 h while for the two antibiotics the cell density at 0 h and after 24 h were very similar. For concentrations of MIC, bacterial growth was observed for perillyl alcohol, a significant reduction of cellular culturability was obtained for amoxicillin (in this case, total reduction) and for hydrocinnamic acid. For chloramphenicol the cell density at 0 h and after 24 h were similar. At $2 \times \text{MIC}$ concentrations, there was a significant reduction in the bacterial population after 24 h for all the compounds (only not total for chloramphenicol). Thus, amoxicillin at MIC and $2 \times \text{MIC}$ and both phytochemicals at $2 \times \text{MIC}$ exhibited bactericidal effect, reducing the starting $\log_{10}$ (CFU/mL) by greater than 3 logs. Chloramphenicol at all tested concentrations, amoxicillin at MIC and hydrocinnamic acid at MIC showed bacteriostatic effect, as the $\log_{10}$ (CFU/mL) over time remained not very different ($< 3 \log_{10}$ (CFU/mL) decrease) from the starting value. Finally, perillyl alcohol at $1/2 \times \text{MIC}$ and MIC and hydrocinnamic acid at $1/2 \times \text{MIC}$ revealed little antimicrobial effect, as the bacteria in the presence of these compound grew over time to a similar level as for the control. These results also confirmed the information contained in the literature that classifies chloramphenicol as a bacteriostatic antibiotic (Rahal and Simberkoff 1979) and amoxicillin as bactericidal (Karaman 2015). It was also observed that the effect exerted by the phytochemicals was highly dependent on their concentration and

the cellular culturability reduction and the efficacy of the compounds were dose and time dependent. In addition, the lowest value of $\log_{10}$ (CFU/mL) for chloramphenicol at $1/2 \times \text{MIC}$ (6.88 after 6 h) and MIC (6.39 after 8 h) and for amoxicillin at $1/2 \times \text{MIC}$ (5.07 after 8 h) was not obtained after 24 h and there was a phenomenon of "recovery" of cellular culturability.

Dual combinations chloramphenicol/perillyl alcohol, chloramphenicol/hydrocinnamic acid, amoxicillin/perillyl alcohol, amoxicillin/hydrocinnamic acid, amoxicillin/metronidazole and perillyl alcohol/hydrocinnamic acid as well as the triple combinations chloramphenicol/perillyl alcohol/hydrocinnamic acid and amoxicillin/perillyl alcohol/hydrocinnamic acid were also evaluated in order to analyse their potential, in this case, with regard to their eventual bactericidal effect. For combinations, the average $\log_{10}$ (CFU/mL) ranged between 0.00 (for treatments that completely reduced the cellular culturability to a null value) and 8.26 after 24 h of exposure to perillyl alcohol/hydrocinnamic acid at $1/2 \times \text{MIC}$ of each compound. Combinations chloramphenicol/perillyl alcohol at $2 \times \text{MIC}$ (of each compound), chloramphenicol/hydrocinnamic acid at $2 \times \text{MIC}$, amoxicillin/perillyl alcohol at MIC and $2 \times \text{MIC}$, amoxicillin/hydrocinnamic acid at MIC and $2 \times \text{MIC}$, amoxicillin/metronidazole for all the concentrations, perillyl alcohol/hydrocinnamic acid at $2 \times \text{MIC}$ and both triple combinations at $2 \times \text{MIC}$ exhibited bactericidal effect. Combinations chloramphenicol/perillyl alcohol at MIC, chloramphenicol/hydrocinnamic acid at MIC, amoxicillin/perillyl alcohol at $1/2 \times \text{MIC}$, amoxicillin/hydrocinnamic acid at $1/2 \times \text{MIC}$, perillyl alcohol/hydrocinnamic acid at MIC and both triple combinations at $1/2 \times \text{MIC}$ and MIC showed bacteriostatic effect. Finally, combinations chloramphenicol/perillyl alcohol at $1/2 \times \text{MIC}$, chloramphenicol/hydrocinnamic acid at $1/2 \times \text{MIC}$ and perillyl alcohol/hydrocinnamic acid at $1/2 \times \text{MIC}$ revealed little antimicrobial effect. Cellular culturability reduction and the efficacy of combinations were dose and time dependent. In addition, the lowest value of $\log_{10}$ (CFU/mL) for chloramphenicol/perillyl alcohol at $1/2 \times \text{MIC}$ (6.90 after 1 h), for amoxicillin/perillyl alcohol at $1/2 \times \text{MIC}$ (5.04 after 6 h), for amoxicillin/metronidazole at $1/2 \times \text{MIC}$ (4.18 after 8 h) and perillyl alcohol/hydrocinnamic acid at $1/2 \times \text{MIC}$ (6.98 after 1 h) was not obtained after 24 h and there was a phenomenon of "recovery" of cellular culturability.

Dual and triple combinations were also evaluated applying the classification proposed by Zhou *et al.* (2020) with some modifications (**Table 12**). Considering both scores obtained for combinations with a classification assigned to $1/2 \times \text{MIC}$ and MIC of each compound, eight combinations resulted in indifference (66.7%), two in additivity (16.7%) and two in synergism (16.7%). In fact, some other authors such as Dhara and Tripathi (2020) also reported interesting results, including synergism, for combinations of antibiotics with natural compounds regarding bactericidal activity assessed by time-kill. Furthermore, it was possible to perform a comparison between the classification attributed to each combination by checkerboard and time-kill (for combinations with a classification assigned for $1/2 \times \text{MIC}$ and MIC of each

compound by time-kill, the one associated to MIC of each compound was considered). From the eight different combinations between compounds that were tested, only one (amoxicillin/hydrocinnamic acid) obtained a distinct classification by checkerboard (synergism) and time-kill (additivity). The percentage of agreement between the two methods was 87.5%.

Table 12. Classification of dual and triple combinations based on time-kill curves. Values are the mean $\pm$ standard deviation for at least two independent experiments.

Combination	$\Delta \log_{10}$ (CFU/mL)	Classification
<i>Chloramphenicol/Perillyl alcohol (1/2×MIC)</i>	0.47	Indifference
<i>Chloramphenicol/Perillyl alcohol (MIC)</i>	-2.29	Synergism
<i>Chloramphenicol/Hydrocinnamic acid (1/2×MIC)</i>	-0.33	Indifference
<i>Chloramphenicol/Hydrocinnamic acid (MIC)</i>	-1.31	Additivity
<i>Amoxicillin/Perillyl alcohol (1/2×MIC)</i>	-0.54	Indifference
<i>Amoxicillin/Hydrocinnamic acid (1/2×MIC)</i>	-1.28	Additivity
<i>Amoxicillin/Metronidazole (1/2×MIC)</i>	-2.67	Synergism
<i>Perillyl alcohol/Hydrocinnamic acid (1/2×MIC)</i>	0.03	Indifference
<i>Perillyl alcohol/Hydrocinnamic acid (MIC)</i>	-0.87	Indifference
<i>Chloramphenicol/Perillyl alcohol/Hydrocinnamic acid (1/2×MIC)</i>	-0.32	Indifference
<i>Chloramphenicol/Perillyl alcohol/Hydrocinnamic acid (MIC)</i>	0.20	Indifference
<i>Amoxicillin/Perillyl alcohol/Hydrocinnamic acid (1/2×MIC)</i>	-0.46	Indifference

Figure A6 in **Appendix F** shows the AUCs values for the time-kill curves. After performing statistical analysis to the AUCs values, it was possible to verify that the curve obtained for *E. coli* exposed to DMSO 5% was not statistically significant different from the curve for *E. coli* ($p > 0.05$). Moreover, all treatments were statistically significant different from controls ($p < 0.05$) with the exception of *E. coli* exposed to perillyl alcohol at 1/2×MIC ($p > 0.05$). It should also be noted that the combination amoxicillin/metronidazole was more effective than amoxicillin/perillyl alcohol and amoxicillin/hydrocinnamic acid, at 1/2×MIC and MIC ($p < 0.05$). This assay has shown that a particular focus should be given to combinations chloramphenicol/perillyl alcohol, chloramphenicol/hydrocinnamic acid, amoxicillin/hydrocinnamic acid and amoxicillin/metronidazole while triple combinations were not particularly effective when compared to dual combinations.

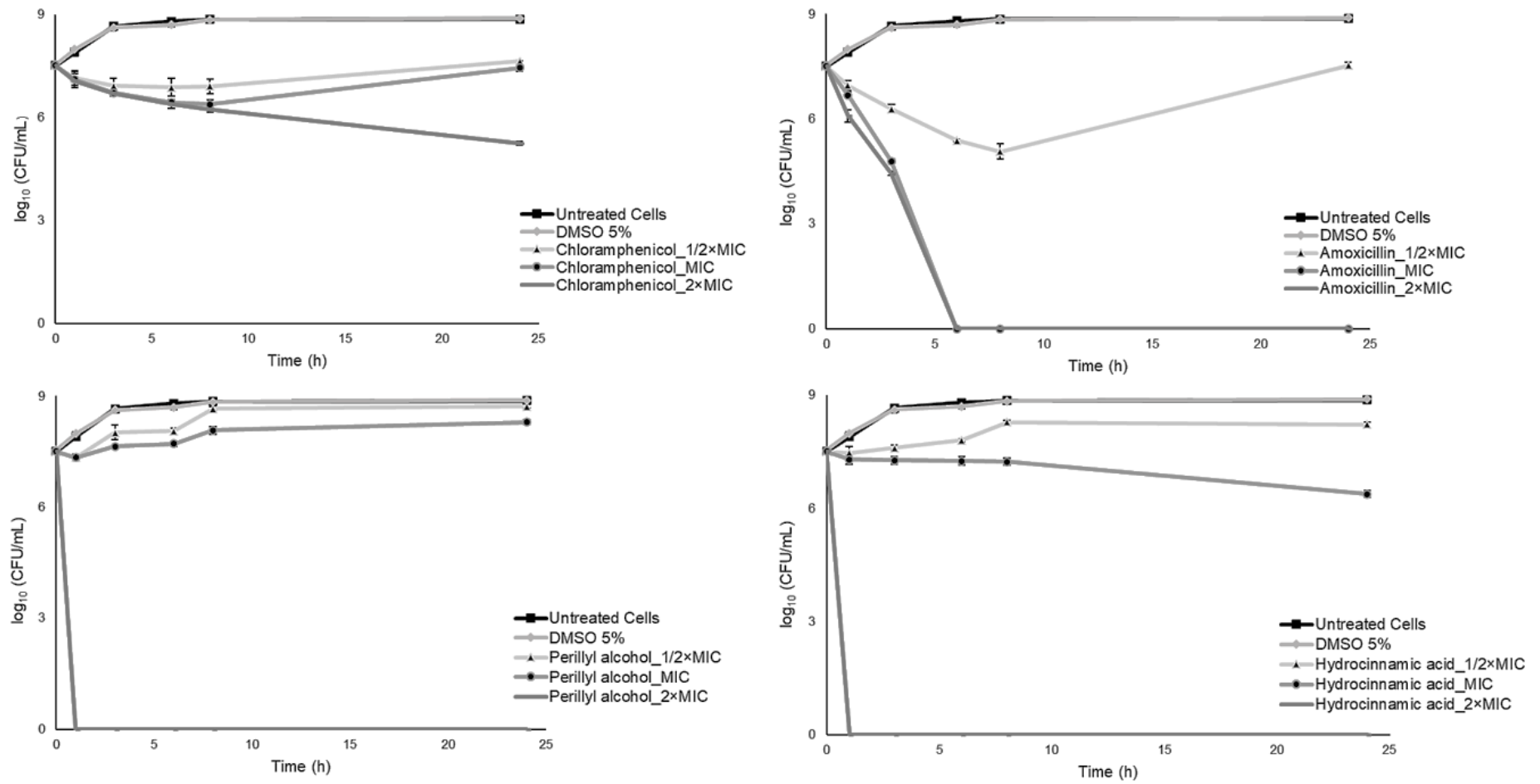


Figure 10. Time-kill curves for individual compounds and two negative controls: *E. coli* and *E. coli* plus DMSO 5%. Values are the mean $\pm$ standard deviation for at least two independent experiments.

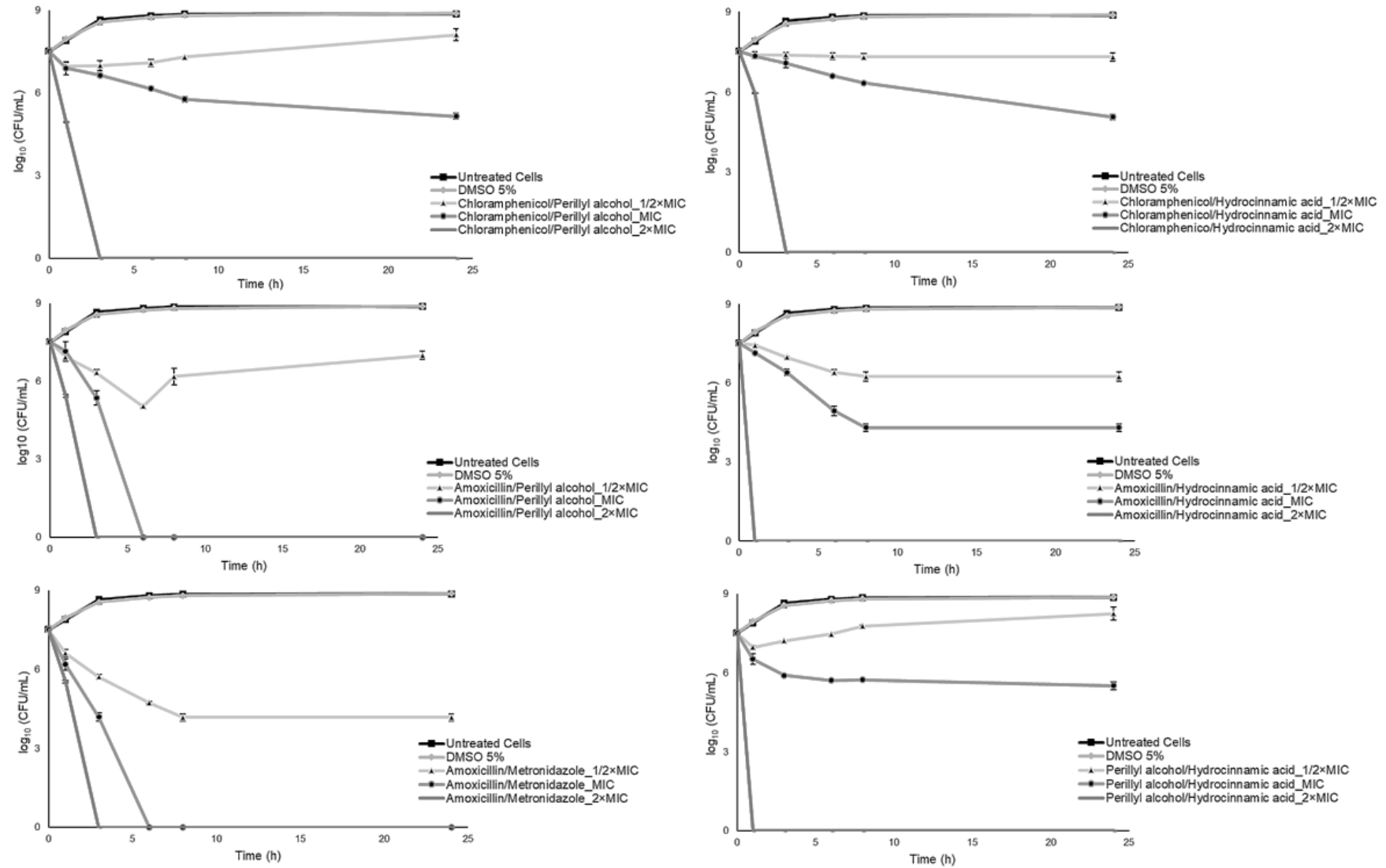


Figure 11. Time-kill curves for dual combinations and two negative controls: *E. coli* and *E. coli* plus DMSO 5%. Values are the mean $\pm$ standard deviation for at least two independent experiments.

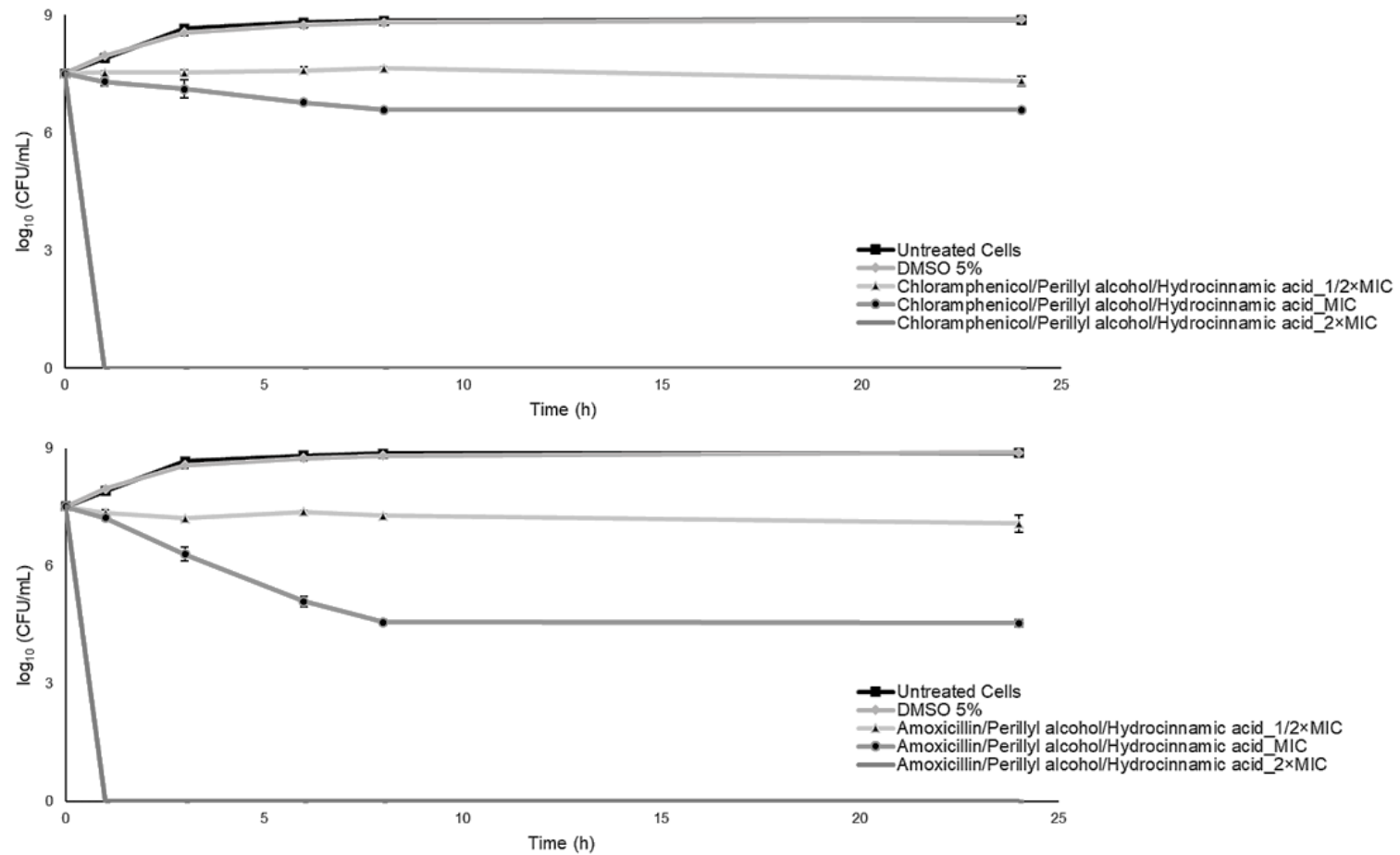


Figure 12. Time-kill curves for triple combinations and two negative controls: *E. coli* and *E. coli* plus DMSO 5%. Values are the mean $\pm$ standard deviation for at least two independent experiments.

4.3.4. Evaluation of biofilm control for dual and triple combinations

Biofilms are known to constitute a severe concern to public health due to the increasing resistance of biofilm-related pathogens (Donlan 2001). However, many studies demonstrated that phytochemicals can be used in co-therapies to find new effective treatments for biofilm-associated infections and reduce the resistance to antibiotics (Soe et al. 2010; Monte et al. 2014; Abreu et al. 2016). In the case of biofilms, there seems to be a particular need for combinatorial therapies as their complex structure requires to harm cells in different metabolic forms (Batoni et al. 2016). The six dual combinations and two triple combinations previously assessed in this study were evaluated for the control of biofilms of *E. coli*, at MIC and 5×MIC.

Figure 13 shows the percentages of metabolic inactivation, for *E. coli* 24 h biofilms, after exposure to dual and triple combinations at MIC and 5×MIC (individual MICs) for 24 h.

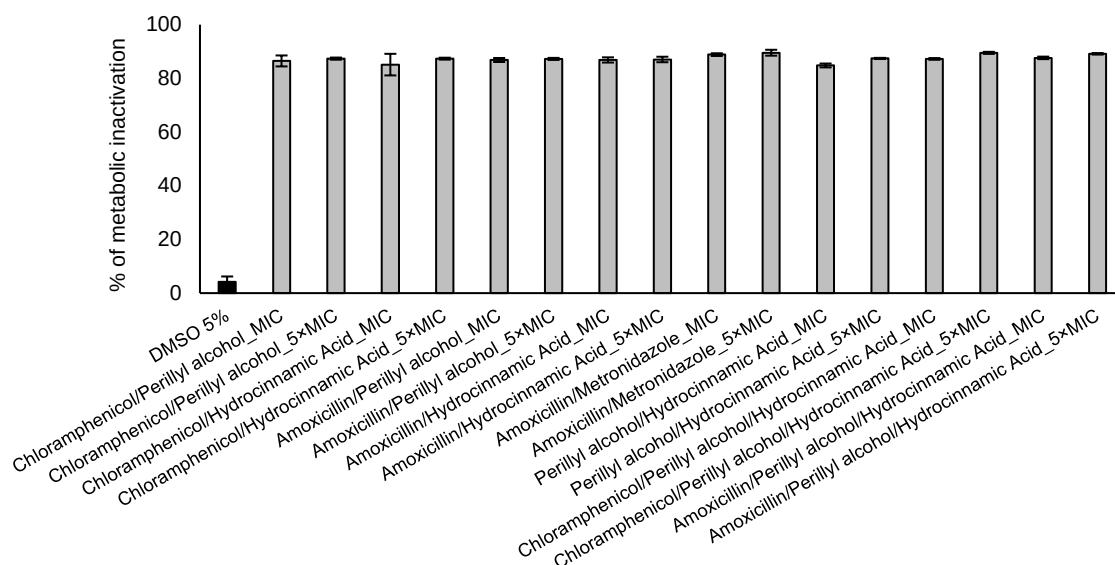


Figure 13. Percentages of percentages of metabolic inactivation, for *E. coli* 24 h biofilms, after exposure to the tested combinations at MIC and 5×MIC for 24 h. Values are the mean $\pm$ standard deviation for at least two independent experiments.

As expected, the negative control with DMSO 5% was not able to cause a remarkable reduction in metabolic activity of biofilms of *E. coli* ($p > 0.05$). The lowest percentage of metabolic inactivation was equal to 84.8% and was obtained for the combination perillyl alcohol/hydrocinnamic acid at MIC while the highest value was equal to 89.5% and was obtained for chloramphenicol/perillyl alcohol/hydrocinnamic acid at 5×MIC. The percentages obtained for treated and untreated biofilms were statistically significant difference ($p < 0.05$). The percentages of metabolic inactivation obtained for all combinations were all very close and between 80.0% and 90.0%, showing there must be a level close to 90.0% of metabolic inactivation for *E. coli* biofilms that probably can not be overtaken (or must be extremely difficult

to). A similar phenomenon was also reported in a study developed by Leão *et al.* (2020), where the antimicrobial and antibiofilm activities of non-steroidal anti-inflammatory drugs against *E. coli* and *S. aureus* were study. However, no justification was advanced for that. Furthermore, the combination amoxicillin/metronidazole did not prove to be significantly more effective than amoxicillin/perillyl alcohol and amoxicillin/hydrocinnamic acid ($p > 0.05$). According to the classification of Lemos *et al.* (2014), all combinations resulted in high efficacy. Looking at the metabolic activity inactivation caused by the individual compounds against *E. coli*, nine treatments showed high efficacy and the other three resulted in moderate efficacy.

Figure 14 shows the percentages of biomass reduction, for *E. coli* 24 h biofilms, after exposure to dual and triple combination at MIC and 5×MIC (individual MICs) for 24 h.

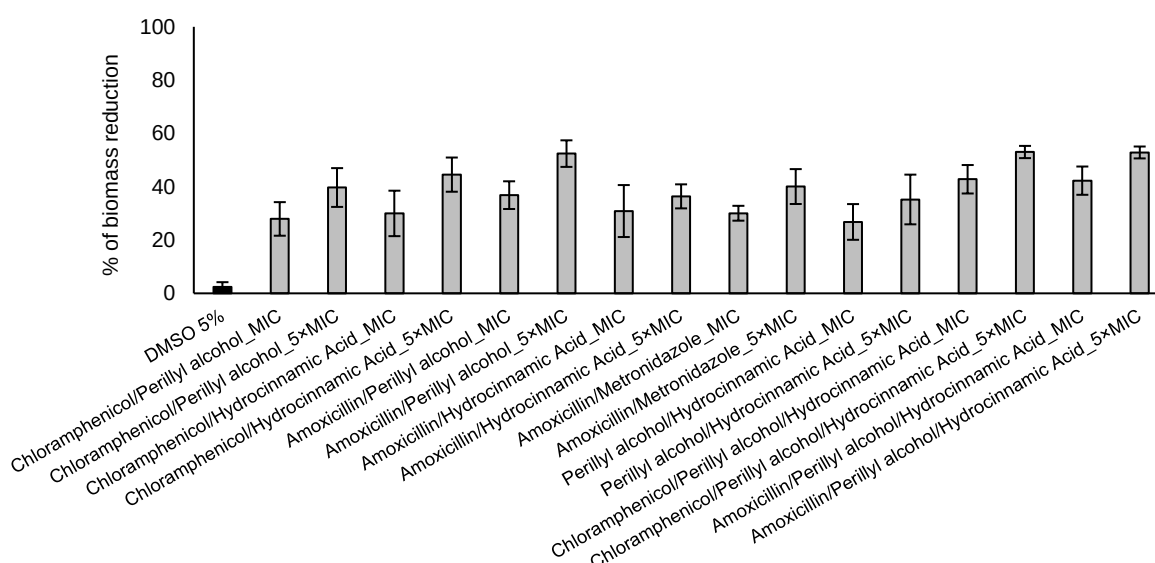


Figure 14. Percentages of biomass reduction, for *E. coli* 24 h biofilms, after exposure to the tested combinations at MIC and 5×MIC for 24 h. Values are the mean ± standard deviation for at least two independent experiments.

As expected, the negative control with DMSO 5% was not able to cause a remarkable reduction in biomass of *E. coli* ($p > 0.05$). The lowest percentage of biomass reduction was equal to 26.8% and was obtained for the combination perillyl alcohol/hydrocinnamic acid at MIC while the highest value was equal to 53.0% and was obtained for chloramphenicol/perillyl alcohol/hydrocinnamic acid at 5×MIC. The percentages obtained for treated and untreated biofilms has always shown to be statistically significant difference ($p < 0.05$). It was observed that increasing the concentrations, the percentages of biomass reduction tends to increase. Also, the triple combinations studied provided higher percentages of biomass reduction than dual combinations ($p < 0.05$). The combination amoxicillin/metronidazole was not statistically significant different from the amoxicillin/hydrocinnamic acid ($p > 0.05$), whereas the combination amoxicillin/perillyl alcohol was more effective than these two ($p < 0.05$). In general,

different combinations provided distinct results. According to the classification of Lemos *et al.* (2014), all combinations resulted in moderate efficacy. Looking at the biomass reduction caused by the individual compounds against *E. coli*, only three treatments showed moderate efficacy and the other nine resulted in low efficacy.

Figure 15 presents the $\log_{10}$ (CFU/cm²) and **Figure 16** shows the $\log_{10}$ reduction, for *E. coli* 24 h biofilms, after exposure to dual and triple combinations at MIC and 5×MIC (individual MICs) for 24 h.

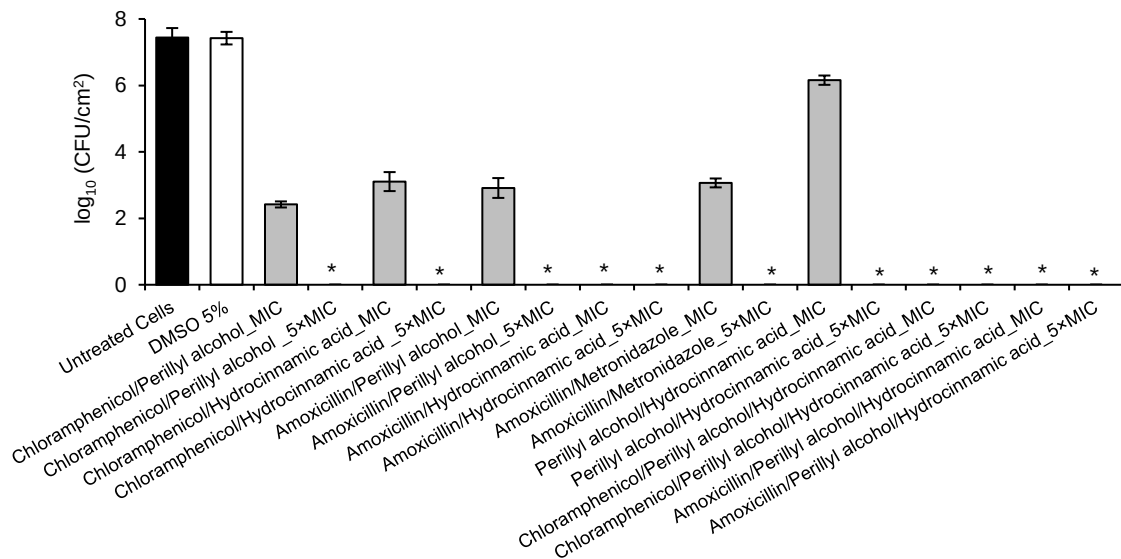


Figure 15. $\log_{10}$ (CFU/cm²), for *E. coli* 24 h biofilms, after exposure to the tested combinations at MIC and 5×MIC for 24 h. (*) means that the result obtained was lower than the detection limit. Values are the mean $\pm$ standard deviation for at least two independent experiments.

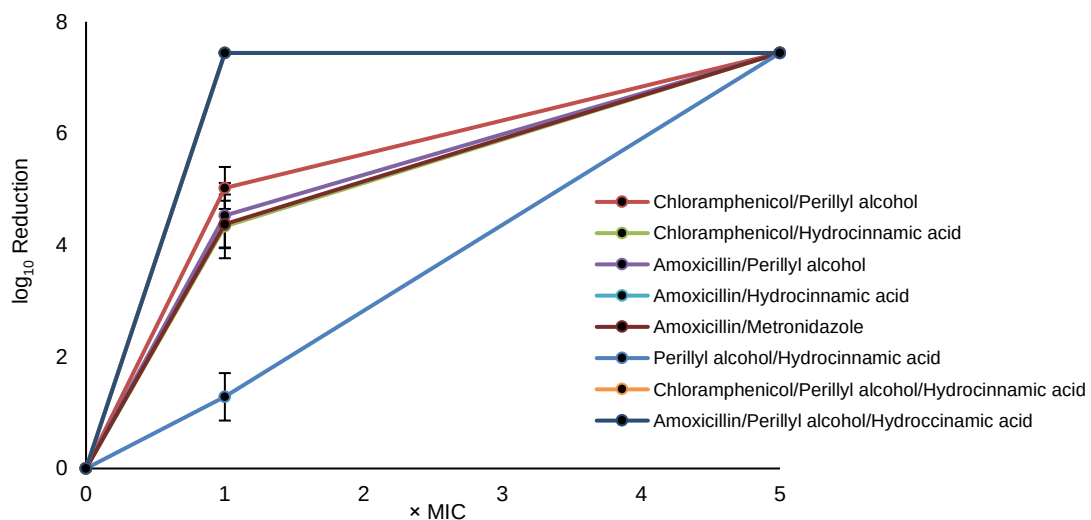


Figure 16. $\log_{10}$ reduction, for *E. coli* 24 h biofilms, after exposure to the tested combination at MIC and 5×MIC for 24 h. Values are the mean $\pm$ standard deviation for at least two independent experiments.

The negative control with DMSO 5% was not able to cause a remarkable reduction in culturability of *E. coli* ($p > 0.05$). The lowest effect was obtained for the combination perillyl alcohol/hydrocinnamic acid at MIC, corresponding to a $\log_{10}$ (CFU/cm²) of 6.16 and a $\log_{10}$ reduction of 1.28 while the highest value, equivalent to a total absence of cell culturability, was obtained for several treatments. The results obtained for treated and untreated biofilms were statistically significant difference ($p < 0.05$). It was also observed that increasing the concentrations, the $\log_{10}$ (CFU/cm²) tends to decrease and $\log_{10}$ reduction tends to increase. The triple combinations were particularly advantageous when compared to most of the dual combinations ($p < 0.05$) since for the lowest concentration tested there was a total absence of cellular culturability (only verified for one of dual combinations: amoxicillin/hydrocinnamic acid). The combination amoxicillin/hydrocinnamic acid caused a greater reduction in cellular culturability than amoxicillin/metronidazole ($p < 0.05$) and there was no statistically significant difference between amoxicillin/perillyl alcohol and amoxicillin/metronidazole ($p > 0.05$). In general, different combinations provided distinct results.

The effect of combinations on the cellular culturability of biofilms was evaluate following the classification proposed by Zhou *et al.* (2020) with some modifications (**Table 13**), also applied in this study to time-kill results. In both cases the values of $\log_{10}$ of CFU per unit of space or time were assessed and the exposure time was equal to 24 h.

Table 13. Classification of combination regarding cellular culturability of biofilms. Values are the mean $\pm$ standard deviation for at least two independent experiments.

Combination (at MIC)	$\Delta \log_{10}$ (CFU/cm²)	Classification
<i>Chloramphenicol/Perillyl alcohol</i>	-2.08	Synergism
<i>Chloramphenicol/Hydrocinnamic acid</i>	-1.55	Additivity
<i>Amoxicillin/Perillyl alcohol</i>	-1.59	Additivity
<i>Amoxicillin/Metronidazole</i>	-1.31	Additivity
<i>Perillyl alcohol/Hydrocinnamic acid</i>	1.66	Indifference

For concentrations equal to MIC of the individual compounds, synergism was found for one combination (20.0%), additivity for three (60.0%), indifference for one (20.0%) and the combination amoxicillin/hydrocinnamic acid led to a total absence of cellular culturability. This results show the potential of these combinations as antibiofilm therapy for the control of sessile *E. coli* infections, especially regarding the combinations of antibiotics with phytochemicals.

Overall, there was a good agreement between the three methods performed concerning the most effective (chloramphenicol/perillyl alcohol/hydrocinnamic acid) and the least active combinations (perillyl alcohol/hydrocinnamic acid) against *E. coli* biofilms. These results also revealed a high potential and success of combinations of antibiotics and phytochemicals against *E. coli* biofilms, as indicated by some other authors, namely Barbieri *et al.* (2017).

Chapter 5 – Conclusions and future perspectives

In the recent decades, the acceptance of non-traditional medicine as an alternative form of healthcare and the emergence of resistance to classic antibiotics were a consequence of the spread of MDR pathogens. As it became a serious and global public health threat, this issue has instigated researchers to study the antibacterial and antibiofilm activity of several medicinal plants used in popular folk medicine and their combination with antibiotics.

The objective of this work was to study the antimicrobial and antibiofilm activity of five phytochemicals: 7-hydroxycoumarin, naringin hydrate, theophylline, perillyl alcohol and hydrocinnamic acid against *S. aureus* CECT 976 and *E. coli* CECT 434. In fact, these bacteria are representative of the strains that most trouble cause within the issue of antibiotic resistance. It was also intended to evaluate the interest of combinations of selected phytochemicals and antibiotics and understand if a potentiation effect could be obtained.

Both bacteria were characterized for their susceptibility profile to various antibiotics. In addition, the MICs and MBCs of the five phytochemicals were also evaluated for both bacteria. Among the phytochemicals, perillyl alcohol (with a MIC equal to 1024 µg/mL for *S. aureus* and 256 µg/mL for *E. coli*) and hydrocinnamic acid (with a MIC of 2048 µg/mL for both bacteria) were the most effective. Furthermore, and according to Lipinski's rule of five, all compounds could be considered “drug-like” molecules with the exception of naringin hydrate. When the assays with biofilm were carried out, despite both bacteria being weak biofilm producers, there were some treatments more effective than others, namely the treatments with both phytochemicals at concentrations equal to 5×MIC and 10×MIC, with metabolic inactivation percentages between 89.54% and 92.47% for *S. aureus* and a total elimination of the CFU of *S. aureus* and *E. coli* biofilms. The highest percentages of biomass reduction, between 41.96% and 59.71%, were obtained with amoxicillin for both bacteria. Since *E. coli* presented the most worrying susceptibility profile, taking into account the results obtained for amoxicillin and chloramphenicol, it was decided to focus this study on that strain. Thus, the antimicrobial and antibiofilm potential of the natural compounds studied and selected became clear.

Looking at the effects of combinations, checkerboard and time-kill demonstrated that combinations chloramphenicol/perillyl alcohol (synergistic for both assays), chloramphenicol/hydrocinnamic acid (additive for both assays), amoxicillin/hydrocinnamic acid (synergistic for checkerboard and additive for time-kill) and amoxicillin/metronidazole (synergistic for both assays) were particularly effective, with a percentage of agreement between the two assays of 87.5%. At the same time, triple combinations did not reveal a special interest (indifferent for both tests). The use of Combeneft to evaluate dual

combinations was an innovation of this work since, as far as it is known, there is only one study on antimicrobial activity where this software was used. This analysis revealed that combinations of chloramphenicol with each selected phytochemical resulted in synergism at lower concentrations of chloramphenicol and a very slight antagonism zone was observed at intermediate or higher concentrations of the antibiotic. Combinations of amoxicillin with each selected phytochemical (perillyl alcohol and hydrocinnamic acid) has shown synergism zones for diverse concentrations being more intense for a concentration of amoxicillin of 2 µg/mL. For *E. coli* biofilms in general, the most active combination was chloramphenicol/perillyl alcohol/hydrocinnamic acid (metabolic inactivation from 87.24% to 89.49% and biomass reduction from 42.81% to 53.04%) and the least effective combination was perillyl alcohol/hydrocinnamic acid (metabolic inactivation from 84.80% to 87.41%, biomass reduction from 26.79% to 35.22% and classified as indifferent based on the results of cellular culturability for biofilm cells). Thereby, the interest of combinatorial therapies based on phytochemicals and antibiotics for the control of healthcare-associated infections has been established.

In the future, it is recommended the study of the bioactivity and targets of the phytochemicals evaluated in this work at the molecular level so that their mechanism of action can be completely elucidated. A detailed and accurate analysis in order to characterize the interactions between phytochemicals and antibiotics using different approaches and methods facilitated by the target profile, molecular information and toxicity data would also be very interesting. The molecular docking approach can be used both to define the targets of natural molecules studied in microorganisms and to study the interaction between the phytochemicals and the antibiotics evaluated at the molecular level. Finally, the experimental results encourage further studies with these and other classes of phytochemicals and *in vivo* experiments to validate the important observations made *in vitro* in this study.

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Appendix

Appendix A – Classes of phytochemicals

Table A1. Classes of phytochemicals.

<i>Class</i>	<i>Examples</i>	<i>Structural Description</i>	<i>Benefits/ Activities</i>	<i>References</i>
<i>Phenolic compounds</i>	Flavonols Flavones Flavanones Isoflavones Flavan-3-ols Anthocyanidins Phenolic Acids Hydroxycinnamate Stilbenes	Aromatic ring(s) with one or more hydroxyl groups linked to linear, highly polymerized chains. Flavonoids are the largest group of phenolic compounds.	Antidiabetic Antioxidant Anticancer Anti-hyperlipidemic Anti-hyperglycemic Anti-hypertensive Antimicrobial	(Hogervorst Cvejić et al. 2017; Farhat et al. 2017; Aguirre and Borneo 2019)
<i>Alkaloids</i>	Indoles Quinolines Isoquinolines Pyrrolidines Pyridines Pyrrolizidines Tropanes Terpenoids Steroids	Heterocyclic ring with a nitrogen atom in a negative oxidation state.	Anti-inflammatory Anticancer Analgesics Local Anesthetic Neuropharmacologic Antimicrobial	(Flieger 2000; Pyne et al. 2016; Shoaib et al. 2016; Kurek 2019)
<i>Organosulfur compounds</i>	Allicin Cysteine Cystine Biotin Glutathione Methionine Penicillin Thiamin Chloride Sulfanilamide	Cyclic or noncyclic molecules with sulfur atoms linked to a cyanate group or a carbon atom.	Antioxidant Anti-inflammatory Anticarcinogenic Anti-hypertensive Anti-hyperlipidemic Anti-atherosclerotic Cardioprotective Antiplatelet Immunomodulatory Antimicrobial	(Tsuji et al. 2016; Bansal et al. 2018; Arya and Saldanha 2019; Walag et al. 2020)
<i>Terpenoids</i>	Hemiterpenoids Monoterpenoids Sesquiterpenoids Diterpenoids Sesterterpenoids Triterpenoids Polyterpenoids	Structural organization of carbons formed by the linear arrangement of isoprene units.	Antioxidant Anti-inflammatory Anticancer Immunomodulatory Antimicrobial	(Bian et al. 2018; Isah et al. 2018; Reyes et al. 2018; Wu et al. 2020)
<i>Carotenoids</i>	Carotenes Cryptoxanthins Lutein Xanthophylls Lycopene	Molecules with a 40-carbon chain backbone.	Antioxidant Anticancer Cardioprotective Antimicrobial	(Cooperstone and Schwartz 2016; Schieber and Weber 2016; Nagarajan et al. 2017)
<i>Phytoestrogens</i>	Isoflavonoids Flavonoids Stilbenes Lignans	Polyphenolic, non-steroidal compounds similar to β -estradiol.	Antidiabetic Antioxidant Anti-inflammatory Antineoplastic Anti-atherogenic Anti-angiogenic Antimicrobial	(Patisaul and Jefferson 2010; Nieves 2013; Darbre 2015; Memariani et al. 2020)
<i>Phytosterols</i>	Desmethyl sterols Monomethyl sterols Dimethyl sterols Sitosterols Campesterols	Tetracyclic cyclopenta- α -phenanthrene ring with a hydroxyl group at C3 and a side chain at C17.	Anti-inflammatory Anticancer Anti-hyperlipidemic Cardioprotective Anti-atherogenic Antimicrobial	(Trautwein and Demonty 2007; Piironen and Lampi 2014; Uddin et al. 2015; Ogbe 2015)

Appendix B – Chemical, structural and molecular properties of the tested compounds

Chloramphenicol is an amphenicol-class, semisynthetic antibiotic presenting a broad-spectrum bacteriostatic activity (Rahal and Simberkoff 1979). It is a organochlorine antibiotic and consists of an acetamide group substituted with a dichloro, having a nitrobenzene ring, an amide bond and two alcohol groups (National Center for Biotechnology Information 2021a). This antibiotic is not photosensitive but is heat sensitive. Chloramphenicol is soluble in lipids, and, in water, its solubility is equal to 2.5 mg/mL at 25 °C being soluble in organic solvents such as ethanol, DMSO, and dimethyl formamide (DMF)(National Center for Biotechnology Information 2021a). Chloramphenicol can bind to the bacterial 50S ribosomal subunit, inhibiting protein synthesis. It is derived from *Streptomyces venequellae* (Scholar 2007). However, chloramphenicol has been little tested and studied so far because of its undesirable adverse effects (Oong and Tadi 2021). Chloramphenicol was obtained from Sigma-Aldrich, Merck KGaA, China (in the form of a white fine crystalline powder).

Amoxicillin is an broad-spectrum, semisynthetic and bactericidal antibiotic used to treat a number of bacterial infections caused by both Gram-negative and Gram-positive bacteria (Karaman 2015; Skarp et al. 2019). It is a β -lactam antibiotic, belonging to the aminopenicillin family, that presents a 2-amino-2-(4-hydroxyphenyl)acetamido group at position 6 of the penam ring (National Center for Biotechnology Information 2022a). This antibiotic inhibits cross-linkage between the linear peptidoglycan polymer chains that constitute the bacterial cell wall during the exponential growth phase (Sezer 2014). Amoxicillin is soluble in organic solvents and lipids, namely DMSO and dimethyl formamide, and, in water, its solubility is equal to 3.43 mg/mL at 25 °C. It is not photosensitive but is heat sensitive (National Center for Biotechnology Information 2022a). Moreover, metronidazole is usually used as adjunct of amoxicillin in periodontal infections, leading to a better clinical outcome when compared to the treatment based on amoxicillin alone. In fact, the development of resistance is very common when it comes to broad spectrum antibiotics. Metronidazole in combination with amoxicillin hinders the emergence of resistance to the second antibiotic mentioned (Yek et al. 2010; Dabija-Wolter et al. 2018). Metronidazole is an imidazole compound that presents three substituent groups: a 2-hydroxyethyl group at C1, a nitro group at C2, and a methyl group at C5 (National Center for Biotechnology Information 2022b). It can inhibit the synthesis of nucleic acid by cells through the formation of nitroso radicals that are toxic to the cells (Dingsdag and Hunter 2018). Since this action requires metronidazole to be partially reduced, which normally only happens in anaerobic bacteria and protozoans, this compound has a weak activity against aerobic microorganisms or human cells (Lamp et al. 1999). Citron *et al.* (2012) tested eighteen isolates of *E. coli* and obtained a range of MICs values for metronidazole between 128 μ g/mL

and 1024 µg/mL. In water, its solubility is equal to 11 mg/mL at 25 °C. Metronidazole is not photosensitive but is heat sensitive (National Center for Biotechnology Information 2022b). Amoxicillin was obtained from Sigma-Aldrich, Merck KGaA, China (in the form of a white crystalline powder) and metronidazole was obtained from Thermo Fisher Scientific, Alfa Aesar, China (in the form of a white crystalline powder).

7-hydroxycoumarin is a natural, organic benzopyrone from the coumarin family. Coumarins are lactones exhibiting a benzene ring linked to an α -pyrone ring. 7-hydroxycoumarin presents one hydroxyl (OH) group linked to the C7 carbon of the coumarin scaffold and absorbs ultraviolet light strongly at several wavelengths. Moreover, there are some indications that suggest that this compound is photomutagenic (Du et al. 2008; Sim et al. 2015; Seoane-Rivero et al. 2020). 7-hydroxycoumarin is also photosensitive, as it can be modified by the effect of natural light, and heat sensitive (Matos et al. 2015). In water, its solubility is equal to 11.3 mg/mL at 25 °C being soluble in organic solvents (National Center for Biotechnology Information 2021b). 7-hydroxycoumarin was obtained from Merck KGaA, Germany (in the form of a white powder/crystal).

Naringin hydrate is a flavonoid, belonging to the class of phenolic compounds. Flavonoids are composed of fifteen carbon atoms distributed by three rings, two of them being benzene rings connected by a 3-carbon chain. Naringin hydrate can be found in citrus fruits, particularly in grapefruit, where it is responsible for the bitter taste (Alam et al. 2014). Flavonoids, in general, are affected by sunlight and are heat sensitive (Zhang et al. 2019). In water, the solubility of naringin is equal to 1.0 mg/mL at 40 °C (National Center for Biotechnology Information 2021c). Naringin hydrate was obtained from Thermo Fisher Scientific, Alfa Aesar, Germany (in the form of a light-yellow powder/crystal).

Theophylline is an alkaloid. It is a derivative from methylxanthine present in tea and coffee. Theophylline has the following functional groups: two amide groups, two methyl groups, an imine group and alkene carbon atoms (Krishna Sri and Padmavathy 2018). The stability of alkaloids depends on light and heat (Dey et al. 2020). In water, its solubility is equal to 7.4 mg/mL at 25 °C (National Center for Biotechnology Information 2021d). Theophylline was obtained from Sigma-Aldrich, Merck KGaA, China (in the form of a white crystalline powder).

Perillyl alcohol is a monocyclic terpene (limonene monoterpenoid) derived from the mevalonate produced by plants (Crowell PL 2000). It has a cyclohexene substituted by a hydroxymethyl group, at C1, and a prop-1-en-2-yl group, at C4. Perillyl alcohol is present in several essential oils such as lavender (National Center for Biotechnology Information 2021e). Moreover, terpenes are noticeably light, oxygen and heat sensitive. In water, its solubility is equal to 1.9 mg/mL at 25 °C (Eichhorn Bilodeau et al. 2019). Perillyl alcohol was obtained from Sigma-Aldrich, Merck KGaA, Japan (in the form of a colourless, dense, oily liquid).

Hydrocinnamic acid, also known as 3-phenylpropionic acid, is a propionic acid, consisting of a monocarboxylic acid and presenting a phenyl group at 3C. It belongs to the class of phenylpropanoids being a phenolic compound, and is produced by humans and plants. Phenylpropanoids possess an aromatic ring as well as a tail with three carbons atoms of the coumaric acid (Kawaguchi et al. 2017; National Center for Biotechnology Information 2021f). This compound should not be in contact with high temperatures and its contact with sunlight should also be void because this substance is sunlight sensitive (echa 2021). In water, its solubility is equal to 5.9 mg/mL at 25 °C (National Center for Biotechnology Information 2021f). Hydrocinnamic acid was obtained from Thermo Fisher Scientific, Acros Organics, USA (in the form of a white crystalline solid).

Appendix C – Microtiter plate layout for dual and triple combinations

For a better and easier understanding of the microtiter plates layout followed for checkerboard assay, **Table A2** and **Table A3** show the scheme used for the evaluating of dual and triple combinations, respectively.

Table A2. Microtiter plates layout for dual combinations.

	1	2	3	4	5	6	7	8	9	10	11	12
A	MHB	0 µg/mL	1/32×MIC of A	1/16×MIC of A	1/8×MIC of A	1/4×MIC of A	1/2×MIC of A	1×MIC of A	2×MIC of A	Bact	–	–
B	MHB	1/32×MIC of B	1/32×MIC of A and 1/32×MIC of B	1/16×MIC of A and 1/32×MIC of B	1/8×MIC of A and 1/32×MIC of B	1/4×MIC of A and 1/32×MIC of B	1/2×MIC of A and 1/32×MIC of B	1×MIC of A and 1/32×MIC of B	2×MIC of A and 1/32×MIC of B	Bact	–	–
C	MHB	1/16×MIC of B	1/32×MIC of A and 1/16×MIC of B	1/16×MIC of A and 1/16×MIC of B	1/8×MIC of A and 1/16×MIC of B	1/4×MIC of A and 1/16×MIC of B	1/2×MIC of A and 1/16×MIC of B	1×MIC of A and 1/16×MIC of B	2×MIC of A and 1/16×MIC of B	Bact	–	–
D	MHB	1/8×MIC of B	1/32×MIC of A and 1/8×MIC of B	1/16×MIC of A and 1/8×MIC of B	1/8×MIC of A and 1/8×MIC of B	1/4×MIC of A and 1/8×MIC of B	1/2×MIC of A and 1/8×MIC of B	1×MIC of A and 1/8×MIC of B	2×MIC of A and 1/8×MIC of B	Bact	–	–
E	MHB	1/4×MIC of B	1/32×MIC of A and 1/4×MIC of B	1/16×MIC of A and 1/4×MIC of B	1/8×MIC of A and 1/4×MIC of B	1/4×MIC of A and 1/4×MIC of B	1/2×MIC of A and 1/4×MIC of B	1×MIC of A and 1/4×MIC of B	2×MIC of A and 1/4×MIC of B	Bact	–	–
F	MHB	1/2×MIC of B	1/32×MIC of A and 1/2×MIC of B	1/16×MIC of A and 1/2×MIC of B	1/8×MIC of A and 1/2×MIC of B	1/4×MIC of A and 1/2×MIC of B	1/2×MIC of A and 1/2×MIC of B	1×MIC of A and 1/2×MIC of B	2×MIC of A and 1/2×MIC of B	Bact	–	–
G	MHB	1×MIC of B	1/32×MIC of A and 1×MIC of B	1/16×MIC of A and 1×MIC of B	1/8×MIC of A and 1×MIC of B	1/4×MIC of A and 1×MIC of B	1/2×MIC of A and 1×MIC of B	1×MIC of A and 1×MIC of B	2×MIC of A and 1×MIC of B	Bact	–	–
H	MHB	2×MIC of B	1/32×MIC of A and 2×MIC of B	1/16×MIC of A and 2×MIC of B	1/8×MIC of A and 2×MIC of B	1/4×MIC of A and 2×MIC of B	1/2×MIC of A and 2×MIC of B	1×MIC of A and 2×MIC of B	2×MIC of A and 2×MIC of B	Bact	–	–

MHB - Culture Media; Bact - Bacteria; – - Empty Wells; A, B and C - Generic Compounds

Table A3. Microtiter plates layout for triple combinations.

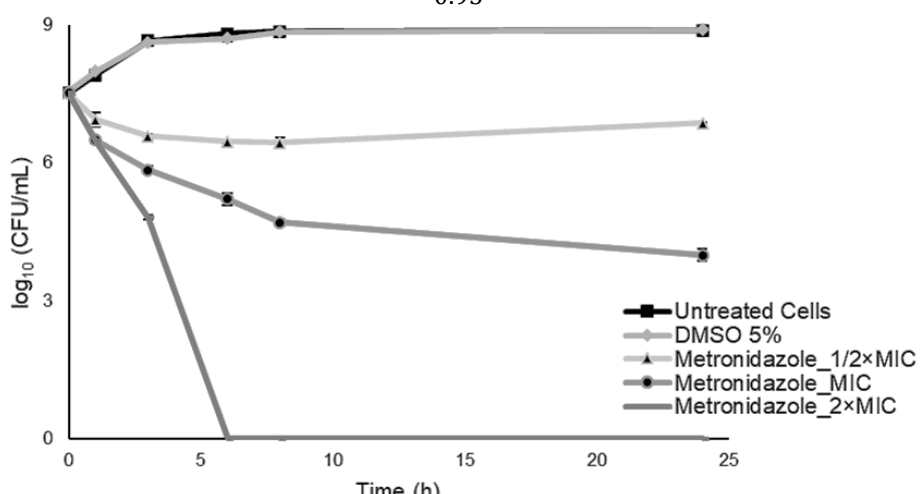
	1	2	3	4	5	6	7	8	9	10	11	12
A	MHB	1/4×MIC of Phy1, 1/4×MIC of Phy2, and 1/4×MIC of Ant	1/2×MIC of Phy1, 1/4×MIC of Phy2, and 1/4×MIC of Ant	1×MIC of Phy1, 1/4×MIC of Phy2, and 1/4×MIC of Ant	2×MIC of Phy1, 1/4×MIC of Phy2, and 1/4×MIC of Ant	–	1/4×MIC of Phy1, 1/4×MIC of Phy2, and 1/2×MIC of Ant	1/2×MIC of Phy1, 1/4×MIC of Phy2, and 1/2×MIC of Ant	1×MIC of Phy1, 1/4×MIC of Phy2, and 1/2×MIC of Ant	2×MIC of Phy1, 1/4×MIC of Phy2, and 1/2×MIC of Ant	Bact	–
B	MHB	1/4×MIC of Phy1, 1/2×MIC of Phy2, and 1/4×MIC of Ant	1/2×MIC of Phy1, 1/2×MIC of Phy2, and 1/4×MIC of Ant	1×MIC of Phy1, 1/2×MIC of Phy2, and 1/4×MIC of Ant	2×MIC of Phy1, 1/2×MIC of Phy2, and 1/4×MIC of Ant	–	1/4×MIC of Phy1, 1/2×MIC of Phy2, and 1/2×MIC of Ant	1/2×MIC of Phy1, 1/2×MIC of Phy2, and 1/2×MIC of Ant	1×MIC of Phy1, 1/2×MIC of Phy2, and 1/2×MIC of Ant	2×MIC of Phy1, 1/2×MIC of Phy2, and 1/2×MIC of Ant	Bact	–
C	MHB	1/4×MIC of Phy1, 1×MIC of Phy2, and 1/4×MIC of Ant	1/2×MIC of Phy1, 1×MIC of Phy2, and 1/4×MIC of Ant	1×MIC of Phy1, 1×MIC of Phy2, and 1/4×MIC of Ant	2×MIC of Phy1, 1×MIC of Phy2, and 1/4×MIC of Ant	–	1/4×MIC of Phy1, 1×MIC of Phy2, and 1/2×MIC of Ant	1/2×MIC of Phy1, 1×MIC of Phy2, and 1/2×MIC of Ant	1×MIC of Phy1, 1×MIC of Phy2, and 1/2×MIC of Ant	2×MIC of Phy1, 1×MIC of Phy2, and 1/2×MIC of Ant	Bact	–
D	MHB	1/4×MIC of Phy1, 2×MIC of Phy2, and 1/4×MIC of Ant	1/2×MIC of Phy1, 2×MIC of Phy2, and 1/4×MIC of Ant	1×MIC of Phy1, 2×MIC of Phy2, and 1/4×MIC of Ant	2×MIC of Phy1, 2×MIC of Phy2, and 1/4×MIC of Ant	–	1/4×MIC of Phy1, 2×MIC of Phy2, and 1/2×MIC of Ant	1/2×MIC of Phy1, 2×MIC of Phy2, and 1/2×MIC of Ant	1×MIC of Phy1, 2×MIC of Phy2, and 1/2×MIC of Ant	2×MIC of Phy1, 2×MIC of Phy2, and 1/2×MIC of Ant	Bact	–
E	MHB	1/4×MIC of Phy1, 1/4×MIC of Phy2, and 1×MIC of Ant	1/2×MIC of Phy1, 1/4×MIC of Phy2, and 1×MIC of Ant	1×MIC of Phy1, 1/4×MIC of Phy2, and 1×MIC of Ant	2×MIC of Phy1, 1/4×MIC of Phy2, and 1×MIC of Ant	–	1/4×MIC of Phy1, 1/4×MIC of Phy2, and 2×MIC of Ant	1/2×MIC of Phy1, 1/4×MIC of Phy2, and 2×MIC of Ant	1×MIC of Phy1, 1/4×MIC of Phy2, and 2×MIC of Ant	2×MIC of Phy1, 1/4×MIC of Phy2, and 2×MIC of Ant	Bact	–
F	MHB	1/4×MIC of Phy1, 1/2×MIC of Phy2, and 1×MIC of Ant	1/2×MIC of Phy1, 1/2×MIC of Phy2, and 1×MIC of Ant	1×MIC of Phy1, 1/2×MIC of Phy2, and 1×MIC of Ant	2×MIC of Phy1, 1/2×MIC of Phy2, and 1×MIC of Ant	–	1/4×MIC of Phy1, 1/2×MIC of Phy2, and 2×MIC of Ant	1/2×MIC of Phy1, 1/2×MIC of Phy2, and 2×MIC of Ant	1×MIC of Phy1, 1/2×MIC of Phy2, and 2×MIC of Ant	2×MIC of Phy1, 1/2×MIC of Phy2, and 2×MIC of Ant	Bact	–
G	MHB	1/4×MIC of Phy1, 1×MIC of Phy2, and 1×MIC of Ant	1/2×MIC of Phy1, 1×MIC of Phy2, and 1×MIC of Ant	1×MIC of Phy1, 1×MIC of Phy2, and 1×MIC of Ant	2×MIC of Phy1, 1×MIC of Phy2, and 1×MIC of Ant	–	1/4×MIC of Phy1, 1×MIC of Phy2, and 2×MIC of Ant	1/2×MIC of Phy1, 1×MIC of Phy2, and 2×MIC of Ant	1×MIC of Phy1, 1×MIC of Phy2, and 2×MIC of Ant	2×MIC of Phy1, 1×MIC of Phy2, and 2×MIC of Ant	Bact	–
H	MHB	1/4×MIC of Phy1, 2×MIC of Phy2, and 1×MIC of Ant	1/2×MIC of Phy1, 2×MIC of Phy2, and 1×MIC of Ant	1×MIC of Phy1, 2×MIC of Phy2, and 1×MIC of Ant	2×MIC of Phy1, 2×MIC of Phy2, and 1×MIC of Ant	–	1/4×MIC of Phy1, 2×MIC of Phy2, and 2×MIC of Ant	1/2×MIC of Phy1, 2×MIC of Phy2, and 2×MIC of Ant	1×MIC of Phy1, 2×MIC of Phy2, and 2×MIC of Ant	2×MIC of Phy1, 2×MIC of Phy2, and 2×MIC of Ant	Bact	–

MHB - Culture Media; Bact - Bacteria; – - Empty Wells; Phy - Phytochemical; Ant - Antibiotic

Appendix D – Results obtained for metronidazole

In order to evaluate in all the assays carried out with combination amoxicillin/metronidazole, it was necessary to evaluate both molecules separately. The results obtained for metronidazole against *E. coli* are shown in **Table A4**.

Table A4. Results obtained for metronidazole against *E. coli*. Values are the mean $\pm$ standard deviation for at least two independent experiments.

Assay	Parameter	Result
MIC determination	MIC	1024 $\mu\text{g/mL}$
Neutralizer efficacy	Log_{10} (CFU/mL) with neutralizer	6.41
	Log_{10} (CFU/mL) without neutralizer	5.41
	Neutralizer efficacy ratio	0.95
Time-kill	Time-kill curves	
Biofilm control	Biofilm mass reduction (%)	(56.30 $\pm$ 7.28)% at MIC; (79.22 $\pm$ 3.27)% at 5 $\times$ MIC; (83.84 $\pm$ 2.41)% at 10 $\times$ MIC
	Biofilm metabolic activity inactivation (%)	(29.73 $\pm$ 6.96)% at MIC; (43.85 $\pm$ 4.75)% at 5 $\times$ MIC; (45.78 $\pm$ 4.04)% at 10 $\times$ MIC
	Quantification of the biofilm culturable cells (log_{10} (CFU/cm ²))	(4.20 $\pm$ 0.12) at MIC; (3.91 $\pm$ 0.07) at 5 $\times$ MIC; (3.37 $\pm$ 0.06) at 10 $\times$ MIC

Appendix E – Single agent dose response and the synergism distribution

Figure A1, Figure A2, Figure A3, Figure A4, Figure A5 and Figure A6 show the single agent dose response and the synergism distribution (matrix synergism plot and synergism mapped to D-R, based on Bliss model).

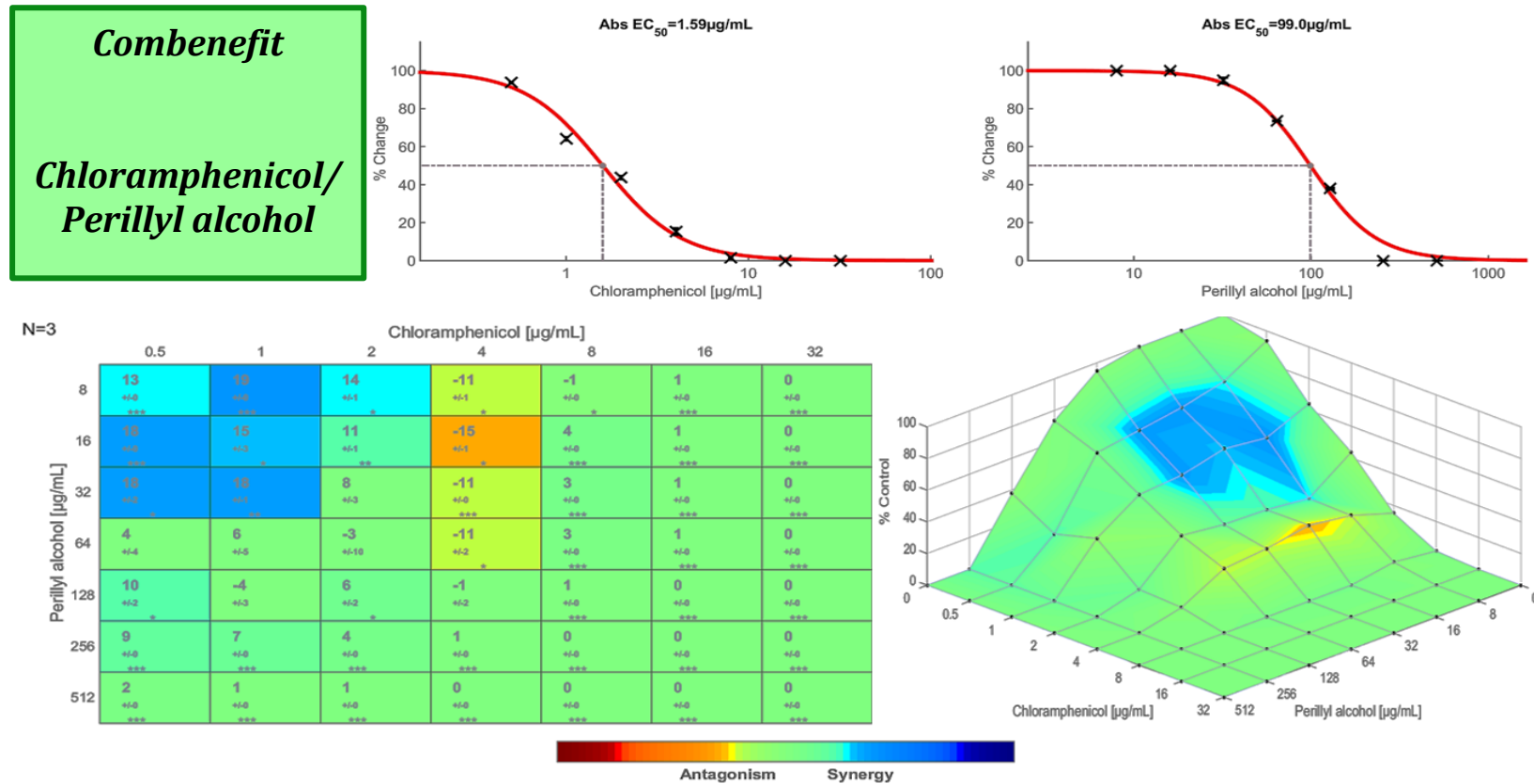


Figure A1. Single agent dose response (at the top), matrix synergism plot (at bottom left) and synergism mapped to D-R (at bottom right) for the combination chloramphenicol/perillyl alcohol. Values are the mean ± standard deviation for three independent experiments.

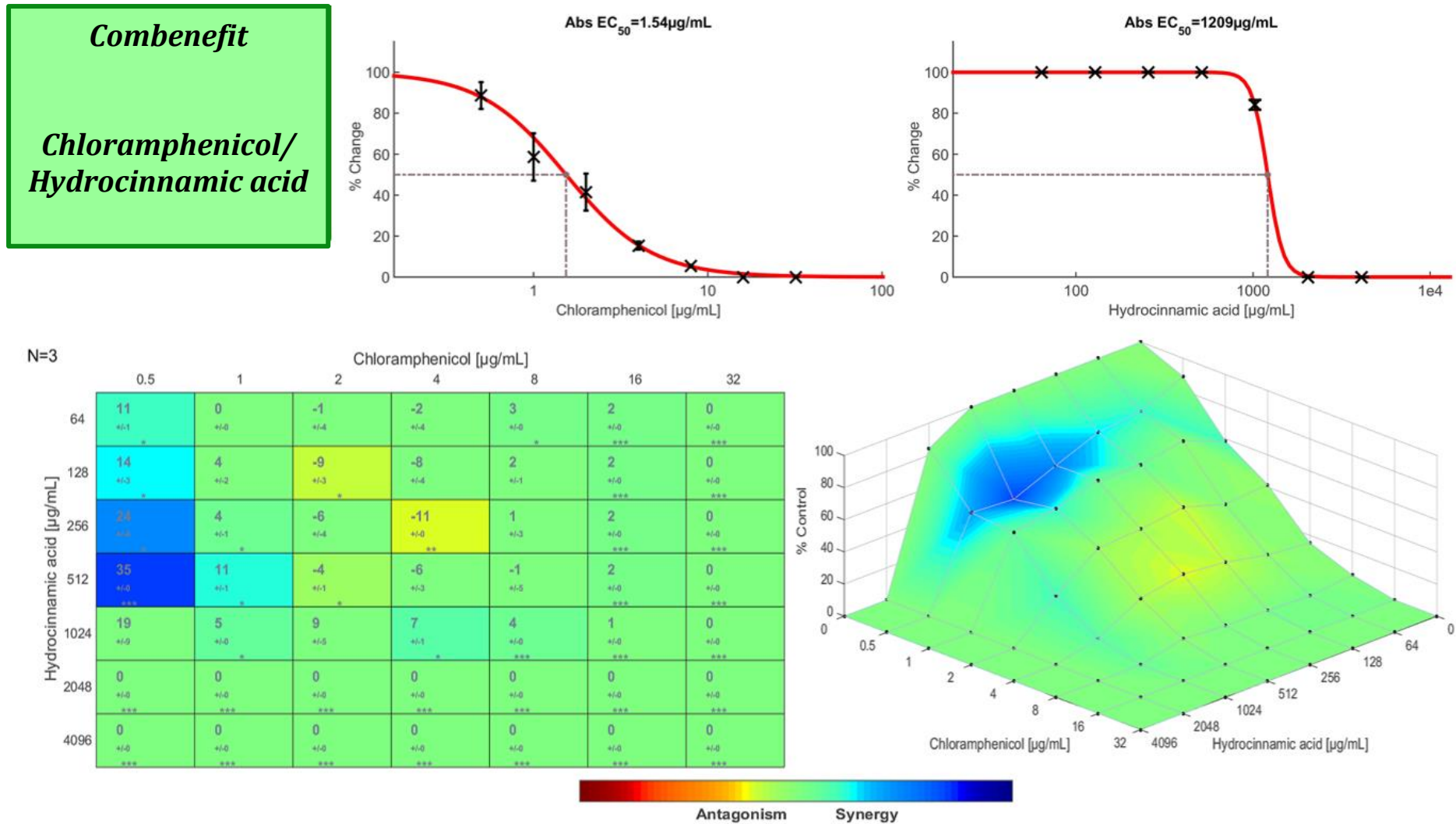


Figure A2. Single agent dose response (at the top), matrix synergism plot (at bottom left) and synergism mapped to D-R (at bottom right) for the combination chloramphenicol/hydrocinnamic acid. Values are the mean $\pm$ standard deviation for three independent experiments.

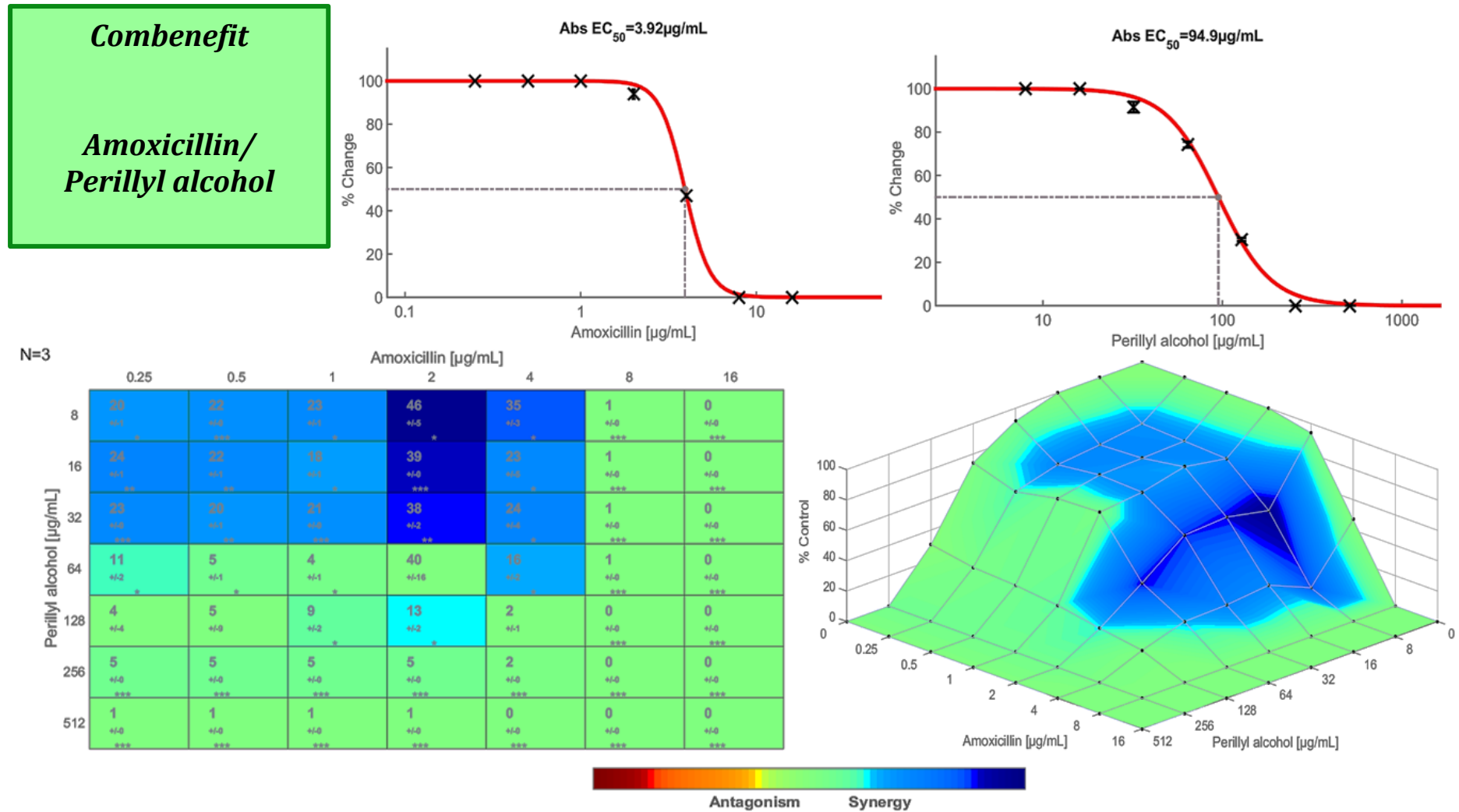


Figure A3. Single agent dose response (at the top), matrix synergism plot (at bottom left) and synergism mapped to D-R (at bottom right) for the combination amoxicillin/perillyl alcohol. Values are the mean $\pm$ standard deviation for three independent experiments.

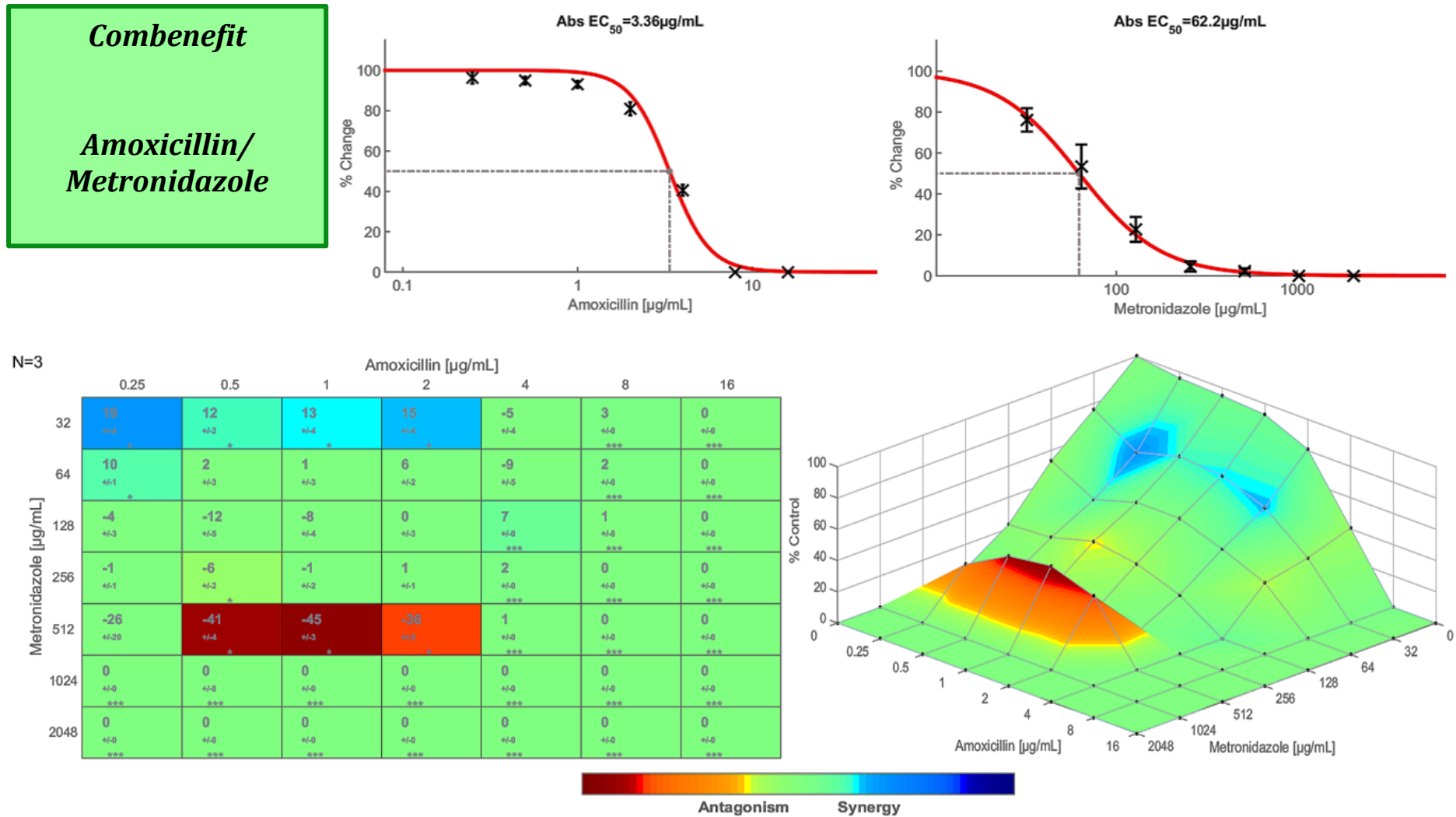


Figure A4. Single agent dose response (at the top), matrix synergism plot (at bottom left) and synergism mapped to D-R (at bottom right) for the combination amoxicillin/metronidazole. Values are the mean ± standard deviation for three independent experiments.

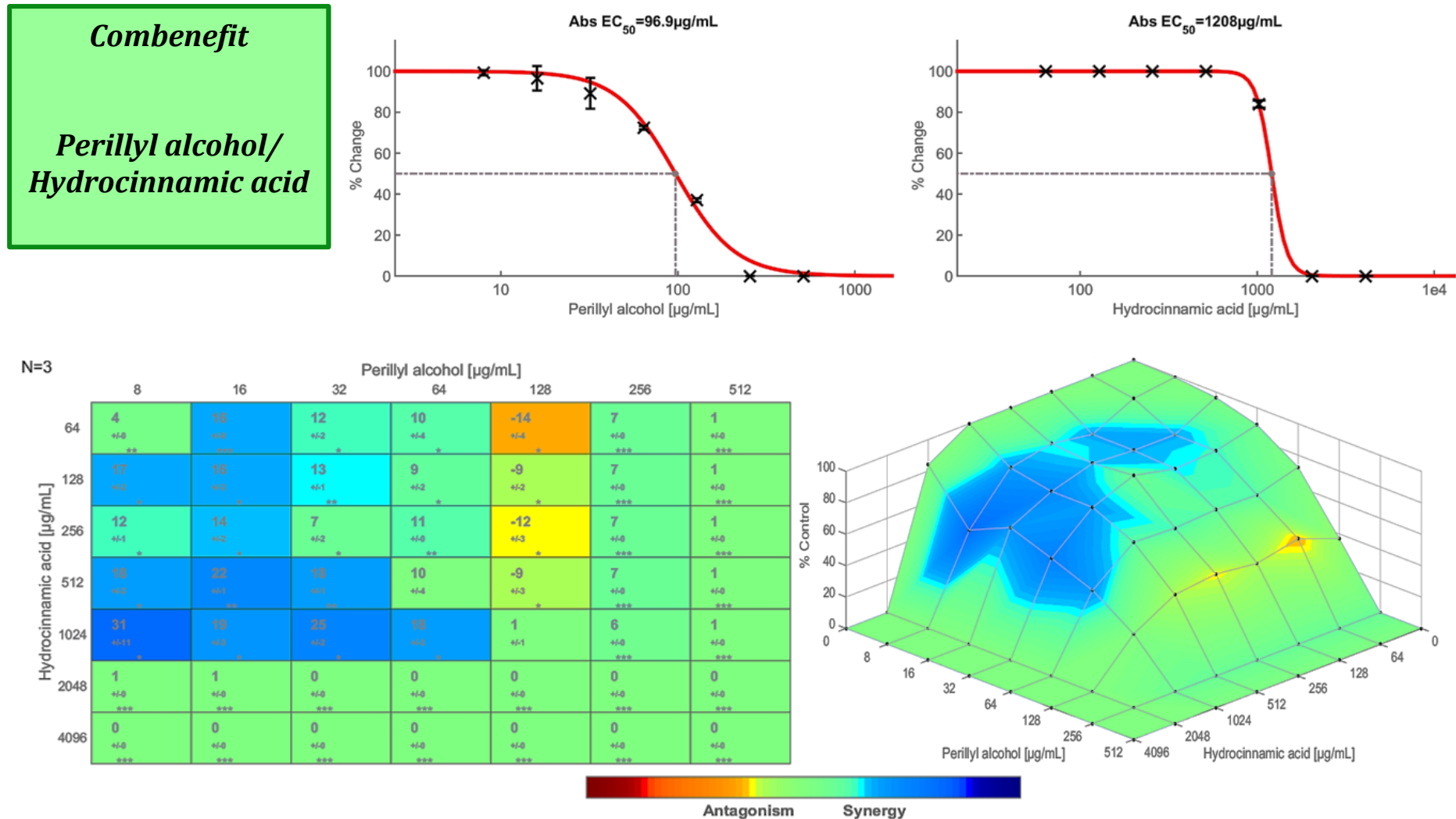


Figure A5. Single agent dose response (at the top), matrix synergism plot (at bottom left) and synergism mapped to D-R (at bottom right) for the combination perillyl alcohol/hydrocinnamic acid. Values are the mean $\pm$ standard deviation for three independent experiments.

Appendix F – AUCs values for the time-kill curves

Figure A6 shows the AUCs values determined for the time-kill curves.

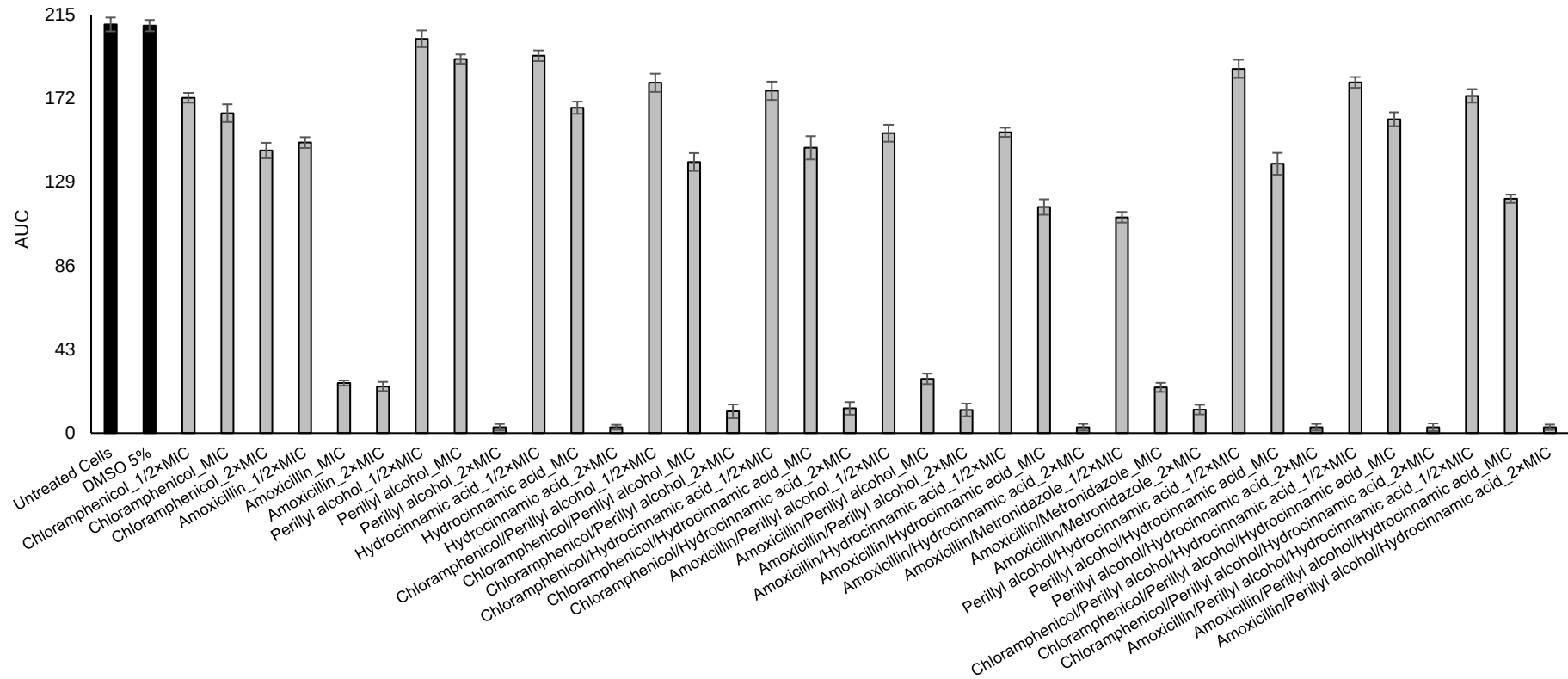


Figure A6. AUCs values for the time-kill curves. Values are the mean $\pm$ standard deviation for at least two independent experiments.