Integrated Master in Bioengineering

Antimicrobial activity of selected phytochemicals against *Escherichia coli* and *Staphylococcus aureus* cells and biofilms

Dissertation for Master Degree in Bioengineering – Specialization in Biological Engineering

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The present thesis was developed for the obtention of Master degree in Bioengineering, in the Faculty of Engineering of University of Porto.

The work was carried out at LEPAE during 6 months. The main objective of the thesis was the evaluation of the efficacy of phytochemicals against *Escherichia coli* and *Staphylococcus aureus* planktonic cells and biofilms.

The judge that approved the present document was composed by three elements: Luís de Melo (Cathedratic Professor), Maria da Conceição Fernandes (PhD), Manuel Simões (PhD).

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“Try to learn something about everything,
and everything about something.”

Thomas H. Huxley

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Abstract

Antimicrobial resistance is one of the biggest problems facing global public health. The effectiveness of antimicrobial drugs has been lost due to the evolution of pathogen resistance. Plants are considered the greatest source to obtain new antimicrobials. They produce secondary metabolites, phytochemicals, which protect the plant against pathogens.

The aim of this study was to assess the antimicrobial activity of four phytochemicals - 7-hydroxycoumarin (7-HC), indole-3-carbinol (I3C), salycilic acid (SA) and saponin (SP) – against *Escherichia coli* and *Staphylococcus aureus* and also understand their ability to control biofilm formation.

Several experiments were carried out in order to: i) test the ability of phytochemicals to control planktonic bacteria growth through the measurement of minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC); ii) evaluate the phytochemicals action in the control of biofilms; iii) understand aspects of the phytochemicals mode of action against the bacteria.

Results have shown that MIC values were higher for *E. coli* than for *S. aureus*. The 7-HC and I3C were the most effective, with MICs of 200 and 400 μg/mL for *S. aureus*, respectively, and 800 μg/mL against *E. coli*. Regarding MBC, 1600 and 5000 μg/mL were obtained for I3C and SA, respectively. It was also observed that 7-HC and SP has no significative effect in surface charge of *E. coli*; in contrast, I3C and SA make the membrane more and less negative, respectively. *S. aureus* surface charge was changed in contact with SA and SP. It was observed that phytochemical concentration did not affect the biofilm removal for both bacteria. *E. coli* biofilms are more susceptible to phytochemicals comparing to *S. aureus* biofilms.

SA and SP promoted the increase and decrease of hydrophilic properties of *E. coli*, respectively. *S. aureus* became less hydrophilic in contact with 7-HC and SA. *E. coli* showed the highest motility and also an increasing in swimming and swarming motility over time. Motility was mostly affected when I3C was added. Swimming and sliding motilities were completely inhibited and swarming motility was not affected by I3C. The quorum-sensing results indicated that inhibition of violacein production was detectable with 7-HC, I3C and SA, with halos ranging from 5 to 19 mm. I3C was also the most effective phytochemical. The increasing of concentration resulted in an

increasing of pigment inhibition zone. The OMPs expression in *E. coli* was not affect after the exposure to phytochemicals. Dual combinations between antibiotics and I3C produced synergictic effects against *S. aureus* resistant strains.

This study suggests that 7-HC and I3C are the most important phytochemicals against *E. coli* and *S. aureus*. Both phytochemicals affected the motility and QS activity, which means that they can play an important role in the interference of cell-cell interactions and in biofilm control.

Resumo

A resistência antimicrobiana é um dos maiores problemas enfrentados pela saúde pública global. A eficácia dos agentes antimicrobianos é cada vez mais inferior devido à evolução de mecanismos de resistência a infecções e microrganismos patogénicos. As plantas são consideradas a melhor fonte para a obtenção de novos agentes antimicrobianos. Estas produzem metabolitos secundários, os fitoquímicos, que fazem parte de mecanismos de proteção da planta contra agentes invasores e patogénicos.

O principal objetivo foi a avaliação da actividade antimicrobiana de 4 fitoquímicos – 7-hidroxicumarina (7-HC), indol-3-carbinol (I3C), ácido salicílico (SA) e saponina (SP) – nas estirpes *Escherichia coli* e *Staphylococcus aureus* e também compreender a sua capacidade de controlo na formação de biofilmes.

Vários estudos foram realizados de modo a: i) testar a capacidade dos fitoquímicos no controlo do crescimento bacteriano de células planctónicas através da deteção da concentração minima inhibitória (CMI) e concentração mínima bacteriana (CMB); ii) avaliar a ação dos fitoquímicos no controlo de biofilmes; iii) entender os aspetos dos fitoquímicos no modo de ação contra as bactérias.

Os resultados mostraram que os valores de CMI são mais elevados para *E. coli* do que para *S. aureus*. A 7-HC e I3C foram os mais eficazes, obtendo-se valores de CIM de 200 e 400 μg/mL para *S. aureus*, respectivamente, e 800 μg/mL para *E. coli*. Relativamente a CMB, obtiveram-se valores de 1600 e 5000 μg/mL para I3C e SA, respectivamente. Foi também observado que 7-HC e SP nao tiveram efeito significativo na carga da membrana da *E. coli*; ao contrário de I3C e SA que tornaram a carga da membrana mais e menos negativa, respectivamente. A carga da membrana de *S. aureus* foi alterada em contato com SA e SP. Foi verificado que a concentração de fitoquímico nao influencia a remoção de biofilmes para ambas as bactérias. Os biofilmes de *E. coli* são mais susceptíveis aos fitoquímicos comparativamente aos biofilmes de *S. aureus*.

O SA e a SP promoveram o aumento e diminuição das propriedades hidrofílicas da *E. coli*, respectivamente. *S. aureus* tornou-se menos hidrofílico em contato com 7-HC e SA. *E. coli* apresentou a mobilidade mais elevada e também um aumento na mobilidade *swimming* e *swarming* ao longo do tempo. A mobilidade foi

maioritariamente afetada pela adição de I3C. A mobilidade *swimming* e *sliding* foi completamente inibida e *swarming* foi afetada após a adição de I3C. Os resultados de quorum-sensing indicaram que a inibição da produção de violaceína foi detetada com 7-HC, I3C e SA, com halos compreendidos entre 5 e 19 mm. I3C foi também o fitoquímico mais eficaz. O aumento da concentração resultou num aumento da zona de inibição de pigmento. A expressão das OMPs na *E. coli* não foi afetada após a exposição aos fitoquímicos. A combinação dupla entre os antibióticos e I3C produziu efeitos sinérgicos contra estirpes resistentes de *S. aureus*.

Este estudo sugere que 7-HC e I3C são os fitoquímicos mais promissores contra *E. coli* e *S. aureus*. Ambos os fitoquímicos afetam a mobilidade e a actividade de quorum-sensing, o que significa que apresentam um papel determinante na interferência de interações célula-célula e no controlo de biofilmes.

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Glossary

Indexes and parameters:

AB – Lewis acid-base component

B – Bacteria

B - α-bromonaphtalene

F – Formamide

LW – Lifshitz-van der Waals component

S – Bubstratum

TOT – Total

W – Water

sws- Between teo entities od a given surface when immersed in water

bws – Between one bacteria and a substratum that are immersed or dissolved in water

bwb – Between two bacterial surfaces, when immersed in water

bw – Between bacteria and substratum

sw – Between substratum and bacteria

TOT – Total

ΔG – Free energy of interaction (mJ/m^2)

γ - Surface free energy (mJ/m^2)

θ - Contact angle (°)

+ - Electron acceptor paramenter of Lewis acid-base component

- - Electron donor parameter of Lewis acid-base component

Abreviations:

AHL - N-acyl-homoserines

AI – Autoinducers

CIP – Ciprofloxacin

CV – Crystal Violet

DMSO – Dimethyl sulfoxide

EPS – Extracellular polymeric substances

ERY - Erythromycin

I3C – Indole-3-carbinol

MIC – Minimum Inhibitory Concentration

MBC – Minimum Bactericidal Concentration

MDR – Multi-drug resistant

OD – Optical Density

OD_C – Cut-off optical density

OMPs – Outer membrane proteins

p – Statistical significance level

PS – Polystyrene

QS – Quorum-sensing

QSI – Quorum-sensing inhibition

RMAs – Resistance-modifying agents

SA – Salycilic Acid

SDS-PAGE – Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SP – Saponin

SPSS - Statistical Package for the Social Sciences

TET - Tetracycline

WHO – World Health Organization

7-HC – 7-hidroxycoumarin

Chapter 1

Work Outline

1.1. Background and Project Presentation

Since the discovery of the first antibiotic, penicillin, the employment of any novel antibiotic has been followed by the appearance of bacterial resistance to that antibiotic in as little time as a few years. Antibiotics have the ability to kill bacteria or inhibit their growth.

Resistance to antibiotics is one of the biggest problems that global public health is facing. Antimicrobial resistance is a natural consequence of the adaption of pathogens to the exposure to antimicrobials used in medicine, food, crop production and to disinfectants in farms and households. Resistant organisms cause infections that are more difficult to treat and more expensive; some strains have become resistant to all available antimicrobial agents (Byarugaba, 2004). Resistant infections affect treatment costs, disease spread and duration of illness (Okeke et al., 2005)

In order to find novel antimicrobial agents with new modes of action, plants have been explored as sources for the identification of new and effective antibacterials. An endless number of plant species have been reported to act against several bacteria *in vitro*, and many medicinal plants produce secondary metabolites (phytochemicals) capable of inhibiting the growth of a wide range of microorganisms including fungi, yeasts and bacteria. Phytochemicals have been studied for the treatment of microbial infections since 1990, due to the increasing inefficacy of conventional antibiotics (Simões et al., 2009).

1.2 Main objectives

The main aim of this work was to assess the antimicrobial efficacy of selected phytochemicals against *Escherichia coli* and *Staphylococcus aureus* planktonic cells and also to evaluate them on biofilm control.

In the present study, four different phytochemicals – 7-hydroxycoumarin (7-HC), indole-3-carbinol (I3C), salicylic acid (SA) and saponin (SP) – were tested against *E. coli* and *S. aureus* in both planktonic and sessile states. The strains tested are considered the most clinical significant bacteria due to their capacity to resist against several antibiotics (Simões et al., 2008; Xu et al., 2006).

To evaluate the antimicrobial activity of the several phytochemicals two experiments were performed to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The biofilm control was also performed to understand the efficacy of phytochemicals to remove biomass and inactivate biofilm cells. The biofilm control assay was studied in 24 h aged biofilms after 1 h in contact with the phytochemicals. The biomass removal and metabolic inactivation were calculated through the optical density (OD) and fluorescence measurements.

Several aspects of planktonic cells were evaluated to understand the mode of action of the selected phytochemicals. The surface charge of bacteria was studied through the measurement of Zeta potential and the hydrophobicity of cells was also assessed. To evaluate the potential activity of the phytochemicals to prevent *E. coli* and *S. aureus* adhesion to polystyrene (PS), the prediction of theoretical adhesion

through the measurement of contact angles was performed. The phytochemicals were also studied on the ability to interfere with bacterial motility and quorum-sensing (QS), two microbial aspects involved in biofilm formation.

The OMPs expression of *E. coli* was studied in contact with phytochemicals through a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in order to detect the possible expression of resistance proteins. Regarding *S. aureus* resistant strains, dual combinations of phytochemicals and antibiotics – tetracycline (TET), ciprofloxacin (CIP) and erythromycin (ERY) - were tested to understand their ability to act in efflux pumps inhibition.

1.3 Thesis Organization

In chapter 1, the context, motivations and main goals for the development of this thesis are explained. This chapter is also a guideline to the overall work, composed by 5 chapters.

Chapter 2 includes the literature review about the main subjects of this work. In this chapter, the main problems associated with the appearance of bacterial resistance are highlighted. Plant products, especially secondary metabolites, are introduced as one of the solution for the antimicrobial resistance. The mode of action of phytochemicals is also developed in this chapter. Finally, it is reported the problem of the higher resistance associated to bacterial growing in biofilms and quorum-sensing inhibition (QSI) is presented as one possible solution for the prevention of biofilm formation.

In the third chapter are described the results of the activity of phytochemicals as antimicrobial agents against *E. coli* and *S. aureus*. The MIC and MBC of the selected phytochemicals are presented. In this chapter are also studied the phytochemicals in the control of biofilm, showing the biofilm removal and metabolic inactivation for each one of the phytochemicals at the MIC and 5 × MIC.

Chapter 4 describes the study of surface charge and hydrophobicity characteristics of *E. coli* and *S. aureus* when exposed to phytochemicals. The influences of phytochemicals in motility and QS are also presented in this chapter. To finalize the chapter, OMPs expression of *E. coli* are studied when exposed to

phytochemicals; and dual combination of phytochemicals and antibiotics are described in order to study the antimicrobial activity and their synergistic effects in *S. aureus* efflux pump inhibition.

Finally, in chapter 5 the main conclusions of the work are referred and some recomendations for future research are given.

Chapter 2

Literature Review

2.1 Antimicrobial resistance and phytochemicals

There is a continuous search for new drugs and antibiotics in order to heal the main infectious diseases. However, the microorganisms have become resistant to most of the antibiotics. The microorganisms are successful when facing adverse conditions, because they seem to sense and respond to the external environment and modulate gene expression accordingly. Antimicrobial resistance is one of the biggest problems threatening global public health (Byarugaba, 2004; Okeke et al., 2005). This problem is a natural consequence of the adaption of infectious agents to antimicrobials used in several areas, including medicine, food animals, crop production and disinfectants in farms, hospitals and households (Bloomfield, 2002; McEwen and Fedorka-Cray, 2002; Vidaver, 2002; Wise and Soulsby, 2002). Resistance allows microorganisms to survive in the presence of toxic conditions. The effectiveness of many antimicrobial drugs has been lost due to the evolution of pathogen resistance. Many of the microorganisms are no longer susceptible to most of the existing antibiotics and therapeutic agents (Byarugaba, 2004). Bacteria generally

acquire drug resistance in a multitude of ways (Saleem et al., 2010). The acquisition can be done by *de novo* mutation or acquisition of resistance genes from other microorganisms and passed on during replication (Fajardo et al., 2008; McManus, 1997; Sefton, 2002; Smith and Lewin, 1993). Resistance genes are able to act in different ways enabling bacterium to: produce the enzymes that inactivate the antimicrobial agent; modify the target site; produce an alternative metabolic pathway that inhibits the antimicrobial agent; express efflux mechanisms, preventing the antimicrobials to reach intracellular targets (Spratt, 1994; Webber and Piddock, 2003; Woodford and Ellington, 2007).

There are several factors influencing the efficacy of antimicrobial agents. Examples include the use of an inefficient product, in other words, of an antimicrobial product that presents an incomplete spectrum of activity; the application of antimicrobial agents at sublethal concentrations, which can allow the adaptation of microorganisms to these new conditions; or an insufficient contact time between the antimicrobial product and microorganisms (Bessems, 1998; Heinzel, 1998; Russel 2003).

Beyond all the environmental factors affecting the resistance explained before, there are cellular mechanisms influencing this process. The Gram-negative bacteria are less susceptible to antimicrobial agents than Gram-positive bacteria, because they present a thick cell wall, more difficult to entry, and an outer membrane. The waxy envelope presented in mycobacteria inhibits the uptake of antimicrobial agents, so they are even more resistant (McDonnell and Russell, 1999). Efflux is another process related with the increasing of resistance. Through efflux pumps, Gram-negative bacteria pump out the antimicrobial, detergents, organic solvents and disinfectant agents, contributing to the resistance (Beumer et al., 2000; Cloete, 2003; Kumar and Schweizer, 2005).

The resistant microorganisms can cause infections that are more difficult to treat, and it is necessary drugs that are more expensive and toxic and also less available (Howard et al., 2001). The effective microbial therapy is usually delayed because of the acquisition of resistance from microorganisms (Ibrahim et al., 2000; Kollef, et al., 1999; Lautenbach et al., 2001). Bacteria have shown resistance with increasing trends. Consequently, the rate of discovery of new antimicrobial agents has decreased since the 1970s (Byarugaba, 2004). The traditional antibiotics have been recognized because they are able to kill bacteria or inhibit their growth, through

inhibition of bacterial functions, such as: cell wall synthesis, DNA replication, RNA transcription and protein synthesis, which are essential for cell growth (Figure 1) (Clatworthy et al., 2007). In order to treat infections caused by antimicrobial-resistant organisms, it is necessary a more effective therapy (Cosgrove and Carmeli, 2003).

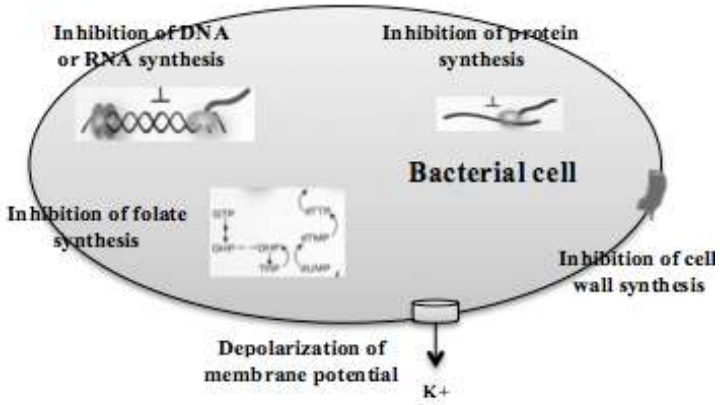

Figure 1. Traditional targets of antimicrobial compounds.

The methods to evaluate antimicrobial activity are essential for the selection of a new drug. However, there are several factors that can affect the antimicrobial susceptibility test: pH, moisture, effects of thymidine or thimine, effects of variation in divalent cations and growth conditions (Lalitha, 2004). Magnesium and calcium are divalent cations that affect the results of aminoglycosides and tetracycline. Finally, the components needed for growth should be fulfilled (Lalitha, 2004). The antimicrobial susceptibility tests are divided into 3 principles: diffusion, dilution and diffusion and dilution. The most popular methods are: the Kirby-Bauer, E-Test, agar dilution, Stokes, microdilution and macrodilution (Anvisa, 2012; Lalitha, 2004; Othman et al., 2011).

2.2. A new therapy against resistance – Phytotherapy

Natural new antimicrobial can be present in several products used by humankind. For example, aromatic plants and spices, used as flavour enhancers, have already been demonstrated to have significant antimicrobial activity (Christaki et al., 2012). Also, antimicrobials from vegetal origin can be obtained from essential oils, seeds, flowers, fruits and roots. These kind of antimicrobial systems have been used

centuries ago, in the preservation of food. Plants produce a wide range of organic compounds. However, the secondary metabolites present special interest because of their importance as pharmaceuticals, venoms, fragrances, industrial materials and cosmetics (Amaral and Silva, 2003; Mendes, 2007). The discovery of all the potential of plants brings a great interest in phytotherapy.

The World Health Organization (WHO) defines medicinal plants those that present traditional use as therapeutic compounds (Severiano et al., 2010). According to the WHO, medicinal plants would be the greatest source to obtain a wide range of drugs with antimicrobial properties (Nascimento et al., 2000). Consequently, interest in medicinal plants has increased in recent years. Indeed, plants were reported to be used by about three quarter of the world population (Rao et al., 2004). The story of medicinal plants says that they are part of the humankind evolution and were the first therapeutic sources used. However, the use of medicinal plants, phytotherapy, had major impact in the beginning of XIX century. There are 250.000-500.000 plant species, however just a small percentage has been investigated phytochemically (Mahesh and Satish, 2008).

It is interesting to realize that most of the medicines used nowadays were identified in plants, and some of the most important pharmaceutical drugs are natural drugs and linked directly to natural product research (Bighetti et al., 2005). Also, most of the commercial drugs were used before in crude form in traditional and folk medicine, suggesting their potential biological activity (Ciocan and Bara, 2007). Also, plants present numerous constituents and their extracts can show synergistic effects between the different active principles due to the presence of classes of compounds or different structures contributing for the same activity (Maciel et al., 2002). The plants can prevent cardiovascular diseases, carcinogenesis, inflammation, atherosclerosis, and others health problems (Albano and Miguel, 2001).

Some plants have potential as remedies for diseases. Examples include the use of bear-berry (*Arctostaphylos uvaursi*) and cranberry juice (*Vaccinium macrocarpon*) to treat urinary tract infections, essential oils of tea tree (*Melaleuca alternifolia*) as active ingredients in many topical formulations to treat cutaneous infections and *Hydrastis canadensis* and *Echinacea* species for tuberculosis infections (Abreu et al., 2012).

Regarding the plants, they can be divided in several families, for example Asteraceae, Lamiaceae, Apeaceae, Euphorbiaceae, Rutaceae and Fabaceae. Regarding

to these families, some of them are more incident in Portugal. Lamiaceae is one of the families found in our country and frequently found in Mediterranean region, Middle East and tropical mountains (Severiano et al., 2010). Currently, there are 3.500 species of this family (Severiano et al., 2010). Lamiaceae are composed of herbs, shrubs and trees. Plants belonging to this family can be applied in medicine, cosmetics and food industry (Mendes, 2007). Asteraceae is another plant family present in Portugal and includes around 23.000 species and 1.500 genus. They can be found in temperate and subtropical climates. Herbs, subshrubs and vines belong to this family (Severiano et al., 2010). Finally, Apiaceae is another family present in Portugal and is composed for 3.000 species and 300 genus (Moreno-Dorado et al., 2000). The plants that belong to this family can be found in tropical or temperate regions (Judd et al., 2002). Such as Lamiaceae, plants of this family are commonly used in medicine and food industry.

2.3. Phytochemicals and their classes

The increasing of antimicrobial resistance has led to the study of plants products for searching new antimicrobials (Clardy et al., 2006). The chemical diversity and structure are the main causes for the success of phytochemicals (Gibbons, 2004). The phytochemicals have become interesting since the 1990's because of the increasing dissemination of bacterial resistance mechanisms worldwide, due to the excessive and inappropriate use of antimicrobials.

Plant products are divided into two classes: phytoanticipins and phytoalexins. Molecules that are present in an inactive form (example: glucosides) belong to the first group; the second group is composed by molecules whose levels increase in response to microbial attack or is generated in response to a specific infection (Tegos et al., 2002). Phytoalexins are a large group of chemically diverse molecules, including: simple phenylpropanoids derivatives, alkaloids, glycosteroids, flavonoids, isoflavonoids, sulfur products, terpenes and polyketides (Hammerschmidt, 1999). The same molecule can be a phytoalexin or a phytoanticipin in different organs of the same plant. Examples of phytoanticipin are terprenoids, quinones and tannins (Abreu et al., 2012).

There are several phytochemical classes with antimicrobial properties; however, the medical community does not recognize them as therapeutics agents. This is mainly explained because the majority of phytochemicals have weak spectra of activities (Tegos et al., 2002) and the concentrations required are too high to be clinically significant (Aeschlimann et al., 1999; Schmitz et al., 1998). The major problem for the identification of antimicrobial agents from plants is the variability in the extraction methods and antibacterial tests used (Simões et al., 2009).

Beyond the antimicrobial properties, phytochemicals are able to present antiviral (Jassim and Naji, 2003; Muhtar et al., 2008; Naithani et al., 2008), antiparasitic (Atawodi and Alafiatayo, 2007; Chan-Bacab and Peña-Rodríguez, 2001; Sriram et al., 2004) and antifungal (Morel et al., 2002; Rahman and Moon, 2007; Treyvaud Amiguet et al., 2006) activities and also exert cytotoxic activity against tumor cells (Rimando and Suh, 2008; Suffredini et al., 2006; Udenigwe et al., 2008).

The groups of secondary metabolites produced by plant include: terpenoids, phenolics, alkaloids, essential oils constituents, lectins polypeptides and polyacetylenes (Mendes, 2007; Simões et al., 2009). The secondary compounds are responsible for defending against external aggressions and the main subclasses are: simple phenols and phenolic acids, quinines, flavones, flavonoids, tannins, coumarins, and others (Simões et al., 2009). Usually, the plants belonging to one family produce the same type of compounds. Normally, alkaloids are produced from the Apocinaceae and Solanaceae families; flavonoids are produced from Rutaceae plants; and terpenoids are produced from Asteraceae and Lamaceae families (Amaral and Silva, 2003). According to the classes that substances belong, they can produce different effects. Flavonoids present anti-inflammatory action, protecting the blood vessels, and are hypotensive and sedative; digestion action is guaranteed by antraquinones; bronchodilator action is made by coumarins; bactericidal action is achieved with tannins and essential oils (Severiano et al., 2010); and essential oils presents, apart from the others already mentioned, sedative, stimulating, analgesic and expectorant properties (Souza et al., 2011). Antimicrobial properties are expressend by most of plants that are composed by: tannins, terpenoids, glycosides, alkaloids, saponnins, flavonoids, polyphenols and coumarins (Das et al., 2010; Hill, 1952; Neelima et al., 2011; Padmini et al., 2010).

The phytochemical compounds are mostly secondary metabolites of plants and deposit in specific parts of the plant (Ciocan and Bara, 2007). Regarding phytochemicals, the main classes with health benefits are present below.

Phenolics and polyphenols form one of the simplest groups of bioactive phytochemicals, consisting of a single substituted phenolic ring (Das et al., 2010). This group seems to be toxic to microorganisms because of the site(s) and number of hydroxyl groups present on the phenolic ring (Scalbert et al., 1991; Urs et al., 1975). Increasing the hydroxylation results in the increasing of toxicity (Ciocan and Bara, 2007). Quinones are composed by an aromatic ring with two ketones substitutions (Das et al., 2010). These molecules react with nucleophilic amino acids in proteins, causing the inactivation of the protein or loss of function (Ciocan and Bara, 2007). Flavones, flavonoids and flavonols belong to a group of molecules with phenolic structures containing one carbonyl group (Ciocan and Bara, 2007; Das et al., 2010). They are active against a wide range of microorganisms, probably because they can react with extracellular and soluble proteins and also react with cell walls (Bennet and Wallsgrove, 1994; Ciocan and Bara, 2007). Tannins are another group that can be divided into two groups: hydrolysable and condensed. The first is based on gallic acid; the second group is composed by numerous condensed tannins derived from flavonoid monomers. The antimicrobial mode of action is related with their ability to inactivate microbial adhesin, enzymes and cell envelope transport proteins (Ciocan and Bara, 2007). Coumarins are derivatives of cinnamic acid and comprise a large class of phenolic substances (Hoult and Payát, 1996; Thuong et al., 2009). These compounds are the simplest members of the group of oxygen heterocyclic, also known as 1,2-benzopyrene, consisting of fused benzene and α-pyrone ring (Hoult and Payát, 1996). More than 1300 coumarins have been studied from natural sources and several properties are related with them, such as: antimicrobial, anti-inflammatory, antioxidant, anticoagulation, antiestrogenic and sedative (Hoult and Payát, 2012; Mello, 2009; Paramjeet et al., 2012). Essential oils and terpenoids are compounds based on an isoprene structure. They occur as diterpenes, triterpenes and tetraterpenes (C_{20}, C_{30}, C_{40}), they are called terpenoids when contain other elements, such as oxygen. These compounds are active against bacteria, fungi, viruses and protozoa (Ciocan and Bara, 2007; Das et al., 2010).

The alkaloids are heterocyclic nitrogenous compounds that present analgesic, antispasmodic and bactericidal action (Ciocan and Bara, 2007; Das et al., 2010; Stary, 1996).

2.4. Mode of Action of Phytochemicals

Bacterial growth can be inhibited by phytochemicals through several mechanisms. These plant products can act on various biochemical targets on the bacterial cells. The mode of action of phytochemicals is not completely understood, neither the phytochemical antibacterial specificity (Simões et al., 2009).

Some experiments have been done to study the mode of action of several phytochemicals. The chemical structure and properties influence the site of action of phytochemicals. The mechanism of action of essential oils against bacteria involves membrane disruption through the lipophilic structure (Griffin et al., 1999; Mendoza et al., 1997). Alkaloids, such as berberine and piperine, interact with bacterial cytoplasmic membrane, intercalate with DNA or inhibit efflux pumps in *S. aureus* (Khan et al., 2006). Phenols act by interruption of energy production due to enzyme inhibition by the oxidized products, which react with sulfhydyl groups or non-specific interaction with proteins (Mason and Wasserman, 1987). In the case of flavonoids, they inhibit the synthesis of nucleic acids of Gram-negative and Gram-positive bacteria (Cushnie and Lamb, 2005; Mori et al., 1987). Other authors, shown that glycoside saponins are able to induce pore-like structures that change the membrane permeability; they can also interfere with energy metabolism (Mandal et al., 2005; Melzig et al., 2001; Sinha Babu et al., 1997).

2.5. Biofilms and phytochemicals:

Natural products have been isolated from plants for usage in biodeterioration control. Biodeterioration is the chemical and physical alteration resulting from

biological activity. The microorganisms associated to biodeterioration growth as biofilms that adhere to substrates. The treatment for this problem involved the use of biocides, however these are chemical agents and most of them are cytotoxic. So, natural compounds from plants with biocidal activity have emerged, and they are promising alternative for the control of biodeterioration without negative impacts of the environment (Guiamet et al., 2006). The chemical biocides are toxic and difficult to degrade, being persistent in the environment, causing chemical contaminations and the spread of resistance. In contrast, natural biocides, extracted from plants, are biodegradable and environmental friendly (Guiamet and Saravia, 2005). The phytochemicals can act as control agents on the bacterial biofilm formation and development (Simões et al., 2009).

Guiamet and co-workers (2006) performed a study with *Cichorium intybus*, *Arctium lappa* and *Centaurea cyanus* from Asteraceae family to test their ability to be used as antimicrobial agents against different microorganisms associated with biodeterioration. These plants showed moderate activity against two species of *Pseudomonas* and no activity against *Bacillus cereus*. *Rosmarinus officinalis* L. extracts act as an antimicrobial agent against *S. aureus*. *Allium sativum* produce allicin, which is one of the most effective antimicrobial products isolated from garlic (Abreu et al., 2012).

Biofilm formation is a feature closely related to pathogenicity (Ren et al., 2005). A biofilm is formed by planktonic bacteria that adhere to a surface and initiate the development of sessile microcolonies surrounded by an extracellular matrix (Otto, 2009) (Figure 2). Bacteria form complex surface-attached communities, also called biofilms (Hentzer and Givskov, 2003). Biofilms develop structures that are morphologically and physiologically differentiated from free-living bacteria (Davies et al., 1998).

The process of biofilm formation includes several steps: preconditioning of the adhesion surface; planktonic cells are transferred from the bulk liquid to the surface; adsorption of cells at the surface; desorption of reversible adsorbed cells; the bacterial cells are adsorbed irreversibly at the surface; transport of substrates to the biofilm; substrate metabolism by the biofilm cells and transport of products out of the biofilm; finally, biofilm is removed by detachment or sloughing (Simões, 2005). The formation of biofilm is dependent of several parameters. Regarding to the surfaces, the attachment is easier on rough, hydrophobic and coated surfaces (Donlan, 2002;

Pereira, 2001). Parameters like flow velocity, water temperature and nutrient concentration also influence the biofilm attachment (Pereira, 2001; Vieira, 1995).

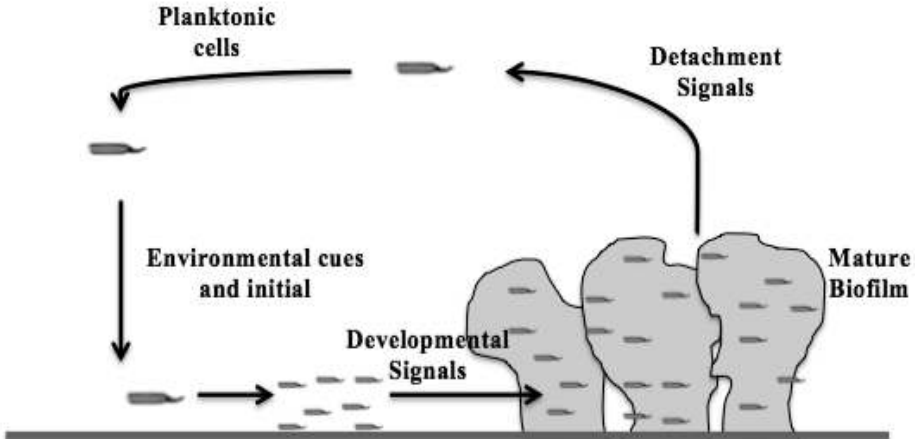

Figure 2. Model of biofilm development. Planktonic cells contact with surface resulting in the formation of microcolonies. Cells in the biofilm can return to a planktonic lifestyle to complete the cycle of biofilm development.

Biofilms are an example of physiological modifications and they also increase the tolerance to antimicrobial therapies and to the host immune response (Hentzer and Givskov, 2003; Simões et al., 2009). Most of the bacterial infections detected in human body involve the formation of biofilms. The biofilm mode of growth permits an increased bacterial survival in hostile conditions, such as in the presence of antibiotics and disinfectants (Hentzer and Givskov, 2003; Trentin et al., 2011). There is an increasing interest in preventing, controlling and eradicating biofilms. Biofilms by bacterial cells are thought to be regulated by autoinducer molecules; in a process called quorum-sensing (Ren et al., 2002). The impairment of bacterial adhesion and biofilm formation by a pathway that does not affect the bacterial death is an important feature of the new concept in antivirulence therapies (Trentin et al., 2011). This alternative should maintain the cells in a planktonic state, switching off the virulence expression and attenuating the pathogen, making the microorganisms more susceptible to antimicrobial agents and immune system (Macedo and Abraham, 2009; Martin et al., 2008).

Bacteria in biofilms present a reduced susceptibility to antimicrobial agents caused by a variety of factors, such as: nutrient depletion within the biofilm, reduced access to cells in the biofilm, production of degradative enzymes and neutralizing

chemicals, between others (Brown and Gilbert, 1993). Biofilms have been reported to be 100-1000 more protectors to bacteria than populations of planktonic cells (Gilbert et al., 2002; Mah and O'Toole, 2001; Stewart and Costerton, 2001). The main difference between planktonic cells and biofilms is the presence of a polysaccharide matrix, delaying the diffusion of antimicrobials into the biofilm (Brooun et al., 2000).

Traditional treatment of infectious diseases is related with compounds that inhibit the growth of bacteria. But, it has been observed that they develop resistance to antimicrobial compounds. So, quorum-sensing seems to be the next opportunity to improve bacterial infection. Quorum-sensing influences bacterial biofilm growth and biofilm development that is related with cell-cell interactions (Simões et al., 2009). Quorum-sensing inhibitory compounds are the new line of antimicrobial agents and can be applied in several areas: medicine, agriculture and aquaculture (Hentzer and Givskov, 2003).

Several biotechnology companies have already tried to develope some strategies to interrupt the bacterial quorum-sensing, such as: inhibition of N-acyl-homoserines (AHL) signal recognition, signal dissemination and signal reception (Hentzer and Givskov, 2003). By interfering with cells communication, it is possible to interfere also in the resistance of biofilms and their ability to form resistant structures, causing cell dispersion. Quorum-sensing systems are involved in a wide range of microbial activities: extracellular enzyme biosynthesis, biofilm development, antibiotic biosynthesis, biosurfactant production, extracellular polymeric substances (EPS) synthesis and production of extracellular virulence factors (Chatterjee et al., 1995; Davies et al., 1998; Daniels et al., 2004; Fux et al., 2005; McGowan et al., 1995; Passador et al., 1993; Pearson et al., 1995; von Bodman and Farrand, 1995).

Autoinducers (AI) are molecules to perceive the size of bacterial population and AHL are the major AI molecules. Several Gram-negative bacteria use AHL signals to coordinate the behaviour of cells in a population. An important achievement was the discovery of molecules produced by plants that mimic AHL signals, affecting quorum-sensing behavior. Several plants of medicinal use demonstrated potential to inhibit quorum-sensing (Hentzer and Givskov, 2003; Waters and Bassler, 2005).

Studying the grapefruit and its furanocoumarins as inhibitors of biofilm formation, it has been shown that dihydroxybergamottin and bergamottin exhibit strong inhibition of both AI-1 and AI-2 activities even at concentrations as low as 1

μg/mL (Girennavar et al., 2008). Furanocoumarins are able to interfere with cell-cell signalling and also inhibit biofilm formation. However, the mechanisms of action are not completely understood (Girennavar et al., 2008). *Delissea pulchra*, an Australian macroalga, produces halogenated furanones, which are inhibitors of AHL, inhibiting bacterial quorum-sensing and biofilm formation (McLean et al., 2004). Auraptene and lacinartin, two compounds belonging to coumarin family, are promising natural compounds that can be used to prevent and treat periodontal diseases. They were also evaluated on the growth, biofilm formation/desorption, and adherence to human oral epithelial cells of *Porphyromonas gingivalis*. Lacinartin was able to inhibit biofilm formation and to cause desorption of a pre-formed biofilm of *P. gingivalis*. This suggests that coumarins may contribute to reducing tissue destruction (Marquis et al., 2012). Some information about the structure-activity relationship of coumarins showed that the group on position 5 and position 2'/3' of the isoamylene chain can affect the antifouling activities against both *Balanus albicostatus* and *Bugula neritina* (Wang et al., 2013).

Therefore, phytochemicals are not only important for the antimicrobial response and the substitution of antibiotics because of bacterial resistance, but also for the control of biofilms formation. Natural products are important sources of bioactive compounds and the medicinal plants used in folk medicine can facilitate the search of new agents.

Chapter 3

Activity of Selected Phytochemical Products as Antimicrobials and in Biofilm Control

3.1 Introduction

Microbiologists have learned to assess antibiotic effects *in vivo* by evaluating the MIC and MBC *in vitro*. These methods assess the influence of antibiotics against planktonic microorganisms in the exponencial phase of growth and predict the efficacy of antibiotcs against bacteria in infections (Ceri et al., 1999; Fux et al., 2005).

Staphylococcus aureus and *Escherichia coli* are two human pathogens that can cause a variety of infections. The main charactheristic of these infections is the formation of biofilms (Beenken et al., 2004).

In order to prevent biofilm formation, several studies have been performed to find antimicrobial agents that affect the viability of bacteria in biofilms. Natural products from plants have been shown to influence microbial biofilm formation (Rasooli et al., 2008). Plants are an important source of disinfection compounds as they produce a wide range of phytochemicals with antimicrobial properties, most of them against microorganisms, insects, nematodes and other plants (Abreu et el., 2013a). Phytochemicals are able to inhibit peptidoglycan synthesis, damage microbial

membrane structures, modify bacterial membrane surface hydrophobicity and also modulate quorum-sensing (Rasooli et al., 2008). These processes can also inhibit or affect biofilm formation.

To reach high killing rates and to avoid resistance and adaption, disinfectants are used at very high concentrations relative to their MICs (Abreu et al., 2013a). So, after the detection of MIC and MBC for the selected phytochemicals, they were tested at MIC and 5×MIC. The effects of phytochemicals on *E. coli* and *S. aureus* biofilms were evaluated through biomass prevention and metabolic inactivation. The quantification of biofilm removal takes into account both live and dead cells assessed by the Crystal Violet method (CV). CV is a dye wich binds to negatively charged surfaces in the extracellular matrix (Extremina et al., 2011; Peeters et al., 2008). Resazurin is used to quantify the viability of cells, based on the live cells. This compound is a blue redox indicator and it reduces to pink by contact with viable bacteria in the biofilm (Extremina et al., 2011).

Pathogens are increasing their capacity to survive after contact with antimicrobials and disinfectants. Many of the existing antibiotics are ineffective due to their extensive and inappropriate use (Abreu et al., 2013a). Plants are important sources for the development of antimicrobials and strategies to control growth and biofilm formation (Abreu et al., 2013a).

The purpose of this study was to assess the antimicrobial efficacy of selected phytochemicals (7-HC, I3C, SA and SP) against *E. coli* and *S. aureus* planktonic cells. Moreover, the effects of these phytochemicals were assessed on biofilm control.

3.2 Matherial and methods

3.2.1. Bacterial Strains

The bacteria used in this study were obtained from the Spanish Type Culture Collection (CECT): the Gram-negative bacterium *Escherichia coli* (CECT 434) and the Gram-positive bacterium *Staphylococcus aureus* (CECT 976). The bacteria were distributed over the surface of Plate Count Agar (PCA – Merck) and incubated for 24 h at 30 ± 3 ºC.

3.2.2. Phytochemicals

The phytochemicals used were: 7-hidroxicoumarin (7-HC), indol-3-carbinol (I3C), salicylic acid (SA) and saponin (SP). These compounds were obtained from Sigma-Aldrich (Portugal) and prepared in dimethyl sulfoxide (DMSO, Sigma).

3.2.3. Determination of Minimum Inhibitory Concentration

The MIC is considered the lowest concentration of an antimicrobial that will maintain or reduce the growth of a microorganism after 24 h incubation (Andrews, 2001). MIC of phytochemicals was determined by microdilution method in sterile 96-wells microtiter plates (McBain et al., 2004). The cell suspensions of *S. aureus* and *E. coli* were obtained by overnight cell cultures in Mueller-Hinton broth (MHB) (Fluka, Portugal) and were adjusted to a OD_{620nm} at 0.1±0.02 (corresponding to approximately 1×10^6 cells/mL) in the spectrophotometer (VWR V-1200). The suspension cells were added to sterile 96-well polystyrene microtiter plates (Orange Scientific) with different phytochemicals in several concentrations (25, 50, 100, 200, 400, 800, 1600, 3200 µg/mL) in a final volume of 200 µL. The volume of 7-HC, I3C, SA and SP added to each well was 10 µL. DMSO was used as a negative controls. No antimicrobial activity was detected by DMSO at this concentration (data not shown). After 24 h at 30 °C, the MIC of each sample was determined by measuring the optical density in the spectrophotometer (620 nm) (SpectraMax M2E, Molecular devices). MIC corresponds to the concentration in which the final OD is inferior or equal to the initial OD.

3.2.4. Determination of Minimum Bactericidal Concentration

The MBC is defined as the lowest concentration of antimicrobial that will prevent the growth of an organism after subculture on to antibiotic-free media (Andrews, 2001). MBC of phytochemicals was determined by the drop method. After measuring the MIC, the wells corresponding to the phytochemicals concentrations equal and above the MIC were added (10 µL) to PCA plates. The drops were drained along the plate. After 24 h at 30 ºC, the plates were analysed and the MBC of each phytochemical corresponding to the concentration which inhibited the growth of the bacteria.

3.2.5. Biofilm formation and control in sterile 96-well polystyrene microtiter plates

Biofilms were developed according to the modified microtiter plate test proposed by Stepanović et al. (2000). For both bacteria, at least 6 wells of a 96-well polysytrene microtiter plate were filled with 200 µL of overnight batch cultures in MHB (OD_{620nm}= 0.04 ± 0.02). The plates were incubated overnight at 30 ºC and 150 rpm. The negative control wells were also placed on the plates, being sterile water and medium. Plates were incubated for 24 h at 30 ºC and agitated at 150 rpm. In order to test the effects of phytochemicals in several steps of the process there are various treatments that can be applied. However, in this case, the treatment applied consisted in incubating overnight the strains without phytochemicals. The biofilms were formed in microtiter plates for 24 h, and subsequently, were incubated with phytochemicals for 1 h.

After biofilm development, the content of wells was removed and the wells were washed three times with 200 µL of NaCl (8.5 g/L) to remove reversibly adherent bacteria. The phytochemicals were added to the wells at the MIC and 5 × MIC. The microtiter plates were incubated for 1 hour. The remaining attached bacteria were analysed by using crystal violet and resazurin.

3.2.6. Biofilm analysis

3.2.6.1 Crystal Violet method

Before phytochemicals application, the inoculum in the walls was removed and the wells were washed with 200 µL of sterile water. Later, 250 µL of ethanol were loaded for 15 minute to promote biofilm fixation. The supernatant was removed and the plates were air-dried. Subsequently, 200 µL of CV solution (Gram color staining set for microscopy, Merck) was added for 10 minutes to stain the fixed bacteria. After washing in water, the plates were dried and finally, the wells were loaded with 200 µL of aceditic acid 33% (v/v) (Merck) to release and dissolve the stain. To analyse the biofilm, the OD of the solutions was measured at 570 nm using a microtiter plates reader (SpectraMax M2E, Molecular Devices). After obtaining the values of absorbance, the percentage of biomass removal is obtained according equation 1:

$$\%\ Biomass\ removal = \frac{OD\ control_{570} - OD\ phytochemical_{570}}{OD\ control_{570}} \times 100 \qquad (1)$$

where OD $control_{570}$ represents the optical density of the control at 570 nm, and OD $phytochemicals_{570}$ is the optical density of the phytochemical.

3.2.6.2 Resazurin Method

In this method, a commercially available resazurin solution (Sigm) was used. The plates were loaded with 190 µL of sterile MH medium and 10 µL of resazurin solution. After 20 minutes of incubation at room temperature, fluorescence (λ_{ex}: 570 nm and λ_{em}: 590 nm) was measured using the microtiter plates reader. After measuring the fluorescence, it is possible to calculate the percentage of metabolic inactivation:

$$\%\ Metabolic\ inactivation = \frac{FLUO\ control - FLUO\ phytochemical}{FLUO_{control}} \times 100 \qquad (2)$$

where $FLUO_{control}$ represents the fluorescence intensity of biofilms not exposed to phytochemicals and $FLUO_{phytochemical}$ represents the fluorescence intensity value for biofilms exposed to phytochemicals.

3.2.7. Classification of biofilm producer bacteria

According to Stepanović et al. (2000), the bacterial strains can be classified using the following groups:

- Non-producer (-): $OD \leq OD_C$;
- Weak producer (+): $OD_C < OD \leq 2xOD_C$;
- Moderate producer (++): $2xOD_C < OD \leq 4xOD_C$;
- Strong producer (+++): $4xOD_C < OD$.

where OD_C represents the cut-off OD for the microtiter plates test as three standard deviations above the mean OD of the negative control. The negative control wells contained broth only.

3.2.8. Statistical analysis

The data was analyzed using One-Way Anova and the statistical program SPSS 21.0 (Statistical Package for the Social Sciences). The results were presented as the means ± standard deviation. Significance level for the differences was set at $p<0.05$ and the calculations were based on confidence level equal or higher than 95%.

3.3 Results and Discussion

3.3.1. Antimicrobial activity of phytochemicals and biofilm control potential

The MIC of 7-OH, I3C, SA and SP were measured for both bacteria (*E. coli* and *S. aureus*). The MIC value was considered to be the lowest concentration of phytochemical able to totally inhibit microbial growth. Table 1 shows the results obtained.

Table 1. MIC for *E. coli* and *S. aureus*.

Strains	Phytochemicals (μg/mL)			
	7-HC	I3C	SA	SP
E. coli	800	800	3200	ND
S. aureus	200	400	1600	ND

ND – Not detectable

The values of MIC for *E. coli* are higher than those for *S. aureus*. This result can be explained because *E. coli* is a Gram-negative bacterium and it is less susceptible to antimicrobials than Gram-positive bacteria. The Gram-negative bacteria tend to be more resistant to lipophilic and amphiphilic inhibitors than those Gram-positive, including dyes, detergents, free fatty acids, antibiotics and chemotherapeutics agents (Nikaido, 1996). In other study, antibiotics from natural origin showed activity against Gram-positive bacteria, but more than 90% of them have no useful effect against *E. coli* (Vaara, 1993). This increased resistance of Gram-negative bacteria can be attributed to the presence of the outer membrane. The porin channels slow down the penetration of small hydrophilic solutes and the low fluidity of the lipopolysaccharide layer decreases the rate of transmembrane diffusion of lipophilic solutes (Nikaido and Vaara, 1985; Plésiat and Nikaido, 1992).

Regarding to the different phytochemicals tested, SP was the unique compound that had no detectable MIC for concentrations lower than 3200 μg/mL. The 7-HC and I3C were the most effective compounds against both bacteria, since they presented the lowest values of MIC.

The literature is full of reports describing natural products and extract with antimicrobial activity with MIC values over 1000 μg/mL, which has little relevance for clinical application (Gibbons, 2004).

After the determination of MIC values for each phytochemical, MBC values were measured, using several concentrations sub and above-MIC. Table 2 presents the values of MBC for both bacteria. Regarding SP, the MBC was not possible to be quantified due to the absence of a MIC value.

Table 2. MBC values for *E. coli* and *S. aureus*.

Phytochemical	Concentration (µg/mL)	
	E. coli	***S. aureus***
7-HC	ND	ND
I3C	1600	800
SA	5000	3200

ND – Not detectable

Similarly to the results of MIC, the values of MBC were higher for *E. coli* than for *S. aureus*. MBC represents the lowest concentration of antimicrobial product necessary to kill a bacterium; so Gram-negative need a higher concentration of antimicrobial, because these bacteria are more resistant (Nikaido, 1996). Regarding to 7-HC, the MBC was not defined for both bacteria, until the maximum value tested (10000 µg/mL). I3C seems to be the most effective phytochemical against both bacteria.

After testing the effects of the selected phytochemicals against the *E. coli* and *S. aureus* strains, the effects of the same phytochemicals on their biofilms were tested. Biofilm formation was performed in sterile 96-well polystyrene microtiter plates.

In this case, the bacteria were incubated overnight without phytochemicals. Biofilms were formed for 24 hours and after that, biofilms were incubated with phytochemicals at the MIC and 5 × MIC for 1 hour. According this procedure, it would be possible to conclude about the efficacy of phytochemicals in control (removal and inactivation) of biofilms. Figure 3 shows the results obtained from this assay.

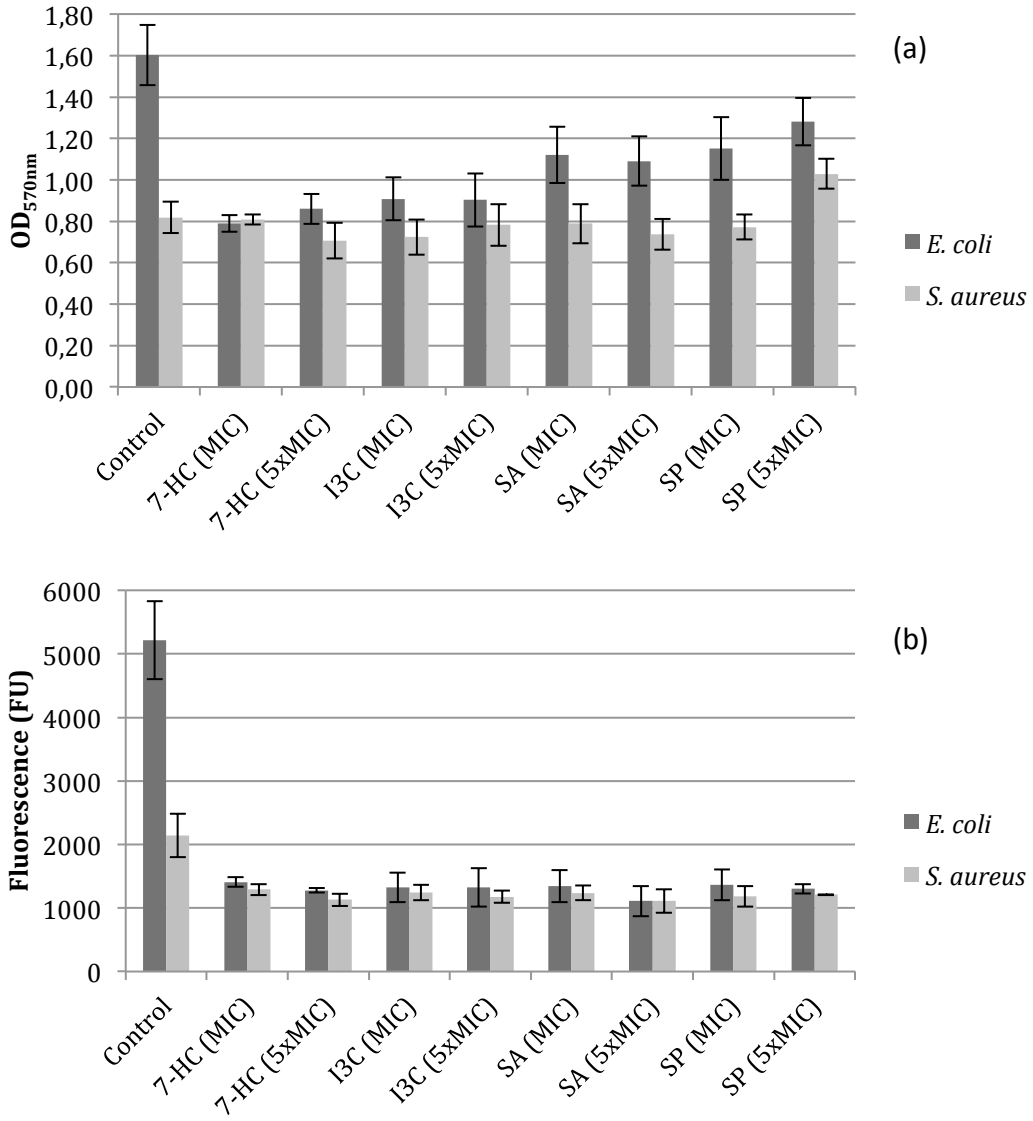

Figure 3. $OD_{570\ nm}$ as a measure of biofilm mass (a) and fluorescence (λ_{ex}: 570 nm and λ_{em}: 590 nm) results as a measure of biofilm viability (b) for *E. coli* and *S. aureus*. The mean ± standard deviation values resultant from three independent experiments are depicted.

According to the classification proposed by Stepanović et al. (2000), both strains are weak biofilm producers (+).

In the Table 3 are presented the percentages of biofilm removal and inactivation with the selected phytochemicals at different concentrations. The ability of phytochemicals to control 24 h aged biofilms was analysed, based on their effects on biomass and metabolic activity.

Table 3. Percentages of biofilm removal and inactivation by the selected phytochemicals against *E. coli* and *S. aureus*.

Phytochemicals	*E. coli*		*S. aureus*	
	% Biomass removal	% Metabolic inactivation	% Biomass removal	% Metabolic inactivation
7-OH (MIC)	51.03±2.53	73.01±1.48	9.63±2.30	39.79±4.15
7-OH (5 × MIC)	46.67±4.47	75.50±0.70	1.68±0.32	47.30±4.59
I3C (MIC)	43.63±6.47	74.67±4.45	0.14±0.04	42.03±5.61
I3C (5 × MIC)	43.98±7.95	74.68±5.78	4.02±0.09	44.99±4.68
SA (MIC)	33.36±5.15	74.24±4.86	0.00±0.00	42.21±5.34
SA (5 × MIC)	34.19±6.52	78.75±4.57	0.00±0.00	48.26±8.55
SP (MIC)	31.80±6.92	73.86±4.62	0.00±0.00	44.71±7.65
SP (5 × MIC)	23.86±5.80	75.09±1.37	0.00±0.00	43.54±0.29

The results show the capacity of phytochemicals to remove/inactivate the biofilms of *E. coli* and *S. aureus*. The biomass removal is dependent on the phytochemical and on its concentration. Comparing the values obtained, it is concluded that the phytochemical concentration (MIC and 5 × MIC) did not influence the removal of the biofilm ($p>0.05$), for both bacteria. Probably, the cells are able to adapt to the phytochemicals and with a higher concentration, the effect is not so noticed. Other possibility is the occurrence of changes in the biofilm phenotype (Cerca et al., 2006). However, the type of bacteria influences the values of biofilm removal. The same phytochemical, at the same concentration, presents a different behaviour in Gram-negative and Gram-positive bacteria ($p<0.05$). The percentages of biomass removal were always higher for *E. coli* than *S. aureus* with all the phytochemicals and concentrations performed. Total biofilm removal was not achieved with any of the selected phytochemicals. The highest reduction in biomass was found for *E. coli* with 7-HC. All the phytochemicals were more active in removing *E. coli* biofilms than *S. aureus* biofilms.

In terms of metabolic activity, the phytochemicals promoted higher reduction for *E. coli* biofilms than *S. aureus* biofilms. The inactivation percentages refer to the quantity of cells not viable present in the biofilm. The selected phytochemicals induced a higher quantity of cells not viable in biofilms of *E. coli* comparing to those of *S. aureus*. The biofilms of *E. coli* treated with phytochemicals at different concentrations did not show significative differences in the percentage of viable cells ($p>0.05$). Probably, the same effect explained before, for the percentage of removal, occurred in this case and the cells are able to adapt or the biofilm phenotype is

changed. However, in the case of *S. aureus*, the biofilms treated with 7-HC and I3C, at different concentrations, were significatively different ($p<0.05$). In these cases, *S. aureus* probably did not develop any mechanism to protect the cells with the lowest concentration, and when it was applied the highest concentration, the cells were inactivated, and the percentage of nonviable cells increased significantly. The inactivation was more significative in the case of *E. coli* biofilm than for *S. aureus* biofilms with all the concentrations performed ($p<0.05$).

Phytochemicals had greater effect in *E. coli* than *S. aureus*. This result is contradictory with the results obtained for MIC and MBC. Gram-negative bacteria should be more resistant to phytochemicals and the removal and inactivation should be higher for *S. aureus* than *E. coli*. According Simões et al. (2008), *E. coli* is less susceptible to antimicrobials because of its outer membrane, which constitutes a barrier for the entrance of molecules. However, the number of resistance mechanisms in biofilms increase significantly and one cannot infer on the biofilm susceptibility based on the results of planktonic cells. Furukawa and co-workers (2010), studied the control of *S. aureus* and *E. coli* biofilms on stainless steel, concluding that the last ones are more susceptible to cleaning by five types of cleaning agents, food additives and other compounds on stainless steel. The morphology of the tested biofilms is known to be different. *S. aureus* biofilms are denser than that of *E. coli* biofilm (Furukawa et al., 2010).

Biofilm eradication is a difficult aspect to be solved due to their inherent resistance to biocides. There are several mechanisms explaining the biocides resistance of biofilms: penetration of chemical agents is delayed in the biofilm matrix; the reaction between the biocides and matrix components; the altered growth rate of biofilm organisms and physiological changues due to the biofilm mode of growth (Sillankorva et al., 2004). So, it is difficult to predict the behaviour of the cells presents in biofilms, because there are several components affecting their resistance. The biofilms also present a different mode of growth from planktonic cells: biofilms individual bacteria are enclosed in a matrix of extracellular polymeric substances; bacteria are not distributed uniformly throughout a biofilm; the cell growth can be lower because of the limited access to nutrients and oxygen (Sillankorva et al., 2004).

Chapter 4

Aspects Underlying the Antibacterial and Biofilm Control Action of Phytochemicals

4.1 Introduction

The use of dual combinations of antimicrobial drugs with positive *in vitro* interactions has become an important parameter to evaluate in clinical applications. The appearance of this practice starts to prevent the emergence and widespread multi-drug resistant (MDR) infections (Sopirala et al., 2010). Several antibiotics have been analysed for their action as resistance-modifying agents (RMAs), i.e. compounds able to modify or inhibit the bacterial resistance, so the antibiotics kill efficiently the resistant bacteria (Abreu et al., 2013b).

The clinical effects and synergism of dual combinations have to be tested *in vivo*. Determination of synergy *in vitro* might not be reflected *in vivo*, due to the achievement of synergic levels of drugs in the tissues, differences in plasma protein binding and drug metabolism (Kalan and Wright, 2011). Dual combinations of phytochemicals and antibiotics were tested to understand their ability to act in efflux pumps of *S. aureus*.

The elucidation of the molecular details of drug resistance is a crucial area of research that crosses many subjects since an understanding of the mechanisms by which drug resistance develops leads to enhancements in extending the efficacy of current antibiotics. Cell envelope is the main target for drug-resistant mechanisms of various pathogens because many drugs need to rapidly diffuse into a cell to meet their targets (Nikaido, 1994; Savage, 2001; Zhang et al., 2001).

Biofilms constitute a threat in the clinical environment by acting as pools of multidrug resistant pathogenic bacteria. Biofilm may be formed in a variety of surfaces including living tissues, indwelling medical devices, portable water system piping or natural water system piping (Kokare et al., 2009).

Diverse mechanisms allow microorganisms to come into closer contact with a surface, attach to it, promote cell-cell interactions and grow as a 3-D structure (Bryers, 2000). There are several mechanisms influencing the attachment of biofilm. The properties of the surface of attachment influence the process. In the case of a rougher, more hydrophobic, and coated surface, the attachment will occur efficiently (Donlan, 2002; Pereira, 2001). Other variable can affect the attachment: flow velocity, water temperature or nutrient concentration (Pereira, 2001; Vieira, 1995). Maximum attachment depends upon high surface free energy or wettability of surfaces. Surfaces with high surface free energies are more hydrophilic and generally show greater bacterial attachment than hydrophobic surfaces.

Quorum sensing, related with cell-cell signalling, play a role in cell attachment and detachment from biofilms (Simões, 2005). This mechanism induces and increased in the intrinsic antimicrobial resistance of biofilm (Brooun et al., 2000). QS regulates a wide number of physiological activities, such as motility, conjugation, competence, sporulation, virulence and biofilm formation. The signal of QS may alter distribution of bacterial species in the biofilm, alter protein expression, introduce new genetic trait and incorporate bacteria in biofilm. The properties of cells, including cell surface hydrophobicity, presence of fimbriae and flagella, and production of EPS influence the attachment of microbial cell (Watnick and Kolter, 2000).

One key element of the adaptability of bacterial cells is their ability to be in a niche where they can propagate. Various mechanisms of motility have been described for bacteria. The most common is flagellar motility, but other mechanisms can be highlighted: twitching, gliding, darting, sliding, swimming and swarming (Davey and O'Toole, 2000).

The aim of this study was to assess aspects of the mode of action of the selected phytochemicals on planktonic cells and in the early stages of biofilm formation. The physicochemical properties of bacterial surface (zeta potential and hydrophobicity) were performed with and without phytochemicals. The evaluation of phytochemicals to inhibit efflux pumps was studied with *S. aureus* while the effects of these products were tested on *E. coli* outer membrane proteins (OMPs) expression. The free energy of adhesion, quorum-sensing inhibition and bacterial motility were assessed in order ascertain the role of phytochemicals on the early stages of biofilm development.

4.2 Materials and methods

4.2.1. Bacterial Strains

Escherichia coli (CECT 434) *Staphylococcus aureus* (CECT 976) were used according to section 3.2.1.. The *Chromobacterium violaceum* (ATCC 12472) were distributed over the surface of Luria–Bertani Agar (Merck) and incubated for 24 h at 30 ± 3 ºC. *S. aureus* RN4220 containing plasmid pUL5054, which carries the gene encoding the MsrA macrolide efflux protein; *S. aureus* SA1199B, which overexpresses the NorA MDR efflux protein and *S. aureus* XU212, which possesses the TetK efflux pump and is also a MRSA strain, were kindly provided by S. Gibbons (University College London, UCL) (Gibbons et al., 2003; Oluwatuyi et al., 2004; Gibbons and Udo; 2000; Smith et al., 2007). Prior to use, these strains at -80ºC were transferred onto Mueller-Hinton (Merck, Germany) agar plate, grown overnight, and inoculated into MH broth at 30 ºC and under agitation (150 rpm).

4.2.2. Phytochemicals and antibiotics

The phytochemicals used were the same referred before and prepared as explained in section 3.2.2. Ciprofloxacin, erythromycin and tetracycline were obtained from Sigma (Portugal). Every antibiotic were prepared in DMSO, which was filtrated before utilization to avoid contamination. After preparation, antibiotics were frozen.

4.2.3. Determination of *Zeta* Potential

The overnight cultures of *E. coli* and *S. aureus* were centrifuged (Eppendorf centrifuge 5810R) at 3777 g for 10 min and washed twice with sterile water. The $OD_{620\ nm}$ of strains was adjusted to 0.2 ± 0.02 and samples were incubated with phytochemicals for 30 minutes at 30 ºC. Phytochemical concentration used was the MIC. Cells suspensions without phytochemicals were used as control. The zeta potential experiments were performed using a Malvern Zetasizer instrument (Nano Zetasizer, Malvern instruments, UK). All experiments were carried out in triplicate at room temperature and were repeated at least at three different occasions.

4.2.4. Physico-chemical charecterization of bacterial surface

The physico-chemical properties were measured using the sessile drop contact angle method. The bacteria cultures were grown overnight in MH medium. The cells suspensions were washed with NaCl (8.5 g/L) and centrifuged (10 minutes at 3777 g) twice. The OD_{620nm} was adjusted to 0.4 ± 0.02. After that, the biocides were applied (at MIC concentration) during 30 minutes. The solutions were filtrated (0.45 μm, Whatman) and placed in microscope slides. The contact angle was measured with 3 different liquids: water, formamide (polar) and α-bromonaphtalene (nonpolar) (Sigma, Portugal).

The measurement of contact angles was performed using a model OCA 15 Plus (Dataphysics, Germany) video based optical contact angle measuring instrument, allowing image acquisition and data analysis. The degree of hidrophobicity of a surface is expressed as the free energy of interaction between entities of that surface, when immersed in water. ΔG_{sws} (mJ/m^2) can be positive or negative according of the interaction between the surfaces. In the case of $\Delta G_{sws} > 0$, the material is considered hydrophilic, because the interaction between the two surfaces is weaker than the interaction of each entity with water. In contrast, when $\Delta G_{sws} < 0$, the interaction between the surfaces is stronger than the interaction of each entity with water and the material is hydrophobic. Hydrophobicity was evaluated after contact angles measurements, following the van Oss approach (van Oss *et al.*, 1987; 1988; 1989).

The degree of hidrophobicity can be calculated through the surface tension components of interacting entities, according to:

$$\Delta G_{sws} = -2\left(\sqrt{\gamma_s^{LW}} - \sqrt{\gamma_w^{LW}}\right)^2 + 4\left(\sqrt{\gamma_s^+\gamma_w^-} + \sqrt{\gamma_s^-\gamma_w^+} - \sqrt{\gamma_s^+\gamma_s^-} - \sqrt{\gamma_w^+\gamma_w^-}\right) \quad (3)$$

Where, γ^{LW} is the Lifshitz-van der Waals component of the surface free energy and γ^+ and γ^- are the electron acceptor and donor, respectively, of the Lewis acid-base parameter (γ^{AB}), being $\gamma^{AB} = \sqrt{\gamma^+\gamma^-}$.

The analysis was performed at room temperature using the three liquids referred before. The surface tension components of liquids were obtained from literature (Janczuk et al., 1993). Subsequently, three equations can be solved:

$$(1+\cos\theta)\gamma_1^{Tot} = 2\left(\sqrt{\gamma_s^{LW}\gamma_w^{LW}} + \sqrt{\gamma_s^+\gamma_w^-} + \sqrt{\gamma_s^-\gamma_w^+}\right) \quad (4)$$

Where θ is the contact angle and $\gamma^{TOT} = \gamma^{LW} + \gamma^{AB}$.

These measurements were performed at least with 12 determinations for each liquid and microorganism.

4.2.5. Free energy of adhesion

The free energy of adhesion between the bacterial cells and polystyrene surfaces was calculated through the surface tension components of the entities involved in the process using the Dupré equation and the procedure described by Simões et al. (2010). The total interaction energy (ΔG_{bws}^{TOT}) is studied by the interaction between one bacteria (b) and a substratum (s) that are immersed or dissolved in water (w) and is expressed by the interfacial tension components:

$$\Delta G_{bws}^{TOT} = \gamma_{bs} - \gamma_{bw} - \gamma_{sw} \quad (5)$$

The thermodynamic theory of the interfacial tension of one system of interaction (for example, bacteria/surface - γ_{bs}) can be defined by the following equations:

$$\gamma_{bs} = \gamma_{bs}^{LW} + \gamma_{bs}^{AB} \quad (6)$$

$$\gamma_{bs}^{LW} = \gamma_{b}^{LW} + \gamma_{s}^{LW} - 2\times\sqrt{\gamma_{b}^{LW}\times\gamma_{s}^{LW}} \quad (7)$$

$$\gamma_{bs}^{AB} = 2\times\left(\sqrt{\gamma_{b}^{+}\times\gamma_{b}^{-}} + \sqrt{\gamma_{s}^{+}\times\gamma_{s}^{-}} - \sqrt{\gamma_{b}^{+}\times\gamma_{s}^{-}} - \sqrt{\gamma_{b}^{-}\times\gamma_{s}^{+}}\right) \quad (8)$$

The two other interfacial tension components, γ_{bw} and γ_{sw}, were calculated in the same way, which permits the assessment of thermodynamic energy of adhesion. The bacterial adhesion to the substratum can be favorable or is not expected to occur, according to the values of ΔG_{bws}^{TOT} if are negative or positive, respectively (Simões et al., 2010).

4.2.6. Motility

Plates containing 1% tryptone (Merck), 0.25% NaCl (Merck) and 0.25% or 0.7% (w/v) agar (Merck) were prepared for swimming/sliding or swarming motilities,

respectively (Butler et al., 2010; Stickland et al., 2010). Phytochemicals at MIC concentration were incorporated in the growth medium after sterilize and cooling the medium, to avoid the deterioration. Overnight cultures of *E. coli* and *S. aureus* grown on LB broth (Merck, Germany) were used to determine bacterial motility. A volume of 15 µL of cell suspension (OD_{620nm}= 0.4 ± 0.02) was placed in the center of the plates. Then, plates were incubated at 30 ºC and the diameter (mm) of the bacterial motility halos were measured at 24, 48 and 72 h (Borges et al., 2012). All experiments were carried out in triplicate. The negative control was performed with DMSO.

4.2.7. Detection of quorum-sensing inhibition

The culture of *Chromobacterium violaceum* (CV12472) was grown overnight in Luria-Bertani (LB) broth at 30 ºC. The OD_{620nm} of the strain was adjusted to 0.1 ± 0.02 (1.4×10^8 CFU/mL). For each phytochemical a sterile 96-well polystyrene microtiter plates were filled with cells (180 µL) and phytochemicals (20 µL). As a negative controls were used cell suspension with DMSO and without phytochemicals. The microtiter plates were incubated during 24 h at 30 ºC in an orbital shaker (150 rpm). Subsequently, the absorbance at 620 nm was measured using a Microplate Reader (Spectramax M2e, Molecular Devices, Inc.). MIC values were determined using the microdilution method, explained above. All tests were performed in triplicate. All the further experiments were performed at sub-MIC concentrations of phytochemicals (Packiavathy et al., 2012).

After these experiments, *C. violaceum* was used to perform the detection of quorum-sensing inhibition (QSI) by the disc diffusion method. The detection of QS activity of phytochemicals was performed at the range of sub-MIC. LB agar (LBA) plates were spread with 100 µL (OD_{620nm} of 0.1 ± 0.02) (1.4×10^8 CFU/mL) of overnight culture of *C. violaceum* CV12472. Sterile paper disks (6 mm diameter) were placed in the plates and impregnated with varios concentrations of each phytochemical (15 µL). DMSO was used as a negative control. The plates were incubated at 30 ºC for 24 h to check the inhibition of pigment production around the disc. The growth inhibtion was also recorded. The zones of QS inhibition were measured from the disks to the edges (Adonizio et al., 2006; Khan et al. 2009). Bacterial growth inhibition by the phytochemicals was measured as radius (r_1) in mm while phytochemicals showing both growth and pigment inhibition was measured as

radius (r_2) in mm. The pigment inhibition (QS) was determined by subtracting bacterial growth inhibition radius (r_1) from the total radius (r_2) thus QS inhibition = (r_2-r_1) in mm (Zahin et al. 2010).

4.2.8. Outer Membrane Proteins

4.2.8.1. Extraction

The Outer Membrane Protein extraction was performed for *E. coli*. The bacterial culture was grown overnight at 30 ºC in an orbital shaker (120 rpm). The inoculum was centrifuged at 3777 g for 15 minutes and washed with twice NaCl (8.5 g/L). After that, the OD was measured and adjusted to 0.4 ± 0.02 (λ = 620 nm). The cell suspension was incubated for 1h with the different phytochemicals at MIC.

After that, the cells suspension were centrifuged and the pellet was ressuspended in 25 mM Tris and 1 mM $MgCl_2$ (Merck) buffer (pH 7.4), twice. Behind this process, each sample was sonicated for 5 times (20 seconds) (Vibracell, 60 W) on ice to promote cell lysis. After sonication the solution was centrifuged (7000 g, 10 min, 4ºC) (Beckman Avanti J25 centrifuge) in order to remove non-lysed cells. The supernatant was collect and 1 mL of Sarcoisine (Sigma) solution was added to obtain a final concentration of 2% (w/v), in order to solubilize the inner membrane proteins. The samples were left on ice for 20 minutes. After incubation time, 25 mL of Tris-HCl 25 mM and 1mM $MgCl_2$ buffer. The solution was centrifuged (13000 g, 1 h, 4 ºC) (Avantis J-25) two times, to recover the pellet containing the OMP. The pellet was ressupended in 300 µL of 25 mM Tris-HCl buffer (pH 7.4) and stored at -20 ºC until required.

4.2.8.2. OMP Analysis

The protein content of OMP samples was determined using Bicinchoninic Acid Protein Assay Kit (BCA) (BCA-PIERCE Cat. No. 23225) with BSA as standard. This procedure was applied in order to insert in the gel cassettes the same OMP concentration for the several samples (3 µg protein in each well).

The OMP samples obtained were subjected to SDS-PAGE, as reported by Laemmli (1970) with 12% (w/v) acrylamide (Bio-rad). Electrophoresis was

performed at a constant current of 10 mA. After electrophoresis, the gel was stained with Coomassie blue (Bio-rad) for protein profile detection.

4.2.9. Antibiotic-Phytochemical Dual Combinations Assay – Efflux Pumps Inhibition

To study the antimicrobial effects of phytochemicals conjugated with antibiotics, they were inserted in MH agar medium (at MIC). The phytochemical was inserted after sterilize and cooling the medium, to avoid the deterioration. Colonies of bacteria were picked from overnight PCA cultures (log phase cultures) in solid medium (Merck, Portugal). The suspension of bacteria was prepared with 0.9% NaCl, which was adjusted to match to 0.5 McFarland turbidity standards. The suspension was spread with a sterile cotton swap into Petri dish (90 mm of diameter) containing 20 ml of Mueller-Hinton Agar. Sterile filter paper discs (with 6 mm in diameter), impregnated with 15 µL of antibiotics, were placed on the agar plate seeded with the respective bacteria. Discs of ciprofloxacin, erythromycin and tetracycline were used as positive controls and discs impregnated with DMSO were used as negative controls. The concentration of antibiotics used was according to Clinical and Laboratory Standards Institute (2005): Performance Standards for Antimicrobial Susceptibility Testing (Fifteen Informational Supplement): ciprofloxacin – 5 µg/disc; erythromycin – 15 µg/disc; and tetracycline – 30 µg/disc. The plates were incubated at 30 °C for 24 hours. After incubation, zones of growth inhibition were measured. All tests were performed in triplicate and the antibacterial activity was expressed as the mean of inhibition diameters (mm).

4.2.9.1. Classification of dual combinations

The effect of dual combinations of antibiotics and phytochemicals can be classified according Saavedra et al. (2010):

- Antagonism (-) – [inhibition halo – (antibiotic inhibition halo + phytochemical inhibition halo)/2] < 0
- Indifference (+) – 0 ≤ [inhibition halo – (antibiotic inhibition halo + phytochemical inhibition halo)/2] < antibiotic inhibition halo or phytochemical inhibition halo

- Additive (++) – antibiotic inhibition halo < [inhibition halo – (antibiotic inhibition halo + phytochemical inhibition halo)/2] < 2x antibiotic inhibition halo or phytochemical inhibition halo
- Synergy (+++) – inhibition halo > 3x antibiotic inhibition halo or phytochemical inhibition halo.

For this classification the highest inhibition halos caused by the antibiotic or phytochemical were used.

In the case of no effect of phytochemicals, the classification of the dual combination is different (Abreu et al., 2013b):

- Additive (++) – 4 mm ≤ inhibition zone diameter combination – inhibition zone diameter most active agent) < 6 mm
- Synergistic (+++) – inhibition zone dizmeter combination – inhibition zone diameter most active agent ≥ 6 mm.

4.2.10. Statistical analysis

The data was analyzed using One-Way Anova and the statistical program SPSS 21.0. The results were presented as the means ± SEM (standard error of the mean). Significance level for the separation was set at $p < 0.05$ and the calculations were based on confidence level equal or higher than 95%.

4.3 Results and Discussion

4.3.1. Surface characterization of cells in presence of phytochemicals

The surface charge of the cells was measured as its zeta potencial, calculated from the mobility of cells in the presence of an electrical field under well-known conditions (Ferreira et al., 2010). It is possible to observe the variations of zeta potencial with several phytochemicals in Table 4.

Table 4. Zeta potential (mV) results of suspensions of *E. coli* and *S. aureus* in contact with phytochemicals at the MIC.

	Zeta Potencial (mV)	
	E. coli	*S. aureus*
Control	-13.0 ± 1.4	-29.8 ± 1.3
7-HC	-13.5 ± 1.8	-26.0 ± 6.2
I3C	-21.0 ± 5.7	-27.3 ± 6.3
SA	-1.8 ± 0.3	1.8 ± 0.5
SP	-12.7 ± 1.6	-19.4 ± 2.2

Both bacterial strains present a negative surface charge. Most of the microorganisms are negatively charged due to the presence of anionic groups, as carboxyl and phosphate, in the membranes (Ferreira et al., 2010).

E. coli presents a less negative surface charge. The constitutive molecules of the membrane are responsible for the surface properties and their thermodynamic and bacterial adhesion. A thin layer of peptidoglican composes the Gram-negative bacteria and proteins, lipopolysaccharides, constitute their external surface. The charge of the bacteria is negative due to carboxyl and phosphoryl groups. So, it is highly charged and interactive with cations. However, the Gram-positive bacteria are composed by a thick layer of peptidoglican, which is covered for neutral and acid polyssacharides, proteins and teichoic acids (Araújo et al., 2010). The magnitude of negative charge on the cell wall varies from strain to strain (Dickson and Koohmaraie, 1989).

Comparing the values of the control with those caused by the phytochemicals exposure, it is possible to see that 7-HC and SP have no significant effect in the charge of *E. coli* ($p>0.05$). In contrast, I3C and SA seem to change the surface charge ($p<0.05$). The contact of I3C with *E. coli* surface makes it more negative, which means that negative charges are transferred for the surface when in contact with this

phytochemicals. In the case of SA, positive charges are transferred for the membrane surface, because *E. coli* surface is less negative. The positive charge of the phytochemical may explain this change in the charge.

The charge of *S. aureus* surface was changed when in contact with SA and SP ($p<0.05$). The first one is able to make the membrane surface positive, which means that positive charges are moved to the surface. The positive charge of the phytochemical may explain the change in the charge, as referred above. The exposure to SP changes the surface charge of cells to less negative values.

The hydrophobicity has been characterized as one important aspect in bacterial adhesion (Chaves, 2004). Hydrophobicity can be studied through the surface tension by measurement of contact angles. The contact angles were measured using the sessile drop method. van Oss and Giese (1995) developed a methodology to calculate the hidrophobicity of matherial surfaces through the surface tension parameters. Table 5 shows the variations in the hydrophobicity parameter according the different phytochemicals tested at the MIC.

Table 5. Hydrophobicity (ΔG^{TOT}), and apolar (γ^{LW}) and polar (γ^{AB}) components of the surface tension of untreated and treated cells. The means ± SDs are illustrated.

Bacteria	Phytochemical	Surface Tension Parameters (mJ/m^2)				Hydrophobicity (mJ/m^2)
		γ^{LW}	γ^{AB}	γ^{+}	γ^{-}	ΔG^{TOT}
E. coli	Control	33.6±5.0	22.4±5.4	2.6±0.5	52.0±4.8	28.9±7.1
	7-HC	30.7±4.8	20.8±4.7	1.5±0.3	44.9±7.1	21.0±5.1
	I3C	20.2±4.8	37.1±7.3	7.8±1.9	55.8±6.7	20.6±3.8
	SA	31.0±5.2	2.07±6.0	1.5±0.3	59.7±11.7	37.6±18.8
	SP	21.0±1.8	40.1±3.1	7.8±1.3	52.1±2.8	19.7±3.3
S. aureus	Control	35.4±5.4	19.7±4.6	2.0±0.4	53.5±3.8	30.2±3.2
	7-HC	36.2±3.4	21.1±3.8	2.7±0.3	47.8±4.2	22.4±4.8
	I3C	34.5±4.2	20.4±4.8	2.2±0.5	55.4±5.1	32.2±7.3
	SA	37.4±3.0	15.2±3.5	1.5±0.3	44.8±7.7	22.6±5.6
	SP	36.1±4.4	18.3±4.4	2.1±0.5	54.4±1.5	30.4±2.9

The hydrophilic and hydrophobic character of surfaces can be determined by the surface tension components:

- γ^{LW} represents the apolar interactions that are established, meaning the higher it is the value, more apolar is the surface.
- γ^{AB} measures the polar interactions and can be attractive or repulsive.

- γ^- is the donor of electrons.
- γ^+ is the electrons receptor.

The hydrophobicity can also be calculated through the energy of hydrophobic attraction ($\Delta \mathbf{G^{TOT}}$). If $\Delta \mathbf{G^{TOT}}<0$ the interaction between the surface molecules is atractive, means that molecules have less affinity for water than among themselves, and it is considered hydrophobic. If $\Delta \mathbf{G^{TOT}} >0$ the surface is considered hydropilic, and the interaction between the surface molecules is repulsive. So, the more negative the value of $\Delta \mathbf{G^{TOT}}$, more hydrophobic is the surface; and the more positive the value of $\Delta \mathbf{G^{TOT}}$, more hydrophilic is the surface (Araújo et al., 2010; Chaves, 2004).

Regarding the several parameters obtained, the apolar interactions (γ^{LW}) are influenced when *E. coli* is treated with I3C and SP ($p<0.05$), making the molecules of surface less apolar. The same phytochemicals are also able to change the molecules surface by making them more hydrophilic (γ^{AB}) ($p<0.05$). *E. coli* is mainly electron donor. However, this ability to give electrons increases with the treatment with SA.

In the case of *S. aureus*, the treatment with the several phytochemicals did not permit variations on the polarity of surface molecules ($p>0.05$). Except in the case of SA, the value of γ^{AB} was lower than the observed in the control, which means that the surface of molecules was less hydrophilic. Regarding their capacity of giving electron, 7-HC and SA varied significatively ($p<0.05$). These phytochemicals decrease the capacity of supply electrons.

Finally, comparing the values obtained for *E. coli* and *S. aureus* of $\Delta \mathbf{G^{TOT}}$ it is concluded that they have hydrophilic character ($\Delta \mathbf{G^{TOT}} > 0$ mJ/m^2). The interaction of bacteria in the surfaces is thermodynamically not favourable. The application of SA and SP influences their hydrophilic character, in the case of *E. coli* ($p<0.05$). SA promotes the increase and SP promotes the decrease of hydrophilic properties. For *S. aureus*, 7-HC and SA decrease their hydrophilic character ($p<0.05$). These phytochemicals significantly interact with bacterial surface constituints, modifying their physico-chemical properties.

4.3.2. Analysis of *E. coli* and *S. aureus* free energy of adhesion

The bacterial adhesion is a complex process that can be influenced by several factors: physico-chemical characteristics of bacteria (hydrophobicity and surface charge), material surface properties and environmental factors (temperature, pH,

exposure time, concentration of bacteria, chemical treatment or the presence of antimicrobials and fluid flow conditions) (Simões et al., 2010). Behind the physic conditions, biological properties of bacteria also influence the attachment to surface, such as: presence of fimbriae and flagella, and the production of EPS (Simões et al., 2010).

The PS microtiter plates are usually used as the standard bioreactor system for adhesion and biofilm formation bacteria. According to the PS surface properties, this matherial was characterized for being hydrophobic ($\Delta \mathbf{G^{TOT}}$ = -44 mJ/m^2) (Simões et al., 2010) (Appendix - A). So, in order to predict the ability of microorganisms to adhere to PS surfaces, the free energy of interaction between the bacterial surface and the PS surface was calculated according to the thermodynamic approach. Table 6 shows the thermodynamical ability of bacteria to adhere to PS.

Table 6. Free energy of adhesion ($\Delta \mathbf{G^{TOT}_{bws}}$) of bacterial cells to polystyrene, treated and untreated with phytochemicals.

Strain	Phytochemical	Free Energy of Adhesion (mJ/m^2)
E. coli	Control	4.4 ± 1.2
	7-HC	3.5 ± 0.5
	I3C	15.3 ± 3.3
	SA	10.3 ± 2.1
	SP	13.5 ± 2.1
S. aureus	Control	5.7 ± 1.2
	7-HC	1.4 ± 0.2
	I3C	6.4 ± 1.2
	SA	-3.2 ± 0.4
	SP	5.4 ± 0.5

According the values of free energy, it is possible to conclude that only *S. aureus* treated with SA have theoretical thermodynamic ability to adhere to PS ($\Delta \mathbf{G^{TOT}}$< 0 mJ/m^2).

Comparing the free energy of adhesion for both bacteria, it is possible to conclude that *S. aureus* and *E. coli* are not significantly different in terms of capacity to adhere to PS ($p>0.05$).

The addition of I3C influences adhesion of *E. coli* to PS ($p<0.05$), the value of free energy increases, so the adhesion is not expected to be favorable. In contrast, 7-HC decreases the value of free energy, meaning that the theoretical adhesion to PS is

more favorable. In the case of *S. aureus* with 7-HC and SA, the bacteria have more ability to adhere to PS ($\Delta \mathbf{G^{TOT}}$ is inferior than control) ($p<0.05$).

It is important to refer that microorganisms have different mechanisms of adhesion and retention, influenced by the substrata, nutrients, ionic strength, pH values and temperatures, and also their phenotype and genotype (Simões et al., 2010).

The ability of microorganisms to attach to the surfaces is crucial for the beginning of bacterial surface colonization. Adhesion is mediated by two mechanisms. Specific bacterial colonization factors, or adhesins, can act as ligands to mediate attachment of the organisms to specific receptors. Bacteria can also bind by a less specific mechanism mediated by hydrophobic domains (Drumm et al., 1989)

The thermodynamic theory of adhesion permits the quantification of the free energy of adhesion and predicts the possibility of establishment of an interface between the surface and microorganism. However, this theory does not quantify the electrostatic interactions and microbiologic aspects of adhesion (Chaves, 2004).

The hydrophobicity is a surface property that plays an important role in the adesion phenomena. Several authors explained that increasing the hydrophobicity produces an increasing extension of adhesion (Ghannoum, 1992; Masuoka and Hazen, 1997; Sinde et al., 2000).

4.3.3. Motility assays

Motility is one of the most important features in microbial physiology. The bacteria show different ways of motility. In the case of swimming and swarming, motility is dependent on flagella. These types of motility contribute to the virulence of pathogens through adhesion and biofilm formation on biotic and abiotic surfaces (Borges et al., 2012). Swarming motility is also important for the early stages of biofilm formation. Sliding motility is produced by the expansive forces of a growing colony in combination with reduced surface tension. This type of motility is important in bacterial surface colonization (Harshey, 2003). The phytochemicals at the MIC were tested for their ability to act in motility inhibition. The results obtained are presented in Table 7.

Table 7. Motility results for bacteria with and with phytochemicals. The drop baseline was 6mm which was subtracted from the results presented.

Time/ Phytochemical	*E. coli* Swimming (mm)	*E. coli* Swarming (mm)	*S. aureus* Sliding (mm)
24h			
Control	79.0 ± 1.2	8.7 ± 0.6	7.0 ± 0.0
7-HC	7.0 ± 1.0	7.7 ± 1.5	5.0 ± 0.0
I3C	4.7 ± 0.6	7.7 ± 2.9	0.0 ± 0.0
SA	3.3 ± 0.9	2.0 ± 0.9	7.7 ± 0.6
SP	80.0 ± 0.0	56.0 ± 2.0	84.0 ± 0.0
48h			
Control	84.7 ± 0.6	13.7 ± 3.8	8.0 ± 1.0
7-HC	43.3 ± 2.9	8.7 ± 1.2	8.3 ± 0.6
I3C	0.0 ± 0.0	10.0 ± 7.8	0.0 ± 0.0
SA	0.0 ± 0.0	55.0 ± 8.7	0.0 ± 0.0
SP	84.0 ± 0.0	61.7 ± 9.1	56.7 ± 5.8
72h			
Control	84.0 ± 0.0	64.3 ± 7.6	7.7 ± 0.6
7-HC	51.3 ± 2.3	8.3 ± 0.6	8.3 ± 0.6
I3C	0.0 ± 0.0	8.7 ± 5.5	0.0 ± 0.0
SA	0.0 ± 0.0	54.3 ± 6.4	2.0 ± 0.6
SP	84.0 ± 0.0	13.3 ± 3.2	55.0 ± 8.7

Swimming and swarming motilities have been documented as two forms of surface motility for *E. coli*, and sliding for *S. aureus* (Borges et al., 2012; Harshey, 2003; Pratt and Kolter, 1998). *E. coli* exhibits flagella; in contrast, *S. aureus* is a non-flagelated bacterium with a motility phenomenum defined as colony spreading (Borges et al., 2012).

The Gram-negative bacteria increased their growth over the time and the Gram-positive bacteria maintained its growth, without the addiction of phytochemicals. In this study, it was verified that *E. coli* presented the highest motility and also showed an increasing in swimming and swarming motility, without the addiction of phytochemicals. However, *S. aureus* had more capacity to adhere to PS, concluding that motility does not regulate adhesion.

Regarding the several phytochemicals performed, motility was mostly affected when I3C is added. In the case of swimming and sliding, the motility was completely inhibited with this phytochemical ($p<0.05$). However, I3C did not influence swarming motility. SA was also able to stop swimming and sliding motilities. Swarming motility was very low in the first 24h, and in the last 48h, increased. Probably, *E. coli* is able to adapt to SA after a long period of exposure. Swimming motilily increased with the addiction of SP after 72h of exposure ($p<0.05$); however, with swarming and

sliding motility, the bacteria showed an increase in the first hours, but after a long period of exposure, the motility decreased. Finally, 7-HC influenced swarming and swimming motility and it was not able to change sliding motility. In the case of swimming motility, the value was very low in the first 24h, after that, it started to increase.

Borges et al. (2012) also studied *E. coli* and *S. aureus* motilities in contact with ferrulic and gallic acids. They concluded that both compounds show potential to inhibit cell motility. Swarming was completely inhibited in *E. coli,* after 24h; Swimming motility of *E. coli* was reduced by the addition of both compounds. *S. aureus* motility was also inhibited by ferrulic acid. Although the compounds tested are not the same, it is possible to conclude that the motility can be changed.

The motility is apparently related with the cell state of the bacteria. The changes in the motility are explained by cell stimuli to alter the funtions of its motility machinery to improve or decrease its chances of migrating to a better location (Jarrell and McBride, 2008). Morphological differentiation in bacteria, which distinguishes them from their planktonic state, occurs as a response of motility (Julkowska et al., 2004).

A relationship between cells surface motility and biofilm formation has been reported, especially in the case of swarming motility. Both processed, biofilm formation and swarming, require production of flagella and surface polyssacharides (Borges et al., 2012). Several authors have been reported mutants with altered swarming motility that present difficulties in biofilm formation, concluding that they can play a role in biofilm development (Pratt and Kolter 1998; Shrout et al., 2006).

4.3.4. Quorum-sensing assays

QS is a mechanism by which a bacterial population senses its cell density (Khan et al., 2009). This mechanism influences bacterial biofilm growth and development and it is related to cell-cell interactions (Simões et al., 2009). This cell-cell communication is dependent of several factors: synthesis, exchange and perception of small signal molecules between bacteria (Khan et al., 2009). The 4 phytochemicals was tested as QS inhibitors, at several concentrations. The Table 8 shows the results obtained.

The MIC of the phytochemicals tested against *C. violaceum* CV12472 ranged from 25 to 3200 μg/mL. This means that phytochemicals are able to inhibit the bacteria growth. QS results show the effect of the phytochemicals in the bacteria growth (inhibition halo) and also the effect of phytochemical in quorum-sensing, through the detection of pigment inhibition (QS halo). Inhibition of pigment production was detected with some phytochemicals at different concentrations. Figure 4 shows several agar plates with different results.

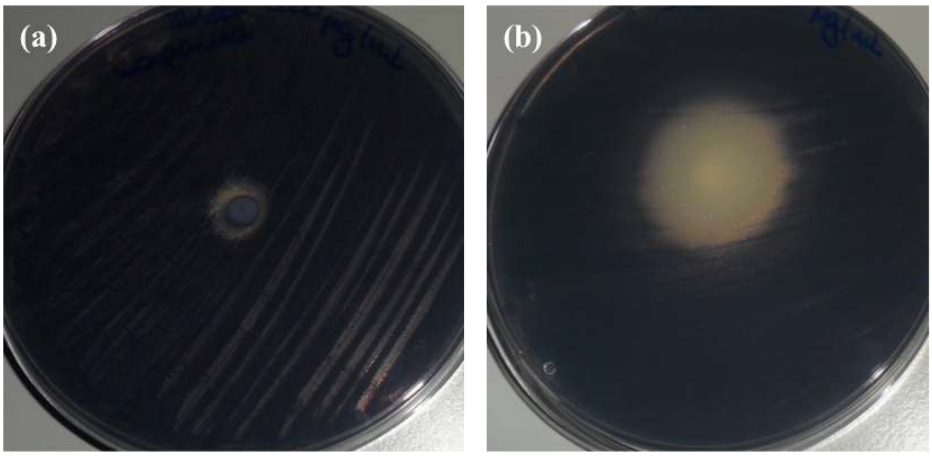

Figure 4. Examples of results obtained with quorum-sensing assay: (a) no bioactivity (SA at 5000 μg/mL); (b) antibacterial and QSI halos are observed (I3C at 5000 μg/mL) with addition of phytochemicals.

Of the 4 phytochemicals tested, inhibition of pigment production was detected with 7-HC, I3C and SA with zones of pigment inhibition ranging 5 to 19 mm. No effect on the pigment was observed with SA at the concentrations tested.

Table 8. Quorum sensing results for several phytochemicals at different concentrations.

Phytochemical	7-HC			I3C			SA			SP		
Concentration (µg/mL)	QSI pigment	Inhibition halo (mm)	QS halo (mm)	QSI pigment	Inhibition halo (mm)	QS halo (mm)	QSI pigment	Inhibition halo (mm)	QS halo (mm)	QSI pigment	Inhibition halo (mm)	QS halo (mm)
Control	+	5	n.d.									
DMSO	+	15	n.d.									
250	+/-	12	n.d.	+	11	n.d.	+	14	n.d.	+	10	n.d.
500	+/-	11	n.d.	+/-	11	5	+	14	n.d.	+	11	n.d.
750	+/-	10	n.d.	+/-	11	6	+	19	n.d.	+	12	n.d.
1000	+/-	11	7	+/-	11	14	+	16	n.d.	+	14	n.d.
1500	+/-	11	7	+/-	12	15	+/-	14	6	+	9	n.d.
2000	+/-	10	5	-	16	9	-	16	8	+	10	n.d.
3000	+/-	10	8	-	20	5	-	16	8	+	10	n.d.
4000	+/-	12	10	-	20	11	-	15	12	+	10	n.d.
5000	+/-	11	5	-	25	9	-	18	9	+	11	n.d.

(+) - There was formation of purple pigment in the plate;(-) - Purple pigment was not formed in the plate; (+/-) - The pigment formed was clearer.
n.d. – not detected.

The I3C is the most effective to inhibit production of pigment. At 500 µg/mL, the inhibition is low, but by increasing the concentration the zone of pigment inhibition is also increasing. Regarding 7-HC and SA, at low concentrations there is no inhibition of pigment production; but from 1000 and 1500 µg/mL of 7-HC and SA, respectively, the QSI halo is detectable. Although, SA shows antimicrobial activity, it was not possible to observe an effect on pigment inhibition at the concentrations tested. Regarding QS halos, the concentration of I3C, 7-HC and SA added influences significantly the quantity of pigment production. So, QS activity of phytochemicals is concentration dependent, as reported by other authors (Khan et al., 2009; Zahin et al., 2010). The same authors identified one compound (clove oil) able to inhibit pigment production with 19 mm of pigment inhibition zone against C. violaceum (CV12472). Also cinnamon, peppermint and lavender present zones of pigment inhibition against the same bacteria (Khan et al., 2009). Al-Hussaini and Mahasneh (2009) reported *T. capensis, Sonchus oleraceus, Pityriasis alba, Pinus nigra, Jasminum sambac, Rosmarinus officinalis, Lavandula angustifolia* and *Laurus nobilis* as great sources of microbial growth and QS inhibitors.

The phytochemicals and other compounds that affect QS can interfere at different levels: inhibition of signal biosynthesis or inhibition of activity of AHL-producing enzymes, enzymatic signal degradation and inhibition of reception signal molecules (Khan et al., 2009).

4.3.5. Characterization of cell membranes

In order to characterize both bacterial cell membranes, two different techniques were performed: outer membrane protein extraction and analysis for *E. coli*, and study of efflux pumps for *S. aureus*.

4.3.5.1. Outer Membrane Proteins

The OMPs are key molecules that are the interface between the cell and the environment. *E. coli* comprises three different layers, as explained before. The external layer consists of lipids, polysaccharides and proteins. Some bacterial proteins are expressed in high copy number, which aid their detection and characterization. These include porins (OmpC and OmpF). Porins are a protein family of OMPs that

form a hydrophilic channel, permiting nonspecific diffusion of small molecules across the outer membrane (Molloy et al., 2000). Due to their location, OMPs are important candidate antigens for the development of strategies to protect against bacterial patogens (Kawahara et al., 1994a; Kawahara et al., 1994b; Negm and Pistole, 1998; Pages et al., 1987). The dominant OMPs of *E. coli* are OmpA, OmpX, OmpF and OmpC (Molloy et al., 2000).

The planktonic cells of *E. coli* were characterized phenotypically in terms of OMPs. The OMPs of *E. coli* strains as planktonic cells were isolated and analysed by SDS-PAGE. The OMP profiles obtained with different phytochemicals (at the MIC) are presented in Figure 5.

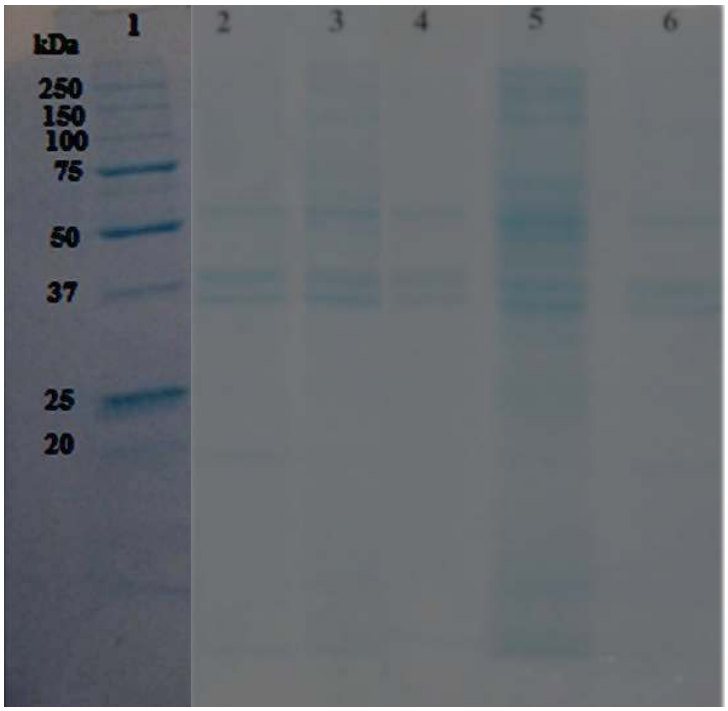

Figure 5. OMPs profiles of *E. coli*. The profile of molecular weight standards (1), control (2), 7-HC (3), I3C (4), SA (5), SP (6) are presented.

According to Figure 3, the OMP profiles obtained with cells and phytochemicals do not differ considerably from the ones obtained with *E. coli* without phytochemicals (well 2). The application of several phytochemicals does not seem to affect OMP expression since, for all lanes, the protein expression is similar. Nevertheless, the outer membrane of cells with SA exhibits a higher intensity than the other OMP. The profile of OMPs shows different proteins. According to the results the phytochemicals applied do not act on membrane proteins.

Regarding the OMPs analysed, there are three proteins that were isolated with all the phytochemicals and also at the control. The molecular weights of them are 55, 38 and 35 kDa. The 35 kDa OMP it was identifiifed as OmpA (Hellman et al., 2000; Molloy et al., 2000), one of the most important OMPs, as referred before. Other studies have been done to study the OMPs of *E. coli.* Proteins with 38 and 55 kDa were identified as being OmpC and ATP synthase alfa subunit (Molloy et al., 2000; Xu et al., 2006)

OMPs have a significant role in the context of biofilm eradication. The bacterial proteins form an adaptative barrier to the external environment, protecting cells from damaging substances, such as biocides and antimicrobial agents, when procedures of desinfections are applied. This permits the selective uptake of nutrients (Simões, 2005). Due to their location, OMPs are important candidates antigens for the development of strategies to protect bacterial cells against pathogens (Molloy et al., 2000).

4.3.5.1. Efflux pumps

Efflux mechanisms are implicated in antimicrobial. Efflux pumps contribute to the resistance of bacteria by pumping out a wide variety of products: dyes, detergents and antibiotics (Simões, 2005). However, efflux pumps have been studied as membrane components in all cell types, from prokaryotes to eukaryotes (van Bambeke et al., 2007). The role of efflux pumps in bacteria has been related to the elimination of metabolites that are poisonous to the cell and in cell stress responses (Costa et al., 2013). The association of antibiotics with phytochemicals can create a synergistic effect against resistant bacteria, creating new choices for the treatment of infectious diseases.

S. aureus genome reveals high potential multidrug efflux-pump-encoding genes (Huet et al., 2008). Several efflux resistance mechanisms have been described for *S. aureus* such as QacA and NorA, which are multidrug transporters, and the more specific MsrA and TetK transport proteins (Gibbons et al., 2003).

In this study, the antimicrobial activity of several phytochemicals was tested in combination with three antibiotics. For this experiments 4 different strains of *S. aureus* were tested. Table 9 shows the antimicrobial activity of antibiotics against *S. aureus* strains.

According to the Clinical and Laboratory Standards Institute (2005), the organisms are susceptible, intermediate or resistant to the agents. *S. aureus* CECT 976 is considered susceptible to all antibiotics tested. *S. aureus* XU212, *S. aureus* RN4220 and *S. aureus* SA1199B present resistance to tetracycline, erythromycin and ciprofloxacin, respectively.

The negative control performed with DMSO in the preparation of phytochemical solutions presented no effects on bacterial growth.

Table 9. Antimicrobial activity of antibiotics. The means (mm) ± standard deviation for at least three replicates are illustrated.

	Diameter of inhibition zone (mm)			
	S. aureus **CECT 976**	***S. aureus*** **XU212**	***S. aureus*** **RN4220**	***S. aureus*** **SA1199B**
TET	41.5±9.2	16.0±4.2	42.5±0.7	46.5±2.1
ERY	37.5±3.5	24.5±4.9	22.0±4.2	35.5±3.5
CIP	40.5±0.7	26.0±2.8	31.5±2.1	18.0±1.4

The antibiotics (tetracycline, erythromycin and ciprofloxacin) had antimicrobial effect against the bacteria tested. Tetracycline was the most effective against all the bacteria, while erythromycin has the lowest antimicrobial activity ($p<0.05$). Regarding to phytochemicals, I3C and SA showed antimicrobial activity against *S. aureus*, with inhibition halos of 20 and 14 mm, respectively. In contrast, 7-HC and SP do not demonstrate antimicrobial properties against the same strain. The most effective phytochemicals was I3C ($p<0.05$).

Dual combinations of antibiotic-phytochemicals were performed. Table 10 shows that the combined application of tetracycline, erythromycin and ciprofloxacin had both negative and positive antimicrobial activities compared to the single application of phytochemicals and antibiotics. The classification presented in Table 10 was done according Saavedra et al. (2010).

The combination of bioactive compounds is expected to exert a synergistic effect or to reduce possible adverse side effects. The development of active compounds, such as phytochemicals, in conjunction with antibiotics could avoid the emergence of resistant variants that might otherwise arise during treatment (Abreu et al., 2013b).

Table 10. Classification of the effect of dual combinations of phytochemicals and antibiotics.

		7-HC	I3C	SA	SP
	TET	+	+++	+	-
***S. aureus* CECT 976**	ERY	-	+++	+	++
	CIP	+	+++	+	-
***S. aureus* XU212**	TET	++	+++	+++	+++
***S. aureus* RN4220**	ERY	-	+++	+++	+++
***S. aureus* SA1199B**	CIP	+	+++	+++	+++

(-) – Antagonist; (+) – Indifference; (++) – Additive; (+++) - Synergistic

The results present dual combinations able to improve the antimicrobial activity against the resistant strains. The combined application of ERY with 7-HC, TET or CIP with SP against *S. aureus* CECT 976 was antagonist. Combination of SA with all antibiotics tested showed to be indifferent against *S. aureus* CECT 976. Regarding 7-HC, the combination of phytochemicals with TET and CIP is also indifferent in the antimicrobial activity when compared with single antibiotic and phytochemicals activities against *S. aureus* CECT 976. The dual combination of ERY and SP showed an additive effect against the growth of *S. aureus* CECT 976. Regarding the resistant strains of *S. aureus*, combined application of TET and 7-HC against *S. aureus* XU212 had an additive effect. The remaining combinations produced a synergistic effect.

Antibiotic synergism occurs when the effects of combination of antimicrobials is greater than the sum of the effects of individual antimicrobials (Saavedra et al., 2010). An additive effect of phytochemical combined with antibiotic may occur due to a double attack of both agents at different target sites of bacteria (Adwan and Mhanna, 2008).

The identification of effective efflux pumps inhibitors of *S. aureus* could restore the clinical utility of pump substrates. Efflux pumps inhibitors could extend the useful lifetime of antibiotics by improving therapeutic efficacy, suppressing the emergence of resistant variants, reducing the effective dose of antibiotics to reduce the adverse toxic effects (Gibbons et al., 2003; Saavedra et al., 2010). Combination therapy with two or more antimicrobials is used to prevent the emergence of resistant strains, to treat emergency cases and to take advantage of antimicrobial products synergy (Hemaiswarya and Doble, 2009).

Chapter 5

Concluding remarks and perspectives for further research

5.1. Conclusions

To find new antimicrobial agents, plant products have been studied as substituints of antibiotics for which bacteria already acquired resistance. So, in this work, the antimicrobial effect of four phytochemicals - 7-HC, I3C, SA and SP – was evaluated. After the development of the experiments, it was possible to conclude about the antimicrobial activity of the selected phytochemicals and their ability to control biofilms of two important pathogens – *E. coli* and *S. aureus*.

The 7-HC was one of the most effective phytochemicals testedagainst *E. coli* and *S. aureus*. The values of MIC obtained were 800 and 200 μg/mL for *E. coli* and *S. aureus*, respectively. However, MBC was not detected for the concentrations tested. Regarding the biofilm control, the exposure of *S. aureus* biofilms to 7-HC at different concentrations, produced significatively different percentages of inactivation. The same phytochemical had no effect in the surface charge of *E. coli*, meaning that this phytochemical did not act in the surface cell membrane. The same phytochemical

decreased the hydrophilic character of *S. aureus* and increase the ability of *S. aureus* to adhere to PS. Swimming and swarming motilities were affected when in contact with 7-HC. However, sliding motility did not change. 7-HC was also able to inhibit the formation of QS pigment (violacein), with halos of 10 mm.

I3C was also effective against both bacteria. The MIC values were 800 and μg/mL for *E. coli* and *S. aureus*, respectively. Regarding the MBC, I3C was also the most effective phytochemicals, with values of 1600 and 800 μg/mL for *E. coli* and *S. aureus*, respectively. By measuring the zeta potential of bacterial strains in contact with I3C, it was concluded that I3C affected the charge of *E. coli*, making the membrane more negative. The values of free energy of adhesion decreased by exposing *E. coli* to I3C, so the adhesion was expected to be more favourable. This phytochemical affected the motility; swimming and sliding motilities were completely inhibited. QSI halos were observed due to I3C, presenting the highest halos (25 mm). Dual combinations of all the antibiotics and I3C presented a synergistic effect against *S. aureus* resistant strains.

SA and SP were the lesst effective phytochemicals. In the case of SP, the MIC value was not detected for concentrations below to 3200 μg/mL.

SA affected the surface charge of *E. coli* and *S. aureus* making the membrane less negative and positive, respectively. This phytochemical influenced the hydrophilic character of *E. coli* and *S. aureus*, increasing and decreasing the character, respectively. SA was the only phytochemical, that when applied to *S. aureus*, was able to make bacteria to adhere to PS, when assessing the free energy of adhesion. Swimming and sliding motilities were inhibited when exposed to SA. Regarding QS, SA presented inhibition zone of pigment production.

Regarding SP, this phytochemical had no significative effect in *E. coli* charge, but influenced the charge of *S. aureus*, making it less negative. The same phytochemical decreased the hydrophilic character of *E. coli* and increased the value of the free energy of adhesion, meaning that the adhesion of *E. coli* to PS was less favourable.

Multi-target antimicrobial products are more effective against Gram-positive bacteria than Gram-negative bacteria (Aires et al., 2009). So, it is important to find antimicrobial products/phytochemicals with significant activity against the more resistant bacteria, which can be applied as therapeutic agents to control infections

caused by them.

This study suggests that 7-HC and I3C are the most promising phytochemicals against *E. coli* and *S. aureus*. Both phytochemicals affected the motility and QS activity, which means that they can play an important role in biofilm prevention and interference with cell-cell interactions. These phytochemicals presented MIC values lower than 1000 μg/mL. The literature has been reported several natural products and extracts with antimicrobial activity with MIC values below 1000 μg/mL, which has important relevance for clinical applications (Gibbons, 2004). In order to apply these phytochemicals to human use, it is necessary the evaluation of their toxicity against mammalian cells. Also, *in vivo* confirmation of the efficacy of these compounds and their side effects would be necessary for a full evaluation of their practical usefulness in the clinical field.

5.2. Perspectives for further research

During this work, the capacity of the selected phytochemicals to control biofilm formation was studied. However, it would be also interesting to study the influence of the selected phytochemicals in biofilm prevention. The same assays could be done, but instead of adding the phytochemicals to the 24-aged biofilms (for 1h), the biofilms should be formed in the presence of the phytochemicals during 24 h.

It would be interesting to develop an assay to evaluate if the phytochemicals can have any influence on the efflux pumps in Gram-negative resistant bacteria and study the dual combinations as it was performed for S. aureus resistant strains. So, it would be possible to understand if there are any synergystic effect between the selected antibiotics and phytochemicals against *E. coli* strains. To study the effects of the phytochemicals in the membrane proteins of *S. aureus*, it would be important the development of a protocol to study their membrane and the influence of phytochemicals. After the performance of these studies, it would be possible to conclude more precisely about the mechanisms of action of the phytochemicals.

Although 7-HC and I3C seems to be important plant compounds able to inhibit microbial growth, these phytochemicals should be tested clinically to evaluate their efficacy and cytotoxicity *in vitro* and *in vivo*.

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Appendix

A. Experimental data

Table A.1. describes the physico-chemical characteristics of polystyrene in order to predict cells adhesion to this surface (Simões et al., 2010).

Table A.1. Physico-chemical characterization of polystyrene (PS).

	Contact Angle (º)			Surface tension parameters (mJ/m^2)			Hydrophobicity (mJ/m^2)	Zeta potential (mV)
	θ_w	θ_F	θ_B	γ^{LW}	γ^+	γ^-	ΔG_{sws}^{TOT}	
PS	83±3	71±2	28±1	39	0.0	9.9	-44	-32±2