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Antimicrobial activity of plants and combinations of
selected phytochemicals against *Escherichia coli* and
Staphylococcus aureus

Master Dissertation

Author: Josué Carvalho

Supervisor: Manuel Simões

Co – Supervisors: Anabela Borges and Marta Ribeiro

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“A ciência? Ao fim e ao cabo, o que é ela senão uma longa e sistemática curiosidade?”

André Maurois

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Abstract

Nowadays, there is a special concern related to the antimicrobial resistance to antibiotics. Investigating the potential of phytochemicals alone or in combination with antibiotics to control planktonic and sessile bacteria is the newest way against resistant bacteria and probably will decrease costs and improve the infections treatment quality.

This work was divided in three main parts. In the first part, the objective was to find autochthonous plants from Portugal with antimicrobial potential not yet studied. Five plants were selected: *Digitalis purpurea* subsp. *purpurea* (stem bark), *Parietaria judaica* (leaves), *Coleostephus myconis* (leaves), *Calystegia sepium* subsp. *sepium* (leaves) and *Oenanthe crocata* (leaves). Water and methanol were the solvents utilized for solid-liquid extraction. The antimicrobial activity of the obtained crude plant extracts against *Staphylococcus aureus* and *Escherichia coli* was evaluated by the disc diffusion method (DDM) and then the extracts were tested in combination with three antibiotics (streptomycin, ampicillin, and ciprofloxacin). It was verified that when tested alone no plant extracts had antimicrobial activity against both bacteria. In the other hand, all extracts increased the effect of antibiotics in at least three combinations. Overall, of the 60 combinations performed, 9 (15%) potentiated the action of the antibiotic and 12 (20%) had an additive effect. The plant extract with the best results was that obtained from leaves of *P. judaica* followed by the stem bark of *D. purpurea*. Leaves of *C. sepium* were the worst plant material tested. *S. aureus* was more susceptible to the action of antibiotics than *E. coli*. Methanol was better solvent than water in extracting compounds with antimicrobial activity.

In the second part, the objective was to assess the antimicrobial capacity of five selected phytochemicals: caffeic acid (CA), ferulic acid (FA), gallic acid (GA), *p*-coumaric-acid (PCA) and saponin (S). The individual molecules were assessed through DDM at varying concentrations and two-to-two, three-to-three, four-to-four, and five-to-five combinations of these compounds were also tested. CA, FA, GA and PCA were analysed based on the Rule of 5 (RO5) and it was verified that the requisites are complied, so they can be considered “drug-like” molecules. It was concluded that they presented antimicrobial activity and probably a similar mode of action. An eventual exception was GA (hydroxybenzoic acid) because showed higher antimicrobial effect than the other phenolics (hydroxycinnamic acids) against *S. aureus*. It was proved that when we combine few phenolic acids we have approximately the same effect that when we test them individually. Nevertheless, if several molecules are combined, the antimicrobial effect may

decrease. That fact may be the cause for no plant had evidenced antimicrobial activity. S didn't showed antimicrobial activity neither potentiating effect.

In the third part, it was evaluated the ability of CA, FA, GA, PCA, S and their combinations to control biofilms of *S. aureus* and *E. coli*. For this the culturability of biofilm's cells after exposition to the phytochemicals, biofilms' metabolic inactivation and biomass removal were evaluated. Gompertz model was also used to fit metabolism curves. The results found revealed some similarities and differences toward the conclusions obtained for planktonic bacteria. It was verified again that when we combine few phenolic acids we have the same effect that when we test that phenolics individually. *E. coli* biofilms seemed to be more susceptible to the selected phytochemicals than *S. aureus* biofilms. PCA and FA were the phenolic acids with better results instead of GA as it was seen with planktonic bacteria. CA, FA, GA, PCA and their combinations inactivated sessile bacterial cells, but don't promoted biofilm removal. The application of Gompertz model to metabolism data confirmed the sigmoidal appearance of the curves. This phenomenon may be caused by persister cells or bacteriostatic effect.

Summing up, the overall results showed that *D. purpurea*, *P. judaica*, *C. myconis*, *C. sepium* and *O. crocata* can be new sources of antimicrobial compounds and/or antibiotics adjuvants. Moreover, CA, FA, GA, PCA and their combinations showed good antimicrobial capacity against planktonic and sessile bacteria and gave new insights for clinical application of phytochemicals in the treatment of infections diseases. In the future, experimental assays should be done in order to corroborate the founds for these molecules and deepen the knowledge about their mode of action.

Resumo

Atualmente, existe uma grande preocupação com a resistência aos antibióticos. Fitoquímicos aplicados individualmente ou em combinação podem ajudar a combater esse problema e provavelmente permitem fazê-lo a custos mais baixos do que outras alternativas, melhorando ao mesmo tempo a qualidade do tratamento médico.

Este trabalho foi dividido em três partes. Na primeira parte, o objetivo foi encontrar plantas autóctones de Portugal com potencial antimicrobiano ainda não explorado. Cinco plantas foram selecionadas: *Digitalis purpurea* subsp. *purpurea* (casca do caule), *Parietaria judaica* (folhas), *Coleostephus myconis* (folhas), *Calystegia sepium* subsp. *sepium* (folhas) e *Oenanthe crocata* (folhas). Água e metanol foram os solventes utilizados para a extração sólido-líquido. A atividade antimicrobiana dos extratos brutos das plantas relativamente a *Staphylococcus aureus* e *Escherichia coli* foi avaliada pelo método de difusão em disco (MDD) e, de seguida, os extratos foram testados em combinação com três antibióticos (streptomicina, ampicilina e ciprofloxacina). Verificou-se que, quando testado isoladamente, nenhum extrato apresentou atividade antimicrobiana para as duas bactérias. Por outro lado, todos os extratos aumentaram o efeito dos antibióticos em pelo menos três combinações. No total, das 60 combinações realizadas, 9 (15%) potenciaram a ação do antibiótico e 12 (20%) tiveram efeito aditivo. O extrato com os melhores resultados foi aquele obtido a partir de folhas de *P. judaica* seguido do de *D. purpurea*. Folhas de *C. sepium* apresentaram os piores resultados. *S. aureus* foi mais suscetível à ação dos antibióticos que *E. coli*. O metanol foi melhor solvente que a água na extração de compostos com atividade antimicrobiana.

Na segunda parte, procurou-se avaliar a capacidade antimicrobiana de cinco fitoquímicos previamente selecionados: ácido cafeico (AC), ácido ferúlico (AF), ácido gálico (AG), ácido *p*-cumárico (APC) e saponina (S). As moléculas individuais foram avaliadas através do MDD a concentrações variáveis e depois combinadas duas a duas, três a três, quatro a quatro e cinco a cinco. AC, AF, AG e APC foram analisados com base na Regra dos 5 e verificou-se que os requisitos são cumpridos, o que indica que as moléculas têm características que as tornam semelhantes a medicamentos. Concluiu-se que os quatro ácidos fenólicos apresentavam atividade antimicrobiana e provavelmente um modo de ação semelhante. Uma eventual exceção foi o AG (ácido hidroxibenzóico), pois apresentou maior efeito antimicrobiano que os demais fenólicos (ácidos hidroxicinâmicos) nos ensaios com *S. aureus*. Ficou provado que quando combinamos poucos ácidos fenólicos, temos aproximadamente o mesmo efeito que

quando os testamos individualmente. No entanto, se várias moléculas são combinadas, o efeito antimicrobiano pode diminuir. Esse facto pode ser a causa de nenhuma planta ter evidenciado atividade antimicrobiana. S não demonstrou atividade antimicrobiana nem efeito potenciador.

Na terceira parte, avaliou-se a capacidade do AC, AF, AG, APC, S e as suas combinações em controlar biofilmes de *S. aureus* e *E. coli*. Para isso, foi avaliada a culturabilidade das células do biofilme após a exposição aos fitoquímicos, a inativação metabólica dos biofilmes e a remoção de biomassa. O modelo de Gompertz também foi usado para ajustar as curvas do metabolismo. Os resultados encontrados revelaram algumas semelhanças e diferenças em relação às conclusões obtidas para as bactérias no estado planctónico. Verificou-se novamente que, quando combinamos poucos ácidos fenólicos, temos o mesmo efeito que quando testamos os fenólicos individualmente. Os biofilmes de *E. coli* pareceram ser mais suscetíveis aos fitoquímicos selecionados do que os biofilmes de *S. aureus*. O APC e o AF foram os ácidos fenólicos com melhores resultados em vez do AG, como se observou com as bactérias planctónicas. AC, AF, AG, APC e as suas combinações inativaram as células bacterianas sésseis, mas não removeram o biofilme. A aplicação do modelo de Gompertz aos dados do metabolismo confirmou a aparência sigmoideal das curvas. Este fenómeno pode ser causado pelas células persistentes ou pelo efeito bacteriostático.

Em resumo, os resultados mostraram que *D. purpurea*, *P. judaica*, *C. myconis*, *C. sepium* e *O. crocata* podem ser novas fontes de compostos antimicrobianos e/ou adjuvantes de antibióticos. Além disso, AC, AF, AG, APC e as suas combinações mostraram boa capacidade antimicrobiana quando testados com bactérias no estado planctónico e sésil e permitiram inferir sobre novas perspetivas para a aplicação clínica de fitoquímicos no tratamento de doenças infecciosas. No futuro, ensaios experimentais devem ser realizados para corroborar o que se descobriu sobre estas moléculas e aprofundar o conhecimento sobre o seu modo de atuação.

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Chapter 1 - Work Outline

1.1 Background

Nowadays, there is a special concern related to the antimicrobial resistance to conventional antibiotics. Several patients have died due to uncontrolled bacterial infections. Moreover, antibacterial resistance isn't limited to healthcare institutions, and is now threatening communities, as well. When bacteria aggregate and form biofilms, resistance increases abruptly [1, 2]. Recent research indicates that phytochemicals may enhance antibiotic's effect and/or act as antimicrobial agents against pathogenic bacterial strains [3-5].

Over the years, bacteria have developed mechanisms of resistance against antibiotics. Some bacteria have intrinsic features that protect them [6]. Others acquire resistance through essentially two ways: mutations and horizontal gene transfer (HGT). Horizontal gene transfer is the movement of genetic information between bacterial cells and occurs by three well-understood genetic mechanisms [7]: conjugation, transformation, and transduction. This process allows the spread of antibiotic resistance genes among bacteria.

Phytochemicals are chemical compounds produced by plants, usually to help them thrive or thwart competitors, predators, or pathogens. At least ten classes of phytochemicals that play an important role in pharmacological issues can be considered [8, 9]: alkaloids, glycosides, flavonoids, phenolics, saponins, tannins, terpenes, anthraquinones, essential oils and steroids. Each class has its proper chemical features and antimicrobial efficacy. Investigating the potential of phytochemicals alone or in combination with antibiotics to control planktonic and sessile bacteria is the newest way against resistant microorganisms and probably will decrease costs and improve the treatment quality.

1.2 Objectives and thesis organization

This thesis is composed by five chapters.

Chapter 1: The objectives and motivations of the thesis are presented.

Chapter 2: A contextualization of the main subjects is made. Firstly, the problematic of antimicrobial resistance to conventional antibiotics and the challenges associated with biofilms uncontrolled growth are described. Then, the resistance mechanisms associated to both planktonic and sessile bacteria are reviewed. Lastly, the main classes of phytochemicals are individually described approaching several topics such like: physical and chemical properties,

therapeutic potential against infectious bacteria and mechanism of action. Some examples and illustrations are given to explain the main issues.

Chapter 3: This chapter is dedicated to antimicrobial tests against planktonic bacteria. One important objective was to find autochthonous plants from Portugal with antimicrobial potential not yet studied. Five plants were selected: *Digitalis purpurea* subsp. *purpurea*, *Parietaria judaica*, *Coleostephus myconis*, *Calystegia sepium* subsp. *sepium* and *Oenanthe crocata*. Then, the crude extracts of all plants were obtained, the antimicrobial activity of the extracts was evaluated by the disc diffusion method (DDM) and, finally, the extracts were tested in combination with commercial antibiotics belonging to different classes (streptomycin, ampicillin, and ciprofloxacin). Other objective was to assess the antimicrobial capacity of five major natural occurring molecules also present at *D. purpurea* plant. Four phenolic acids were selected: caffeic acid (CA), ferulic acid (FA), gallic acid (GA) and *p*-coumaric-acid (PCA). Saponin (S) was also analysed. DDM was utilized again to test the antimicrobial activity of individual molecules and their combinations. The individual molecules were assessed at varying concentrations: 200, 250, 333, 500 and 1 000 µg/disc. Two-to-two, three-to-three, four-to-four, and five-to-five combinations of the selected compounds were tested in such a way that for all tests 1 000 µg was the total mass of phytochemicals per disc. These five molecules were also analysed in terms of their “drug – likeness” properties considering the Rule of 5 (RO5) requisites.

Chapter 4: In this chapter, it was decided to test again the five molecules of the chapter 3 but this time against 24 h old biofilms of *E. coli* and *S. aureus*. The objective was to verify if these phytochemicals and their combinations were able to control *S. aureus* and *E. coli* biofilms. Thus, experiences were made in order to assess the culturability of biofilm’s cells after exposition to the phytochemicals, the biofilm’s metabolic inactivation and the biomass removal. The culturability of biofilm’s cells was assessed following the traditional method by colony forming units (CFU) counts. Biofilm’s metabolic inactivation and biomass removal were experimental tested using resazurin and crystal violet (CV) staining, respectively. At last, Gompertz model was used to fit metabolism curves.

Chapter 5: The main conclusions are highlighted, and ideas are given for future work.

Chapter 2 – Literature Review

2.1 Introduction

Resistant bacteria, particularly *Staphylococcus* sp., *Enterococcus* sp., *Klebsiella pneumoniae*, and *Pseudomonas* spp. are becoming commonplace in healthcare institutions [10]. Bacterial resistance often results in treatment failure, which can have serious consequences, especially in critically ill patients. The initial use of an antibacterial agent to which the causative pathogen is not susceptible and prolonged therapy with antibiotics, have been associated with increased antimicrobial resistance. Resistant bacteria may also spread and become broader infection-control problems, not only within healthcare institutions, but in communities as well. Clinically important bacteria, such as methicillin resistant *Staphylococcus aureus* (MRSA) and extended-spectrum β -lactamase (ESBL)-producing *Escherichia coli* are increasingly observed in the community [11, 12].

While planktonic bacteria are already resistant to many antimicrobials, in biofilms this resistance can increase several times [5]. Biofilms are aggregates of microorganisms in which cells are frequently embedded in a self-produced matrix of extracellular polymeric substances (EPS) that are adherent to each other and/or a surface [1]. All higher organisms, including humans, are colonized by microorganisms that form biofilms, which can be associated with chronic infections (periodontitis, cystic fibrosis pneumonia) and with the contamination of medical devices such as catheters, heart valves, and prostheses [2, 13]. Furthermore, biofilms are responsible for biofouling and contamination of process water, deterioration of the hygienic quality of drinking water and corrosion [1].

Therefore, pharmaceutical companies have been motivated to develop new antimicrobial drugs in recent years, especially due to the constant emergence of microorganisms resistant to conventional antimicrobials and to biofilms threat. Moreover, with respect to the cost-containment pressures of today's healthcare environment, antibacterial drug resistance places an added burden on healthcare costs although its full economic impact remains to be determined [10]. So, it's an actual problem to find valid alternatives to conventional antibiotics and biocides.

At the same time, 250 to 500 thousand plant species are estimated to exist on the planet, but only 20% have been submitted to pharmaceutical or biological tests [3, 14]. Thus, numerous studies on the pharmacology of plants have been accomplished, since they constitute a potential source to produce new medicines and may enhance the effects of conventional antimicrobials against planktonic and sessile bacteria, which will probably decrease costs and

improve the treatment quality [4]. For example, Kardong *et al.* [15] discovered antimicrobial activity of ethanol and petroleum ether extracts of *Pteridium aquilinum* against *Bacillus subtilis*, *S. aureus*, *Proteus vulgaris* and *E. coli*. In this case, the extracts of the plant were better and/or equally effective against tested bacteria as compared to antibiotic streptomycin except *P. vulgaris*. In other study, Ruberto *et al.* [16] reported the antibacterial effects of essential oils obtained from *Foeniculum vulgare*. Indeed, phytotherapeutic compounds entered the market promising a shorter and cheaper production, since basic requirements to use medicinal plants do not involve strict quality control regarding safety and efficacy compared to the other types of drugs [3].

2.2 Mechanisms of bacterial resistance

Resistance mechanisms allow bacteria to survive in the presence of toxic conditions [17]. Some species of bacteria are innately resistant to antimicrobial agents. Others may become resistant through mutation or by acquiring from other bacteria the genetic information that encodes resistance [10]. The last event, also called horizontal gene transfer (HGT), may occur through transformation (via bacteriophages), conjugation (via plasmids and conjugative transposons), or transduction (via incorporation into the chromosome of chromosomal DNA or plasmids) [7, 10, 18]. Through genetic exchange mechanisms, many bacteria have become resistant to multiple classes of antibacterial agents, and these bacteria with multidrug resistance (defined as resistance to ≥ 3 antibacterial drug classes) have become a cause for serious concern, particularly in hospitals and other healthcare institutions where they tend to occur most commonly [10].

The most common mechanisms of resistance usually involve production of enzymes that inactivate the antibacterial product, target alteration, impermeability and efflux to prevent the antibacterial to reach its intracellular target [19].

Efflux pumps are widely involved in antibiotic resistance. There are 5 major families of efflux pumps, including i) the major facilitator superfamily (MFS), ii) the small multidrug resistance family (SMR), iii) the resistance-nodulation-cell-division family (RND), iv) the ATP-binding cassette family (ABC), and v) the multidrug and toxic compound extrusion family (MATE) [7, 20]. These families differ in terms of structural conformation, energy source, range of substrates that they can extrude and in the type of bacterial microorganisms in which they are distributed. Efflux is most commonly associated with the tetracyclines (e.g. TetA, TetB, TetK pumps) and the fluoroquinolones in both Gram-positive and Gram-negative bacteria [19, 21].

For antibiotics to exert their bacteriostatic or bactericidal actions on bacteria they sometimes need to access intracellular targets. For instance, in Gram-negative bacteria, they need to cross the outer membrane, which constitute a substantial permeability barrier and, thus, a major determinant of antimicrobial resistance in these bacteria [19]. The outer membrane of Gram-negative bacteria acts as a permeable barrier as the narrow porin channels limit the penetration of hydrophobic molecules and the low fluidity of the lipopolysaccharide leaflet slows down the inward diffusion of lipophilic products [22, 23]. Indeed, the outer membrane barrier explains, at least in part, the enhanced resistance of Gram-negative vs Gram-positive bacteria to many antibacterials. The intrinsic resistance of many Gram-negative organisms to macrolides, for example, is probably explained by the limited permeability of this membrane to macrolides [19]. Acquired resistance to β -lactams in several Gram-negative organisms, too, has been attributable to outer membrane changes that correlate with reduced permeability [24]. Still, the outer membrane barrier as a resistance mechanism is only significant in the context of additional resistance mechanisms (i.e. efflux and β -lactamases) that work synergistically with it to promote resistance. Gram-negative bacteria that show a high level of resistance include *Pseudomonas aeruginosa*, *Burkholderia cepacia*, *Proteus spp.*, and *Providencia stuartii* [6]. Mycobacteria and bacterial spores are among the least susceptible cell types, due to the innate presence of a waxy cell envelope and a spore coat, respectively [17].

Physiological adaptation of microorganisms induces the development of intrinsic resistance [17]. Biofilms are the leading example of physiological adaptation and are one of the most important sources of bacterial resistance to antimicrobial products. Biofilms can consist of monocultures, of several diverse species, or of mixed phenotypes of a given species [6]. Bacteria in different parts of a biofilm experience different nutrient environments, and their physiological properties are affected. Within the depths of a biofilm, for example, nutrient limitation is likely to reduce growth rates, which can affect the susceptibility to antimicrobial agents. Thus, the phenotypes of sessile microorganisms within biofilms differ considerably from the planktonic cells found in laboratory cultures. Slow-growing bacteria are particularly insusceptible. Several reasons can account for the reduced sensitivity of bacteria within a biofilm [25]: i) reduced access of an antimicrobial agent to the cells within the biofilm, ii) chemical interaction between the antibiotic and the biofilm itself, iii) modulation of the microenvironment, iv) production of degradative enzymes (and neutralizing chemicals), or v) genetic exchange between cells in a biofilm. Biofilms afford bacteria a 100-to 1000-fold increase in antimicrobials resistance compared to their equivalent populations of free-floating counterparts [17]. Lewis [26] explained yet other hypotheses for the considerable resistance of biofilms related to the

potential of damaged bacterial cells to undergo apoptosis or programmed cell death (PDC). In the case of serious injury by antimicrobial agents, damaged cells will donate their nutrients to their neighbors instead of draining resources from their kin in a futile attempt to repair themselves. Then, the persister cells would grow rapidly in the presence of nutrients released from their lysed community partners and the biofilm would become restored. It appears that biofilm resistance to antimicrobial agents can be largely explained by the increased production of persister cells. It is known that small fractions of persistent bacteria resist to killing when exposed to antimicrobials. These persister cells are not believed to be mutants. Rather it has been hypothesized that they are phenotypic variants and can exist in both planktonic and sessile populations. However, while planktonic persisters are antimicrobial susceptible, biofilm persister cells are protected by the EPS.

2.3 Classes of phytochemicals: Basic aspects and antimicrobial potential

Plants produce many secondary metabolites that protect them against pathogenic microorganisms. These can be found in leaves, roots, stems, flowers, barks, and their derivatives, and are distributed in different phytochemical classes of pharmacological interest. The main active compounds of plants are alkaloids, glycosides, flavonoids, phenolics, saponins, tannins, terpenes, anthraquinones, essential oils and steroids [8, 9]. These metabolites are responsible for the antimicrobial activity of plants.

2.3.1 Alkaloids

They occur in approximately 300 plant families and in any part of the plant [27]. Alkaloids function in the defence of plants against herbivores and pathogens. Amino acids act as precursors for biosynthesis of alkaloids with ornithine and lysine commonly used as starting materials. Despite their structural diversity, alkaloids share many physical and chemical properties. Because they possess a nitrogen atom with an unshared pair of electrons synthesized from amino acid building blocks, alkaloids are basic (hence their name, which literally means alkali-like) [8]. Most alkaloids possess just one nitrogen atom, but some have up to five. This nitrogen may occur in the form of a primary amine (RNH_2), a secondary amine (R_2NH) or a tertiary amine (R_3N). In addition to carbon, hydrogen and nitrogen, most alkaloids contain oxygen [28]. Alkaloids can occur as monomers or they may form dimers (also known as bisalkaloids), trimers or tetramers. Majority of alkaloids exist in solid such as atropine, some as liquids containing carbon, hydrogen, and nitrogen [8, 27]. Most alkaloids are readily soluble in

alcohol and though they are sparingly soluble in water, their salts are usually soluble. The name alkaloid ends with the suffix *-ine*.

Possessing a proton-accepting nitrogen atom and one or more proton-donating amine hydrogen atoms, alkaloids readily form hydrogen bonds with proteins, enzymes, and receptors [29]. This, coupled with the frequent presence of proton-accepting and -donating functional groups such as phenolic hydroxyl and polycyclic moieties, explains the exceptional bioactivity of the alkaloids. Plant alkaloids, including berberine (Figure 1 - left), found in *Berberis* species, and piperine (Figure 1 - right), found in *Piper* species, can interact with the bacterial cytoplasmic membrane, intercalate with DNA, and inhibit efflux pumps in *S. aureus* [17]. Moreover, numerous alkaloids inhibit the formation of (and/or disperse) bacterial biofilms, including imidazoles, isoquinolines, piperidines, pyrrolidines, pyrrole-imidazoles and cinchona alkaloids [27, 30]. In some cases, this inhibition has been attributed to quorum sensing (QS) disruption. Quorum sensing is a process of cell–cell communication that allows bacteria to share information about cell density and adjust gene expression accordingly [31]. Phytochemicals, including some classes of alkaloids, can interfere and break that communication in such a way that biofilms will not develop their intrinsic resistance and the ability to form robust biofilms is compromised, causing cell dispersion [17, 31, 32].

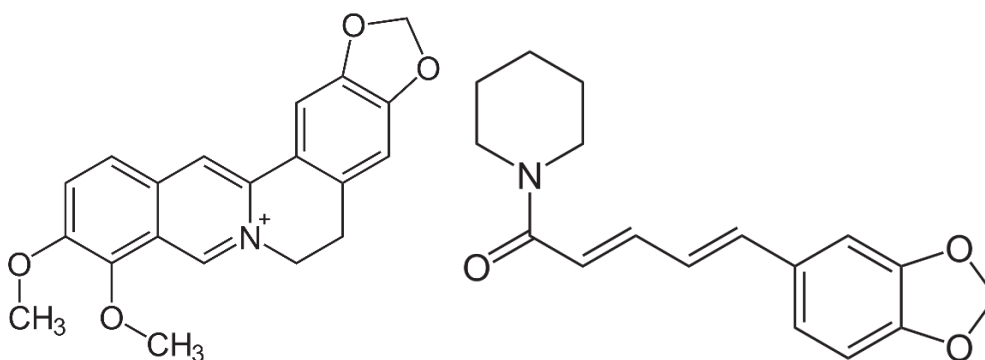


Figure 1: Structure of berberine (left) and piperine (right).

2.3.2 Glycosides

Glycosides in general, are defined as the condensation products of sugars with a host of different varieties of organic hydroxy compounds [8]. They are crystalline carbon, hydrogen and oxygen-containing (some contain nitrogen and sulfur) water-soluble phytoconstituents. Glycosides are comprised of two chemically and functionally independent parts: the aglycone (genin) and the glycone (saccharide) parts [33]. The glycone part is linked to the aglycone portion by a glycosidic bond. Alcohol, glycerol, or phenol represents aglycones. The glycone is most frequently a monosaccharide, the most common being glucose. Glycosides can be readily hydrolyzed into its components by diluted acids or by enzymes, e.g., β -glucosidases. The trivial

name of glycosides usually has a suffix “in” and the names essentially include the source of the glycoside, for instance: strophanthidin from *Strophanthus*, digitoxin (Figure 2) from *Digitalis*, barbaloin from *Aloes*, salicin from *Salix*, cantharidin from *Cantharides*, and prunasin from *Prunus* [8]. However, the systematic names are invariably coined by replacing the “ose” suffix of the parent sugar with “oside”.

The overall antimicrobial effect of glycosides is dependent on their two components: the aglycone portion interferes more with the mode of antibacterial action, while the saccharide part (sugar chain/s) increases water solubility and influences kinetics and dynamics properties [33]. Glycosides can have antibacterial action solely and in combination with other phytochemicals of different classes. For instance, Zearah *et al.* [34] found that the glycosides of the *Citrus laurantiifolia* L. fruits had antimicrobial activity against *Staphylococcus* sp., *Streptococcus* sp., *Pseudomonas* sp. and *E. coli*, what means a broad spectrum antibiotic effect.

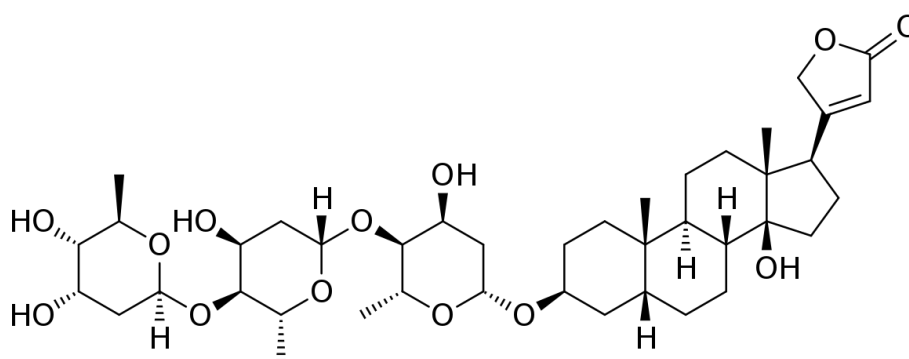


Figure 2: Structure of digitoxin.

2.3.3 Flavonoids

Flavonoids are an important group of polyphenols widely distributed among the plant flora [8]. The basic structural feature of flavonoid compounds is the 2-phenyl-benzo[α]pyrane or flavane nucleus, which consists of two benzene rings (A and B) linked through a heterocyclic pyrane ring (C), as represented in Figure 3 [35]. Quercetin, kaempferol and quercitrin are common flavonoids present in nearly all the plants. Other groups of flavonoids include flavones, dihydroflavons, flavans, flavonols, anthocyanidins, proanthocyanidins, calchones and catechin and leucoanthocyanidins [36].

Flavonoids act as bactericidal and bacteriostatic compounds by damaging cytoplasmic membrane, inhibiting energy metabolism, and inhibiting synthesis of nucleic acids against different microorganisms [17, 37, 38]. The activity of quercetin, for example, has been at least partially attributed to inhibition of DNA gyrase [35]. It has also been proposed that sophoraflavone G and (–)-epigallocatechin gallate inhibit cytoplasmic membrane function, and

that licochalcones A and C inhibit energy metabolism. In addition, flavonoids inhibit quorum-sensing signal receptors, what indicates their capacity in targeting biofilms [39].

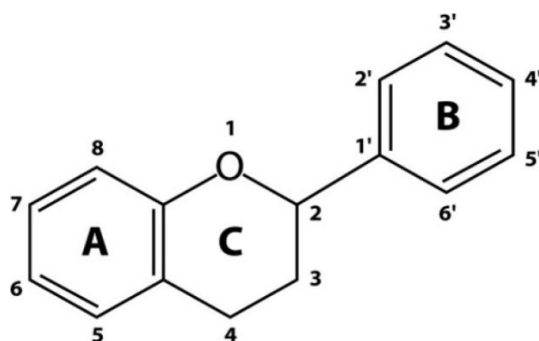


Figure 3: Basic structure of flavonoids [40].

2.3.4 Phenolics

Phenolics, phenols or polyphenolics (or polyphenol extracts) are chemical components that occur ubiquitously as natural colour pigments responsible for the colour of fruits of plants [8]. Phenolics in plants are mostly synthesized from the shikimate pathway from L-phenylalanine or L-tyrosine [41]. They are classified into i) phenolic acids and ii) flavonoid polyphenolics (flavonones, flavones, xanthenes and catechins) and iii) non-flavonoid polyphenolics [8, 9, 42]. Caffeic acid, chlorogenic acid, rutin and naringin are well known phenolic compounds.

Several authors have described the antimicrobial potential of phenolic compounds, mainly phenolic acids, against pathogenic microorganisms like *Salmonella*, *E. coli*, *Lactobacillus* spp., *S. aureus*, *P. aeruginosa* and *Candida albicans* [17, 43-47]. The mechanisms thought to be responsible for phenolic toxicity to microorganisms include enzyme inhibition by the oxidized compounds (possibly through reaction with sulfhydryl groups) and disruption of the lipid-protein interface, altering membrane permeabilization [9, 17, 48]. The site(s) and number of hydroxyl groups on the phenol group are thought to be related to their relative toxicity to microorganisms, with evidence that increased hydroxylation results in increased toxicity. In addition, some authors have found that more highly oxidized phenols are more inhibitory [9, 48].

2.3.5 Saponins

The term saponin is derived from *Saponaria vaccaria* (*Quillaja saponaria*), a plant, which abounds in saponins and it was once used as soap [8, 49]. Therefore, saponins possess “soaplike” behaviour in water, i.e. they produce foam. All saponins contain an aglycone (hydrophobic part), which is linked to one or more sugars or oligosaccharides (hydrophilic part) [50-53]. There are two major groups of saponins: steroid and triterpenoid saponins (Figure 4). Steroidal saponins consist of a steroidal aglycone, a C₂₇ spirostane skeleton, generally comprising of a six-ring

structure [54]. Triterpenoid saponins consist of a triterpenoid aglycone, which consists of a C_{30} skeleton, comprising of a pentacyclic structure.

Saponin's structure is complex and depends on the variation in the aglycone structure and the position and nature of attachment of the glycosides to the molecule [52]. This diversity in structure leads to a diversity of biological activity. Concerning to antimicrobial action, Avato *et al.* [55] studied the antimicrobial activity of saponins from *Medicago* species. They concluded that the sugar moiety is not particularly important for the antimicrobial efficacy and that Gram-positive bacteria (*Bacillus cereus*, *B. subtilis*, *S. aureus* and *Enterococcus faecalis*) are more susceptible to saponins instead of Gram-negative bacteria. The observed antimicrobial properties were related to the content of medicagenic acid and hederagenin [55]. Nevertheless, in most cases, saponins present weak antibacterial action, especially in the presence of dense microbial populations, such as biofilms [50, 56].

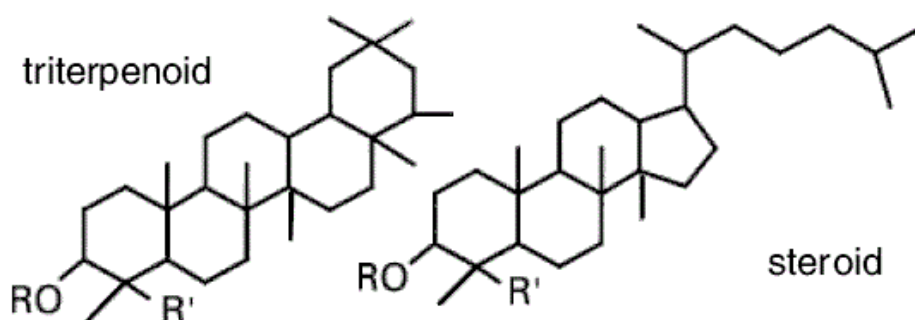


Figure 4: Basic structures of steroid and triterpenoid saponins.

2.3.6 Tannins

Tannins are phenolic compounds that can tan or convert animal skin into leather [8, 57, 58]. They are acidic in reaction and the acidic reaction is attributed to the presence of phenolics or carboxylic group. They form complexes with proteins, carbohydrates, gelatin and alkaloids. Tannins are divided into hydrolysable tannins and nonhydrolyzable/condensed tannins (Figure 5). Hydrolysable tannins, upon hydrolysis, produce gallic acid and ellagic acid and depending on the type of acid produced, the hydrolysable tannins are called gallotannins or egallitannins. Common examples of hydrolysable tannins include theaflavins, daidzein, genistein and glycitein. Condensed tannins include the oligomers and polymers composed of flavan-3-ol nuclei (proanthocyanidins).

Tannins have been reported to be bacteriostatic or bactericidal against *E. coli*, *P. aeruginosa*, *S. aureus* and *Listeria monocytogenes* and their biofilms [59-61]. The different mechanisms proposed to explain tannin antimicrobial activity include inhibition of extracellular

microbial enzymes, deprivation of the substrates required for microbial growth, inhibition of oxidative phosphorylation and iron deprivation [62].

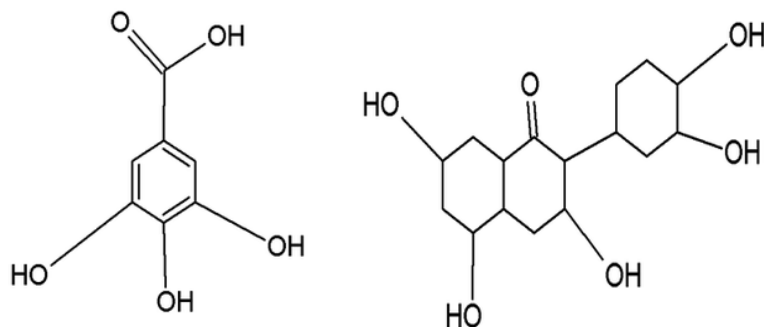


Figure 5: Basic structures of hydrolysable tannins (left) and nonhydrolyzable/condensed tannins (right) [63].

2.3.7 Terpenes

Terpenes are unsaturated hydrocarbons commonly found in essential oils and resins [8]. The generic name “terpene” was originally applied to the hydrocarbons found in turpentine, the suffix “ene” indicating the presence of olefinic bounds [64]. Terpenes are classified based on the number and structural organization of carbons formed by the arrangement of isoprene units, the “building blocks” (Figure 6). So, they keep the general formula $(C_5H_8)_n$ and are classified as mono-, di-, tri- and sesquiterpenes depending on the number of carbon atoms. When the compounds contain additional elements, usually oxygen, they are termed terpenoids [9].

Terpenes and terpenoids are active against bacteria. For example, Trombetta *et al.* [65] reported the antimicrobial efficacy of three monoterpenes [linalyl acetate, (+)menthol, and thymol] against the Gram-positive bacterium *S. aureus* and the Gram-negative bacterium *E. coli*. Gallucci *et al.* [66] also studied the synergistic or antagonistic associations between nine monoterpenes. They concluded that the associations geraniol/menthol against *S. aureus* and *B. cereus* and thymol/menthol against *B. cereus* were synergistic. The mechanism of action of terpenes is speculated to involve membrane disruption by the lipophilic compounds [9, 65].

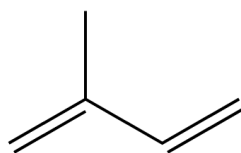


Figure 6: Structure of isoprene, the “building block” of terpenes.

2.3.8 Anthraquinones

Anthraquinones are derivatives of phenolic and glycosidic compounds [8]. They are derived from anthracene (Figure 7) giving variable oxidized derivatives such as anthrones and anthranols [67]. Other derivatives such as chrysophanol, aloe-emodin, rhein, salinos poramide,

luteolin and emodin have in common a double hydroxylation at positions C-1 and C-8. Comini *et al.* [68] studied photosensitizing anthraquinones isolated from *Heterophyllaea pustulata* (Rubiaceae), namely soranjidiol, rubiadin, damnacanthal and 5,5'-bisoranjidiol and discovered antibacterial activity (bacteriostatic/bactericide) on *S. aureus*. The mechanism of action seems to involve an increase in the levels of superoxide anion and/or singlet molecular oxygen.

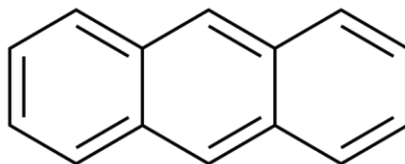


Figure 7: Anthracene structure.

2.3.9 Essential oils

Essential oils (EOs) are complex mixtures of low molecular weight compounds extracted from plants by steam distillation and various solvents [8]. Chemically, a single volatile oil comprises of more than 200 different chemical components, essentially terpenoids and phenylpropanoids. Examples of volatile oils include amygdaline, sinigrin, and eugenol occurring as gein. Various pharmaceutical and biological activities like, antibacterial, antifungal, and antiprotozoal are assigned to them [69-73]. Most of the time the bioactivities of an EO is decided by either one or two of its main components [70, 74]. Primary mode of action of EOs is the membrane destabilization [17, 70, 72]. Essential oils are lipophilic in nature and hence easily permeable through the cell wall and cell membrane. Interactions of EOs and their components with polysaccharides, fatty acids and phospholipids make the bacterial membranes more permeable, so that loss of ions and cellular contents leads to cell death.

2.3.10 Steroids

Plant steroids (or steroid glycosides) also referred to as “cardiac glycosides” are basically steroids with an inherent ability to afford a very specific and powerful action mainly on the cardiac muscle [8]. Diosgenin and cevadine (from *Veratrum veride*) are examples of plant steroids (Figure 8). There are steroids with antimicrobial activity. For instance, Taleb-Contini *et al.* [75] tested five steroids (stigmasterol, β -sitosterol, campesterol, espinasterol and Δ^7 -stigmastenol) isolated from *Chromolaena squalida* and *Chromolaena hirsuta* against 22 strains of microorganisms including bacteria (Gram-positive and Gram-negative). They concluded that steroids showed antimicrobial activity mainly against Gram-positive bacteria. The immense chemical diversity exhibited by steroids arises because of various oxidation states of the carbons of its tetracyclic core and methyl groups, and the structure of the side-chain [76].

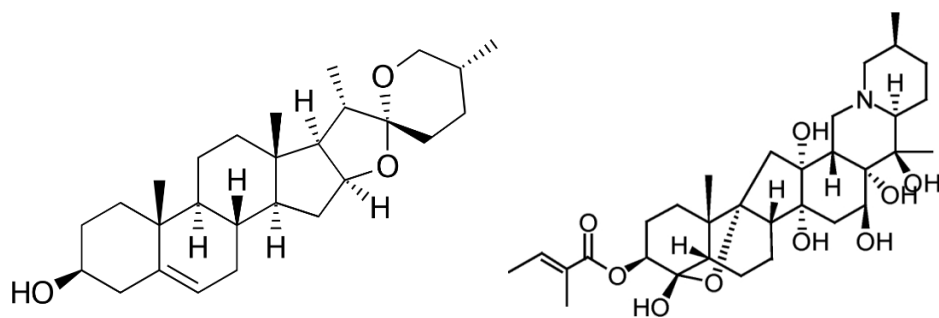


Figure 8: Diosgenin (left) and cevadine (right).

Chapter 3– Antibacterial activity of new plant extracts and phytochemicals' combinations

3.1 Introduction

Bacterial resistance to antibiotics represents a major health problem. Solving this problem and search for new sources of antimicrobial agents is a worldwide challenge and the aim of many research teams in science, academy institutions and pharmaceutical companies. One of the approaches in solving this issue is testing the biologically active compounds of plant origin. Plants produce compounds that are not important for primary metabolism but act as protective agents against adverse abiotic and biotic environmental conditions [3, 4, 77, 78]. These secondary metabolites are biologically active organic compounds that usually have antimicrobial activity. They can be divided in three large molecule families: phenolics, terpenes and alkaloids [8, 9, 78]. The phenolics and polyphenols are one of the largest groups of phytochemicals. Important subclasses in this group of compounds include phenols, phenolic acids, quinones, flavones, flavonoids, flavonols, tannins and coumarins.

While several bioactive compounds have a significant antimicrobial activity, other compounds have been found to be synergistic enhancers of antibiotics, despite they may not have any antimicrobial properties alone [78, 79]. The modes of action underlying the synergistic activity of these antibiotic adjuvants can be diverse. Two important mechanisms include the serial or orthogonal inhibition of vital physiological pathways or the dispersion of a biofilm to planktonic cells, resulting in an increased susceptibility to antibiotics [79]. Also, several antibiotic adjuvants have been evaluated for their action as resistance-modifying agents (RMAs), i.e., compounds that can modify or inhibit the bacterial mechanisms of resistance, so that antibiotics can efficiently counteract the resistant bacteria. Efflux pump inhibitors (EPIs) are important examples of RMAs [80].

Preparation of plant material usually includes the following steps [81]: pre-washing, drying (or freeze drying) and grinding to obtain a homogenous sample and increase the contact of sample surface with the solvent system. Then, extraction plays a significant and crucial role on the final result and outcome. Several extraction techniques are available [82]: solid-liquid extraction, soxhlet, microwave assisted extraction (MAE), supercritical fluid extraction (SFE) and ultrasounds extraction. During extraction, solvents diffuse into the plant material and solubilise compounds with similar polarity. The most often tested extracts are [9, 83]: water extract and

extracts from organic solvents such as methanol, ethanol as well as ethyl acetate, acetone, chloroform, and dichloromethane. At the end of the extraction, solvents can be evaporated, so that a sticky mass is obtained with a concentrated mixture of plant active compounds. Thereafter, the stock solutions are prepared resuspending that sticky mass using a solvent, being one of the most common, dimethyl sulfoxide (DMSO).

Subsequently, exist several methods for *in vitro* evaluating antimicrobial activity. Diffusion and dilution methods are two types of susceptibility tests commonly used [78, 84]. Diffusion method is a qualitative test that allows classify the bacteria as susceptible or resistant to the tested plant extract according to the diameter size of the inhibition zone. In the dilution method, the activity of plant extracts is determined as Minimum Inhibitory Concentration (MIC). MIC is defined as the lowest concentration able to inhibit bacterial growth. In broth-dilution methods, turbidity and redox-indicators are most frequently used for results reading. Turbidity can be estimated visually or spectrophotometrically while change of indicator colour indicate inhibition of bacterial growth.

The edible plants are already widely studied and therefore it was opted to study the antimicrobial potential of invasive and autochthonous plants of Portugal. Autochthonous, indigenous, or native plants are formed or originated in the place where are found. Invasive plants are non-native plants that cause negative environmental and economic impacts. This chapter resumes the experimental tests performed to find new Portuguese plants with antimicrobial potential. Additionally, the effect of combinations of some phytochemicals was assessed and compared with the effect of each compound tested alone against the same pathogenic bacteria.

3.2 Material & Methods

3.2.1 Plants origin and sample collection

Plant research was carried out through the Flora-On database provided by the Portuguese Botany Society [85]. Flora-On presents photographic, geographic, morphological, and ecological information of all species of autochthonous plants in Portugal and is constantly updated.

In Portugal there are approximately 2666 species of native plants. In the research for this project we looked for herbaceous plants. This allowed to refine the research to 1705 species of plants. Then, those that could be collected in Perafita - Matosinhos were selected. Finally, a literature review was done to ensure that the plants to be studied had not been tested for their antimicrobial potential. It was found that some plants were already exploited, for example

Plantago and *Urtica* species [86-89]. In contrast, for the following five native plants, *Digitalis purpurea* subsp. *purpurea*, *Parietaria judaica*, *Coleostephus myconis*, *Calystegia sepium* subsp. *sepium* and *Oenanthe crocata*, the antimicrobial activity was unknown and so these plants were selected to be studied.

3.2.1.1 Preparation of the extracts

First, the plant material was collected: leaves of *Parietaria judaica*, *Coleostephus myconis*, *Calystegia sepium* and *Oenanthe crocata*. In the case of *D. purpurea*, the stem bark was used. The plants were washed, dried at $(47 \pm 3) ^\circ\text{C}$ for 48 hours, and then powdered [15]. For the extraction procedure, the obtained powder was maintained in contact with two solvents, sterile water or methanol (0,0275 g/mL), at room temperature for 2 h. Thereafter, the extraction product was decanted and centrifuged at 3220 G for 10 + 10 minutes, the pellet was removed, and the extracts of sterile water and methanol were stored in the refrigerator until removal of the solvent.

Methanol was evaporated using a rotary evaporator (Büchi B-490) at $40 ^\circ\text{C}$ under reduced pressure during 1 h. The flask with the powder of the methanolic extract was placed in a desiccator with silica to remove the moisture. To ensure drying the product was still passed through a stream of nitrogen.

The aqueous extracts were kept up in the freezer ($-80 ^\circ\text{C}$) and then in the lyophilizer (Labconco) at $-80 ^\circ\text{C}$ and 0.3 mBar for 5 days.

After the evaporation and lyophilization processes, the sticky mass was dissolved in the lowest volume of DMSO (stock solution).

3.2.2 Phytochemicals

It is known that phenolics and polyphenols are one of the largest groups of phytochemicals. An important subclass in this group of compounds include phenolic acids and most of them have well documented antimicrobial activity. Nevertheless, they are usually tested individually and there are relatively few studies upon the effect of their combination. So, four common phenolic acids with described antimicrobial activity and present at *D. purpurea* plant were selected in order to analyse their effect when tested together: caffeic-acid (CA), ferulic-acid (FA), gallic-acid (GA) and *p* – coumaric-acid (PCA). Saponin (S) was also tested individually and combined with the four phenolic acids. Research of phytochemicals was carried out mostly through the Dr Duke's Phytochemical and Ethnobotanical Databases [90]. Further studies were made to corroborate the data obtained from Dr Duke's Databases using other chemoinformatic tools described by Lagunin *et al.* [91].

3.2.2.1 “Drug-likeness” prediction

The drug-likeness properties of the selected phenolic acids were analysed according to the Lipinski's “rule of five” [92], using the Molinspiration calculation software for parameters determination.

3.2.3 Bacterial strains and culture conditions

The bacteria used were obtained from the CECT (Spanish Type Culture Collection): Gram-negative bacterium, *Escherichia coli* (CECT 434), and Gram-positive bacterium, *Staphylococcus aureus* (CECT 976). The bacteria were preserved at - 80 °C in Mueller Hinton Broth (MHB, Oxoid) containing 30% (v/v) glycerol (Panreac, Barcelona, Spain). The bacterial cultures were grown overnight in Mueller-Hinton Agar (MHA, Oxoid) at 37 °C before the experiments. The bacterial suspensions were prepared in sterile saline solution by adjusting the turbidity to match 0.5 McFarland standards (optical density (OD) = 0.132 ± 0.002 at $\lambda_{600 \text{ nm}}$) (VWR V-1200 spectrophotometer).

3.2.4 Antibacterial Activity Assessment

The antibacterial activity assessment was performed using the disc diffusion method according to Saavedra *et al.* [93].

3.2.4.1 Plant extracts

The extracts used were the stock solutions (without concentration standardization).

Sterile filter paper discs (5 mm diameter) were placed on the agar plate seeded with the respective bacteria. A volume of 10 μL of extract, 10 μL of DMSO and 10 μL of ciprofloxacin (5 $\mu\text{g}/\text{disc}$) was added to the blank discs. The plates were incubated at 37 °C for 24 hours. After incubation, the diameter in mm of the inhibitory zones around the disks was recorded. Independent experiments were repeated at least two times. In each time, all tests were performed in duplicate. The antibacterial activity was expressed as the mean of inhibition diameters (mm) produced and the disc diameter was subtracted.

The antibacterial effects of the tested extracts were classified as follows [93]:

Non-effective (-): inhibition halo = 0;

Moderate efficacy (+): $0 < \text{inhibition halo} < \text{antibiotic inhibition halo}$;

Good efficacy (++): $\text{antibiotic inhibition halo} < \text{inhibition halo} < 2 \times \text{antibiotic inhibition halo}$;

Strong efficacy (+++): $\text{inhibition halo} > 2 \times \text{antibiotic inhibition halo}$.

3.2.4.2 Selected phytochemicals

The different phenolic acids (CA, FA, GA and PCA) used in the *in vitro* assays were obtained from Sigma-Aldrich (Portugal) and S was obtained from VWR (Portugal). All the selected phytochemicals were dissolved in DMSO. Sterile filter paper discs (5 mm diameter) were placed on the agar plate seeded with the respective bacteria. Each product was tested individually at a concentration of 200, 250, 333, 500 and 1 000 µg/disc and for that the discs were impregnated with 15 µL of each phytochemical. Afterwards, several combinations were performed.

Two-to-two combinations: CA+FA, CA+GA, CA+PCA, CA+S, FA+GA, FA+PCA, FA+S, GA+PCA, GA+S and PCA+S. Consider, for example, CA+FA. In this case, discs were firstly impregnated with 7.5 µL of CA (500 µg/disc) and secondly with 7.5 µL of FA (500 µg/disc), that correspond to 15 µL of phytochemical solution in the total (1 000 µg/disc). This process was then repeated equally to the other two-to-two combinations.

Three-to-three combinations: CA+FA+GA, CA+FA+PCA, CA+FA+S, CA+GA+PCA, CA+GA+S, CA+PCA+S, FA+GA+PCA, FA+GA+S, FA+PCA+S and GA+PCA+S. Consider, for example, CA+FA+GA. Discs were firstly impregnated with 5 µL of CA (333.3 µg/disc), secondly with 5 µL of FA (333.3 µg/disc) and finally with 5 µL of GA (333.3 µg/disc), that correspond to 15 µL of phytochemical solution in the total (1 000 µg/disc). This process was then repeated equally to the other three-to-three combinations.

Four-to-four combinations: CA+FA+GA+PCA, CA+FA+GA+S, CA+GA+PCA+S and FA+GA+PCA+S. Following the same procedure, discs were impregnated like this: (3.75 µL + 3.75 µL + 3.75 µL + 3.75 µL = 15 µL) corresponding to (250 µg/disc + 250 µg/disc + 250 µg/disc + 250 µg/disc = 1 000 µg/disc).

Five-to-five combination: CA+FA+GA+PCA+S. (3 µL + 3 µL + 3 µL + 3 µL + 3 µL = 15 µL) corresponding to (200 µg/disc + 200 µg/disc + 200 µg/disc + 200 µg/disc + 200 µg/disc = 1 000 µg/disc)

The effect of the phytochemicals combinations was classified as follows (adapted from Abreu *et al.* [79]):

Antagonism (--): (control halo - combination halo) ≥ 6 mm;

Indifference (-): Indifferent interactions are considered between the limits proposed for additive and antagonistic interactions;

Potentiation (+): Potentiating interactions are applicable only if one of the phytochemicals don't has antimicrobial activity when applied individually, but increases the

antimicrobial capacity of other molecules when combined: (combination halo - control halo) ≥ 4 mm;

Additive (++): $4 \leq (\text{combination halo} - \text{control halo}) < 6$ mm;

Synergism (+++): (combination halo - control halo) ≥ 6 mm.

It was used as control, inhibition halos obtained to phytochemicals when tested individually at 1 000 $\mu\text{g}/\text{disc}$.

3.2.5 Antibiotic-Extracts Dual Combinations Assay

The extract (dissolved in DMSO) was inserted into MHA medium [79]. It was not possible to establish the same final concentration for all extracts given the considerable discrepancies in the volumes of each stock solution. The uniformity of concentrations would require setting an even lower concentration than that used, i.e. without any scientific significance.

Sterile filter paper discs (5 mm diameter) were placed on the agar plate seeded with the respective bacteria. A volume of 10 μL of streptomycin (STR, 10 $\mu\text{g}/\text{disc}$), 10 μL of ampicillin (AMP, 10 $\mu\text{g}/\text{disc}$) and 10 μL of ciprofloxacin (CIP, 5 $\mu\text{g}/\text{disc}$) prepared according to the Clinical Laboratory Standards Institute (CLSI) standards [94] was added to the blank discs. The plates were incubated at 37 °C for 24 hours. After incubation, the diameter in mm of the inhibitory zones around the discs was recorded.

The effect of the dual combinations of antibiotics and extracts was classified as follows [79]:

Antagonism (-): (antibiotic halo - antibiotic halo with phytochemical) ≥ 6 mm;

Indifference (+): Indifferent interactions are considered between the limits proposed for additive and antagonistic interactions;

Additive (++): $4 \leq (\text{antibiotic halo with phytochemical} - \text{antibiotic halo}) < 6$ mm;

Potentiation (+++): (antibiotic halo with phytochemical - antibiotic halo) ≥ 6 mm.

3.2.6 Statistical Analysis

The data were analysed using Anova (Two-factor with replication) from the Microsoft Excel 2016. Statistical analysis was calculated based on a confidence level of $\geq 95\%$, where $p < 0.05$ was considered statistically significant.

3.3 Results and Discussion

3.3.1 Antibacterial Activity Assessment

3.3.1.1 Plant extracts

Cowan [9] reviewed the main classes of phytochemicals that each solvent can dissolve and concluded that water, ethanol, and methanol were the solvents that guarantee better efficient extractions, extracting more quantity of compounds than the others. Since ancient times water extracts have been studied for their medicinal properties, so water was the first solvent to be selected. Between ethanol and methanol other physical characteristics were reviewed. It was found that methanol is more volatile and has lower boiling point than ethanol, which is very important to the evaporation process that follows extraction [83]. Thus, methanol was preferred instead of ethanol. Perhaps other solvents also fitted the requirements but probably were more expensive than those that were selected. Therefore, water and methanol were selected as extraction solvents. Tables 1 and 2 show the antimicrobial activity of water and methanol extracts evaluated through the disc diffusion method against *E. coli* and *S. aureus* strains.

Table 1: Classification of the antibacterial activity of aqueous extracts: Strong efficacy (+++), Good efficacy (++), Moderate efficacy (+), Non-effective (-).

	<i>D. purpurea</i> (bark)	<i>C. myconis</i> (leaves)	<i>O. crocata</i> (leaves)	<i>P. judaica</i> (leaves)	<i>C. sepium</i> (leaves)
Extract concentration (mg/mL)	143.8	120.6	30.1	70.7	93.2
<i>E. coli</i>	-	-	-	-	-
<i>S. aureus</i>	-	-	-	-	-

Table 2: Classification of the antibacterial activity of methanolic extracts: Strong efficacy (+++), Good efficacy (++), Moderate efficacy (+), Non-effective (-).

	<i>D. purpurea</i> (bark)	<i>C. myconis</i> (leaves)	<i>O. crocata</i> (leaves)	<i>P. judaica</i> (leaves)	<i>C. sepium</i> (leaves)
Extract concentration (mg/mL)	118.1	32.1	26.6	13.0	32.9
<i>E. coli</i>	-	-	-	-	-
<i>S. aureus</i>	-	-	-	-	-

It was observed that no extract showed antimicrobial activity. This was particularly impressive and somehow unexpected. These plants weren't studied yet for their antimicrobial

potential, but they did concerning other medicinal properties. For example, *D. purpurea* is known as the original source of the heart medicine digitoxin and similarly other therapeutic characteristics are related to the other four species [95-100]. So, it was expected that they also had influence in antibacterial susceptibility. However, the aqueous and methanolic extracts weren't effective against the bacterial strains tested. Two problems that may have made the results unfeasible are the concentration of the extract, which was relatively low [101] and the extraction time of 2 h, which was relatively short [15].

3.3.1.2 Selected phytochemicals

Individual Tests

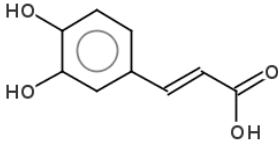
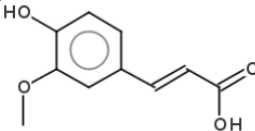
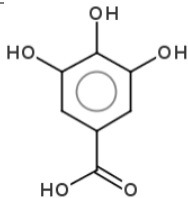
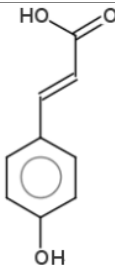
Through Dr Duke's Phytochemical and Ethnobotanical Databases, it was discovered that CA, FA, GA, PCA and S are very common phytochemicals, being present respectively in at least 301, 196, 151, 204 and 85 different plants including *D. purpurea*, studied in section 3.3.1.1. Moreover, they have known antibacterial potential, excluding S, which antimicrobial activity is usually weak.

First of all, it was decided to investigate about the “drug likeness” properties of the four phenolic acids according the rule of five (RO5) as described by Lipinski [92]. The original RO5 deals with orally active compounds and defines four simple physicochemical parameter ranges associated with 90% of orally active drugs that have achieved phase II clinical status: molecular weight (MWT) ≤ 500 (g/mol), octanol-water partition coefficient ($\log P$) ≤ 5 , number of hydrogen bond acceptors ≤ 10 and number of hydrogen bond donors ≤ 5 . These physicochemical parameters are associated with acceptable aqueous solubility and intestinal permeability and comprise the first steps in oral bioavailability. The results obtained concerning to CA, FA, GA and PCA are presented in Table 3.

If a compound fails more than one of the rules there is a high probability that oral activity problems will be encountered. In this case, all the phenolic acids meet the requisites and therefore, these molecules can be considered “drug-like” compounds. However, this prediction is limitative in a sense that it cannot predict biological activity or toxicity to the organism.

Thus, it was decided to study the antibacterial activity of these molecules against *E. coli* and *S. aureus* bacteria. Firstly, the five molecules (CA, FA, GA, PCA and S) were tested individually by disc diffusion method at varying concentrations: 200, 250, 333, 500 and 1 000 $\mu\text{g}/\text{disc}$. The results are illustrated in Figure 9.

Table 3: Structural and molecular properties of selected phenolic acids determined with Molinspiration calculation software. MWT, molecular weight; Log *P*, octanol-water partition coefficient; *n*-ON, number of hydrogen bond acceptors; *n*-OHNH, number of hydrogen bond donors.

Phenolic acids	Chemical structure	MWT (g mol ⁻¹)	Log <i>P</i>	<i>n</i> -ON acceptors	<i>n</i> -OHNH donors
Caffeic acid (CA)		180.16	0.94	4	3
Ferulic acid (FA)		194.19	1.25	4	2
Gallic acid (GA)		170.12	0.59	5	4
<i>p</i> -Coumaric acid (PCA)		164.16	1.43	3	2

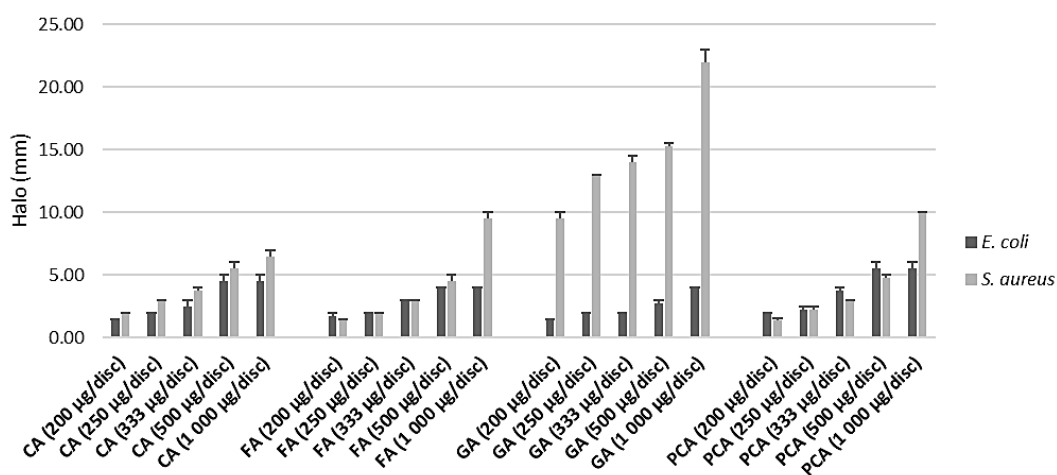


Figure 9: Diameter of the inhibition halo for each phytochemical when tested against *E. coli* and *S. aureus* determined by disc diffusion assay. The halo size was calculated by deducting the size of the disc (5 mm). Values are the means \pm SD of at least two independent experiments.

Figure 9 shows the antimicrobial activity for all the phenolic acids (CA, FA, GA and PCA). This result was expected because several authors have described the antibacterial potential of these molecules [41, 46, 60, 102-106]. Usually, antimicrobial activities of phenolics involves destabilization of cytoplasmatic membrane and enzyme inhibition by the oxidized products [48, 93]. A possible mechanism to explain the destabilization of internal membrane is the hyperacidification observed at the plasma membrane interphase because of dissociation of phenolic acids [44, 107]. This hyperacidification alters cell membrane potential, making it more permeable. Enzyme inhibition is possibly related to the formation of reactive quinones that can react with amino acids and proteins, exercising antimicrobial activity [93]. In contrast, bacterial strains weren't inhibited to grow in the presence of S (data not shown), which is in agreement with the conclusions of other studies [50, 56].

The results showed significant differences between the various concentrations ($p < 0.05$). It was observed that bacteria susceptibility increases with increasing concentration of phenolics. This was especially true with Gram-positive bacteria *S. aureus*.

S. aureus was more affected by the presence of phytochemicals than *E. coli*, mainly when applying higher concentrations such as 1 000 $\mu\text{g}/\text{disc}$ ($p < 0.05$). This may indicate different modes of action of phenolic acids when tested against different types of bacteria (Gram-positive or -negative). It is known that the Gram-positive bacterium *S. aureus* lacks an outer membrane, which facilitates diffusion of the phenolic acids through the cell wall and intracellular acidification, as well as irreversible alterations in the sodium-potassium ATPase pumps, consequently leading to cell death [44]. Gram-negative bacteria like *E. coli* are expected to be more protected because of their outer membrane. So, it is common that Gram-positive bacteria present higher susceptibility to phenolic acids than Gram-negative ones [101].

Concerning to *E. coli*, it seems that the bacterial susceptibility isn't affected by the compounds tested. Moreover, at 500 $\mu\text{g}/\text{disc}$ the antibacterial potential of phenolic acids stabilizes near the same value for all the molecules ($p > 0.05$). This behaviour was observed by other authors and suggests that CA, FA, GA and PCA have a similar mode of action with respect to antibacterial activity against *E. coli* [93]. Concerning to *S. aureus* bacteria, the results were also similar between CA, FA and PCA ($p > 0.05$). However, an interesting observation was that GA showed a much higher antimicrobial effect than the other phenolics against this bacterium ($p < 0.05$). It is known that phenolic acids are hydroxylated derivatives of benzoic and cinnamic acids [41, 60]. Although CA, FA and PCA are hydroxycinnamic acids, GA is a hydroxybenzoic acid. This could explain the differences observed with *S. aureus* Gram-positive bacteria. Indeed, Borges *et al.* [60] when studying the mode of action of FA and GA against pathogenic bacteria

verified that GA (hydroxybenzoic acid) caused more significant changes on the bacterial physicochemical properties than FA (hydroxycinnamic acid). Cho *et al.* [108] also concluded that the antibacterial mode of action of hydroxybenzoic and hydroxycinnamic acids differs. It seems that antibacterial activity of hydroxybenzoic acids decreases with an increasing number of hydroxyl groups and is thus primarily correlated with their lipophilicity [109]. On the other hand, the antibacterial activity of hydroxycinnamic acids depends to a much lesser extent on the substitutions of the aromatic ring with hydroxyl or methoxy groups, but it is strongly dependent on the double bond of the side chain. The reduction of the double bond of hydroxycinnamic acids substantially decreased their antibacterial activity. Perhaps, for that reason, in this experience, the bacteria strains presented similar susceptibility to the three hydroxycinnamic acids tested. CA, FA and PCA have different aromatic ring substituents, but have exactly the same side chain with its double bond in the same position.

Combined Tests

Several methods such disc-diffusion, well diffusion and broth or agar dilution are well known and commonly used when assessing antimicrobial activity of phytochemicals individually or in combination with conventional antibiotics [84]. However, this area of combinations between phytochemicals is relatively recent and scientific community is still developing new experimental procedures to test that. For example, Ziaei-Daroukalei *et al.* [110] developed AZDAST, a novel method to detect antimicrobial synergism.

In this regard, to analyse the combinations of phenolics, one could opt by diffusion or dilution methods. It was decided to adapt the well-known disc diffusion method to assess the synergism of the five phytochemicals. Indeed, disc diffusion method is appropriate as a preliminary screening test prior to other quantitative determinations with dilution methods [111].

The results of the two-to-two, three-to-three, four-to-four, and five-to-five combinations are presented in tables 4, 5 and 6, respectively. The halos of the combinations were compared with that obtained to phytochemicals when tested individually at 1 000 µg/disc and classified.

Table 4: Classification of two-to-two combinations of selected phytochemicals as synergetic (+++), additive (++), potentiating (+), indifference (-) or antagonistic (--). Values are the means \pm SD of at least two independent experiments.

	CA+FA	CA+GA	CA+PCA	CA+S	FA+GA	FA+PCA	FA+S	GA+PCA	GA+S	PCA+S
<i>E. coli</i>	5.50 \pm 1.50 (-)	6.00 \pm 2.00 (-)	6.00 \pm 1.00 (-)	2.75 \pm 0.75 (-)	5.50 \pm 1.50 (-)	5.50 \pm 1.50 (-)	3.00 \pm 0.00 (-)	5.50 \pm 0.50 (-)	2.50 \pm 0.50 (-)	4.00 \pm 0.00 (-)
<i>S. aureus</i>	4.00 \pm 0.00 (-)	16.50 \pm 0.50 (-)	5.50 \pm 1.50 (-)	2.00 \pm 0.00 (-)	7.00 \pm 0.00 (-)	9.75 \pm 0.25 (-)	3.00 \pm 0.00 (--)	8.50 \pm 0.50 (-)	19.00 \pm 1.00 (-)	5.50 \pm 0.50 (-)

Table 5: Classification of three-to-three combinations of selected phytochemicals as synergetic (+++), additive (++), potentiating (+), indifference (-) or antagonistic (--). Values are the means \pm SD of at least two independent experiments.

	CA+FA+GA	CA+FA+PCA	CA+FA+S	CA+GA+PCA	CA+GA+S	CA+PCA+S	FA+GA+PCA	FA+GA+S	FA+PCA+S	GA+PCA+S
<i>E. coli</i>	4.50 \pm 0.50 (-)	3.50 \pm 0.50 (-)	2.50 \pm 0.50 (-)	4.50 \pm 0.50 (-)	2.00 \pm 0.00 (-)	3.00 \pm 0.00 (-)	5.00 \pm 0.00 (-)	2.50 \pm 0.50 (-)	2.00 \pm 0.00 (-)	2.50 \pm 0.50 (-)
<i>S. aureus</i>	2.00 \pm 0.00 (-)	4.50 \pm 0.50 (-)	2.00 \pm 0.00 (-)	2.00 \pm 0.00 (-)	7.00 \pm 1.00 (-)	2.00 \pm 0.00 (-)	6.00 \pm 1.00 (-)	2.50 \pm 0.50 (-)	5.00 \pm 0.00 (-)	4.00 \pm 0.00 (-)

Table 6: Classification of four-to-four combinations and five-to-five combination of selected phytochemicals as synergetic (+++), additive (++), potentiating (+), indifference (-) or antagonistic (--). Values are the means \pm SD of at least two independent experiments.

	CA+FA+GA+PCA	CA+FA+GA+S	CA+GA+PCA+S	FA+GA+PCA+S	CA+FA+GA+PCA+S
<i>E. coli</i>	5.00 \pm 1.00 (-)	3.50 \pm 0.50 (-)	4.00 \pm 1.00 (-)	3.50 \pm 1.00 (-)	4.00 \pm 0.00 (-)
<i>S. aureus</i>	8.50 \pm 1.00 (-)	6.00 \pm 0.50 (-)	4.50 \pm 0.00 (-)	7.00 \pm 0.50 (-)	8.00 \pm 1.00 (-)

It was observed, that most of the combinations were indifferent, with exception of FA+S, FA+GA+S and GA+PCA+S against *S. aureus* that were antagonistic. Therefore, it wasn't found potentiating effect when combining S with phenolic acids. On the contrary, S can mitigate the effect of these molecules.

Combinations between CA, FA, GA and PCA were classified as indifferent. There was visible inhibition halo, however the effect wasn't superior to that observed previously when the phytochemicals were tested individually at 1 000 μ g/disc. This may indicate again that the mode of action of the four phenolic acids is quite similar. For example, it was not observed any difference between 1 000 μ g/disc of CA or 1 000 μ g/disc of CA+FA, CA+GA, CA+PCA or other of the combinations. Nevertheless, it seems that sometimes how much molecules we combine, minor is the bacterial susceptibility. This diminution is very slightly and usually doesn't interfere with the overall classification of the combinations. However, this may be the reason why in section 3.3.1.1 no plant (including *D. purpurea*) had evidenced antimicrobial activity.

So, it can be hypothesized that the four phenolic acids (CA, FA, GA and PCA) have antimicrobial activity and a very similar mode of action against pathogenic bacteria [93]. When we combine relatively few phenolic acids we have approximately the same effect that when we test that phenolics individually. However, in the case that we combine many molecules, the entropy increases, and the antimicrobial effect seems to decrease proportionally.

Despite that, we cannot forget that these are preliminary assays on the combination of phytochemicals. Therefore, in the future further tests against planktonic bacteria must be done in order to clarify these results.

3.3.2 Antibiotic-Extracts Dual Combinations Assay

Tables 7 and 8 summarize all the results of antibiotic potentiation by phytochemicals.

Table 7: Classification of the combinations with aqueous extracts as potentiating (+++), additive (++), indifferent (+) or antagonistic (-). CIP, ciprofloxacin; STR, streptomycin; AMP, ampicillin.

		<i>D. purpurea</i> (bark)	<i>C. myconis</i> (leaves)	<i>O. crocata</i> (leaves)	<i>P. judaica</i> (leaves)	<i>C. sepium</i> (leaves)
Concentration (mg/mL)		1.80	0.38	1.51	0.88	1.17
<i>E. coli</i>	CIP	+	+	+	+	+
	STR	+	+	+	+	+
	AMP	+	+	+	+	+
<i>S. aureus</i>	CIP	++	++	+	++	+
	STR	+	+	+	+	+
	AMP	+++	+	+	+	++

Table 8: Classification of the combinations with methanolic extracts as potentiating (+++), additive (++), indifferent (+) or antagonistic (-). CIP, ciprofloxacin; STR, streptomycin; AMP, ampicillin.

		<i>D. purpurea</i> (bark)	<i>C. myconis</i> (leaves)	<i>O. crocata</i> (leaves)	<i>P. judaica</i> (leaves)	<i>C. sepium</i> (leaves)
Concentration (mg/mL)		1.48	1.61	1.33	0.65	1.65
<i>E. coli</i>	CIP	+	+++	+++	++	+++
	STR	+	+	+	+	+
	AMP	+	++	++	++	++
<i>S. aureus</i>	CIP	+++	++	++	+++	+
	STR	+	+	+	+++	+
	AMP	+++	+	++	+++	+

The results of tables 7 and 8 showed that of the 60 combinations, 9 (15%) potentiated the action of the antibiotic, 12 (20%) had an additive effect, and 39 (65%) were indifferent. No combination was antagonistic. These results are in agreement with the research carried out in this area. For example, Abreu *et al.* [79] when studied the potentiation of antibiotics by phytochemical agents, tested 190 combinations using different strains of *S. aureus* and only obtained potentiation and additive effects in 5,8% and 7,4% of the results, respectively.

Analysis of the results allowed to infer that all plant extracts, without exception, increased the effect of antibiotics in at least three combinations. However, of the five types of plant material under study there were some that presented better results in detriment of others. Figure 10 represents these differences by ranking plant materials for number of 'winning' combinations. By 'winning' combination is meant a combination with additive or potentiating

effect. In the case of the plants that obtained the same number of 'winning combinations', it was firstly considered that it had the highest number of combinations with a potentiating effect. In this study, twelve combinations were performed per plant type, which indicates that the maximum number of 'winning combinations' is twelve.

Figure 10 showed that the plant extract with the highest antimicrobial potential was obtained from *P. judaica* (six 'winning' combinations, three of which are potentiations). After *P. judaica*, the stem bark extract of *D. purpurea* obtained four 'winning' combinations, of which three were potentiations. *O. crocata* and *C. myconis* extracts presented similar results, both achieved four 'winning' combinations including one potentiation. Finally, *C. sepium*, obtained two combinations with additive effect and one with potentiating effect, which makes a total of three 'winning' combinations.

1 st <i>P. judaica</i> (leaves)
•6 combinations (3 potentiations)
2 nd <i>D. purpurea</i> (bark)
•4 combinations (3 potentiations)
3 rd <i>O. crocata</i> (leaves); <i>C. myconis</i> (leaves)
•4 combinations (1 potentiation)
4 th <i>C. sepium</i> (leaves)
•3 combinations (1 potentiation)

Figure 10: Rank by plants after antibiotic-extracts dual combinations assay.

Comparing the two solvents used for the extraction, water and methanol, it was verified that methanol was the best solvent to extract compounds with antimicrobial activity. Of the 6 combinations tested, the methanolic extracts had at least two combinations in which there was an increase in the antibiotic effect. Concerning to aqueous extracts, most of the times, none of the 6 combinations had additive or potentiating effects. The fact that methanol was a better solvent than water was confirmed even by only analysing the potentiating combinations. A more careful analysis allowed to infer that of these nine potentiating combinations only one was obtained with an aqueous extract. All other eight were due to methanolic extracts. Furthermore, of the twelve combinations with additive effect, eight were due to the methanolic extracts and only four to the aqueous ones.

Eloff [83] extracted phytochemicals from leaves of *Anthocleista grandiflora* and *Combretum erythrophyllum*, two plants with antimicrobial activity. The extraction solvents studied were: water, ethanol, methanol, methylene dichloride, a mixture of

chloroform/methanol/water (12:5:3) and acetone. For each solvent, the author evaluated the amount and diversity of the extracted compounds, the number of phytochemicals with antimicrobial activity, extraction efficiency, toxicity in bioassays, ease of solvent removal and biological hazard. Moreover, a scoring system that considered these parameters was developed. For the plants in study, acetone obtained the highest score (102), followed by chloroform/methanol/water (81), methylene dichloride (79), methanol (71), ethanol (58) and water (47). This study corroborates our results as it was concluded that methanol was better than water for the extraction of phytochemicals with antimicrobial potential.

It was also clear that *S. aureus* (Gram-positive) was more susceptible to the action of antibiotics than *E. coli* (Gram-negative). Note, for example, that of the nine potentiations, six were with *S. aureus* and only three with *E. coli*. Of the twelve combinations with additive effect, seven were with *S. aureus* and only five with *E. coli*. As previously referred, the cell wall of *S. aureus* lacks the peripheral/external membrane present in the Gram-negative bacteria, making it more vulnerable to antibiotics [101].

Finally, by evaluating the behaviour of the three antibiotics under study, quinolone CIP and penicillin AMP obtained satisfactory results, instead of STR (aminoglycoside), which of the twenty combinations only one isn't considered indifferent. In the future, it is recommended to test other groups of antibiotics.

3.4 Conclusions

It can be concluded that no plant crude extracts had antimicrobial activity. On the other hand, the results showed that all plant extracts increased the effect of antibiotics in at least three combinations. The plant extract with the best results was those obtained with *P. judaica* followed by the stem bark of *D. purpurea*. Then, leaves extracts of *O. crocata* and *C. myconis* presented similar results and finally leaves extracts of *C. sepium* were the worst plant material tested. Overall, of the 60 combinations, 9 (15%) potentiated the action of the antibiotics and 12 (20%) had an additive effect. Ciprofloxacin and ampicillin had satisfactory results, rather than streptomycin. Methanol was better solvent than water in extracting compounds with antimicrobial activity. *S. aureus* (Gram-positive) was more susceptible to the action of antibiotics than *E. coli* (Gram-negative).

Caffeic acid (CA), ferulic acid (FA), gallic acid (GA) and *p*-coumaric-acid (PCA) comply the rule of 5 (RO5) requisites. Therefore, these molecules can be considered "drug-like" compounds.

The experiments developed showed that the four phenolic acids had antimicrobial activity and a very similar mode of action against bacterial strains studied. An eventual exception

was GA because showed a much higher antimicrobial effect than the other phenolics against *S. aureus*. This may be because GA is a hydroxybenzoic acid and CA, FA and PCA are hydroxycinnamic acids.

Furthermore, it was proved that when we combine relatively few phenolic acids we have approximately the same effect that when we test that phenolics individually. However, in the case that we combine many molecules, the antimicrobial effect may decrease. This fact may be the reason why no plant extract (including *D. purpurea*) showed antimicrobial activity.

Lastly, saponin (S) didn't showed antimicrobial activity neither potentiating effect when combined with the other four phenolic acids selected.

Chapter 4- Biofilm control using phytochemicals' combinations

4.1 Introduction

Biofilms are aggregates of microorganisms in which cells are embedded in a matrix of extracellular polymeric substances (EPS) produced by their one [1]. EPS has physical and chemical properties that allow the bacterial cells to adhere to each other and/or a solid surface. When bacteria growth as biofilm, they experience a diversified spectrum of interactions between themselves and the environment that are different from that studied on planktonic cells [1, 112, 113]. Some of that mechanisms are responsible for the increased antibiotic resistance in biofilms compared to that of free-living bacteria.

There have been appointed at least four main causes for the antibiotic resistance in bacterial biofilms [25]: i) poor antibiotic penetration, ii) nutrient limitation and slow growth, iii) adaptive stress responses, and iv) formation of persister cells. Biofilms usually have cell densities ranging from 10^8 to 10^{11} cells g^{-1} wet weight [1]. So, when antibiotic diffuses into the biofilm, it can be inactivated by reaction, or sequestered by binding, and thus it cannot reach the inner parts of the cellular community. Beta-lactamases, aminoglycoside-modifying enzymes and chloramphenicol acetyltransferases are some enzymes that inactivate or modify the antibiotics, while they penetrate the biofilm [2, 25]. Other factor that is related with biofilm's resistance is that antibiotics activity is mostly growth-dependent. Penicillins, for example, only kill growing bacteria [2]. This may be a problem particularly in thick biofilms, because inner cells may don't have access to nutrients and for that reason may be metabolically inactive or in a dormant state, being less susceptible to antibiotics. Other problem when targeting biofilms is the induction of the general stress response by the threatening bacteria. For example, RpoS is a sigma factor expressed in Gram-negative bacteria and that play an important role in protecting biofilms [114]. Other stress responses such as increasing expression of multidrug efflux pumps, activating quorum-sensing (QS) systems and changing profiles of outer membrane proteins (OMP) have been reported to drug resistance on biofilms [32, 114-116]. Finally, it is known that some cells within the biofilm have the ability to survive to the antibacterial action and acquire resistance, being commonly named as persisters [26, 117]. They utilize the residues left by dead bacteria to their proper metabolic pathway and continue to thrive after the antibiotic's action.

Sadly, biofilms' resistance is becoming an increasingly bigger problem. It has been estimated that biofilms are associated with more than 65% of nosocomial infections and the

economic burden associated with the treatment of these biofilm-based infections is high [13]. Hence, phytochemicals appear as the new approach to target bacterial biofilms. Several natural origin compounds have showed good antimicrobial potential against biofilms [115, 118, 119]. On the other hand, there are relatively few studies about the antibacterial potential of combinations of these molecules.

Therefore, it was decided to test again the five molecules of the previous chapter, caffeic acid (CA), ferulic acid (FA), gallic acid (GA), *p*-coumaric acid (PCA) and saponin (S), against 24 h old biofilms of *E. coli* and *S. aureus*. The objective was to verify if these phytochemicals and their combinations were able to control *S. aureus* and *E. coli* biofilms. Thus, experiences were made in order to assess the culturability of biofilm's cells after exposition to the phytochemicals, the biofilm's metabolic inactivation and the biomass removal. Moreover, Gompertz model was used to analyse metabolism data.

4.2 Material & Methods

4.2.1 Biofilm formation

Biofilm formation was performed according to a modification of the method proposed by Stepanovic' *et al.* [120]. Bacterial suspensions were adjusted to an OD of 0.04 ± 0.02 ($\lambda=620$ nm) and 200 μ L per well were added to sterile 96-well polystyrene microtiter plates (Orange Scientific, Belgium). Plates were covered and incubated for 24 h at 37 °C under 150 rpm of agitation.

4.2.2 Addition of phytochemicals

The effect of the selected phytochemicals and combinations was evaluated according to Baptista *et al.* [121]. After biofilm development during 24 h, the culture medium was removed, and the wells were washed twice with 200 μ L of sterile saline solution (NaCl; 8.5 g/L) to remove non-adherent cells. Subsequently, for the individual tests of the phytochemicals, 150 μ L of sterile saline and 50 μ L of each compound were applied at 1 000 μ g/well for CA, FA, GA, PCA and S. For dual-combinations, 25 μ L of each phytochemical was added to the wells (25 μ L + 25 μ L) maintaining at 1 000 μ g/well the overall concentration (500 μ g + 500 μ g). The combinations tested were: CA+FA, CA+GA, CA+PCA, CA+S, FA+GA, FA+PCA, FA+S, GA+PCA, GA+S and PCA+S. The microtiter plates were incubated at 37 °C and 150 rpm during 1 h. Biofilms without treatment (200 μ L of sterile saline) were used as negative control. The effect of DMSO was also verified (150 μ L of saline and 50 μ L of DMSO). After the exposure period, the biofilms were analysed in terms of produced mass, metabolic activity, and cultivability on solid medium.

4.2.3 Biofilm Analysis

4.2.3.1 Biofilm culturable cells' quantification

The sessile biofilm cells were analysed in terms of their culturability in solid medium after exposure to the selected phytochemicals. The biofilms were washed twice with sterile NaCl to remove weakly adherent bacteria. Afterwards, biofilm cells were obtained by wells' scrapping (three times, 1 min periods, with 200 μ L of sterile NaCl) and 10-fold serial dilutions in sterile saline solution were performed. Then, 10 μ L of each dilution was plated in PCA plates and incubated at 37 °C for 24 h. All tests were performed in duplicate. The number of colony forming units (CFU) was visually counted ($10 < \text{CFU} < 100$) and expressed per square centimeter of the microtiter plates' well (CFU/cm²), according to Eqs. (1) and (2):

$$\frac{\text{CFU}}{\text{mL}} = \frac{N}{SV \times \text{Dilution}} \quad (1)$$

where N is the number of CFU in the PCA plates and SV is the sample volume in mL.

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

where WV is the well's working volume (0.2 mL) and 1.53 is the well area in cm². The results were then expressed in terms of the log (CFU/cm²).

4.2.3.2 Biofilm metabolic activity quantification by resazurin assay

The quantification of the biofilms metabolic activity was assessed by resazurin/alarmar blue (Sigma-Aldrich, Portugal) assay. The microtiter plates' content was removed, and the wells were washed with 200 μ L of sterile saline (NaCl; 8.5 g/L). After that, 180 μ L of MHB medium and 20 μ L of resazurin solution at 0.4 mM were added to the microtiter plates. The resazurin solution was added in darkness. Thereafter, the fluorescence ($\lambda_{\text{excitation}} = 570 \text{ nm}$; $\lambda_{\text{emission}} = 590 \text{ nm}$) was measured in a microtiter plate reader (FLUOstar Omega; BMG LABTECH, Germany) during 20 h. All tests were performed with three replicates and a minimum of two independent repeats. The obtained data was analysed in terms of percentage of biofilm metabolic activity reduction when exposed to the selected compounds and combinations, according to Eq. (3):

$$\%B_{\text{MAR}} = \frac{FL_C - FL_W}{FL_C} \times 100 \quad (3)$$

where %B_{MAR} is the percentage of biofilm metabolic activity reduction, FL_C is the fluorescence intensity of biofilms not exposed to phytochemicals and FL_W is the fluorescence intensity for biofilms exposed to the selected compounds. The hour at which was verified the maximum value of metabolic activity to the biofilms not exposed to phytochemicals was considered to read FL_C and FL_W.

4.2.3.2.1 Application of Gompertz's model

Gompertz's model is described in Eq. (4):

$$f(t) = a \cdot e^{-e^{(b-ct)}} \quad (4)$$

where $f(t)$ is the variable measured (number of bacteria, density of microorganisms, etc; in this case the fluorescence), t is the independent variable of time (h), $e = \exp(1)$, a , b and c are mathematical parameters.

The first step was to find the constants a , b and c in the Gompertz model that best fit the fluorescence curves given by the resazurin data (Appendix A), according to Carey [122]. Microsoft Excel 2016 was used by inputting the given data and a guess was made as to the three constants. The square in the error of the guesses was calculated. The Solver application was then used by manipulating the constants until the sum of the squared errors (sos) was as small as possible. Thus, a , b and c were calculated for each different graph.

Using calculus, it is possible to derive biological parameters from the mathematical constants a , b , and c [123]. Three biological parameters commonly calculated are represented in Figure 11:

- Maximum specific growth rate (μ_m): defined as the tangent in the inflection point;
- Lag time (λ): defined as the x-axis intercept of this tangent;
- Asymptote (A): the maximal value reached.

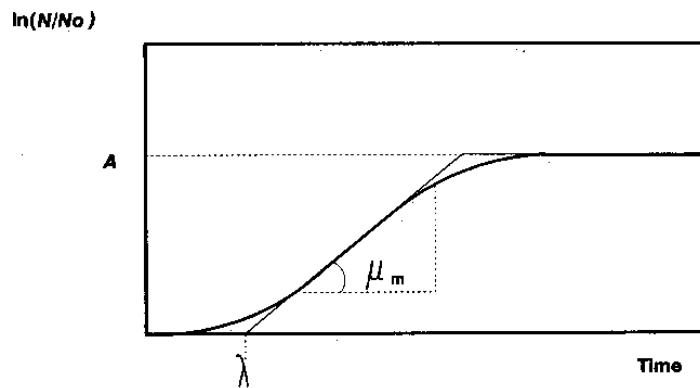


Figure 11: Three biological parameters useful in characterizing the growth curve [123].

Lag time (λ) was the biological parameter considered to analyse the results of resazurin method and can be related to mathematical parameters b and c , according to Eq. (5):

$$\lambda = \frac{(b-1)}{c} \quad (5)$$

Thus, lag time (λ , h) was calculated for each graph (Appendix A), according to Gompertz's model.

4.2.3.3 Biofilm mass quantification by crystal violet staining

Finally, the biofilm mass was quantified using crystal violet (CV; Merck, Germany) assay. The microtiter plates' wells were emptied and washed with 200 μ L of sterile water to remove non-adherent or weakly adherent cells. The remaining bacteria were fixed with 250 μ L of 99% (v/v) ethanol (NeoLar; Portugal) for 15 min. Plates were emptied and left to dry for 10 min. Then, the fixed bacteria were stained for 10 min with 200 μ L of 1% (v/v) CV solution. The wells were emptied and washed with 200 μ L of sterile water to remove exceeding stain. The dye bound to the adherent cells was resolubilized with 200 μ L of 33% (v/v) glacial acetic acid (Chem-Lab, Belgium). Afterwards, the absorbance was measured at 570 nm in a microtiter plate reader. All tests were performed with three replicates and a minimum of two independent repeats.

4.2.4 Statistical Analysis

The data were analysed using Anova (Single factor, Two-factor with replication and Two-factor without replication, as required) from the Microsoft Excel 2016. For all tests, statistical analysis was calculated based on a confidence level of $\geq 95\%$, where $p < 0.05$ was considered statistically significant.

4.3 Results and Discussion

4.3.1 Biofilm culturable cells' quantification

Firstly, the sessile biofilm cells were analysed in terms of their culturability in solid medium after exposure to the selected compounds. Figures 12 and 13 summarize the results obtained for *S. aureus* and *E. coli* bacterium, respectively.

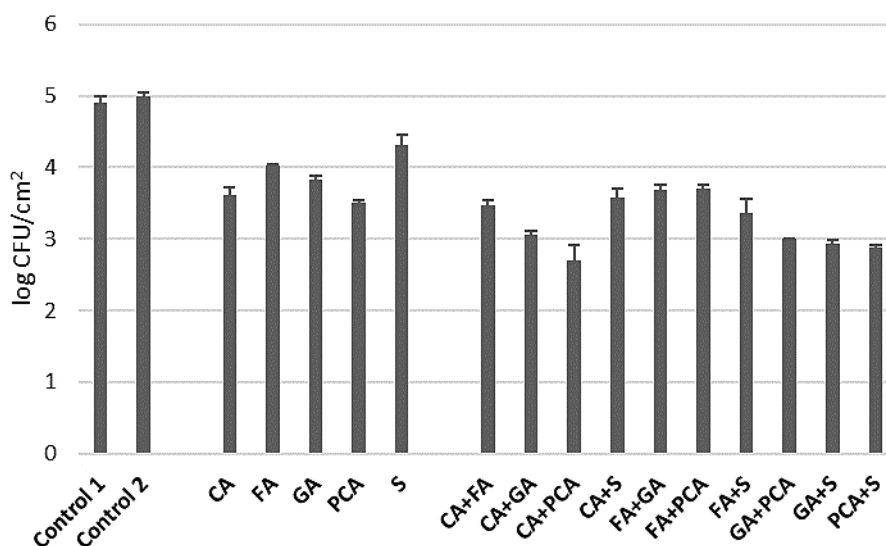


Figure 12: Log CFU/cm² reduction of *S. aureus* biofilm cells after 1 h of exposure to the selected phytochemicals and negative controls: Control 1 (saline) and Control 2 (DMSO).

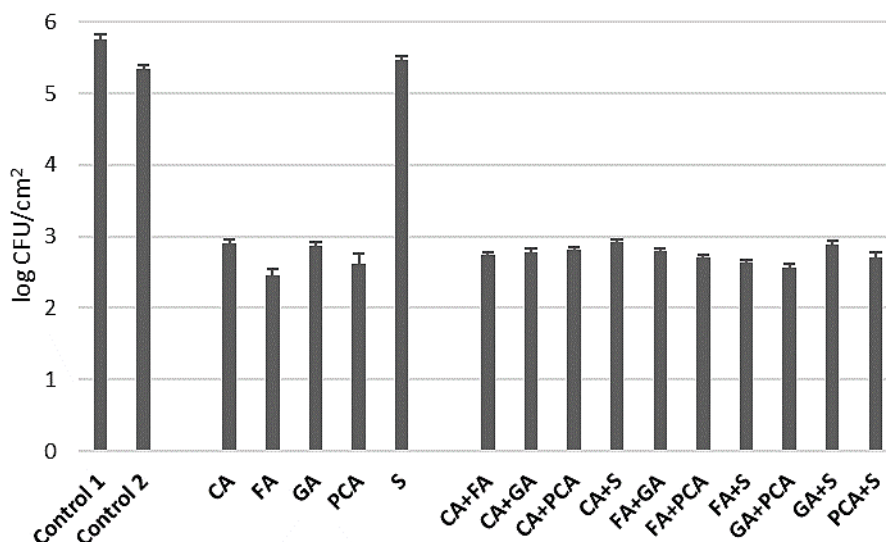


Figure 13: Log CFU/cm² reduction of *E. coli* biofilm cells after 1 h of exposure to the selected phytochemicals and negative controls: Control 1 (saline) and Control 2 (DMSO).

First of all, it was observed that there were no significant differences between control 1 and control 2 for both bacteria ($p>0.05$), indicating that DMSO didn't influence the culturability of the biofilm cells.

On the other hand, Figures 12 and 13 show significant differences between the results obtained for *S. aureus* and *E. coli* biofilms ($p<0.05$). *E. coli* biofilms seem to be more susceptible to the selected phytochemicals than *S. aureus* biofilms. This contradicts what was concluded for planktonic cells in chapter 3 (3.3.1.2). Indeed, we cannot infer on the biofilm susceptibility based on the results of planktonic cells, because the morphology of the biofilms is different [124, 125]. However, these results are in agreement with other studies in the literature. For example, Monte *et al.* [5] studied the antimicrobial potential of selected phytochemicals against planktonic and sessile cells and also found contradictory results. They observed that *E. coli* biofilms were more susceptible to phytochemicals instead of those of *S. aureus*. This contradicted with the results that they obtained about the effect of the same phytochemicals against planktonic cells, where *S. aureus* was more susceptible than *E. coli*. Indeed, the number of resistance mechanisms in biofilms increase significantly from the planktonic state. The protective mechanisms commonly encountered at planktonic bacteria such as target mutations, low cell permeability, efflux pumps, and modifying enzymes do not appear to be at the root of the reduced antimicrobial susceptibility in biofilms [2, 113]. In biofilms, poor antibiotic penetration, nutrient limitation and slow growth, adaptive stress responses, and formation of persister cells are the main causes for antibiotic resistance.

The Figures 12 and 13 also indicated significant differences between the five selected phytochemicals ($p < 0.05$). For both bacteria, *S* demonstrated poor ability to reduce the biofilm cells culturability compared with the four phenolic acids (CA, FA, GA and PCA), which is in agreement with the results found in the chapter 3 (3.3.1.2). On the other hand, PCA and FA were the phenolic acids with better results instead of GA as it was seen in the previous chapter. As stated in the previous chapter, the mechanisms that are described for the antimicrobial activity of phenolic acids include destabilization and permeabilization of cytoplasmatic membranes, efflux pump inhibition, bacterial type II fatty acid synthesis inhibition, and enzyme inhibition by the oxidized products [9, 17, 48]. It is known too that hydroxycinnamic acids like CA, FA and PCA have chemical and physical differences from hydroxybenzoic acids like GA, which has implications on their mode of antimicrobial action [108, 109]. However, when characterizing the antimicrobial mode of action on biofilms, the heterogeneity of their biological structure must be taken into account and this sometimes leads to different results between planktonic and sessile bacteria [1]. Perhaps for that reasons, in this study, it was observed a special antimicrobial action of FA and PCA against biofilms instead of GA that presented better results against planktonic Gram-positive bacteria, *S. aureus*. Recently, experiments have been done in order to clarify the causes for these differences, but it is recognized that there is much yet to be discovered regarding this subject [60].

Finally, it was found significant differences between the ten dual combinations of the selected compounds ($p < 0.05$). Some combinations seem to have better log CFU/cm² reductions than others. However, comparing the results of the combinations with those of the individual tests we don't find any synergistic interaction. This proves again what we have concluded in chapter 3. It was proved that when we combine relatively few phenolic acids we have approximately the same effect that when we test that phenolics individually. However, this is another topic that is underexploited. More experimental work is required to support such a claim.

4.3.2 Biofilm metabolic activity quantification by resazurin assay

Figures 14 and 15 show the percentages of metabolic inactivation against *S. aureus* and *E. coli* biofilms, respectively. In Appendix A, all the results of resazurin assay are presented. The hour at which was verified the maximum value of metabolic activity to the biofilms not exposed to phytochemicals was considered to read the fluorescence of the other biofilms. For *S. aureus*, the values of fluorescence at 12 h were selected whereas for *E. coli* the values were chosen at 5 h. Then, the percentages of inactivation were calculated.

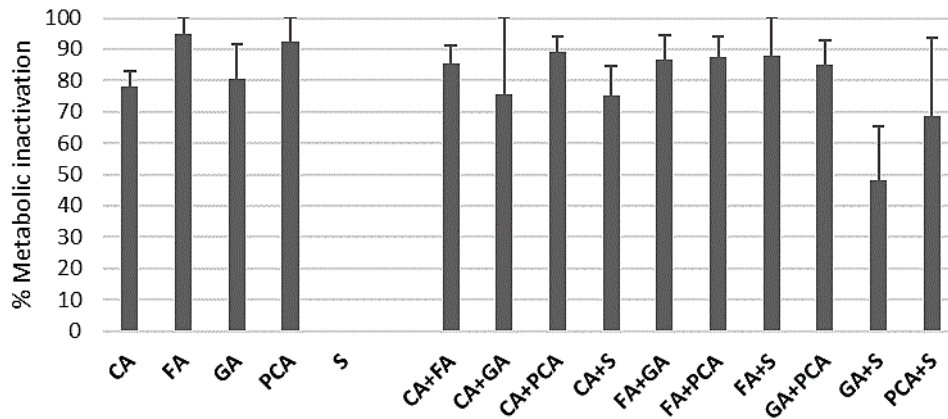


Figure 14: Percentages of inactivation of *S. aureus* biofilm cells after 1 h of exposure to the selected phytochemicals.

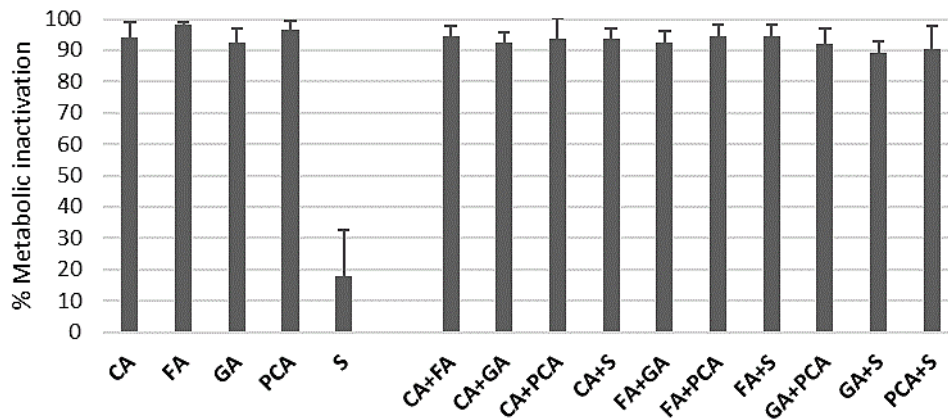


Figure 15: Percentages of inactivation of *E. coli* biofilm cells after 1 h of exposure to the selected phytochemicals.

Primarily, it was observed that there were significant differences between the results obtained for the two bacteria studied ($p < 0.05$). Once again, *E. coli* biofilms present higher susceptibility to the selected molecules than those of *S. aureus*.

S presented poor metabolic inactivation against both bacteria as were expected. Concerning to *S. aureus*, there were significant differences between the four phenolic acids ($p < 0.05$). FA and PCA were the compounds that promoted higher percentages of metabolic inactivation as it was concluded when assessing the culturability of biofilm's cells after exposition to the phytochemicals. Concerning to *E. coli*, there were no significant differences between CA, FA, GA and PCA ($p > 0.05$). The four phenolic acids achieved almost total inactivation of *E. coli* biofilm's cells.

The combinations of the selected phytochemicals also presented high percentages of metabolic inactivation. However, as expected, when comparing the results of the combinations

with those of the individual tests for both types of bacteria tested we didn't find any synergetic interaction.

Thus, the results of Figures 14 and 15 indicated a strong metabolic inactivation by the selected phytochemicals and their combinations. However, if we take into account the overall measures of fluorescence over time (Appendix A), it is observed that the graphical representation is quite similar of that of characterized by Gompertz model.

4.3.2.1 Application of Gompertz model

The Gompertz model is one of the most frequently used sigmoid models fitted to growth data and other data [126]. In this study, the similarities with the Gompertz model were analysed through the calculus of the lag time. At the same time, lag time of each nonlinear approximation was used to compare the metabolic inactivation achieved by the selected molecules and their combinations against *S. aureus* and *E. coli* biofilms. The results are presented in Tables 9 and 10.

Table 9: Lag time (h) for control (DMSO) and the five selected phytochemicals.

	Control	CA	FA	GA	PCA	S
<i>S. aureus</i>	4.0	12.0	*	11.2	*	6.3
<i>E. coli</i>	1.0	7.3	*	12.7	*	0.7

Table 10: Lag time (h) calculated for the ten two-to-two combinations of the selected phytochemicals.
* Gompertz's model couldn't fit the experimental data.

	CA+FA	CA+GA	CA+PCA	CA+S	FA+GA	FA+PCA	FA+S	GA+PCA	GA+S	PCA+S
<i>S. aureus</i>	*	*	*	11.5	*	*	*	*	7.2	9.5
<i>E. coli</i>	11.9	9.9	10.8	10.6	9.4	12.8	11.8	12.8	12.7	8.9

First of all, Gompertz model didn't fit very well to *S. aureus* experimental data. Moreover, when it was possible to apply the theoretical model, the values for *S. aureus* were usually higher than those obtained for *E. coli*. This means that the lag time is much higher in a such a way that the graphical representation looks like a straight line instead of a sigmoidal curve. This allowed to infer that despite the higher values of metabolic inactivation obtained for *E. coli*, this bacterium recovered faster or the remain persister cells have grown faster than those of *S. aureus*. Indeed, reporting *E. coli*, only for FA and PCA Gompertz model wasn't applicable. This corroborates what was concluded previously in this chapter, because attests the exceptional potential of FA and PCA to target both *S. aureus* and *E. coli* biofilms. S were again the phytochemical with the worst results, presenting a lag time substantially inferior than those of the other molecules and, consequently, a poor metabolic inactivation. Concerning to the combinations of the selected phytochemicals, it was concluded that there wasn't any special synergistic effect, as it was described previously.

However, the main advantage of doing this analysis through the Gompertz model is that can confirm the sigmoidal appearance of the fluorescence curves. It is interesting to note that there were differences between the lag time, but the maximum value reached for each graph is quite similar. This behaviour may be due to essential two distinct factors: the existence of persister cells that have the ability to survive against the antimicrobial attack and then proliferate or the bacteriostatic effect of phytochemicals instead of bactericide effect.

Recently, persister cells have been appointed to one of the major causes of antibiotic resistance of bacterial biofilms. Several studies have been performed about this subject [127, 128]. It has been proved that there are small populations of biofilm cells, named persisters, that remain alive after antibiotic's action even when there is an increase in antibiotics' concentration. This phenomenon also occurs with planktonic bacteria [17]. However, planktonic persisters are more susceptible to antimicrobial action and can be easily controlled by immune system, for example. When persisters arise in biofilm cultures their susceptibility is reduced because of the EPS structure. Persisters usually are phenotypic variants instead of genetic mutants of their bacterial strains [26, 129]. These phenotypic variants seem to stay in a dormant state throughout the antibiotic attack. Apparently, during the attack, these cells have little cell wall synthesis and reduced translation or topoisomerase activity, which allows antibiotics bind to their targets, but enables them to corrupt the bacteria [26, 117]. It is like if the antibacterial potential of antibiotics was shutted down. This dormant state prevents the proliferation of persisters during the attack. In this way, persisters are cells that temporarily forfeit propagation in favor of survival. Nevertheless, when the antibiotics' concentration drops, persister cells recover their metabolism and repropagate the bacterial biofilm. It seems that persisters are produced by a stochastic process, because all the cells in a population are genetically identical kin [130]. Persister cells must be controlled in order to mitigate biofilm negative impacts on health system. Combination of a conventional antibiotic with a compound inhibiting persister formation or maintenance may produce an effective therapeutic. Other approaches to the problem have been studied and include pulse-dosing with conventional antimicrobials [117].

Relatively to the hypothesis of the sigmoidal appearance of the fluorescence curves may be related to the bacteriostatic action of the phytochemicals instead of their bactericide action, other authors have also appointed the same suggestion [61, 119]. What may be happening is that cells stay alive at a dormant state during the phytochemicals' attack. Then, when the antimicrobial attack ends the bacterial cells recover their natural state and metabolism.

It is not possible with certainty to infer about what really happens. It is believed that persister cells and the bacteriostatic effect are the two main hypothesis. Further experimental tests may help to clarify these aspects.

4.3.3 Biofilm mass quantification by crystal violet staining

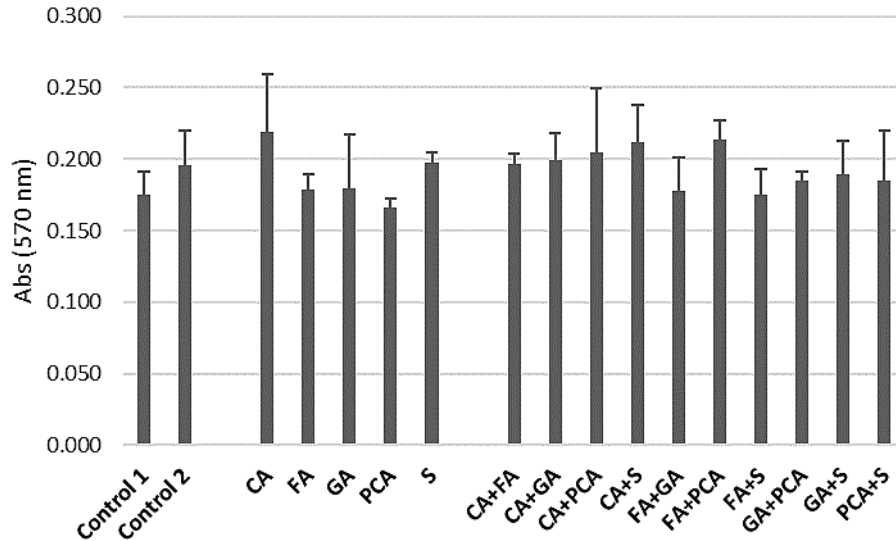


Figure 16: Biofilm mass quantification of *S. aureus* after 1 h of exposure to the selected phytochemicals and negative controls: Control 1 (saline) and Control 2 (DMSO).

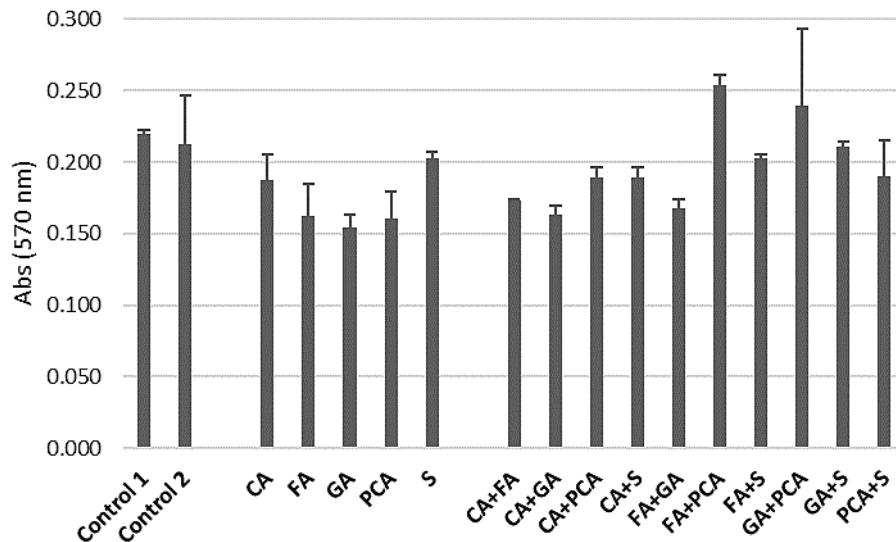


Figure 17: Biofilm mass quantification of *E. coli* after 1 h of exposure to the selected phytochemicals and negative controls: Control 1 (saline) and Control 2 (DMSO).

Figures 16 and 17 showed no significant differences between control 1 (saline) and control 2 (DMSO) as it was observed in the previous experiences ($p > 0.05$). For both types of

bacteria, it was also observed that any phytochemical neither any combination promoted complete removal of the biofilm's cells ($p>0.05$). Previous studies also demonstrated that biofilm removal and inactivation are distinct processes [5, 61]. Therefore, it is possible to infer that CA, FA, GA, PCA, S and their combinations showed good antibacterial inactivation and at the same time don't removed the biofilm, which included both bacteria and their extracellular matrix (EPS).

4.4 Conclusions

In conclusion, biofilm experiences revealed some similarities as well as differences toward the results obtained for planktonic bacteria in chapter 3.

Concerning to the similarities, saponin (S) demonstrated poor antimicrobial activity compared with the four phenolic acids (CA, FA, GA and PCA). Moreover, it was proved that when we combine relatively few phenolic acids we have approximately the same effect that when we test that phenolics individually.

Resuming the differences, *E. coli* biofilms seemed to be more susceptible to the selected phytochemicals than *S. aureus* biofilms. This contradicted with the results obtained for planktonic cells. Furthermore, PCA and FA were the phenolic acids with better results instead of GA as it was seen in the previous chapter. Further experiments must be done in order to clarify the causes for these differences, but it is recognized that when characterizing the antimicrobial mode of action on biofilms, the heterogeneity of their biological structure has to be taken into account and this sometimes leads to different results between planktonic and sessile bacteria.

Additionally, biofilm analysis showed that CA, FA, GA, PCA and their combinations inactivated sessile bacteria. On the contrary, none of the phytochemicals removed the biofilm mass, showing that biofilm removal and inactivation are distinct processes.

Ultimately, for metabolism data the similarities with the Gompertz model were analysed through the calculus of the lag time. The model confirmed the sigmoidal appearance of the fluorescence curves. This behaviour may be mainly related with two distinct factors: the existence of persister cells that have the ability to survive against the antimicrobial attack and then proliferate or the bacteriostatic effect of phytochemicals instead of bactericide effect. At last, the lag time values for *S. aureus* were usually higher than those obtained for *E. coli*. So, despite the higher values of metabolic inactivation obtained for *E. coli*, this bacterium recovered faster or the remain persister cells have grown faster than those of *S. aureus*.

Chapter 5- Conclusions and Future Work

The antimicrobial capacity of *Digitalis purpurea*, *Parietaria judaica*, *Coleostephus myconis*, *Calystegia sepium* and *Oenanthe crocata* was assessed against planktonic bacteria and it was observed that no plant extract presented antimicrobial activity. On the other hand, the results showed that all plant extracts increased the effect of antibiotics in at least three combinations. Methanol was better solvent than water in extracting compounds with antimicrobial activity. *S. aureus* (Gram-positive) was more susceptible to the action of antibiotics than *E. coli* (Gram-negative). For the future, it is recommended to test other solvents and extraction techniques, which can allow better extraction efficiency and recoveries of the interested compounds whereas rejects molecules with no relevant therapeutic properties. It can be also interesting select other antibiotics to test in combination with plant extracts.

Further experiences against planktonic bacteria were conducted with four major natural occurring phenolic acids: caffeic acid (CA), ferulic acid (FA), gallic acid (GA) and *p*-coumaric-acid (PCA). Saponin (S) was also analysed. The phenolic acids tested full fit the rule of 5 (RO5) requisites and, therefore, these molecules can be considered “drug-like” compounds. Then, it was concluded that the four phenolic acids presented antimicrobial activity and a very similar mode of action against the pathogenic bacteria tested. It was proved that when we combine relatively few phenolic acids we have approximately the same effect that when we test that phenolics individually. However, when many molecules were combined, the antimicrobial effect decreased. This fact may be the reason why no plant extract evidenced antimicrobial activity. S didn't show antimicrobial activity neither potentiating effect when combined with the four phenolic acids. In the future, it is recommended to utilize broth dilution methods to determine minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of the tested combinations in order to corroborate these results obtained through the disc diffusion method (DDM). Similarities and differences between hydroxybenzoic and hydroxycinnamic acids should also be better investigated.

CA, FA, GA, PCA and S and their combinations were also tested against *S. aureus* and *E. coli* biofilms'. The results revealed some similarities as well as differences toward the conclusions obtained for planktonic bacteria. It was proved again that when combined relatively few phenolic acids it was obtained approximately the same effect that when that phenolics were tested individually. However, *E. coli* biofilms seemed to be more susceptible to the selected phytochemicals than *S. aureus* biofilms. Additionally, biofilm analysis demonstrated that CA, FA,

GA, PCA and their combinations inactivated sessile bacteria, but don't removed the biofilm. The application of Gompertz model to metabolism data confirmed the sigmoidal appearance of the experimental curves. It is believed that persister cells and the bacteriostatic effect are the two main causes that explain this behaviour. Further experiences regarding the evaluation of sessile cells' membrane integrity and cell surface hydrophobicity (CSH) may help to clarify these aspects.

Summing up, this work provided further information about the important role played by natural occurring molecules on the control of planktonic and sessile pathogenic bacteria. However, phytochemicals also have important properties that enable them to target resistant bacterial strains. Sometimes, phytochemicals act as resistance modifying agents (RMAs), modifying or inhibiting the bacterial mechanism of resistance, so that antibiotics can efficiently kill bacteria. So, it would be interesting to combine these phytochemicals with antibiotics against resistant bacteria like MRSA (methicillin-resistant *S. aureus*) using disc diffusion method, for example. Other suggestion is use acridine orange/ethidium bromide (AO/EB) staining to approach these molecules and their combinations also as efflux pump inhibitors (EPIs), a special kind of RMAs.

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Appendix

A. Overall results of resazurin assay

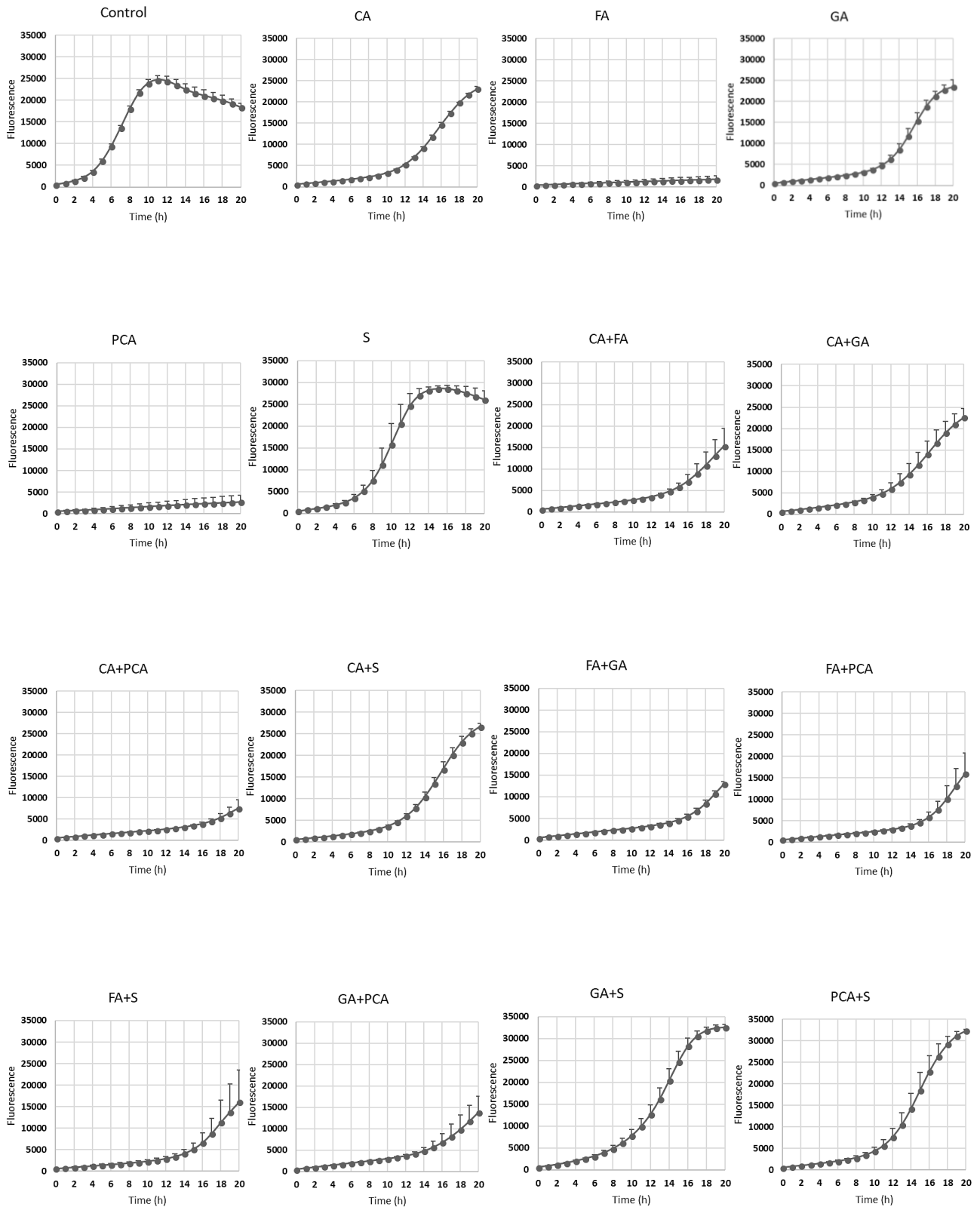


Figure A.1: Fluorescence data of *S. aureus* biofilm cells after 1 h of exposure to the selected phytochemicals and control (DMSO).

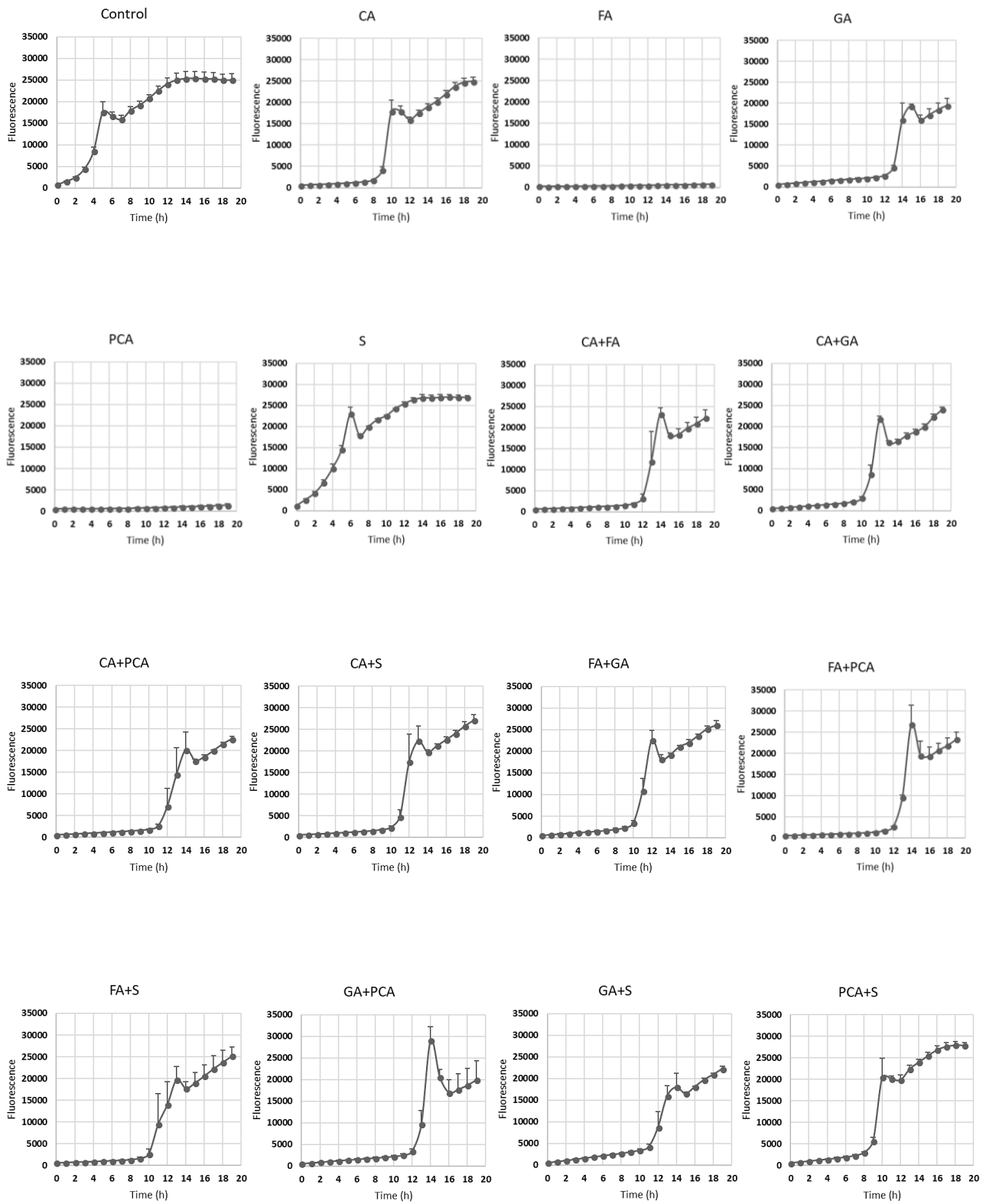


Figure A. 2: Fluorescence data of *E. coli* biofilm cells after 1 h of exposure to the selected phytochemicals and control (DMSO).