Integrated Master in Bioengineering

Phytochemical-based strategies for the treatment of chronic wound biofilms

Dissertation for Master's degree in Bioengineering
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Abstract

Chronic wounds (CWs) represent a heavy burden for the healthcare system and have a negative impact on the quality of life of patients. They are often characterized by an impaired healing, which is mostly related to the establishment of bacterial biofilms. This is particularly problematic since the bacteria embedded in biofilms have been shown to be 10-1000 times more resistant to antimicrobial agents than their planktonic counterparts. Moreover, the increasing bacterial resistance to conventional antibiotics is one of the major causes of failure in the treatment of CWs. Thus, more efficient antimicrobial agents are urgently needed. Within this context, phytochemicals, in particular essential oils (EOs), have already proven to be interesting compounds in the antimicrobial field, making them promising candidates to prevent and control biofilms. They can also circumvent the emergence of resistant bacteria due to their multiple modes of action. Therefore, different phytochemical-based strategies to deal with biofilms were studied in the present work.

The antimicrobial and antibiofilm potential of selected phytochemicals (citronellol, cis-6-nonen-1-ol, 3,7-dimethyl-1-octanol and citronellic acid) alone and in combination with standard antibiotics (gentamicin, mupirocin and fusidic acid) against *Staphylococcus aureus*, including a methicillin-resistant strain (MRSA), and *Escherichia coli* was studied. Based on the determination of the minimum bactericidal concentration, it was found that citronellol presented the highest antimicrobial activity against *S. aureus* and cis-6-nonen-1-ol displayed the highest antimicrobial activity against MRSA and *E. coli*. According to the Chick-Watson model, citronellol and cis-6-nonen-1-ol interacted chemically with cell targets of all bacteria, except citronellol that stablished physicochemical interactions with *S. aureus*. Synergism between antibiotics and phytochemicals was found against both planktonic and sessile bacteria, demonstrating the potential of this combinatorial strategy to increase the antimicrobial and antibiofilm activity. EOs are unstable and susceptible to degradation when exposed to environmental stresses. Therefore, the encapsulation of these natural compounds into drug delivery formulations could also be a good strategy to enhance their antibiofilm activity, ensuring their increased bioavailability in the infected site. In this sense, chitosan beads loaded with citronellol and cis-6-nonen-1-ol were tested on the prevention and control of *E. coli*, *S. aureus* and MRSA biofilms. These loaded beads exhibited equal or enhanced antibiofilm activity compared to pure compounds. Overall, these results revealed the prominent antimicrobial and antibiofilm properties of citronellol and cis-6-nonen-1-ol against pathogenic bacteria commonly encountered in infected CWs. Their combination with conventional antibiotics or encapsulation into chitosan beads may be promising approaches for dealing with biofilm infections in CWs.

Keywords: antibiofilm; antibiotics; antimicrobial; chitosan beads; chronic wounds; phytochemicals.

Resumo

As feridas crónicas representam um fardo para o sistema de saúde e têm um impacto negativo na qualidade de vida dos pacientes. Estas são frequentemente caracterizadas por uma cicatrização prejudicada, que está principalmente relacionada com a formação de biofilmes bacterianos. Isto é particularmente problemático, uma vez que as bactérias na forma de biofilmes demonstraram ser 10-1000 vezes mais resistentes aos agentes antimicrobianos do que as mesmas bactérias em estado planctónico. Além disso, o aumento da resistência bacteriana aos antibióticos convencionais é uma das principais causas da falha no tratamento das feridas crónicas. Assim, agentes antimicrobianos mais eficientes são urgentemente necessários. Nesse contexto, os fitoquímicos, em particular os óleos essenciais (EOs), já mostraram ser compostos interessantes na área dos agentes antimicrobianos, tornando-os candidatos promissores na prevenção e no controlo de biofilmes. Eles também podem contornar o surgimento de bactérias resistentes devido aos seus múltiplos modos de ação. Assim, no presente trabalho, foram estudadas diferentes estratégias baseadas em fitoquímicos para lidar com biofilmes.

O potencial antimicrobiano e antibiofilme de fitoquímicos (citronelol, cis-6-nonen-1-ol, 3,7-dimetil-1-octanol e ácido citronélico) individualmente e em combinação com antibióticos convencionais (gentamicina, mupirocina e ácido fusídico) foi estudado em *Staphylococcus aureus*, incluindo uma estirpe resistente à meticilina (MRSA), e *Escherichia coli*. Com base na determinação da concentração bactericida mínima, constatou-se que o citronelol apresentou maior atividade antimicrobiana contra *S. aureus* e o cis-6-nonen-1-ol mostrou maior atividade contra MRSA e *E. coli*. De acordo com o modelo Chick-Watson, o citronelol e o cis-6-nonen-1-ol interagiram quimicamente com alvos celulares de todas as bactérias, exceto o citronelol que estabeleceu interações físico-químicas com *S. aureus*. Foram encontrados efeitos sinérgicos entre os antibióticos e os fitoquímicos tanto contra bactérias no estado planctónico como no estado séssil, demonstrando o potencial desta estratégia para aumentar a atividade antimicrobiana e antibiofilme. Os EOs são instáveis e suscetíveis à degradação com a temperatura e a exposição à luz. Desta forma, o encapsulamento desses compostos naturais em formulações de liberação de fármacos também pode ser uma boa estratégia para aumentar a sua atividade antibiofilme, garantindo a sua maior biodisponibilidade no local infetado. Nesse sentido, um suporte de quitosano contendo citronelol e cis-6-nonen-1-ol imobilizado foi testado na prevenção e no controlo de biofilmes de *E. coli*, *S. aureus* e MRSA. Este sistema teve atividade antibiofilme igual ou mesmo superior ao efeito dos compostos em solução. Em geral, estes resultados revelaram as propriedades antimicrobianas e antibiofilme proeminentes do citronelol e do cis-6-nonen-1-ol contra bactérias patogénicas comummente encontradas em feridas crónicas. A sua

combinação com antibióticos convencionais ou o seu encapsulamento num suporte de quitosano podem ser abordagens promissoras para lidar com biofilmes em feridas crónicas.

Declaration

Declara, sob compromisso de honra, que este trabalho é original e que todas as contribuições não originais foram devidamente referenciadas com identificação da fonte.

Declare, under oath, that this work is original and that all non-original contributions were properly referenced with the source identification.

September 24th, 2021

Maria Beatriz Silva

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Glossary

ADD	Additive
ANT	Antagonism
CA	Citronellic acid
CAGR	Compound Annual Growth Rate
CECT	Spanish Type Culture Collection
CFU	Colony forming unit
CIS	Cis-6-nonen-1-ol
CITRO	Citronellol
CLSI	Clinical and Laboratory Standards Institute
CS	Chitosan
CW	Chronic wound
DDS	Drug delivery system
DMSO	Dimethyl sulfoxide
EO	Essential oil
EPS	Extracellular polymeric substance
FBCI	Fractional bactericidal concentration index
FDA	Food and Drug Administration
FUS	Fusidic acid
GEN	Gentamicin
IND	Indifference
LPS	Lipopolysaccharide
MBC	Minimum bactericidal concentration
MDR	Multidrug resistant
MHB	Mueller–Hinton broth
MRSA	Methicillin-resistant *S. aureus*
MSSA	Methicillin-sensitive *S. aureus*
MUP	Mupirocin
NA	No activity
NP	Nanoparticle
OM	Outer membrane
PCA	Plate count agar
PHMB	Polyhexamethylene biguanide
QS	Quorum sensing
RO5	Rule of five

SD	Standard deviation
SYN	Synergy
TPP	Tripolyphosphate
VBNC	Viable but nonculturable
WHO	World Health Organization
3,7DOC	3,7-Dimethyl-1-octanol

Chapter 1 | Work outline

1.1 Background and project presentation

Chronic wounds (CWs) are a progressively common type of skin injury that affects a large fraction of the world [1]. They represent a significant economic burden for the healthcare system and are related to high rates of morbidity and mortality [2]. Most CWs contain bacteria living within a biofilm that prolong the inflammatory phase of wound healing [3]. Biofilm treatment has been increasingly troubled due to the antibiotic use and abuse in recent years, which has promoted the emergence of resistant bacteria [4]. In fact, the antibiotic resistance crisis is one of the biggest problems that global public health is facing, being expected to become more lethal than cancer by 2050 if no alternative therapies are found [5]. In addition, biofilms are known to be 10-1000 times more resistant to antibiotics than their planktonic counterparts [6]. Therefore, it becomes imperative to search for new antibiofilm strategies to treat infected CWs.

Phytochemicals (bioactive compounds from plant metabolism), in particular essential oils (EOs), used alone or in combination with antibiotics have been thoroughly investigated as a means to circumvent the emergence of resistant bacteria due to their multiple action mechanisms [7]. Besides that, they are an attractive source of eco-friendly, relatively inexpensive, and broad-spectrum antimicrobials [8]. Furthermore, the encapsulation of EOs into drug delivery formulations may be responsible for several advantages, as for instance improve their solubility, stability and bioavailability, as well as reduce doses of these natural compounds with controlled release levels over an extended period of time [9].

1.2 Main objectives

The overall objective of this thesis focuses on discovering new strategies based on phytochemicals for the topical treatment of biofilms in CWs. For the achievement of this general objective, a set of sub-objectives were proposed: i) investigate the antimicrobial potential of selected phytochemicals, in particular EOs components, against *S. aureus*, including a MRSA, and *E. coli* in planktonic form, which are bacteria that normally occur in patients with infected wounds; ii) understand the properties of the tested phytochemicals, taking into account their chemical structure, which provide them antimicrobial activity; iii) assess the antimicrobial efficacy of selected dual combinations of phytochemicals and antibiotics, commonly used to treat skin infections, against planktonic *S. aureus*, MRSA, and *E. coli* in order to discover synergistic effects; iv) evaluate the antibiofilm potential of selected phytochemical-antibiotic combinations on the control of pre-established biofilms of *S. aureus*, MRSA, and *E. coli* since biofilms are a major concern in infected

CWs and v) evaluate the antibiofilm capacity of chitosan beads loaded with selected EOs components on the prevention and control of biofilms of *S. aureus*, MRSA, and *E. coli*.

1.3 Thesis organization

This thesis is divided into 6 Chapters. In Chapter 1, the context, motivation and main goals for the development of this study are presented. Furthermore, this chapter provides a guideline of the work described in the following chapters.

Chapter 2 consists in a short review of the literature. In this chapter, the problem of CWs as well as the strong impact of biofilms as the main responsible for their delayed healing are highlighted. The stages involved in biofilm development in the wound and the problem of the higher resistance associated with bacterial growing in biofilms are also summarized. Additionally, the current approaches used to treat these biofilms in CWs are described, showing that the repeated and improper usage of conventional antibiotics can initiate bacterial resistance that leads to an urgent demand for discovering new healing substitutes. Plant-based products (phytochemicals), in particular EOs and their components, are introduced as an alternative antimicrobial approach to conventional antibiotics. At last, hydrogel-based drug delivery systems, composed particularly of chitosan, are presented as a good strategy to avoid the degradation of EOs, increasing their bioavailability.

Chapter 3 describes the results of the antimicrobial activity of selected EOs components, particularly citronellol, cis-6-nonen-1-ol, 3,7-dimethyl-1-octanol and citronellic acid, and their combinations with traditional antibiotics, namely gentamicin, mupirocin and fusidic acid, against *S. aureus*, MRSA, and *E. coli* in planktonic state. The minimum bactericidal concentration of these compounds and dose-response curves for citronellol and cis-6-nonen-1-ol were determined. The interactions between phytochemicals and antibiotics were evaluated by the determination of fractional bactericidal concentration index. In this chapter, a structure activity relationship study and drug-likeness of the phytochemicals by Lipinski's rule of five were also applied.

Chapter 4 focuses on the study of the best phytochemical-antibiotic combinations, obtained in Chapter 3, on the control of biofilms of *S. aureus*, MRSA and *E. coli* by biofilm cells culturability method.

Chapter 5 provides the study of the antibiofilm activity of chitosan beads loaded with phytochemicals citronellol and cis-6-nonen-1-ol. The ability of these beads to control and prevent biofilms of *S. aureus*, MRSA and *E. coli* was assessed also by biofilm cells culturability method.

Finally, in Chapter 6, the main conclusions of the work, some recommendations/perspectives for future research and thesis outputs are mentioned.

Chapter 2 | Literature review

2.1 Chronic wounds

A wound may be defined as damage or disruption to the normal anatomic structure of the skin cells and a disturbance to its function in connecting and protecting underlying tissues and organs [10,11]. Wounds can be caused by surgery, accidental blow or cut, chemicals, friction shear force, extreme temperatures, pressure, electrical current, or as a result of underlying pathologies, such as diabetes, immunological or dermatological diseases and chronic venous/arterial insufficiency [11–13].

The largest organ of the human body and the most exposed to injuries is, in fact, the skin [14]. The skin consists of three main multi-histological layers: epidermis, dermis and subcutaneous tissue, and skin adjuncts like hairs and glands [14]. When the epithelium and connective structures are damaged, the human body starts a dynamic and complex biological process called wound healing, which involves a cascade of carefully regulated steps that can be divided into four main stages: hemostasis/coagulation, inflammation, proliferation and restoration (Figure 1) [14,15].

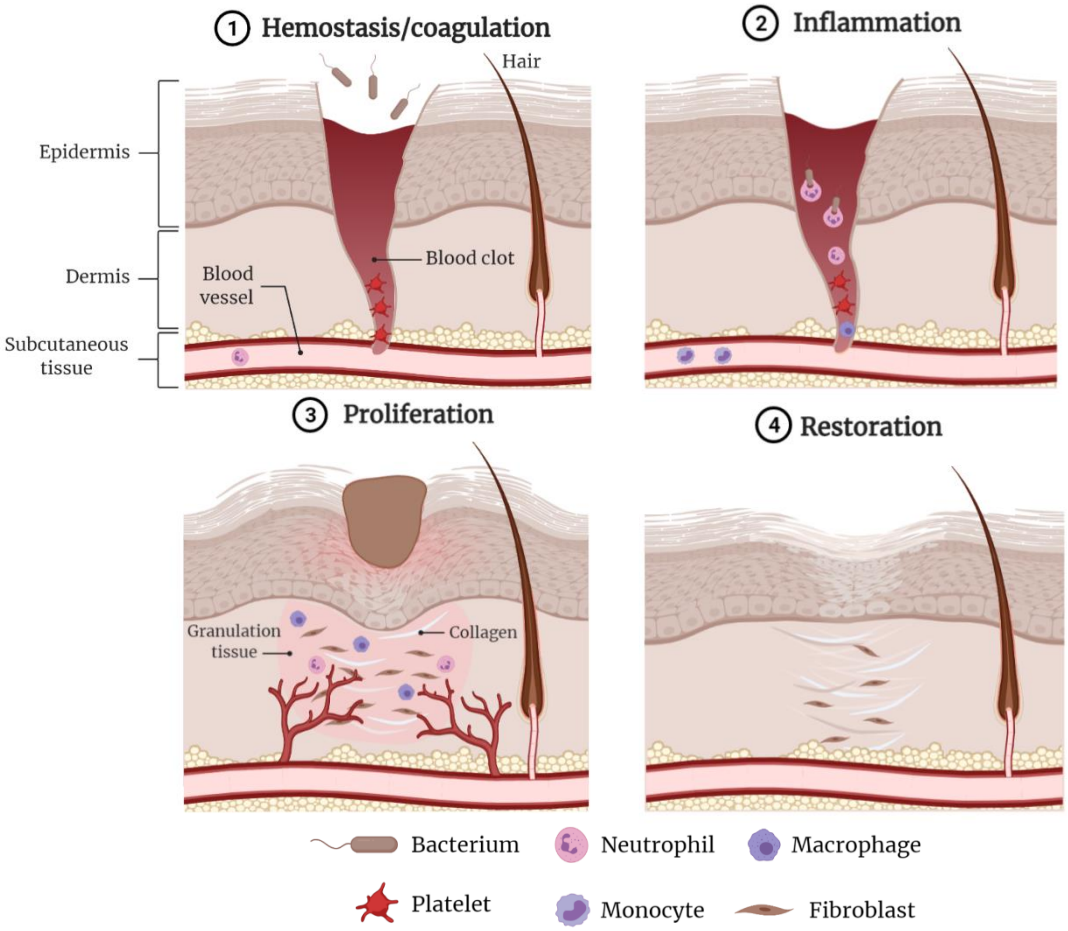


Figure 1. Schematic representation of the four steps of wound healing. Adapted from Negut *et al.* [14].

Coagulation and hemostasis take place immediately after damage [15]. This first stage of wound repair aims to prevent exsanguination by platelet aggregation and clot formation [15,16]. Furthermore,

the blood clot and platelets trapped within it also provide a matrix for invading cells required in the subsequent phases of healing [15]. The platelets secrete several growth factors that initiate the wound healing cascade by attracting and activating neutrophils, macrophages, fibroblasts, and endothelial cells [15,16]. The inflammatory phase begins at the same moment as hemostasis, but it may last for up to 3 days [11]. The main purpose of this phase is to clear pathogens and foreign material from the wound [17]. Firstly, neutrophils infiltrate the wound site and start the mechanism of phagocytosis in order to prevent infection [15]. They destroy bacteria by releasing proteolytic enzymes and free radicals species derived from oxygen [16]. In the later stages of the inflammatory phase, macrophages (blood monocytes that undergo phenotypic changes) appear in the wound and act as the key regulatory cells of repair [15,16]. They not only phagocytose and digest tissue debris and remaining neutrophils, but also provide a reservoir of tissue growth factors that promote tissue proliferation [15–17]. The proliferative phase starts about 3 days after the initial injury and lasts for approximately 2–4 weeks [16]. This step centers around fibroblast migration, deposition of the extracellular matrix constituted by fibrous structural proteins (elastin, collagen) and an interstitial matrix and formation of granulation tissue [16]. Endothelial cells enter a phase of fast growth and angiogenesis occurs within the granulation tissue, forming a rich vascular network that is essential to keep an adequate blood supply within the wound bed [11,17]. The final stage of the proliferative phase is the epithelialization of the wound, which is characterized by the migration of the epithelial cells over the provisional matrix formed [18]. Finally, the restoration phase takes place and may possibly last up to 1 or 2 years [15]. In this stage, a final scar tissue, characterized by a mature avascular and acellular environment, is formed [19]. This phase is responsible for the achievement of the maximum tensile strength through reorganization, degradation, and resynthesis of the extracellular matrix [19]. However, the original strength of the tissue can never be recovered. In fact, the healed skin can only achieve about 80% of the original strength [20].

Wounds can be classified as acute and chronic [14]. Acute wounds are these that heal through the normal phases of wound repair above-mentioned, resulting in the restoration of anatomic and functional integrity [10,14]. On the contrary, chronic wounds (CWs) fail to proceed through the natural phases of wound healing and normally stall in the inflammation stage [21]. Regarding the duration, there is no consensus between the authors. Some authors consider that if the wound does not progress to healing or shows no sign of recovery within 12 weeks (3 months), it is considered a CW [11,22,23]. However, others consider a wound to be chronic if it fails to show any signs of healing in 4 to 6 weeks [1,24,25]. Common CWs include diabetic ulcers, pressure ulcers, vasculitic ulcers, venous stasis ulcers, and chronic nonhealing wounds resulting from trauma or dehisced surgical wounds [26].

The delayed healing of CWs can be attributed to many factors, which can be categorized into local (external influences) and systemic (internal factors) [27]. Systemic factors are associated with the

patient's general condition, such as age and gender, medications, poor nutrition, obesity, stress, and underlying comorbidities like diabetes, fibrosis, jaundice, etc. [27]. On the other hand, local factors are related to the circumstances in the wound and include, for instance, poor oxygenation, poor blood supply, foreign bodies and infection [27]. In fact, bacterial colonization and the consequent infection of the wound is the most frequent and unavoidable impediment to wound healing [14].

CWs correspond to a silent epidemic that affects a significant part of the world [28]. In developed countries, it is even estimated that 1 to 2% of the population will face a CW during their lifetime [29]. They represent a severe problem, not only because of their negative effect on the quality of life of patients but also because they represent a substantial burden for the healthcare system [2]. Furthermore, this burden grew considerably in recent years due to population aging and the increasing incidence of obesity and chronic diseases [30]. For instance, CWs cost the medical system approximately $50 billion per year in the United States [31]. Wound healing is, actually, an enormous commercial enterprise, with the global market for wound care products projected to reach $18.7 billion by 2027, growing at a Compound Annual Growth Rate (CAGR) of 6.6% over the analysis period 2020–2027 [32]. Based on these facts, it is clear that CWs are an extremely serious health care problem and their incidence is constantly growing. Thus, wound research is an important field to find new therapies, diagnostics, clinical practices and procedures with the goal of promoting wound healing and combat this epidemic.

2.2 Biofilms in chronic wound infections

Wound infection occurs when the number of microbes, mainly bacteria, or their virulence is sufficient to cause a host response locally and/or systemically [33]. As long as the wound is infected, it will revert or remain in the inflammatory phase of the healing process [21]. The wound infection continuum characterizes the progression of an infection in a wound and is described by the gradual increase in the number and virulence of microorganisms, together with the response they invoke within the host (Figure 2) [33,34]. According to the International Wound Infection Institute, the wound infection continuum can be divided into five stages: contamination, colonization, local infection, spreading infection and systemic infection [33]. Contamination is characterized by non-proliferating microorganisms in the wound that do not promote the immune response or the delayed healing [33,34]. On the other hand, colonization is represented by the existence of proliferating microorganisms within the wound, but they are not still in enough levels or virulence to impede wound healing or provoke a host response [33,34]. When bacteria move deeper into the wound and proliferate at a faster rate triggering a host reaction, a local wound infection has occurred. This infection is localized in a structure or system and is characterized by delays in wound healing [33,34]. Spreading infection occurs when pathogens proliferate beyond the wound border, including surrounding and deeper tissue,

muscle or even organs [33,34]. At last, systemic infection represents the most advanced phase that affects the whole body via vascular or lymphatic systems [33,34].

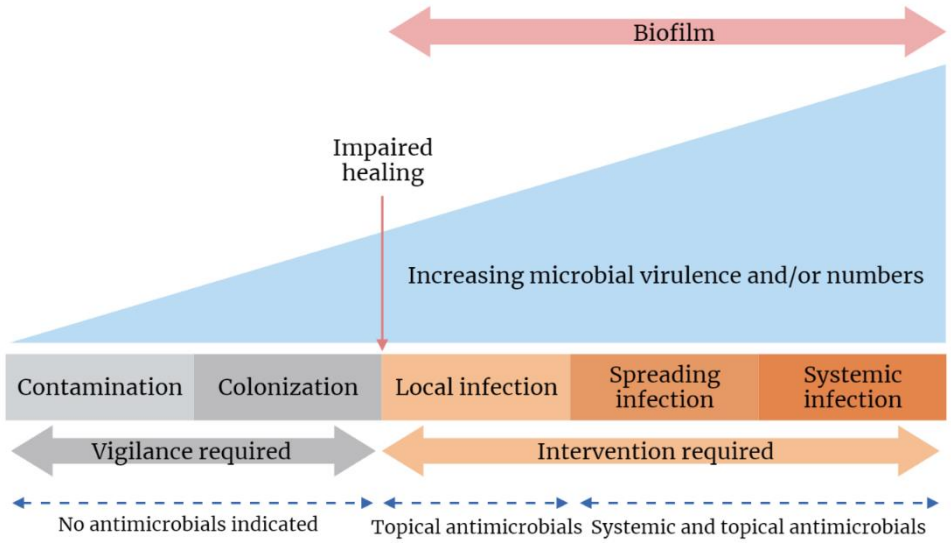


Figure 2. Wound infection continuum. Adapted from Swanson *et al.* [33] and Farhan and Jeffery [35].

As a matter of fact, all open skin wounds contain bacteria (bioburden), however a critical threshold of existing bacteria has been proposed as the delineation between colonization and infection [14,17,36]. This threshold depends on the type and number of bacterial species present and the status of the host immune system, but most studies suggest loads between 10^4 CFU/g and 10^5 CFU/g (CFU - colony forming unit) [35,37]. The wounds are usually contaminated by bacteria from the surrounding skin, endogenous patient sources and the local environment [37]. Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria correspond to the four major genera of bacteria found in the normal skin flora [14]. CWs have a complex colonizing flora, containing multiple diverse species, which alter over time. In fact, Cowan proposed that it is not uncommon for 60 distinct types of microorganisms to be in a wound [38]. At the beginning of CW development, Gram-positive microorganisms, such as coagulase-negative staphylococci, *Corynebacterium* spp, *Streptococcus* spp and *S. aureus*, are predominant. In the later phases, Gram-negative microorganisms, such as *Pseudomonas* spp, *Proteus* spp and *E. coli*, are common and have a propensity to invade deeper layers in the wound causing considerable tissue damage [11,39]. Normally, once oxygenation of the wound environment decreases, the anaerobes proliferate more, being found in higher numbers than aerobes in CWs [40]. The colonization with resistant microorganisms, including methicillin-resistant *S. aureus* (MRSA) or vancomycin-resistant enterococci, can also occur, mostly due to hospitalization, surgical procedures and prolonged antibiotic therapy [37].

Analogous to humans and other social beings, microbes do not live in isolation [41]. In natural ecosystems, bacteria live preferentially on surfaces and assemble into complex aggregated

communities known as biofilms [42]. Biofilms have been defined as a community of microorganisms attached to a substratum or interface or to each other in which cells are frequently embedded in a self-produced matrix of extracellular polymeric substances (EPSs) [43]. There are several possible advantages imparted by the biofilm mode of growth that explains the preference of bacteria to form biofilms, which include: higher protection against environmental stresses, predators and antimicrobial agents; improved access to nutrients; reduce the possibility of dehydration; closer proximity between cells, enabling mutualistic or synergistic associations and amplified expression of beneficial genes [7].

The concept of biofilm infections and their importance in medicine was only introduced in the early 1970s and the term biofilm was then instituted in 1985 by J. W. Costerton [44]. In fact, it is now estimated by the National Institutes of Health that biofilms occur in 80% of all known infections [45], including catheter infections, urinary tract infections, rhinosinusitis, osteomyelitis, endocarditis, cystic fibrosis lung infection, ocular infections, infections related to surgical implants/indwelling medical devices and CWs [6]. It is now well recognized that bacteria in infected CWs occur often in the form of biofilms [27] and it is even estimated that more than 90% of CWs carry bacteria living cooperatively in highly organized biofilms [3].

Even though the evident crucial role of bacterial biofilms in infectious diseases, the studies are mostly developed with cells in the planktonic state (free-living bacteria) [46]. However, in the last years, the literature on biofilms associated with CWs has increased in terms of publications (Figure 3). Performing the following term search on PubMed: “biofilm AND chronic wounds”, there are only 6 studies available on the database published between 1988 and 1999. Nevertheless, from 2000 until 2020, the number of publications per 2 years has been increasing considerably. This may represent a recent increase in the importance given to the role of biofilms in CW infections, as they are more and more recognized and understood.

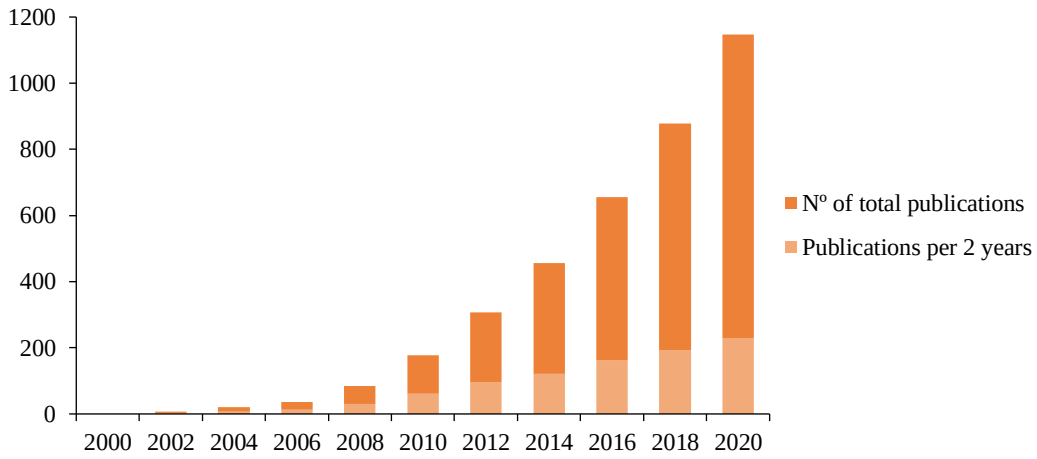


Figure 3. Cumulative and 2 year publications of biofilms in CWs in the PubMed database.

Biofilm formation is a dynamic process often described as multi-stage and it can be formed within only 24 h in a wound [47]. In fact, its formation in wounds is boosted by the hostile environment created by the presence of necrotic tissue, foreign material, and bacteria that render inert or destroy the building blocks like chemotactants and growth factors responsible for the normal healing of the wound [3]. The biofilm development can be influenced by diverse factors, such as bacteria strain, surface properties, pH, temperature, nutrient levels, humidity, hydrodynamic conditions, among others [48]. Commonly, biofilm formation involves five main stages (Figure 4): (1) reversible attachment; (2) irreversible attachment; (3) cell proliferation; (4) biofilm maturation and (5) detachment [49].

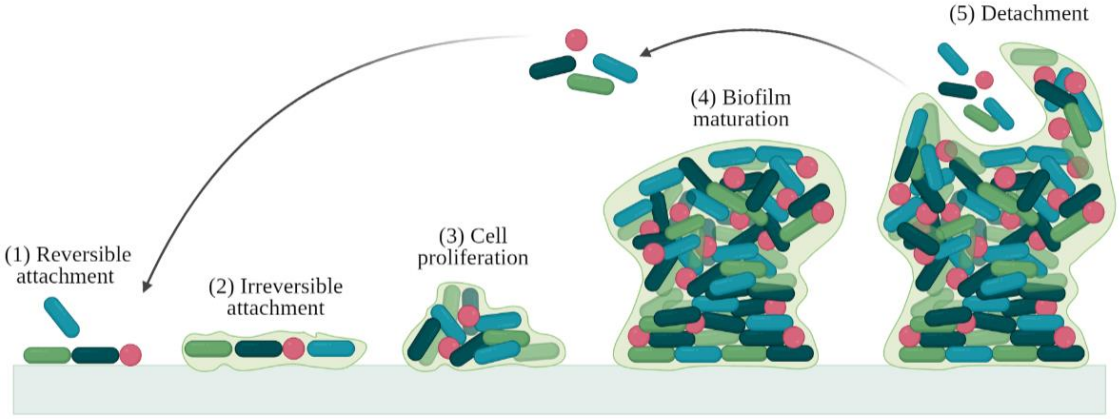


Figure 4. Sequence of the main steps involved in biofilm formation. Adapted from Monroe [49].

First, planktonic bacteria reversibly attach to the wound (biotic surface) and this attachment is influenced by the properties of the cell surface (e.g., presence of flagella, pili, fimbriae, or glycocalyx) and the substratum itself [50]. Initial physical contact of bacteria to biotic surfaces will face repulsive electrostatic forces and the cell surface properties may be important to overcome this force [51]. Then, the weak interactions turn into a permanent bond and the bacteria start to form a monolayer and produce a matrix of EPSs [52,53]. This matrix is mainly composed of polysaccharides, proteins, glycolipids and bacterial DNA, however, its exact composition varies according to the microorganisms present and the growth conditions [54]. Once the bacteria are irreversibly attached to the wound, they start to multiply and form microcolonies that exhibit significant cell-cell communication, a phenomenon recognized as quorum sensing (QS) [52,53]. QS is a communication process between bacterial cells involving the production, detection and response to extracellular signaling molecules known as autoinducers. Thus, this system allows bacteria to obtain information about the environment, inhabit inhospitable environments, verify population density and, mainly, regulate gene expression [55]. As cells replicate and the EPSs accumulate, a fully mature biofilm (three-dimensional structure) is formed and is characterized by a permeate structure that contains a network of channels and pores [52,53]. At this stage, host defenses are insufficient to eradicate the biofilm, but recognize its presence by inappropriate excessive recruitment of neutrophils, pro-inflammatory

cytokines, and excessive host-derived proteases. This leads to tissue destruction and increased capillary permeability, which in turn provides nutrition to the biofilm, resulting in the interruption of the healing process in the inflammatory phase [33]. Lastly, a small cluster of bacteria is isolated from the biofilm, being able to disperse and attach to other sections of the wound, forming new biofilm colonies [47,54]. These detached cells can even spread the infection to other locations [52].

Currently, there is no specific clinical manifestation for the diagnosis of biofilm in wounds. The presence of biofilms is not easily detectable since they are often less than 100 μm in size, not being possible to see them by naked eye [47]. Clinicians have used some visual "signs" to identify the presence of biofilm in wounds, such as shiny, translucent and slimy layer on the non-healing wound surface [54]. However, the World Union of Wound Healing Societies states that "all non-healing CWs potentially harbor biofilms", so doctors should assume that "all non-healing, CWs that have failed to respond to standard care have biofilms" [56]. Thus, treatments should be directed towards the effective disruption of biofilms and preventing their formation and reseed [56].

2.3 Biofilm treatment of chronic wounds

The treatment of biofilm infections in CWs is challenging because, as already mentioned, biofilm-associated bacteria can withstand host defenses, antimicrobials and other stresses more easily than the analogous free-living bacteria [6]. Bacteria embedded in biofilms can be 10-1000 times more resistant to antimicrobial agents than their planktonic counterparts [57]. Indeed, susceptible bacteria that do not have a known genetic basis for resistance can still be highly resistant when they are in a sessile form [58]. This resistance has been associated with several factors. One of the main factors is the difficulty of penetration of antimicrobial agents into the biofilm since it is a complex and compact structure made up of multiple layers of cells and the matrix of EPSs, resulting in a decreased intracellular concentration of the antimicrobials [59]. The slow or stationary growth of cells in the biofilm, a consequence of the altered metabolic activity by the decrease of oxygen and the nutrient gradient that exists on the deeper layers, can also reduce the antimicrobial susceptibility of bacteria in biofilms [58]. This is a problem for the action of antimicrobial agents since the effects of most agents depend on active metabolism and bacterial growth [58]. Another relevant factor is the formation of persister cells (dormant variants of regular cells), which are highly tolerant to antimicrobial agents also due to the fact that antimicrobials generally work on growing cells [60]. Other hypotheses for this resistance can be associated with chemical interactions between the antimicrobials and biofilm constituents (e.g., the EPSs matrix) or the ability of cells to execute programmed apoptosis when subjected to antimicrobials [61]. The release of nutrients from the dead cells would allow the community to grow rapidly after adverse conditions [61]. Furthermore, the rate of mutation in biofilms is substantially more recurrent in comparison to planktonic cells [58]. The horizontal gene transmission is

even promoted by biofilms, as they form an ideal niche for the exchange of genetic material, allowing the acquisition of antimicrobial resistance genes [58].

The concept of wound bed preparation has achieved international recognition as a structured approach to wound care in order to accelerate endogenous healing or to facilitate the efficacy of other therapeutic measures [62]. Therefore, the "TIME" (Tissue management; Infection/Inflammation control; Moisture balance; Edge advancement) acronym was created as a practical guide for assessing a wound correctly and formulating a treatment plan [62]. By this approach, the main therapeutic strategies options to treat biofilms in CWs include debridement and adjunctive use of antimicrobial/antibiofilm compounds [40].

The first key step to remove the biofilm is wound debridement, as this allows for the physical disruption of the biofilm [47,54,63]. Since biofilm resistance increases with its maturation, any action that physically disrupts it can generate a "window of opportunity" [64]. Bacteria return to their vulnerable planktonic state, leading to a possible improvement of antimicrobial susceptibility post-debridement [64]. This clinical practice also removes inactivated, poor healing or necrotic tissue and foreign bodies, which offer an attachment point for biofilm formation and consequent delayed wound healing [47]. Therefore, wound debridement prepares the wound bed for re-epithelialization [65]. This can be achieved through surgical, autolytic, enzymatic, biological or mechanical mechanisms [66]. In the surgical debridement, the inactive tissue is removed using sharp instruments that can lead to adverse events like bleeding or general complications from the anesthesia [65]. Hydrosurgical debridement is a type of surgical debridement that causes less pain for the patient and it is based on liquid jet technology to remove bacteria, tissue fragments, etc. [47]. The autolytic debridement is a body's natural defense process by which endogenous phagocytic cells and proteolytic enzymes break down necrotic tissue [65]. On other hand, the enzymatic debridement method uses exogenous proteolytic enzymes, such as collagenase, which digests the collagen in the necrotic tissue [65]. The biological debridement is associated with the application of larvae/maggots that release proteolytic enzymes containing secretions and excretions, responsible to dissolving necrotic tissue [65]. This technique does not affect the healthy tissue around the wound and it is a painless debridement method [47]. Lastly, mechanical debridement is associated with the application of a mechanical force that will remove both devitalized and viable tissues [65]. Wet-to-dry dressing and pressure irrigation are examples of this type of debridement [66]. The debridement method to be applied to each patient must take into account several factors, from the type and condition of the wound to the skills of the clinician and the available resources [66]. Furthermore, the use of topical surfactant-based wound cleansing solutions may improve the debridement process by lowering the surface tension between a liquid and a solid and aid removal [54].

Another approach to treat infected CWs is the use of antimicrobial agents commonly incorporated into dressings used against biofilms to prevent reformation or as primary bactericidal agents [54,67]. Therefore, they can be used not only to prevent the biofilm reformation after debridement, but also to kill microbial cells of the residual biofilm that may still exist [54]. In fact, debridement does not necessarily remove all biofilm and prevent its recurrence, as the microbial cells dispersed into the wound are able to quickly reattach to the wound surface, allowing the biofilm to regenerate rapidly [40,63]. However, it is important to note that these antimicrobial wound dressings are not intended to remove a spreading infection [14]. When the infected area spreads beyond the wound border, involving underlying bone or tissues, or becoming a systemic infection, systemic antibiotic treatment is needed [67]. Even so, in these cases, wound dressings that contain antimicrobial agents can be used in conjunction with systemic antibiotics as an adjunctive therapy (see Figure 2) [68].

Wound dressings have evolved over the years, from crude applications of animal fat, plant herbs and honey to tissue engineering scaffolds [69]. There are many types of dressings available today that can integrate antimicrobial compounds, such as gauzes, alginates, foams, hydrogels, hydrocolloids, films, etc. [17]. The different types and characteristics of the wounds and the fact that there is no dressing that can be suitable in all circumstances contributed to this wide range of wound dressings [14]. These dressings must provide a moist environment, maintain an appropriate tissue temperature to improve the blood flow, promote angiogenesis and tissue renewal processes, be semi-permeable to oxygen and water, enhance epidermal migration, be biocompatible and, at the same time, be a barrier to the entry of harmful external bacteria [14,70]. In addition, they should be easy to remove after healing and inexpensive [14,70].

Despite the lack of evidence regarding the efficacy, optimal regimens or clinical indications for treatment with antibiotics, several topical formulations of antibiotics have been developed to apply to the wound site [17,71]. The main classes of antibiotics that have been used to produce antimicrobial wound dressings are tetracyclines, quinolones, aminoglycosides and cephalosporins [14]. Antibiotics interact very specifically with some unique features of the bacterial structure or their metabolic processes [72]. Different antibiotics have different modes of action, including breakdown of cell membrane structure or function, inhibition of cell wall synthesis, inhibition of nucleic acids structure and function, blocking of key metabolic pathways or inhibition of protein synthesis [73]. This selectivity of antibiotics increases the probability of bacteria developing mechanisms to escape their effects and, consequently, develop resistance [74]. Although antibiotics are one of the main weapons for the control of biofilms, high concentrations are often needed, which remain ineffective, leading to the emergence of resistant strains and the occurrence of dose-dependent cytotoxic effects in patients [6]. It is even

estimated that about 70% of bacteria that trigger wound infections are resistant to minimum one of the most frequently used antibiotics [75].

Bacterial resistance to antibiotics is a well-established and widely studied worldwide problem and is responsible for about 700 000 deaths each year [76]. This resistance can be intrinsic or obtained via mutations in chromosomal genes and by horizontal gene transfer (transduction, conjugation, transformation) [58]. The misuse and overuse of antibiotics, as well as poor infection prevention and control, favors the selection of resistant strains and the consequent transmission of their resistant features to succeeding generations [4]. There are several mechanisms that provide resistance to bacteria, such as increased efflux pumps expression, prevention of the access to the target by reducing the permeability, degradation/modification of the antibiotics by enzymatic hydrolysis, group transfer or redox process and modification of the molecular target [58]. These main mechanisms are illustrated in Figure 5. Moreover, there is variation in the types of mechanisms used by Gram-positive and Gram-negative bacteria because of the differences in their structure. For instance, the lipopolysaccharides (LPSs) layer in Gram-negative bacteria offers a barrier to some types of molecules, giving those bacteria innate resistance to certain antimicrobial agents [77].

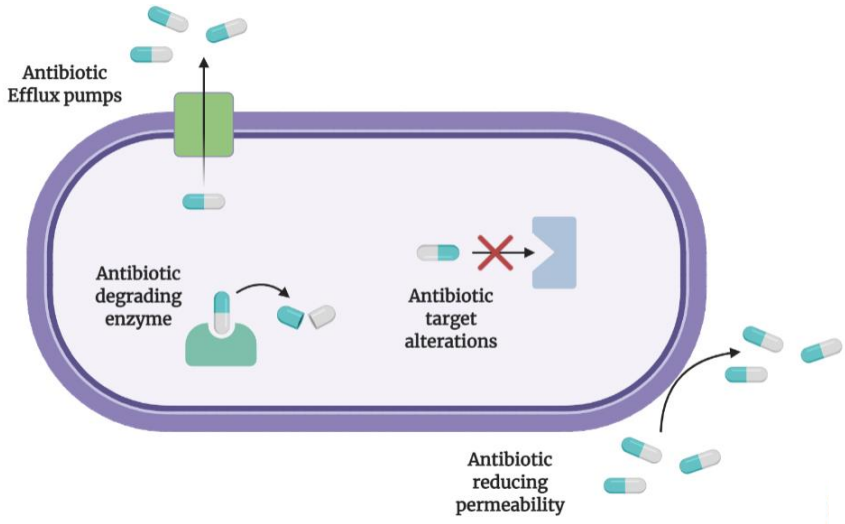


Figure 5. Main mechanisms that provide bacterial resistance to antimicrobial agents. Adapted from Reygaert [77].

The development of new antibiotics by the pharmaceutical industry has slowed over the last years due to challenging regulatory requirements and reduced economic incentives [78]. In fact, the number of new antibiotics approved by the Food and Drug Administration (FDA) has been declining since the 1980s [76]. Therefore, because of this antibiotic crisis, it is imperative to find new therapeutic agents outside the constricted antibiotics box to control biofilm infections and promote wound healing.

Currently, there are several wound dressings available on the market with antimicrobial agents used to treat infected wounds as a viable alternative to traditional antibiotics, which are summarized in Table 1. Unlike antibiotics, these antimicrobial dressings employ a broad-spectrum of non-selective antibacterial action, acting at multiple sites within microbial cells, thus lowering the probability of bacteria developing resistance [74]. However, it is important to note that some traditional antimicrobials already used in the treatment of wounds, like iodine, exhibit relatively high cytotoxicity against human cells, leading to an increasing pressure to replace these antimicrobials for others with low levels of cytotoxicity [74,79]. Furthermore, the incorporation of heavy metals, like silver, into a wound dressing has been described to origin skin allergy and inhibit fibroblast growth [80].

Table 1. Examples of some antimicrobial dressings available on the market.

Antimicrobial	Effect(s) on biofilm	Products
Honey	Inhibits biofilm formation; remove or destroy an established biofilm; interfere with QS; reduce bacterial adhesion [33,47].	Medihoney; Mesitran; Activon Tulle [68]
Silver	90% or 100% of the bacteria in the wound biofilm could be cleared within 24 h or 48 h, respectively, in concentrations as high as 5 to 10 g/mL [81].	Silverlon; Acticoat; Mepilex Ag [82]
Iodine	Eradicates young biofilm colonies; significantly reduces mature biofilm colonies; inhibits new biofilm development [33].	Inadine; Iodosorb; Iodozyme [83]
Polyhexamethylene biguanide (PHMB)	Disrupt biofilm attachments [33].	Suprasorb X + PHMB; Kendall AMD; Activheal® PHMB foam [68]

2.4 Phytotherapy as an alternative antimicrobial approach

Plants have been used as a natural source for healing purposes in traditional medicine for centuries, although the emergence of antibiotics in the 1950s considerably reduced their use [84,85]. Nevertheless, antibiotics are progressively becoming tolerated by infection-producing strains leading to resurgence of plants as new antimicrobial products. Indeed, many studies have been developed regarding the use of antimicrobial agents produced by plants since they exhibit a diversity of medicinal properties, attracting attention to incorporate these compounds in wound dressings [8,14]. Plants synthesize a substantial variety of compounds that are classified as primary and secondary metabolites [86]. Secondary metabolites, also known as phytochemicals, are mainly involved in plant defense against other microorganisms, competitors, and herbivores, thus protecting the plant against infections and other external stress conditions [86,87]. Their successful defense mechanisms is proven by the scarcity of infectious diseases in wild plants [7]. In addition, they have proven to be outstanding broad-spectrum antimicrobial compounds, in parallel with other unique characteristics, such as anti-

inflammatory, antioxidant, anticancer and regenerative activities [8]. Honey, already mentioned as an antimicrobial agent used in wound dressings (Table 1), is an example of a natural antimicrobial that contains phytochemicals. The existence of a wide variety of plant species, around 250 000 – 500 000 worldwide, and the fact that just a small percentage has been phytochemically explored to date, opens new doors for the discovery of new bioactive compounds [86]. Phytochemicals may circumvent the problem of developing bacterial resistance, as they can kill microorganisms with multiple mechanisms of action (multiple molecular targets and/or targets encoded by multiple genes), including damage microbial membrane structures, inhibit peptidoglycan synthesis, modify bacterial membrane surface hydrophobicity, coagulation of cytoplasm and depletion of proton [7,47,88]. Indeed, there is still no evidence about the emergence of bacterial resistance to phytochemicals [7,58]. Besides that, they are eco-friendly, reasonably inexpensive, widely available and associated with low levels of environmental toxicity and cutaneous cytotoxicity [8]. In addition to the considerable impact of phytochemicals against free-living bacteria, they have also been shown to be effective in biofilm prevention and control [61]. There are evidences that phytochemicals may interfere with various biofilm formation processes like motility, adhesion, EPSs production and QS [7,89,90].

The major groups that are responsible for antimicrobial activity from plants comprise phenolics (45%), terpenoids (27%) and alkaloids (18%) [86]. Essential oils (EOs) are mixtures of hydrophobic substances produced by aromatic plants [6]. They are mainly synthesized from vegetable parts of plants, such as seeds, leaves, barks, roots, twigs, fruits and flowers [14]. The extraction procedure (e.g., water or steam distillation, expression under pressure or supercritical fluid extraction) will influence the amount and the presence of the various constituents of the EOs [14,91]. It is true that their bioactivity is strictly correlated with their constituents, in particular with their structural arrangement and functional groups, which can be divided into four groups according to their chemical structure: terpenes, terpenoids, phenylpropenes, and “others”, being terpenoids and phenylpropenes those that have demonstrated higher antimicrobial activity [6,86]. Terpenes are hydrocarbons produced from the combination of several isoprene units and their hydrocarbon backbone can be rearranged into cyclic structures [86]. Terpenoids are terpenes with additional oxygen molecules or that have had their methyl groups moved or removed at various positions [86]. At last, phenylpropenes are characterized by a six-carbon aromatic phenol group and a three-carbon propene tail from cinnamic acid [86]. The mode of action of EOs is centered essentially on their ability to disrupt the cell wall and cytoplasmic membrane, leading to the lysis and release of internal cellular components [86]. In addition, in a work developed by Borges *et al.*, it has also been stated that this antimicrobial action can result in increased membrane permeability/fluidity, disruption of membrane embedded proteins, and alteration of ion transport processes in both Gram-positive and Gram-negative bacteria [7].

There are already several studies demonstrating the antibiofilm potential of EOs or their isolated components on different microorganisms [6–8,88]. Due to the impact of biofilms in CWs, these compounds have also been investigated with the prospect of being used in the treatment of infected wounds. Some of these studies are presented in Table 2.

Table 2. EOs and their components with potential for biofilm prevention and/or control in infected wounds.

Phytochemical(s)	Microorganism(s)	Effect(s) on biofilm	Other relevant results	References
Pimenta dioica leaf EO	Multidrug resistant (MDR) clinical isolates of *Acinetobacter baumanii*	Inhibition of biofilm formation (85% at 0.05 µg/mL); partial eradication of the biofilm (34% at 0.05 µg/mL).	*In vivo* experiments in a mouse model of wound infection showed a significant reduction of *A. baumannii* microbial load (3 log cycle reduction).	92
Thymol (loaded in polycaprolactone nanofibers)	*S. aureus* ATCC 25923; GFP-expressing antibiotic-sensitive *S. aureus* (methicillin-sensitive strain, MSSA); *S. aureus* Newman (MSSA clinical strain); *S. aureus* USA300 (MRSA clinical strain)	Removal of the already formed biofilms; inhibition of the first stages of bacterial adhesion and biofilm formation.	Dressing at reduced doses and short contact times did not show cytotoxic effects against macrophages. Thymol loaded dressings were able to eliminate pathogenic bacteria in *in vitro* co-culture models based on infected murine macrophages.	93
Thymus vulgaris (thyme) and *Origanum vulgare* (oregano) EOs (loaded in collagen hydrolysate-based nanofibers)	*S. aureus* ATCC 25923; *Pseudomonas aeruginosa* ATCC 27853	Total inhibition of biofilm formation (at 31.25 and 1.953 µg/mL for thyme and oregano, respectively, against *S. aureus* and 1.953 and 7.8125 µg/mL for thyme and oregano, respectively, against *P. aeruginosa*).	Composites of collagen and thyme or oregano oil had no cytotoxicity on fibroblastic cells up to concentrations of 1000 µg/mL and 500 µg/mL, respectively.	94
Salvia rosmarinus (rosemary) and oregano EOs (loaded in cellulose acetate nanofibers)	*S. aureus* ATCC 25923; *E. coli* ATCC 25922; *Candida albicans* ATCC 10231	Inhibition of microbial attachment and biofilm formation.	-	95
Satureja hortensis EO (loaded in functionalized magnetite nanoparticles (NPs))	*C. albicans* ATCC 10231	Inhibition of adhesion and subsequent biofilm development (biofilm impaired in its early as well as mature phases, quantified at 24 h, 48 h and 72 h)	-	96

- These studies did not perform cytotoxicity or *in vivo* tests; however they were also developed in the context of creating new strategies mainly for the treatment of biofilms in wounds.

Table 2. EOs and their components with potential for biofilm prevention and/or control in infected wounds (cont.).

Phytochemical(s)	Microorganism(s)	Effect(s) on biofilm	Other relevant results	References
Carvacrol (loaded in crosslinked polymer nanocomposite)	*P. aeruginosa* CD-1006; *S. aureus* CD-489 (MRSA); *E. coli* CD-2; *Enterobacter cloacae* complex CD-1412	Diffusion and dispersal throughout the biofilm matrix; killed bacterial cells in 1 day-old biofilms within 3 h.	*In vitro* co-culture model composed of fibroblasts and a *P. aeruginosa* biofilm showed that nanocomposites effectively treated the biofilm infection while fibroblast viability was largely unaffected (4-fold log reduction (~ 99.5%) in biofilm).	97
Carvacrol (loaded in poly(Ɛ-caprolactone) NPs)	*S. aureus* NCTC 10788; *S. aureus* [MW2] ATCC BAA-170; *S. aureus* ATCC 33593; *P. aeruginosa* ATCC 9027*; P. aeruginosa* ATCC BAA- 47	Eradication of pre-established biofilms (kill rates of 88-100% for Gram-positive and 80-99.9% for Gram-negative bacteria at concentrations between 156 to 1250 µg/mL).	Hemolytic activity analysis against erythrocytes demonstrated a hemolysis index less than 5% at all tested concentrations, which is considered safe. NPs were able to inhibit bacterial burden (> 99% decrease in bacterial load) in an *ex vivo* neonatal porcine skin wound infection model. Successful *in vivo* delivery of the NPs to the skin.	98
Foeniculum vulgare Mill. (fennel) EO	*S. aureus* ATCC 29213 and *S. aureus* ATCC 6538 (MSSA); *S. aureus* ATCC 43300 (MRSA)	Biofilm prevention at subinhibitory concentrations; synergism with hydrogen peroxide.	-	99
Mentha piperita (peppermint) EO combined with cinnamaldehyde (loaded in amine functionalized silica NPs)	*E. coli* DH5α; *P. aeruginosa* CD-1006, *S. aureus* CD-489 (MRSA); *E. cloacae* complex CD-1412	Diffusion and dispersal throughout the biofilm matrix; killed bacterial cells in 1 day-old biofilms within 3 h.	*In vitro* co-culture model consisting of fibroblasts and an *E. coli* biofilm showed that NPs effectively eliminated the biofilm infection while promoting fibroblast viability.	100

- These studies did not perform cytotoxicity or *in vivo* tests; however they were also developed in the context of creating new strategies mainly for the treatment of biofilms in wounds.

Table 2. EOs and their components with potential for biofilm prevention and/or control in infected wounds (cont.).

Phytochemical(s)	Microorganism(s)	Effect(s) on biofilm	Other relevant results	References
Carvacrol (loaded in poly(lactic acid) nanofibers)	*S. aureus* ATCC 6538; *C. albicans* ATCC 10231	Reduction of the biofilm production by 92–96 and 88–95% of *S. aureus* and *C. albicans*, respectively, in single and mixed cultures; decrease of cell count, biomass, metabolic activity, and vitality of established 24 and 48 h-old biofilms.	-	101
Carvacrol, cinnamaldehyde and thymol	*S. aureus* ATCC 25923	Partial eradication of preformed biofilms (2 log reduction with cinnamaldehyde and 3 log reductions with carvacrol and thymol at 500 µg/mL); hampers biofilm formation (4, 5 and 6 log reductions with cinnamaldehyde, thymol and carvacrol, respectively, at 500 µg/mL).	Cytotoxicity assay with fibroblasts, macrophages, and keratinocytes demonstrated that although the phytochemicals had cytotoxic activity at doses above 60–90 mg/mL they are less harmful against eukaryotic cells than conventional antiseptics.	102
Eugenol, methyl eugenol, carvacrol, linalool, (+)-limonene, p-cymene and α-pinene (loaded in crosslinked polymer nanocomposite)	*E. coli* CD2; *P. aeruginosa* CD1006; *E. cloacae* complex CD1412; *S. aureus* CD489 (MRSA)	Eradication of 90% of Gram-negative bacteria in biofilms; nanocomposites (loaded with low logP oils) removed biofilm biomass up to 70%; eugenol, linalool and carvacrol nanocomposites eliminated 90% of bacteria in *S. aureus* biofilm.	Cytotoxicity assay towards fibroblast cells showed that phytochemicals with lower logP and no phenolic hydroxyl groups provide particularly low cytotoxicity nanocomposites.	103

- These studies did not perform cytotoxicity or *in vivo* tests; however they were also developed in the context of creating new strategies mainly for the treatment of biofilms in wounds.

Another approach to overcome the accelerated rate of adaptive resistance in pathogenic bacteria is the use of phytochemicals in combination with well-known and clinically approved antibiotics in order to discover potential synergistic effects. Indeed, several studies have already suggested that natural compounds in combination with antibiotics are a good strategy to develop therapies for bacterial infections [104–106]. Since phytochemicals usually have higher minimum inhibitory concentrations than antibiotics, they often cannot be used in monotherapy [104]. This combinatorial strategy may increase the efficacy of the treatment, dealing simultaneously with the microbial resistance and toxicity, since lower concentrations of both compounds can be used [107]. Phytochemicals are also known to modulate or modify resistance mechanisms in bacteria allowing the recycling of less effective or even inactive antibiotics for which resistance mechanisms are already disseminated [108]. Efflux pump inhibitors are an example of resistance-modifying agents because they can avoid the extrusion of an antibiotic to the exterior of the cell, allowing the antibiotic to act against bacteria [108].

2.5 Hydrogel-based drug delivery systems for phytochemicals administration

A drug delivery system (DDS) is defined as a formulation or a device used for the administration of a therapeutic substance in the body, improving its efficacy and safety by controlling the time, rate, and place of release of drugs in the body [109]. Delivery strategies have significantly contributed to convert promising therapeutics into truly effective therapies [110]. Indeed, the fundamental advantage related with the use of drug carriers is their ability to protect drugs during the administration time by enclosing them in external protective barriers [111]. This avoids losses of active molecules and side effects in patients [111]. DDSs have also been developed for the eradication of bacteria in biofilms while mitigating the effects of antimicrobials on normal tissues [112]. The global DDSs market size was $26.08 billion in 2019 and is expected to reach $45.20 billion by 2027 (CAGR of 6.8% during the forecast period), thus showing their considerable clinical use [113].

Hydrogels are an attractive type of DDS and have been used in several medicine fields, including oncology, cardiology, wound healing, immunology and pain management [114]. Hydrogels are three-dimensional crosslinked polymeric networks capable of absorbing a large amount of water or biological fluids [115,116]. This growing interest in hydrogels as DDSs is related to their outstanding biocompatibility and biodegradability, easy preparation and capacity to be simply conjugated with hydrophobic and hydrophilic therapeutic compounds [116,117]. Differences in size (from macro- to nano-scale), architecture and function of hydrogels determine how they are used for drug delivery [114,115]. Depending on the polymerization technique, they can be produced in many different sizes and shapes, as for instance as a matrix, films, macro-beads, microparticles, nanoparticles, coatings or membranes [115]. They can be classified by the origin of the materials used for their preparation into natural (e.g.,

proteins, proteoglycans, polysaccharides, etc.) or synthetic (e.g., polyacrylic acid, polyvinyl alcohol, polyacrylamide, etc.) [115,118]. Synthetic hydrogels usually lack bioactivity and have little biodegradability [115].

Chitosan (CS, Figure 6) is an example of a common natural polymer obtained by the deacetylation of chitin, widely found in insects, crustacean shell and fungal cell wall [119]. It is a linear polysaccharide composed of β-(1–4)-2-acetamido-2-deoxy-β-d-glucopyranose and 2-amino-2-deoxy-β-d-glucopyranose [117]. Besides its non-toxic, biocompatible, biodegradable, non-immunogenic, non-carcinogenic and bioadherent properties, CS also has antimicrobial activity [117,120]. The precise mechanism of its antimicrobial action is not yet fully understood. However, its proposed mode of action is related to the interaction between the positive surface charge from the amino groups in CS with negatively charged microbial cell membranes, which leads to the leakage of intracellular constituents [117].

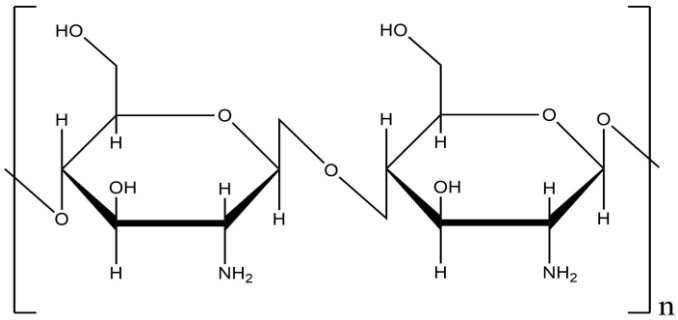


Figure 6. Chemical structure of CS (ChemDraw software).

Hydrogels can also be classified based on the type of interactions involved in the formation of the crosslinked structures: physical or chemical hydrogels [115]. Physical hydrogels are based on non-covalent interactions, such as ionic bonds, weak hydrogen bonds and dipolar or hydrophobic interactions [115]. In contrast, chemical hydrogels are characterized by the formation of permanent covalent bonds [115].

Drug loading in hydrogel matrices can be achieved by two methods: *in situ* loading and post-loading [115]. Briefly, *in situ* loading is associated with simultaneous incorporation of the drug inside the carrier and the carrier formation [121]. Post-loading consists of a two-step preparation: forming the hydrogel matrix and then immobilizing the drug in that polymeric network [121]. The drug release from hydrogels depends on the polymeric composition, extent of crosslinking within the polymer, the size and density of the carrier, and the physicochemical properties of the encapsulated drug itself [119]. Besides that, the local environmental conditions, such as temperature, pH, composition of the release medium, effect of enzymes, and immune responses also affect the drug release [119]. The main release

mechanisms of hydrogels include: drug diffusion, degradation of the polymeric matrix (erosion) and swelling [115].

Despite the vast studies that prove the antimicrobial activity of phytochemicals, this is not enough to satisfy the medical community. The compounds must also have high bioavailability at the target site [122]. Indeed, the complex wound environment represents a significant challenge for delivering antimicrobial agents to the locally infected site (logistical issues, pain, heterogeneity of the wound, etc.) [123]. Notwithstanding the promising properties of EOs already described, these compounds also have some limitations. EOs are unstable and easily degrade due to their high volatility and photosensitivity [9,124]. Therefore, some environmental factors like light, temperature, humidity and accessibility to atmospheric oxygen can decrease their bioavailability [9,124]. Actually, upon exposure to atmospheric conditions, some terpenoids can be converted to new products and result in skin hypersensitivity reaction and allergic dermatitis [125]. Furthermore, their low water solubility and low permeation in biological fluids and tissues represent other factors that limit their use [9]. For effective eradication of microorganisms in the infected wounds, antimicrobials must reach the anatomical site of infection in suitable concentrations, being this frequently hindered by the mentioned instability of EOs [79]. Therefore, their encapsulation into DDSs, like CS hydrogels, can be a useful alternative to overcome these limitations. EOs are retained within a matrix leading to their slow release and ensuring their increased duration availability on the target [9]. This capability to control EO release will confer better efficacy, reduced toxicity and improved patient compliance and convenience [9]. Curiously, in Table 2 (Section 2.4), it is possible to verify that in most of the studies drug delivery strategies were used to avoid the degradation and increase the effectiveness of EOs or their compounds.

Chapter 3 | Antimicrobial activity of phytochemical-antibiotic combinations against planktonic bacteria

3.1 Introduction

E. coli and *S. aureus* are the most common and versatile opportunistic pathogens responsible to cause a variety of infections, including CWs [126]. In fact, it is well recognized that *S. aureus* is one of the most frequent bacteria isolated from CWs [14]. *S. aureus* is a Gram-positive, coccus-shaped and facultative anaerobic microorganism, while *E. coli* is a Gram-negative, rod-shaped and chemo-organotrophic facultatively anaerobic bacterium [126].

Fusidic acid, mupirocin and gentamicin are three topical antibiotics available for the treatment of CWs [68]. There are also some studies regarding the incorporation of gentamicin in wound dressings, namely films prepared from collagen, chitosan and hyaluronic acid, sodium carboxymethyl cellulose hydrogels and chitosan nanofiber meshes with liposomes immobilized [127–129]. However, there is some controversy about the use of topical antibiotics alone, since their indiscriminate use may contribute to the spread of MDR bacteria [130].

Therefore, the use of plant-based molecules/phytochemicals, is being currently studied as an alternative to antibiotics [8]. In particular, EOs have been shown to exhibit great antimicrobial activity against both Gram-positive and Gram-negative bacteria, besides their good wound repairing properties like antioxidant, anti-inflammatory, anti-allergic and regenerative [8,87]. It is important to note that EOs cannot replace antibiotics in the treatment of systemic infections due to the limited level of clinical efficiency and the lack of clinically applicable pharmaceutical forms [107]. Nevertheless, they are being investigated for the treatment of local infections [107]. Citronellol, cis-6-nonen-1-ol, 3,7-dimethyl-1-octanol and citronellic acid are some of the compounds normally found in EOs. They are acyclic phytochemicals that belong to the class of terpenoids. Furthermore, they are natural flavoring ingredients, generally recognized as safe by the FDA, being typically applied as food additives for human consumption [131–134].

A significant number of studies have also reported interactions between EOs and antibiotics as a strategy to prevent the development of resistance, while increasing the antimicrobial activity of both agents and reducing the toxic effects against mammalian cells [107,135]. However, it is also important to mention that not all EO-antibiotic combinations show good results. In fact, the combinations can have distinct types of effects, including indifferent, additive, antagonistic and synergistic [135,136]. The indifferent effect is observed when the combination has an identical effect to that of the most active component [135,136]. On the other hand, the additive effect occurs when the

combination has an effect equal to the sum of the effects of each component [135,136]. Antagonism is present when a reduced activity is observed relative to the effect of the most efficient individual compound [135,136]. Finally, the synergistic effect is detected when the combination has a greater effect than the added effects of each constituent [135,136].

There are few or no studies on the antimicrobial ability of the above-stated phytochemicals, as well as on their effects in combination with the aforementioned antibiotics. Thus, the antimicrobial efficacy of these phytochemicals against planktonic cells of *E. coli* and two different strains of *S. aureus*, including a MRSA, was evaluated. Combinations of these phytochemicals with antibiotics, commonly used to treat skin infections, were also studied in order to understand whether there is an improved antimicrobial effect.

3.2 Materials and methods

3.2.1 Bacterial strains and culture conditions

The microorganisms *S. aureus* CECT 976 and *E. coli* CECT 434, obtained from the Spanish Type Culture Collection (CECT), and *S. aureus* XU212 (MRSA strain), kindly provided by Simon Gibbons (University College London), were used in all experiments. The bacteria were preserved at -80 ºC in Mueller–Hinton broth (MHB, Oxoid) containing 30% (v/v) glycerol (Panreac). For the inoculum preparation, the bacterial cultures were grown overnight in MHB at 37 ºC under 160 rpm of agitation before all the experiments.

3.2.2 Phytochemicals and antibiotics

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

3.2.3 Minimum bactericidal concentration

Minimum bactericidal concentration (MBC) was assessed for all phytochemicals and antibiotics by the broth microdilution method according to Ribeiro *et al.* [8] with some modifications. Overnight cultures were adjusted to a cell density of approximately 10^6 cells/mL in fresh MHB. Then, 180 µL of the adjusted bacterial suspensions were added to sterile 96-well polystyrene microtiter

plates (VWR) with 20 µL of 2-fold dilutions of each compound to test. Bacterial suspensions without phytochemicals or antibiotics and bacterial suspensions with the solvent of each compound (DMSO or sterile water (10%, v/v)) were used as negative controls. The plate was incubated at 37 ºC for 1 h and 160 rpm. Afterwards, 180 µL of the content of wells were removed and 180 µL of antimicrobial neutralizer composed by lecithin (3 g/L, ThermoFisher), tween 80 (30 g/L, VWR), sodium thiosulphate (5 g/L, labKem), α-histidine (1 g/L, Merck KGaA), and saponin (30 g/L, ThermoFisher) in phosphate buffer 0.25 M (previously prepared) at 1% were added to each well and allowed to act for 15 min. After that, 10 µL of each well were dropped on plate count agar (PCA, Merck) plates. Lastly, after 24 h of incubation at 37 ºC, the plates were analyzed, and the MBC of each compound corresponded to the minimum concentration causing no bacterial growth on the PCA plates. The experiments were performed in triplicate and repeated three times.

3.2.4 Dose-response curves

Dose-response curves were performed only for the phytochemicals CITRO and CIS and were determined through the quantification of culturable cells. The initial procedure was the same as mentioned in the current chapter, Section 3.2.3. However, after the neutralization step, serial dilutions were performed from 10^{-1} to 10^{-5} in NaCl solution (8.5 g/L, Merck) and 10 µL of the diluted samples were plated on PCA plates. After 24 h of incubation at 37 ºC, the colony forming units (CFUs) were determined and expressed in log CFU/mL. The limit of detection was 2.3 log CFU/mL [137]. At least two replicates were performed for each phytochemical.

To analyze the experimental dose-response data, the Chick-Watson mathematical model (Eq. 1) was assumed [138].

$$ln\left(\frac{X}{X_0}\right) = -k_{dis}C^n t \qquad \text{(Eq. 1)}$$

where X_0 and X correspond to the counts of CFU/mL before and after phytochemical exposure, respectively, k_{dis} to a disinfection rate coefficient, C to the phytochemical concentration (µg/mL), t to the exposure time (h), and n to concentration exponent.

For the determination of the parameter n, an apparent rate of disinfection, r (h^{-1}), was previously determined for different phytochemical concentrations after 1 h exposure by Eq. 2.

$$r = \frac{-\ln(10^{-red})}{t} \qquad \text{(Eq. 2)}$$

where *red* is the logarithmic reduction after 1 h exposure *i.e.*, the subtraction between the log CFU/mL of cells not exposed to EOs components and the log CFU/mL of the remaining cells following the exposure to phytochemicals.

Finally, a linear regression with slope *n* and y-interception ln(k_{dis}) was fitted (Eq. 3) using a toolbox for linear regression of GraphPad Prism 9.1.2 for Windows (GraphPad Software, La Jolla California, USA). The quality of the model was assessed through the coefficient of determination (r^2).

$$\ln(r) = \ln(k_{dis}) + n\ln(C) \quad \text{(Eq. 3)}$$

3.2.5 Determination of the fractional bactericidal concentration index

Fractional bactericidal concentration index (FBCI) was established to understand the effect between combinations of phytochemicals (CITRO and CIS) and antibiotics under investigation. For that, this was assessed by microdilution chequerboard method performed in 96-well microtiter plates via MBC determination according to Abreu *et al.* [108] with some modifications. Firstly, 180 µL of the bacterial suspensions (~ 10^6 cells/mL) were added to each well, along with 10 µL of the antibiotics and 10 µL of the phytochemicals in different concentrations ranging from MBC/16 to 2×MBC. However, when the MBC of a determined phytochemical or antibiotic was not found, concentrations were tested in the range of 32-1024 and 256-8192 µg/mL for antibiotics and phytochemicals, respectively. Each antibiotic was 2-fold diluted along the x axis (rows) while the phytochemicals were 2-fold diluted along the y axis (columns) as illustrated in Figure A.1 in Appendix A. Bacterial suspensions without the phytochemical and the antibiotic and bacterial suspensions with the solvent used to dissolve each compound (DMSO and/or sterile water (10%, v/v)) were used as negative controls. The MBC of each combination was then determined as previously described in the current chapter, Section 3.2.3. At least three replicates and two independent assays were performed for each combination.

The FBCI for all combinations was determined using the following equations [136]:

$$FBCI = FBC_{Phytochemical} + FBC_{Antibiotic} \quad \text{(Eq. 4)}$$

where,

$$FBC_{Phytochemical} = \frac{MBC_{(Phytochemical\ in\ the\ presence\ of\ antibiotic)}}{MBC_{(Phytochemical\ alone)}} \quad \text{(Eq. 5)}$$

$$FBC_{Antibiotic} = \frac{MBC_{(Antibiotic\ in\ the\ presence\ of\ phytochemical)}}{MBC_{(Antibiotic\ alone)}} \quad \text{(Eq. 6)}$$

The FBCI value was then used to categorize the interaction of the two compounds, according to Table 3.

Table 3. Correlation between FBCI and the effect of the combination of compounds [136].

	Synergy	Additive	Indifference	Antagonism
FBCI	≤ 0.5	> 0.5 to ≤ 1	> 1 to < 2	≥ 2

3.2.6 Statistical analysis

The results were expressed as the average ± standard deviation (SD). The statistical analysis of the results was performed using One-Way Anova in excel and was based on a confidence level ≥ 95% ($p < 0.05$ was considered statistically significant).

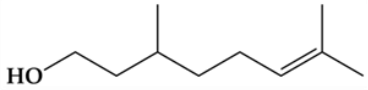
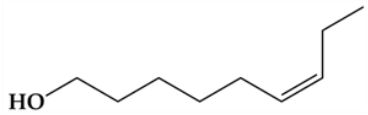
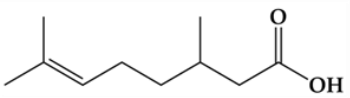
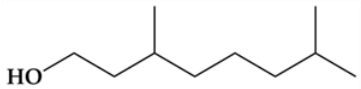
3.3 Results and discussion

The antimicrobial activity of selected phytochemicals alone and in combination with GEN, MUP and FUS against planktonic *S. aureus* CECT 976, MRSA XU212 and *E. coli* CECT 434 was investigated by MBC and FBCI determination. The establishment of these parameters is considered a great starting point, as they allow the selection of suitable concentrations for further analysis in the biofilm prevention and control.

3.3.1 Properties and drug-likeness of phytochemicals

A structure activity relationship analysis was also performed as an attempt to correlate the antimicrobial activity of the EOs components tested in this study with their chemical structure (Table 4), since variations in the structure of phytochemicals can result in differences in their antimicrobial effects [139]. For instance, the presence of functional groups, such as hydroxyl (OH), oxygenated and double bond, can affect the antimicrobial activity on multiple levels, having an essential role in the polarity, solubility, hydrogen bonding capacity and pKa of the compounds [139].

Table 4. Chemical structure (ChemDraw software) and molecular properties (Molinspiration calculation software) of the selected phytochemicals. LogP, octanol-water partition coefficient; n-OH, number of hydrogen bond acceptors; n-OHNH, number of hydrogen bond donors.

Phytochemical	Abbreviation	Chemical structure	Molecular weight (g/mol)	logP	n-OH acceptors	n-OHNH donors
Citronellol	CITRO		156.27	3.15	1	1
Cis-6-nonen-1-ol	CIS		142.24	2.87	1	1
Citronellic acid	CA		170.25	3.03	2	1
3-7-Dimethyl-1-octanol	3,7DOC		158.28	3.13	1	1

The molecular properties of the phytochemicals shown in Table 4 correspond to the physicochemical parameters that are assessed in Lipinski's rule of five (RO5) [140,141]. This rule evaluates the drug-likeness of a molecule, predicting its absorption, distribution, metabolism and excretion. According to this rule, drug-like molecules should have an octanol-water partition coefficient (logP) ≤ 5. The logP is applied as a measure of hydrophobicity and consequently affects, among others, the drug bioavailability and mode of action. Moreover, according to the RO5, drug-like compounds should also have a molecular weight ≤ 500 g/mol, present a number of hydrogen bond acceptors ≤ 10 and a number of hydrogen bond donors ≤ 5. Accordingly, by analyzing Table 4, it is possible to observe that all the compounds fulfill all the requirements of the RO5. Nevertheless, RO5 does not evaluate the biological activity or toxicity of the molecules, so further analysis is mandatory before considering their possible use for clinical purposes.

It is important to clarify that the focus of the present study is a topical application and RO5 was conceived to aid the development of orally bioavailable drugs. Nevertheless, other studies also tested whether the RO5 predicts drugs for delivery via non-oral routes. For instance, Choy and Prausnitz demonstrated that > 98% of 111 non-oral drugs currently approved by the FDA had physicochemical properties within the limits of the RO5 [142].

3.3.2 Bactericidal activity of phytochemicals and antibiotics

Before the MBC determination, an attempt to determine the minimum inhibitory concentration was made. However, it was not possible to obtain reliable conclusions since the wells of the microplate became turbid. This is a problem because the method is based on measuring the optical density of the wells. This fact may be related to a possible reaction or interference of phytochemicals with the MHB medium. Therefore, the evaluation of the susceptibility of planktonic cells to the antimicrobial compounds was only assessed by MBC determination. MBC corresponds to the lowest concentration of the compound showing complete growth absence in solid medium. MBC values of all tested compounds for the three different bacteria are summarized in Table 5.

Table 5. MBC (µg/mL) of selected phytochemicals and standard antibiotics against pathogenic bacteria.

		***S. aureus* CECT 976**	**MRSA XU212**	***E. coli* CECT 434**
Antibiotics	**MUP**	32	64	NA
	FUS	128	NA	NA
	GEN	8	NA	64
Phytochemicals	**CITRO**	512	NA	2048
	CIS	1024	2048	1024
	CA	2048	NA	4096
	3,7DOC	NA	NA	NA

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

According to the results shown in Table 5, GEN was the most effective antibiotic against both *S. aureus* and *E. coli*, with MBC values of 8 µg/mL and 64 µg/mL, respectively. GEN is an aminoglycoside antibiotic that binds to the 16S rRNA component of the 30S ribosomal subunit, disturbing the mRNA and, thus, leading to the formation of truncated or nonfunctional proteins [143]. In fact, GEN is described in the literature as a broad-spectrum antibiotic i.e., it is effective against both Gram-positive and Gram-negative bacteria [144]. However, the lower antimicrobial activity of GEN against *E. coli* could be related to the more complex cell wall of Gram-negative bacteria. It is constituted by a thin peptidoglycan layer adjacent to cytoplasmatic membrane, and an outer membrane (OM) composed by phospholipids and lipopolysaccharides (LPSs), which serves as a selective barrier inhibiting the penetration of antimicrobials, such as antibiotics [145]. Interestingly, MUP was the only antibiotic presenting bactericidal activity against MRSA (MBC of 64 µg/mL), besides its activity against *S. aureus* (MBC of 32 µg/mL). In fact, this antibiotic has been demonstrated to be highly active against staphylococci, including MRSA strains, and much less active against most Gram-negative bacilli, including *E. coli* (no activity found in the present study) [146,147]. This fact can also be explained by the OM of Gram-negative bacteria that may prevent the effective penetration of MUP. MUP exerts its antimicrobial action by inhibiting bacterial RNA and protein synthesis through binding to bacterial isoleucyl-tRNA synthetase, ultimately leading to bacterial death [146]. The antibiotic FUS also exhibited bactericidal activity against *S. aureus* (MBC of 128

µg/mL), yet at higher doses compared to GEN and MUP and no antimicrobial activity was found against MRSA and *E. coli*. In fact, FUS has limited activity against Gram-negative bacteria due to its large size and lipophilicity, which does not favor transport through porins in the OM of these bacteria [148]. FUS inhibits bacterial protein synthesis by biding with the elongation factor G, which is an indispensable protein that promotes translocation on the ribosome after the formation of the peptide bond [149]. Effectively, it is possible to verify, through the above-referenced mechanism of action of the antibiotics GEN, MUP and FUS, that these are focused at intracellular processes and, therefore, must be able to penetrate the bacterial cell envelope, which is usually hindered in Gram-negative bacteria.

It is generally accepted that more lipophilic molecules (higher logP) will interact more easily with the fatty acid tails of the lipid bilayer, allowing the molecule to cross cell membranes [150]. For this reason, the antimicrobial activity will possibly increase with an increase in hydrophobicity. In fact, CITRO that is the compound with the higher hydrophobicity (logP = 3.15) was found to be the most efficient antimicrobial phytochemical against planktonic *S. aureus* (MBC of 512 µg/mL). However, CIS was the compound with the smallest hydrophobicity (logP = 2.87) and presented the highest bactericidal effect against both *E. coli* and MRSA (MBC ranging from 1024 to 2048 µg/mL). This fact suggests that additional factors besides hydrophobicity must be involved. Indeed, other studies have also revealed the existence of molecules that do not follow the relationship between hydrophobicity and antimicrobial activity [8,151]. Compared to CA and 3,7DOC, the chemical structures of CITRO and CIS are characterized by the C=C double bond and OH group (Table 4), which could also be responsible for the higher antimicrobial activity of these compounds. The OH groups are thought to interact with the cell membrane of bacteria, causing the destabilization of the cytoplasmic membrane and reducing the pH gradients across the membrane, which eventually leads to the leakage of cellular components and cell death [139]. Furthermore, it has been demonstrated that the number of double bonds is also significant in relation to antimicrobial effectiveness, in which the increasing number of double bonds may enhance the antimicrobial effect [152].

No antimicrobial studies with CIS were found in the literature, which demonstrates the relevance of the results obtained in this study for this phytochemical. Regarding CITRO, some studies have also shown its antimicrobial activity against different bacteria and their potential use in other applications, as for instance for food preservation [153] and for the control of oral infectious diseases [8]. Kotan *et al.* studied the antibacterial activity of twenty-one oxygenated monoterpenes, including CITRO, against some food-borne pathogens, such as *Bacillus coagulans*, *E. cloacae*, *Enterococcus fecalis*, *E. coli*, *Klebsiella pneumoniae*, *P. aeruginosa* and *Salmonella enteritidis* [153]. By using a disc diffusion method, they demonstrated that CITRO exhibited antimicrobial activity against some of

these pathogens. In addition, in a work developed by Ribeiro *et al.*, the effect of CITRO against *Streptococcus mutans*, an oral pathogen, in planktonic and biofilm states was assessed [8]. CITRO was shown to be very effective in inhibiting the growth of planktonic *S. mutans*, also causing significant biofilm inactivation. Moreover, they also evaluated the cytotoxicity of CITRO against fibroblast cells in order to understand its effects on mammalian cells viability. At a concentration of 3 mM (469 μg/mL) of CITRO, no obvious cytotoxic effects were observed, highlighting that CITRO may be a candidate for the control of wound infections without compromising cell viability. Sinha *et al.* also stated that EOs and their compounds may be commonly considered safe for humans when some precautions are taken with the concentrations used [154].

It should also be noted that CIS was the compound that showed the highest spectrum of action, having bactericidal activity against the three different bacteria. This is a very interesting result, demonstrating the great potential of this phytochemical for the treatment of wound biofilms since they are normally polymicrobial, i.e. contain multiple diverse species [155]. Although CITRO has not demonstrated bactericidal activity against the MRSA strain in the present work, Mulyaningsih *et al.* showed that CITRO has bactericidal activity (MBC values between 125-8000 μg/mL) against different clinical isolates of MRSA [156]. Furthermore, the phytochemicals were also shown to have generally a lower efficacy against *E. coli* compared to *S. aureus*, except for CIS that had a similar MBC value for both bacteria. This behavior is in agreement with the results obtained by Griffin *et al.* for CITRO [157]. They found that CITRO exhibited lower antimicrobial activity against *E. coli* compared with *S. aureus.* In fact, most studies reveal that in general EOs and their components are more effective against Gram-positive bacteria due to the complexity of the cell wall of Gram-negative bacteria, as previously explained [87]. The saturated fatty acid chains present in LPSs make the OM more rigid [158]. This low fluidity can also be explained by the fact that LPS molecule contains six or seven covalently linked fatty acid chains, in contrast to the glycerophospholipid found in cytoplasmatic membrane that contains only two fatty acid residues [158]. These facts decrease the rate of transmembrane diffusion of lipophilic solutes. Furthermore, the hydrophilic polysaccharide chains on LPSs and the abundant presence of purines, which serve as hydrophilic transmembrane channels, may also be other reasons that Gram-negative bacteria are relatively resistant to hydrophobic compounds [159,160]. On the other hand, in Gram-positive bacteria, the OM is not present and the peptidoglycan wall is not dense enough to resist small antimicrobial molecules, facilitating the access to the cell membrane [161]. Additionally, Gram-positive bacteria may facilitate the penetration of hydrophobic compounds of EOs due to the lipophilic ends of lipoteichoic acid present in cell membrane [161].

Comparing the MBC values found for the antibiotics with those obtained for the phytochemicals, it was possible to verify that the antibiotics presented bactericidal effects at lower concentrations. While the MBC for phytochemicals was found between 512 to 4096 µg/mL, the MBC for antibiotics ranged from 8 to 128 µg/mL, showing a poorer antimicrobial activity of phytochemicals. Nevertheless, as antibiotic resistance is dramatically increasing, the combinatorial approach of phytochemicals with already available antibiotics may be a great strategy.

3.3.3 Effect of different doses of citronellol and cis-6-nonen-1-ol on bacteria growth

CITRO and CIS presented considerably more effective antimicrobial activity against pathogenic bacteria compared to the other phytochemicals. Thus, they deserve further investigation, to better understand their modes of action. Therefore, in order to assess the correlation between the culturability of the bacterial cells and different concentrations of these phytochemicals, CFUs was determined after 1 h exposure of *S. aureus*, *E. coli* and MRSA to CITRO and CIS (Figure 7).

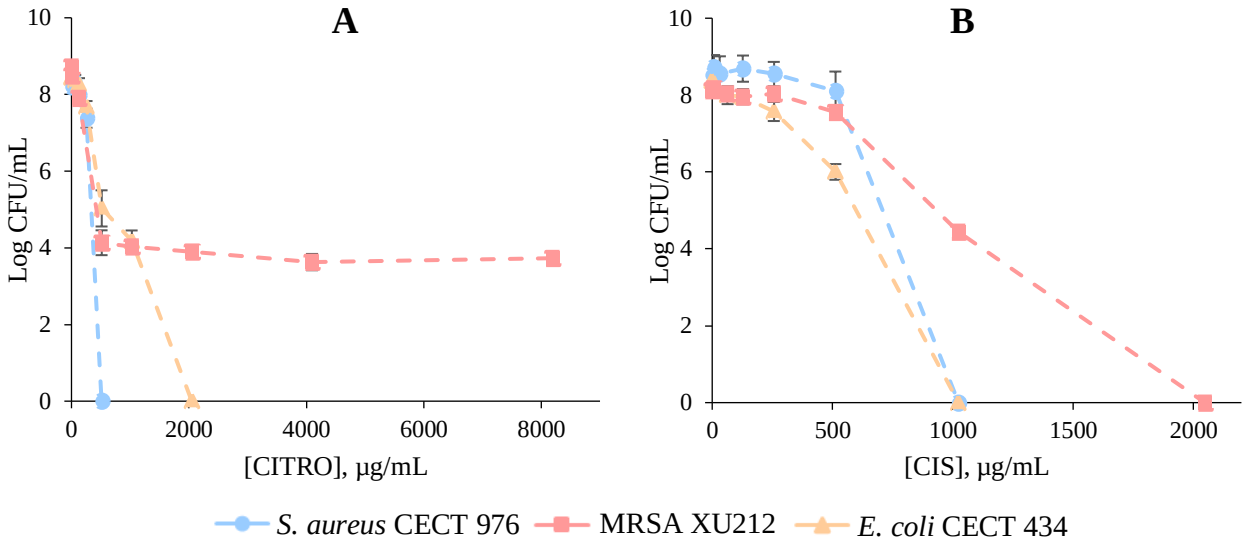


Figure 7. Log CFU/mL of planktonic bacteria as a function of CITRO (A) and CIS (B) concentration (in µg/mL) after 1 h of exposure against *S. aureus*, *E. coli* and MRSA. When log CFU/mL is equal to zero, cells were below the detection limit of the plate counting method (2.3 log CFU/mL). Points represent mean ± SD.

Through the analysis of Figure 7, it was possible to verify that the bacterial culturability, after exposure to both phytochemicals, remained constant ($p > 0.05$) for all strains until a certain concentration is reached. From this concentration, an increase in the phytochemical concentration led to an increase in antimicrobial activity until total or partial reduction of cell culturability was obtained, showing a dose-dependent effect. This correlation was noticed to be dependent on the phytochemical used and the strain tested. The compound concentration leading to the total reduction of cells corresponded to the MBC previously obtained (Table 5). It was found that CITRO caused only a partial reduction against MRSA, even at high phytochemical concentrations. This is in agreement

with the result obtained in MBC determination in the current chapter, Section 3.3.2 (MBC > 8192 µg/ml against MRSA). Thus, a maximum reduction of 4.6 log CFU/mL was obtained from the concentration of 512 µg/mL tested (similar log reductions for the higher concentrations tested, $p > 0.05$).

An analysis of concentration dependence of bacterial killing was performed by adjusting the experimental dose-response data to the Chick-Watson mathematical model. It is believed that this is one of the first works that demonstrates the application of this mathematical model to understand the mode of action of CITRO and CIS. The parameter *n* is a numerical indicator of phytochemical behavior that describes the dependence of the killing rate on antimicrobial concentration and can be obtained from the referred model. The estimated values of this phytochemical concentration dependence parameter (*n*) are presented in Table 6. If *n*=1, log CFU/mL reduction increases in direct linear proportion to the phytochemical concentration [137,138]. In contrast, if *n*=2 log CFU/mL reduction is proportional to the square of the phytochemical concentration [137,138]. Thus, compounds with a high *n* value are significantly influenced by changes in concentration, whereas those with a low *n* value are affected to a minor extent (less dose-dependent).

Table 6. Estimated values of phytochemical concentration dependence parameter, *n*, considering the experimental points before achieving the maximum log CFU/mL reduction. The uncertainty given is the SD of the mean.

	***S. aureus* CECT 976**	**MRSA XU212**	***E. coli* CECT 434**
CITRO	2.6 ± 0.4	0.7 ± 0.2	0.8 ± 0.2
CIS	1.0 ± 0.4	1.1 ± 0.2	0.8 ± 0.2

According to Table 6, both terpenoids showed *n* values close to 1 against all bacterial strains, except for CITRO against *S. aureus* ($2 < n < 4$). Moreover, this higher *n* value obtained for CITRO against *S. aureus* demonstrates that this compound is less dose-dependent against MRSA and *E. coli*. A correlation between the *n* value and the type of interaction among the phytochemical and its cellular targets can be made. Usually, when $n < 2$ the compounds interact strongly with their target by chemical interactions, while $n > 4$ is associated with physical interactions [162]. Compounds with *n* values between this range are considered to have a dual action [162]. Therefore, CITRO potentially has both chemical and physical interactions between the *S. aureus* cells, whereas in the other cases only chemical interactions are present. Nuñez and D'Aquino also calculated the parameter *n* for clove EO against the three distinct bacteria *E. coli*, *S. aureus* and *P. aeruginosa* [163]. They obtained *n* values between 0.22 and 1.29, which also demonstrates the existence of essentially chemical interaction of EOs with the cells.

3.3.4 Combinatorial activity between phytochemicals and antibiotics

The combination of antimicrobial agents can provide many benefits, particularly increased antimicrobial activity and reduced toxicity effects of the combined compounds. Accordingly, in order to check the combinatorial effects between the phytochemicals CITRO and CIS and the standard antibiotics MUP, FUS and GEN against pathogenic bacteria, the FBCI was adopted and the data are shown in Table 7. The combination of phytochemicals with these specific antibiotics was due to the fact that they are already clinically approved for the treatment of skin infections. To the best of the author's knowledge, no study has been performed to investigate the antimicrobial effects based on these combinations. Therefore, this investigation can be an asset to the scientific community, since the use of these combinations for the treatment of infected wounds could be an interesting approach to replace the use of antibiotics alone, avoiding the emergence of resistant bacteria.

Table 7. FBCI of the different combinations between selected phytochemicals and standard antibiotics against pathogenic bacteria.

		***S. aureus* CECT 976**	**MRSA XU212**	***E. coli* CECT 434**
CITRO	**MUP**	0.562 (ADD)	*	-
	FUS	0.312 (SYN)	NA	-
	GEN	1.125 (IND)	NA	0.188 (SYN)
CIS	**MUP**	0.750 (ADD)	0.562 (ADD)	-
	FUS	0.375 (SYN)	-	-
	GEN	0.750 (ADD)	-	0.250 (SYN)

NA: no activity; SYN: synergy; ADD: additive; IND: indifference; - no significant bactericidal effect when combining the compounds (compared with the compound alone at MBC); * the concentration of MUP was reduced from 64 µg/mL (MUP alone) to 32 µg/mL in the presence of CITRO (NA alone against MRSA XU212).

The bactericidal potential of GEN against *E. coli* was significantly enhanced by its combination with both CITRO and CIS, with a clearly synergistic activity between the compounds (FBCI of 0.188 for the combination CITRO-GEN and 0.250 for the combination CIS-GEN). Noteworthy is the fact that when the bacterial cells were treated with these phytochemical-antibiotic combinations, the bactericidal effect was observed at significantly lower concentrations. Indeed, the effective doses of GEN and CIS were reduced by 8-fold in *E. coli*, while doses of CITRO were reduced by 16-fold. This outcome is particularly interesting since, due to the distinctive structure of Gram-negative bacteria, they are often more resistant than Gram-positive bacteria. In fact, the majority of the World Health Organization (WHO) list of priority pathogens is composed of Gram-negative bacteria, which have been associated with substantial morbidity and mortality worldwide [164]. In addition, the combination of CIS with GEN caused an additive effect against *S. aureus*, whereas the interaction between CITRO and GEN was indifferent against this bacterial strain. Regarding the combinatorial effects of phytochemicals with FUS, synergistic effects were found against *S. aureus* (FBCI of 0.312 for the combination CITRO-FUS and 0.375 for the combination CIS-FUS). The

bactericidal doses of FUS were reduced by 4-fold, while doses of CITRO and CIS were 16- and 8-fold reduced, respectively. Concerning the effects of combining the phytochemicals CITRO and CIS with MUP, FBCI values revealed that these combinations act additively against both *S. aureus* and MRSA. Indeed, the most interesting effect was observed for the combination CITRO-MUP against MRSA, in which the concentration of MUP needed to have bactericidal activity against this bacterial pathogen was reduced from 64 µg/mL (MUP alone) to 32 µg/mL in the presence of CITRO. Herein, it is important to highlight that the CITRO alone did not demonstrate bactericidal activity against MRSA, showing the potential of these natural products as drug modulating or modifying agents when combined with antibiotics. As a result, the use of phytochemicals in association with antibiotics could be an effective tool for the management of MDR bacteria.

It has been proposed that phytochemicals and antibiotics can act synergistically mostly by multi-target effect where the compounds target different bacterial sites, by pharmacokinetic or physicochemical effects (e.g., enhancement of solubility or bioavailability), or by targeting a specific resistance mechanism of the bacteria [165]. Furthermore, as EOs are known to disturb the structure of the cell membrane and rendering it more permeable, that interaction could be used to improve the activity of antibiotics, facilitating their penetration with a reduction of therapeutic doses [166].

3.4 Conclusions

In conclusion, the results obtained in this chapter shown that some EOs components have great antimicrobial action against planktonic *S. aureus*, MRSA and *E. coli*. Generally, *E. coli* showed to be more resistant to phytochemical action than *S. aureus*, which can be associated with the complexity of the cell wall of Gram-negative bacteria. The phytochemicals CITRO and CIS showed the highest bactericidal effects, may be due to the presence of the C=C double bond and the OH group. Moreover, CIS was the compound that exhibited the highest and broad spectrum of action, presenting bactericidal effect against the three different bacteria. According to the Chick-Watson model, these compounds proved to have essentially a chemical interaction with the bacterial cells, with CITRO also showing to have a physical interaction between the *S. aureus* cells.

The combinatorial approach between CITRO and CIS with traditional antibiotics used in the treatment of skin infections showed synergistic effects against *S. aureus* and *E. coli*. It was verified an increase in antimicrobial activity by the decrease in the bactericidal concentrations of each compound in combination. Therefore, the use of phytochemicals in combination with antibiotics could be an effective approach to increase antimicrobial activity, dealing at same time with the problem of microbial resistance and toxicity.

Chapter 4 | Action of selected phytochemical-antibiotic combinations on bacterial biofilm control

4.1 Introduction

Biofilm development is a crucial virulence factor in the pathogenesis of several medically important bacteria, including *S. aureus* and *E. coli*, and their prevalence in microbial infections in humans, in particular CWs, is now well recognized [45,167,168]. Biofilm cells are known to be physiologically distinct from their planktonic counterparts and they are normally more resistant to the effect of the antimicrobial agents [57]. The numerous mechanisms explaining the resistance of biofilms to antimicrobials (e.g., decreased growth rates and metabolism, restricted antimicrobial penetration, and differentiation into persistent cells) make it difficult to predict the behavior of the biofilm cells [58]. Thus, despite antimicrobial studies using planktonic cells have provided relevant information on the mechanisms inhibiting bacterial growth, the impact of antimicrobials on planktonic cells cannot be compared to the effects on biofilm cells [106].

Induction of biofilm dispersal, eradication of persisters, target QS signaling, use of divalent cation chelators and use of bacteriophages are some approaches to eradicate established biofilms [169]. Nonantibiotic compounds, used alone or in combination with antibiotics to potentiate biofilm eradication or to reduce the necessary concentrations of both compounds, are viewed as modern promising strategies [169]. For instance, phytochemicals, in particular EOs and their components, as well as phytochemical-antibiotic combinations have already demonstrated their antibiofilm activity against numerous pathogens [92–103,106]. However, among EOs and their constituents, little or none is known about the antibiofilm activity of CITRO and CIS in combination with standard antibiotics. Therefore, this chapter was intended to evaluate the effects of selected antibiotics (MUP, GEN and FUS) and phytochemicals (CITRO and CIS) individually and in combination against biofilms of *E. coli* and *S. aureus*, including a MRSA.

4.2 Materials and methods

4.2.1 Bacterial strains and culture conditions

The bacteria *S. aureus* CECT 976, *E. coli* CECT 434 and MRSA XU212 were used for the biofilm control studies by phytochemical-antibiotic combinations and they were preserved at -80 ºC in MHB containing 30% (v/v) glycerol. For the inoculum preparation, the bacterial cultures were grown overnight in MHB at 37 ºC under 160 rpm of agitation before all the experiments.

4.2.2 Phytochemicals and antibiotics

The phytochemicals CITRO and CIS and the antibiotics MUP, GEN, and FUS were tested. Solutions of FUS and GEN were prepared in sterile water and CITRO, CIS and MUP was dissolved in DMSO.

4.2.3 Biofilm formation and control

The best combinations obtained in the determination of FBCI (Chapter 3) were tested for their ability to control biofilms. Biofilm formation and control were executed according to the procedure described by Borges *et al.* [6] with some modifications. Firstly, 200 µL of adjusted bacterial suspensions (~ 10^6 cells/mL) were added to 96-wells polystyrene microtiter plates and incubated at 37 ºC for 24 h under 160 rpm of agitation. After biofilm development, the medium was removed and the wells were washed twice with sterile NaCl solution (8.5 g/L) in order to remove loosely attached bacteria. Then, 180 µL of NaCl solution (8.5 g/L) were added to each well with 20 µL of the phytochemical-antibiotic combinations. The concentrations of each compound in the combination were 1, 10, and 100×MBC of the compound in the presence of the other. In addition, the effects of individual compounds at concentrations similar to those tested in the combinations were also assessed. Sessile bacteria without the compounds and sessile bacteria with respective solvents (DMSO or sterile water (10%, v/v)) were used as negative controls. The microtiter plates were incubated at 37 ºC and 160 rpm for 1 h. After that, the remaining attached bacteria were analyzed in terms of CFUs. At least two replicates and two independent assays were performed.

4.2.3.1 Quantification of the biofilm culturable cells

The culturability of the biofilm cells was assessed as described by Borges *et al.* [6] with a few modifications. The content of the wells was removed and 200 µL of neutralizer were added to each well and allowed to act for 15 min. Then, the wells were washed twice with sterile NaCl solution (8.5 g/L), and biofilms were scrapped from the microtiter plate with a pipette tip (three times for periods of around 1 min each) and resuspended in 1 mL of NaCl solution (8.5 g/L). Serial dilutions were made from 10^{-1} to 10^{-5} in NaCl solution (8.5 g/L) and then 10 µL of the diluted samples were plated on PCA plates. After 24 h of incubation at 37 ºC, the CFUs were determined and expressed per square centimeter of microtiter plate well (CFU/cm^2). The limit of detection was 2.1 log CFU/cm^2 [137]. Log CFU/cm^2 reduction was then determined by subtracting the log CFU/cm^2 of the biofilm cells not exposed to the antimicrobial compounds by the log CFU/cm^2 of the remaining biofilm cells after the exposure to the compounds (Tables B.1, B.2 and B.3 in Appendix B). The interaction of the antibiotics and phytochemicals was categorized according to Abreu *et al.* [106]. The combination was considered synergic when the log CFU/cm^2 reduction caused by a combination was significantly higher ($p < 0.05$) than the sum of reductions by the individual compounds. On other hand, an

antagonistic combination was characterized when the log CFU/cm^2 reduction of the combination was significantly lower ($p < 0.05$) than that obtained with the most effective individual substance. The remaining combinations were considered to have an indifferent effect (distinction between an indifferent or an additive effect was not made).

4.2.4 Statistical analysis

The results were expressed as the average ± SD. The statistical analysis of the results was performed using the Student's t-test for independent samples in excel. Levels of $p < 0.05$ were considered to be statistically significant (confidence level ≥ 95%).

4.3 Results and discussion

It is essential to study the effect of the phytochemical-antibiotic combinations, already tested in planktonic cells, on biofilms since they are the main reason for the delayed healing of CWs. In fact, an accurate characterization of the antimicrobial action of a compound must focus on cells in both planktonic and sessile states. Therefore, this chapter focused on the ability of selected phytochemical-antibiotic combinations in the control (removal and/or inactivation) of biofilms of *S. aureus* CECT 976, MRSA XU212 and *E. coli* CECT 434.

4.3.1 Eradication of pre-formed biofilms with selected phytochemical-antibiotic combinations

Based on the results of FBCI determination against planktonic cells (Chapter 3), the best combinations obtained for each bacterium were tested on their biofilm state and are illustrated in Table 8.

Table 8. Best phytochemical-antibiotic combinations obtained against planktonic cells.

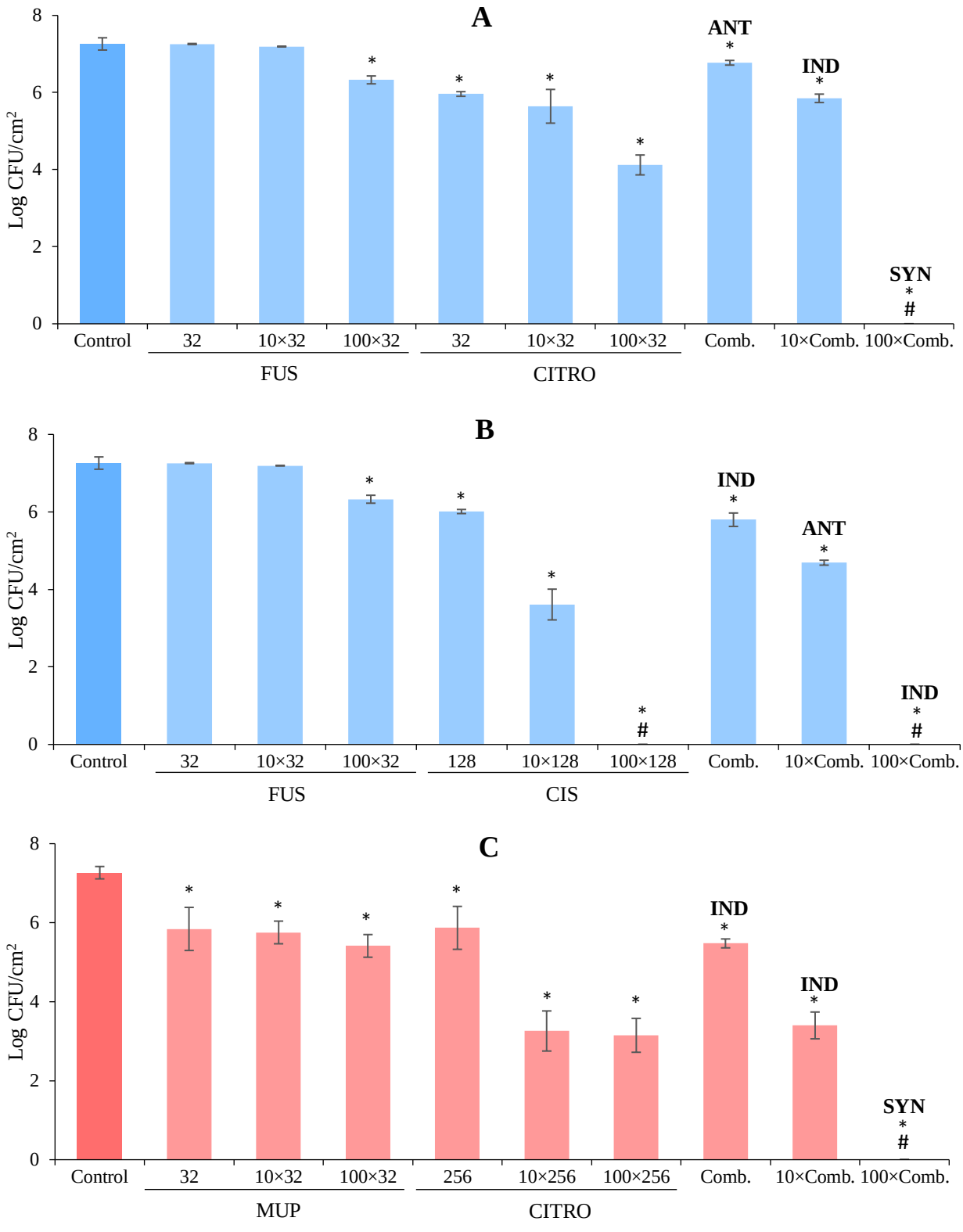
	Combination	Concentration (µg/ml)	Effect
***S. aureus* CECT 976**	FUS CITRO	32 32	SYN
	FUS CIS	32 128	SYN
MRSA XU212	MUP CITRO	32 256	*
	MUP CIS	32 128	ADD
***E. coli* CECT 434**	GEN CITRO	8 128	SYN
	GEN CIS	8 128	SYN

SYN: synergy; ADD: additive; * the concentration of MUP was reduced from 64 µg/mL (MUP alone) to 32 µg/mL in the presence of CITRO (no activity alone against MRSA XU212).

In addition, 10- and 100-times higher concentrations of the two compounds in combination were also evaluated, as biofilms are often more resistant to antimicrobial agents, being expected that

the bactericidal combinations tested on planktonic cells will not completely eradicate biofilm cells. Antibiotics and phytochemicals were also tested individually in order to assess whether their effects in the combination in controlling biofilms were, in fact, improved.

Figure 8 represents the effects of these phytochemical-antibiotic combinations, as well as their individual compounds, against pre-established 24 h-old biofilms within 1 h of exposure, evaluated in terms of remaining culturable biofilm cells.



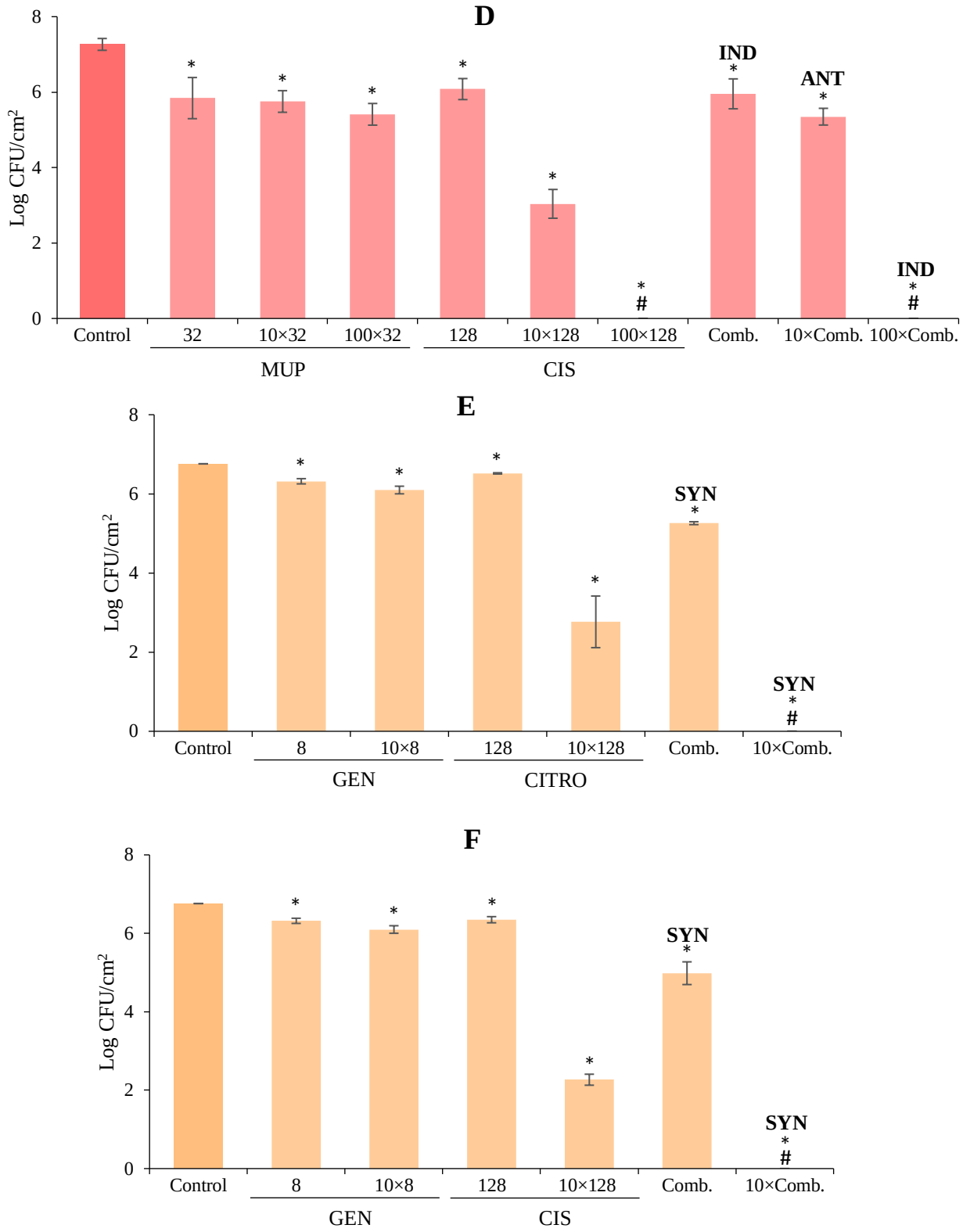


Figure 8. Antibiofilm activity of selected antibiotics and phytochemicals individually and in combination (comb.) on 24 h-old biofilms of *S. aureus* (A, B), MRSA (C, D) and *E. coli* (E, F) after 1 h of exposure. Compounds in the combination were applied at 1, 10, and 100×MBC of the compound in the presence of the other. Control represents sessile bacteria without the antimicrobial compounds. Control experiments were performed on antibiofilm activity of the solvents and no activity was found at 10% (v/v) (data not shown). Values represent mean ± SD. # Remaining culturable biofilm cells were below the detection limit of the plate

counting method (i.e., 2.1 log CFU/cm^2); * statistically different from the control ($p < 0.05$); SYN: synergy; ANT: antagonism; IND: indifference.

Regarding the ability of the three bacterial strains to produce biofilm, it was possible to observe that *S. aureus* and MRSA biofilms had slightly higher cell densities compared to *E. coli* biofilms ($p < 0.05$). As shown in Figure 8, most of the compounds and their combinations were able to reduce the number of culturable cells compared to the control, regardless of the concentration tested. However, FUS at concentrations of 32 and 10×32 µg/mL against *S. aureus* did not show statistical differences compared to control. In general, a dose-dependent behavior was observed, in which for higher concentrations of phytochemicals, antibiotics and their combinations, the number of remaining culturable biofilm cells decreased ($p < 0.05$). This is in line with previous studies which showed the same antibiofilm pattern of EOs and their components [6,98,170]. However, when the antibiotic MUP was tested against MRSA, there was no significant difference ($p > 0.05$) in the number of culturable cells for the three concentrations tested. This may be due to the existence of a very cohesive biofilm that strongly hinders the penetration of this antibiotic, leading to a low antimicrobial action regardless of the concentration used. In fact, the architecture of MRSA biofilms can strongly influence the effectiveness of compounds. Interestingly, Teixeira *et al.* proved that MRSA biofilms tended to be less susceptible to antimicrobial photodynamic therapy mediated by curcumin than MSSA biofilms [171]. They verified that MSSA biofilms presented a structure with peaks and valleys that could have led to better distribution and penetration of the curcumin, while MRSA biofilms presented a more uniform architecture, without cells conglomerations. Curiously, CITRO at 10×256 and 100×256 µg/mL against MRSA also demonstrated statistically similar log CFU/cm^2 reductions ($p > 0.05$). This result corroborates with the previous study for planktonic cells, where for concentrations above 512 µg/mL similar log CFU/mL reductions were obtained with this phytochemical against MRSA (Figure 7 in Chapter 3).

Antibiotics when used alone demonstrated low log reductions, even at higher concentrations. They showed maximum log CFU/cm^2 reductions of 0.9, 1.9 and 0.7 against *S. aureus*, MRSA and *E. coli*, respectively (see support information in Tables B.1, B.2 and B.3 in Appendix B). However, it is important to note that some of the concentrations tested were below the MBC of the antibiotic alone against planktonic bacterial cells (Chapter 3). The phytochemicals tested alone exhibited generally more significant log reductions (see details in Tables B.1, B.2 and B.3 in Appendix B) than those obtained with antibiotics. This was particularly evident when CIS was applied at 100×128 µg/mL against *S. aureus* and MRSA biofilm cells, where no remaining culturable cells were observed (7.3 log CFU/cm^2 reduction for both strains). Indeed, phytochemicals had generally greater antibiofilm activity than antibiotics, as they usually showed higher log reductions at lower concentrations ($p <$

0.05). For example, FUS at 100×32 µg/mL revealed a lower log reduction (0.9 log CFU/cm^2 reduction) when compared to CIS at 10×128 µg/mL (3.6 log CFU/cm^2 reduction) against *S. aureus*. This result is particularly interesting, as antibiotics have shown to have greater antimicrobial activity against planktonic cells (lower MBC values, Chapter 3) compared to phytochemicals. The ability of phytochemicals to control biofilms can be correlated with an increased capacity to penetrate the EPS matrix. In fact, the amphipathic nature of most EOs components has been suggested to be responsible not only for the disruption of the bacterial cell wall and cytoplasmic membrane, but also for the good diffusion throughout the EPS matrix [6]. Another possible reason may be related to a greater capacity of EOs components to eradicate persister cells (non-growing or slow-growing bacterial cells), which are not exclusive to biofilms, but their frequency of occurrence is much higher within sessile communities than in planktonic [58]. As already mentioned, these cells are difficult to kill since the effectiveness of most antimicrobials is strongly influenced by the bacterial growth phase. They often require ongoing metabolic activity and division of bacterial cells, so slow bacterial growth is frequently associated with decreased susceptibility [58,172]. In fact, some studies have already demonstrated the existence of EOs with activity against this stationary phase bacteria [173–175]. Furthermore, these phytochemicals can also have a role as QS inhibitors, interfering with bacterial communication. Indeed, phytochemicals have been recognized as an interesting source of QS inhibitors [58]. Some QS inhibitors and signals can be used to trigger biofilm dispersal, leading to an increase in biofilm susceptibility and facilitating its eradication [58,169]. Kavanaugh and Ribbeck also demonstrated that selected antimicrobial EOs could eradicate bacteria within biofilms with higher efficiency than certain important antibiotics [176].

The selected phytochemical-antibiotic combinations caused a total log reduction in culturable cells of the biofilm at concentrations 100 times higher against *S. aureus* and MRSA. Regarding *E. coli*, total eradication of the biofilm was achieved at concentrations 10 times higher. These results demonstrated the greater resistance of biofilms to the action of antimicrobial agents, as expected, showing that experiments with planktonic cells cannot be taken alone as a reference to developing biofilm control strategies. Furthermore, this difference between *E. coli* and *S. aureus* strains may be related to the fact that the antibiotics present in the combinations tested for each strain were not the same.

In addition, the combinatorial effects between phytochemicals and antibiotics against biofilms compared to the effect of the compounds alone were also analyzed. Figure 8 also presents the respective characterization of the interaction between the compounds (synergy, antagonism or indifference). It was verified the existence of synergistic effects only for 100×Comb. with CITRO against *S. aureus* and MRSA (total log reductions for both strains) and for all combinations, regardless

of the concentration tested, against *E. coli*. This result against staphylococci bacteria showed that the effects of the combinations are dose-dependent, i.e. the same compounds at different concentrations in the combination resulted in distinct effects. For instance, CITRO-FUS combination resulted in an indifferent effect at 10×Comb., but at 100×Comb. the interaction was synergistic. The CIS-FUS and CIS-MUP combinations against *S. aureus* and MRSA, respectively, also showed distinct effects (indifferent or antagonistic) depending on the concentration tested. The antagonistic results obtained with the antibiotics FUS and MUP demonstrate that these compounds can somehow disturb the activity of the phytochemicals, which in those cases were more effective individually than in combination with antibiotics. The environmental stress caused by the presence of antibiotics, at certain concentrations, may promote the switch of the cells to more tolerant phenotypes, reducing the antimicrobial capacity of phytochemicals [106]. These concentration-dependent interactions would need a more integrated approach in order to be fully understood. Furthermore, the results demonstrated that the data obtained regarding the effects of the combinations on planktonic cells (Chapter 3) may not be enough to predict therapeutic success, since combinations considered to be synergistic against planktonic cells may not have synergistic effects when applied to cells in the sessile state. Abreu *et al.* also obtained some antagonistic effects after biofilm exposure to combinations that had shown good results against planktonic cells in previous studies [106]. In other words, phytochemicals that have previously been shown to potentiate the effects of antibiotics against planktonic bacteria, demonstrated antagonistic effects when tested on biofilms.

4.4 Conclusions

In this chapter, the effect of selected phytochemical-antibiotic combinations on sessile cells of *S. aureus*, including a MRSA, and *E. coli* to control 24 h-old biofilms was evaluated through the quantification of CFUs.

Overall, the results reinforced the higher resistance of biofilm cells compared to their planktonic counterparts, since total reductions of the pre-established biofilms were obtained only at concentrations higher than the bactericidal concentrations found against planktonic cells (Chapter 3). When used individually, the phytochemicals proved to have, in most cases, a greater antibiofilm activity compared to antibiotics (higher log reductions in bacteria culturability at lower concentrations). CIS at 100×128 µg/mL was even able to eradicate all culturable cells of *S. aureus* and MRSA biofilms. Regarding the phytochemical-antibiotic combinations, the results demonstrated concentration-dependent interactions (synergy, antagonism or indifference). Finally, the synergistic effects obtained, mainly against *E. coli*, highlight the potential of using this combinatorial approach to control biofilms.

Chapter 5 | Evaluation of the potential of phytochemical-based controlled drug delivery formulations on the prevention and control of biofilms

5.1 Introduction

Biofilm management in CWs is becoming a primary goal of wound care, as its presence is recognized as a major cause of delayed wound healing [56]. Besides the importance of the removal of a pre-established biofilm, prevent its reformation is also crucial [56]. In fact, one potential and promising strategy against bacterial biofilms is to prevent their formation or reformation, by killing bacteria in planktonic state before biofilm development, while they are still vulnerable to antimicrobial action [54]. Biofilm prevention can also be achieved by the inhibition of the attachment of bacterial cells to a substratum or interface, as for instance by inhibiting the production of adhesins [169]. Furthermore, the alteration of the progression from initial attachment to microcolonies and mature biofilms by interfering with biofilm communication (QS inhibition) is also a good prevention approach [169].

Although EOs have already shown great results as antimicrobial compounds for the prevention and control of biofilms, their poor water solubility and stability can considerably limit their application [100]. Therefore, their encapsulation in drug delivery systems, particularly chitosan (CS) beads, could be a good strategy to overcome these limitations. CS is an FDA-approved biopolymer and can be an asset in CWs treatment due to its hydrating effect, anti-inflammatory and angiogenic activity and hemostatic action [120]. CS facilitates dermal regeneration and skin re-epithelialization [120]. In fact, CS beads have received significant attention for their use in many fields like drug delivery, adsorption and protein release [177].

Ionotropic gelation of CS has been used mainly for the production of gel beads [178]. This method is based on the electrostatic interactions between two ionic species of opposite charge [178,179]. The most used ionic pairs are CS and sodium tripolyphosphate (TPP) [178]. Cationic CS in the presence of diluted acid solution (positively charged amino groups) interacts with TPP due to its polyanionic character (negatively charged phosphate groups), forming a hydrogel [179]. Bioactive molecules are added to the reaction and become trapped within the physical network [178,179]. Usually these beads are synthesized by the addition of drug-loaded CS solution drops into the TPP solution [178]. Indeed, this method has gained much interest because is a simple procedure, allows the production of particles in an extensive range of sizes and has a medium to high drug encapsulation efficiency [178].

Many EOs have been encapsulated into CS platforms with different sizes and shapes, as for instance as microcapsules, films, nanoparticles and nanocapsules [180–183]. However, research regarding EOs-based CS beads on the treatment of biofilms for their potential use as wound dressings is quite limited. Thus, this chapter aims to evaluate the activity of CS beads loaded with CITRO or CIS on the prevention and control of biofilms of *S. aureus*, MRSA, and *E. coli*.

5.2 Materials and methods

5.2.1 Bacterial strains and culture conditions

The bacteria *S. aureus* CECT 976, *E. coli* CECT 434 and MRSA XU212 were used for the biofilm prevention and control studies through the encapsulation strategy and they were preserved at -80 ºC in MHB containing 30% (v/v) glycerol. For the inoculum preparation, the bacterial cultures were grown overnight in MHB at 37 ºC under 160 rpm of agitation before all the experiments.

5.2.2 Phytochemicals

The phytochemicals CITRO and CIS were selected for studies related to the effectiveness of their encapsulation on biofilm prevention and control. Solutions of these phytochemicals were prepared in DMSO for their study in their free form.

5.2.3 Preparation of chitosan beads loaded with selected phytochemicals

Chitosan (medium molecular weight 190,000-310,000 Da; deacetylation degree 75-85%) and sodium tripolyphosphate were purchased from Sigma Aldrich. CS-TPP beads loaded with the phytochemicals CIS and CITRO were prepared by ionic gelation method according to Yuan *et al.* [184] and Zhang *et al.* [185] with some modifications. Firstly, 1% (w/v) CS solution was prepared by dissolving CS (1 g) in 100 mL of an aqueous acetic acid solution (1% v/v, Fisher Chemical) and kept under gentle agitation overnight. After that, this solution was filtered through a 1.00 µm glass fiber (GF) syringe filter to remove any impurity. Tween 80 (equivalent to 10% of the phytochemical) was added to 10 mL of the prepared CS solution to serve as a surfactant and favor the dispersion of the phytochemical. Then, the phytochemical was added to the mixture and stirred until the formation of an oil/water (O/W) emulsion. A 0.5% (w/v) TPP solution was prepared by dissolving TPP (0.25 g) in 50 mL of distilled water. Gelled spheres were formed instantaneously by dropping 10 mL of the prepared CS solution into 10 mL of the TPP aqueous solution. The mixture was kept under gentle agitation for about 1 h. Finally, the beads were washed three times with distilled water, collected and stored at 4 °C. CS beads without phytochemical were also prepared.

5.2.4 Biofilm formation and control

The ability of CS beads loaded with selected phytochemicals to control biofilms was investigated and compared with the activity of the free phytochemical compound. The procedure was the same as described in Chapter 4, Section 4.2.3. However, the concentrations of the phytochemicals tested were selected based upon the results obtained in the biofilm control assays in Chapter 4. Moreover, CS beads without the phytochemicals were also tested and their mass was calculated in order to be equal to the mass of CS beads loaded with phytochemicals, for comparative purposes. Sessile bacteria with DMSO (10%, v/v) and sessile bacteria without phytochemicals or CS beads were used as negative controls. The biofilm was then analyzed in terms of CFUs as described in Chapter 4, Section 4.2.3.1. Log CFU/cm^2 reduction was determined by subtracting the log CFU/cm^2 of the biofilm cells not exposed to phytochemicals or CS beads by the log CFU/cm^2 of the remaining biofilm cells after the exposure to them (Tables C.1 and C.3 in Appendix C). At least two replicates and two independent assays were performed.

5.2.5 Biofilm prevention

The ability of the encapsulated phytochemicals to prevent biofilm formation was also tested according to Baptista *et al.* [186] with some modifications. Briefly, 180 µL of adjusted bacterial suspensions (~ 10^6 cells/mL) were added to 96-wells microtiter plates. Then, 20 µL of each phytochemical at MBC/2 and MBC/4 and the CS beads loaded with the same corresponding mass of phytochemical were added to the bacterial suspension. Moreover, CS beads without the phytochemicals were also tested and their mass was calculated in order to be equal to the mass of CS beads loaded with phytochemicals, for comparative purposes. Plates were incubated at 37 ºC for 24 h under 160 rpm of agitation. Bacterial suspensions with DMSO (10%, v/v) and bacterial suspensions without phytochemicals or CS beads were used as negative controls. After 24 h of exposure, the formed biofilm was analyzed according to the quantification of the culturable cells, as previously stated in Chapter 4, Section 4.2.3.1. Log CFU/cm^2 reduction was determined by subtraction the log CFU/cm^2 of the formed biofilm cells after cell growth in the presence of phytochemicals or CS beads from the log CFU/cm^2 of the formed biofilm cells after cell growth in the absence of those compounds (Tables C.2, C.4 and C.5 in Appendix C). At least two replicates and two repeats were performed.

5.2.6 Statistical analysis

The results were expressed as the average ± SD. The statistical analysis of the results was performed using the Student's t-test for independent samples in excel. Calculations were based on a confidence level of ≥ 95%, where $p < 0.05$ was considered statistically significant.

5.3 Results and discussion

EOs or their individual compounds are known to degrade easily due to their high volatility and photosensitivity, potentially compromising their topical bioavailability on wound site. Therefore, the encapsulation of these compounds can protect them from degradation and it may be possible to reduce the compound concentrations needed to eradicate a biofilm in the wound, as its biological properties are maintained over time. Furthermore, in addition to eradicating a pre-established biofilm in a CW, it is also important to prevent it from reforming. Thus, the encapsulation of EOs can be an interesting strategy to not only improve the biofilm eradication, but also prevent its formation, as it will allow a controlled release of the compound in the wound over time.

Hence, this part of the work aimed to evaluate the antibiofilm potential of CS beads loaded with selected EOs components (CITRO and CIS) on the prevention and control of biofilms of *S. aureus* CECT 976, MRSA XU212 and *E. coli* CECT 434.

CS beads loaded with and without CITRO or CIS were successfully prepared by ionic gelation. They displayed relatively uniform morphology and mean diameters between 2.3 ± 0.1 mm and 2.6 ± 0.1 mm (Figure C.1 in Appendix C). It seems that the presence of phytochemicals influenced the particle size. Indeed, the size of the droplets depends not only on the diameter of the Pasteur pipette (used to form the particles), flow rate of the solution and concentration of the ionic solution in the hardening bath, but also on the viscosity of the polymer solution [187], which may be altered by the presence of the phytochemicals.

5.3.1 Eradication of pre-formed biofilms with chitosan beads loaded with selected phytochemicals

The compounds were encapsulated for the eradication of pre-formed biofilms taking into account the results obtained in Chapter 4. Concerning *E. coli*, since the results obtained from the combination of phytochemicals with antibiotics were already quite promising, the encapsulation of compounds in the control of *E. coli* biofilms was not evaluated. These combinations showed synergistic effects and it was possible to eradicate the biofilm at low concentrations (10×Comb.). It was verified that only the combinations 100×CITRO-FUS and 100×CITRO-MUP presented synergistic effects and were able to completely eradicate the biofilm of *S. aureus* and MRSA, respectively. However, the concentrations of the compounds in those combinations are quite high and can be associated with a higher probability of being toxic against mammalian cells. Thus, the encapsulation strategy was used to understand if CS beads are able to reduce the concentrations of compounds needed to eradicate a biofilm. Since at a concentration of 10×CITRO-antibiotic the combinations had similar effects to the individually tested CITRO (indifferent effect), its individual

encapsulation seems to be a more promising strategy. Therefore, CITRO was encapsulated at a concentration of 10×32 and 10×256 µg/mL against *S. aureus* and MRSA, respectively. Regarding the combinations with CIS against *S. aureus* and MRSA, no synergistic effects were obtained. However, individually CIS was able to completely eradicate the biofilm. Since this was observed only at relatively high doses (100×128 µg/mL against both strains), encapsulation strategy of CIS at a low concentration (10×128 µg/mL for both strains) was used. Thereby, the remaining culturable biofilm cells after exposure to CS beads encapsulated with CITRO or CIS for 1 h were determined and the data are shown in Figure 9.

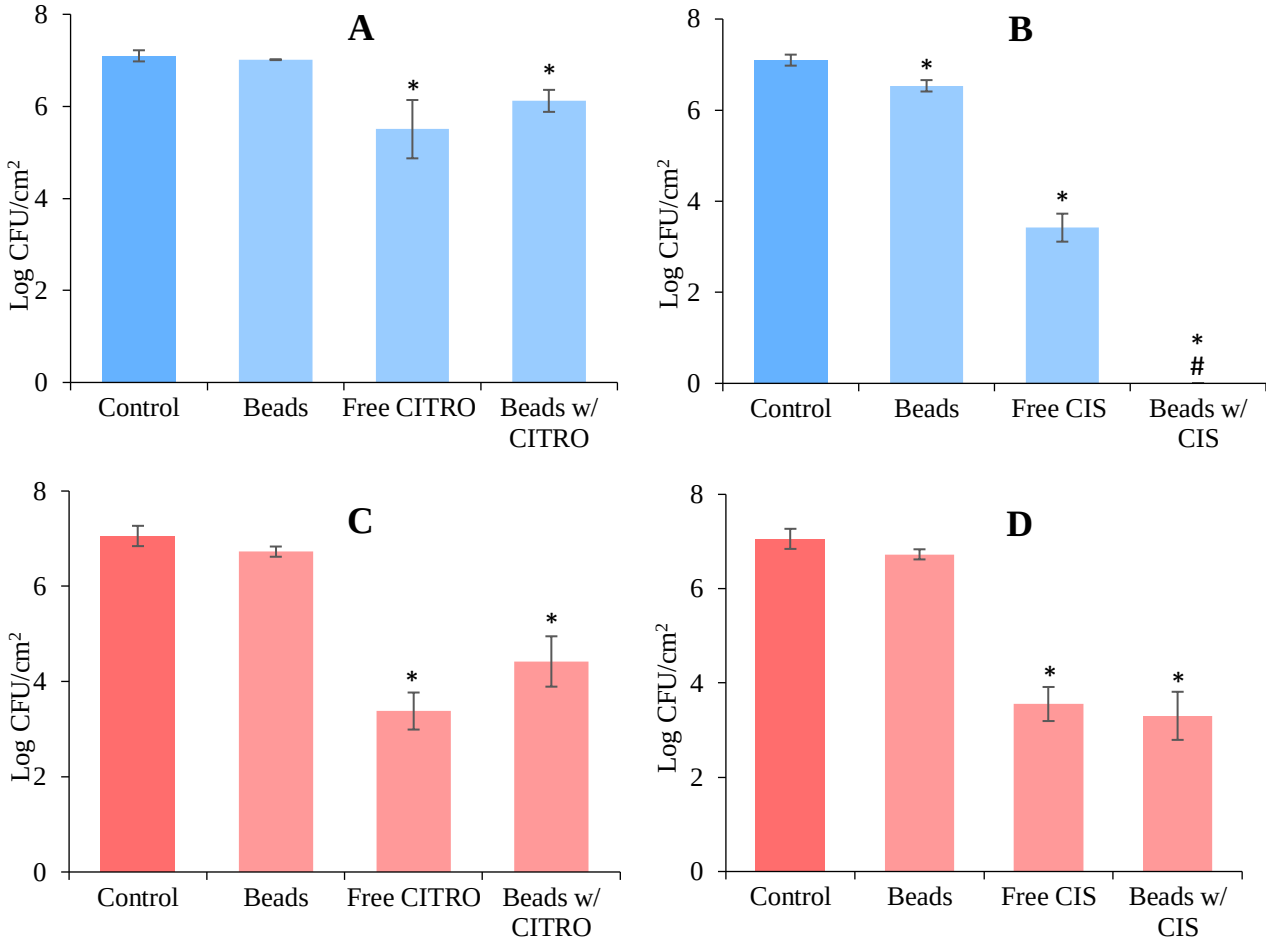


Figure 9. Antibiofilm activity of CS beads loaded with and without CITRO or CIS and phytochemicals in their free form on 24 h-old biofilms of *S. aureus* (A, B) and MRSA (C, D) after 1 h of exposure. Control represents sessile bacteria without phytochemicals or CS beads. Control experiments were performed on antibiofilm activity of DMSO and no activity was found at 10% (v/v) (data not shown). Values represent mean ± SD. # Remaining culturable biofilm cells were below the detection limit of the plate counting method (i.e., 2.1 log CFU/cm^2); * statistically different from the control ($p < 0.05$).

As CS is well recognized for its antimicrobial properties, CS beads without phytochemicals were also tested [117]. Interestingly, unloaded CS beads (graph B of Figure 9) were able to reduce the

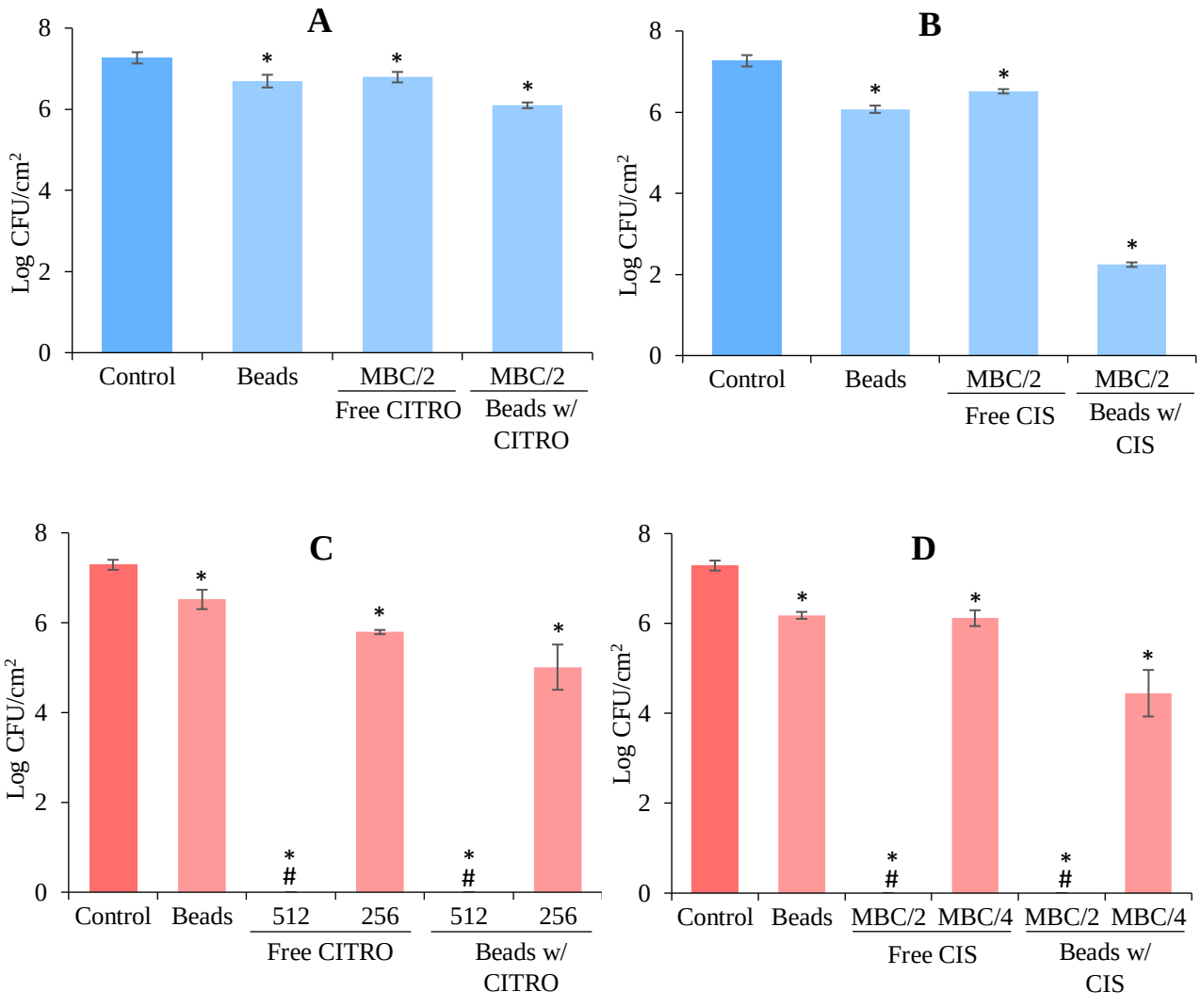
cells on the biofilm of *S. aureus*. Notably, the presence of CIS in those CS beads had an improved antibiofilm effect (higher log CFU/cm^2 reduction, $p < 0.05$). This result shows that selecting this polymer is a good approach, as in addition to its possible ability to protect the phytochemical from degradation, it also has some antibiofilm activity itself. CS beads may even facilitate CIS diffusion through the bacterial cell membrane due to interactions between the CS cationic group and the anionic components on the cell membrane surface [188]. All CS beads loaded with phytochemicals were able to reduce the number of biofilm cells compared to the control. Log CFU/cm^2 reductions of 1.0 and 7.1 for CITRO and CIS, respectively, against *S. aureus* and 2.6 and 3.8 for CITRO and CIS, respectively, against MRSA were obtained. Interestingly, the beads loaded with CIS were able to completely eradicate *S. aureus* biofilm, showing a significant improvement ($p < 0.05$) in the antibiofilm activity when compared to pure CIS. Noteworthy is the fact that the encapsulation of CIS allowed to eradicate the *S. aureus* biofilm at a concentration 10-times lower than the compound in its free form. Comparing this result with the one obtained through the combination 10×CIS-FUS against *S. aureus*, which presented a log CFU/cm^2 reduction of only 2.6, it is readily apparent that, in this case, the encapsulation strategy was much more promising. This result shows that the encapsulation of a natural compound in a polymer also of natural origin can avoid the need to use antibiotics in combination, being also a good strategy to deal with the problem of antibiotic resistance. Regarding CITRO against *S. aureus* and MRSA and CIS against MRSA, it was found that the effects of the loaded beads on the pre-established biofilm were similar to those obtained with the compounds in their free form ($p > 0.05$). In fact, the antimicrobial/antibiofilm activity of the CS beads loaded with phytochemicals is due to the contribution of several factors that may not always act in an additive or synergistic way. However, if the exposure time of biofilm cells to these beads is increased, their antibiofilm effects may be improved. Considering that the compound is encapsulated, its release profile may not be immediate and, therefore, it will not be fully available in just 1 h of exposure. Having said that, phytochemical release studies must be carried out and, according to those results, evaluations of the antibiofilm capacity of those beads for exposure times different from 1 h must be implemented.

Although the encapsulation of phytochemicals has not always shown improved effects over pure compounds, it is important to keep in mind that the wound environment is very different from the one where compounds are subjected to *in vitro* experiments. The high amount of reactive oxygen species in CWs and the wound exudate, which contains salts and proteases, may change the wound microenvironment and ultimately impact the drug and the effectiveness of the treatment [123]. Therefore, due to the complexity of the wound environment, this could easily be responsible for the degradation of EOs, especially when they are used in their free form. Thus, encapsulation may show

more significant effects in experiments that actually mimic the heterogeneity of the wound environment.

5.3.2 Biofilm prevention using chitosan beads loaded with selected phytochemicals

The ability of CS beads loaded with CITRO or CIS to prevent biofilm formation within 24 h was also tested at sub-bactericidal concentrations. The results, in terms of culturable biofilm cells, that were able to adhere to polystyrene, are represented in Figure 10. While the compounds were only tested at MBC/2 against *S. aureus*, they were tested at MBC/2 and MBC/4 against MRSA and *E. coli*. This is due to the fact that both free and encapsulated phytochemicals at MBC/2 totally inhibited MRSA and *E. coli* biofilm formation. Thus, at a concentration of MBC/2 against these strains, it would not be possible to draw conclusions regarding the effectiveness of the loaded beads compared to the free compounds. In addition, as CITRO did not show bactericidal activity against MRSA in the range of concentrations tested in this study (Table 5 in Chapter 3), it was tested at the concentration that caused the maximum log CFU/mL reduction (512 µg/mL, Figure 7 in Chapter 3) and at half that concentration.



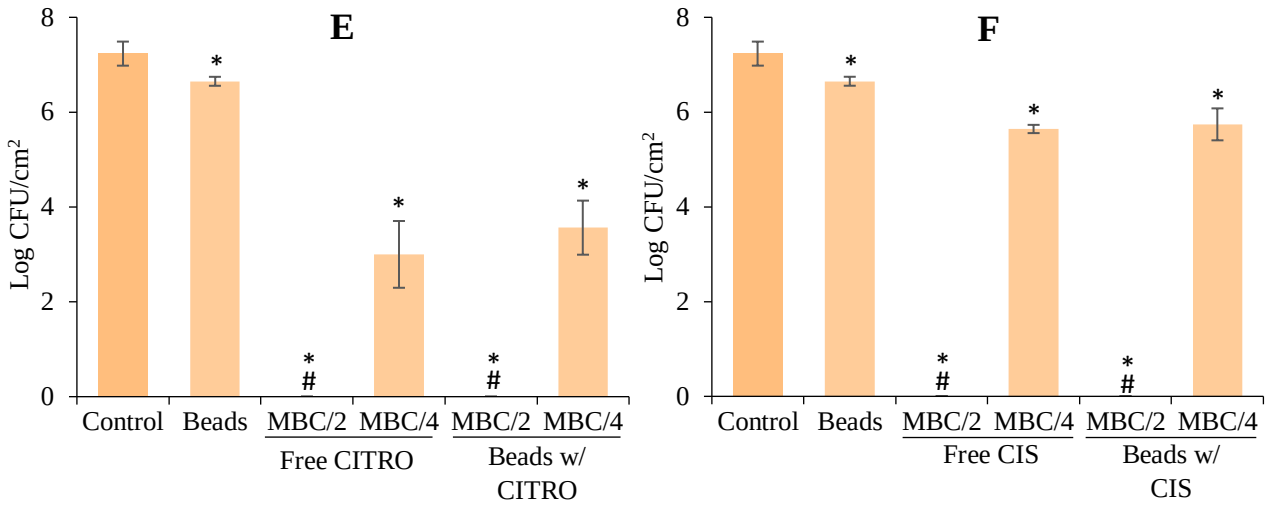


Figure 10. Antibiofilm activity of CS beads loaded with and without CITRO or CIS or compounds in their free form at MBC/2 and MBC/4 on *S. aureus* (A, B), MRSA (C, D) and *E. coli* (E, F) after 24 h growth in their presence. Control represents sessile bacteria without phytochemicals or CS beads. Control experiments were performed on antibiofilm activity of DMSO and no activity was found at 10% (v/v) (data not shown). Values represent mean ± SD. # Remaining culturable biofilm cells were below the detection limit of the plate counting method (i.e., 2.1 log CFU/cm^2); * statistically different from the control ($p < 0.05$).

Unloaded CS beads were able to reduce the number of biofilm cells for all bacterial strains tested, showing, once again, the antibiofilm capacity of CS. They exhibited greater antibiofilm potential in the prevention of biofilm formation than at their control. It is not surprising that the eradication of an established biofilm is usually more difficult to achieve than its prevention, as biofilms are well known to resist the effects of antimicrobials more easily than the free-living bacteria [6]. All CS beads loaded with CITRO or CIS were able to prevent biofilm formation, showing even better results than unloaded beads ($p < 0.05$). Beads loaded with CIS or CITRO at MBC/2 tested against *S. aureus* and beads loaded with CIS at MBC/4 tested against MRSA also demonstrated improved antibiofilm activity than the compounds in their free form ($p < 0.05$). Interestingly, CIS against *S. aureus* showed the most significant increase in antibiofilm activity after encapsulation (0.8 log CFU/cm^2 reduction for free CIS compared to 5.0 log CFU/cm^2 reduction for encapsulated CIS). On the other hand, the effects of beads loaded with CITRO at 256 µg/mL against MRSA and beads with CIS or CITRO at MBC/4 against *E. coli* in the prevention of biofilm formation were similar to those obtained with the pure compounds ($p > 0.05$).

Free compounds tested at MBC/2 were able to completely inhibit the formation of MRSA and *E. coli* biofilms (no CFU/cm^2 detected). Interestingly, sub-bactericidal concentrations were able to fully prevent biofilm formation, but doses above the bactericidal concentrations were needed for its total eradication (Chapter 4). This result demonstrates, once again, the greater capacity to prevent

biofilm formation than to eradicate it. These biofilm prevention results of pure compounds at sub-lethal doses can be strongly related to the characteristics of the bacterial cells involved in biofilm formation. For instance, the flagella production by *E. coli* can be compromised in the presence of these EOs components, preventing biofilm formation, as demonstrated by Burt *et al.* [189]. Furthermore, the ability of microorganisms to adhere to surfaces is influenced, to a certain degree, by the hydrophobicity of the cell surface [6]. Thus, the preventive effects of EOs components, at sub-bactericidal concentrations, may also be related to the capacity of these compounds to alter the cell surface hydrophobicity, leading to a weaker interaction with the substratum or between the cells themselves. Indeed, Lopez-Romero *et al.* showed that CITRO was able to modify the cell surface hydrophobicity of both *E. coli* and *S. aureus* [87].

5.4 Conclusions

This study showed the antibiofilm potential of CS beads loaded with CITRO or CIS. CS presented an intrinsic antibiofilm activity alone. Beads loaded with those phytochemicals were able to prevent the biofilm formation of all bacterial strains and control a pre-established biofilm of *S. aureus*, including a resistant strain (MRSA). They showed an enhanced effect compared to empty CS beads. Furthermore, beads loaded with CIS at 10×128 µg/mL demonstrated more effective antibiofilm properties on pre-established *S. aureus* biofilms than pure CIS. Regarding the effectiveness of loaded beads on the prevention of biofilm formation, it was verified that beads with CIS or CITRO at MBC/2 tested against *S. aureus* and beads loaded with CIS at MBC/4 tested against MRSA also had greater activity than free compounds. Indeed, CIS against *S. aureus* showed the most significant increase in its activity, both in the control and prevention of the biofilm, after its encapsulation. These results suggest that synergistic interaction occurred between CS beads and EOs components. Moreover, overall results confirmed that the eradication of a pre-established biofilm is generally more difficult to achieve than its prevention, as it was possible to completely avoid the formation of a biofilm at sub-bactericidal concentrations.

Therefore, the encapsulation could be used as a promising strategy to protect EO components from degradation, improving their bioavailability in the wound site and consequently their antibiofilm activity.

Chapter 6 | Final remarks

6.1 General conclusions

Pathogenic bacteria, including *E. coli* and *S. aureus*, are known to be related with several infectious diseases. CW infections are an important cause of delayed wound healing, being associated with a substantial healthcare burden and a reduction in quality of life of patients. In addition, the impaired healing in CWs is mostly associated with the capacity of bacteria to establish biofilms, which are notably recalcitrant to antimicrobial agents and immune clearance. Furthermore, bacteria are increasingly acquiring resistance to conventional antibiotic treatments, representing a significant public health threat. Thus, in order to overcome the development of resistant strains, there is an urgent need to find new molecules with antimicrobial/antibiofilm properties or agents capable of recycling antibiotics for which bacteria already acquired resistance. Therefore, this work evaluated the antimicrobial and antibiofilm potential of selected phytochemicals, particularly EOs components (CITRO, CIS, CA and 3,7DOC), to be used as alternative and/or in combination with standard antibiotics (GEN, FUS and MUP) against planktonic cells and biofilms of *E. coli* and *S. aureus*, including a MRSA. In addition to the antimicrobial activity of some phytochemicals already proven and their multiple mechanisms of action, the side effects associated with them are often less as they are derived from plants. Moreover, since EOs are easily degradable due to their high volatility and photosensitivity, the effects of CS beads loaded with these molecules on the control and prevention of biofilms were also studied.

Firstly, all phytochemicals evaluated in this work proved to be drug-like compounds by RO5. CITRO and CIS were the most effective phytochemicals, as they exhibited lower MBC values. Based on a structure activity relationship analysis, it was found that the presence of double bonds and the OH group may be responsible for their higher bactericidal activity. Regarding their mode of action, according to the Chick-Watson model, these compounds may interact chemically with cells, except CITRO that may also interact physically with *S. aureus* cells. In addition, dual combinations between phytochemicals and antibiotics were tested against both planktonic and sessile cells. The results indicated that careful management in the combination of the compounds is needed since the resulted interactions have shown to be dependent on the state of the cell (planktonic or sessile) and the concentration of the compounds in combination. The bactericidal effect on planktonic cells was observed at significantly lower concentrations of the selected phytochemicals and antibiotics when combined (i.e., MBC of the compounds in combination was lower than the MBC of the compounds when tested individually). Thus, the combination of phytochemicals and antibiotics can result in a reduction of their toxicity effects. Regarding sessile cells, the synergistic effects obtained, mostly against *E. coli* biofilms, highpoint that those combinatorial approaches could be a good strategy to

deal with biofilm infections. Synergism is associated with an increase of the antibiofilm effect, which was confirmed by the fact that reductions in culturability of cells caused by the combinations were significantly higher than the sum of reductions by the individual treatments. In addition to this strategy circumventing the problem of bacteria resistance, by extending the effective life of antibiotics or reversing antibiotic resistance, the cost of developing a new antimicrobial is also substantially higher than the cost of finding a combination between known ones. For the phytochemical encapsulation strategy, interesting results were also obtained. Beads with CITRO or CIS were able not only to control a pre-established biofilm, but also to prevent their formation. Remarkably, the encapsulated beads showed equal or increased antibiofilm activity compared to pure compounds. These results prove that the encapsulation of these compounds in CS beads may prevent their degradation and increase their bioavailability. Furthermore, empty CS beads also proved to have some antibiofilm activity themselves.

Overall, the results of this thesis showed that phytochemicals, particularly CITRO and CIS, are promising molecules that could be used as an alternative or complementary approach to conventional antibiotic therapies for the treatment of biofilm infections in CWs. The encapsulation of phytochemicals into delivery formulations could also be a great strategy to treat and prevent biofilm reseed in the wound bed.

6.2 Future research perspectives

Based on the results obtained in this thesis, several interesting aspects were worthy of further investigation. First of all, although the mode of action of phytochemicals regarding their type of interaction with cells (chemical and/or physical) was studied, further research could be made in order to complete an insightful understanding of its action. For instance, understanding if these phytochemicals interfere with membrane integrity of cells, by using, for example, the Live/Dead® BacLight™ Bacterial Viability kit or the transmission electron microscopy (TEM) could be interesting. The hydrophobicity of cells or the surface charge of bacteria, evaluated through the measurement of zeta potential could also be assessed.

This work allowed to determine the antibiofilm capacity of selected antibiotics, phytochemicals and their combinations and CS beads loaded with phytochemicals through the quantification of CFUs. The application of other methods to validate the effects observed by these compounds could be of great interest. For instance, the biofilm mass removal evaluated in terms of biomass content and the biofilm inactivation by measuring the metabolic activity of biofilm cells could be assessed. Viable cells that are not culturable (VBNC) have been defined as cells that, induced by some stress like the presence of antimicrobial agents, become nonculturable on media, but which

can be demonstrated by various methods to be alive and capable of returning to a metabolically active and culturable state (resuscitation) [190]. Thus, the antibiofilm activity of the compounds under study could also be studied by other methods, as for instance Live/Dead® BacLight™ Bacterial Viability kit, in order to understand whether the cells were killed or induced in VBNC. At last, it would be essential to evaluate the ability of the compounds to fight mixed-species microbial biofilms since the biofilms present in wounds are constituted by a large diversity of species.

It would also be important to proceed with the characterization of CS beads loaded with phytochemicals obtained in this work. In that context, studies related to its encapsulation efficiency and drug release should be performed. Moreover, understands the effects of some parameters like pH and TPP concentration on the entrapment of phytochemicals in CS-TPP beads and the subsequent release would also be interesting.

Finally, further investigation is needed regarding the possibility of the antibiofilm strategies presented here being used for clinical purposes, in particular for the treatment of CWs. Therefore, it is essential to understand their cytotoxicity before being applied in humans. Besides that, antimicrobial, antibiofilm and cytotoxicity studies using *in vivo* models should also be taken to evaluate their true behavior in therapeutics, since *in vitro* studies do not necessarily predict *in vivo* outcomes.

6.3 Thesis outputs

The results presented in this thesis are partially published in:

Paper in conference proceedings

Ribeiro, M. *, Silva, M.B.*, Simões, M. Antimicrobial activity of phytochemical-antibiotic combinations against pathogenic bacteria. Proceedings of the 1st International Electronic Conference on Antibiotics—The Equal Power of Antibiotics And Antimicrobial Resistance, 8–17 May 2021, MDPI: Basel, Switzerland, doi:10.3390/ECA2021-09622 * These authors contributed equally to this work (it is possible to find the published PDF paper in Appendix D).

Oral presentation in conference (abstract publication)

Silva, M.B., Ribeiro, M., Simões, M. Antibacterial activity of citronellol on *Staphylococcus aureus* biofilms as a potential treatment for chronic wounds. Conference: IJUP'21 – 14th Meeting of Young Researchers of the University of Porto, 2021, Online, Porto, Portugal.

References

1. Zeng, Q. *et al.* 6.20 Skin Tissue Engineering☆. in: ed. Ducheyne, P. - *Comprehensive Biomaterials II*, 334–382 (Elsevier, 2017).

2. Ermolaeva, S. A. *et al.* 10.18 - Cold Plasma Therapy. in: ed. Brahme, A. - *Comprehensive Biomedical Physics*, 343–367 (Elsevier, 2014).

3. Attinger, C. & Wolcott, R. Clinically Addressing Biofilm in Chronic Wounds. *Adv. wound care* **1**, 127–132 (2012).

4. French, G. L. The continuing crisis in antibiotic resistance. *Int. J. Antimicrob. Agents* **36**, S3–S7 (2010).

5. Nothias, L.-F., Knight, R. & Dorrestein, P. C. Antibiotic discovery is a walk in the park. *Proc. Natl. Acad. Sci.* **113**, 14477 LP – 14479 (2016).

6. Borges, A., Lopez-Romero, J. C., Oliveira, D., Giaouris, E. & Simões, M. Prevention, removal and inactivation of *Escherichia coli* and *Staphylococcus aureus* biofilms using selected monoterpenes of essential oils. *J. Appl. Microbiol.* **123**, 104–115 (2017).

7. Borges, A., Saavedra, M. J. & Simões, M. Insights on antimicrobial resistance, biofilms and the use of phytochemicals as new antimicrobial agents. *Curr. Med. Chem.* **22**, 2590–2614 (2015).

8. Ribeiro, M., Malheiro, J., Grenho, L., Fernandes, M. H. & Simões, M. Cytotoxicity and antimicrobial action of selected phytochemicals against planktonic and sessile *Streptococcus mutans*. *PeerJ* **6**, e4872 (2018).

9. Lammari, N., Louaer, O., Meniai, A. H., Fessi, H. & Elaissari, A. Plant oils: From chemical composition to encapsulated form use. *Int. J. Pharm.* **601**, 120538 (2021).

10. Lazarus, G. S. *et al.* Definitions and guidelines for assessment of wounds and evaluation of healing. *Wound Repair Regen.* **2**, 165–170 (1994).

11. Sarheed, O., Ahmed, A., Shouqair, D. & Boateng, J. Antimicrobial dressings for improving wound healing. in: ed. Alexandrescu, V. - *Wound Heal. Insights into Anc. Challenges*, 373–398 (2016).

12. Mani, S. WOUND HEALING AND IT'S IMPORTANCE- A REVIEW. *Der Pharm. Sin.* **1**, 24 (2014).

13. Kujath, P. & Michelsen, A. Wounds - from physiology to wound dressing. *Dtsch. Arztebl. Int.* **105**, 239–248 (2008).

14. Negut, I., Grumezescu, V. & Grumezescu, A. M. Treatment Strategies for Infected Wounds. *Molecules* **23**, 2392 (2018).

15. Velnar, T., Bailey, T. & Smrkolj, V. The wound healing process: an overview of the cellular and molecular mechanisms. *J. Int. Med. Res.* **37**, 1528–1542 (2009).

16. Enoch, S. & Leaper, D. J. Basic science of wound healing. *Surg.* **26**, 31–37 (2008).

17. Han, G. & Ceilley, R. Chronic Wound Healing: A Review of Current Management and Treatments. *Adv. Ther.* **34**, 599–610 (2017).

18. Cundell, J. Chapter Two - Diabetic Foot Ulcers: Assessment, Treatment, and Management. in: eds.

Davis, J., McLister, A., Cundell, J. & Finlay, D. - *Smart Bandage Technologies*, 37–61 (Academic Press, 2016).

19. Gonzalez, A. C. de O., Costa, T. F., Andrade, Z. de A. & Medrado, A. R. A. P. Wound healing - A literature review. *An. Bras. Dermatol.* **91**, 614–620 (2016).

20. Landén, N. X., Li, D. & Ståhle, M. Transition from inflammation to proliferation: a critical step during wound healing. *Cell. Mol. Life Sci.* **73**, 3861–3885 (2016).

21. Frykberg, R. G. & Banks, J. Challenges in the Treatment of Chronic Wounds. *Adv. wound care* **4**, 560–582 (2015).

22. Avishai, E., Yeghiazaryan, K. & Golubnitschaja, O. Impaired wound healing: facts and hypotheses for multi-professional considerations in predictive, preventive and personalised medicine. *EPMA J.* **8**, 23–33 (2017).

23. Billiar, T. *et al. Schwartz's principles of surgery*. (McGraw-Hill Professional, 2004).

24. Singh, A. *et al.* Meta-analysis of randomized controlled trials on hydrocolloid occlusive dressing versus conventional gauze dressing in the healing of chronic wounds. *Asian J. Surg.* **27**, 326–332 (2004).

25. Fowler, E. Chronic wounds: an overview. *Chronic wound care a Clin. source B. Healthc. Prof.* 12–18 (1990).

26. Marks, M. W., Argenta, L. C. & DeFranzo, A. J. Chapter 7 - Principles and Applications of Vacuum-Assisted Closure (VAC). in: ed. Weinzweig, J. - *Plastic Surgery Secrets*, 38–44 (Mosby, 2010).

27. Guo, S. & Dipietro, L. A. Factors affecting wound healing. *J. Dent. Res.* **89**, 219–229 (2010).

28. Sen, C. K. *et al.* Human skin wounds: a major and snowballing threat to public health and the economy. *Wound Repair Regen.* **17**, 763–771 (2009).

29. Gottrup, F. A specialized wound-healing center concept: importance of a multidisciplinary department structure and surgical treatment facilities in the treatment of chronic wounds. *Am. J. Surg.* **187**, 38S-43S (2004).

30. Kuehn, B. M. Chronic wound care guidelines issued. *JAMA.* **297**, 938–939 (2007).

31. Raziyeva, K. *et al.* Immunology of Acute and Chronic Wound Healing. *Biomolecules.* **11**, 700 (2021).

32. Reportlinker. Global Advanced Wound Care Products Industry. https://www.reportlinker.com/p05961345/Global-Advanced-Wound-Care-Products-Industry.html?utm_source=gnw (2021).

33. Swanson, T. *et al.* Wound infection in clinical practice: principles of best practice. *Wounds Int.* (2016).

34. Haesler, E. & Ousey, K. Evolution of the wound infection continuum. *Wounds Int.* **9**, 6–10 (2018).

35. Farhan, N. & Jeffery, S. Diagnosing Burn Wounds Infection: The Practice Gap & Advances with MolecuLight Bacterial Imaging. *Diagnostics* **11**, 268 (2021).

36. Hernandez, R. The use of systemic antibiotics in the treatment of chronic wounds. *Dermatol. Ther.*

19, 326–337 (2006).

37. Siddiqui, A. R. & Bernstein, J. M. Chronic wound infection: facts and controversies. *Clin. Dermatol.* **28**, 519–526 (2010).

38. Cowan, T. Biofilms and their management: from concept to clinical reality. *J. Wound Care* **20**, 220,222-226 (2011).

39. Landis, S. J. Chronic Wound Infection and Antimicrobial Use. *Adv. Skin Wound Care* **21**, (2008).

40. Debrah, P., Kwapong, A. A. & Fredua-Agyeman, M. Treatment of Biofilms in Infected Wounds. in: ed. Boateng, J. - *Therapeutic Dressings and Wound Healing Applications*, 115–136 (Wiley, 2020)

41. Kalan, L. R. & Brennan, M. B. The role of the microbiome in nonhealing diabetic wounds. *Ann. N. Y. Acad. Sci.* **1435**, 79–92 (2019).

42. Costerton, J. W. Introduction to biofilm. *Int. J. Antimicrob. Agents* **11**, 217–219 (1999).

43. Flemming, H.-C. *et al.* Biofilms: an emergent form of bacterial life. *Nat. Rev. Microbiol.* **14**, 563–575 (2016).

44. Høiby, N. A short history of microbial biofilms and biofilm infections. *APMIS* **125**, 272–275 (2017).

45. Percival, S. L., Bowler, P. & Woods, E. J. Assessing the effect of an antimicrobial wound dressing on biofilms. *Wound repair Regen. Off. Publ. Wound Heal. Soc. [and] Eur. Tissue Repair Soc.* **16**, 52–57 (2008).

46. Dias, C. *et al.* Biofilms and antibiotic susceptibility of multidrug-resistant bacteria from wild animals. *PeerJ* **6**, e4974 (2018).

47. Wei, D. *et al.* Chronic wound biofilms: diagnosis and therapeutic strategies. *Chin. Med. J. (Engl).* **132**, 2737–2744 (2019).

48. Fysun, O., Kern, H., Wilke, B. & Langowski, H.-C. Evaluation of factors influencing dairy biofilm formation in filling hoses of food-processing equipment. *Food Bioprod. Process.* **113**, 39–48 (2019).

49. Monroe, D. Looking for Chinks in the Armor of Bacterial Biofilms. *PLOS Biol.* **5**, e307 (2007).

50. Donlan, R. M. Biofilm Formation: A Clinically Relevant Microbiological Process. *Clin. Infect. Dis.* **33**, 1387–1392 (2001).

51. Wu, Y.-K., Cheng, N.-C. & Cheng, C.-M. Biofilms in Chronic Wounds: Pathogenesis and Diagnosis. *Trends Biotechnol.* **37**, 505–517 (2019).

52. Verderosa, A. D., Totsika, M. & Fairfull-Smith, K. E. Bacterial Biofilm Eradication Agents: A Current Review. *Front. Chem.* **7**, 824 (2019).

53. Renner, L. D. & Weibel, D. B. Physicochemical regulation of biofilm formation. *MRS Bull.* **36**, 347–355 (2011).

54. Bjarnsholt, T., Eberlein, T., Malone, M. & Shultz, G. S. Management of wound biofilm made easy. *Wounds Int.* **8**, 1–6 (2017).

55. Rutherford, S. T. & Bassler, B. L. Bacterial quorum sensing: its role in virulence and possibilities for its control. *Cold Spring Harb. Perspect. Med.* **2**, a012427 (2012).

56. World Union of Wound Healing Societies. Florence Congress, Position Document. *Manag. Biofilm. Wounds Int.* (2016).

57. Davies, D. Understanding biofilm resistance to antibacterial agents. *Nat. Rev. Drug Discov.* **2**, 114–122 (2003).

58. Borges, A. *et al.* New Perspectives on the Use of Phytochemicals as an Emergent Strategy to Control Bacterial Infections Including Biofilms. *Molecules* **21**, 877 (2016).

59. Fish, K. E., Osborn, A. M. & Boxall, J. Characterising and understanding the impact of microbial biofilms and the extracellular polymeric substance (EPS) matrix in drinking water distribution systems. *Environ. Sci. Water Res. Technol.* **2**, 614–630 (2016).

60. Miyaue, S. *et al.* Bacterial Memory of Persisters: Bacterial Persister Cells Can Retain Their Phenotype for Days or Weeks After Withdrawal From Colony–Biofilm Culture. *Frontiers in Microbiology* **9,** 1396 (2018).

61. Simões, M., Bennett, R. N. & Rosa, E. A. S. Understanding antimicrobial activities of phytochemicals against multidrug resistant bacteria and biofilms. *Nat. Prod. Rep.* **26**, 746–757 (2009).

62. Dowsett, C. & Newton, H. Wound bed preparation: TIME in practice. *WOUNDS UK* **1**, 58 (2005).

63. Percival, S. L., Vuotto, C., Donelli, G. & Lipsky, B. A. Biofilms and Wounds: An Identification Algorithm and Potential Treatment Options. *Adv. wound care* **4**, 389–397 (2015).

64. Wolcott, R. *et al.* Biofilm maturity studies indicate sharp debridement opens a timedependent therapeutic window. *J. Wound Care* **19**, 320–328 (2010).

65. Manna, B. & Morrison, C. A. Wound debridement. in *StatPearls [Internet]* (StatPearls Publishing, 2019).

66. Halim, A. S., Khoo, T. L. & Saad, A. Z. M. Wound bed preparation from a clinical perspective. *Indian J. Plast. Surg.* **45**, 193–202 (2012).

67. Martin, J. M., Zenilman, J. M. & Lazarus, G. S. Molecular microbiology: new dimensions for cutaneous biology and wound healing. *J. Invest. Dermatol.* **130**, 38–48 (2010).

68. Ousey, K. & McIntosh, C. Topical antimicrobial agents for the treatment of chronic wounds. *Br. J. Community Nurs.* **14**, S6, S8, S10 passim (2009).

69. Boateng, J. S., Matthews, K. H., Stevens, H. N. E. & Eccleston, G. M. Wound healing dressings and drug delivery systems: a review. *J. Pharm. Sci.* **97**, 2892–2923 (2008).

70. Dhivya, S., Padma, V. V. & Santhini, E. Wound dressings - a review. *BioMedicine* **5**, 22 (2015).

71. Tzaneva, V., Mladenova, I., Todorova, G. & Petkov, D. Antibiotic treatment and resistance in chronic wounds of vascular origin. *Clujul Med.* **89**, 365–370 (2016).

72. Meyer, B. & Cookson, B. Does microbial resistance or adaptation to biocides create a hazard in infection prevention and control? *J. Hosp. Infect.* **76**, 200–205 (2010).

73. Etebu, E. & Arikekpar, I. Antibiotics: Classification and mechanisms of action with emphasis on

molecular perspectives. *Int. J. Appl. Microbiol. Biotechnol. Res.* **4**, 90–101 (2016).

74. Vowden, P., Vowden, K. & Carville, K. Antimicrobial dressings made easy. *Wounds Int.* **2**, (2011).

75. Friedman, N. D., Temkin, E. & Carmeli, Y. The negative impact of antibiotic resistance. *Clin. Microbiol. Infect. Off. Publ. Eur. Soc. Clin. Microbiol. Infect. Dis.* **22**, 416–422 (2016).

76. Chahine, E. B., Dougherty, J. A., Thornby, K.-A. & Guirguis, E. H. Antibiotic Approvals in the Last Decade: Are We Keeping Up With Resistance? *Ann. Pharmacother.* (2021).

77. Reygaert, W. C. An overview of the antimicrobial resistance mechanisms of bacteria. *AIMS Microbiol.* **4**, 482–501 (2018).

78. Ventola, C. L. The antibiotic resistance crisis: part 1: causes and threats. *P T* **40**, 277–283 (2015).

79. Kaiser, P., Wächter, J. & Windbergs, M. Therapy of infected wounds: overcoming clinical challenges by advanced drug delivery systems. *Drug Deliv. Transl. Res.* **11**, 1545–1567 (2021).

80. Rozman, N. A. S. *et al. Homalomena pineodora* essential oil nanoparticle inhibits diabetic wound pathogens. *Sci. Rep.* **10**, 3307 (2020).

81. Toy, L. W. & Macera, L. Evidence-based review of silver dressing use on chronic wounds. *J. Am. Acad. Nurse Pract.* **23**, 183–192 (2011).

82. Khansa, I., Schoenbrunner, A. R., Kraft, C. T. & Janis, J. E. Silver in Wound Care-Friend or Foe?: A Comprehensive Review. *Plast. Reconstr. surgery. Glob. open* **7**, e2390–e2390 (2019).

83. Sibbald, R. G., Leaper, D. J. & Queen, D. Iodine made easy. *Wounds Int* **2**, 1–4 (2011).

84. Newman, D. J., Cragg, G. M. & Snader, K. M. The influence of natural products upon drug discovery. *Nat. Prod. Rep.* **17**, 215–234 (2000).

85. Murphy, C. M. Plant Products as Antimicrobial Agents. *Clin. Microbiol. Rev.* **12**, 564–582 (1999).

86. Ribeiro, M., Simões, L. C. & Simões, M. Biocides. in: ed. Schmidt, T. M. - *Encyclopedia of Microbiology*, 478–490 (Academic Press, 2018).

87. Lopez-Romero, J. C., González-Ríos, H., Borges, A. & Simões, M. Antibacterial Effects and Mode of Action of Selected Essential Oils Components against *Escherichia coli* and *Staphylococcus aureus*. *Evidence-Based Complement. Altern. Med.* **2015**, 795435 (2015).

88. Rasooli, I., Shayegh, S., Taghizadeh, M. & Astaneh, S. D. A. Phytotherapeutic prevention of dental biofilm formation. *Phytother. Res.* **22**, 1162–1167 (2008).

89. Sybiya Vasantha Packiavathy, I. A., Agilandeswari, P., Musthafa, K. S., Karutha Pandian, S. & Veera Ravi, A. Antibiofilm and quorum sensing inhibitory potential of *Cuminum cyminum* and its secondary metabolite methyl eugenol against Gram-negative bacterial pathogens. *Food Res. Int.* **45**, 85–92 (2012).

90. Niu, C., Afre, S. & Gilbert, E. S. Subinhibitory concentrations of cinnamaldehyde interfere with quorum sensing. *Lett. Appl. Microbiol.* **43**, 489–494 (2006).

91. Semeniuc, C., Pop, C. & Rotar, A. Antibacterial activity and interactions of plant essential oil combinations against Gram-positive and Gram-negative bacteria. *J. Food Drug Anal.* **25**, 403–408 (2017).

92. Ismail, M. M., Samir, R., Saber, F. R., Ahmed, S. R. & Farag, M. A. Pimenta Oil as a Potential Treatment for *Acinetobacter baumannii* Wound Infection: *In Vitro* and *In Vivo* Bioassays in Relation to Its Chemical Composition. *Antibiotics* **9**, 1–16 (2020).

93. Garcia-Salinas, S. *et al.* Antimicrobial Wound Dressings against Fluorescent and Methicillin-Sensitive Intracellular Pathogenic Bacteria. *ACS Appl. Mater. Interfaces* **12**, 51302–51313 (2020).

94. Berechet, M. D. *et al.* Bioactive Properties of Nanofibres Based on Concentrated Collagen Hydrolysate Loaded with Thyme and Oregano Essential Oils. *Materials* **13**, 1618 (2020).

95. Liakos, I. L., Holban, A. M., Carzino, R., Lauciello, S. & Grumezescu, A. M. Electrospun Fiber Pads of Cellulose Acetate and Essential Oils with Antimicrobial Activity. *Nanomater. (Basel, Switzerland)* **7**, 84 (2017).

96. Anghel, I. *et al.* Biohybrid nanostructured iron oxide nanoparticles and *Satureja hortensis* to prevent fungal biofilm development. *Int. J. Mol. Sci.* **14**, 18110–18123 (2013).

97. Landis, R. F. *et al.* Cross-Linked Polymer-Stabilized Nanocomposites for the Treatment of Bacterial Biofilms. *ACS Nano* **11**, 946–952 (2017).

98. Mir, M. *et al.* Microneedle liquid injection system assisted delivery of infection responsive nanoparticles: A promising approach for enhanced site-specific delivery of carvacrol against polymicrobial biofilms-infected wounds. *Int. J. Pharm.* **587**, 119643 (2020).

99. Kwiatkowski, P. *et al.* Synergistic effect of fennel essential oil and hydrogen peroxide on bacterial biofilm. *Adv. Dermatology Allergol. Dermatologii i Alergol.* **37**, 690–698 (2020).

100. Duncan, B. *et al.* Nanoparticle-Stabilized Capsules for the Treatment of Bacterial Biofilms. *ACS Nano* **9**, 7775–7782 (2015).

101. Scaffaro, R., Lopresti, F., D'Arrigo, M., Marino, A. & Nostro, A. Efficacy of poly(lactic acid)/carvacrol electrospun membranes against *Staphylococcus aureus* and *Candida albicans* in single and mixed cultures. *Appl. Microbiol. Biotechnol.* **102**, 4171–4181 (2018).

102. García-Salinas, S., Elizondo-Castillo, H., Arruebo, M., Mendoza, G. & Irusta, S. Evaluation of the Antimicrobial Activity and Cytotoxicity of Different Components of Natural Origin Present in Essential Oils. *Molecules* **23**, 1399 (2018).

103. Li, C.-H. *et al.* Phytochemical-Based Nanocomposites for the Treatment of Bacterial Biofilms. *ACS Infect. Dis.* **5**, 1590–1596 (2019).

104. Kyaw, B. M., arora, S. & Lim, C. S. Bactericidal antibiotic-phytochemical combinations against methicillin resistant *Staphylococcus aureus*. *Brazilian Journal of Microbiology* **43**, 938–945 (2012).

105. Jayaraman, P., Sakharkar, M. K., Lim, C. S., Tang, T. H. & Sakharkar, K. R. Activity and interactions of antibiotic and phytochemical combinations against *Pseudomonas aeruginosa in vitro*. *Int. J. Biol. Sci.* **6**, 556–568 (2010).

106. Abreu, A. C., Saavedra, M. J., Simões, L. C. & Simões, M. Combinatorial approaches with selected phytochemicals to increase antibiotic efficacy against *Staphylococcus aureus* biofilms. *Biofouling* **32**, 1103–1114 (2016).

107. Kon, K. V. & Rai, M. K. Chapter 10 - Combining Essential Oils with Antibiotics and other

Antimicrobial Agents to Overcome Multidrug-Resistant Bacteria. in: eds. Rai, M. K. & Kon, K. V. - *Fighting Multidrug Resistance with Herbal Extracts, Essential Oils and Their Components*, 149–164 (Academic Press, 2013).

108. Abreu, A. C. *et al.* Evaluation of the best method to assess antibiotic potentiation by phytochemicals against *Staphylococcus aureus*. *Diagn. Microbiol. Infect. Dis.* **79**, 125–134 (2014).

109. Jain, K. K. *Drug delivery systems*. vol. 251 (Springer, 2008).

110. Vargason, A. M., Anselmo, A. C. & Mitragotri, S. The evolution of commercial drug delivery technologies. *Nat. Biomed. Eng.* (2021).

111. Trucillo, P. Drug Carriers: Classification, Administration, Release Profiles, and Industrial Approach. *Processes* **9**, 470 (2021).

112. Liu, Y., Li, Y. & Shi, L. Controlled drug delivery systems in eradicating bacterial biofilm-associated infections. *J. Control. Release* **329**, 1102–1116 (2021).

113. Fortune Business Insights. Drug Delivery Systems Market Size, Share & Industry Analysis, By Type (Inhalation, Transdermal, Injectables and Others), By Device Type (Conventional and Advanced), By Distribution Channel (Hospital Pharmacies, Retail Pharmacies, and Others) and Regional. https://www.fortunebusinessinsights.com/drug-delivery-systems-market-103070 (2020).

114. Li, J. & Mooney, D. J. Designing hydrogels for controlled drug delivery. *Nat. Rev. Mater.* **1**, 16071 (2016).

115. Chyzy, A., Tomczykowa, M. & Plonska-Brzezinska, M. E. Hydrogels as Potential Nano-, Micro- and Macro-Scale Systems for Controlled Drug Delivery. *Materials*. **13**, 188 (2020).

116. Onaciu, A., Munteanu, R. A., Moldovan, A. I., Moldovan, C. S. & Berindan-Neagoe, I. Hydrogels Based Drug Delivery Synthesis, Characterization and Administration. *Pharmaceutics* **11**, 432 (2019).

117. Qu, B. & Luo, Y. Chitosan-based hydrogel beads: Preparations, modifications and applications in food and agriculture sectors – A review. *Int. J. Biol. Macromol.* **152**, 437–448 (2020).

118. Irmukhametova, G. S., Mun, G. A. & Khutoryanskiy, V. V. Hydrogel Dressings. in: ed. Boateng, J. - *Therapeutic Dressings and Wound Healing Applications*, 185–207 (Wiley, 2020)

119. Rani, M., Agarwal, A. & Negi, Y. S. Review: Chitosan based hydrogel polymeric beads - As drug delivery system. *BioResources* **5**, (2010).

120. Pereira Dos Santos, E. *et al.* Chitosan/Essential Oils Formulations for Potential Use as Wound Dressing: Physical and Antimicrobial Properties. *Mater. (Basel, Switzerland)* **12**, 2223 (2019).

121. Rezaie, H. R., Esnaashary, M., arjmand, A. A. & Öchsner, A. *A Review of Biomaterials and Their Applications in Drug Delivery.* (2018).

122. Aqil, F., Munagala, R., Jeyabalan, J. & Vadhanam, M. V. Bioavailability of phytochemicals and its enhancement by drug delivery systems. *Cancer Lett.* **334**, 133–141 (2013).

123. Smith, R., Russo, J., Fiegel, J. & Brogden, N. Antibiotic Delivery Strategies to Treat Skin Infections When Innate Antimicrobial Defense Fails. *Antibiot. (Basel, Switzerland)* **9**, 56 (2020).

124. Garcia, L. G. S. *et al.* Essential oils encapsulated in chitosan microparticles against *Candida albicans* biofilms. *Int. J. Biol. Macromol.* **166**, 621–632 (2021).

125. Turek, C. & Stintzing, F. C. Stability of Essential Oils: A Review. *Compr. Rev. Food Sci. Food Saf.* **12**, 40–53 (2013).

126. Bachir, R. *Escherichia coli* and *Staphylococcus aureus* most common source of infection. in: ed. Méndez-Vilas, A. - *The Battle Against Microbial Pathogens: Basic Science, Technological Advances and Educational*, 637–648 (FORMATEX, 2015).

127. Michalska-Sionkowska, M., Kaczmarek, B., Walczak, M. & Sionkowska, A. Antimicrobial activity of new materials based on the blends of collagen/chitosan/hyaluronic acid with gentamicin sulfate addition. *Mater. Sci. Eng. C. Mater. Biol. Appl.* **86**, 103–108 (2018).

128. Anjum, A., Sim, C.-H. & Ng, S.-F. Hydrogels Containing Antibiofilm and Antimicrobial Agents Beneficial for Biofilm-Associated Wound Infection: Formulation Characterizations and *In vitro* Study. *AAPS PharmSciTech* **19**, 1219–1230 (2018).

129. Monteiro, N. *et al.* Antibacterial activity of chitosan nanofiber meshes with liposomes immobilized releasing gentamicin. *Acta Biomater.* **18**, 196–205 (2015).

130. Falcone, M. *et al.* Challenges in the management of chronic wound infections. *J. Glob. Antimicrob. Resist.* **26**, 140–147 (2021).

131. National Center for Biotechnology Information. PubChem Compound Summary for CID 8842, Citronellol. https://pubchem.ncbi.nlm.nih.gov/compound/3_7-dimethyloct-6-en-1-ol (2021).

132. National Center for Biotechnology Information. PubChem Compound Summary for CID 5362792, (Z)-Non-6-en-1-ol. https://pubchem.ncbi.nlm.nih.gov/compound/Z_-Non-6-en-1-ol (2021).

133. National Center for Biotechnology Information. PubChem Compound Summary for CID 7792, 3,7-Dimethyloctan-1-ol. https://pubchem.ncbi.nlm.nih.gov/compound/3_7-Dimethyloctan-1-ol. (2021).

134. National Center for Biotechnology Information. PubChem Compound Summary for CID 10402, Citronellic acid. https://pubchem.ncbi.nlm.nih.gov/compound/Citronellic-acid (2021).

135. Faleiro, M. L. & Miguel, M. G. Chapter 6 - Use of Essential Oils and Their Components against Multidrug-Resistant Bacteria. in: eds. Rai, M. K. & Kon K. V. - *Fighting Multidrug Resistance with Herbal Extracts, Essential Oils and Their Component*s, 65–94 (Academic Press, 2013).

136. European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society of Clinical Microbiology and Infectious Dieases (ESCMID). EUCAST Definitive Document E.Def 1.2, May 2000: Terminology relating to methods for the determination of susceptibility of bacteria to antimicrobial agents. *Clin. Microbiol. Infect.* **6**, 503–508 (2000).

137. Fernandes, S., Gomes, I. B. & Simões, M. Antimicrobial activity of glycolic acid and glyoxal against *Bacillus cereus* and *Pseudomonas fluorescens*. *Food Res. Int.* **136**, 109346 (2020).

138. Grobe, K. J., Zahller, J. & Stewart, P. S. Role of dose concentration in biocide efficacy against *Pseudomonas aeruginosa* biofilms. *J. Ind. Microbiol. Biotechnol.* **29**, 10–15 (2002).

139. Fan, X., Ngo, H. & Wu, C. Natural and Bio-based Antimicrobials: A Review. in: *Natural and Bio-Based Antimicrobials for Food Applications*, 1–24 (American Chemical Society, 2018).

140. Lipinski, C. A. Lead- and drug-like compounds: the rule-of-five revolution. *Drug Discov. Today Technol.* **1**, 337–341 (2004).

141. Lipinski, C. A., Lombardo, F., Dominy, B. W. & Feeney, P. J. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv. Drug Deliv. Rev.* **23**, 3–25 (1997).

142. Choy, Y. Bin & Prausnitz, M. R. The rule of five for non-oral routes of drug delivery: ophthalmic, inhalation and transdermal. *Pharm. Res.* **28**, 943–948 (2011).

143. Kohanski, M. A., Dwyer, D. J. & Collins, J. J. How antibiotics kill bacteria: from targets to networks. *Nat. Rev. Microbiol.* **8**, 423–435 (2010).

144. Krause, K. M., Serio, A. W., Kane, T. R. & Connolly, L. E. Aminoglycosides: An Overview. *Cold Spring Harb. Perspect. Med.* **6**, a027029 (2016).

145. Blair, J. M. A., Webber, M. A., Baylay, A. J., Ogbolu, D. O. & Piddock, L. J. V. Molecular mechanisms of antibiotic resistance. *Nat. Rev. Microbiol.* **13**, 42–51 (2015).

146. Hetem, D. J. & Bonten, M. J. M. Clinical relevance of mupirocin resistance in *Staphylococcus aureus*. *J. Hosp. Infect.* **85**, 249–256 (2013).

147. Sutherland, R. *et al.* Antibacterial activity of mupirocin (pseudomonic acid), a new antibiotic for topical use. *Antimicrob. Agents Chemother.* **27**, 495–498 (1985).

148. Fernandes, P. Fusidic Acid: A Bacterial Elongation Factor Inhibitor for the Oral Treatment of Acute and Chronic Staphylococcal Infections. *Cold Spring Harb. Perspect. Med.* **6**, a025437–a025437 (2016).

149. Collignon, P. & Turnidge, J. Fusidic acid *in vitro* activity. *Int. J. Antimicrob. Agents* **12**, S45–S58 (1999).

150. Alyar, S. & Karacan, N. Synthesis, characterization, antimicrobial activity and structure-activity relationships of new aryldisulfonamides. *J. Enzyme Inhib. Med. Chem.* **24**, 986–992 (2009).

151. Ullah, A. *et al.* Amino acid conjugated antimicrobial drugs: Synthesis, lipophilicity- activity relationship, antibacterial and urease inhibition activity. *Eur. J. Med. Chem.* **145**, 140–153 (2017).

152. Gochev, V., Dobreva, A., Girova, T. & Stoyanova, A. Antimicrobial Activity of Essential Oil from *Rosa Alba*. *Biotechnol. Biotechnol. Equip.* **24**, 512–515 (2010).

153. Kotan, R., Kordali, S. & Cakir, A. Screening of Antibacterial Activities of Twenty-One Oxygenated Monoterpenes. *Zeitschrift für Naturforsch. C* **62**, 507–513 (2007).

154. Sinha, S., Jothiramajayam, M., Ghosh, M. & Mukherjee, A. Evaluation of toxicity of essential oils palmarosa, citronella, lemongrass and vetiver in human lymphocytes. *Food Chem. Toxicol. an Int. J. Publ. Br. Ind. Biol. Res. Assoc.* **68**, 71–77 (2014).

155. Phillips, P. L., Wolcott, R. D., Fletcher, J. & Schultz, G. S. Biofilms made easy. *Wounds Int.* **3**, (2010).

156. Mulyaningsih, S., Sporer, F., Reichling, J. & Wink, M. Antibacterial activity of essential oils from Eucalyptus and of selected components against multidrug-resistant bacterial pathogens. *Pharm. Biol.* **49**, 893–899 (2011).

157. Griffin, S. G., Wyllie, S. G., Markham, J. L. & Leach, D. N. The role of structure and molecular properties of terpenoids in determining their antimicrobial activity. *Flavour Fragr. J.* **14**, 322–332 (1999).

158. Nikaido, H. Prevention of drug access to bacterial targets: permeability barriers and active efflux. *Science* **264**, 382–388 (1994).

159. Inouye, S., Takizawa, T. & Yamaguchi, H. Antibacterial activity of essential oils and their major constituents against respiratory tract pathogens by gaseous contact. *J. Antimicrob. Chemother.* **47**, 565–573 (2001).

160. Nazzaro, F., Fratianni, F., De Martino, L., Coppola, R. & De Feo, V. Effect of essential oils on pathogenic bacteria. *Pharmaceuticals (Basel).* **6**, 1451–1474 (2013).

161. Chouhan, S., Sharma, K. & Guleria, S. Antimicrobial Activity of Some Essential Oils-Present Status and Future Perspectives. *Med. (Basel, Switzerland)* **4**, 58 (2017).

162. Russell, A. D. & McDonnell, G. Concentration: a major factor in studying biocidal action. *J. Hosp. Infect.* **44**, 1–3 (2000).

163. Nuñez, L. & D'Aquino, M. Microbicide activity of clove essential oil (*Eugenia caryophyllata*). *Brazilian J. Microbiol.* **43**, 1255–1260 (2012).

164. Breijyeh, Z., Jubeh, B. & Karaman, R. Resistance of Gram-Negative Bacteria to Current Antibacterial Agents and Approaches to Resolve It. *Molecules* **25**, 1340 (2020).

165. Langeveld, W. T., Veldhuizen, E. J. A. & Burt, S. A. Synergy between essential oil components and antibiotics: a review. *Crit. Rev. Microbiol.* **40**, 76–94 (2014).

166. Iseppi, R., Mariani, M., Condò, C., Sabia, C. & Messi, P. Essential Oils: A Natural Weapon against Antibiotic-Resistant Bacteria Responsible for Nosocomial Infections. *Antibiotics* **10**, 417 (2021).

167. Gordon, R. J. & Lowy, F. D. Pathogenesis of Methicillin-Resistant *Staphylococcus aureus* Infection. *Clin. Infect. Dis.* **46**, S350–S359 (2008).

168. Beloin, C., Roux, A. & Ghigo, J. M. *Escherichia coli* biofilms. *Curr. Top. Microbiol. Immunol.* **322**, 249–289 (2008).

169. Lebeaux, D., Ghigo, J.-M. & Beloin, C. Biofilm-related infections: bridging the gap between clinical management and fundamental aspects of recalcitrance toward antibiotics. *Microbiol. Mol. Biol. Rev.* **78**, 510–543 (2014).

170. Husain, F. Antibacterial and antibiofilm activity of some essential oils and compounds against clinical strains of *Staphylococcus aureus*. *J. Biomed. Ther. Sci.* **1**, 65–71 (2014).

171. Teixeira, C. G. de S. *et al.* Antimicrobial photodynamic therapy effectiveness against susceptible and methicillin-resistant *Staphylococcus aureus* biofilms. *Photodiagnosis Photodyn. Ther.* **30**, 101760 (2020).

172. Greulich, P., Scott, M., Evans, M. R. & Allen, R. J. Growth-dependent bacterial susceptibility to ribosome-targeting antibiotics. *Mol. Syst. Biol.* **11**, 796 (2015).

173. Xiao, S., Cui, P., Shi, W. & Zhang, Y. Identification of essential oils with strong activity against stationary phase uropathogenic *Escherichia coli*. *Discovery medicine* **28**, 179–188 (2019).

174. Vasireddy, L., Bingle, L. E. H. & Davies, M. S. Antimicrobial activity of essential oils against multidrug-resistant clinical isolates of the *Burkholderia cepacia* complex. *PLoS One* **13**, e0201835 (2018).

175. Xiao, S., Cui, P., Shi, W. & Zhang, Y. Identification of essential oils with activity against stationary phase *Staphylococcus aureus*. *BMC Complement. Med. Ther.* **20**, 99 (2020).

176. Kavanaugh, N. L. & Ribbeck, K. Selected antimicrobial essential oils eradicate *Pseudomonas* spp. and *Staphylococcus aureus* biofilms. *Appl. Environ. Microbiol.* **78**, 4057–4061 (2012).

177. Li, B., Wang, J., Gui, Q. & Yang, H. Continuous production of uniform chitosan beads as hemostatic dressings by a facile flow injection method. *J. Mater. Chem. B* **8**, 7941–7946 (2020).

178. Sacco, P. *et al.* Ionotropic Gelation of Chitosan Flat Structures and Potential Applications. *Molecules* **26**, 660 (2021).

179. Pedroso-Santana, S. & Fleitas-Salazar, N. Ionotropic gelation method in the synthesis of nanoparticles/microparticles for biomedical purposes. *Polym. Int.* **69**, 443–447 (2020).

180. Hsieh, W.-C., Chang, C.-P. & Gao, Y.-L. Controlled release properties of Chitosan encapsulated volatile Citronella Oil microcapsules by thermal treatments. *Colloids Surf. B. Biointerfaces* **53**, 209–214 (2006).

181. Bonilla, J., Vargas, M., Atarés, L. & Chiralt, A. Physical properties of chitosan-basil essential oil edible films as affected by oil content and homogenization conditions. *Procedia Food Sci.* **1**, 50–56 (2011).

182. Keawchaoon, L. & Yoksan, R. Preparation, characterization and *in vitro* release study of carvacrol-loaded chitosan nanoparticles. *Colloids Surf. B. Biointerfaces* **84**, 163–171 (2011).

183. Sotelo-Boyás, M., Correa-Pacheco, Z., Bautista-Baños, S. & Gómez Y Gómez, Y. Release study and inhibitory activity of thyme essential oil-loaded chitosan nanoparticles and nanocapsules against foodborne bacteria. *Int. J. Biol. Macromol.* **103**, 409–414 (2017).

184. Yuan, D., Jacquier, J. C. & O'Riordan, E. D. Entrapment of protein in chitosan-tripolyphosphate beads and its release in an *in vitro* digestive model. *Food Chem.* **229**, 495–501 (2017).

185. Zhang, H., Jung, J. & Zhao, Y. Preparation and characterization of cellulose nanocrystals films incorporated with essential oil loaded β-chitosan beads. *Food Hydrocoll.* **69**, 164–172 (2017).

186. Baptista, J., Simões, M. & Borges, A. Effect of plant-based catecholic molecules on the prevention and eradication of *Escherichia coli* biofilms: A structure activity relationship study. *Int. Biodeterior. Biodegradation* **141**, 101–113 (2019).

187. Burey, P., Bhandari, B., Howes, T. & Gidley, M. Hydrocolloid Gel Particles: Formation, Characterization, and Application. *Crit. Rev. Food Sci. Nutr.* **48**, 361–377 (2008).

188. Granata, G. *et al.* Oregano and Thyme Essential Oils Encapsulated in Chitosan Nanoparticles as Effective Antimicrobial Agents against Foodborne Pathogens. *Molecules* **26**, 4055 (2021).

189. Burt, S. A. *et al.* Carvacrol induces heat shock protein 60 and inhibits synthesis of flagellin in *Escherichia coli* O157:H7. *Appl. Environ. Microbiol.* **73**, 4484–4490 (2007).

190. Li, L., Mendis, N., Trigui, H., Oliver, J. D. & Faucher, S. P. The importance of the viable but non-culturable state in human bacterial pathogens. *Front. Microbiol.* **5**, 258 (2014).

Appendix

A. Antimicrobial activity of phytochemical-antibiotic combinations against planktonic bacteria

A schematic representation of the checkerboard plate containing the FBCI values for each well and the respective characterization of the interaction of the two antimicrobial agents according to Table 3 in Chapter 3 are shown in Figure A.1. It is important to note that for all tested combinations the FBCI values are the same in each well, since the compounds were always 2-fold diluted.

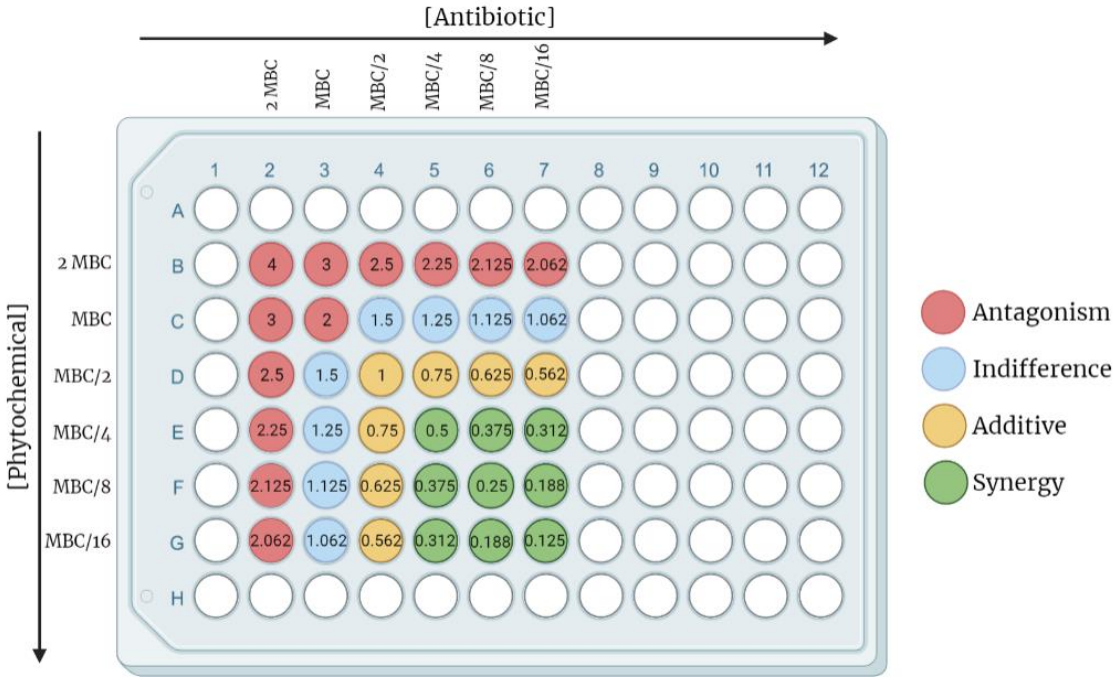


Figure A.1. Schematic representation of the interaction between antibiotics and phytochemicals. Numbers in the checkerboard represent FBCI for each combined concentration of compounds.

B. Action of selected phytochemical-antibiotic combinations on bacterial biofilm control

Tables B.1, B.2 and B.3 present the log CFU/cm^2 reductions caused by the selected antibiotics, phytochemicals and their respective combinations at different concentrations, applied to *S. aureus*, MRSA and *E. coli* pre-established biofilms, respectively, after 1 h exposure.

Table B.1. Log CFU/cm^2 reductions after *S. aureus* biofilm exposure to antibiotics, phytochemicals and their respective combinations.

	FUS (32 µg/mL)			**CITRO** (32 µg/mL)			**CIS** (128 µg/mL)			**Combination**					
										CITRO-FUS			**CIS-FUS**		
	×1	×10	×100	×1	×10	×100	×1	×10	×100	×1	×10	×100	×1	×10	×100
log CFU/cm^2 reductions	0	0	0.9	1.3	1.6	3.1	1.3	3.6	7.3	0.5 (ANT)	1.4 (IND)	7.3 (SYN)	1.5 (IND)	2.6 (ANT)	7.3 (IND)

SYN: synergy; ANT: antagonism; IND: indifference.

Table B.2. Log CFU/cm^2 reductions after MRSA biofilm exposure to antibiotics, phytochemicals and their respective combinations.

	MUP (32 µg/mL)			**CITRO** (256 µg/mL)			**CIS** (128 µg/mL)			**Combination**					
										CITRO-MUP			**CIS-MUP**		
	×1	×10	×100	×1	×10	×100	×1	×10	×100	×1	×10	×100	×1	×10	×100
log CFU/cm^2 reductions	1.4	1.5	1.9	1.4	4.0	4.1	1.2	4.2	7.3	1.8 (IND)	3.9 (IND)	7.3 (SYN)	1.3 (IND)	1.9 (ANT)	7.3 (IND)

SYN: synergy; ANT: antagonism; IND: indifference.

Table B.3. Log CFU/cm^2 reductions after *E. coli* biofilm exposure to antibiotics, phytochemicals and their respective combinations.

	GEN (8 µg/mL)		**CITRO** (128 µg/mL)		**CIS** (128 µg/mL)		**Combination**			
							CITRO-GEN		**CIS-GEN**	
	×1	×10	×1	×10	×1	×10	×1	×10	×1	×10
log CFU/cm^2 reductions	0.4	0.7	0.2	4.0	0.4	4.5	1.5 (SYN)	6.8 (SYN)	1.8 (SYN)	6.8 (SYN)

SYN: synergy.

C. Evaluation of the potential of phytochemical-based controlled drug delivery formulations on the prevention and control of biofilms

An example of CS beads obtained with and without phytochemical is shown in Figure C.1.

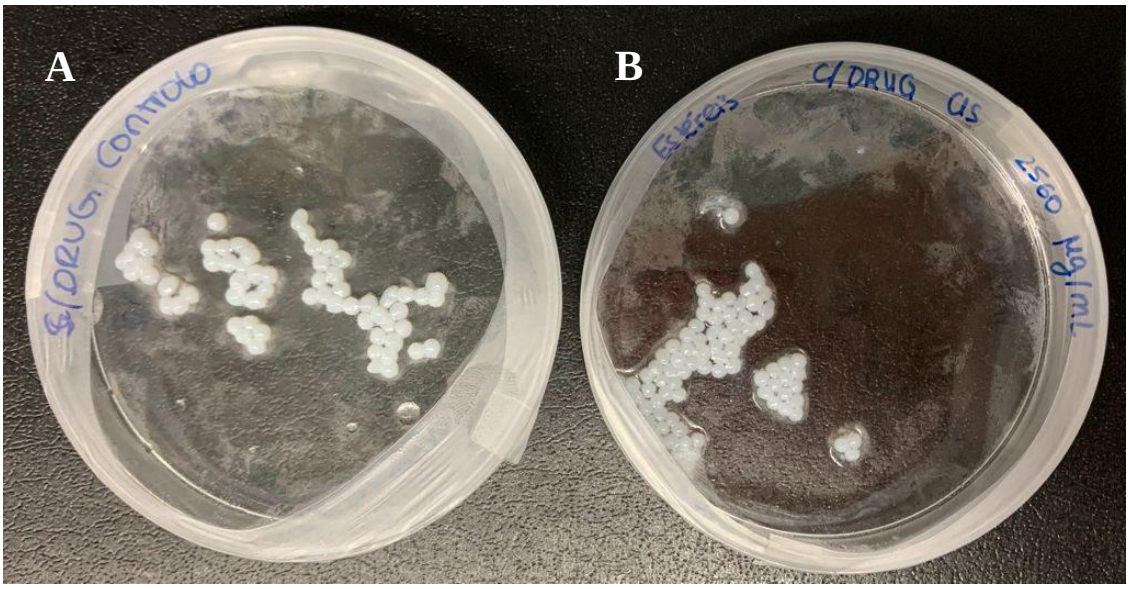


Figure C.1. Photo image of CS beads unloaded (A) and loaded (B) with phytochemical CIS.

Tables C.1 and C.3 present the log CFU/cm^2 reductions caused by the CS beads loaded with and without CITRO or CIS and phytochemicals in their free form against *S. aureus* and MRSA pre-established biofilms, respectively, after 1 h exposure (biofilm control). While in Tables C.2, C.4 and C.5 are represented the log CFU/cm^2 biofilm reductions after 24 h of exposure *S. aureus*, MRSA and *E. coli* planktonic cells, respectively, to the CS beads loaded with and without CITRO or CIS and phytochemicals in their free form (biofilm prevention).

Table C.1. Log CFU/cm^2 reductions after 1 h *S. aureus* biofilm exposure to the CS beads loaded with or without selected phytochemicals and phytochemicals in their free form.

	CITRO (10×32 µg/mL)			**CIS** (10×128 µg/mL)		
	Unloaded beads	Loaded beads	Free	Unloaded beads	Loaded beads	Free
log CFU/cm^2 reductions	0	1.0	1.6	0.6	7.1	3.7

Table C.2. Log CFU/cm^2 biofilm reductions after 24 h of exposure *S. aureus* cells to the CS beads loaded with or without selected phytochemicals and phytochemicals in their free form.

	CITRO (MBC/2)			**CIS** (MBC/2)		
	Unloaded beads	Loaded beads	Free	Unloaded beads	Loaded beads	Free
log CFU/cm^2 reductions	0.6	1.2	0.5	1.2	5.0	0.8

Table C.3. Log CFU/cm^2 reductions after 1 h MRSA biofilm exposure to the CS beads loaded with or without selected phytochemicals and phytochemicals in their free form.

	CITRO (10×256 µg/mL)			**CIS** (10×128 µg/mL)		
	Unloaded beads	Loaded beads	Free	Unloaded beads	Loaded beads	Free
log CFU/cm^2 reductions	0	2.6	3.7	0	3.8	3.5

Table C.4. Log CFU/cm^2 biofilm reductions after 24 h of exposure MRSA cells to the CS beads loaded with or without selected phytochemicals and phytochemicals in their free form.

	CITRO					**CIS**				
	Unloaded beads	512 µg/mL		256 µg/mL		Unloaded beads	(MBC/2)		(MBC/4)	
		Loaded beads	Free	Loaded beads	Free		Loaded beads	Free	Loaded beads	Free
log CFU/cm^2 reductions	0.8	7.3	7.3	2.3	1.5	1.1	7.3	7.3	2.8	1.2

Table C.5. Log CFU/cm^2 biofilm reductions after 24 h of exposure *E. coli* cells to the CS beads loaded with or without selected phytochemicals and phytochemicals in their free form.

	CITRO					**CIS**				
	Unloaded beads	(MBC/2)		(MBC/4)		Unloaded beads	(MBC/2)		(MBC/4)	
		Loaded beads	Free	Loaded beads	Free		Loaded beads	Free	Loaded beads	Free
log CFU/cm^2 reductions	0.6	7.2	7.2	3.7	4.2	0.6	7.2	7.2	1.5	1.6

D. Paper in conference proceedings

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Antimicrobial activity of phytochemical-antibiotic combinations against pathogenic bacteria †

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

Keywords: biofilms; antimicrobial; antibiotics; phytochemicals.

1. Introduction

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

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

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

2. Materials and Methods

2.1. Bacteria and Culture Conditions

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

2.2. Phytochemicals and Antibiotics

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

2.3. Determination of Minimum Bactericidal Concentration (MBC)

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

2.4. Determination of Fractional Bactericidal Concentration (FBC) Index

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

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

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

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

3. Results and Discussion

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

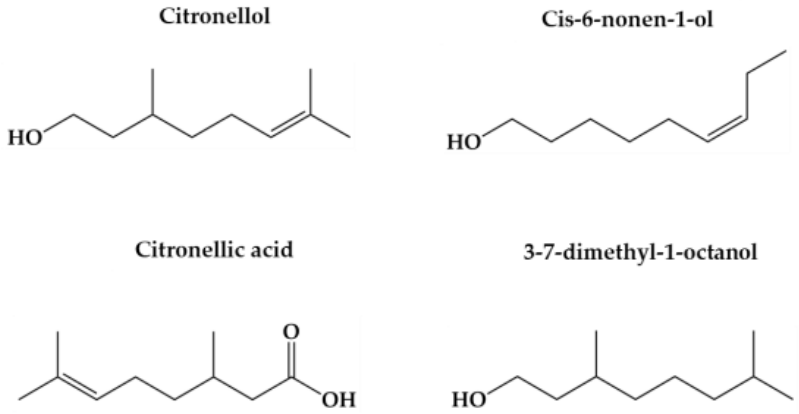

Figure 1. Chemical structure of selected phytochemicals tested in this study. The structures were created using ChemDraw software.

3.1. Bactericidal Activity of Phytochemicals and Antibiotics

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

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

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

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

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

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

3.2. Combinatorial Activity between Phytochemicals and Antibiotics

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

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

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

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

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

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

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

4. Conclusions

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

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

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References

1. R.J. Gordon, F.D. Lowy, Pathogenesis of Methicillin-Resistant *Staphylococcus aureus* Infection, Clin. Infect. Dis. 46 (2008) S350–S359. https://doi.org/10.1086/533591.
2. C. Beloin, A. Roux, J.M. Ghigo, *Escherichia coli* biofilms, Curr. Top. Microbiol. Immunol. 322 (2008) 249–289. https://doi.org/10.1007/978-3-540-75418-3_12.
3. D. Davies, Understanding biofilm resistance to antibacterial agents, Nat. Rev. Drug Discov. 2 (2003) 114–122. https://doi.org/10.1038/nrd1008.
4. A.C. Abreu, A.J. McBain, M. Simões, Plants as sources of new antimicrobials and resistance-modifying agents., Nat. Prod. Rep. 29 (2012) 1007–1021. https://doi.org/10.1039/c2np20035j.
5. M. Ribeiro, J. Malheiro, L. Grenho, M.H. Fernandes, M. Simões, Cytotoxicity and antimicrobial action of selected phytochemicals against planktonic and sessile *Streptococcus mutans*., PeerJ. 6 (2018) e4872. https://doi.org/10.7717/peerj.4872.
6. C. Forni, F. Facchiano, M. Bartoli, S. Pieretti, A. Facchiano, D. D'Arcangelo, S. Norelli, G. Valle, R. Nisini, S. Beninati, C. Tabolacci, R.N. Jadeja, Beneficial Role of Phytochemicals on Oxidative Stress and Age-Related Diseases, Biomed Res. Int. 2019 (2019) 8748253. https://doi.org/10.1155/2019/8748253.
7. EUCAST Definitive Document E.Def 1.2, May 2000: Terminology relating to methods for the determination of susceptibility of bacteria to antimicrobial agents., Clin. Microbiol. Infect. Off. Publ. Eur. Soc. Clin. Microbiol. Infect. Dis. 6 (2000) 503–508. https://doi.org/10.1046/j.1469-0691.2000.00149.x.
8. X. Fan, H. Ngo, C. Wu, Natural and Bio-based Antimicrobials: A Review, in: Nat. Bio-Based Antimicrob. Food Appl., American Chemical Society, 2018: P. 1. https://doi.org/doi:10.1021/bk-2018-1287.ch001.
9. J.M.A. Blair, M.A. Webber, A.J. Baylay, D.O. Ogbolu, L.J. V Piddock, Molecular mechanisms of antibiotic resistance., Nat. Rev. Microbiol. 13 (2015) 42–51. https://doi.org/10.1038/nrmicro3380.

10. M.A. Kohanski, D.J. Dwyer, J.J. Collins, How antibiotics kill bacteria: From targets to networks., Nat. Rev. Microbiol. 8 (2010) 423–435. https://doi.org/10.1038/nrmicro2333.
11. D.J. Hetem, M.J.M. Bonten, Clinical relevance of mupirocin resistance in *Staphylococcus aureus*, J. Hosp. Infect. 85 (2013) 249–256. https://doi.org/https://doi.org/10.1016/j.jhin.2013.09.006.
12. V. Gochev, A. Dobreva, T. Girova, A. Stoyanova, Antimicrobial Activity of Essential Oil from *Rosa alba*, Biotechnol. Biotechnol. Equip. 24 (2010) 512–515. https://doi.org/10.1080/13102818.2010.10817892.
13. W.T. Langeveld, E.J.A. Veldhuizen, S.A. Burt, Synergy between essential oil components and antibiotics: A review., Crit. Rev. Microbiol. 40 (2014) 76–94. https://doi.org/10.3109/1040841X.2013.763219.
14. Z. Breijyeh, B. Jubeh, R. Karaman, Resistance of Gram-Negative Bacteria to Current Antibacterial Agents and Approaches to Resolve It., Molecules. 25 (2020). https://doi.org/10.3390/molecules25061340.