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Insights on Antimicrobial Resistance, Biofilms and the Use of Phytochemicals as New Antimicrobial Agents

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Abstract: Antimicrobial resistance is one of the most serious public health problems. This is of particular concern when bacteria become resistant to various antimicrobial agents simultaneously and when they form biofilms. Consequently, therapeutic options for the treatment of infections have become limited, leading frequently to recurrent infections, treatment failure and increase of morbidity and mortality. Both, persistence and spread of antibiotic resistance, in combination with decreased effectiveness and increased toxicity of current antibiotics have emphasized the urgent need to search alternative sources of antimicrobial substances. Plants are recognized as a source of unexplored chemical structures with high therapeutic potential, including antimicrobial activity against clinically important microorganisms. Additionally, phytochemicals (plant secondary metabolites) present several advantages over synthetic molecules, including green status and different mechanisms of action from antibiotics which could help to overcome the resistance problem. In this study, an overview of the main classes of phytochemicals with antimicrobial properties and their mode of action is presented. A revision about the application of phytochemicals for biofilm prevention and control is also done. Moreover, the use of phytochemicals as scaffolds of new functional molecules to expand the antibiotics pipeline is reviewed.

Keywords: Antibiotic resistance, biofilm control, infectious biofilms, mode of action, natural products, phytochemicals

RESISTANCE TO ANTIBIOTICS: AN EMERGENT PROBLEM

The discovery of antibiotics was considered one of the major advances in the history of medical science due to their role in the control of infectious diseases, which were previously untreatable and fatal [1]. However, the excessive and incorrect use of antibiotics has contributed to the development of antibacterial resistance [2, 3]. These inadequate practices are commonly performed not only in human medicine, but also in veterinary and in agriculture (**Error! Reference source not found.**) [4-6]. Consequently, during the last decades a rapid evolution and spread of resistance among clinically important bacterial species has been observed, which can be manifested through various mechanisms (**Error! Reference source not found.**). This problem becomes more serious when microorganisms, develop resistance not only to a single antimicrobial agent, but also to several antimicrobials or chemical classes available in the market. These microorganisms are often referred as multidrug-resistant (MDR) [7, 8]. Some of them have become so resistant that the therapeutic options are reduced and, sometimes, no commercial antibiotic is effective.

This leads to the increase of treatment failures and severity of infections, and also the emergence of untreatable cases of infectious diseases [7-9].

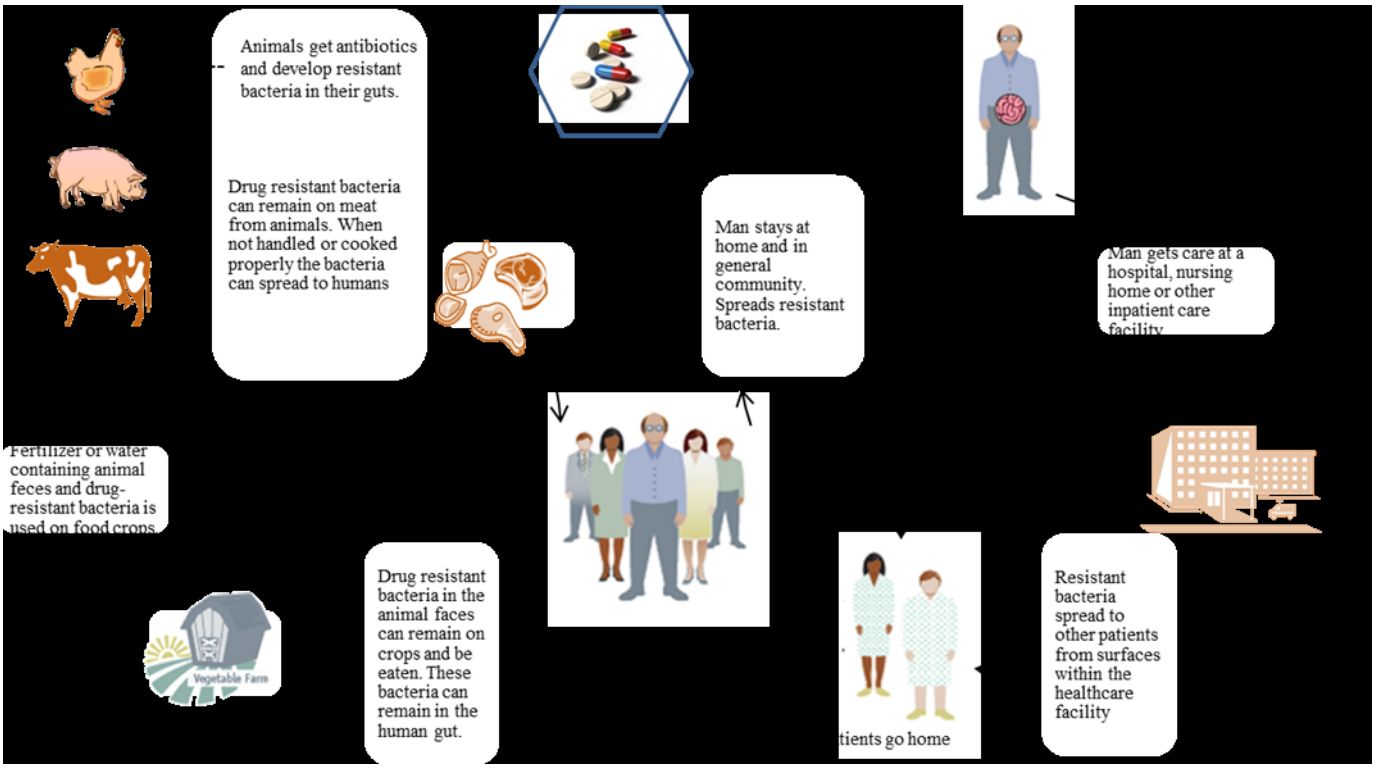

Fig. (1). Scheme on the spread of antibiotic resistance. Antibiotics should be used prudently in the treatment of human/animal infections or as growth promoters, as their prudent usage can generate resistance. Adapted from Center for Disease Control and Prevention (CDC) [10].

Moreover, the periods of hospital care are extended and more costly when treating antibiotic resistant infections [8, 9]. Indeed, when the treatment options are limited (first-line and second-line antibiotic) due to resistance, it is mandatory the use of antibiotics that may be more toxic to the patient and often more costly. Some investigations has shown that even when alternative treatments exist, the probability of dying of patients with resistant infections is frequently higher [10]. Although antibiotic resistance has a considerable and undesirable economical cost, the most dramatic effect is the large morbidity and mortality worldwide. The pathogens of most current concern include: *Enterococcus faecium*, *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa*, *Mycobacterium tuberculosis* and *Enterobacter* species. In particular, multi- and methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant *Enterococcus* (VRE), *P. aeruginosa*, *E. coli* and *K. pneumoniae* producing extended-spectrum β-lactamases (ESBL) and carbapenemases [9, 11].

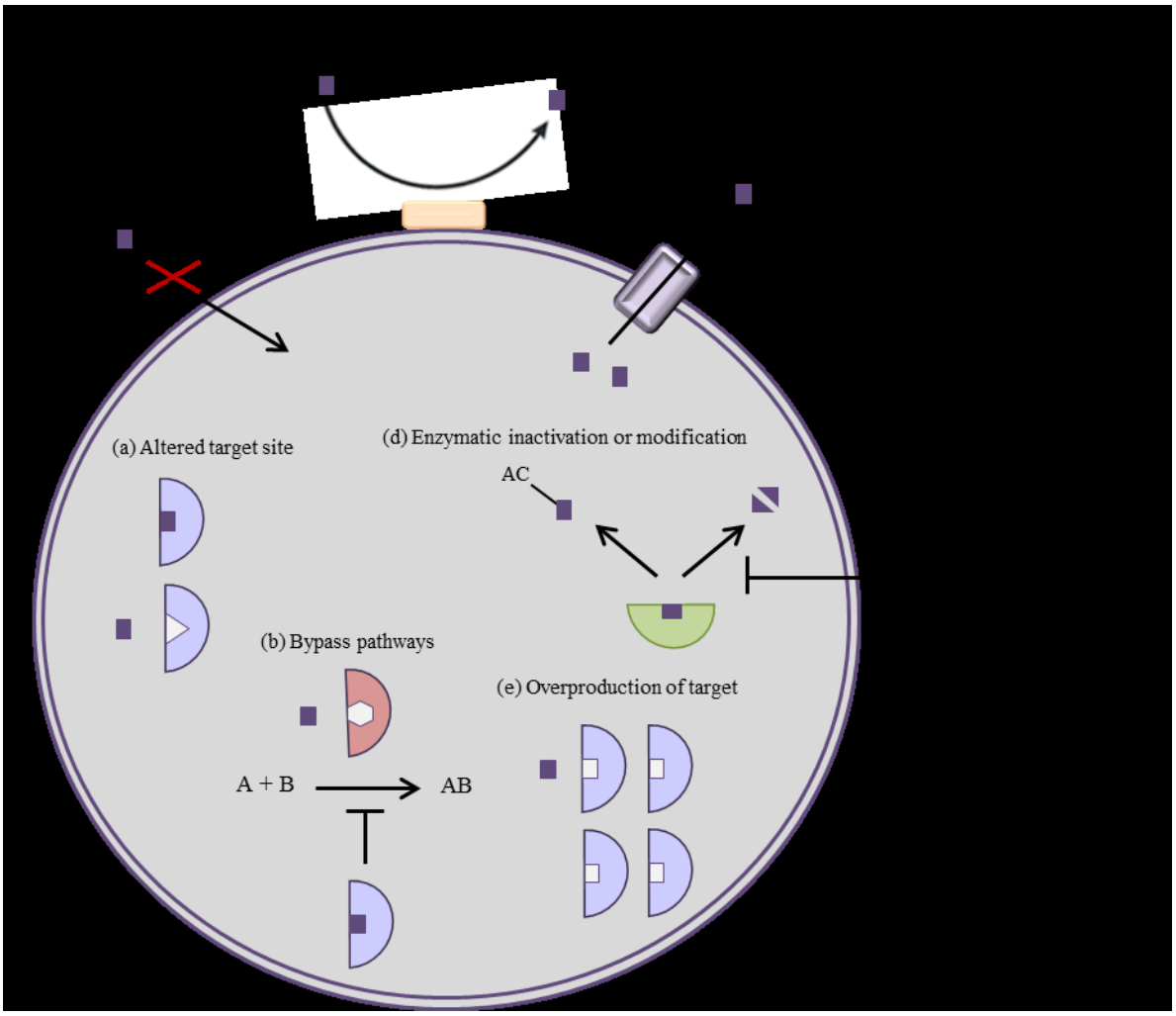

Fig. (2). Mechanisms of bacterial resistance to antimicrobials. (a) modification of the target site; (b) acquisition of alternative metabolic pathways to those inhibited by the drug; (c) alteration of permeability of the bacterial cell wall/membrane that restrict antibacterial agent access to target sites; (d) enzymatic modification or degradation of the antimicrobial agent; (e) over-expression of the drug target; and (f) active efflux pumps that extrude the antibiotic from the cell. Adapted from Coates *et al*. [12].

The understanding of the evolutionary process that is behind resistance requires a global knowledge not only of the genetic causes but also on the physiological consequences of its acquisition [13]. Mechanisms that lead to antibiotic resistance occur in genes that usually play an important role in bacterial physiology and hence in their metabolism (fitness cost) [14]. In this way, the resistance mechanisms are included in the physiology of the bacteria and can be controlled by their metabolic condition [15]. This generally confers a reduction in fitness, expressed as reduced growth rate. A good example is the insusceptibility of various antibiotics against cells that are not actively dividing (dormant cells). The occurrence of dormant

cells aids to elucidate the presence of persistent subpopulations in antibiotic-susceptible bacterial populations. Moreover, they explain the phenotypic resistance demonstrated by certain bacterial modes of life, such as biofilms. Many bacteria in nature and in persistent infections grow in biofilm communities [16, 17]. Drug resistance is also becoming a major problem in infections involving biofilms. In fact, considering the increased rate of resistance development to last option antibiotics and the slow introduction of new molecules, it is expectable that in the coming years serious public health problems may occur if no dramatic changes in antibiotics usage and development are implemented [18].

BIOFILM: AN ADVANTAGEOUS MICROBIAL LIFESTYLE

Biofilms are structured microbial communities of surface-attached cells embedded in a self-produced matrix of extracellular polymeric substances (EPS) composed of proteins, lipids, nucleic acids, polysaccharides, and other components [19, 20]. This lifestyle differs considerably from the planktonic mode of growth as regards to behavior, structure and physiology [21]. Biofilm formation is a phenomenon that occurs in both natural and man-made environments on a wide variety of surfaces, including living tissues, indwelling medical devices, industrial/potable water system piping and natural aquatic systems [22, 23]. There are a number of possible advantages of living in a biofilm community that help to explain what leads a microorganism to form biofilms (Box **1**) [20, 24-26]. Indeed, the microbial cells in biofilms undertake several functions that are not possible to occur when the cells are alone or outside of this sessile community [27].

Box 1. What leads bacteria to produce biofilms?

- Higher protection against environmental stress, predators and antimicrobial agents (e.g. antibiotics and disinfectants);
- Increased access to nutrients;
- Enhanced binding of water molecules, reducing the possibility of dehydration;
- Closer proximity between cells, conferring protection, facilitating mutualistic or synergistic associations (community benefits), and also plasmid transfer that permit the acquisition of antibiotic resistance genes;
- Increased expression of beneficial genes [20, 24-26].

Biofilm Formation

The widespread recognition that biofilms are the predominant mode of life in nature, industrial processes and in infections increased the interest to investigate the mechanisms underlying their formation and maintenance [20]. The development of a mature biofilm is a dynamic and multicellular process that depends enormously on the characteristics of the surface to which attachment occurs, on the bacterial cells involved, on the environmental conditions (e.g. oxygen level, shear force, nutrients) and on the genetic factors (expression of biofilm specific genes) [19, 23]. Biofilm formation is achieved through several steps namely (Fig. **3**): (1) development of a conditioning film; (2) transport of planktonic cells from the surrounding medium to the surface; (3) adhesion of microorganisms; (4) microcolony and biofilm formation; (5) dynamic surface growth and detachment [28, 29].

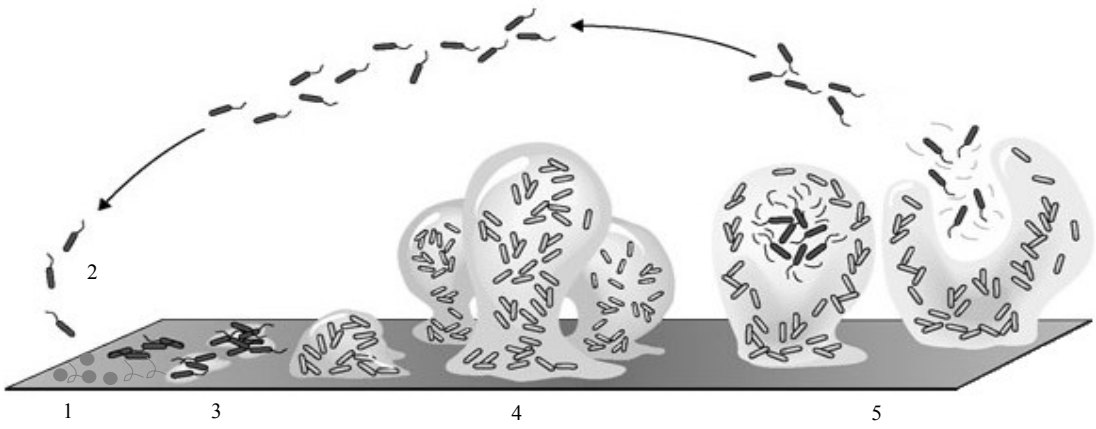

Fig. (3). Scheme of the five steps involved in bacterial biofilm development. Adapted from Stoodley *et al*. [21].

Formation of Conditioning Film and Microbial Mass Transport

Biofilm formation starts with the adsorption of layers of macro/micromolecules (glyproteins, polysaccharides, humic acids, fatty acids and lipids) on the surface, forming a conditioning film. The type and composition of absorbed molecules is dependent on the surface characteristics, nature of the molecules and environmental factors. Both the molecules and the cells are transported to the surface by means of mass transport (combination of convection, diffusion and sedimentation events) [23, 30]. The surface conditioning step alters the physicochemical characteristics of the interface, including surface hydrophobicity and electrical charge and enables the attachment of the cells [31]. Therefore, surface

147 conditioning that prepares the substratum for microbial colonization is an important phenomenon in the
148 early steps of adhesion of microorganisms.
149
150 **Adhesion and Microcolony Formation**
151 The adhesion occurs after surface conditioning and transport of bacteria to one area near the substratum.
152 This is a very complex process that is affected by several variables. In general, it will occur most readily
153 on surfaces that are rougher, more hydrophobic, and coated by surface conditioning films. Adhesion can be
154 divided into two phases involving reversible (mediated by hydrophobic and electrostatic interactions, and
155 non-specific attractive Lifshitz-van der Waals forces – DLVO - Derjaguin-Landau-Verwey-Overbeek
156 forces) and irreversible processes (mediated by dipole-dipole, hydrophobic, ion-dipole, ion-ion, covalent
157 bonds and hydrogen interaction) [31]. Reversible or primary adhesion is the initial weak attachment of
158 microbial cells to a conditioned surface and irreversible or secondary adhesion is the permanent bonding
159 of the microorganisms to a surface.
160 The surface of a microbial cell has a major impact on adhesion. This process is conducted through the
161 expression of bacterial adhesins, which bind to receptors on the substratum and in the EPS matrix [23]. It
162 has been shown that proteinaceous cell surface structures, such as pili, fimbriae, flagella and curli are crucial
163 for the early attachment processes [32, 33]. These are structural components that serve as sensory systems
164 for the environmental cues leading to biofilm formation. Flagella and type IV pili mediate motility (which
165 will be discussed in more detail below), which has been proven to be essential for initial biofilm formation,
166 increasing the chance of adhesion [34]. While initial contact of the cells with surface is dependent of the
167 flagella-mediated motility (e.g. swarming), microcolonies formation and three-dimensional architecture are
168 dependent of the type IV pili associated surface motility (twitching) [34]. Cell surface hydrophobicity, the
169 presence of extracellular appendages, and principally the quantity and composition of produced EPS, are
170 the main factors that influence the rate and degree of microbial adhesion [22, 23].
171 The adhesion of microbial cells to the substratum is followed by formation of microcolonies (cell-to-
172 cell adhesion), which involves the initial production of EPS matrix and multiplication of the attached
173 organisms and/or attachment of other bacteria to already adhered cells, in a phenomenon known as
174 coadhesion [35]. The coaggregation and coadhesion of cells is influenced by temperature, pH, and ionic
175 strength [30]. Within these microcolonies extensive cellular differentiation begins to be observed.
176 Irreversible attachment and EPS production represent the onset steps of biofilm maturation [30].

177

178 **Dynamic Surface Growth and Detachment**

179 As the cells are growing, the biofilm develops complex three-dimensional structures (with water
180 channels and pores) that provide niches with distinct physicochemical conditions. Thus, cells in different
181 regions of the biofilm can exhibit different patterns of gene expression [36]. This process is regulated by
182 production of signaling molecules in a phenomenon known as quorum sensing (QS) (discussed later below)
183 [37]. The cells start to differentiate within the biofilm community and acquire specialized functions,
184 comparable with multicellular organisms [21]. After the full development of a biofilm is achieved, cells
185 begin to senesce and detach. Cells can detach from biofilm by physical (erosion, shear forces, sloughing or
186 abrasion and human intervention) or physiological factors (activation of specific enzymes, for example
187 proteases produced by the biofilm cells) [27, 34]. Nutrient and oxygen depletion, temperature, pH, and the
188 presence of organic molecules are other factors that can lead to biofilm detachment [23]. From an
189 evolutionary point of view, biofilm detachment is beneficial in order to increase genetic diversity, and the
190 colonization of new niches. Conversely, this process has very important implications to public health in
191 particular for the medical sector, increasing the incidence of hospital-acquired infections.

192

193 ## The Role of EPS, Bacterial Motility and QS in Biofilm Formation

194 Biofilms are primarily constituted by microbial cells and a matrix of EPS. The quantity of EPS in
195 biofilms represents about 50-90% of the total organic matter, being a complex mixture of high-molecular
196 mass polymers (>10,000 Da) produced by bacterial cells and products resulting from their lysis/hydrolysis.
197 Although, EPS may vary in terms of chemical and physical properties, they are mainly constituted by
198 polysaccharides. The other components are proteins, nucleic acids, lipids, phospholipids, and humic
199 substances [22, 38].

200 The EPS molecules are regarded as the major factor influencing the biofilm structure. They provide the
201 mechanical stability of biofilms that permits the building of structured and complex communities, within
202 which can occur extensive cellular differentiation [38]. Moreover, the biosynthesis of EPS is believed to
203 serve many functions concerning: promotion of the initial attachment of cells to solid surfaces (adhesion);
204 formation and maintenance of microcolony (cohesion) and mature biofilm structure (three-dimensional);
205 and enhanced biofilm resistance to environmental stress (extreme pH, extreme temperatures and
206 dehydration), disinfectants and antibiotics. In some cases, the EPS matrix also enables the bacteria to

capture nutrients [31, 39, 40]. The highest productivity of EPS compounds is observed during the early stages of the biofilm formation process [31]. The correlation between production of exopolysaccharides and biofilm density was noticed by Tsuneda *et al*. [41]. The role of EPS constituents other than polysaccharides remains to be established. Lipids and nucleic acids (other of the major components of EPS) might significantly influence the stability and integrity of biofilms [42]. For example, extracellular DNA (eDNA) is required for the initial establishment of biofilms of *P. aeruginosa*, *Streptococcus intermedius*, *S. mutans*, *Enterococcus faecalis*, *Bacillus cereus* and staphylococci [43]. The biosynthesis of EPS may reflect not only the attachment and aggregation processes but also provide an ideal environment for the exchange of genetic material between the cells, with eDNA having an important role. Horizontal gene transfer (HGT) is facilitated, since the cells are maintained in close proximity to each other and are not fully immobilized. This enhanced HGT within biofilms directly determines the antimicrobial resistance of the attached cells [23, 24, 43].

Motility plays a major role in the transition from planktonic to surface-associated lifestyle [32]. In addition, bacteria in a motile state suffer alterations in their morphology which distinguish them from their planktonic state [44]. Bacterial motility has been implicated in the process of biofilm formation for a great number of microorganisms. However, both motile and non-motile species can form biofilms [34, 45]. Six different types of motility have been described for microorganism upon surface attachment, namely, swimming, swarming, gliding, twitching, sliding and darting [46]. During swimming, swarming and darting motilities the bacteria use flagella. Twitching has been shown to require type IV pili. However, gliding and sliding are surface movements that do not require flagella or pili [47].

The swarming and twitching are the types of bacterial motility more often involved in biofilm formation. It has been shown that swarming motility has a key role on the early stages of biofilm formation, being important for both initial interaction with the surface and for the movement along it [48]. The major role of flagella-mediated swarming motility in biofilm formation is to promote initial attachment. This is possible because the force-generating motion helps to overcome bacterium-substratum electrostatic repulsive forces. Therefore, the initial interactions between the two surfaces are improved [45]. Shrout *et al*. [49] demonstrated that differences in surface motility could explain differences in biofilm structure at initial phases of development. Moreover, previous reports have demonstrated that many mutants with altered swarming motility were also defective in biofilm formation [45, 49]. It has been shown that biofilm

formation and swarming motility are strictly linked. Besides, these two processes are regulated by a large group of overlapping genes [42].

In addition to swarming, twitching motility has also been shown to be important for initial biofilm structural development. As mentioned, twitching refers to a flagella-independent form of surface translocation mediated by the active extension and retraction of polar type IV pili [50, 51]. The type IV pili-mediated twitching motility is important for the formation of microcolonies and the stabilization of the biofilm [34]. Without type IV pili, bacterial cells are still capable to attach to solid surfaces but ca not build up multicellular layers of the biofilm structure [52]. For example, in *P. aeruginosa* biofilms, microcolonies were produced by the aggregation of individually attached cells *via* twitching motility [32]. Likewise, type IV pili may play a role in subsequent *P. aeruginosa* biofilm development. It was also demonstrated that strains of *P. aeruginosa* type IV pili mutants produced biofilms consisting of a dense cell monolayer with small aggregates, while the wild-type strain produced a characteristic biofilm architecture with a mound-like structure. This suggested that the type IV pili mutants are defective in the developmental events that lead to the formation of mature *P. aeruginosa* biofilm structures [34]. Taking into account the previous information, motility inhibition can be correlated with a decreased ability of bacteria to form biofilms. Therefore, the inhibition of bacterial motility can represent an important strategy to control biofilms. In addition to their role in biofilm formation, it is well established that flagella and pili-mediated motility may also contribute to the virulence of pathogenic bacteria [52, 53].

It is known that populations of bacteria sense and respond to their environments, exhibit intercellular signaling and also interact with cells of their hosts [32]. These characteristics are also likely to be expressed by individual populations localized within biofilm communities, and can be achieved by cell-to-cell interaction also known as QS. QS is mediated by production, release and detection of signaling molecules called autoinducers (AIs) [54, 55]. Therefore, using QS bacterial populations can change from acting as individual cells to functioning in a concerted multi-cellular manner. This system of intercellular communication was first described in marine bacterium *Vibrio fischeri*, in that the production of their bioluminescence is QS dependent, and occurs in response to the increase of cell density [55, 56]. QS can be considered as a complex gene regulatory circuit, dependent on the bacterial cell density, consisting of three components: a small signalling molecule called autoinducer, the gene coding for the autoinducer synthase protein and the gene for a response regulator protein [57]. During QS, AIs are produced and secreted by the bacterial cells. At low cell population density, the concentration of AIs is also low. The

level of released signaling molecules increases, with the increase of the number of cells. Hence, the AIs begin to accumulate in the surrounding environment and when their concentration reaches a critical threshold level (quorum), the QS system is activated and initiates a concerted response that changes the behavior of the bacterial population. This sequence of events lastly leads to the control of gene expression [58, 59].

Although regulation by QS is highly conserved in bacteria its molecular mechanism, as well as the chemical nature of the AIs, differ significantly between Gram-negative and Gram-positive bacteria and is species dependent [57]. Several chemical classes of microbial-derived signalling molecules have now been identified, based upon on shared molecular features. Broadly, these can be split into three categories: *N*-acyl homoserine lactones (AHLs – AI-1), that are predominantly employed by Gram-negative bacteria; autoinducer peptides (AIPs), that are produced by Gram-positive bacteria; and autoinducer-2 (AI-2), a furanosyl borate diester, that is considered a "universal signal" involved in inter-specific communication in both Gram-negative and -positive bacteria [57, 60]. In addition, to these signalling molecules other type called autoinducer-3 (AI-3) has been described. AI-3 is used as an inter-kingdom chemical signalling system between microbes and their hosts [61]. Recent advances indicate that cell-cell communication *via* AIs occurs both within (AHLs and AIPs) and between bacterial species (AI-2).

As the QS controlling pathways are activated when bacteria reach high cell densities, it is expected that QS is induced in biofilms, where the local concentration of cells are generally higher than in planktonic cultures [62]. It is well known that QS is an important event that is linked with the different steps of bacterial biofilm formation [22, 37, 58]. QS systems are almost always integrated into some processes important to initiate biofilm formation, namely bacterial adhesion (e.g. secretion of adhesins) and bacterial motility [63-65]. For example, QS-regulated motility has been demonstrated for several microorganisms, *Serratia liquefaciens*, *Bacillus subtilis*, *B. cepacia*, *P. aeruginosa*, *E. coli* and *Proteus mirabilis* [58, 63, 64]. Biofilm-related characteristics such as formation of microcolonies and EPS production are also often QS regulated [39]. Several aspect of biofilm dynamic including heterogeneity, architecture, stress resistance, maintenance and sloughing has been documented that are mediated by signaling molecules of the type of AHLs. The role of the AHLs in the regulation of colonization events and in the differentiation of microcolonies was also recognized [37]. The production of the EPS is known to be AHL-dependent in some bacteria [55, 63]. Indeed, the role of the AIs, such as AHL and AI-2 in biofilm formation has been shown

by diverse authors [37, 66, 67]. Previous studies showed that mutants lacking QS genes formed biofilms more unstructured and susceptible to chemical agents compared to those formed by wild type strains [37, 68]. Therefore, the interference with the communication systems of microorganisms is a promising target to tackle biofilms [62].

In addition to its role in biofilms, QS regulate the expression of various genes that are involved in many physiological processes such as: bioluminescence, pigment and antibiotic production, conjugation and sporulation [59, 69]. Moreover, it has been shown that QS control the production of virulence factors in both Gram-negative and -positive bacteria [55, 62]. Virulence factors that are QS controlled play an important role in infectious diseases caused by pathogenic bacteria. So, QS systems are potential drug targets for the treatment of infectious diseases [69, 70]. In fact, various pathogenic bacteria such as *P. aeruginosa*, *Vibrio* sp., *B. cepacia* and *Yersinia enterocolitica* employed QS to regulate their virulence and pathogenicity [71].

Mechanisms of Bacterial Resistance in Biofilms

Bacteria embedded in biofilms experiment numerous changes in gene regulation that lead biofilm cells to become phenotypically and metabolically different from their planktonic counterparts [21, 72]. Biofilms are the leading example of physiological adaptation and are one of the main sources of bacterial resistance to antimicrobial products, host defense mechanisms and environmental stress conditions [29, 73, 74]. This bacterial phenotype can be 10-1000 times less susceptible to antimicrobials than the same bacterial population growing in the planktonic state [19, 28, 40]. Consequently, efficient treatment based on conventional antibacterials is hard to achieve, exceeding often the highest deliverable doses [75]. This is particularly worrying, since the National Institute of Health (NIH) estimated that over 80% of microbial infections that occur in the human body involve biofilms. The most common diseases associated with biofilm formation are presented in Table **1**.

Table 1. Common biofilm-associated diseases. Adapted from [73, 76, 77].

Organism	Biofilm-associated disease

Pseudomonas aeruginosa	Cystic fibrosis lung infection
Burkholderia cepacia	Cystic fibrosis lung infection
Acinetobacter baumannii	Burn wound, trauma infection
Helicobacter pylori	Gastrointestinal infection
Escherichia coli	Urinary, catheter infection
Klebsiella pneumoniae	Urinary tract infections
Haemophilus influenzae	Otitis media
Bordetella pertussis	Respiratory infection
Legionella pneumophila	Legionnaires' disease
Staphylococcus aureus	Burn wound, catheter, trauma infection
Staphylococcus epidermidis	Sepsis, catheter infection
Streptococcus mutans	Dental plaques, gingivitis
vancomycin-resistant enterococci (VRE)	Nosocomial infections

The recalcitrant resistance of bacterial biofilms to antibiotic treatment holds serious consequences for the therapy of infections that involve biofilms, leading to increased morbidity and mortality of affected individuals [78]. Nevertheless, the reasons for this much higher resistance are not entirely clear [40]. The conventional mechanisms of antibiotic resistance, referred above, do not seem to be the only responsible for the protection of bacteria in biofilms [72]. Possible explanations for the improved resistance of bacteria in biofilm comprise, innate and induced resistance factors namely (Fig. **4**):

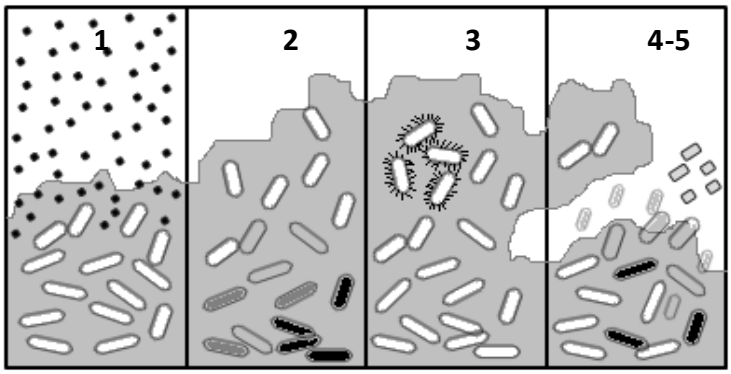

Fig. (4). Illustration of the hypothesized mechanisms of biofilm resistance. 1 – The penetration of antibiotic (squares) is slow and/or incomplete; 2 – Along the biofilm there is heterogeneity – some cells are in a dormant state (shaded cells); 3 – Some cells (marked cells) express different phenotypes as stress response; 4 – Altruist comportment of bacteria (apoptosis) that leads to generation of public goods (gray squares); 5 – A small number of cells differentiate into a more protected state (dark cells) which allows them to survive in adverse condition. Adapted from Stewart [79].

(1) **Limited diffusion/interaction** – Reduced access of the antimicrobials to cells due to their poor penetration of EPS matrix [80]. It has therefore been suggested that the EPS acts as a diffusion barrier which limits the penetration of antimicrobial agents to the surface of biofilm cells by combination of ionic interactions and molecular-sieving events (size exclusion) [36, 79, 81]. In addition to their action as a physical barrier, the antimicrobial agents may be inactivated due to chemical interaction with the components of the EPS, thereby reducing its availability to the underlying cells. This reaction-diffusion limitation property of the EPS matrix can be further enhanced through the production of extracellular enzymes capable of degrading/neutralizing the antimicrobial agents, which can get accumulate within the biofilm matrix and increase resistance [28, 73, 82, 83];

(2) **Reduced growth rate** – An altered bacterial metabolic state within the biofilm leading to areas of reduced or no growth (dormant cells). Slow-growing or non-growing cells are less susceptible to almost all chemical antimicrobial agents, some of which have a requirement for cell replication. Reduction in growth rate and even growth cessation are frequently related to stress response and associated with survival responses [17, 40, 84]. Nutrient and oxygen limitation are two factors that can cause stress in microorganisms. Cells growing in biofilms, particularly the deeply placed cells experiment these limitations that generate a concomitant decrease in growth rates. So, it has been suggested that this physiological change can works favorably for sessile microorganism and account for their resistance [40, 82, 85]. However, the difference between peripheral and inner cells produces physiological gradients across biofilms. Peripheral cells having greater access to nutrients are expected to have growth rates close to planktonic cells, making them more susceptible to antimicrobial treatment, and allow the existence of physiological heterogeneity within the biofilm [81];

(3) **Induction of biofilm specific phenotypes** – While the reaction-diffusion limitation attribute of the EPS and the existence of heterogeneous growth rates within biofilms provide some degree of insusceptibility, they cannot explain completely their tolerance to antimicrobial treatment. It is currently supposed that these two mechanisms delay the action of antimicrobial treatment, and permit the selection of more protected and tolerant biofilm phenotypes, by genetic adaptation. This

mechanism is important because it implies that reduced susceptibility of biofilm bacteria is genetically programmed [81, 83]. As a consequence of this buffering effect, the concentration of antimicrobial available for biofilm cells inactivation is reduced, particularly in the deeper zones. So, the cells may be exposed to sub-inhibitory dosages of the antimicrobial agent for an extended period of time, allowing the emergence of resistance scenarios within the biofilm population [78, 82]. Also, the upregulation of efflux pumps contribute to resistance phenotype [78, 83]. Furthermore, some microorganisms in biofilms have demonstrated the ability to express specific genes of antimicrobial resistance [36, 72];

(4) **Apoptosis or programmed cell death (PCD)** – PCD is a genetically encoded process that conducts to cell death, playing an important function in the life cycle of diverse bacterial species (survival and pathogenesis) [86, 87]. Although the PCD in bacteria is apparently a paradoxical behavior considering that no direct benefit is acquired by the bacterial cells sacrificed, growing evidences suggest that this mechanism represent a potential "altruistic" trait. PCD are a form of cooperation, because survivors are benefited by dead cells through "public goods" production [87]. Sometimes, the observation of cell death after treatment with antimicrobials, is a consequence of this mechanism of programmed suicide and not due to direct action of the compound. In the absence of adverse conditions, the damaged cells can use the nutrients released from their lysed partners, restoring the community. The survival capability of these cells to treatment phases, associated to their proliferation proficiency in the post-treatment phase, confers resistance to the biofilm community [88];

(5) **Persister cells** – The existence of persistent cells is the most recent explanation for decreased biofilm susceptibility to antimicrobials. It has been known for many years that small numbers of persistent bacteria resist killing when exposed to antimicrobials [85, 89]. These so called persister cells, survive to lethal concentrations of antimicrobial agents without undergone mutations that confer resistance. Hence, these subpopulations are not considered to be mutants. Instead, it has been hypothesized that they are phenotypic variants of the wild type that can exist in both planktonic and biofilm populations. Unlike planktonic persisters, biofilm persister cells are protected by EPS, and the remaining persisters will be responsible for biofilm regrowth [73, 89]. However, in a recent study

it was demonstrated that biofilm persister cells may survive to biocide treatment, even in the absence of EPS [74].

Individually, each mechanism is insufficient to explain bofilm resistance. It is thus probable that they complement one another to create insusceptibility and an environment suitable for the emergence of antimicrobial tolerant cells. In fact, biofilm antimicrobial resistance is the result of a complex mixture of innate and induced factors.

NATURAL PRODUCTS AS SOURCE OF NEW DRUGS

Natural products (NPs) are ubiquitous chemical compounds, typically produced by living organisms (plants, fungi, bacteria, insects and other animals) in response to external stimuli that usually have biological and/or pharmacological activities [90]. For thousands of years, NPs and medicinal agents have been closely linked through the use of remedies, ointment, potions and infusions of these bioactive compounds in traditional medicine [91, 92]. According to the World Health Organization (WHO) [93], 70-95% of the world's population depends on traditional medicines for primary health care needs. Traditional medicinal practices provided the basis of most of the early medicines (derived predominantly from plants) followed by subsequent clinical, pharmacological and chemical studies [92]. An notable amount of modern drugs have been obtained from natural sources [94]. The most exemplificative and well-known cases include, acetylsalicylic acid – aspirin (anti-inflammatory agent) isolated from the bark of the willow tree *Salix alba* L.; morphine isolated from *Papaver somniferum* L. (opium poppy) and quinine (anti-malarial drug) isolated from the bark of *Cinchona succirubra* Pav. [91, 92].

NPs traditionally have played an important role in drug discovery. There are innumerous advantages of NPs-based drug discovery compared to its synthetic chemistry counterparts as stated by Knight et *al*. [170]. Currently, it is known that NPs have been the most productive source of active principles for the development of new therapeutic agents, given that more than 80% of drug substances in use today are NPs or based on natural scaffolds [95, 96]. This is especially true for anti-infective agents as recently surveyed by Newman and Cragg [94]. So, these compounds have played an important role in treatment and prevention of wide range of diseases included in diverse areas: infectious diseases (antibacterial, antifungal, antiparasitic and antiviral); cardiovascular and metabolic diseases; neurological diseases (central nervous system - CNS); neoplastic and oncological diseases; immunological, inflammatory and related diseases [94,

97, 98]. In addition to the role that NPs have as drug templates, in many cases they also provide additional information about the targets and pathways involved in the disease process [99].

Numerous reviews about important NPs used to treat diseases have been described extensively. They include compounds derived from microbes (fungi and bacteria), plants, animals and marine sources [92, 94, 97]. Currently, the majority of compounds that are in development are originating from both plant and microbial sources. It has been estimated that only a small part of the world's plant biodiversity has been explored and/or are available for screening [95, 100]. Hence, despite decades of investigation, all evidences suggest that there is still many interesting undiscovered natural molecules with potential therapeutic application.

NATURAL PRODUCTS FROM PLANTS - PHYTOCHEMICALS

Plants have been well documented for their versatile applications and particularly for their medicinal use [91, 100, 101]. They have the capacity to produce an enormous array of natural secondary metabolites (phytochemicals), many of which play a key role in plants defense and have evolve to confer selective advantage against several microorganisms, insects, nematodes and even other plants. The scarcity of infectious diseases in wild plants is itself an indication of the successful defense mechanisms [102-104]. In addition of their activity against pathogenic invaders it is assumed that they have other functions in plant physiology and functionality [29].

The study of pathways involved in production of plant secondary metabolites and their role in plant defense mechanisms against pathogens and also infections has led the scientific community to explore the biological properties of these compounds. Their use in traditional medicine also contributed for this interest. In fact, in many countries (e.g. India, Africa, China) plants are used for thousands of years, as a source of medicines to treat infections caused by microorganisms and other disorders [97, 103, 105, 106]. Likewise, clinical studies have proved the therapeutic value of molecules of plant origin [107]. Hence, in recent years, a large number of plants have been investigated for their antimicrobial properties. The major reasons that have emphasized the research aiming the discovery of antibacterial agents derived from phytochemicals are related with some aspects presented in Box **2** [101, 106, 108].

Box 2. Main reasons that have been leading to explore NPs from plants as source of new antibacterial agents

- Development of MDR by pathogenic microorganisms as consequence of widespread and uncontrolled use of traditional antibiotics;
- High popularity and general acceptance of NPs as tools for disease prevention and health maintenance;
- Plants are considered the major source of chemical diversity;
- Numerous reports on phytochemicals with antibacterial activity, when used alone and as synergists of less effective products, against a wide variety of pathogenic bacteria [102, 107, 109];
- Evidences that phytochemical products can be used as resistance-modifying agents (RMAs), which represent an attractive strategy to mitigate the spread of bacterial drug resistance, since it could facilitate the recycling of ineffective antibiotic that are often cheaper and less toxic than new antimicrobials [110].
- Evident lack of development of new antibacterial products. In fact, only six new antibiotics have been approved over the last decade, and the success of these has been compromised due to the emergence of resistance. The scarce number of novel structural classes combined with the inconsequent management of the use of drugs makes this therapeutic area more susceptible to the emergent of resistant microorganisms [99].

Antibacterial Phytochemicals and Their Mode of Action

In general, current therapies rely on the inhibition of microbial growth, imposing thus a strong selective pressure on the cells and inducing the development of resistance [111]. Unlike synthetic molecules, phytochemical products display an unmatched structural diversity with complex and novel multilayer mechanisms of action. In fact, although some currently used antibiotics act also through multiple modes of action (multiple molecular targets and/or targets encoded by multiple genes) [112], phytochemicals have demonstrated distinctive properties [29]. Therefore, compounds that inhibit bacterial growth by different mechanisms than the presently used by conventional antibiotics, can provide an interesting approach to control drug-resistant infections. Moreover, contrarily to the previously considered strategy “one drug, one target, one disease”, it is now extensively recognized that the use of a single molecule able to operate simultaneously in various targets is more advantageous for the treatment of complex infectious diseases [113]. The use of differential multi-target compounds is an emerging strategy that is widely appreciated. In fact, it is theoretically more difficult for the pathogen to develop resistance when an inhibitor has activity against multiple targets [114]. Therefore, the well-know multi-faceted mode of action of phytochemicals can probably hinder the ability of pathogens to develop resistance. In fact, there are no evidences on the emergence of resistance to phytochemicals.

The antibacterial mechanism of action of phytochemicals is not completely understood [29]. Hence, more studies are needed in order to know their exact antimicrobial targets. Degradation of the cell wall, disruption of cytoplasmatic membrane, damage of membrane proteins, leakage of intracellular contents, coagulation of cytoplasm and depletion of proton have been currently reported as the mechanisms responsible for cell death, caused by some of these compounds [101, 103, 106, 111, 115].

Useful phytochemicals with antimicrobial activity can be divided into several classes that include: phenolics and polyphenolics, terpenoids and other essential oils constituents, alkaloids, lectins and peptides, and polyacetylenes. The major subclasses are: simple phenols and phenolic acids, quinones, flavones, flavonoids and flavonols, tannins, coumarins, terpenoids, alkaloids, lectins and polyketides, polyamines, isothiocyanates, sulfides, thiosulfinates, glycosides, phenanthrenes and stilbenes, among much others [97, 101, 116]. The antimicrobial activity of the main classes/subclasses of phytochemicals, focusing their mechanisms of action will be presented below and are summarized in Table **2**.

Phenolics and Polyphenolics

Phenolic compounds constitute one of the most diverse groups of phytochemicals, being widely distributed in plants and protecting them from microbial infections. They have antioxidant properties but are also potent anti-infectives [111, 117]. The antimicrobial activity of plant phenolics has been extensively studied against human pathogens, to characterize and develop new healthy food ingredients, medical compounds and pharmaceuticals [111, 118]. Phenolics are a large group of aromatic compounds consisting of flavones, flavanones, flavanols and flavonols (one carbonyl group), quinones (two carbonyl groups), tannins (polymeric phenolic substances), and coumarins (phenolic compounds with fused benzene and pyrone groups) [101, 103, 106]. However, based solely on their number of phenol subunits they can be subdivided into three main categories: phenolic acids, flavonoids and tannins [119].

- Phenolic acids

Phenolic acids are one of the major classes of phenolic compounds, that occur with frequency in plant-derived foods [120]. Substituted derivatives of hydroxybenzoic and hydroxycinnamic acids are the predominant phenolic acids in plants, with hydroxycinnamic acids being the mot common. These derivatives differ in the patterns of the hydroxylations and methoxylations of their aromatic rings. The most common hydroxycinnamic acids are caffeic, *p*-coumaric, sinapic and ferulic acids, which frequently occur

505 in foods as simple esters with quinic acid or sugars. Probably, the most well-known bound hydroxycinnamic
506 acid is cholorogenic acid, which is a combination of caffeic and quinic acids. Unlike hydroxycinnamics,
507 hydroxybenzoic acid derivatives are mainly present in foods in glycosylated forms (gallic, *p*-
508 hydroxybenzoic, vanillic, syringic and protocatechuic acids) [119, 121].

509 Phenolic acids have attracted considerable interest in the past few years due to their potential health
510 benefits such as, antioxidant, antibacterial, antiviral, anticarcinogenic, anti-inflammatory and vasodilatory
511 actions [117, 122]. Their antimicrobial activity can be due to their ability to destabilize and permeabilize
512 the cytoplasmatic membrane, inhibition of enzymes involved in radical generation (cytochrome P_{450}
513 isoforms, lipoxygenases, cyclooxygenase and xanthine oxidase) and also the inhibition of the synthesis of
514 nucleic acids of bacteria [101, 118, 123-125]. The potential of phenolic acids to inhibit microbial growth is
515 dependent on the concentration of the undissociated acid and the number and positions of the hydroxyl
516 groups on the aromatic ring [101, 103, 119, 126].

517 In a study performed by Sánchez-Maldonado *et al*. [127], hydroxybenzoic and hydroxycinnamic acids
518 (p-hydroxybenzoic, protocatechuic, gallic, syringic, p-coumaric, caffeic and ferulic acids) exhibited
519 antimicrobial activity against lactic acid bacteria (*Lactobacillus plantarum* and *L. hammesii*), *E. coli* and
520 *B. subtilis*. In addition, these authors found that the activity of phenolic acids was dependent on the number
521 of hydroxyl groups and their substituents. Protocatechuic and gallic acids demonstrated inhibitory activity
522 against five strains of *P. aeruginosa* including clinical isolates. In addition, some of these compounds
523 showed synergistic action with antibiotics [122]. Antibacterial activity was also obtained with gallic and
524 ferulic acids against *E. coli*, *P. aeruginosa*, *S. aureus* (including MRSA) and *L. monocytogenes* [115, 128-
525 132]. Moreover, it was observed synergistic effects between these compounds and the antibiotic
526 streptomycin [132].
527
528
529
530
531
532
533

534 **Table 2.** Main classes and subclasses of phytochemicals with antibacterial properties and description of

535 their mechanisms of action

Class	Subclass	Example(s)	Mechanism of action	Reference(s)
Phenolic and polyphenolics	Phenolic acids	Benzoic (e.g. gallic acid) and cinnamic acids (e.g. ferulic acid)	Destabilize and increase the permeability of the bacterial cytoplasmatic membrane/cell wall; form complexes with extracellular proteins and with the cell wall; interfere with the metabolism of bacterial cells; inhibit enzymes and nucleic acid synthesis; inactivate microbial adhesins	[101, 118, 123-125]
	Flavonoids	Catechin, quercetin and robinetin		[133] [103, 111] [125]
	Tannis	Ellagitannin		
Terpenoids and essential oils	Monoterpenoids	Thymol	Increase the membrane fluidity and permeability; disturb the membrane embedded proteins; inhibit the respiration and alter of ion transport processes in both Gram-positive and -negative bacteria	[134-136]
	Sesquiterpenoids	Farnesol, nerolidol		[105, 136]
	Diterpenoids	Totarol		[137]
	Sesterterpenoids	Oleanolic acid		[138]
Alkaloids		Berberine, piperine and stephanine	Increase the membrane/cell wall permeability and intercalation with DNA	[139] [140] [141, 142]
Peptides	Thionins	Fabatin	Disrupt the cell membranes; inhibit the nucleic acids and protein synthesis	[143-146]
	Plant defensins	Pp-Defensin		
	Lipid transfer proteins	Ace-AMP1		
	Hevein-and knottin-like proteins	Ac-AMP1, Mj-AMP1		
	Snakins	Snakin-1		
Lectins	Legume lectins	Phytohemagglutinin, concanavalin A, isolectin I	Interact with components of the bacterial cell wall (teicoic, teicuronic acids, peptidoglycans and lipopolysaccharides)	[147-149]
	Chitin-binding lectins	Wheat germ agglutinin (WGA)		
	Type 2 ribosome-inactivating proteins	Ricin		
	Jacalin-related lectins	Jacalin (JAC)		
	Amaranthus lectins	Amaranthin		
Polyacetylenes	Falcarinol-type	C17-acetylene and diacetylene falcarindiol	Disrupt the cell membranes	[150]
Glucosinolate hydrolysis products	Isothiocyanates	Allylisothiocyanate, benzyl-isothiocyanate and 2-phenylethylisothiocyanate	Bind to sulfhydryl groups of external proteins of cell membranes	[151-153]
	Nitriles	Indole-3-acetonitrile		

536

537

538 ▪ Flavonoids

539 Flavonoids are one of the biggest classes of secondary metabolites found in various types of edible
540 plants, especially in vegetables, fruits, tea and wine [154]. Flavonoids, share a common structure that
541 comprises two aromatic rings linked by three carbon atoms that form an oxygenated heterocyclic. They can
542 be separated into six subclasses as a function of the type of heterocyclic involved: flavonols, flavones,
543 isoflavones, flavanones, anthocyanidins, and flavan-3-ols (catechins and proanthocyanidins) [121]. They
544 have been identified as potent antimicrobial agents and were suggested as a therapeutic possibility [155].
545 Their activity is arguably due to the ability to form a complex with extracellular proteins, which then binds
546 to the bacterial cell wall, increasing their permeability. Flavonoids with greater lipophilic character may
547 also disrupt microbial membranes [133]. Flavonoids with less hydroxyl groups on their *β*-rings are more
548 active against microorganisms and its target are the membranes with -OH groups [103, 111]. Interference
549 with metabolism and inhibition of nucleic acid synthesis was also reported as possible mechanisms of action
550 [125].
551 Catechin, a component present in different plants, particularly in the tea-plant *Camelia sinensis*, forms
552 complexes with the bacterial cell wall of intestinal microorganisms [156]. Quercetin, a flavonoid found in
553 propolis causes an increase in permeability of the inner membrane of *E. coli* and also dissipation of
554 membrane potential [157]. This flavonoid can inhibit DNA gyrase. Also, rutin demonstrated the potential
555 to inactivate specific bacterial enzymes [125]. Moreover, other flavonoids such as (-)-epigallocatechin
556 gallate (EGCG), myricetin and robinetin, from *Elaegnus glabra* can inhibit the synthesis of nucleic acids
557 of both Gram-negative and -positive bacteria [125]. Also, it was reported that EGCG inhibits antibiotic
558 efflux in MRSA [158]. An amino-coumarin, 7-amino-4-methylcoumarin, from *Ginkgo biloba*, had broad-
559 spectrum antibacterial activities against *S. aureus*, *E. coli*, *S. typhimurium*, *Salmonella enteritidis*, *A.*
560 *hydrophila*, *Yersinia* sp., *Shigella* sp. and *Vibrio parahaemolyticus* [111]. Some researchers reported the
561 synergy between active flavonoids as well as between flavonoids and antibiotics (e.g. vancomycin,
562 fosfomycin, minocycline, rifampicin and oxacillin) against resistant strains [125]. For example, significant
563 synergy was observed between theaflavin and epicatechin against important nosocomial Gram-negative
564 pathogens [159]. Moreover, recovery of *β*-lactam activity against MRSA was also observed with some
565 catechins and gallates [160].
566
567 ▪ Tannins

Tannins are found in almost every plant part (bark, wood, leaves, fruits, and roots), and can be divided into two groups, hydrolysable (based on gallic acid moiety) and non-hydrolysable (condensed) tannins (derived from flavonoid monomers and called proanthocyanidins) [101, 161]. Nutritional and biological properties of tannins have been described previously [162]. In addition, antibacterial actions of tannins have been reported as bacteriostatic and bactericidal against different harmful bacteria, including *A. hydrophila*, *E. coli*, *Listeria*, *Pseudomonas* spp., *Salmonella* spp., *Staphylococcus* spp. and *Streptococcus* spp. [163-165]. Their mode of action is apparently related to their ability to inactivate microbial adhesins, enzymes, membrane proteins and formation of complexes with cell wall. Also, they can complex with polysaccharide, which is suggested to be the main reason for their inhibitory effects on bacteria [166, 167].

Terpenoids and Essential Oils

Terpenoids, also referred as terpenes (compounds based in an isoprene structure with additional elements such as oxygen), are the largest group of natural compounds. All terpenoids are synthesized from two to five-carbon building blocks. Based on the number of the building blocks, terpenoids are commonly classified as monoterpenoids (C_{10}), sesquiterpenoids (C_{15}), diterpenoids (C_{20}), and sesterterpenoids (C_{25}) [168]. Terpenoids are one of the main classes of constituents of essential oils (EO) and are present as either monoterpenoids or sesquiterpenoids, and their derivatives [169]. These bioactive products have a lot of biological properties, including antioxidant and antimicrobial activities. Due to their recognized antimicrobial potential, terpenoids has been the subject of several studies along the years [170]. The most prominent activity is exhibited by the oils that contain phenols such as thymol, carvacrol and eugenol [171].

The mechanism of action of terpenoids is not fully understood, but it is speculated that involves membrane disruption by the lipophilic compounds and their activity depend largely of the structure of the compound, as recently demonstrated by some authors [134-136]. This antibacterial action can result in the increase of membrane fluidity/permeability, disruption of membrane embedded proteins, and change of ion transport processes in both Gram-positive and -negative bacteria [134-136].

Sesquiterpenoids isolated from different plants exhibited antimicrobial activity against Gram-positive and -negative bacteria and inhibited the growth of *M. tuberculosis* [172-174]. It was demonstrated that six diterpenoids isolated from the bark of *Podocarpus nagi*, of which the most abundant compound was totarol, exhibited potent bactericidal activity against the Gram-positive bacteria *Propionobacterium acnes*, *S. mutans* and *S. aureus* [137]. Similarly, bactericidal and bacteriostatic activity of diterpenoids isolated from

598 roots of *Salvia sclarea* L. was also observed against *S. aureus* and *Staphylococcus epidermidis* [175].
599 Antimicrobial properties against oral pathogens (*Streptococcus sobrinus*, *Streptococcus mutans*,
600 *Streptococcus mitis* and *Streptococcus sanguinis*) was also observed with some diterpenoids [176].
601 Oleanolic acid, a triterpenoid from leaves of *Salvia officinalis* exhibit potent activity against *Streptococcus*
602 *pneumoniae*, VRE and MRSA [138]. In a study of Togashi *et al*. [177] farnesol the sesquiterpene alcohol
603 found in EOs, showed antimicrobial activity against *S. aureus*. It has also been verified synergic effect
604 between the major classes of clinically relevant antibacterials and sesquiterpenoids such as farnesol and
605 nerolidol [105, 136]. Moreover, salvipisone and aethiopinone from *Salvia sclarea* hairy roots, showed
606 synergy with several classes of antibiotics [178]. In the case of *β*-lactams class this phenomenon was due
607 to the probable alternation of cell surface hydrophobicity and cell envelopes permeability [178]. Eugenol
608 (a constituent of clove oil) demonstrated synergistic activity with ampicillin and gentamicin against various
609 cariogenic/periodontopathogenic bacteria (*Streptococcus criceti* and *Streptococcus gordonii*, *Streptococcus*
610 sanguinis and *Porphyromonas gingivalis*) [179]. Gossypol (a bissesquiterpene from cotton seeds) and some
611 of its derivatives have demonstrated several biological activities, including antimicrobial [180-182].
612 Przybylski and coworkers [183] obtained an interesting antimicrobial activity with gossypol against
613 several strains of Gram-negative (*E. coli*, *Proteus vulgaris*, *P. aeruginosa*, *Bordetella bronchiseptica*) and
614 -positive bacteria (*S. aureus*, including MRSA strains, *S. epidermidis*, *B. cereus*, *B. subtilis*, *Enterococcus*
615 *hirae* and *Micrococcus luteus*), including clinical isolates. Gossypol and its isomers also exhibited
616 antimicrobial activity against *Edwardsiella ictaluri* [184].
617
618 **Alkaloids**
619 Alkaloids represent a highly diverse group of compounds with a nitrogen atom in a heterocyclic ring
620 [185]. They are historically known since the isolation of morphine from *P. somniferum,* which is probably
621 the first reported clinically important alkaloid [103, 111].
622 Numerous plant families are known to produce alkaloids and has been reported that several of them
623 possess high antimicrobial activity and could therefore be a good alternative for actual drugs [111]. Extracts
624 from different parts of *Terminalia chebula* containing alkaloids showed antimicrobial activity against nine
625 MDR bacteria, namely *E. coli*, *P. aeruginosa*, *P. mirabilis, S. aureus, B. subtilis, Raoultella planticola*,
626 *Enterobacter aerogens, Agrobacterium tumefaciens,* and *K. pneumonia* [186]. Likewise, ethanolic extracts
627 of *Tabernaemontana catharinensis* root bark that contain indole alkaloids revealed antibacterial activity

[187]. Diterpenoid alkaloids found in plants of the *Ranunculaceae* family are frequently reported for their antimicrobial properties [188]. Berberine, an isoquinolone alkaloid isolated from *Mahonia aquifolium,* has activity against Gram-positive bacteria [140]. Moreover, canthin-6-one (from *Allium neapolitanum*) inhibited several strains of *Mycobacterium smegmatis* and *S. aureus* [189]. Stephanine and crebanine two alkaloids isolated from tubers of the traditional Chinese medicinal plant *Stephania dielsiana*, showed antimicrobial activity against animal pathogenic bacteria [139]. Their mechanism of action can be attributed to their ability to increase membrane permeability and to intercalate with DNA. RNA polymerase, DNA gyrase and topoisomerase IV are also possible targets [141, 142].

Peptides

Short-length peptides (between 15 and 30 amino acids) with microbicidal activity are commonly named as antimicrobial peptides (AMPs) [144]. These biologically active molecules are an important component of the innate immune system of a wide variety of organisms (plants, mammals, insects, marine invertebrates and microorganisms) against invading pathogens [190, 191]. They comprise several protein groups with different features, as regard to the total charge of the molecule and the content of disulphide bonds [146]. Presently, more than 2,000 AMPs have been reported and most of them are cationic peptides, and only a few are anionic [191].

Peptides with antimicrobial properties are present in all organs of a variety of plant species constitutively or in response to microbial infections [77, 192, 193]. Plant AMPs can be classified into distinct families comprising thionins, plant defensins, lipid transfer proteins, hevein-and knottin-like proteins and snakins, based on the primary structure, size and cysteine content [191-193].

AMPs are effective against a wide range of microorganisms, namely Gram-negative and Gram-positive bacteria, including multidrug-resistant strains, parasites, yeast, fungi and some viruses [145, 194]. Their mechanism of action is believed be the damage or destabilization of the microbial cell membranes by formation of ion channels, transmembrane pores or extensive membrane rupture [143, 144]. Different models have been proposed for the mechanism of membrane disruption by AMPs, namely the barrel-stave model, the carpet model, the toroidal model and the aggregate channel model [143, 195]. In addition to cell membrane permeabilization, AMPs can also act on intracellular targets, inhibiting nucleic acids and protein synthesis, and enzymatic activity [143, 145]. Competitive inhibition of adhesion of microbial proteins to host polysaccharide receptors has also been observed [101].

The precise nature of the mechanism of action of AMPs is still uncertain, however, some studies have shown that their mode of action is related with their structural properties and sequence diversity. Besides, certain factors such as size, cationic nature, hydrophobicity and amphipathicity play an crucial role for their interaction with target cells [77, 190, 191]. Due to their cationic and hydrophobic features AMPs interact primarily with negatively charged components of the bacterial envelope, such as lipopolysaccharides (LPS) of the outer membrane (OM) of Gram-negative bacteria or lipoteichoic acids present on the cell wall of both Gram-negative and Gram-positive bacteria [77, 143, 144, 196]. The difference in the lipid composition between prokaryotic (higher proportion of negatively charged lipids) and eukaryotic (uncharged lipids predominate) cell membranes plays an important role in the selectivity of AMPs for microorganisms and reduces toxic side effects against host cells [77, 191].

Due to their broad-spectrum of antimicrobial activity, selectivity, lower toxicity, rapid action and low propensity for developing bacterial resistance (probably due to their distinct mode of action compared to traditional antibiotics), AMPs represent a promising class of molecules for the development of new antimicrobial agents [145, 190, 194]. Moreover, they show antimicrobial activity at low concentration [195].

AMPs have demonstrated activity not only against phytopathogens, but also against bacteria pathogenic to humans. Antibacterial activity against human pathogenic bacteria such as, *S. aureus*, *M. luteus*, *E. coli*, *P. aeruginosa*, *P. vulgaris* and *Klebsiella oxytoca* was observed in some studies with circulins A-B and cyclopsychotride A from *Chassalia parviflora* and *Psychotria longipes*, respectively. These effects were displayed at micromolar concentrations [197-199]. Additionally, the thionin fabatin from *Vicia faba* also inhibited the growth of *E. coli*, *P. aeruginosa*, and *E. hirae* [200].

Ib-AMP1 and Ib-AMP4, two AMPs from *Impatiens balsamina* were capable to inhibit the growth of *B. subtilis*, *M. luteus*, *S. aureus* and *Streptococcus faecalis* at very low concentrations [201]. Moreover, hevein-like proteins such as, Ac-AMP1 and Ac-AMP1, from *Amaranthus caudatus* promoted growth inhibition of *Bacillus megaterium* and *Sarcina lutea,* also at low concentrations [202]. The same results were observed previously with peptides from *Mirabilis jalapa* such as Mj-AMP1 and Mj-AMP2, belonging to the knottin family [203].

In addition to their bactericidal, fungicidal and virucidal activity, AMPs also possess other biological properties, being of interest as drug delivery vectors, antitumor agents, mitogenic agents, immune modulators, contraceptive agents and signalling molecules in transduction pathways [191, 196].

688

689 **Lectins**

690 Many plants contain an important group of biologically active proteins or glycoproteins that are
691 commonly designated as lectins, agglutinins or hemagglutinins [204]. The major role of lectins may be
692 related to the protection of plants from attack by insects and other predators, and against pathogenic
693 microorganisms [205]. Lectins can be found in a variety of tissues (leaves, stems, bark, bulbs, tubers, corms,
694 rhizomes, phloem, fruits and flowers) of a large number of plants [149, 206]. The most known plant lectins
695 are included in four families, namely the legume lectins, the chitin-binding lectins composed of hevein
696 domains, the type 2 ribosome-inactivating proteins, and the monocot mannose-binding lectins. Moreover,
697 the jacalin-related lectins, the amaranthin, and the Cucurbitaceae phloem lectins, are also other recognized
698 families [204].

699 In general, there are no structural features common to all lectin families. Indeed, lectins are a
700 heterogeneous group of proteins that have a common activity, but different sizes, structures, molecular
701 organization and active sites. [149]. They are a class of proteins of nonimmune origin, and their main
702 characteristic is the capability to bind with carbohydrates, without catalytic function, promoting
703 hemagglutination and antimicrobial effect [149, 205].

704 The antibacterial mode of action of lectins on Gram-negative and -positive bacteria, occurs through
705 interaction with components of the bacterial cell wall namely, teicoic and teicuronic acids, peptidoglycans
706 and lipopolysaccharides [147-149]. Bourne *et al*. [147], demonstrated that isolectin I from *Lathyrus ochrus*
707 seeds had capability for bind to muramic acid and muramyl dipeptide.

708 Lectin from *Myracrodruon urundeuva* showed antimicrobial activity against several Gram-negative (*E.*
709 *coli*, *K. pneumoniae* and *P. aeruginosa*) and -positive (*B. subtilis*, *Corynebacterium callunae*, *S. aureus* and
710 *S. faecalis*) bacteria. Its antimicrobial effects are related with their specificity for *N*-acetylglucosamine, and
711 were more evident against Gram-positive than on Gram-negative bacteria [206]. Moreover, inhibition of
712 *K. pneumoniae*, *S. epidermidis*, *Streptococcus faecalis* and *B. subtilis* was observed with *Phthirusa pyrifolia*
713 leaf lectin that has affinity for fructose-1-6-biphosphate. This lectin was also more active against Gram-
714 positive bacteria [207]. The EuniSL lectin isolated from *Eugenia uniflora* seeds demonstrated nonselective
715 antibacterial activity against Gram-negative and –positive pathogenic bacteria, such as: *S. aureus*, *B.*
716 *subtilis*, *Streptococcus* sp., *Klebsiella* sp., *P. aeruginosa* and *E. coli* [208]. *Schinus terebinthifolius* leaf
717 lectin (SteLL) inhibited the growth of *E. coli* [209].

718 Lectins from the seeds of *Archidendron jiringa* Nielsen and *Curcuma longa* inhibited the growth of
719 *B. subtilis*, *S. aureus*, *E. coli* and *P. aeruginosa* [210, 211]. A lectin from *Curcuma amarissima*
720 demonstrated antibacterial activity against *E. coli*, *S. aureus* and *B. subtilis*, but had no capability to inhibit
721 the growth of *P. aeruginosa*. This was due to the absence of polysaccharide ligands to interact with this
722 lectin [212].
723
724 **Polyacetylenes**
725 Polyacetylenes are derivatives of fatty acids that are characterized by one or more acetylenic groups in
726 their structures. These bioactive secondary metabolites are wide-spread among diverse plant families
727 (Apiaceae, Araliaceae, and Asteraceae), which protect them from attack by insects, viruses and bacteria
728 [213]. Polyacetylenes possess also beneficial effects for human health due to their biological properties,
729 such as: anti-inflammatory, antiallergenic, anticancer, antifungal, antimycobacterial and antibacterial
730 activity [214, 215]. Their antifungal and antimicrobial properties have been known for centuries [111]. C_{17}-
731 acetylene isolated from *Bupleurum salicifolium*, a plant native from the Canary Islands, shown
732 antimicrobial activity against *S. aureus* and *B. subtilis.* [216] Moreover, C_{17}-acetylene and
733 diacetylene falcarindiol have had antimycobacterial effects. Interesting is the fact that these effects occur
734 at non-toxic concentrations [217-219]. Many polyacetylenes from the Asteraceae have also demonstrated
735 antibacterial properties against various strains of Gram-positive and -negative bacteria (e.g. *Bacillus* spp.,
736 *Staphylococcus* spp., *Streptococcus* spp., *Escherichia* spp. and *Pseudomonas* spp.) [214].
737 The mechanism of action of acetylenes has been poorly studied, but is speculated that it involves the
738 disruption of the cell membranes, through the interference with energy metabolism of the bacterial cell
739 [150].
740
741 **Glucosinolates**
742 Glucosinolates (GLS) are an important group of phytochemicals that can be found in large numbers of
743 edible plants, particularly members of Brassicaceae family (i.e. cabbage, broccoli, cauliflower, mustard,
744 horseradish, watercress, Brussels sprouts, kohlrabi and wasabi). More than 120 different GLS are known
745 to occur naturally in plants. They are organosulfur compounds and, based on their chemical structure, can
746 be grouped into aliphatic, aromatic and indole [220]. These compounds are degraded when tissue disruption
747 occurs during consumption of cruciferous vegetables or through attack of insects and herbivores, due to

748 hydrolysis by myrosinase enzyme (*β*-thioglucosidase enzyme, EC 3.2.3.1) [221]. Intact GLS are relatively
749 biologically inactive, but their hydrolysis products such as, isothiocyanates (ITCs), nitriles, thiocyanates,
750 epithionitriles and oxazolidinethiones have numerous properties including anticarcinogenic, antioxidant
751 and antimicrobial [221, 222]. Hence, their therapeutic properties, including antimicrobial activity are being
752 actively explored. Among glucosinolate hydrolysis products (GHP), ITCs are considered the most potent
753 inhibitors of bacterial activity [223]. Their antimicrobial potential has been demonstrated against several
754 pathogens [224, 225].

755 The binding of sulfhydryl groups to enzymes that are important to microbial growth and survival
756 appears to be the mode of action of ITCs. This leads to reductions in the cellular levels of important thiol
757 groups conducting to reactive radicals formation [151-153]. Indeed, the binding of ITCs to external proteins
758 of cell membranes is well known [226, 227]. Moreover, some researchers have shown the capacity of some
759 ITCs to cross the plasma membrane and achieve the cytoplasm of cells [228, 229].

760 ITCs from seeds of *Sinapis alba* L. (white mustard), which comprise phenethyl, benzyl and benzoyl
761 groups exhibited good antimicrobial activity against intestinal bacteria, namely *Clostridium difficile*,
762 *Clostridium perfringens* and *E. coli* [222]. Allylisothiocyanate, an aliphatic ITC, showed high bactericidal
763 activity against many foodborne pathogens, including *L. monocytogenes*, *S. aureus*, *Salmonella enterica*
764 serovar Typhimurium, and *E. coli* O157:H7 [225, 230-233]. Furthermore, high activity was obtained with
765 allylisothiocyanate from roots of wasabi against six foodborne pathogenic bacteria (*E. coli* O157:H7, *S.*
766 *aureus*, *V. parahaemolyticus*, *S. typhimurium*, *B. cereus* and *S. mutans*) [234]. Growth inhibitory effects
767 against several bacterial pathogens, namely *E. coli*, *P. aeruginosa* and *L. monocytogenes* were also obtained
768 with allylisothiocyanate and aromatic the ITC 2-phenylethylisothiocyanate [224, 235-237]. A mixture of
769 ITCs (allylisothiocyanate, benzylisothiocyanate and 2-phenylethylisothiocyanate) was tested against
770 clinical important bacterial pathogens including antimicrobial resistant isolates (*Haemophilus influenzae*,
771 *Moraxella catarrhalis*, *Serratia marcescens, P. vulgaris*, *S. aureus*, *S. pyogenes*, *S. pneumoniae*, *K.*
772 *pneumoniae*, *E. coli* and *P. aeruginosa*) and showed positive inhibitory activity [238]. Moreover,
773 antimicrobial activity of some ITCs and synergy with commercial antibiotics against Gram-negative (*E.*
774 *coli* and *P. aeruginosa*) and -positive *(E. faecalis*, *S. aureus* and *L. monocytogenes*) pathogens was also
775 observed [132, 239].

776

777 **PHYTOCHEMICALS TO PREVENT AND CONTROL BIOFILM FORMATION**

778 Antibiotic resistance is a significant public health problem that is worsened when microorganisms are
779 in biofilms [36, 40, 78]. The main weapons used to control harmful biofilms have been the antimicrobial
780 products, nonetheless there are no antimicrobials with ensured efficacy [28]. Consequently, with the
781 presently used therapies, the treatment of infections associated to biofilms remains a hard task. Moreover,
782 for treating these infections it is frequently needed to reach distinct bacterial targets using combinations of
783 antimicrobials [28]. Thus, due to the tolerance of sessile bacteria to antimicrobial agents and the severity
784 of biofilm infections, the development of new antimicrobials and approaches for their effective control has
785 been a priority to the pharmaceutical industry and to the medical community [240]. Interesting strategies to
786 combat the resistance problem involve the search of new molecules with the capacity to suppress the
787 bacterial resistance mechanisms, and/or act synergistically with the existing antimicrobials. The use of
788 compounds with different modes of action on biofilm cells is another conceivable alternative [62, 110, 111].
789 Biofilm formation is regulated by combination of several mechanisms that are intrinsically related, such
790 as adhesion, EPS synthesis, bacterial motility and QS [62, 241]. Therefore, these cellular processes can be
791 possible targets for the discovery of new drugs. Moreover, as the eradication of an established biofilm is
792 more difficult to achieve than their prevention, it is preferable to implement preventive strategies [242].
793 This led to an increased interest in the search of natural products that have been proven to be able to restrict
794 the capability of bacteria to adhere, communicate, and form complex biofilms [237].
795 Diverse researchers already identified new strategies for biofilm control [28, 29, 62]. The use of
796 phytochemicals in biofilm prevention and control is a relevant strategy. According to Simões *et al*. [29],
797 phytochemicals may represent a natural antimicrobial strategy with considerable impact not only against
798 free-living bacteria but also on bacterial biofilm formation. Nevertheless, studies on biofilm prevention and
799 control with phytochemicals are scarce. In addition, antibacterial studies are mainly focused on the potential
800 of plant extracts and few studies exist with pure compounds. There are evidences that phytochemicals can
801 interfere with diverse biofilm formation processes (e.g. motility, EPS production, adhesion and QS) (Table
802 **3**).
803 Polyphenolics demonstrated ability to interfere with the adhesion potential of *Streptococcus mutans* to
804 saliva-coated hydroxyapatite and glass [243-245]. Sendamangalam *et al*. [246] verified that the inhibition
805 of enzymes produced by *Streptococcus mutans* affected their ability to form biofilms. Extracts of *Rubus*
806 *ulmifolius* Schott., Rosaceae (Elmleaf blackberry) that are rich in the polyphenol ellagic acid and
807 glycosylated derivatives inhibited biofilm formation of *S. aureus* [247]. Pure ellagic acid also displayed

antibiofilm properties against *S. aureus* and *E. coli* [247, 248]. In another study, eight selected natural phenolic compounds (anacardic acid, polyanacardic acid, salicylic acid, polysalicylic acid, polyphenol, catechin, epigallocatechin and tannic acid) were able to promote a significant reduction in biofilm formation by *P. aeruginosa* [249]. Polyphenol rich extract from *Rosa rugosa* tea, inhibited QS controlled violacein production in *Chromobacterium violaceum* CV026. This extract inhibited swarming motility and biofilm formation of *E. coli* K-12 and *P. aeruginosa* PAO1 in a concentration-dependent manner [250]. In a study performed by Packiavathy and coworkers [251], curcumin, the major constituent of turmeric (*Curcuma longa* L.) rhizomes, exhibited antibiofilm potential against some uropathogens (*E. coli*, *P. aeruginosa* PAO1, *Proteus mirabilis* and *Serratia marcescens*) by interfering with their QS system. Curcumin demonstrated also capacity to attenuate QS-dependent factors and to enhance the susceptibility of uropathogens to conventional antibiotics. This phytochemical inhibited biofilm development and the production of virulence factors in *Vibrio* spp. [252]. Marked reduction of enterohemorrhagic *E. coli* O157:H7 biofilm formation was found with the flavonoid phloretin (frequently found in apples) through repression of several genes, including those encoding for toxins, curli, fimbria and AI-2 production [253]. Vikram and coworkers [254], showed biofilm inhibitory activity against *V. harveyi* BB120 and *E. coli* O157:H7 with the flavonoids naringenin and quercetin (found in citrus species) in a concentration-dependent manner. These compounds, are antagonists of AHLs and AI-2-mediated cell-cell signaling in *V. harveyi* [240]. Inactivation of *S. sobrinus* biofilms in dental plaque of rats was observed with a biologically active compound of propolis, the flavonoid apigenin [255].

Prominent antibiofilm activity of *Polygonum cuspidatu* extracts, as well as their active compound resveratrol, was verified against of *Propionibacterium acnes* at subinhibitory concentrations [256]. Subinhibitory concentrations of resveratrol, protocatechuic/p-hydroxybenzoic acids and genistein showed antibiofilm activity against *S. aureus* [257]. Some polyphenolic compounds having a gallic acid moiety ((-)-epigallocatechin gallate, (+)-catechin and tannic acid) were able to block AHLs synthesis [258] and biofilm formation [259] of *E. coli* and *P. putida*. Moreover, (-)-epigallocatechin gallate inhibited biofilm formation of *Staphylococcus* spp. by reduction of EPS production [260]. It was also reported that combination of the antibiotic tetracycline with (-)-epicatechin gallate and ethyl gallate demonstrated higher efficiency on biofilm inhibition of *S. aureus* methicillin-sensitive (MSSA) and MRSA than the single molecules [261]. The tannin hamamelitannin that occur on the bark of *Hamamelis virginiana* significantly reduced biofilm metabolic activity of some strains of *S. aureus*, *S. epidermidis* and *Acinetobacter baumanii*,

838 *in vitro* and *in vivo* [262, 263]. Additionally, QS inhibition (QSI) was also observed with this compound
839 [264]. Santiago and coworkers [265] found that a bioactive fraction isolated from leaves of *Duabanga*
840 *grandiflora* containing alkaloids, tannins, saponins, steroids, glycosides and flavonoids inhibited MRSA
841 biofilm formation. Moreover, these authors correlated the antibiofilm activity with the ability of
842 phytochemicals to reduce cell-surface adhesion and attenuate the level of penicillin-binding protein 2a
843 (PBP2a). Borges *et al*. [242], demonstrated the potential of gallic and ferulic acids to inhibit bacterial
844 motility, adhesion and to prevent and control biofilms of *E. coli*, *P. aeruginosa*, *S. aureus* and *L.*
845 *monocytogenes*. Gallic acid was also identified as a molecule with sigificant antimicrobial and antibiofilm
846 activity against oral pathogens such as *Streptococcus mutans* [266].
847
848 **Table 3.** Phytochemicals with biofilm prevention and control potential and their mode of action on the
849 sessile cells

	Plant extract/Phytochemical	Biofilm action	References
Essential oils (EO)	*Cuminum cyminum*: methyl eugenol	Inhibition of motility (swimming and swarming), EPS production and biofilm formation by *P. aeruginosa*, *P. mirabilis* and *S. marcescens*	[63]
	Cinnamomum cassia: cinnamaldehyde, derivatives and eugenol	Interference with motility, adhesion and biofilm formation by *E. coli*; QSI of *E. coli* and *V. harveyi*; Biofilm mass reduction of *V. anguillarum* and *V. vulnificus*; QSI	[64, 67, 267]
	Extracts of *Curcuma xanthorrhiza* and *C. longa*: sesquiterpenoid xanthorrhizol, α-turmerone, germacrone, α-zingiberene, αr-turmerone, trans-*β*-elemenone, curlone, and *β*-sesquiphellandrene	Inhibition of adhesion and biofilms of *S. mutans* and alteration of their structure	[268-270]
	Salvia sclarea: diterpenoid salvipisone	Inhibition of cell viability of biofilms of *S. aureus* and *S. epidermidis*	[175]
	Clove, cinnamon, peppermint and lavender	QSI	[71]
	Thyme and oregano: carvacrol and thymol	Control of dual-species biofilm formation by *S. aureus* and *S. enterica* Typhimurium; Suppress of *Salmonella* spp., *S. aureus*, *S. epidermidis* and C. violaceum biofilms	[271-274]
	Farnesol	Biofilm inhibition of *S. aureus*	[275]
	6-gingerol	Reduces biofilm formation and virulence of *P. aeruginosa*	[276]

	Plant extract/Phytochemical	Biofilm action	References
Phenolics	Polyphenolics/polyphenols, polyanacardic acid, polysalicylic acid, catechin, epigallocatechin and tannic acid	Anti-adhesive properties and inhibition of biofilm formation of *S. mutans*; Inhibition of biofilm formation by *P. aeruginosa*	[243-246, 249]
	(-)-epigallocatechin gallate, (+)-catechin, (-)-epicatechin gallate, ethyl gallate, hamamelitannin and tannic acid	Interference with QS and inhibition of biofilm formation by *E. coli* and *P. putida*. Decrease of EPS production by *Staphylococcus* spp.; Biofilm inhibition of *S. aureus* (MRSA and MSSA); Reduction of metabolic activity of biofilm cells of *S. aureus*, *S. epidermidis* and *A. baumanii*	[258-264]
	Extracts of *Rubus ulmifolius*: ellagic acid	Inhibition of biofilm formation of *S. aureus* and *E. coli*	[247, 248]
	Extracts of *Polygonum cuspidatu*: resveratrol	Antibiofilm activity against *Propionibacterium acnes* and *S. aureus*	[256, 257]
	Extract of Rosa rugosa tea: polyphenols and flavonoids	Inhibition of QS controlled violacein production in *C. violaceum* CV026; Inhibition of motility and biofilm formation by *P. aeruginosa*	[250]
	Curcumin	Inhibition of biofilm formation by *E. coli*, *P. aeruginosa*, *P. mirabilis* and *S. marcescens*; Biofilm inhibition and interference with virulence factors production of *Vibrio* spp.	[251, 252]
	Phloretin	Reduction in biofilm formation by enterohemorrhagic *E. coli* O157:H7	[253]
	Naringenin and quercetin	Inhibition of biofilm formation by *V. harveyi* and *E. coli* O157:H7	[254]
	Apigenin	Inactivation of biofilms of *S. sobrinus*	[255]
	Gallic, ferulic and salicylic acids	Inhibition of motility and adhesion, biofilm prevention and control for *E. coli*, *P. aeruginosa*, *S. aureus*, *L. monocytogenes*; Biofilm inhibition of *S. mutans*; Inhibition of swarming motility and QS of *B. cereus* and *P. fluorescens*; QSI of *P. aeruginosa*	[242, 266, 277-279]
Alkaloids	Berberine	Reduction of viable bacterial cells counts of multispecies biofilms (*Fusobacterium nucleatum*, *E. faecalis* and *Prevotella intermedia*)	[280]
	Cinchona officinalis: 11-triphenylsilyl-10,11-dihydrocinchonidine	Biofilm prevention of *S. aureus*	[281]
	Macleya cordata: chelerythrine and sanguinarine	Antibiofilm activity against strains of *S. aureus* and *S. epidermidis*	[282]
Isothiocyanates (ITCs)	Allylisothiocyanate and 2-phenylethilisotiocyanate	Interference with adhesion of *S. aureus*; Inhibition of motility and adhesion, biofilm prevention and control of *E. coli*, *P. aeruginosa*, *S. aureus* and *L. monocytogenes*	[65, 237]

	Plant extract/Phytochemical	Biofilm action	References
	Extracts of *Brassica nigra*: allylisothiocyanate	Interference with adhesion of *Pseudomonas* sp.	[226, 283]
	Iberin	QSI of *P. aeruginosa*	[284]
Organosulfur compounds	Garlic (*Allium sativum*): allicin and ajoene	QSI of *P. aeruginosa* and *E. coli*	[68, 285-289]

Antibiofilm properties were observed with gallic, caffeic and chlorogenic acids against strains of *S. aureus,* including MRSA. Gallic acid interfered with the adhesion of *S. aureus* [290]. At low concentrations, simple aromatic esters of ferulic acid were able to inhibit biofilm formation of *S. aureus* [277]. Lemos *et al*. [279] shown that ferulic and salicylic acids can inhibit swimming motility and QS of *B. cereus* and *P. fluorescens*. Additionally, the development of biofilms in the presence of these phenolic acids increased the susceptibility of dual-species biofilms (*B. cereus*-*P. fluorescens*) to a second exposure to the chemicals. Salicylic acid was also identified as QS inhibitor of *P. aeruginosa* and therefore inhibitor of virulence factors QS-regulated [278].

As stated by Girennavar *et al*. [291], the presence of furocoumarins (dihydroxybergamottin and bergamottin) in grapefruit provides interesting inhibitory properties against pathogenic biofilms of *E. coli*, *S. typhimurium* and *P. aeruginosa*, as well as the ability to inhibit the activities of AI-1 and AI-2. Methyl eugenol, an EO found in methanolic extracts of *Cuminum cyminum*, inhibited swimming and swarming motilities, QS, EPS production and biofilm formation by *P. aeruginosa*, *Proteus mirabilis* and *Serratia marcescens* [63]. EOs from *Cinnamomum cassia* and their components (cinnamaldehyde and eugenol) affected the formation and structure of *E. coli* biofilms [64]. This was due to their interference with swimming motility and adhesion. Furthermore, the signaling molecules AHLs and AI-2 that mediate QS in *E. coli* and *V. harveyi* were affected by cinnamaldehyde [67]. Also, reduced biofilm formation by *V. anguillarum* LMG 4411 and *V. vulnificus* LMG 16867 was verified in the presence of cinnamaldehyde and some derivatives. This effect on biofilm formation is apparently related with reduced production of EPS and/or accumulation through QSI [267]. EO from plants, such as clove, cinnamon, peppermint and lavender also exhibited QSI [71]. Xanthorrhizol, and EO isolated from the methanolic extract of the rhizome of *Curcuma xanthorrhiza* Roxb. showed potential activity to reduce adherent cells in the process of *Streptococcus mutans* biofilm formation [268]. This sesquiterpenoid demonstrated also potential to alter the microstructure of *Streptococcus mutans* biofilms [269]. Moreover, EO of *Curcuma longa* in that α-turmerone, germacrone, α-zingiberene, αr-turmerone, trans-β-elemenone, curlone,

876 and β-sesquiphellandrene are the main components, inhibited the growth, attachment and biofilms of
877 *Streptococcus mutans*, at concentrations higher than 0.5 mg/mL [270]. In a study performed by Knowles *et*
878 *al*. [271], another EO component, the carvacrol, demonstrated potential to control dual-species biofilm
879 formation by *S. aureus* and *Salmonella enterica* Typhimurium, at different phases of maturation.
880 Repression of biofilm formation of *Salmonella* spp. strains by EOs of thyme and oregano and their natural
881 constituent carvacrol was also achieved [272]. Carvacrol was also able to inhibit biofilm formation of *C.*
882 *violaceum* ATCC 12472, *Salmonella enterica* Typhimurium DT104 and *S. aureus* 0074, in a study
883 conducted by Burt *et al*. [274]. Reduction of the expression of *cviI* gene, production of violacein and
884 chitinase activity of *C. violaceum*, with carvacrol at subinhibitory concentrations was also observed by
885 these authors. Furthermore, attenuation of biofilm formation by *S. aureus* and *S. epidermidis* with oregano
886 oil and its major phenolic components, monoterpene carvacrol and thymol was also demonstrated [273,
887 292]. In addition, the diterpenoid salvipisone isolated from acetone extract of transformed roots of *Salvia*
888 *sclarea* decreased significantly the cell viability of biofilms of antibiotic resistant *S. aureus* and *S.*
889 *epidermidis* [175]. Moreover, farnesol a sesquiterpene found in essential oils of citrus fruits, showed
890 antimicrobial properties (at high concentration, 30 mM), against bacterial biofilms of *S. aureus* [275]. The
891 phytochemical 6-gingerol, a pungent oil of fresh ginger (*Zingiber officinale*), reduced biofilm formation
892 and virulence in *P. aeruginosa* by binding to their QS receptor LasR [276].

893 Berberine, a plant alkaloid isolated from many medicinal plants reduced the viable bacterial counts in
894 the *in vitro* multispecies biofilm of endodontic pathogens (*Fusobacterium nucleatum, E. faecalis* and
895 *Prevotella intermedia*) [280]. In a work performed by Skogman and co-authors [281], synthetic derivative
896 of alkaloid cinchonidine found in *Cinchona officinalis*, 11-triphenylsilyl-10,11-dihydrocinchonidine (11-
897 TPSCD), prevented biofilm formation of *S. aureus* at low micromolar concentrations. However, higher
898 concentrations were required to eradicate mature biofilms. Two alkaloids, chelerythrine and sanguinarine,
899 obtained from *Macleya cordata*, showed antibiofilm activity against strains of *S. aureus* and
900 *S. epidermidis* [282].

901 Biofilm control with ITCs was demonstrated by Lee and coworkers [65]. Those authors found that some
902 genes related to adhesion of *S. aureus* were down-regulated after exposure to allylisothiocyanate. Aqueous
903 extracts of *Brassica nigra* and its main constituent allylisothiocyanate reduced the number of adhered cells
904 of *Pseudomonas* sp. [283]. Gómez De Saravia and Gaylarde [226] found similar results with these
905 molecules. Prevention and control of *E. coli*, *P. aeruginosa*, *S. aureus* and *L. monocytogenes* biofilms was

attained with allylisothiocyanate and 2-phenylethilisotiocyanate. Moreover, these molecules were also capable to interfere with motility and adhesion [237]. The blockade of the expression of genes involved in QS in *P. aeruginosa* was observed with iberin ITC, an organosulfur compound produced by horseradish and many other members of the Brassicaceae family [284]. The antimicrobial properties attributed to garlic is due to the presence of allicin [285]. Increased susceptibility to antibiotic tobramycin and to graze by polymorphonuclear leukocytes of biofilms of *P. aeruginosa* previously treated with garlic extracts was verified in some studies [68, 286-288]. These extracts demonstrated ability to treat *P. aeruginosa* lung infections in a mouse model. All of these effects were apparently due to QSI [68, 286-288]. Inhibition of biofilm formation by *P. aeruginosa* and *E. coli* was also obtained with molecules structurally similar to those found in garlic. Its activity was attributed to QSI [289]. Natural compounds with capability for QSI can be used in combination with less effective antibiotics [289]. Brackman *et al*. [293] demonstrated that the susceptibility of bacterial biofilms to several types of antibiotics was enhanced with QS inhibitors.

DEVELOPMENT OF NEW ANTIMICROBIALS USING PHYTOCHEMICALS AS SCAFFOLDS

The pharmaceutical industry is constantly under pressure to bring new drugs for the market timely [92, 294]. However, the process of discovery and development of safe and effective anti-infective compounds is hard, time consuming and expensive. Recent advances in genomics and other omic technologies, and the use of bioinformatic tools have significantly contributed to speed up the drug discovery process [295]. Despite the efforts of pharmaceutical companies to identify new antibiotics, only few candidates entered in preclinical tests and appeared in clinical trials. The main factors that are indispensable to attain with any potential drug candidate, before proceeding to clinical studies, are efficacy and safety. The concept of safety covers not only the absence of toxicity but also the ability to avoid adverse reactions and therapeutic failure, minimizing risk-benefit ratio associated with its use. A successful drug must comply, high efficacy *in vivo* against a broad spectrum of pathogens, with minimal burdens against mammalian cells. For this, the pharmacokinetic and pharmacodynamic properties required for a molecule to be considered clinically usable should be characterized. The most valuable drugs must be chemically stable, water soluble and capable to cross the biological membranes/tissues within the body [296].

There are no doubts on the role that phytochemicals have played in the history of medicine and that continue to have as basis of many drugs and medical formulations. However, despite the high number of

compounds with antimicrobial activity found in plants many of them may not be usable due to inappropriate characteristics to be considered as drugs. For instance, the concentrations required for therapeutic activity are too high to be clinically relevant; they do not display selective toxicity to bacteria or lack the desired pharmacokinetic properties [29]. In this context, one possible strategy is to improve the potency, selectivity and drug-like properties of phytochemicals by tailored structural modification in order to be translated into more functional drugs. In fact, phytochemicals provide one excellent source of scaffolds for novel antimicrobials [297, 298]. Many of the current pharmaceutical products in clinical use have plant origins (with new drugs being either synthetic/semisynthetic derivatives or synthetic mimetics of pharmacophores found in plant products), a fact that illustrate the usefulness of these molecules [111].

For the fine-tuning of antimicrobial/antibiofilm activities and drug-like properties of phytochemicals it is necessary to perform structure activity relationship (SAR) studies. Based on medicinal chemistry studies it is possible identify the structural variables that improve the efficacy of the molecule in terms of potency, selective and drug-like properties. In fact, these parameters are extremely dependent of the physical/chemical properties of phytochemicals that is related with the type/number of functional groups and their location in the molecule. For example, the properties of phenolic products vary according to the type of substituents, and with the number and positions of the hydroxyl groups in the aromatic ring [119, 299, 300]. Ergün *et al*. [277] studied the antioxidant and antimicrobial activity of ferulic acid and its aromatic esters derivatives. They found that 3-(4-hydroxy-3-methoxyphen-yl)-2-propenoate and 3-(4-hydroxy-3-meth-oxyphenyl)-2-propenoate, compounds bearing free phenolic hydroxyl groups, demonstrated the most prominent antioxidant and antimicrobial activities. In another study, a SAR analysis was performed with different phenolic compounds in order to verify the structure variables responsible for antimicrobial activity against MRSA. The authors verified that the presence of carboxylic acid (COOH) group, two hydroxyl (OH) groups in the *para* and *ortho* positions of the benzene ring and also a methoxyl (OCH3) group in the *meta* position seems to be fundamental for anti-MRSA activity [301]. A study performed with arylspiroborate salts derived from caffeic acid phenethyl ester revealed that these derivatives increased the antioxidant/antimicrobial properties and their capability to inhibit 5-lipoxygenase, compared to caffeic acid phenethyl ester [302]. It was also verified that the sodium salt was more active than its corresponding ammonium salt, and this difference was probably due to the low water solubility of the ammonium salt [302].

Numerous studies has been developed on the antimicrobial/antibiofilm potential of phytochemicals, as illustrated above, but only few explore their toxicity to mammalian cells and drug-like properties, and thus deserve further investigation. As examples, the administration of oral curcumin for the treatment of dermatitis caused by radiation therapy, was approved by Food and Drug Administration (FDA), being in phase 3 of clinical trials [303]. In the same way curcumin demonstrated potent antiproliferative effect and capability to improve the efficacy of the standard chemotherapy gemcitabine in patients with advanced pancreatic cancer, being in phase 3 of clinical trials [304]. Moreover, resveratrol revealed an interesting effect in patients with metabolic syndrome, being in phase 2 of clinical trials [305].

CONCLUSIONS AND FUTURE PERSPECTIVES

In the current scenario of antibiotic resistance, emergence of MDR pathogenic bacteria, and problems with the use of traditional antibiotics to treat infections caused by bacterial biofilms, scientists and the medical community consider that we are approaching a post-antibiotic era [1]. Moreover, it has been observed a decreased interest of pharmaceutical industries to search and develop new antimicrobials, due to the increased costs and complexity involved in drug discovery and development [306]. Thus, novel strategies aiming at discovering and developing effective alternatives should be encouraged. These measures should include approaches that permit the eradication of MDR pathogens, including their biofilms. Novel molecules with new mechanisms of action and multiple targets are the preferred candidates, including the interference with cellular processes involved in biofilm formation.

Although the recognized activity of phytochemicals, conventional screenings for identifying and characterizing the activity of secondary metabolites have been often inefficient, fastidious, expensive and involve pharmacological time-consuming assays [295, 307]. Consequently, a large number of natural compounds remain unexplored. In this context, in the past few years most of the pharmaceutical companies, ended or significantly scaled down their NPs investigations [92, 95, 98]. In order to continue with successful and competitive research on NPs from plants, new and innovative approaches are required particularly the use of genomics and other omic technologies (proteomics, transcriptomics and metabolomics) and the application of new screening tools [92, 99, 100]. Indeed, the use of bioinformatics tools has accentuated significantly the speed of drug discovery from plants [295]. Computational methodologies like molecular docking allows the prediction on the affinity/interaction of compounds toward different targets and therefore their biological activity, constituting a crucial component of many drug discovery programs [307,

308]. The simultaneous use of high-throughput screening with synthesis techniques and computational design of new molecules, using phytochemicals as scaffolds will accelerate and improve the discovery of new effective antimicrobial and antibiofilm products. In order to systematize and facilitate the interpretation of results, it would be advantageous to standardize the *in vitro* methods to characterize the antimicrobial activity of phytochemicals. Because *in vitro* studies do not necessarily predict *in vivo* outcomes, more pharmacological assays using *in vivo* models including studies on pharmacokinetic, pharmacodynamic and toxicology should be performed in order to validate phytochemical molecules for clinical usage.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

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