Resurgence of the interest in plants as sources of medicines and resistance-modifying agents

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In the last decades, bacterial resistance to antimicrobials has become a serious threat to clinical and public health. Traditional methods of antibiotic discovery have failed to get new alternatives and solutions to face the fast rhythms of resistance evolution. Therefore, new strategies to control bacterial infections are highly desirable. The therapeutic value of plant extracts is well-known for centuries. Indeed, medicinal plants always had an important role on the treatment of several illnesses and infections. Plants can produce complex mixtures of structural different compounds. Some of them are being reported to have significant antimicrobial activity against several important clinical pathogens or for their action as synergists or potentiators of other antibacterial agents, by interacting with them or with the pathogens in several ways. This chapter aims to present an overview about the current state of the use of antibiotic coadjuvants and resistance-modifying agents in clinical treatments. Also, the use of plants as promising sources of novel compounds with clinical interest will be highlighted.

Keywords antimicrobial combinations; antibiotic coadjuvants; multi-drug resistance; resistance-modifying agents

1. Introduction

The beginning of the era of antibiotics was one of the most important events and a turning point in the history of medicine. The introduction of antimicrobial drugs in clinical treatments saved countless lives and gave hope for a future in which all infectious diseases could be controlled [1]. Even now, decades later, we are totally dependent on the use of antibiotics, not only for the treatment of infectious diseases but also for guarantee the success of advanced surgical procedures, including organ and prosthetic transplants [2]. Since their introduction, millions of tons of antibiotics have been produced and employed for a wide variety of purposes. The excessive and, sometimes, inappropriate use of these drugs has been accompanied by the rapid appearance of resistant strains. Bacteria possess an impressive ability to adapt to environmental challenges and develop resistance by altering the expression or function of their own genes or by acquiring new genes. The explosive spread of antibiotic resistance determinants among pathogenic, commensal, and environmental bacteria has reached a global dimension. Thus classical measures trying to contain or slow the evolution of antibiotic resistance have clearly become insufficient [3]. Consequently, newer pathogens with multidrug-resistance profiles such as Acinetobacter baumannii have emerged, as well as “old” pathogens such as Mycobacterium tuberculosis and Neisseria gonorrhoeae which are now resistant to frontline antibiotics [4].

Antibiotic resistance is an enormous challenge that will require several strategies to address. The most important obstacle is finding new effective antimicrobials, which has proven to be difficult since few new classes of antibiotics have been described over the past 40 years. This is not surprising since identifying a new chemical matter that is effective and nontoxic is hard, considering the actual even more complex regulatory environment [4]. Also, there is little economic and medical justification for the development of new antibiotics that do not solve relevant resistance problems [5]. All of these facts make the discovery of novel agents against new bacterial targets a risky business. As so, major pharmaceutical companies have tended to concentrate their efforts on improving antimicrobial agents in established classes. However, it has been acknowledged that researchers are getting close to the end of the possible alternatives in terms of parent structure alterations [6]. Therefore, new strategies to control bacterial infections are highly desirable.

The use of two or more bioactive compounds simultaneously has been a promising solution to the clinical setting. Antimicrobial combinations can interfere with several targets, which is thought to intensify their efficiency, increase the pharmacological action and/or reduce adverse side effects. Also, antimicrobial combinations could probably avoid the emergence of resistant bacteria that might otherwise arise during treatment [7]. Many studies report that the use of drug combinations against multi-drug resistant bacterial pathogens have much better efficacy compared to monotherapies [8]. It is a fact that this approach has financial benefits since developing a new drug requires extensive clinical trials [9]. However, the need for new antibacterial drugs should not be address by an exclusive focus on combinations of antimicrobial agents. Instead, a more comprehensive approach is the development of antibiotic coadjuvants that include not only antibiotics but also other bioactive molecules.

New antimicrobial combination drugs which include natural product combinations have recently become a research priority. The plant kingdom is recognized as a source of new chemical compounds, which may be important due to their potential uses in medicine and, specifically, for developing novel therapeutic agents. Some plant compounds have been reported as antibacterials and, despite being less potent compared with antibiotics, plants can fight off infections...
successively [10]. Other compounds have direct activity against many species of bacteria, by enhancing the activity of a
specific antibiotic, reversing the natural resistance of bacteria to antibiotics, promoting the elimination of bacterial
plasmids or by inhibiting transport functions of the plasma membrane in regard to given antibiotics [11]. Natural or
synthetic bioactive compounds that promote the enhancement of antibiotic activity or the reversal of antibiotic
resistance afford the classification of resistance-modifying agents (RMAs).

In this chapter, it is intended to give an overview about the importance and the potential of antimicrobial coadjuvants
in clinical therapies. Several groups of different antimicrobial coadjuvants will be addressed and discussed in more
detail. The prospects of using plants as source of this type of compounds will also be discussed.

2. Antibiotic resistome

Selection for antibiotic resistance in bacteria provides one of the most well-documented examples of an evolutionary
response to natural selection [12]. The development of antibiotic-resistant microbes is the consequence of decades of
constant selective pressure from human applications of antibiotics, via underuse, overuse, and misuse. Similar
resistance development has also been observed in viruses, fungi, plants and insects towards antiviral drugs, antifungals,
herbicides and insecticides, respectively, creating increasing medical and economic problems [13]. Even before the first
clinical use of antibiotics more than 60 years ago, resistant organisms had been isolated, which proves that this is not a
recent problem [14]. Indeed, antibiotic resistant genes have been characterized in coliforms from glacial water and ice
in the Arctic estimated at 2000 years old [15]. And despite years of intense investigations, there is no doubt that the
situation with respect to antibiotic resistance is pandemic and there are no simple solutions to the problem.

The term ‘antibiotic resistome’ was proposed for the collection of all antibiotic resistance genes in microorganisms,
including those from pathogenic and non-pathogenic bacteria [16]. This resistome has suffered several alterations over
time, mainly in the last years with the introduction of new resistance genes. Generally, the failure of susceptibility of
microorganisms to antimicrobial treatments arises through: an inherent insusceptibility to the agents employed; the
acquisition of resistance, by previously susceptible strains, either by mutation or by transfer of genetic material from
another species or genus; or the emergence of pre-existing, but unexpressed, resistance phenotypes [17-19]. The
exchange and acquisition of new genetic material, by transduction, transformation or conjugation, contributes to the
rapid horizontal dissemination of resistance determinants. The localization of resistance determinants on mobile
elements, such as genomic islands, transposons, plasmids, or phages, greatly enhances their dispersion [20]. The major
mechanisms of bacterial resistance to antimicrobials include drug inactivation, target modification, alteration in the
accessibility to the target through drug efflux and decreased uptake, and over-expression of the drug target [21]. All of
these mechanisms require new genetic programming by the cell in response to the presence of antibiotics [22]. The most
relevant examples of bacterial resistance mechanisms are briefly described in this section.

2.1. Inactivation of antibiotics

There are several strategies of antibiotic inactivation. Hydrolysis, group transfer, and redox enzymes are involved in this
type of resistance. In Gram-negative pathogens, the production of β-lactamases is a major resistance mechanism in β-
lactam antibiotics. Penicillins, cephalosporins and carbapenems antibiotics with higher stability to hydrolysis were
developed to circumvent the inactivating activity of these enzymes [23]. However, extended-spectrum β-lactamases
(ESBLs) have been reported to confer resistance to newer generations of cephalosporins in *Escherichia coli*,
*Enterobacter* and *Klebsiella* species [24]. Regarding to carbapenemases, KPC, OXA-48, VIM and IMP-1 producers are
currently the most widespread types of carbapenemase in *Enterobacteriaceae*. More recently, a new type of carbapenem
resistance gene was reported – NDM [25]. NDM producers are becoming highly prevalent and several cases have been
identified in many countries all over the world [26].

2.2. Target modification

Other mechanism of bacterial resistance is related to the alteration of the antibiotic target. The most common example
involves the clinically relevant resistance in staphylococci, which is based on the acquisition of the modified penicillin-
binding-protein (PBP) 2a that mediates resistance to all known β-lactam antibiotics, particularly the cephalosporins [5].
Alterations in PBPs are known to be responsible for specific resistances, namely, methicillin-resistant *Staphylococcus
aureus* (MRSA) and penicillin-resistant *Streptococcus pneumoniae* (PRP) [15]. This PBP 2a protein is encoded by
mecA gene, which is carried on the staphylococcal cassette chromosome mec (SCCmec) that is integrated into MRSA.
Five different types of SCCmec, designated types I-V, are currently recognized, together with some variants [27]. Other
equally important examples are presented in Table 1.
Table 1 Examples of antibiotic resistance mediated by target modification [15, 24, 27, 28].

<table>
<thead>
<tr>
<th>Antibiotic class</th>
<th>Target</th>
<th>Resistance mechanism</th>
<th>Examples of bacteria</th>
</tr>
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<tbody>
<tr>
<td>Quinolones</td>
<td>DNA gyrase or topoisomerase IV, (inhibition of DNA synthesis)</td>
<td>Chromosomal mutations in both target enzymes which promotes lower affinity for quinolones</td>
<td>Gram-positive bacteria, particularly S. aureus and S. pneumoniae</td>
</tr>
<tr>
<td>Glycopeptides (e.g. vancomycin, teicoplanin)</td>
<td>Peptidyl-d-alanyl-d-alanine (d-Ala-d-Ala) termini of peptidoglycan precursors (inhibition of cell wall synthesis)</td>
<td>Biosynthesis of peptidoglycan with altered glycopeptide recognition sites</td>
<td>Vancomycin-resistant S. aureus (VRSA), vancomycin-intermediate S. aureus, glycopeptide-intermediate S. aureus</td>
</tr>
<tr>
<td>Macrolides, lincosamides and streptogramin B</td>
<td>50S ribosomal subunit (block protein synthesis)</td>
<td>Post-transcriptional modification of the 23S rRNA component of the 50S ribosomal subunit involving methylation or dimethylation of key adenine bases in the peptidyl transferase functional domain</td>
<td>Acquisition of VanA and VanB, which encode for enzymes that produce a modified peptidoglycan precursor terminating in d-Ala-d-Lac</td>
</tr>
<tr>
<td>Oxazolidinones (e.g. linezolid)</td>
<td>50S subunit (inhibition of formation of the initiation complex and interference with translocation of peptidyl-tRNA from A to P site)</td>
<td>Mutations in the 23S rRNA resulting in decreased affinity for binding; most mutations involve G to U substitutions in the peptidyl transferase region of 23S rRNA</td>
<td>Vancomycin-resistant enterococci (VRE), such as E. faecium and E. faecalis</td>
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2.3. Alteration in the accessibility to the target

This can be performed by two ways: alteration in the accessibility to the target through drug efflux or through decreased uptake.

Energy-driven drug efflux systems are increasingly recognized as mechanisms of antibiotic resistance. These efflux systems are either intrinsic to bacteria and activated in response to environmental signals, or acquired by a mutation in a gene which regulates their expression [14]. Reduced drug accumulation causing increased minimum inhibitory concentrations (MICs) of antibiotics is well described for macrolides, tetracyclines and quinolones [15]. Bacterial drug efflux transporters are currently classified into five families: (1) the major facilitator superfamily (MFS); (2) the adenosine triphosphate (ATP)-binding cassette (ABC) superfamily; (3) the small multidrug resistance (SMR) family; (4) the resistance-nodulation-cell division (RND) superfamily; and (5) the multidrug and toxic compound extrusion (MATE) family [29]. Four different antibiotic efflux systems have been described in *Pseudomonas aeruginosa*: mexAB-oprM, mexXY-oprM, mexCD-oprJ and mexEF-oprN10. In *S. aureus*, several efflux pumps include QacA (MFS family), Smr (SMR family) and NorA (MFS family) [29]. The TetA(B) protein, among Gram-negative bacteria, and the Tet(K) from *S. aureus* among Gram-positive bacteria confer resistance to tetracyclines and is also one of the most extensively studied members of the MF family [26, 30]. For *E. coli*, the best-studied members of these pumps are the AcrAB–ToIC system (RND superfamily) and also AcrEF, AcrD, YhiUV and MdtABC. Multidrug transporter systems, which may arise from deregulation of chromosomal genes governing flux of drugs across the bacterial cell wall, also result in resistance to multiple antimicrobials [24].

The outer membrane of bacteria serves as a protective barrier from the environment, and alterations in its permeability can confer antibiotic resistance [24]. The import of an antibiotic can also be inhibited by mutations that down-regulate, delete or modify outer-membrane porins [25]. Porin mutations in Gram-negative bacteria may confer
resistance to β-lactams, tetracyclines, chloramphenicol, sulfonamides, and fluoroquinolones [24]. As such, changes in porin copy number, size and selectivity will alter the rate of diffusion of these antibiotics.

2.4. Increase of bacterial resistance by over-expression of the drug target or stress-induced modifications

The over-expression of the drug target is also responsible for an increasing bacterial resistance. Hyperproduction of TEM β-lactamases leading to clavulenate resistance has been reported, and could be considered over-expression of drug target [15]. Overexpression of the transcriptional activator MarA from mar operon in E. coli, which confers drug resistance by altering the expression of multiple genes located on the bacterial chromosome, affected the expression of more than 60 genes [14]. Some of these genes have well-known functions: activation of AcrAB/TolC efflux, repression of the synthesis of OmpF, the point of entry for some antibiotics, and alteration of the expression of other membrane proteins [31]. Others presumably have something to do with stress because they are affected by the stress locus [14].

Stress derived from nutrient starvation, hypoxia, low pH, increased osmotic pressure, extreme temperature shifts or antimicrobial exposure, can also affect the gene expression patterns and cell physiology of bacteria in ways that can influence their susceptibility to antimicrobials. This occurs indirectly, as a result of stress-induced growth cessation, since antimicrobials typically act on growing cells, or directly as a result of the stress-dependent recruitment of resistance determinants (e.g. antimicrobial efflux), changes to antimicrobial targets, generation of resistance mutations, promotion of resistant growth modes (biofilms), etc. [32].

3. Antibiotic coadjuvants: how to increase the activity of a selected antibiotic?

New classes of antibiotics and more effective antimicrobial agents are needed. High-throughput methodologies combined with traditional molecular biology techniques have enabled the discovery of potential drug targets for new antibiotics and antibiotic potentiators. However, translating these targets from identification to actual drug compounds requires a significant amount of additional work and financial investment [33]. Also, a successful antibiotic must satisfy a perplexing number of demands: it should be able to cross bacterial cell membranes at high rates, avoid bacterial efflux pumps, not be a target for bacterial modifying or hydrolyzing enzymes, reach its target at a sufficiently high concentration, have a broad spectrum of antibacterial activity, have little or no toxicity and minimal side effects in humans [25]. There is no perfect antibiotic, and even if such antibiotic was discovered, it would be essential that prescription of this antibiotic would be restricted to very specific uses.

Recently, multidrug therapy has gained a wider acceptance in the fight against multidrug resistant microbial strains. The therapeutic value of synergistic interactions has been known since antiquity, and many different cultural healing systems (such as Ayurveda and traditional Chinese herbal medicine) have relied on this principle in the belief that combination therapy may enhance efficacy [9]. The use of drug combinations rather than single drugs provide better clinical outcomes, as the use of single agent is highly associated with occurrence of resistance [8]. Antimicrobial combinations are employed in order to prevent the emergence of resistant strains or to increase activity, in cases of mixed infections or to reduce the toxicity of a substance without compromising the antimicrobial action. The World Health Organization, for example, has urged pharmaceutical companies to stop promoting the use of artemisinin derivatives in monotherapy [9]. Also, with respect to tuberculosis, streptomycin was highly effective, until the pathogen acquired a mutation in the 30S ribosomal protein RpsL. So, new anti-TB drugs were introduced, but the microorganisms acquired mutations in the targets for each of these drugs which led to its resistance. It has become clear that a multidrug approach must be used to treat tuberculosis (e.g. isoniazid, rifampicin, pyrazinamide, and ethambutol), in order to have a realistic chance of success [34].

Several additional approaches to antibiotic discovery have been pursued, including targeting virulence factors, antimicrobial peptides and phage therapy [34]. Also, the use of vaccines, monoclonal antibodies, immuno-regulatory cytokines and hematopoiesis-stimulating factors may have utility in the control of antibiotic resistant infectious diseases [35].

Due to the reduction in the number of new antimicrobial agents, there has been a resurgence of interest in the search for compounds that will restore the activity of licensed antimicrobial agents. Antibiotic adjuvants offer an orthogonal approach to addressing the emergency of multi-drug-resistant pathogens [36]. Potentiation of antibiotic activity can occur through several mechanisms, including complex multi-target effects, serial or orthogonal inhibition of vital physiological pathways, inhibition of resistance enzymes that degrade or covalently modify an antibiotic to a nonactive form or of compounds that block antibiotic efflux, enhancement of the permeability of the cell for antibiotics causing improved solubility, resorption rate and enhanced bioavailability, and dispersal of a biofilm to planktonically growing cells, resulting in increased susceptibility to antibiotics [4, 37, 38]. Fig. 1 presents several schematic explanations of potentiation between antibiotics and other bioactive compounds.
Fig. 1 Several possible mechanisms explaining a potentiating activity between two compounds (two antibiotics or an antibiotic and a bioactive coadjuvant): A – Multi-target effect; B - serial or orthogonal inhibition of vital physiological pathways; C - inhibition of resistance enzymes, of compounds that block antibiotic efflux or that decrease the uptake into the cell; D - compounds that enhance the permeability of the cell for antibiotics; E - dispersal of a biofilm to planktonically growing cells, resulting in increased susceptibility to antibiotics.

4. Plants as source of antibiotic coadjuvants

Natural products have served as an important source of drugs for combating diseases since ancient times. In modern pharmaceutical industries, they continue to be a major resource for the generation of lead compounds, and half the drugs on the market are direct descendants of natural products [39]. Plants are a rich source of useful secondary metabolites of different chemical structures, such as tannins, terpenoids, alkaloids, flavonoids and polyphenols, that are used as plant defense mechanisms against pathogenic invaders [8, 40]. The number of characterized phytochemical compounds has been estimated to be at least 200,000 and still represents only a fraction of the compounds produced by the around 250,000 plant species growing on Earth [41]. Interest in medicinal plants has increased in recent years. It is encouraging to observe that there has been a recent increase in the number of publications reporting on plant-based pharmacological interactions and synergistic principles [9]. This interest has led to the discovery of new biologically-active molecules by the pharmaceutical industry and the adoption of crude extracts of plants for self-medication by the general public. Examples include the use of bear-berry (Arctostaphylos uva-ursi) and cranberry juice (Vaccinium macrocarpon) to treat urinary tract infections, essential oils of Tea Tree (Melaleuca alternifolia) as active ingredients in many topical formulations to treat cutaneous infections and Hydrastis canadensis and Echinacea species for tuberculosis infections [42-44]. Also, many plant extracts were reported to enhance the activity of several antibiotics [45-49].

Based on their biological activities, secondary plant metabolites were reported to be highly active with a great selectivity for cellular targets and others are weakly active compounds, which attack various cellular targets [41]. Some of them have been found to have effective antibacterial properties in vitro against both Gram-positive and Gram-negative bacteria. However, phytochemicals have higher MICs (100-5000 μg/ml) than antibiotics and cannot be used in monotherapy as sole agents [8]. Active ingredients derived from these natural products can serve as a model in semi-synthesis and total syntheses and their activities can be enhanced [50]. Many plant-derived compounds have been evaluated not only for their inherent antimicrobial activity, but also for their action as potentiators of antibiotics. RMAs can enhance the antibiotic activity by reversing antibiotic resistance. RMAs and other type of antibiotic coadjuvants or
bioactive compounds will be discussed in this section, according to their specific function. Also, it is intended to succinctly collate some the available literature that has focused on interactive plant-derived compounds that have a positive interaction with antibiotics against resistant pathogens.

4.1. Potentiation of antibiotics due to a multi-target effect

A group of drugs or multicomponent mixtures are expected to have multiples targets. This multiplicity of targets and the subsequent unpredictable mode of action has been over many years the main argument against the use of phyto preparations [51]. Multi-target combinations provide evolutionary advantages, since plants can protect themselves against a wide variety of predators. Interestingly, 90% of all thoroughly described medicinal plants contain broad spectrum compounds with rather weak or moderate bioactivity [41]. It is also interesting to note that phytochemicals that have different antibacterial modes of action can potentiate the activity of the same antibiotic classes [52]. Multi-target effects caused by mixtures of phytochemicals can modulate the three-dimensional structure of proteins (and thus their function), by interfering with DNA or RNA (especially gene expression), can target and disrupt the cell membrane, inhibit cytochrome P450 or enhance absorption and thus bioavailability of active metabolites [41, 53]. For example, myricetin, found in many fruits, vegetables and herbs was found to inhibit DNA B helicase [54, 55]. Allicin from *Allium sativum* was reported to inhibit RNA synthesis and to interact with important thiol-containing enzymes [54, 56-58].

The interactions may be synergistic, neutral or antagonistic [59]. Positive interactions that intensify the potency of a bioactive product by an inactive adjuvant are generally called potentiating. According to European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) there are four possible outcomes:

- **synergism** is present when the combination of antibacterials exceeds the additive effects of the individual products;
- **an additive effect** is observed when a combination of antibacterial products is equal to that of the sum of the effects of the individual products;
- **indifference** is present when a combination of antibacterial products promotes equal effects to those obtained with the most active product;
- **antagonism** interactions occur when certain components of the mixture inhibit full biological activity of pharmacologically-active compounds by reducing their stability or bioavailability or by enhancing their metabolism and, for that reason, promotes a reduced effect comparatively to that of the most effective individual product [52, 60].

However, the fact that a combination of two antibiotics is more effective than either agent alone does not necessarily mean that the combination has synergistic activity *in vivo*, but it could reflect an additive effect. In practice, most investigators use statistical methods to evaluate the *in vivo* effectiveness of combinations, and they call *in vivo* synergism a statistically significant difference between the activity of a combination and that of the most effective agent alone [61]. Also, the FIC index (FICI) is usually calculated in order to characterize a combination as synergistic (FIC (drug A) + FIC (drug B), where FIC (drug A/B) = MIC (drug A/B in combination) / MIC (drug A/B)) [18]. Synergy has generally been defined as a FICI ≤ 0.5 and antagonism as a FICI > 4 [62, 63].

4.2. Potentiation of antibiotics by resistance-modifying agents

The concept of using a compound that inhibits resistance in a bacterium which may be employed with a conventional antibiotic is well proven [64]. Important examples of these inhibitors are clavulanic acid, which binds with high affinity to many bacterial β-lactamases and is available commercially in combination with amoxicillin (as Augmentin®) and ticarcillin (Timentin®) [35, 65, 66]; sulbactam, marketed in combination with ampicillin (as Unasyn®), and tazobactam, marketed in combination with piperacillin (as Tasocin®) [35, 65, 67]. There have been considerable efforts to discover other inhibitors of β-lactamases and, in particular, molecules that target the emerging metallo-β-lactamases [22]. Expanding this strategy to other antibiotics is a possibility that should be explored in order to maintain the effectiveness of our current arsenal of antibiotics [22].

Many plants have been evaluated not only for direct antimicrobial activity, but also as RMAs [18, 68-71]. Their potential use in combinations can help to recycle older antibiotics for which resistance mechanisms are greatly disseminated. There is a significant interest in plant compounds that inhibit bacterial efflux pumps. Ideally, a good bacterial efflux inhibitor (EPI) must not inhibit the human P-glycoprotein involved in the xenobiotic efflux of normal tissues [72]. Numerous phytochemicals have been shown to have activity against *S. aureus* or other Gram-positive bacteria, or to act as potential EPIs with antimicrobials for Gram-positive bacteria [73]. Some of them are: carnosol and carnosic acid from *Rosmarinus officinalis*, reserpine from *Rauwolfia serpentina*, piperine from *Piper nigrum*, sylbin from *Silybum marianum*, chalcone from *Dalea versicolor*, chrysosplenol-D and chrysoplenetin from *Artemisia annua* [6, 7, 18, 47, 74-76]. The toxicity of reserpine and its adverse effect to humans, even at low concentrations, limit its usage thereby warranting the quest for alternative EPIs [72]. Other phytochemicals were reported for their action as
inhibitors of PBP 2a; such as baicalein from *Scutellaria* species, tellimagrandin I and rugosin B from *Rosa canina* L., corilagin from *Arctostaphylos uva-ursi* [18, 77, 78].

4.3. Synergism between active compounds due to enhanced cell permeability effects

Another strategy to overcome resistance is to improve the delivery or enhance the accessibility of antibiotics to their sites of action. Many compounds have been reported to affect membrane permeability of a diverse range of microorganisms [79], mainly due to the perturbation of the lipid fraction of the cell membrane. Also, owing to their lipophilic character, they can increase membrane permeability [80]. Such permeabilizers, as they have been termed, can non-specifically enhance the permeability of bacterial cells to exogenous products, including antimicrobial agents, and may therefore potentiate the antibacterial activity of antibiotics that interact with intracellular targets [52].

Carvacrol and thymol are two main products of the essential oil of *Thymus vulgaris*, and were reported to disintegrate the outer membrane and thus to increase membrane permeability and fluidity in Gram-negative bacteria, facilitating the penetration of antibiotics [37, 42, 52, 79, 81]. Gallic acid from berry extracts has proven to be an efficient permeabilizer for several *Salmonella* strains due to a disintegrating activity of the outer membrane based on the chelation of divalent cations and to the partial hydrophobicity of this product, which promote the membrane destabilization [82]. Also, this compound was reported to cause irreversible changes in membrane properties through hydrophobicity changes, decrease of negative surface charge, and occurrence of local rupture or pore formation in the cell membranes [83]. Xanthohumol and lupulon, from *Humulus lupulus*, also promote changes in the properties and permeability of the membrane [84]. For this reason, potentiation of several antibiotics by these compounds was reported.

4.4. Potentiation of antibiotics by promoting the dispersal of a biofilm to planktonically growing cells

It is well known that bacteria in biofilms are much more resistant compared with planktonic cells. The microorganisms generate physiological changes when cells attach to a surface by expressing a biofilm phenotype that can confer resistance face to stress environmental conditions such as nutrient limitation, heat and cold shocks, changes in pH and to chemical agents. Chronic infections in which biofilms have been demonstrated to be involved are many and include periodontitis, cystic fibrosis pneumonia, and numerous infections associated with indwelling devices such as catheters, heart valves, and prostheses [85]. Also, biofilms constitute a major threat in the clinical environment by acting as reservoirs of multidrug resistant pathogenic bacteria.

Biofilm resistance involves multiple mechanisms [86]. One obvious difference between planktonic cells and biofilms is the presence of a polymeric matrix enveloping the community that retards diffusion of antimicrobials into the biofilm [87-89]. Many studies have investigated the formation of biofilms as an explanation for microbial resistance [90-93]. Antibiotics have been shown to penetrate biofilms readily in some cases and poorly in others, depending on the particular agent and biofilm [87]. However, given that, in many cases, biofilms consist of stacks of cells with aqueous channels flowing in between, only impenetrability seems unlikely [93]. Because of the special structure of biofilms, there are gradients of nutrients and oxygen and, for this reason, cells can be in distinct growth states. Consequently, cells in different layers of the biofilm will be affected differently by different types of antimicrobials depending on their mechanism of action [1]. For example, penicillin antibiotics, which target cell-wall synthesis, kill only growing bacteria [94]. Other theories include a reduced susceptibility of biofilm microorganisms compared to their freely suspended counterparts [90]; and the existence of persister cells, a small population of cells with a highly protected phenotype [85, 88, 95]. Fig. 2 represents some hypotheses explaining the higher resistance to antibiotics in biofilms.

![Fig. 2](image_url) Four hypothesized biofilm resistance mechanisms: A – The antibiotic (black points) penetrates slowly or incompletely the biofilm; B – an adaptive stress response is expressed by some cells (marked cells); C – a concentration gradient of a metabolic substrate or product leads to zones of slow non-growing bacteria (shaded cells); D – a small fraction of the cells differentiate into a highly protected persister state (dark cells).
Plant-based antimicrobial studies on planktonic microorganisms have been given extensive priority. The inhibition of biofilms, however, has been largely neglected [9].

The rapid reversal of resistance upon dispersion from a biofilm suggests that this is an adaptive resistance mechanism rather than a genetic alteration [85]. Cells in the biofilm can return to a planktonic lifestyle by two possible ways. In order to colonize new areas or to environmental cues, such as starvation, a programmed set of events leads to hydrolysis of the extracellular polysaccharide matrix and conversion of a subpopulation of cells into motile planktonic cells, which can rapidly multiply and leave the sessile communities [87]. Also, biofilm dispersal can occur as a consequence of physical detachment or mechanical breakage due to flow or shear stresses [96].

Potential therapies include enzymes that dissolve the matrix polymers of the biofilm, chemical reactions that block biofilm matrix synthesis, and analogues of microbial signaling molecules that interfere with cell-to-cell communication, required for normal biofilm formation [94].

Many bacteria are known to regulate diverse physiological processes through a mechanism called quorum sensing (QS) which is accomplished through the production, secretion and subsequent detection of extracellular signal molecules called autoinducers (AIs). When these molecules reach a particular threshold concentration, they are taken up by other microbes and trigger adaptive changes appropriate to the community of organisms [1, 97, 98]. QS has been found to regulate a number of physiological activities, including motility, conjugation, competence, sporulation, virulence and biofilm formation. Some of these quorum sensing molecules, such as the Pseudomonas quinolone signal (PQS) and the homoserine lactones (AHL) from P. aeruginosa, possess antimicrobial activity at very high concentrations [1]. Consequently, QS pathways of competing bacteria are potential targets for such nontoxic chemical defenses. Screening for natural products able to promote biofilm dispersal has led to the identification of inhibitors of AHL-based QS, such as bromoageliferin and oroidin [96]. The aqueous extract of Moringa oleifera was found to inhibit violacein production, a QS-regulated behavior in Chromobacterium violaceum [99]. In other study, thehexane, chloroform and methanol extracts of an Ayurveda spice, namely clove (Syzygium aromaticum), shown anti-QS activity by inhibiting the response of C. violaceum CV026 to exogenously supplied N-hexanoyl-homoserine lactone, in turn preventing violacein production [100]. Aqueous extracts of six plants, Conocarpus erectus, Chamaesyce hypericifolia, Callistemon viminalis, Bucida buceras, Tetrazygia bicolor and Quercus virginiana, caused the inhibition of QS genes and QS-controlled factors, with marginal effects on bacterial growth of P. aeruginos [101]. Also, common dietary fruit, herb and spice extracts also significantly inhibited QS [102].

5. Conclusions

Our planet is saturated with antibiotics, which had a major contribution to the selection of resistant strains. Resistance mechanisms are pandemic and create an enormous clinical and financial burden on health care systems worldwide. Resistance remains as a primary driver for antibacterial R&D. Plant-based medicines are important therapeutic weapons to cure human diseases, and are of extremely relevance to pharmacology. Indeed, it has been shown that resistance to crude extracts occurs less than resistance to single actives. The concept of antimicrobial synergy is based on the principle that, in combination, the formulation may enhance efficacy, reduce toxicity, decrease adverse side effects, increase bioavailability, lower the dose and reduce the advance of antimicrobial resistance [9].

Research into antimicrobial combinations may yield new developments that may address the increasing concern towards antimicrobial resistance. Recent advances in combinatorial chemistry have allowed the synthesis of large number of compounds by automated high-throughput synthesis. Also, new techniques, such as metabolomics and other “omics”, are providing new tools to explore the mechanism of action of complex herbal preparations. Understanding the molecular diversity that underlies resistance using up-to-date technology will guide new efforts to develop effective antibiotics [14]. Exploration of structure-activity relationships can lead to the rational preparation and evaluation of new analogs that offer some protection against one or more of the known resistance mechanisms. More studies of inhibitory-target and inhibitory-resistance interactions will also provide new leads. However, any new chemical that is initially identified as having an interesting antimicrobial activity in a screening process will have a long way before being approved as an antibiotic.

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