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Review paper

Plant-derived secondary metabolites as the main source of efflux pump inhibitors and methods for identification

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ABSTRACT

The upsurge of multiple drug resistance (MDR) bacteria substantially diminishes the effectiveness of antibiotic arsenal and therefore intensifies the rate of therapeutic failure. The major factor in MDR is efflux pump-mediated resistance. A unique pump can make bacteria withstand a wide range of structurally diverse compounds. Therefore, their inhibition is a promising route to eliminate resistance phenomenon in bacteria. Phytochemicals are excellent alternatives as resistance-modifying agents. They can directly kill bacteria or interact with the crucial events of pathogenicity, thereby decreasing the ability of bacteria to develop resistance. Numerous botanicals display noteworthy efflux pumps inhibitory activities. Edible plants are of growing interest. Likewise, some plant families would be excellent sources of efflux pump inhibitors (EPIs) including Apocynaceae, Berberidaceae, Convolvulaceae, Cucurbitaceae, Fabaceae, Lamiaceae, and Zingiberaceae. Easily applicable methods for screening plant-derived EPIs include checkerboard synergy test, berberine uptake assay and ethidium bromide test. In silico high-throughput virtual detection can be evaluated as a criterion of excluding compounds with efflux substrate-like characteristics, thereby improving the selection process and extending the identification of EPIs. To ascertain the efflux activity inhibition, real-time PCR and quantitative mass spectrometry can be applied. This review emphasizes on efflux pumps and their roles in transmitting bacterial resistance and an update plant-derived EPIs and strategies for identification.

1. Introduction

Global health is under constant threat, that of the recrudescence of bacteria resistant to most or all conventional antibacterial agents. There is an upsurge in microbial strains with a high rate of antimicrobial resistance (AMR) in both Gram-negative and Gram-positive bacteria. Each antibiotic category can be exposed to greater than one single mechanism of resistance. The infective bacteria can develop non-susceptibility to numerous antimicrobial drugs, leading to multidrug-resistance (MDR) [1]. Some highly resistant pathogenic bacteria, grouped under the acronym ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.), are related to healthcare-associated AMR. The World Health Organization (WHO) has classified these microorganisms as a worldwide priority pathogen list of resistant bacteria for prioritization of research and improvement of novel and efficient antibiotics. The risk is considered "critical" for three genera of bacteria: *Acinetobacter*, *Pseudomonas*, and *Enterobacter* [2]. Yet, alternative strains like *Escherichia coli*, *Proteus mirabilis*, and *Shigella* spp. depicted a significant level of AMR. The Center for Disease Control and Prevention (CDC) estimates that antibiotic-resistant ESKAPE pathogens cause over 2 million illnesses and approximately 23,000 deaths annually [3]. The MDR emerged in response to selective pressures. Several risk factors might embrace

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unsuitable prescription practices, inadequate patient education, restricted diagnostic facilities, unauthorized sale of antimicrobials, lack of acceptable functioning drug restrictive mechanisms, and non-human use of antimicrobials [4].

Antibiotics act at different levels on bacterial cell structure components (membrane, cell wall, genetic material, protein synthesis, metabolism), which defines their modes of action. The active principles targeting the bacterial cell wall must find complementary receptors for their binding and appropriate for their action, while those acting inside the cell must be able to cross the cell membrane and reach target elements into the cell. Various mechanisms of resistance developed by the bacteria target one or more of these abilities to prevent the antibacterial from exerting its inhibitory or bactericidal action. Among these mechanisms, the over-expression of efflux pump systems, which consists of extruding the antibacterial molecules out of the bacterial cell, thereby reducing their concentrations to an insufficient value for a proven effect, is the main mechanism giving rise to MDR [5]. Efflux pumps are membrane-spanning proteins located in the cytoplasmic membrane of both prokaryotic and eukaryotic cells. They are active transporters, meaning that they require a source of chemical energy to execute their function. The active efflux mechanism can also be engaged in other resistance strategies including reduction of cell membrane permeability, enzymatic drug lysis/inactivation, drug/target modification, biofilm formation and quorum sensing (QS) [6], which are various cellular processes leading to passive resistance in bacteria. A unique pump can give the bacteria the ability to withstand a wide range of structurally diverse compounds, leading to superbugs bacteria. The “superbugs” bacteria pose the threat of a worldwide resurgence of deadly infections. Yet, the search for innovative therapeutic solutions is struggling to take off. The hurdle of bacteria resistance points out the imperative need for novel medication, with new mechanisms of action and/or combination therapy to treat infections bring about by resistant bacteria. Existing studies from the literature suggest that efflux pumps play a major role in the development of AMR in bacteria [7]. Therefore, there is a need to grasp their structural elements and physiology to reveal the attention-grabbing views for the event of specific inhibitors, which will be employed in combination therapy at the side of commonly used antibiotics. Thus, the scarcity of new antibiotics promotes the development of alternative treatments, including herbal therapy. In addition to low cost, availability, and accessibility, all together to their use since ancient time, medicinal plants are important sources of a broad variety of secondary metabolites (terpenoid, phenolic compounds, and alkaloids) of therapeutic value [8–12]. This explains the growing interest in medicinal plants as about 40% of current medicines are derived from phytochemicals [13]. Phytochemicals may have a direct antimicrobial effect. Moreover, they can be used to improve the effectiveness of the usual antibiotics after combination [14,15]. Plant-derived compounds can interact with the key events of the pathogenic process, thereby decreasing potentiality of bacteria to develop resistance. Thus, using these compounds in combination with traditional antibiotics is promising as it allows the reuse of antibiotics that have lost their effectiveness due to MDR pumps system in both Gram-positive and Gram-negative bacteria [16]. Reversal, modulating, resistance modifying agents are various expressions used to qualify compounds that have activity against resistant bacteria. As the efflux pumps inhibitors (EPIs) do not have any direct effect on the bacterial structures, this could minimize the appearance and propagation of resistance [17]. From about 250,000 species of higher plants worldwide, about 14%–28% have been scholarly investigated for a medical purpose [18]. Thus, by adequately exploring medicinal plants considered as an immensely unexploited resource, there is an increasing expectation of having novel effective therapeutic compounds.

During the antimicrobial screening of medicinal plants, priority is usually given to plant extracts or derived compounds showing direct and significant inhibitory effects on microbes. A review of this approach would be important, as most inhibitors of bacterial resistance strategies do not have a direct inhibitory effect on the bacteria cell. Indeed, without having a direct inhibitory effect on the bacterium, in addition to acting as EPIs, phytochemicals could be, for example, excellent membrane permeabilizer (useful against Gram-negative bacteria) or inhibitors of inactivating enzymes such as beta-lactamases. A restraint number of research teams are working to characterize MDR inhibitors from natural sources, time and cost for identifying and characterizing are other problems [19]. In this review, we describe major efflux pumps and their roles in transmitting bacterial AMR, update plant-derived EPIs and strategies for identification.

2. Overcoming antibiotic effects: bacterial strategies

The ultimate goal of all living beings is survival. Microorganisms such as bacteria are no exceptions. These microscopic beings developed over time multiple strategies to ensure their survival against hostile conditions. The hope born of the advent of antibiotic therapy soon faded with the increased ability of pathogens to resist antimicrobial agents. In addition to the natural or intrinsic capacity of certain bacterial species to oppose the inhibitory or killing effects of usual antimicrobial agents, it is nowadays widely accepted that the use of antibiotics makes the occurrence of resistance inevitable. As aforementioned, inappropriate consumption is one of the fundamental causes. Despite a large number of antibiotic resistance phenotypes identified in bacteria, few investigations describe the strategies by which resistance genes are acquired. The genes coding for the determinants of antibacterial resistance are localized, either on the bacterial chromosome or on mobile genetic elements such as plasmids or transposons, to be transmitted vertically and horizontally [20]. The ease with which bacterial populations adapt to a hostile environment, coupled with their high capacity for exchanging genetic material, underscores the inevitability of the biological phenomenon of antibiotic resistance. Mastering the mechanisms of AMR is a significant step in the discovery of effective ways to counteract them (Fig. 1). On the phylogenetic plan, two types of resistance are distinguished: intrinsic or natural resistance results from a functional process or structural characteristic common to bacteria of the same species, whereas acquired or clinical resistance results from numerous mutations of genes encoding target structures. As described in Fig. 1, common strategies of antibiotic resistance comprise: (i) reduction of cell membrane permeability; (ii) drug lysis/inactivation by hydrolytic enzymes; (iii) drug modification; (iv) cellular target mutation enabling antibiotics to effectively bind to its specific target; (v) active drug extrusion by membrane-spanning efflux pumps system [21]; and the most recent strategy (vi) drug suppression [22].

The existence of an outer membrane in Gram-negative bacteria and mycobacteria confers to them a reduced permeability to many molecules. The highly hydrophobic property of this membrane justifies this characteristic [7]. This structural feature partly explains the increased resistance noted in Gram-negative bacteria compared to Gram-positive ones. The increased impermeability is considered as a passive mechanism of resistance. This cannot be absolute because activation of efflux pumps could also cause a reduction in membrane permeability. In that context, the process is considered active [23].

Enzymatic inactivation, involving enzymes such as hydrolases, transferases and/or redox enzymes, consists of an alteration leading to inactivation of the antimicrobial agents’ structure. The main

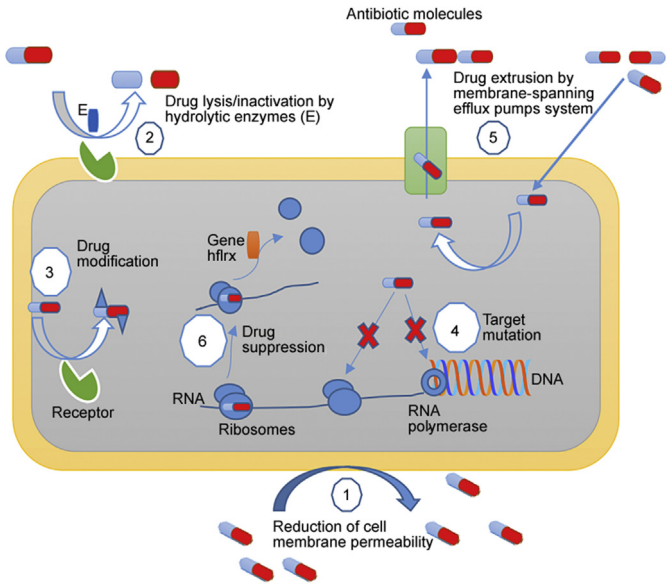


Fig. 1. Scheme of main bacteria strategies of antibiotic resistance.

mechanisms include hydrolysis and transfer of functional groups (thiol-, acyl-, nucleotidil-, phosphoryl-, ribosyl-, glycosyl-) [24]. Of the known inactivating enzymes, β-lactamases are the major problem in the management of infections caused by Gram-negative bacteria [25].

The modification of the cellular targets of antibiotics is a common mechanism of resistance. Examples of clinical concern bacteria expressing resistance can be found for every class of antibiotic, regardless of the mode of action. Target site alterations often originate from spontaneous mutation of a bacterial gene on the chromosome and selection in the presence of the antibiotic. As examples, mutations in RNA polymerase and DNA gyrase lead to the resistance to the rifamycins and quinolones, respectively. In other cases, acquisition of resistance may indicate the relocation of resistance genes from other organisms by conjugation, transduction, or transformation. Examples of these mechanisms include acquisition of the mecA genes encoding methicillin resistance in *Staphylococcus aureus* and the various van genes in enterococci encoding resistance to glycopeptides [26]. Furthermore, the ability of bacterial species to over-express efflux pumps confers them a great ability to oppose the effects of numerous or even all classes of available antimicrobial molecules.

The aforementioned bacterial resistance mechanisms reinforce the idea that research to overcome bacterial MDR should prioritize membrane permeabilizers, β-lactamase inhibitors along with inhibitors of efflux pump systems. Duval and collaborators [22] highlighted a novel resistance mechanism, expressed by *Listeria monocytogenes*. Indeed, in the presence of antibiotics that target ribosomes and block protein synthesis, these bacteria can split their ribosomes in two to boost protein production (step 6 Fig. 1). The results demonstrate the existence of an unsuspected number of resistance mechanisms that bacteria can develop.

3. Active efflux phenomenon: the main mechanism leading to MDR

3.1. Physiology of the main efflux pump systems and role in the occurrence of MDR

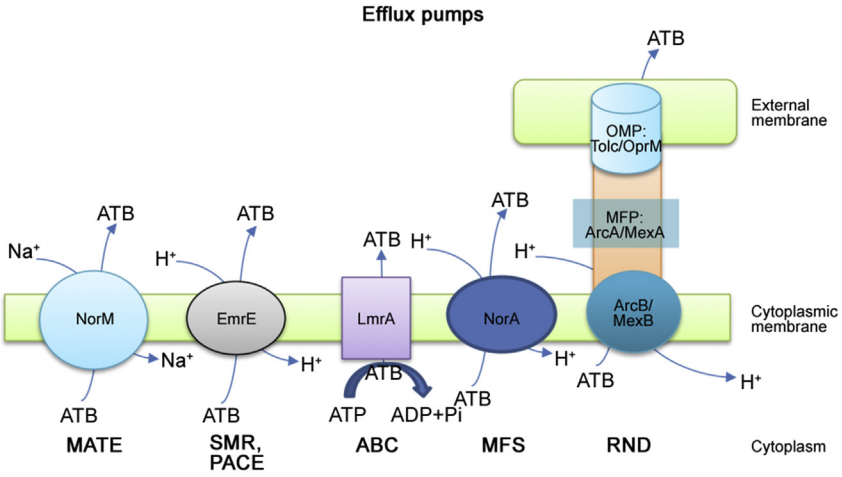
Active efflux is the main phenomenon by which bacteria release substances harmful to their survival, including antibiotics, outside their cells. This is the main bacterial strategy leading to MDR [27]. Efflux-pump mediated resistance depends on energy and transportation systems. The pumps provide recognition, fixation, and transport of efflux substrates. A pump may be specific to a given antibiotic or otherwise, it may carry several types of therapeutic molecules with different structural characteristics, leading to the development of MDR phenotypes [28].

These transmembrane transporters are classified into five families based on their primary structure including ATP-Binding Cassette (ABC), Multidrug And Toxic compound Extrusion (MATE), Major Facilitator Superfamily (MFS), Resistance Nodulation cell Division (RND), Small Multidrug Resistance (SMR) (Fig. 2). In addition to these carriers, another efflux protein has been described in *A. baumannii* called Proteobacterial Antimicrobial Compound Efflux (PACE) superfamily. PACE is structurally close to the SMR family [29]. Primary carriers (case of ABC pumps) use energy from ATP hydrolysis to extrude compounds outside the bacteria cell, whilst secondary transporters (MFS, SMR, RND, and MATE) use energy from membrane-spanning electrochemical gradients of H^+ (proton motive force, case of MFS, SMR, RND) and Na^+ ions (sodium motive force, case of MATE) [30]. In Gram-positive bacteria, there is a single carrier protein, located at the cytoplasmic membrane of MFS, SMR or ABC families, which is responsible for the expression of drugs extrusion. Gram-negative bacteria show greater complexity due to the presence of a lipophilic outer membrane.

The MFS proteins consist of a sequence of 400–600 amino acids in their primary structure arranged in 12 or 14 membrane-spanning α-helical. Widely found in the two groups of bacteria, they are mainly involved in the uniport, the antiport and the symport of several substances [31]. They can adopt a tripartite pump structure in Gram-negative bacteria due to the presence of the outer membrane. However, this family is the main vector of Gram-positive bacteria. NorA, QacA, and QacB from *S. aureus* and LmrP from *Lactococcus lactis* are the well-characterized MFS pumps in Gram-positive bacteria involved in MDR [32]. Moreover, some other examples including CraA in *A. baumannii* [33], MdfA in *E. coli* [30], KpnGH in *K. pneumoniae* [34] mediated resistance to several antibiotics comprising chloramphenicol, norfloxacin, tetracycline, ceftazidime, cefepime, and streptomycin.

Compared to other carriers, SMR transport proteins are the smallest (100–120 amino acids and 4 transmembrane helices). This family has three subclasses: small multidrug pumps, groEL mutation proteins suppressor, and paired SMR proteins. The first, small multidrug pumps, has been identified as having the ability to confer MDR in both groups of bacteria from the expression of a single gene [35]. The EmrE protein from *E. coli* is the best characterized SMR model, consists of 110 amino acid residues [36], and usually extrudes acriflavine and quaternary ammonium compounds [37]. Other well-known models of the SMR efflux transporters include SepA and QacC from *S. aureus* and EbrAB from *Bacillus subtilis* [32].

Resistance Nodulation cell Division (RND) proteins are capable of recognizing and transporting a broad spectrum of molecules regardless of their physicochemical properties (hydrophilic, hydrophobic or amphiphilic). RND proteins are large and can contain more than 1000 amino acid residues. In their functional state, these carriers form multimeric assemblies (tripartite pumps) traversing both the inner membrane, the periplasm, and the outer membrane. Thus, the transporter embedded in the inner membrane (AcrB and MexB) associates with an outer membrane protein (TolC and OprM) and a periplasmic protein (AcrA and MexA) which consolidates the building (Fig. 2). As examples, AcrAB-TolC from *E. coli* and MexAB-OprM from *P. aeruginosa* are the well-known RND transporters [30]. The ArcB transporter binds therapeutic molecules from the



MATE: Multidrug And Toxic compound Extrusion; **SMR**: Small Multidrug Resistance; **PACE**: Proteobacterial Antimicrobial Compound Efflux; **ABC**: ATP-Binding Cassette; **MFS**: Major Facilitator Superfamily; **RND**: Resistance Nodulation cell Division.
ATB: Antibiotic. MFP: Membrane Fusion Protein. OMP: Outer Membrane Protein.

Fig. 2. Scheme of main families of the transmembrane transporters.

periplasmic space, leading to a broad substrate specificity that includes, in addition to detergents and organic solvents (as hexane), several classes of antibiotics (phenicols, cyclin, β-lactams, fluoroquinolones) [38]. The arrangement of RND as tripartite pumps favors the recognition of a large number of low molecular weight compounds [39]. Some hypotheses underlie that each subunit goes through a cycle of conformational changes, which in turn are favored by complementary modifications of adjacent subunits. Likewise, the deactivation of a single subunit resulted in a loss of operation of the entire trimer and that, if there was a defect in the proton relay network of a subunit, the whole pumping would be compromised [39].

ATP-Binding Cassette (ABC) transporters are ATP-dependent membrane-spanning pumps widely represented in both eukaryotes and prokaryotes [40]. Therefore, they have a broad spectrum of substrates including metal ions, sugars, amino acids, peptides, and protein. Structurally, ABC transporter is the largest membrane-spanning protein (contain ~1200 amino acid residues) arranged in four domains. The two transmembrane hydrophobic domains are involved in substrate recognition and transport (TransMembrane Domain or TMD), whereas two cytoplasmic domains in binding and hydrolyzing ATP (Nucleotide-Binding Domain or NBD) [41]. In Gram-negative bacteria, some ABC transporters interact with an outer membrane protein (OMP) and a periplasmic adapter protein (Membrane Fusion Protein (MFP)) to allow substrates to cross the outer membrane and be released into the outer membrane extracellular compartment. The typical example is that of MacAB-TolC from *E. coli*. ABC efflux pumps operate in one direction. They are reorganized after the output of the pharmaceuticals and the hydrolysis of ATP [42].

Made up of approximately 450 amino acids, MATE transporters form a structure of 12 membrane-spanning segments. This causes resistance to some classes of hydrophilic antibiotics such as fluoroquinolones and aminoglycosides [43]. Although most use a Na^+ motive force as a source of energy, some of them such as PmpM from *P. aeruginosa* and AbeM from *A. baumannii* operate with the proton motive force [44]. Others most studied transporters of MATE include NorM and MepA from *S. aureus* [45].

3.2. The interconnection between active efflux systems and communication processes in bacteria accentuate the spread of MDR phenotypes

3.2.1. Interconnection between active efflux and quorum sensing

During some biological cellular process, bacteria use various molecules called autoinducers with the aim of generating communication between two adjacent cells. This process, known as quorum sensing (QS), plays a significant role in controlling the expression of certain genes and their capacity to suit to the nature of their environment, to control the size of the bacterial population in a given environment while securing the availability of nutrients [46]. Numerous studies demonstrated a correlation between the QS signaling system and the control of the expression of the genes involved in active efflux systems. Besides, efflux pumps extrude autoinducers out of the bacterial cell, so these molecules may be considered as a major determinant in cell-cell and host-bacteria interactions [47]. Therefore, changes in the expression of efflux pumps leading to MDR phenotypes may compromise the effectiveness of QS signaling. A typical example is the over-expression of MexAB-OprM and MexEF-OprN pumps from *P. aeruginosa*, which results in an alteration of the infectious process in humans, the response to QS, resulting in a decreased susceptibility to several classes of antibiotics [48], whilst isolates of the same infectious agent not expressing MexHI-OpmD pumps were in the inability to synthesize several autoinducers [49]. A QS-dependent cell division regulator, the SdiA (suppressor of division inhibition) protein from *E. coli*, positively affects the expression of ArcAB pumps, resulting in increased resistance of several antibiotics [50], and obviously to MDR. Investigations conducted by Xu [47] revealed that efflux pumps and QS receptors share transcription regulators. Interestingly, EPIs inhibited the secretion of QS signals [51]. QS signals degradation may prevent signal propagation from one region of a biofilm to another. This suggests the significance of efflux pumps system in the QS response of the bacterial community.

3.2.2. Interconnection between active efflux and biofilm formation

Another challenge facing the MDR crisis is the management of chronic diseases following biofilms-associated infections. Biofilms-

Table 1
Plant-derived efflux pump inhibitors.

Plant family	Bioactive compounds	Plant source	Pharmacological activity against MDR bacteria over-expressing efflux pumps	References
Aizoaceae	Uvaol Oleanolic acid	*Carpobrotus edulis*	MRSA COLOXA efflux systems *E. coli* efflux pumps	[73]
Apiaceae	Osthol	*Cnidii monnieri*	*P. aeruginosa* efflux pumps; *S. aureus* NorA, MdeA, TetK and MsrA efflux pumps	[74,75]
	Cumin	*Cuminum cyminum*	*S. aureus* LmrS efflux pump	[76]
	Falcarindiol	*Levisticum officinale*	Gram-negative bacteria efflux pumps	[77]
Apocynaceae	Reserpine	*Rauwolfia serpentina, Rauwolfia vomitoria*	Bmr-mediated MDR in *B. subtilis*, MDR *S. aureus* and *S. pneumoniae*, NorA and TetK-mediated MDR in MRSA, and LmrA of *Lactococcus lactis*, PmrA efflux protein in *S. pneumoniae*	[78]
	Conessine	*Holarrhena antidysenterica*	MexAB-OprM of *P. aeruginosa*	[79]
	Indirubin	*Wrightia tinctoria*	NorA of *S. aureus* and *Staphylococcus epidermidis*	[80]
	Catharanthine	*Catharanthus roseus*	Superbug *P. aeruginosa*	[81]
Asteraceae	Silybin	*Silybum marianum*	Synergistic activity against *S. aureus*	[82]
	Chrysoplenetin Chrysosplenol-D Chrysoplenetin	*Artemisia annua*	NorA over-expressing *S. aureus*	[83]
	Ethanolic leaf extracts	*Vernonia adoensis*		
	Caffeoylquinic acids	*Artemisia absinthium*	*S.aureus* and *E. faecalis* efflux pumps	[84]
	Crysoplenol Crysoplenetin	*Artemissia annua*	NorA of *S. aureus*	[85]
Berberidaceae	Berberine	*Berberis* spp.	NorA activity in *S. aureus* and MexAB-OprM of *P. aeruginosa*	[86]
	Palmatine		MexAB-OprM of *P.aeruginosa*	
	Porphyrin pheophorbide A Flavonolignan 5′- methoxyhydnocarpin (5′-MHC)		*S. aureus* NorA efflux pumps	[87]
	Pheophorbide	*Berberis aetnensis*	*S. aureus* efflux pumps	[88]
Boraginaceae	Sarothrin (5,7,4'-trihydroxy-3,6,8-trimethoxyflavone)	*Alkanna orientalis* (L.) Boiss.	*S. aureus* NorA efflux pumps	[89]
Burseraceae	Murucoidins	*Ipomoea murucoides*		[90]
Combretaceae	Gallotannin	*Terminalia chebula*	*E. coli* efflux pumps	[91]
Convolvulaceae	Orizabin XIX	*Ipomoea tricolor*	NorA over-expressing *S. aureus*	[92]
	Orizabin XIX and orizabin IX, orizabin XV	Mexican Morning Glory species		
	Lysergol and 17-O-3′′,4′′,5′′-trimethoxybenzoyllysergol	*Ipomoea muricata*	*E. coli* ABC pump YojI	[93]
Cucurbitaceae	Karavilagenin C	*Momordica balsamina L*	Fluoroquinolone transporters in *E. coli;* Efflux pumps of MRSA COLOXA;	[94]
	Balsaminagenin B		*E. faecalis* efflux systems;	
	Balsaminol A		*S. aureus* and *E. faecalis* efflux pumps; NorA and AcrAB-TolC efflux pumps	
Cupressaceae	Ferruginol	*Chamaecyparis lawsoniana*	*S. aureus* NorA efflux pumps	[95]
	Totarol	*Chamaecyparis nootkatensis*		
Ebenaceae	Diospyrin	*Diospyros montana*	*Mycobacterium aurum* efflux pumps	[96]
Euphorbiaceae	Jatropholone A and B	*Jatropha gossypiifolia*	NorA efflux pumps	[97,98]
	2,6-dimethyl-4-phenylpyridine-3,5-dicarboxylic acid diethyl ester	*Jatropha elliptica*	*S. aureus* efflux pumps	
	Terpenoids	*Euphorbia hirta*	Membrane and biofilm in *P. aeruginosa*	
	Propacine	*Jatropha elliptica*	*S. aureus* efflux pumps	
Fabaceae	Liquiritin	*Glycyrrhiza uralensis*	Transporters of fluoroquinolone in *E. coli*	[99]
	Arylbenzofuran aldehyde (Spinosan A)	*Dalea spinosa*	NorA over-expressing *S. aureus*	[100]
	Pterocarpan Isoflavone			
	Juliflorine	*Prosopis juliflora*	NorA over-expressing *S. aureus*	[101]
	Genistein Orobol Biochanin A	*Lupinus argenteus*		[102]
	Flavanoid/phenolic compounds	*Dalea versicolor*	NorA of *S. aureus* and *Bacillus cereus*	[103]
Geraniaceae	Polyacylated neohesperidosides	*Geranium caespitosum*	*S. aureus* NorA efflux pumps	[104]
Guttiferae	Coumarins	*Mesua ferrea*	*S. aureus* NorA efflux pumps	[105]
Hypericaceae	Olympicin A 4-hydroxy-α-tetralone	*Hypericum olympicum*	*S. aureus* NorA efflux pumps	[106]
Lamiaceae	Carnosic acid	*Rosmarinus officinalis*	*S. aureus* NorA efflux pumps, potentiates tetracycline against *S. aureus* strains possessing MsrA efflux pump	[107]
	Carnosol		Potentiates tetracycline against *S. aureus* strains possessing MsrA efflux pump	

(*continued on next page*)

Table 1 (*continued*)

Plant family	Bioactive compounds	Plant source	Pharmacological activity against MDR bacteria over-expressing efflux pumps	References
	Baicalein	*Thymus vulgaris*	MDR pumps in MRSA and NorA of *Salmonella enteridis*	[108,109]
	Isopimarane diterpenes Geranylgeranyl diterpenes Abietane diterpenes	*Lycopus europaeus*	TetK and MsrA efflux pumps of *S. aureus*	[110]
	Essential oils	*Salvia fruticosa*	TetK of *S. aureus*	[111]
Lauraceae	Kaempferol rhamnoside	*Persea lingue*	NorA of *S. aureus*	[112]
Leguminosae-mimosoideae	3-O-[β-xylopyranosyl-(1 → 4)-β-galactopyranosyl]-oleanolic acid; 3-O-[β-galactopyranosyl-(1 → 4)-β-galactopyranosyl]-oleanolic acid	*Acacia polyacantha* Willd.	MDR Gram-negative bacteria over-expressing efflux pumps	[113]
Lythraceae	Gallic acid	*Punica granatum*	NorA over-expressing *S. aureus*	[114]
	4-hydroxy-α-tetralone and semisynthetic derivatives	*Ammannia spp*	ABC-type efflux pump gene yojI of *E. coli*	[115]
Malvaceae	Kaempferol glycoside Tiliroside	*Herissantia tiubae*	NorA efflux pump in *S. aureus*	[116]
	Theobromine	*Theobroma cacao*	AcrAB-TolC and MexAB-OprM efflux systems	[117]
Menispermaceae	Tetrandrine	*Stephania tetrandra* S. Moore	Fluoroquinolone transporters in *E. coli*	[99]
Myrtaceae	Ethanolic leaf extracts	*Callistemon citrinus*	ATP-dependent efflux pump of *S. aureus*	[118]
	Ursolic acid and derivatives (3-O-acetyl-urs-12-en-28-isopropyl ester and 3-O-acetyl-urs-12-en-28-n-butyl ester)	*Eucalyptus tereticornis*	*E. coli* AcrAB, TolC, MacB, and YojI efflux systems	[119]
Nyctaginaceae	Polyphenolic amide: N-trans-feruloyl 4'-Omethyldopamine	*Mirabilis jalapa*	NorA efflux pump of *S. aureus*	[120]
Pinaceae	Isopimaric acid	*Pinus nigra*	NorA efflux pump of MRSA	[121]
Piperaceae	Piperine	*Piper nigrum*	MFS efflux systems	[122]
Portulacaceae	Linoleic acid	*Portulaca oleracea*	MRSA overexpressing MsrA; ABC efflux pump	[123]
Rubiaceae	Quinine	Cinchona tree	MDR in MRSA strains	[124]
	Resveratrol	*Nauclea pobeguinii*	MDR Gram-negative efflux pumps	[11]
Rutaceae	Bergamottin epoxide derivative Citropten and furocoumarins derivatives	*Citrus paradisi*	MDR in MRSA strains; NorA, ermA, ermB efflux pumps	[125]
	Phenylpropanoid (+) ailanthoidiol	*Zanthoxylum capense*	*S. aureus* efflux pumps	[126]
Salicaceae	Salicylic acid	*Salix alba*		[127]
Solanaceae	Capsaicin	*Capsicum annuum* L.		[128]
Theaceae	Epigallocatechin gallate	*Camellia sinensis*	*S. aureus* NorA, TetB and TetK efflux systems	[129]
Zingiberaceae	*Trans,trans*-1,7-diphenylhepta-4,6-dien-3-one	*Alpinia katsumadai*	*Mycobacterium smegmatis* efflux pumps	[130]
	Phenylpropanoids (1′-S-1′-acetoxyeugenol acetate)	*Alpinia galanga*	*Mycobacterium smegmatis* efflux pumps	[131]
	Curcumin	*Curcuma longa*	*P. aeruginosa* efflux pumps; NorA, MdeA, TetK and MsrA efflux pumps of *S. aureus*	[74,75]
Zygophyllaceae	Harmaline	*Peganum harmala*	MDR in MRSA strains	[124]
Others compounds from various families	Caffeic acid	Variety of plants	NorA and MsrA efflux pumps of *S. aureus*	[132]
	Tannic acids	*Caesalpinia spinosa*, *Rhus semialata*, *Quercus infectoria* or *Rhus coriaria*.	*A. baumannii* efflux pumps	[133]
	Ellagic acid	Fruits and vegetables		
	Thymol and carvacrol	Aromatic plants	MDR food-borne pathogens	[134]
	Farnesol	Dietary aromatic plants	*M. smegmatis* efflux pumps	[135]
	Geraniol	Rose oil, palmarosa oil, and citronella oil		
	p-coumaric acid and derivatives	Variety of edible plants	MexAB-OprM overexpressing *P. aeruginosa*	[136]

ABC: ATP Binding Cassettes. MDR: Multidrug-resistant. MFS: Major Facilitator Superfamily. MRSA: Methicillin-resistant *Staphylococcus aureus*.

associated infections are involved on average in 72% of infections in developed countries. The non-susceptibility of bacteria grouped as a community to high doses of antibiotics is linked to the existence of a distinct phenotype embedded in biofilms leading to MDR strategies. These strategies include, among others, the selection of persistent cells of latency and stationary phases of bacterial growth, which is widely responsible for the ineffectiveness of antibiotics in eliminating infections [52]. In general, biofilms-producing bacteria are recurrent in hospitals, colonizing and persisting on materials such as catheters and common implants. Their eradication remains a major challenge clinically [53]. The production of biofilms is a passive resistance strategy characterized by the formation of a polysaccharide matrix around the bacterium, which consequently leads to an obstruction of the passage of antibiotics, thus making the bacterium highly resistant to these molecules. The elimination of biofilms is all the more difficult because, in addition to obstructing the diffusion of antibiotics, they promote the entry into the dormant state of the bacterium rendering ineffective the inhibitory effect of therapeutic agents [54]. There is an interrelationship between biofilm development and the operation of efflux pumps, indicating the intervention of multiple mechanisms at the same time contributes to this event. Among the aforementioned families of efflux pumps, RND transporters are the most frequent, proven to be involved in this cellular process in bacteria

[55]. Studies focusing on biofilm-forming *P. aeruginosa* revealed the role of MexAB-OprM and MexCD-OprJ efflux pumps in the biofilm production and increased resistance to ciprofloxacin [54], azithromycin [56], colistin [57], and aminoglycosides [58]. Therefore, the inactivation of efflux pumps by EPIs could completely decrease or eradicate biofilm formation.

4. Fighting against multidrug-resistance: the alternative measures

4.1. Development of new antibiotic molecules with new targets

Currently, resistance mechanisms are reported for all antibiotics available for clinical use in the fields of human medicine and veterinary medicine. This is why effective management of the available molecules, as well as the research and development of new compounds, are essential to preserving human and animal health. Indeed, a plethora of new compounds is in clinical phases [59,60]. The concern, however, lies in the fact that these products act on bacterial targets already known so far, in view, that most of these new molecules are made from chemical derivatization. To the best of our knowledge, to date, only two promising antimicrobial compounds have action on new targets: Brilacidin and ACHN-975. The first is a synthetic mimetic of host defense protein, which is the first line of defense against infectious diseases in most species, clinical studies are in phase 2, whereas ACHN-975 (phase 1 clinical study) acts by blocking the action of an enzyme, LpxC, implicated in the biosynthesis of lipid A in bacteria [60].

4.2. Restriction of selective pressure and frequency of resistance occurrence by antipathogenic compounds

Nowadays, investigations are conducted aiming at developing therapies that play a dual role: interaction with the virulence factors of bacteria and/or direct inhibition of the pathogen. Because of non-based interference with bacterial growth, active principles that interfere only with virulence factors are called antipathogenic. Indeed, antipathogenic substances act only by interfering with the ability of bacteria to induce disease in a host. While altering the regulation of the virulence factors expression in cases of biofilms-associated chronic infections, for example, the advantage here lies in the possibility of effective control of the severity of the clinical manifestations following infections [61]. A blockage of the communication between cells could influence at different levels of the signaling pathway including inhibition of chemomediators synthesis, the deactivation of signaling mediators and the obstructing of signal receptors or transmission [62]. In this case, both synthetic compounds and those derived from natural sources are investigated for their antipathogenic effects.

Several other alternative actions at different levels of study and development are to be considered including (i) phagotherapy [63], (ii) QS inhibitors [64], (iii) use of probiotics [65], and (iv) the development of nanoparticles with antimicrobial activities [66]. These strategies are considered to have an increased benefit in comparison to the usual antibiotics. Indeed, in most cases, only the antipathogenic activity is observed with an action consisting of an alteration of communication mechanisms between bacteria without killing single cells. The immediate consequence would be a restricted selective pressure and a decrease in the frequency of resistance occurred during the treatment [46]. Furthermore, other means of dealing with bacterial infections such as vaccine therapy, immunotherapy or targeting genes engaged in the anti-infectious response, also have a significant advantage in the measure where they also limit the probability of occurrence of resistant mutants [67].

4.3. A combined approach for the identification of new antimicrobial molecules

Identification of new antimicrobial drugs or new targets would require a combined approach to proteomics, genomics along with functional databases and bioinformatics tools [68]. Chemoinformatics may make use of the current chemical knowledge provided by high-throughput screening, proteins structure arsenal, structure-activity relationship studies, ligand-binding affinity, microbial genome project, and substantiated biological activity of a large variety of molecules to produce a rational and targeted choice of compounds with properties similar to those of a drug [69]. Another investigation focus is the development of novel aminoglycosides that are forbearing to aminoglycoside-inactivating enzymes, notably in Gram-negative bacterium [70]. Low molecular weight (below 50 kDa) antimicrobial peptides (AMPs) of natural or synthetic origin that target the bacterial membrane, genetic material synthesis (DNA) and metabolism, would represent a promising class of antimicrobial agents [71]. However, the challenges related to their sensitiveness to the degrading action of proteolytic enzymes, their toxicity to human cells, as well as the cost of production are to be taken into account. Major progress in nanotechnology could be successfully implemented to enhance the ability of AMPs to oppose the effects of these enzymes, increase their bioavailability and contribute to reducing toxicity [72]. These measures, while promising are expensive and require special equipments and high-level laboratories, which therefore limits their applications.

5. Medicinal plants: the main natural source for specific and potent MDR inhibitors

5.1. Potent EPIs from medicinal plants

Medicinal plants are the main promising natural sources of EPIs, because of the presence of chemically and structurally diverse secondary metabolites with multiple pharmacological properties. Many studies on medicinal plant extracts showed the existence of putative molecules that block efflux pumps in both Gram-negative and Gram-positive bacteria and potentially restore the efficacy of antibiotics, thus allowing the antibiotics to reach the sufficient concentration inside the bacteria for a bactericidal effect. Many plant species of various families have been investigated so far, leading to numerous molecules with efflux pump inhibitory activity (Table 1). Some families would be excellent sources of EPIs, according to a considerable number of isolated compounds with potent EPIs activities. These include Apocynaceae, Berberidaceae, Convolvulaceae, Cucurbitaceae, Fabaceae, Lamiaceae, and Zingiberaceae. Because of common consumption as food by population, edible plants are of growing interest. Indeed, investigations on some fruits (examples of grapefruits, grapes, pomegranate), seeds (examples of coffee and cocoa seeds), vegetables (examples of lemongrass, tealeaves, and condiments), and spices (example of pepper), commonly used in traditional diet led to the identification of potent EPIs. Some of these dietary phytochemicals including farnesol and geraniol; thymol and carvacrol ellagic acid, piperine, theobromine, *p*-coumaric acid and derivatives, and resveratrol; have been reported to act against efflux pump systems in both Gram-negative and Gram-positive bacteria (Table 1). Some of the aforementioned compounds such as farnesol, geraniol, thymol, and carvacrol are also common components of essential oils derived from aromatic plants, suggesting that essential oils would also be

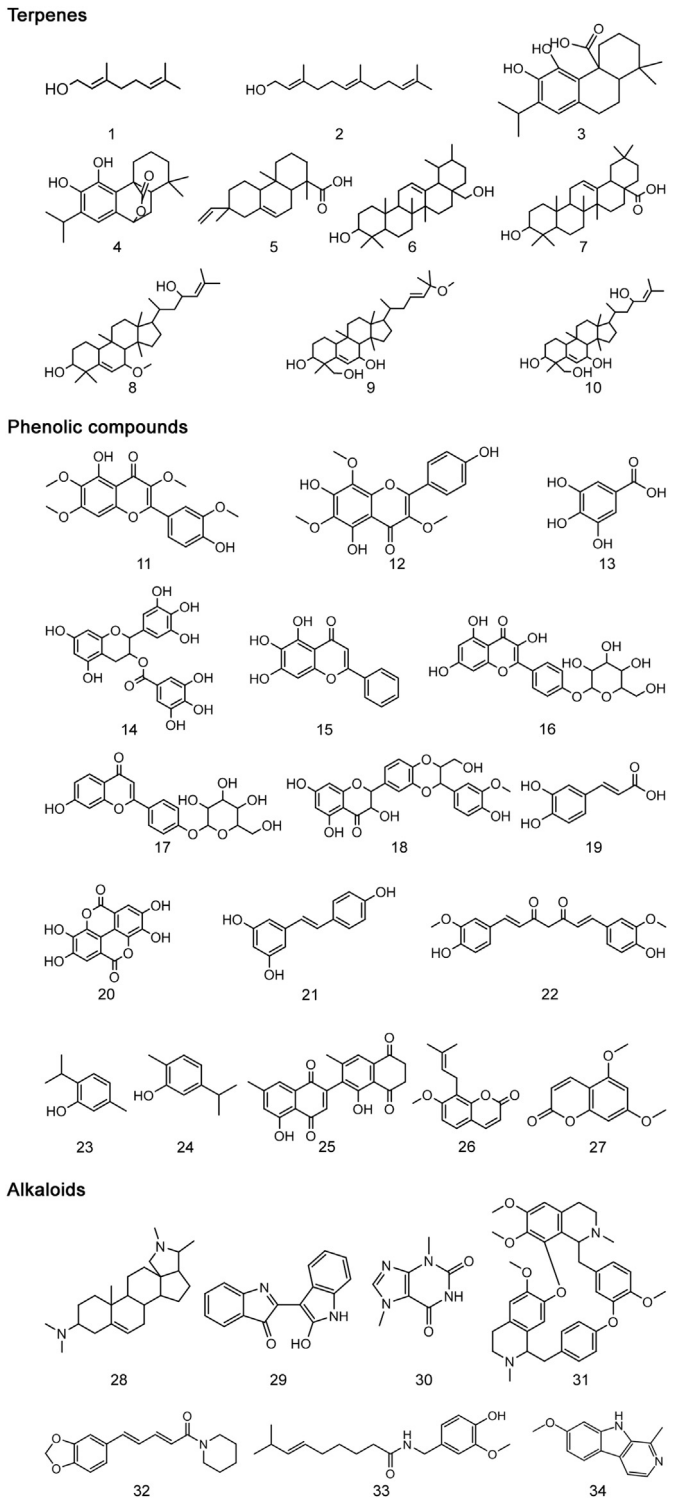


Fig. 3. Chemical structures of some potent efflux pump inhibitors derived from edible plants.

Terpenes

Geraniol (**1,** monoterpenoid) and **Farnesol** (**2,** acyclic sesquiterpene alcohol) from a variety of fruits and aromatic plants acting on MDR food-borne pathogens, *M. smegmatis* efflux pumps and *P. aeruginosa* MexAB-OprM efflux pumps. **Carnosic acid** (**3**, abietane diterpenoid from *Rosmarinus officinalis*, blocks *S. aureus* NorA efflux pumps and potentiates tetracycline against *S. aureus* strains possessing MsrA efflux pump). **Carnosol** (**4**, phenolic diterpene from *Rosmarinus officinalis*, potentiates tetracycline against *S. aureus* strains possessing MsrA efflux pump). **Isopimaric acid** (**5**, diterpenoid from *Pinus nigra*, blocks NorA efflux pump of MRSA). **Uvaol** (**6**, triterpenoid from *Carpobrotus edulis*, acts on MRSA COLOXA efflux systems). **Oleanolic acid** (**7**, pentacyclic triterpenoid from *Carpobrotus edulis*, acts on *E. coli* efflux pumps). Cucurbitane-type triterpenoids from *Momordica balsamina* acting against *E. coli* (AcrAB-TolC), *S. aureus* (NorA) and *E. faecalis* efflux pump systems [(**Karavilagenin** (**8**), **Balsaminagenin B** (**9**), **Balsaminol A** (**10**)].

Phenolic compounds

Chrysoplenetin (**11**, flavonoid from *Artemisia annua*, blocks NorA over-expressing *S. aureus*). **Sarothrin** (**12**, flavonoid from *Alkanna orientalis*, blocks *S. aureus* NorA efflux pumps). **Gallic acid** (**13**, phenolic acid from *Punica granatum*, blocks NorA over-expressing *S. aureus*). **Epigallocatechin gallate** (**14**, flavonoid from *Camellia sinensis*, blocks *S. aureus* NorA, TetB and TetK efflux systems). **Baicalein** (**15**, flavone from *Scutellaria baicalensis*, blocks MDR pumps in MRSA and NorA of *Salmonella enteridis*). **Kaempferol glycoside** (**16**, flavonol from *Herissantia tiubae*, blocks NorA efflux pump in *S. aureus*). **Liquiritin** (**17**, flavanone glycoside from *Glycyrrhiza uralensis*, transporters of fluoroquinolone in *E. coli*). **Silybin** (**18**, flavonolignan from *Silybum marianum*, blocks efflux activity in *S. aureus*). **Caffeic acid** (**19**, polyphenol from a variety of plants, blocks NorA and MsrA efflux pumps of *S. aureus*). **Ellagic acid** (**20,** phenol, from a variety of fruits and aromatic plants acting on MDR food-borne pathogens, *M. smegmatis* efflux pumps and *P. aeruginosa* MexAB-OprM efflux pumps). **Resveratrol** (**21**, polyphenol stilbenoid from various fruits and vegetables, blocks MDR in Gram-negative bacteria overexpressing efflux pumps). **Curcumin** (**22**, polyphenol curcuminoids from *Curcuma longa*, blocks MexAB-OprM of *P. aeruginosa*, NorA, MdeA, TetK and MsrA efflux pumps from *S. aureus*). **Thymol** (**23**, monoterpenoid phenol) and **carvacrol** (**24,** monoterpenoid phenol) from a variety of fruits and aromatic plants acting on MDR food-borne pathogens, *M. smegmatis* efflux pumps and *P. aeruginosa* MexAB-OprM efflux pumps. **Diospyrin** (**25**, naphthoquinone from *Diospyros montana*, blocks *Mycobacterium aurum* efflux pumps). **Osthol** (**26**, coumarin from *Cnidii monnieri*, blocks MexAB-OprM of *P. aeruginosa*, NorA, MdeA, TetK and MsrA efflux pumps from *S. aureus*). **Citropten** (**27**, coumarin from *Citrus paradisi*, blocks *S. aureus* efflux pumps).

Alkaloids

Conessine (**28**, steroid alkaloid from *Holarrhena antidysenterica*, blocks MexAB-OprM of *P. aeruginosa*). **Indirubin** (**29**, bisindole alkaloid from *Wrightia tinctoria*, blocks NorA efflux pump of *S. aureus* and *Staphylococcus epidermidis*). **Theobromine** (**30**, alkaloid from *Theobroma cacao*, blocks AcrAB-TolC and MexAB-OprM efflux pumps). **Tetrandrine** (**31**, bis-benzylisoquinoline alkaloid from *Stephania tetrandra*, fluoroquinolone transporters in *E. coli*). **Piperine** (**32**, alkaloid from *Piper nigrum*, blocks MFS efflux systems). **Capsaicin** (**33**, alkaloid from *Capsicum annuum*, blocks *S. aureus* NorA, TetB and TetK efflux systems). **Harmaline** (**34**, indole alkaloid from *Peganum harmala*, against MRSA).

excellent sources of EPIs. These food constituents would be less toxic or not at all on normal eukaryotic cells, which would favor their exploitation in drugs development and therefore their clinical use. Chemical structures of some potent EPIs derived from edible plants are presented in Fig. 3.

Most of the plant-derived EPIs act on Gram-positive bacteria efflux pumps (Table 1). The difficulty of finding effective EPIs against Gram-negative bacteria is due to the structural complexity of their efflux pump systems (tripartite pump arrangement) link to the existence of an outer membrane. In spite of that, investigations have shown significant inhibitory effects of several phytochemicals against some highly resistant Gram-negative bacteria of clinical concerns. Some examples of compounds acting on Gram-negative bacteria efflux pumps include oleanolic acid (from *Carpobrotus edulis*) [73], gallotannin (from *Terminalia chebula*) [91], karavilagenin (from *Momordica balsamnia*) [94]; all against *E. coli* ArcAB-TolC efflux pumps. Other compounds including Osthol (from *Cnidii monnieri*) [74], falcarindiol (from *Levisticum officinale*) [77], conessine (from *Holarrhena antidysenterica*), catharanthine (from *Catharanthus roseus*) [79], berberine and palmatine (from *Berberis vulgaris*) [87], resveratrol (from *Nauclea pobeguinii*) [11], curcumin (from *Curcuma longa*) [75], *p*-coumaric acid and derivatives (from variety of edible plants), act against *P. aeruginosa* MexAB-OprM efflux pumps, whereas tannic acids and ellagic acid (from various fruits and vegetables) [136] act against *A. baumannii* efflux pumps. Theobromine (from *Theobroma cacao*) has been reported to act on both AcrAB-TolC and MexAB-OprM efflux pumps [117] of Gram-negative bacteria.

5.2. Modes of action of plant-derived efflux pump inhibitors

Since most efflux systems use the energy from H^+ and Na^+

electrochemical gradients for their operation, the main modes of action of EPIs consist of targeting of H^+/Na^+ motive force of the efflux mechanism or by competitive or non-competitive inhibition with the binding substrate. Other mechanisms comprise: (i) ionic gradient dissipation through the cell membrane; (ii) decreasing regulation of the transcription pathway of the genes encoding the efflux pumps; (iii) interference with the hydrolysis of ATP which compromises activation of the efflux pumps and increases permeability of the outer membrane in the structure of Gram-negative bacteria; and finally (iv) conformational changes in efflux protein structures or compromising of assembly of multi-component pumps such as tripartite RND pumps [137].

5.3. Other potential natural sources of EPIs

In addition to medicinal plants, as the main source of effective new EPIs, some microorganisms can produce substances capable of altering the functions of efflux pumps [138]. Some examples including two compounds from the microbial origin, EA-371α and EA-371δ, produced by *Streptomyces vellosus* MF-EA-371-NS1 b, have been reported to inhibit *P. aeruginosa* MexAB-OprM efflux pumps [139]. Likewise, some living beings also evolved to produce potential molecules able to reverse bacterial resistance to antibiotics. Indeed, the ethyl alcohol extract of *Nasutitermes corniger* (termites) enhanced the effectiveness of antibiotics, by a significant reduction of antibiotics MIC value against *S. aureus* and *E. coli* [140,141]. Two compounds, ilicicolinic acids and ilicicolinal derivatives obtained from *Neonectria discophora* (isolated from the nest of *N. corniger*), depicted significant antibacterial activity (MIC <16 mg/L) against *T. rubrum, S. aureus* and MRSA [142].

6. Screening for EPIs from plants

6.1. Overview of methods of the efflux activity assay

EPIs screening is carried out in several ways to determine the effect of a potential efflux inhibitor on a bacterial strain expressing efflux activity. This could consist of incorporating an efflux inhibitor midway via a time-course test to measure variance in fluorescence. An alternative technique is to conduct two distinct time-course tests, one in the absence and the other in the presence of an inhibitor to establish any effect that a test compound may have as a promising inhibitor. In the presence of an EPI only, an increase in drug accumulation proves that the inhibitor is a blocker of efflux machinery [143].

The modulation assay (example of checkerboard synergy testing) is a rapid and simple technique for potential EPIs identification both in Gram-positive and Gram-negative bacteria [144]. The MIC (Minimal Inhibitory Concentration) determination of a crude plant extract or derived compounds appears as a prerequisite with the aim of preventing false-positive results. A reduction of concentration, normally 4-fold lower than the MIC value, is chosen when evaluating a potentiation testing. The modulation assay requires a sub-inhibitory concentration of a crude extract and is usually investigated using 96-well microplates broth microdilution. Efflux inhibition assays can afford information about a molecule whether it is a substrate or not. Besides, the kinetic information of real-time transport and competition for efflux pump binding sites can be readily measured as well [145].

The development of effective EPIs requires the measurement of kinetic parameters of both inhibitor and substrate and their relationships to the structure of the efflux pump component. This will ascertain a clear dissimilarity between a true EPI and a substrate (which is not an inhibitor). Thus, one of the most crucial issues in searching for novel EPIs is the understanding of how EPIs block the transport of antibacterial molecules out of the cell [146]. Molecular simulations can provide information about the ligand-binding process in efflux pumps such as AcrB and possible mode of action of inhibitors [147]. Furthermore, EPIs should have a very low effect or no direct antibacterial activity when applied alone, but they should be able to act synergistically with antibiotics in combination assay.

Techniques for studying the activity of efflux pumps can be divided into two groups. These comprise direct measurement of efflux pump substrate extruded from the bacteria cells and accumulation assay, which measured the amount of efflux pump substrate accumulated into the bacterium [145]. In general, an increase in the activity of the tested compounds in the presence of EPIs reflects the existence of efflux pumps in the studied pathogen. In addition, in the context of accumulation assay, an increase in the accumulation of the therapeutic substance only in the presence of an inhibitor is indicative that the inhibitor can block the efflux activity [148].

6.2. Direct measurement of efflux activity

6.2.1. Fluorescent dyes

Several types of fluorescent dyes can be used for direct measurement of the efflux activity. (i) Alanine-β-napthylamide (Ala-Nap) dye (excitation wavelength, 335 nm; emission wavelength, 410 nm) and (ii) rhodamine 160 6G (R6G) (excitation wavelength, 524 nm; emission wavelength, 547 nm) are used to specifically detect the efflux activities of the MFS and ABC efflux pumps, respectively [149,150]. (iii) The lipophilic dye 1,20-dinaphthylamine (excitation wavelength, 370 nm; emission wavelength, 810 nm) fluoresces in nonpolar environments and is well retained in membranes [151]. (iv) Nile Red (excitation wavelength, 552 nm; emission wavelength, 636 nm) is a periplasmic lipophilic dye that binds to phospholipids of the membrane [152]. Regarding the above-mentioned dyes, 1,20-dinaphthylamine is the most sensitive, and more lipophilic than Nile Red, so perfect for assaying RND efflux pumps since phenylalanines are significant for substrate interaction in the binding pocket. Another substrate for direct efflux measurement is (v) Ethidium bromide (EtBr). EtBr is a DNA-intercalating dye, which fluoresces when bound to DNA (excitation wavelength, 530 nm; emission wavelength, 600 nm). EtBr is a well-validated substrate of many efflux pumps such as the RND pump AcrB [153]. Another DNA intercalating agent, (vi) Doxorubicin, fluoresces more extracellularly than intracellularly, so fluorescence rises upon efflux (excitation wavelength, 450 nm; emission wavelength, 600 nm) [145].

6.2.2. MC-207110, CCCP and reserpine assay

The peptidomimetic compound MC-207110 (phenylalanine arginyl-β-naphthylamide or PaβN) can be used for efflux activity. This synthetic EPI has a competitive mechanism of inhibition and is mostly specific to RND efflux pumps of Gram-negative bacteria such as MexAB-OprM and AcrAB-TolC of *P. aeruginosa* and *E.coli* respectively. Another synthetic EPI, carbonyl cyanide m-chlorophenylhydrazone (CCCP) is an energy-dependent EPI that de-energizes membranes unlike PAβN, which is more substrate-specific [154,155]. Reserpine assay can be used to evaluate the efflux activity of Gram-positive bacteria. This plant-derived alkaloid was shown to be an inhibitor of the *Bmr* efflux pump of *Bacillus subtilis* [156] as well as *PmrA* in *Streptococcus pneumoniae* [157]. In general, an increase in the activity of the tested compounds in the presence of these EPIs reflects the existence of efflux pumps in the studied microorganism.

6.3. Accumulation assay

6.3.1. Checkerboard synergy assay

The checkerboard synergy assay is used to ascertain the interaction and effectiveness of two test compounds when used simultaneously. Checkerboard assay has been used to screen for potential EPIs [158]. Serial 2-fold dilutions of a pump substrate as well as 2-fold dilutions of a test compound will result in microtitre wells with a different combination of pump substrate and test compound concentration. This technique is part of the *in vitro* validation of the potential MDR inhibitors. Results are expressed in terms of FICi (Fractional Inhibitory Concentration index) after determination of FIC (Fractional Inhibitory Concentration) of each product in combination. Considering two substances A and B in combination, FICi values are calculated using the following formula:

$$FICi = FIC_A + FIC_B$$

Where $FIC_A = MIC_A$ in combination/MIC_A alone and $FIC_B = MIC_B$ in combination/MIC_B alone.

The interpretive criteria are as follows: according to Odds' study [159], Synergy is defined when $FICi \leq 0.5$; Indifference when $0.5 < FICi \leq 4$ and Antagonism when $FICi > 4$. Another interpretation made by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [160] defined Synergy when $FICi \leq 0.5$; Additivity when $0.5 < FICi \leq 1$; Indifference when $1 < FICi < 2$ and an Antagonism when $FICi \geq 2$. Most recently Fratini and co-workers [161] have proposed new cut-off points for FICi interpretation where Synergy is observed when $FICi < 1$; Commutativity (or Additivity) when $FICi = 1$; Indifference when $1 < FICi \leq 2$ and Antagonism when $FICi > 2$.

6.3.2. Berberine uptake assay

The berberine uptake assay is used in bioassay-guided isolation of MDR inhibitors [20]. Tested compounds are evaluated in the presence and absence of a sub-inhibitory concentration of this plant alkaloid. Bacterial growth in the absence of berberine and no growth in its presence can be considered as an indicator of the presence of an EPI in the extract [87]. This is a substantial evaluating tool enabling many plant-derived constituents to be assayed rapidly and readily.

6.3.3. Ethidium bromide, Bisbenzimide H33342, and acriflavine (pyronin Y) accumulation assays coupled to flow cytometry

The EtBr accumulation study is a more detailed study of the potentiation activity of a test compound. EtBr is a DNA-intercalating dye that produces fluorescence when it accumulates within the cell. For this reason, the intracellular fluorescence is considerably higher than the fluorescence in the extracellular milieu. EtBr is a substrate for several MDR efflux pumps such as the RND pump AcrB. The activity of putative inhibitors can be measured fluorometrically due to the retention of fluorescence over time if efflux is reduced. Bisbenzimide H33342 (excitation wavelength, 355 nm; emission wavelength, 460 nm) accumulation assay and acriflavine or pyronin Y (excitation wavelength, 463 nm; emission wavelength, 490 nm) assay can be performed in a similar way as that of EtBr efflux inhibition assay. To determine the different bacterial cell subpopulations showing the various amounts of EtBr accumulated inside of the cells, flow cytometry is applicable. In this case, the fluorescence of the cell population will be determined by flow cytometer [162,163].

6.3.4. Liquid chromatography/mass spectrometry-based assay

A liquid chromatography/mass spectrometry-based assay was established to monitor cold or non-fluorescent compounds in a drug discovery setting. It was applied for the first time regarding the efflux pump activity of *P. aeruginosa* with ciprofloxacin and other fluoroquinolones. The drug is allowed to accumulate inside cells, cells are washed and then lysed, and the concentration of drug in solution is measured and related to dry cell weight to estimate the amount of drug inside each cell [164,165].

6.3.5. Fluorescein-di-β-D-galactopyranoside (FDG) based assay

Regarding the rapid development of microfabrication techniques in biology, an FDG based assay was described using a microfluidic channel material with a fluorescence microscope to study the RND pumps in *E. coli*. Since FDG is hydrolyzed by β-galactosidase in the cytoplasm of *E. coli*, the fluorescent dye, fluorescein will be produced and the signal can be recorded and measured [166].

6.3.6. Agar-based method

To enable rapid and cheap detection, a simple, instrument-free, agar-based method utilizes EtBr for the demonstration of efflux pump activity in bacteria. The technique is applied concurrently to up to twelve bacterial strains with the purpose of identifying clinical isolates over-expressing efflux activity [73,167].

6.3.7. In silico high-throughput virtual screening

In silico high-throughput virtual screening can be applied as a criterion for exclusion of substances with efflux substrate-like characteristics, thereby improving the selection process and enhancing the identification of inhibitor. The technique requires an in-house database of botanicals against specific efflux transporters and the process can be filtered by assorting with the conventional pharmacophore models yielded using known efflux substrates. The method can be combined with XP ligand docking of potential hits against specific carriers such as AcrB and MexB proteins of *P. aeruginosa* and *E. coli* respectively. The benefit of *in silico* docking testing is the possibility to identify the molecular interactions between the EPIs and their targets. Understanding the molecular interactions will help in the modification of EPIs for enhancing binding with their target efflux pumps and therefrom, more effective inhibition of efflux pumps [168]. Generally, the method requires in vitro validation assay including MIC determination, checkerboard synergy testing of natural compounds and antibiotics, fluorescence-based ethidium accumulation assay [169]. Further, molecular docking is a powerful tool to understand the interactions of natural compounds with the efflux pumps and to identify the actual target sites of inhibitory compounds [170].

6.3.8. Real-time PCR

While the inhibition of efflux activity and potentiation of antibiotics could be ascertained by MIC and EtBr efflux assays, it is relatively difficult to determine if one or more efflux pumps are inhibited and to identify the target efflux pumps. The real-time PCR would help to determine if known efflux pumps are underexpressed due to the activities of inhibitory compounds [168]. Likewise, a bacterium may possess many efflux pumps including several uncharacterized or even unidentified efflux pumps. Microarray is an alternative approach, which can help to identify unknown efflux pump genes modulated by the activities of inhibitory compounds [170].

6.3.9. Quantitative mass spectrometry

Detection of natural compounds or crude mixtures for efflux inhibitory assay can be complex, as they can contain constituents that cause optical interference when used with fluorescence-based methods. One method to solve this question is to use quantitative

mass spectrometry with the aim of quantifying the concentration to which a substrate has accumulated within cells by measuring the reduction of the substrate from a spent liquid medium. For example, Brown and collaborators [171] have used HPLC-ESI/MS (high-performance liquid chromatography electrospray ionization mass spectrometry) to quantify EtBr uptake to carry out efflux inhibitory activity in *S. aureus* by a crude plant extract and pure flavonoids. Many of the techniques for efflux measurement or accumulation depend on the drugs being either fluorescent or radioactive so that their concentrations can be measured. However, mass spectrometry-based methods of measuring drug accumulation are a significant improvement since they can be used to measure the concentration of any substrate, comprising drugs.

6.4. Constraints in the development of EPIs

To define a clear dissimilarity between the inhibitor of efflux systems and substrate (which has no inhibitory effect), the characterization of efficient EPIs requires the determination of kinetic parameters of the inhibitor and those of the substrate as well as the correlation with the structure of the efflux pump components [146]. Thus, one of the major concerns in the search for new EPIs is to understand how these interfere with the transport of pharmaceuticals outside the bacterial cell [147]. The development of EPIs remains a major challenge and requires the overcoming of many barriers including the choice of antibiotics to potentiate and the adequacy of the pharmacological effects of the combination EPIs-antibiotic [172]. Despite the hope based on the use of effective EPIs, the major concern remains over their toxicity that impedes their clinical application. Unfortunately, no active EPI has been introduced in clinical use nowadays. The cause lies in the low selectivity coupled with the low level of stability and raised toxicity of these inhibitors on human cells [173], although research continues. Mpex Pharmaceuticals reported a promising formulation (in phase 2 clinical trials) of an EPI (MC-601,205) combined with a fluoroquinolone (ciprofloxacin) in the management of pulmonary infections in patients suffering from cystic fibrosis [174]. However, no structure or mechanism of action of this substance has been disclosed.

Considerable efforts have led to the characterization of many efflux pumps as well as potential active EPIs. However, the molecular bases of active extrusion transport and blocking of efflux, necessary for the development of drugs, remain indeterminate. The progress remains unsatisfactory, insofar as the investigations have not yet made it possible to describe the physicochemical characteristics for an effective inhibitory potential. Likewise, more and more experimentation is needed to quantify the effects of EPIs on efflux pump systems. The development of new EPIs, therefore, requires a definition of the kinetic parameters and the structural relationship with the components of the pumps likely to be affected. Integration of quantitative structure-activity relationship (QSAR) analysis would make it possible to establish the correlation between the biological effects of a compound with the various physicochemical parameters. This technique could be used to predict EPI activity and quantize important regions in molecules [175]. The production of broad-spectrum EPIs is more complicated and would also constitute a real risk (toxicity) for human cells, while selective inhibitors will tend to generate mutants in which a secondary pump will assume the functions of the inhibited ones.

Another challenge to consider is that anti-infective treatment with EPIs is combination therapy. To improve the synergistic interactions between the two combined substances, the pharmacokinetic properties (ADME: Absorption, Distribution, Metabolism, Excretion) of EPIs must be suitably adapted to the pharmacokinetics of the second (antibiotic) compound of the combination [176]. In the case where an EPI and the compound with which it has been combined are substrates of the same efflux pump, its use can lead to the development of resistance to EPIs and cross-resistance to antibiotics. Therefore, it would be appropriate to carry out the experiments with the aim of differentiating antibiotic resistance from those affecting the action of EPIs. Besides, for future clinical applications, EPIs should be selective concerning human cellular components.

7. Conclusion

The resurgence of bacterial resistance has led to an unprecedented stalemate in the field of antibiotic research. Technological advances have provided the essential tools for finding new classes of antibiotics and improving the ones already known to avoid escalating therapeutic impasses. Likewise, there is a need to detect and validate new antibiotic agents with uncommon activities, less toxic and against which the probability of developing resistant mutants would be limited. Active efflux is the main mechanism that leads to bacterial MDR. Inhibition of these pumps by effective EPIs would probably lead to a regression of the rise of MDR. Plants constitute a vast source of bioactive molecules, with potential EPIs, so they play a major role as an alternative in the discovery of new antimicrobial agents. The identification, design, and production of the EPIs are a promising way leading to the past destination. Indeed, the blocking of the efflux pumps would allow re-use of the antibiotics that were no longer used clinically because of the resistance. Due to their usual consumption, edible plants deserve more attention and could result in low- or non-toxic compounds. To date, no natural or synthetic EPIs have yet passed the clinical trial stages. However, the scientific community is carrying out continuous investigations to identify and develop less toxic and therapeutically effective EPIs.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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