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Polyphenols as antimicrobial agents

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Polyphenols are secondary metabolites produced by higher plants, which play multiple essential roles in plant physiology and have potential healthy properties on human organism, mainly as antioxidants, anti-allergic, anti-inflammatory, anticancer, antihypertensive, and antimicrobial agents. In the present review the antibacterial, antiviral, and antifungal activities of the most active polyphenol classes are reported, highlighting, where investigated, the mechanisms of action and the structure–activity relationship.Moreover, considering that the microbial resistance has become an increasing global problem, and there is a compulsory need to find out new potent antimicrobial agents as accessories to antibiotic therapy, the synergistic effect of polyphenols in combination with conventional antimicrobial agents against clinical multidrug-resistant microorganisms is discussed.

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Introduction

Polyphenols are secondary metabolites ubiquitously distributed in all higher plants, which have important roles as defense against plant pathogens and animal herbivore aggression and as response to various abiotic stress conditions, such as rainfall and ultraviolet radiation. As regard to chemical structure, they comprise a wide variety of molecules with polyphenol structure and are generally divided into flavonoids and nonflavonoids. Flavonoids share a common carbon skeleton of diphenyl propanes, two benzene rings (ring A and B) joined by a linear three-carbon chain. The central three-carbon chain forms a closed pyran ring (ring C) with A benzene ring. More than 4000 flavonoids have been identified in fruits, vegetables, and plant-derived beverages, such as tea and wine, and the list is constantly growing. Depending on the oxidation state of the central pyran ring, flavonoids can themselves be subdivided into many subclasses: flavonols, flavones, flavanones, anthocyanidins, flavanols, and also isoflavones (Figure 1).

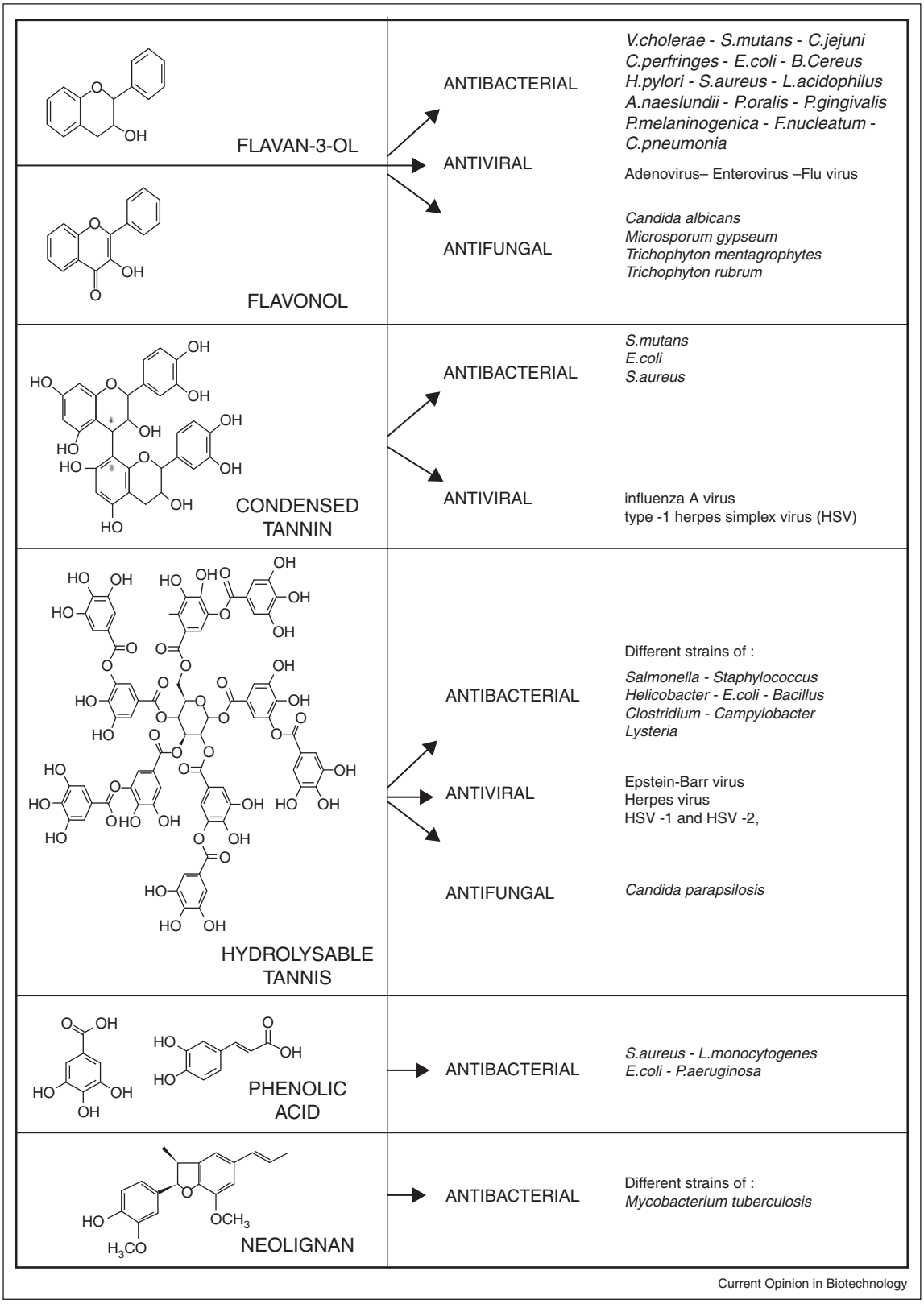
The main groups of nonflavonoids are first phenolic acids, that can be subdivided into derivatives of benzoic acid, such as gallic acid and protocatechuic acid, and derivatives of cinnamic acid, that consist chiefly of coumaric, caffeic, and ferulic acid, second stilbenes, whose main representative is resveratrol, that exists in both *cis* and *trans* isomeric forms, and third lignans, produced by oxidative dimerization of two phenylpropane units (Figure 1).

In addition to this diversity, polyphenols are present in plant tissues mainly as glycosides and/or associated with various organic acids and/or as complex polymerized molecules with high molecular weights, such as tannins [1••,2].

Over the course of the last 20 years, polyphenols have been studied for their potential involvement in the prevention of chronic diseases, such as cardiovascular disease, cancer, osteoporosis, diabetes mellitus, and neurodegenerative diseases. Their protective activity has been attributed initially to their antioxidant, free radical scavenger, and metal chelator properties, then to the capability of inhibiting or reducing different enzymes, such as telomerase [3], cycloxygenase [4,5], or lipoxygenase [6,7], and in more recent years, to the interaction with signal transduction pathways and cell receptors [8–10].

Moreover, the antimicrobial activity of polyphenols occurring in vegetable foods and medicinal plants has been extensively investigated against a wide range of microorganisms. Among polyphenols, flavan-3-ols, flavonols, and tannins received most attention due to their wide spectrum and higher antimicrobial activity in comparison with other polyphenols, and to the fact that most of them are able to suppress a number of microbial virulence factors (such as inhibition of biofilm formation, reduction of host ligands adhesion, and neutralization of bacterial toxins) and show synergism with antibiotics. The antimicrobial properties of certain classes of polyphenols have been proposed either to develop new food preservatives [11], due to the increasing consumer pressure on the food industry to avoid synthetic preservatives, or to develop innovative therapies for the treatment of various microbial infections [12•,13•], considering the increase in microbial resistance against conventional antibiotic therapy.

Figure 1



Chemical structure of the polyphenol classes and microorganisms sensitive to them, as reported in the review.

This review reports the results of the latest investigations regarding the most active antibacterial, antiviral, and antifungal polyphenols and brings to focus the synergistic activity of some of them in combination with conventional antimicrobials against multiresistance microbial pathogens.

Antimicrobial activity of flavan-3-ols

Considering flavan-3-ols, the antibacterial activity of catechins is known from the 1990s, when it was demonstrated that these compounds, largely present in oolong tea and above all green tea (*Camellia sinensis*), inhibited the *in vitro* growth of several bacterial species, such as *Vibrio cholerae*, *Streptococcus mutans*, *Campilobacter jejuni*, *Clostridium perfringes*, and *Escherichia coli* [14–18].

More recently, it was demonstrated that some tea catechins, such as (−)-gallocatechin-3-gallate, (−)-epigallocatechin-3-gallate, (−)-catechin-3-gallate, and (−)-epicatechin-3-gallate, are active at nanomolar levels against some other food-borne pathogenic bacteria, such as *Bacillus cereus*. Most of these compounds were found to be more active than antibiotics, such as tetracycline or vancomycin, at comparable concentrations: this suggested that the tested tea catechins could exert a positive effect against gastrointestinal diseases [19].

Among tea catechins, epigallocatechin gallate (EGCG) has received the most attention and has been investigated more deeply in its antibacterial, antiviral, and antifungal activities. As far as the antibacterial activity is concerned, 56 clinical isolates of *Helicobacter pylori*, a urease producing gastric pathogen that may contribute to the formation of ulcers and gastric cancer in humans, including 19 isolates highly resistant to metronidazole and/or clarithromycin, were used to determine their *in vitro* EGCG sensitivity. The minimum inhibitory concentration (MIC) required to inhibit the growth of 90% of organisms was found to be 100 μg/ml. It is interesting to underline that those clinical isolates highly resistant against antibiotics also showed a similar EGCG sensitivity [20].

As reported above, tea catechins are active against *E. coli*. In particular, EGCG at sub-MIC (25 μg/ml) did not affect *E. coli* O157:H7 growth rate, but showed significant antipathogenic effect because it decreased some virulence factors such as biofilm formation and bacterial swarm motility [21••].

Also EGCG antiviral activity was discovered in the 1990s. EGCG was found to prevent infection caused by flu virus by binding to the viral hemagglutinin, thereby preventing the attachment of viral particles to the target receptor cells [22]. Other studies showed that modifications of viral membrane properties contributed to tea catechin's antiviral effect against flu virus while, at the same time, structure–activity studies showed that the 3-galloyl side chain potentiates the parent catechin molecule antiviral activity. In fact, both EGCG and epicatechin gallate (ECG) were found to be 10–15 times more active against flu virus than epigallocatechin (EGC) [23]. Other investigations confirmed EGCG antiviral activity against adenovirus and enterovirus infections [24,25].

EGCG also exhibited variable time-dependent and concentration-dependent fungicidal activities. Several fungi, including *Candida albicans*, proved sensitive to this compound, suggesting that flavan-3-ols may be useful in the treatment of *C. albicans* superinfections of the oral cavities, intestine, and vagina, which may result from an excessive use of antibiotics [26].

Flavonols antimicrobial activity

As far as flavonols are concerned, we can see a remarkable activity against several Gram-positive bacteria, such as *Staphylococcus aureus*, *Lactobacillus acidophilus*, and *Actinomyces naeslundii* and Gram-negative bacteria, such as *Prevotella oralis*, *Prevotella melaninogenica*, *Porphyromonas gingivalis*, and *Fusobacterium nucleatum*, probably due to different mechanisms of action, among which the most convincingly identified is the aggregatory effect on all the bacterial cells [27].

It is worth mentioning the following investigation reporting that rhamnetin, myricetin, morin, and quercetin showed high activity against *Chlamydia pneumoniae*, an obligate intracellular Gram-negative pathogen, which is a common cause of acute upper and lower respiratory infections, including pharyngitis, sinusitis, and pneumonia. In this study, the pretreatment of a human cultured cell line (HL cells), which is conventionally used in *C. pneumoniae* cultivation, with flavonols, decreased the infectivity of *C. pneumoniae* by 50% compared to the percentage seen in untreated controls, at polyphenol concentrations ranging from 0.5 to 50 μM [28]. When the compounds were continuously present in cell cultures, infectivity was clearly lower, varying from 0 to 32%. All compounds also decreased the infective yields, and the most chlamydiosidic compound was rhamnetin, which killed *C. pneumoniae* at the tested concentrations. As the opportunity to use polyphenols as therapeutic agents is often limited by their bioavailability [29], it is interesting to highlight that due to their hydrophobicity, flavonols are capable of penetrating cell phospholipid membranes, being therefore able to exert their antibacterial activity also inside the cell. Moreover, rhamnetin resulted to be more active than quercetin and morin, probably because of the methoxy group in the A-ring, which makes this molecule more hydrophobic [28].

Recent investigations also pointed out the fungicidal activity of flavonols. It was shown that propolis, which

is recommended worldwide for external topical use as it relieves various types of bacterial and fungal dermatitis, possessed antifungal activity (against *Microsporum gypseum*, *Trichophyton mentagrophytes*, and *Trichophyton rubrum*), and the main responsible agents for this activity were identified as flavonols (galangin, izalpinin, and rhamoncitrin) [30••]. Among the other propolis polyphenols found active, there were flavanone (pinocembrin and pinostrobin) and chalcones (2,4-dihydroxychalcone and 2,4-dihydroxy-3-methoxychalcone), that other studies reported to show antimicrobial activity [31,32].

Tannins antimicrobial activity

Tannins are subclassified into proanthocyanidins (condensed tannins) and gallotannins and ellagitannins (hydrolyzable tannins).

The proanthocyanidins occur in fruits, bark, leaves, and seeds of many plants. They are dimers, oligomers, and polymers of catechins that are bound together by links between C4 and C8 (or C6) and are composed of a myriad of oligomeric products that differ first, in region and stereochemical configuration of the flavanol interlinkages, second, in the phenolic hydroxylation pattern, and third, in the configuration of the hydroxylated C-ring C3 center of the flavan-3-ol building block. These oligoflavanols are further subdivided into two basic types, A-type and B-type, which are characterized by the occurrence of either a double or a single linkage connecting two flavanol units [1••]. These differences in the chemical structures make investigations directed toward their biological properties, or to their structure–activity relationships, quite challenging. The most studied proanthocyanidins are those derived from berries that inhibit the growth of several pathogenic bacteria, such as uropathogenic *E. coli*, cariogenic *S. mutans*, and oxacillin-resistant *S. aureus* [33]. The cranberry proanthocyanidins, consisting primarily of epicatechin tetramers and pentamers with at least one A-type linkage, were found to be active against the reported pathogenic bacteria. Several mechanisms could explain the effect of the A-type proanthocyanidin in the bacterial growth inhibition, such as the destabilization of the cytoplasmic membrane, the permeabilization of the cell membrane, the inhibition of extracellular microbial enzymes, direct actions on microbial metabolism, or the deprivation of the substrates required for microbial growth, especially essential mineral micronutrients such as iron and zinc (via proanthocyanidin chelation with the metals), whose depletion can severely limit bacterial growth [34,35].

Besides antibacterial activity, proanthocyanidins showed antiviral effects against influenza A virus and type-1 herpes simplex virus (HSV). In this case the mechanism of action seems to consist in preventing the entry of the virus into the host cell, which is the first critical step in primary HSV-1 infection [36].

Gallotannins and ellagitannins derived from the metabolism of the shikimate-derived gallic acid (3,4,5-trihydroxybenzoic acid) follow through various esterification and phenolic oxidative coupling reactions to yield numerous (near 1000) monomeric and oligomeric polyphenolic galloyl ester derivatives of sugar, mainly D-glucose [1••]. The antimicrobial activity of hydrolysable tannins is well known. Ellagitannins, the main phenolic compounds of *Rubus* and *Fragaria* genus (raspberry, cloudberry, and strawberry) show very interesting properties because they inhibit to different extents the growth of selected Gram-negative intestinal bacteria (strains of *Salmonella*, *Staphylococcus*, *Helicobacter*, *E. coli*, *Clostridium*, *Campylobacter*, and *Bacillus*), but they are not active against Gram-positive beneficial probiotic lactic acid bacteria [37]. Unfortunately, *Listeria monocytogenes*, a common bacterium found in the environment and associated with animals that may cause meningitis, sepsis, or abortion, is not affected by these berry compounds [38].

As far as gallotannins are concerned, penta-O-galloylglucose, hexa-O-galloylglucose, hepta-O-galloylglucose, octa-O-galloylglucose, nona-O-galloylglucose, and deca-O-galloylglucose isolated from mango kernels showed antibacterial activity against food-borne bacteria. Gram-positive bacteria were generally more susceptible than Gram-negative, in fact the MICs against *Bacillus subtilis*, *B. cereus*, *Clostridium botulinum*, *C. jejuni*, *L. monocytogenes*, and *S. aureus* were 0.2 mg/ml or less; enterotoxigenic *E. coli* and *Salmonella enterica* were inhibited by 0.5–1 mg/ml. Also in this case, lactic acid bacteria exhibited strong resistance [39].

The activity of gallotannins is attributable to their strong affinity for iron and it is also related to the inactivation of membrane-bound proteins.

Considering the antifungal activity of ellagitannins, the discovery of this property derives from the observation that the durability of hardwoods, such as oaks and chestnuts is thought to owe much to the deposition of ellagitannins which are able to precipitate protein and/or remove metal cofactors through their strong affinity for metal ions, acting as a microbial barrier. A recent investigation showed that ellagitannins isolated from *Ocotea odorifera*, a medicinal plant commonly used in Brazil, have potent activity against *Candida parapsilosis* at a concentration level of 1.6 μM [40]. Ellagitannins possess antiviral activities, in particular against HIV infection [41,42] and manifest inhibitory effects on HSV-1 and/or HSV-2 replication, as well as Epstein-Barr virus [43]. Ellagitannins activity against Herpes virus seems due to a marked inhibitory effect on the replication of both HSV-1 and HSV-2, including acyclovir-resistant strains, with acyclovir being the first effective specific drug against Herpes virus made available [44•].

Nonflavonoid compounds antimicrobial activity

As reported above, nonflavonoids show weaker antimicrobial activity in comparison with flavonoids; nevertheless, some investigations are worth mentioning. Some phenolic acids (gallic, caffeic, and ferulic acids) showed antibacterial activity against Gram-positive (*S. aureus* and *L. monocytogenes*) and Gram-negative bacteria (*E. coli* and *Pseudomonas aeruginosa*). These compounds were found to be more efficient against the reported bacteria than conventional antibiotics such as gentamicin and streptomycin. Differently, chlorogenic acid showed no activity against Gram-positive bacteria [13•].

Considering another nonflavonoid class of compounds, lignans, a recent investigation showed that the hexane extract obtained from *Aristolochia taliscana* roots, a plant used in traditional Mexican medicine, contains neolignans, among which Licarin A was found to be the most active, with MICs ranging from 3.12 to 12.5 μg/ml against four mono-resistant variants and 12 clinical isolates of *Mycobacterium tuberculosis* strains [45]. These results confirm previous investigations on lignans biological properties [46] and suggest that these compounds represent a potentially active agent to fight tuberculosis, a pathology that, in recent years, has become more of a worldwide concern as one-third of the world's population is currently infected with *M. tuberculosis*.

Use of polyphenols as a new strategy to fight microbial resistance in combination with anti-infective drugs

Infectious diseases remain among the leading causes of morbidity and mortality both in developed and developing countries. The selective pressure exerted by the use, misuse, and overuse of anti-infective drugs has raised the problem of antibiotic resistant microbes (bacteria, viruses, or parasites) that have acquired the ability to survive existing drugs at clinically relevant concentrations and are responsible for very serious diseases, such as AIDS, tuberculosis, gonorrhea, malaria, influenza, pneumonia, diarrhea, and the chronic infections caused by bacterial biofilms. Therapeutic options for these so-called community-acquired pathogens (such as penicillin-resistant, methicillin-resistant and vancomycin-resistant *S. aureus* or multidrug-resistant *V. cholera*) are extremely limited, as are prospects for the next generation of antimicrobial drug development.

Recently, by considering that antibiotics are biological compounds that are produced by bacteria or other microorganisms and are capable of killing or suppressing the growth and reproduction of other bacteria [47], several investigations have proposed that polyphenols, secondary metabolites developed by plants as a strategy of defense against phytophagous insects, fungi or bacteria, could be used in combination with antibiotics in order to potentiate their efficacy, to lower antibiotic dose, and therefore to reduce antibiotic adverse reactions [48–51].

The *in vitro* synergistic effect of two flavonols (kaempferol and quercetin), in combination with rifampicin (a complex macrocyclic antibiotic), was demonstrated against clinical rifampicin-resistant methicillin-resistant *S. aureus* (MRSA) isolates [52]. As regard to the mechanism of action, quercetin and kaempferol alone showed slight β-lactamase inhibition, but when combined with rifampicin, the complex exhibited good β-lactamase inhibitory effect (57.8 and 75.8%, respectively). In the same study, the authors showed that the bactericidal action of ciprofloxacin, a fluoroquinolone derivative, commonly used in Australia to treat staphylococcal infections and associated with a rapid emergence of resistance in Gram-positive bacteria, was greatly enhanced by the sub-MIC addition of the two polyphenols. Quinolones mechanism of action is the bond with *S. aureus* topoisomerase IV, which causes cell death, mainly thanks to DNA synthesis inhibition, cessation of growth, and numerous double-stranded DNA breaks in the bacterial chromosome. Both quercetin and kaempferol inhibit the catalytic activity of different bacterial topoisomerases [53] and this might explain some of the synergistic activities between ciprofloxacin and quercetin/kaempferol [52]. These results were confirmed by a more recent investigation in which kaempferol glycosides isolated from *Laurus nobilis* L. reduced the MIC of some fluoroquinolones registered against MRSA [54].

Many papers reported that EGCG act synergistically with various β-lactam antibiotics against MRSA [55–58]. More recently, a Korean green tea polyphenol extract containing five main compounds occurring in different percentages (EGCG, EGC, gallocatechin gallate, epicatechin, and ECG), resulted to have antibacterial effects against 13 strains of MRSA clinical isolates and 17 strains of methicillin-susceptible *S. aureus* (MSSA). The MICs of oxacillin for each of the 13 MRSA strains were reduced between 8-fold and 128-fold when these strains were coincubated with sub-MIC ($<0.5\times$ MIC) levels of tea polyphenols, demonstrating that the combination of tea polyphenols plus oxacillin was synergistic for all the clinical MRSA isolates. As for the mechanism of action, two-dimensional polyacrylamide gel electrophoresis identified 13 downregulated proteins and three upregulated ones by exposure to polyphenols, demonstrating that flavan-3-ols can stimulate in a different way the expression of various proteins in these bacteria [59].

The combination of conventional antimicrobials and proanthocyanidins isolated from *Quercus ilex* L., not only showed antimicrobial effects against human bacterial species, but also against fungal species; proanthocyanidins combined with bifonazole and ketoconazole

increased the activity of both of these conventional fungicides [60].

Conclusions

The *in vitro* antimicrobial activity of some polyphenol classes has been widely shown by the scientific literature published over the last two decades. This activity can be attributable both to direct action against bacteria, virus and fungi, as well as to the suppression of microbial virulence factors. There is growing evidence that flavonoids act synergistically with various antibiotics against multidrug-resistant microorganisms. These findings suggest that future investigations should be carried out in order to study their *in vivo* activity, toxicity, and bioavailability, and therefore to determine their actual relevance for treatment of human and animal infection diseases.

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