Recent advances in understanding the antibacterial properties of flavonoids

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Abstract

Antibiotic resistance is a major global problem and there is a pressing need to develop new therapeutic agents. Flavonoids are a family of plant-derived compounds with potentially exploitable activities including direct antibacterial activity, synergism with antibiotics, and suppression of bacterial virulence. In this review, recent advances towards understanding these properties are described. Information is presented on the ten most potently antibacterial flavonoids, and the five most synergistic flavonoid-antibiotic combinations tested in the last six years (identified from PubMed and ScienceDirect). Top of these respective lists are panduratin A with MICs of 0.06 to 2.0 μg/mL against Staphylococcus aureus, and epicatechin gallate which reduces oxacillin MICs as much as 512-fold. Research seeking to improve such activity, and understand structure-activity relationships is discussed. Proposed mechanisms of action are discussed too. In addition to direct and synergistic activities, flavonoids inhibit a number of bacterial virulence factors including quorum sensing signal receptors, enzymes, and toxins. Evidence of these molecular effects at the cellular level include in vitro inhibition of biofilm formation, inhibition of bacterial attachment to host ligands, and neutralisation of toxicity toward cultured human cells. In vivo evidence of bacterial pathogenesis being disrupted includes demonstrated efficacy against Helicobacter pylori infection and S. aureus α-toxin intoxication.

Keywords: flavonoids; antibacterial; structure-activity; mechanism of action; synergy; antivirulence

1. Introduction

With antibiotic resistance reaching crisis point in many hospitals around the world [1] and resistance increasing in community acquired infections also [2], there is an urgent need to replenish our arsenal of anti-infective agents. Ideally, this should be in the form of new classes of antibacterial agent [3], as the structural alteration of drugs to which resistance has already developed rarely provides a major solution [4]. Inhibition of resistance mechanisms through the development of novel adjuncts represents an important strategy also. The β-lactamase inhibitor clavulanate, launched in 1981, remains effective today in spite of many years of extensive use [5, 6]. A third promising but unproven approach is the development of drugs that target bacterial virulence factors. Rather than inhibiting cellular components necessary for growth or viability, these compounds would ameliorate infection by interfering with aspects of bacterial pathogenesis eg. attachment to host tissue [7].

Natural products are a major source of chemical diversity and have provided important therapeutic agents for many bacterial diseases [8]. Most of these agents have been of microbial origin, but the antibacterial properties of plant-derived compounds are attracting increasing attention [9, 10]. This is attributed, in part, to the fact that plants can be rationally selected for antibacterial testing based on ethnomedicinal use [11]. Flavonoids are a group of heterocyclic organic compounds present in plants and related products eg. propolis and honey [12]. Poultices, infusions, balms, and spices containing flavonoids as active constituents have been used in many cultures for centuries. Traditional uses include treatment and prevention of various infectious and toxin-mediated diseases eg. sores, wound infections [13], acne, respiratory infections [14], gastrointestinal disease [15], and urinary tract infections [16]. Not surprisingly, this family of compounds is the subject of much antibacterial research.

There are fourteen classes of flavonoid in total, differentiated on the basis of the chemical nature and position of substituents on the A, B and C rings [17]. The skeleton structures of six of these classes are shown in Figure 1 with rings named and positions numbered. Most of the reports of flavonoids possessing antibacterial properties can be attributed to these six structures or their isoflavonoid counterparts (flavonoids where ring B is

joined at position 3 of ring C instead of position 2). Potential applications for these compounds include modern agents [18] and adjuncts [19] for the treatment of bacterial infection, drugs for treating toxin-mediated disease [20], antivirulence therapies [21], and capture molecules for removing endotoxin from pharmaceutical preparations [22].

In this paper, reports on the diverse range of antibacterial properties exhibited by flavonoids are reviewed. Emphasis is on important developments in the last six years as earlier research has already been discussed [13, 23]. The activity of naturally occurring flavonoids is covered, as well as that of semi-synthetic and synthetic flavonoids. Proposed structure-activity relationships and mechanisms of action (MOAs) are reviewed too. The structures of all the flavonoids discussed are presented in Supplementary Table 1. Readers interested in the more specific topic of antibacterial tea flavonoids or broader topic of medicinal flavonoids are directed to reviews by Friedman [24] and Cazarolli et al. [25].

2. Direct antibacterial activity

2.1 Naturally occurring flavonoids

For several decades, the antibacterial activity of flavonoid-rich natural products has been reported in the scientific literature. This has continued in recent years, with some plant and propolis extracts being identified with MICs <100 μg/mL [26-28] or in one case <10 μg/mL [29]. Antibacterial flavonoids have been successfully isolated in over 50 such studies, and a list of compounds with the lowest reported MICs is presented (Table 1). To put these values into context, compounds with MICs ≤100 μg/mL are considered noteworthy, and those with MICs ≤10 μg/mL, very interesting [30]. Caution is always necessary when comparing flavonoid MICs determined in different laboratories [13] but, this caveat notwithstanding, some of the flavonoids isolated since 2005 have very impressive antibacterial activity.

2.2 Semi-synthetic and synthetic flavonoids

Synthetic modification of natural flavonoid structures has been reported as early as 1981 [31], but it is only in recent years that there has been a real surge of work in this area.

Particularly successful alterations include linking an N-heterocyclic ring to the A ring of chrysin (position 7). This derivative was 16- to 32-fold more active than its parent compound, with MICs of 1.56 and 3.13 µg/mL against Escherichia coli and Staphylococcus aureus respectively [32]. Similar enhancements of activity were observed when dibutylamine was linked to the A ring of genistein (position 7) [18]. Alkylation of (-)-epigallocatechin gallate dramatically improved the activity of this compound against Gram positive pathogens. The derivative 3-O-decyl-(+)-catechin was 64- to 128-fold more active than its parent structure, with MICs of 1.0 and 2.0 µg/mL against S. aureus and Enterococcus faecalis [33].

2.3 Structure-activity relationship

The findings of recent structure-activity investigations are summarised below. These correspond well with relationships summarised previously [13], and shed further light on this subject. Structural components which improve the activity of open chain flavonoids (chalcones) generally improve the activity of other flavonoids. However, in the interests of clarity these two groups are discussed separately.

2.3.1 Chalcones

For the A ring, Avila et al. have confirmed that hydroxylation at position 2' is important for antibacterial activity, though it is hypothesised that this feature indirectly affects activity by promoting structural stability [34]. Hydroxylation at other A ring positions [35] including position 4' [36] improve activity too. Interestingly, carboxylation at position 4' has been shown to cause a 60-fold improvement in aqueous solubility with negligible loss of antibacterial activity [37]. Reports by Avila [34] and Batovska [38] suggest that A ring lipophilicity is important however, with chalcones possessing prenyl or geranyl groups at position 3' displaying good activity [34]. Substitutions which decrease activity include acetoxylation or methoxylation at position 2' [34] and fluorination at positions 3' and 5' [37].

On the B ring, substitution at position 4 is important for antibacterial activity. For example, chalcones with a 6-carbon alkyl chain and piperidine group at this position have

good activity [39] as do compounds with a hydroxyl group [34]. Batovska et al. report that hydroxylation of the B ring is not sufficient for activity on its own though, and suggest that a lipophilic A ring is necessary [38]. Presence of the lipophilic substituents trifluoromethyl or bromo at position 3 of the B ring has also been reported to improve antibacterial activity, with activity increasing further if one of these groups is present at position 5 [37].

2.3.2 Other flavonoid classes

On the A ring, the presence of an O-acyl [40] or O-alkylamino chain [41] at position 7 improves the antibacterial activity of compounds in the flavone class. Šmejkal and colleagues report that hydroxylation at position 5 is also important [42], a finding which corresponds well with previous studies of flavones and flavanones [43, 44]. The presence of a lipophilic group (eg. geranyl) at position 6 or 8 of the A ring improves activity too [42]. This supports previous findings by Tsuchiya and colleagues [44].

There has been comparatively little work on the B ring but Šmejkal et al. report that, as is the case with chalcones [34], methoxylation decreases activity [42]. This correlates well with previous work by Alcaraz and colleagues, who found that 4'-oxymethylflavanone had an MIC in excess of 1000 µg/mL against S. aureus [43]. Older research, worth mentioning here because it corresponds well with recent data for the chalcones [37], indicates that bromo- (and chloro-) groups at positions 2', 3' and 4' improve flavanone activity [31].

For the C ring, hydroxylation at position 3 improves the activity of flavanones [42]. There is growing evidence to suggest that an O-acyl or O-alkyl chain at this position improves activity even further, at least in the case of flavonols [45] and flavan-3-ols [33, 46]. Recent work by Mughal et al., which sought to improve the activity of flavones, found that replacement of the oxygen atom at position 4 with sulphur or nitrogen was effective [47].

2.4 Identification of flavonoid activity as bacteriostatic or bactericidal

With increasing numbers of immunocompromised patients [48], there is understandable interest in the identification of compounds which kill bacteria rather than just inhibiting their

growth. Bactericidal activity in this context is usually defined as activity resulting in a 99.9% reduction in bacterial numbers, and is tested for using the time-kill method or minimum bactericidal concentration (MBC) assay [49, 50]. Such methods have frequently been used to test flavonoids, and on many occasions bactericidal activity was reportedly detected [51-53]. Studies with model membranes indicate that flavonoids cause aggregation though [54, 55], and in 2007 it was confirmed that the flavonol galangin has this effect on bacterial cells [56]. The flavan-3-ol epicatechin gallate has also been reported to cause bacterial aggregation [19], though it is not yet clear if this observation was due to genuine aggregation (cells clumping together) or pseudomulticellular bacteria (cells failing to separate following binary fission [46]). An important ramification of this aggregatory effect is that conventional methods are no longer sufficient for demonstrating the bactericidal activity of flavonoids. This is because decreases in colony forming unit (CFU) numbers may be attributable to bacteria clumping together and not cell death (Figure 2). There are no immediately apparent solutions to this methodological problem, but an interim approach might be to cease MBC testing in favour of time-kill studies, with microscopic analysis of treated bacteria [57].

2.5 Mechanism(s) of action

Early flavonoid research (1987 to 2004; reviewed previously [13]) suggested that their direct antibacterial activity may be attributable to up to three mechanisms. These were cytoplasmic membrane damage (caused by perforation [54] and / or a reduction in membrane fluidity [58]), inhibition of nucleic acid synthesis [59] (caused by topoisomerase inhibition [60, 61]), and inhibition of energy metabolism (caused by NADH-cytochrome c reductase inhibition [62]). In the period since (2005 to 2010), additional evidence has been presented in support of each of the proposed mechanisms. Work with compounds in the flavonol [63], flavan-3-ol [64, 65], and flavolan classes [66] suggests these damage the cytoplasmic membrane (possibly by generating hydrogen peroxide [67]), and work with flavan-3-ols [68, 69] and isoflavones [70] suggests these inhibit nucleic acid synthesis (through topoisomerase [68, 71] and / or dihydrofolate reductase [69] inhibition). In addition, compounds in the

flavonol, flavan-3-ol, and flavone classes have been shown to inhibit energy metabolism (through ATP synthase inhibition [72]). Evidence has also been presented for two new mechanisms. These are inhibition of cell wall synthesis (caused by D-alanine:D-alanine ligase inhibition [73]) and inhibition of cell membrane synthesis (caused by inhibition of FabG [74-76], FabI [74], FabZ [77], Rv0636 [78] or KAS III [79]).

Probably for logistical reasons, most of the above studies were conducted with just one or two compounds. For a long time, this meant it was not clear whether the findings from these studies were due to (a) flavonoids of one structure having a single MOA and flavonoids of a different structure having a different MOA, (b) all flavonoids having multiple MOAs, or (c) all flavonoids having the same single MOA, with the suggestion of multiple MOAs attributable to errors in experimental design, data interpretation etc. The possibility that different flavonoids have different MOAs [option (a)] was always the least probable of the three hypotheses because all flavonoids share broad structural similarity. Furthermore, the number of flavonoid studies has now grown to the extent that the activity of some compounds eg. quercetin [61, 73, 80] has been investigated several times and attributed to numerous mechanisms. Essentially, this leaves two viable hypotheses: either flavonoids have multiple MOAs, or flavonoids have a single MOA which remains to be convincingly identified.

Several reports suggest flavonoids have multiple MOAs [45, 73, 81] and, on face value, that is what the evidence suggests. Recent developments mean the findings of some MOA studies are not as reliable as first thought though. One such development is the discovery that epigallocatechin gallate causes aggregation of FabG enzyme purified from E. coli [75]. It is not yet clear if this effect occurs with other enzymes, or if other flavonoids induce similar enzyme aggregation. Importantly though, this finding raises doubts about conclusions drawn by studies which examined the inhibitory effect of flavonoids on purified bacterial enzymes [60-62]. Perceived inhibitory effects may have been due to enzyme aggregation rather than specific inhibition, so the MOA of the tested flavonoids may not involve enzymes at all.

A second development is the finding that flavonoids have an aggregatory effect on whole bacterial cells. This was shown to occur in cells treated with flavonol [56] and

possibly flavan-3-ol [19] compounds. Prior to this, research was conducted on the basis that decreases in CFU numbers equated to decreases in viability. This can no longer be assumed (Figure 2). Therefore, studies where supposed decreases in bacterial viability were correlated with events like potassium leakage [63], inhibition of nucleic acid synthesis [70] or inhibition of dihydrofolate reductase [69] to draw inferences about MOA may require re-examination. Another consideration is that this aggregatory effect may have directly interfered with the results of some MOA assays. If bacterial cells clump together when treated with flavonoids, this will decrease the surface area of the bacterial population. This, in turn, is likely to result in decreased oxygen consumption by the bacteria, an observation previously thought to indicate disruption to the electron transport chain [62]. Decreased surface area is also likely to result in decreased uptake of nutrients such as uridine and thymidine, an observation previously thought to indicate inhibition of nucleic acid synthesis [70].

In addition, the possibility exists that 'cause' and 'effect' have been confused in some MOA studies. If an antibacterial agent damages the cytoplasmic membrane, for example, this will disrupt the proton-motive force. This, in turn, will affect ATP generation and transport of solutes into the bacterial cell [82]. If the cell's ability to generate energy and acquire nutrients is impaired, then it follows that the bacterium's ability to synthesise DNA, peptidoglycan etc. will also be impaired. In this way, a single MOA may be misinterpreted as multiple MOAs. Similarly, if an agent inhibits a bacterial enzyme like DNA gyrase, then this may trigger programmed cell death and lysis [83]. In this way an antibacterial agent which inhibits nucleic acid synthesis could be mistaken for one that damages the cytoplasmic membrane.

3. Synergistic and antibiotic resistance modulating activity

3.1 Naturally occurring flavonoids

There have been many recent reports of flavonoids increasing the activity of antibiotics, and information on the five most potent combinations is presented (Table 2). Methods used in these studies have varied, but investigations which determined FIC index values concluded the effect is genuinely synergistic as opposed to just additive [84-86]. Of all the flavonoid

classes reported to have synergistic activity, it is the flavan-3-ols which have received most attention and been investigated in greatest depth. Galloyl flavan-3-ols such as (-)-epicatechin gallate reduce the MICs of β-lactam antibiotics against some strains of methicillin resistant *Staphylococcus aureus* (MRSA) more than 512-fold [87]. A recent development in this area is the finding that nongalloylated flavan-3-ols, which are themselves unable to sensitise strains of MRSA to β-lactams, can potentiate galloyl flavan-3-ol mediated sensitisation [88].

3.2 Semi-synthetic flavonoids

(-)-Epicatechin gallate is known to sensitise MRSA isolates to a range of β-lactam antibiotics [87, 89, 90], but the susceptibility of this flavan-3-ol to bacterial esterases had raised doubts about its clinical usefulness. Recently, a hydrolytically more stable structure was prepared by substituting the ester linkage between the C-ring and the galloyl D-ring with an amide. This semi-synthetic flavonoid possesses a similar level of synergistic activity to its parent compound, reducing oxacillin MICs against strains of MRSA 32- to 512-fold [91].

3.3 Structure-activity relationship

Structure-activity relationships for synergism and antibiotic resistance modulation are less well characterised than for direct antibacterial activity. However, there is compelling evidence to suggest that flavan-3-ols require a gallo- or gallate group to potentiate β-lactam antibiotics against MRSA [87]. There has also been a study into the ability of flavones, flavonols, flavanones and flavan-3-ols to potentiate the effect of isoniazid against different *Mycobacterium* spp. [86]. Results from this suggest that hydroxylation of the A ring at positions 5 and 7 is important. Hydroxyl groups in ring B are also thought to contribute.

3.4 Mechanism(s) of action

Several MOAs have been proposed for the synergistic and antibiotic resistance modulating activity of flavonoids. For the galloyl flavan-3-ols, it has been suggested these modulate β-lactam resistance by reducing D-alanylation of cell wall teichoic acid [resulting in

inactivation of penicillin binding protein 2a (PBP2a)] [92], or by intercalating into the cytoplasmic membrane [19] and inducing structural changes that result in delocalisation of PBP2a [93]. Mechanisms which have been discounted are inhibition of PBP2a expression, and binding of the flavan-3-ol to peptidoglycan [19].

For less studied compounds in the flavone [94, 95], isoflavone [96], flavonol [95, 97], and flavolan [66] classes, it has been suggested these increase antibiotic efficacy through β-lactamase inhibition [66, 95], efflux pump inactivation [94, 96], cytoplasmic membrane destabilisation [66, 95], disruption of PBP2a synthesis [95], and topoisomerase inhibition [97]. On the basis that some of these mechanisms do not fully account for detected activity, it has been proposed that these flavonoids exert their effect via multiple mechanisms [66, 94].

4. Attenuation of bacterial pathogenicity by flavonoids

4.1 Inhibition of the quorum sensing signal receptors TraR and RhlR

Quorum sensing is a cell-to-cell communication system bacteria use to regulate aspects of virulence including biofilm formation. Bacteria release signal molecules which bind to cell density-responsive receptors in neighbouring cells, resulting in activation of virulence genes [98]. Two recent studies suggest flavonoids disrupt the interaction between acyl-homoserine lactones (AHLs; signal molecules used by Gram negative bacteria) and their receptors. Zeng et al. report that baicalein inhibits the cytoplasmic membrane-associated [99] receptor TraR [100]. Evidence presented included docking scores from computer modelling, and bioassay data showing receptor degradation [100]. In the second study, catechin was shown to inhibit the cytoplasm-associated [101] receptor RhlR [21]. This study used an RhlR-based biosensor to show catechin affects the rhlRI system, and P. aeruginosa reporter strains to demonstrate reduced expression of associated genes [21]. In both studies, sub-MIC levels of flavonoid reduced P. aeruginosa adhesion and biofilm formation, and this was attributed to quorum sensing inhibition [21, 100]. Other studies have shown flavonoids to inhibit surface adhesion by Gram positive bacteria [19] and even latex microspheres though [102]. Inhibition of biofilm formation by flavonoids must therefore be attributable to an additional mechanism(s).

4.2 Inhibition of sortase

Sortases are enzymes found in the cytoplasmic membrane of Gram positive bacteria which catalyse the assembly of surface proteins eg. adhesins and internalins [103]. Studies with knockout mutants suggest that sortases are important for the establishment of infection, but not bacterial viability [104]. Using purified S. aureus enzymes, Kang and colleagues recently demonstrated that sub-MIC quantities of morin inhibit sortases A and B [105]. Encouragingly, there were indications of this activity at the cellular level. Whole cells of *S. aureus* treated with morin exhibited decreased binding to fibrinogen, one of the host ligands to which bacteria attach during infection [105]. This suggests that the enzyme inhibition detected by Kang et al. is specific and not due to aggregation. If this is the case, then sortase inhibition (and its knock-on effect on cell surface proteins eg. FruA and WapA [106]) may contribute to the ability of some flavonoids eg. flavan-3-ols [107] and flavolans [108] to inhibit biofilm formation by Gram positive bacteria.

4.3 Inhibition of urease

The gastric pathogen Helicobacter pylori secretes urease during infection to survive the low pH of the stomach. Recent reports suggest that compounds in the isoflavone [109] and chalcone [110] classes inhibit this enzyme. This may, to some extent, explain the in vivo activity of the flavonol quercetin against H. pylori in guinea pigs [111] and the clinical efficacy of sofalcone (a chalcone derivative) in multidrug treatment of human H. pylori infection [112]. Other flavonoid effects may be responsible for this in vivo activity too though. These include neutralisation of VacA [113] and interference with TLR-4 signalling [114]. It is also possible that some of the tested flavonoids have direct antibacterial activity against H. pylori or work synergistically with antibiotics used against this bacterium.

4.4 Inhibition of listeriolysin O

Listeriolysin O (LLO) is a virulence factor of the intracellular pathogen Listeria monocytogenes. Secretion of this protein enables bacteria to escape from phagosomes and

enter the cytosol of host cells, where they can multiply [115]. Kohda et al. recently showed that sub-MIC levels of epigallocatechin gallate inhibit growth of L. monocytogenes within macrophages [116]. This was attributed to LLO inhibition, following observation that the flavan-3-ol prevented LLO from binding to membrane lipid and inhibited LLO-induced lysis [116]. If this is the case, then LLO inhibition may also be partially responsible for the activity recently detected from β-naphthoflavone against L. monocytogenes in hepatocytes [117].

4.5 Neutralisation of bacterial toxins

Toxins play an important role in bacterial pathogenesis, sometimes causing fatal disease long after the bacteria themselves have been killed [118]. Recent studies indicate flavonoids can neutralise these virulence factors. Choi et al. demonstrated that polymerised catechin negates the effect of S. aureus α-toxin both in vitro and in vivo [20]. The isoflavone genistein inhibits exotoxin too [119]. Oh et al. showed that pretreatment of Hela cells with genistein protected them from the Vibrio vulnificus toxin RtxA1. Genistein also had a protective effect against V. vulnificus infection in vivo, as demonstrated using CD-1 mice [119].

In addition, Delehanty et al. [22] have shown that polymers of catechin and epicatechin neutralise endotoxin (lipopolysaccharide; LPS). This effect was detected by incubating LPS with the flavonoids, then demonstrating a decrease in the quantity of LPS attaching to beads coated with binding agent. Activity was detected against LPS from multiple species [22], and was retained when the flavolans were immobilised on beads [120], suggesting a possible use for these compounds in removing endotoxin from pharmaceutical preparations. Furthermore, the flavonoids blocked interaction between LPS and its receptors TLR4/MD2 and CD14, an early step in the development of septic shock. Possibly, therefore, these compounds could also be employed for in vivo treatment of Gram negative bacterial infections [22].

4.6 Inhibition of virulence factor secretion

The capacity of S. aureus to cause disease is largely attributable to its ability to secrete enzymes and toxins. Recent studies have shown that sub-MIC levels of flavonoid inhibit

release of this bacterium's virulence factors. Shah et al. found that epicatechin gallate prevents secretion of coagulase and α-toxin [121]. This was demonstrated by examining supernatants from treated S. aureus cultures for the ability to coagulate blood plasma and lyse erythrocytes. As in the Choi et al. study [20], some direct activity was detected from the flavonoid against α-toxin, but not enough to account for the results obtained [121]. The decrease in secretion is unlikely to be related to cell aggregation (and diminished surface area) either, because secretion of the enzyme protease was not inhibited.

More recent studies by Qiu et al. show that S. aureus α-toxin secretion is also reduced by licochalcone A [122]. Decreased secretion of enterotoxins was noted too [123]. Real time PCR indicated these effects were accompanied by a decrease in transcription of the agrA gene, suggesting the observed results may actually be due to inhibition of virulence factor synthesis rather than virulence factor secretion [122, 123]. In light of these PCR results, and the recent demonstration that epicatechin gallate binds predominantly to the cytoplasmic membrane [93], it seems plausible that the above flavonoids may interfere with AgrC (a cytoplasmic membrane-associated [101] quorum sensing signal receptor found in S. aureus).

5. Concluding remarks

There have been considerable advances in antibacterial flavonoid research since 2005, and it is important we take stock of these developments and move forward effectively. Recent studies have identified some flavonoids with MICs as low as 0.06 μg/mL, and others with impressive levels of synergistic activity. Whilst promising, many of these compounds will require further analysis to determine if the detected activity is selective. Improvements in the way this fundamental research is performed would facilitate the development process. The importance of various experimental parameters, in particular inoculum size [49, 50], is not always recognised. In some laboratories, the bacterial cell density being used is too low ($<10^5$ CFU/mL), and in others it is not being reported. Details of such variables and other pertinent aspects of antibacterial screening are discussed in a review by Cos et al. [11].

Recent medicinal chemistry studies have identified several structural features which

improve the antibacterial properties of flavonoids. The establishment of such relationships is essential if flavonoid activity is to be optimised. As with MIC testing however, there is room for improvement in the way these studies are executed. It is important to bear in mind that increases in antibacterial activity can be accompanied by decreases in selectivity. Future structure-activity studies should therefore perform cytotoxicity testing in parallel with antibacterial testing.

Definitive identification of the antibacterial MOA of flavonoids is key to the development of these compounds. Identification of their cellular target(s) would permit anticipation of problems relating to clinical safety and drug resistance [8], and facilitate optimisation by means of ligand-target structure activity relationship and cocrystallography [124]. It has been proposed by some groups that the antibacterial properties of flavonoids are due to interference with specific intracellular or surface enzymes. Future studies examining this possibility using cell-free assays should be wary of false positive results due to promiscuous inhibition of the purified enzymes via aggregation. MOA studies with whole bacterial cells should be similarly wary of cell aggregation and the manifold ways this could influence results. Where possible, new technologies such as genetically engineered target-specific screening [125], 'reporter' strains of bacteria [126], and gene overexpression and inactivation studies [127] should be used to facilitate identification of flavonoid MOA.

In addition to direct and synergistic antibacterial activity, there is growing evidence that flavonoids interfere with various bacterial virulence factors including enzymes, toxins, and signal receptors. This opens the possibility of flavonoids being developed as antivirulence therapies. It should be noted that there are inherent limitations with this as-yet-theoretical approach to infection treatment. For example, opsonophagocytosis would need to take place for host clearance, so patients would need to be immune-competent [103]. Nevertheless, this finding does add a new dimension to antibacterial flavonoid research. Importantly, in vitro assays currently used to assess direct and synergistic antibacterial activity of flavonoids are likely to be underestimating the in vivo potential of compounds possessing these additional activities. This point underscores recent concerns raised regarding the limitations of

pharmacology studies performed with single bioassays [128], and adds weight to wider calls [129] for a less reductionist approach to drug development.

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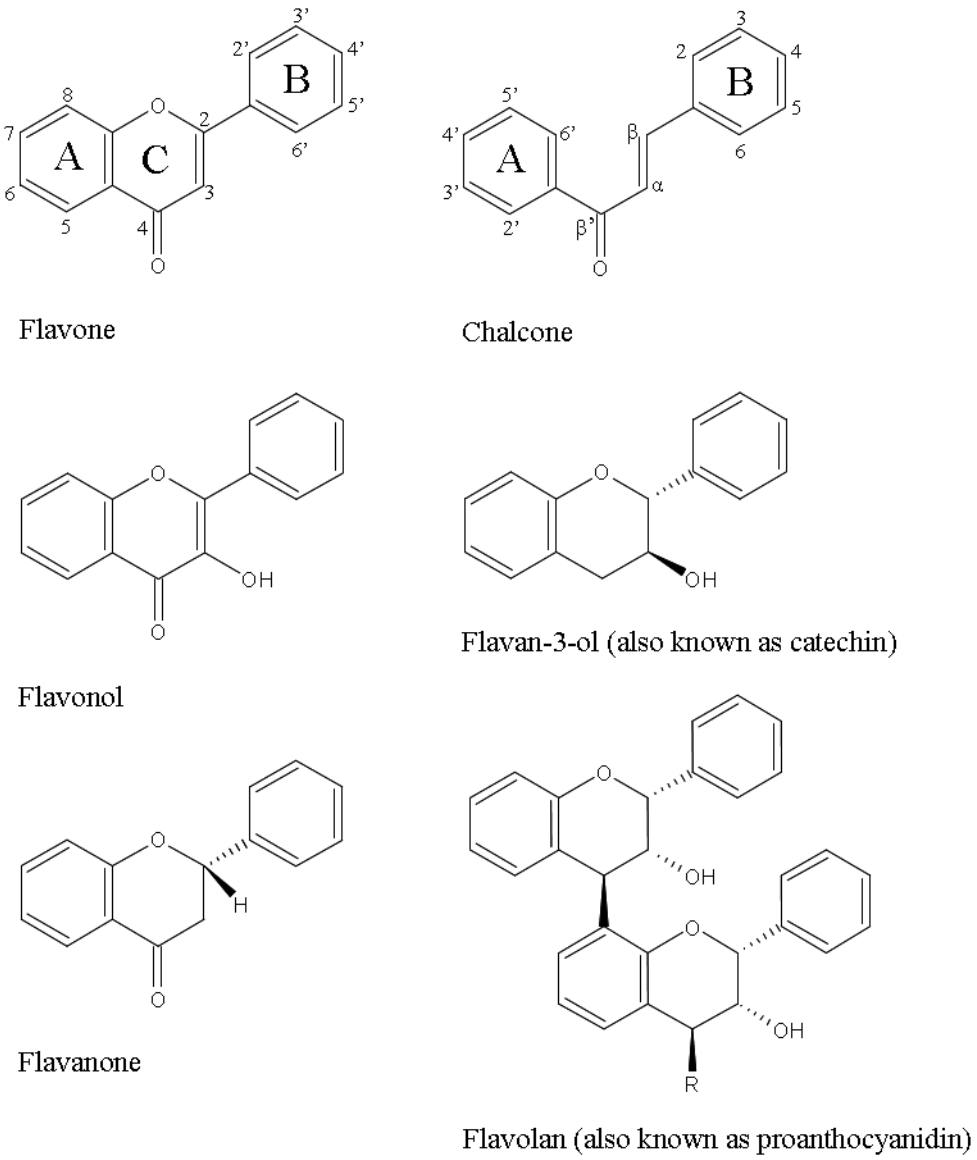

Figure 1 The skeleton structures of six of the main classes of antibacterial flavonoids: flavones, flavonols, flavanones, chalcones, flavan-3-ols (chiral centres at C-2 and C-3 mean that these compounds have 4 diastereoisomers; the 2R3S diastereoisomer is depicted above) and flavolans [occur as oligomers or polymers; R = 0, 1, or >1 flavan-3-ol unit(s)]. Note: The convention for numbering chalcones is different to that of the other five flavonoid classes shown (in chalcones, the A ring positions are primed instead of the B ring positions).

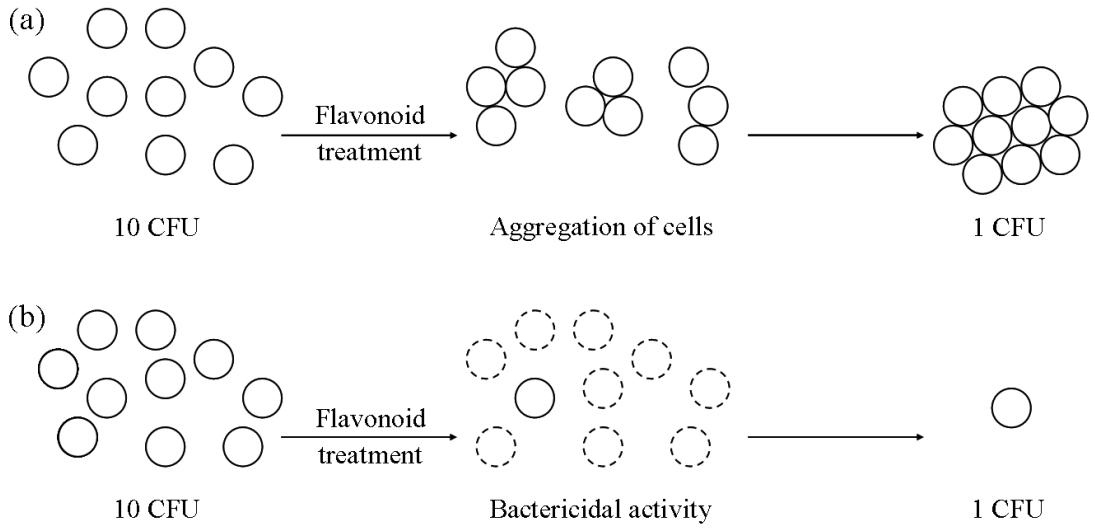

Figure 2 Two mechanisms by which flavonoids may reduce colony forming unit (CFU) numbers of bacteria in time-kill and MBC assays.

Table 1 Information on the ten most potently antibacterial natural flavonoids tested in recent years as identified by PubMed and ScienceDirect searches for studies published between January 2005 and December 2010 (studies marked with an asterisk isolated more than one highly antibacterial flavonoid).

Flavonoid	MIC assay	Cell density (CFU/mL)	MIC (μg/mL)		Reference
			Gram positive	Gram negative	
Panduratin A	BMID	5×10^5	0.06 to 2.0	NT	[10, 53]
Isobavachalcone	BMID	3.75×10^4	0.3 to 0.6	0.3 to >39.1	[130]*
Bartericin A	BMAD	NS	0.6 to 2.4	0.3 to 39.1	[52]*
Scandenone	BMID	1×10^5	0.5 to 8	2 to 32	[131]*
Kaempferol 3-O-α-L-(2'',4''-di-E-p-coumaroyl)-rhamnoside	BMID	1×10^5	0.5 to >16	>16	[45]
Sepicanin A	BMID	5×10^5	1.2	NT	[132]
Isolupalbigenin	BMAD	1×10^5	1.6 to 3.1	NT	[133]
Flavone	BMID	5×10^5	7.8 to 31.3	1.95 to 31.3	[134]
3'-O-methyldiplacol	BMID	5×10^5	2 to 4	>32	[42]
Licochalcone A	BMID	5×10^5	2 to 8	NT	[122]

BMID, broth microdilution assay; BMAD, broth macrodilution assay; NS, not stated; NT, not tested

Table 2 Information on the five most potently synergistic flavonoid-antibiotic combinations tested in recent years as identified by PubMed and ScienceDirect searches for studies published between January 2005 and December 2010 (studies marked with an asterisk identified more than one highly synergistic flavonoid).

Flavonoid	Antibiotic or antibiotic class	Test bacteria	Reduction in antibiotic MIC	Reference
Epicatechin gallate	Oxacillin	MRSA	256- to 512-fold	[92]
Quercetin	β-lactam	Penicillin resistant S. aureus	>20- to >80-fold	[95]*
Baicalein	β-lactam	MRSA	16- to 1024-fold	[94]
Myricetin	Isoniazid	Mycobacterium spp.	8- to 16-fold	[86]
ZP-CT-A	Oxacillin	MRSA	4- to 256-fold	[66]

Note: To take into account the fact that MICs can vary by a factor of 2 during testing [135], data from studies where flavonoids were used at a concentration just 2-fold lower than their MIC were excluded. All of the above studies tested for synergy using variations of the broth microdilution method.

Supplementary Table 1 Structures of flavonoids discussed within the review (compiled from individual research papers)

Compound	Substituents at carbon position											
	2	3	4	5	6	7	8	2’	3’	4’	5’	6’
Flavone:												
Baicalein	-	-	-	OH	OH	OH	-	-	-	-	-	-
Chrysin	-	-	-	OH	-	OH	-	-	-	-	-	-
Flavone	-	-	-	-	-	-	-	-	-	-	-	-
β-naphthoflavone	-	-	-	*	*	-	-	-	-	-	-	-
Isoflavone:												
Genistein	-	-	-	OH	-	OH	-	-	-	OH	-	-
Isolupalbigenin	-	-	-	OH	-	OH	R1	-	R1	OH	-	-
Scandenone	-	-	-	OH	Φ	Φ	R1	-	-	OH	-	-
Flavonol :												
Kaempferol 3-O-α-L-(2’’,4’’-di-E-p-coumaroyl)-rhamnoside	-	R2	-	OH	-	OH	-	-	-	OH	-	-
Galangin	-	OH	-	OH	-	OH	-	-	-	-	-	-
Morin	-	OH	-	OH	-	OH	-	OH	-	OH	-	-
Myricetin	-	OH	-	OH	-	OH	-	-	OH	OH	OH	-
Quercetin	-	OH	-	OH	-	OH	-	-	OH	OH	-	-
Flavanone:												
3’-O-methyldiplacol	-	OH	-	OH	R3	OH	-	-	OCH_3	OH	-	-
Hesperetin	-	-	-	OH	-	OH	-	-	OH	OCH_3	-	-
Naringenin	-	-	-	OH	-	OH	-	-	-	OH	-	-
Pinocembrin	-	-	-	OH	-	OH	-	-	-	-	-	-
Sepicanin A	-	-	-	OH	R3	OH	-	OH	-	OH	-	-
Flavan-3-ol:												
Catechin	-	OH	-	OH	-	OH	-	-	OH	OH	-	-
Epicatechin	-	OH	-	OH	-	OH	-	-	OH	OH	-	-
Epicatechin gallate	-	R4	-	OH	-	OH	-	-	OH	OH	-	-
Epigallocatechin gallate	-	R4	-	OH	-	OH	-	-	OH	OH	OH	-
Flavolan:												
ZP-CT-A	-	OH	R6	OH	-	OH	-	-	OH	OH	-	-

Compound	Substituents at carbon position												
	2	3	4	5	6	α	β	β'	2’	3’	4’	5’	6’
Chalcone:													
Bartericin A	-	R1	OH	R7	-	-	-	O	OH	-	OH	-	-
Isobavachalcone	-	-	OH	-	-	-	-	O	OH	R1	OH	-	-
Licochalcone A	OCH_3	-	OH	R8	-	-	-	O	-	-	OH	-	-
Panduratin A	-	-	-	-	-	Ψ	Ψ	O	OH	-	OCH_3	-	OH
Sofalcone	-	-	R9	-	-	-	-	O	R10	-	R9	-	-

-: no substitution; *: benzene ring attached at positions C-5 and C-6; Φ: 2,2-dimethylpyran ring attached at positions C-6 and C-7; Ψ: 1-methyl-2-(3-methyl-2-butenyl)-benzene ring attached at positions α and β; R1: prenyl group; R2: O-α-L-(2’’,4’’-di-E-p-coumaroyl)-rhamnoside group; R3: geranyl group; R4: gallate group; R5: octanoyl group; R6: 15 epicatechin units with catechin or epicatechin as the terminal unit; R7: 2-hydroxy-3-methylbut-3-enyl group; R8: 3,3-dimethyl-1-butene group; R9: prenyloxy group; R10: carboxymethoxy group