

Antibacterial potential of flavonoids with different hydroxylation patterns

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Abstract: The antibacterial activity of ten flavonoids, with variations in the hydroxylation pattern, was assayed in this study with the aim of investigating the influence of the hydroxyl groups of flavonoids on the evaluated activity. The evaluated degree of hydroxylation of the flavonoid molecules appears to have a crucial role in antibacterial effects. Kaempferol, the most active compound against *Staphylococcus aureus*, has a hydroxyl group in the B ring, a double bond between carbons 2 and 3 in conjunction with a 4-carbonyl group and hydroxyl groups at positions 3, 5 and 7, showing that the hydrophilic / lipophilic balance appears to be an important variable for antibacterial activity. With respect to the strain of *Escherichia coli*, the compounds evaluated did not inhibit completely bacterial growth; however they reduced the percentage of Gram-negative cells, under the conditions used in this work. These studies contribute to clarify the mechanisms by which these compounds act in the evaluated activity, since flavonoids can act through different mechanisms other than conventional antibiotics and could, therefore, be of use in the treatment of resistant bacteria.

Keywords: flavonoids; antibacterial activity; structure-activity relationships; degree of hydroxylation

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INTRODUCTION

Antibiotics have been proven to be powerful drugs for the control of infectious diseases and remain as one of the most significant discoveries of modern medicine. Their extensive and unrestricted use has, however, imposed a selective pressure upon bacteria, leading to the development of antimicrobial resistance. Antibiotic resistance is recognized by the World Health Organization (WHO) as the greatest threat in the treatment of infectious diseases [1].

An important potential strategy to help combat the resistance problem involves the discovery and development of new active agents capable of partly or completely suppressing bacterial resistance mechanisms [2].

Flavonoids are a family of plant-derived compounds with potentially exploitable activities, including direct antibacterial activity, synergism with antibiotics, and suppression of bacterial virulence [3]. Some of these substances produced by plants can successfully fight infections [1]. Thus, in this study, the determination of *in vitro* antibacterial activity of ten flavonoids was also assessed.

The compounds assessed were: quercetin, kaempferol, luteolin, fisetin, chrysin, galangin, flavone, 3-hydroxyflavone, 5-hydroxyflavone and 7-hydroxyflavone. These compounds have differences in the hydroxylation pattern of their molecule. These differences allow an investigation on the influence of the hydroxyl groups of flavonoids on the evaluated activity, since the propensity of the flavonoids biological activity is governed by its chemical structure.

MATERIAL AND METHODS

Chemicals and Culture Media

Quercetin, kaempferol, fisetin, luteolin, flavone, 3-hydroxyflavone, 5-hydroxyflavone, 7-hydroxyflavone, chrysin, galangin (minimum 90-98% HPLC for the flavonoids), dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Difco Bacto Agar (Difco, USA) was used as bacterial media.

Antibacterial assay

The bacteria utilized were *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922. The bacterial stock was kept in 50 vol% glycerol at a temperature of -20°C . Cultures were prepared from the stock by inoculation of Müller-Hinton broth (MHB) and incubation at 37°C for 24 h.

The minimum inhibitory concentrations (MICs) of flavonoids were determined in MHB by a broth dilution micromethod in 96-well round-bottomed polystyrene microtiter plates, following the guideline procedures of the Clinical Laboratory Standards Institute [4]. Briefly, bacterial strains were cultured overnight at 37°C in MHB, adjusted to a final density of 10^6 CFU/mL and used as the inoculum. Flavonoids were dissolved in DMSO at a concentration of 1000 $\mu\text{g}/\text{mL}$. The final solvent concentration (DMSO) did not exceed 5% of the final volume / well. Serial twofold dilutions of these stock solutions were done in a concentration range from 3.9 - 500 $\mu\text{g}/\text{mL}$. The 96-well plates were prepared by dispensing into each well 80 μL of MHB and 20 μL of the bacterial inoculum. An aliquot of 100 μL of each test solution was then pipetted into the first test well of each microliter row, and then 100 μL was transferred from well to well along the row, to make the series of concentrations. Each microliter plate had one column with the broad-spectrum antibiotic used for positive control (ciprofloxacin chloride at 35 $\mu\text{g}/\text{mL}$).

In addition to the test substance and positive control, each plate carried a sterility control of medium alone, bacterial growth control, color and sterility control of the sample and a negative control (solvent) (5% DMSO in MHB).

The lowest concentration of the compounds that showed no growth was taken as the MICs. All tests were done in triplicate wells.

After 24 h of incubation, the absorbance at 620 nm (A_{620}) was determined for each well. The MIC was determined for each strain as the lowest concentration of flavonoid that completely inhibits measurable growth ($A_{620} = 0$) [5].

The determination of bacterial growth for each microorganism was calculated as follows:

$$\text{Bacterial growth (\%)} = (\text{Abs A} - \text{Abs B} / \text{Abs C}) \times 100$$

where *Abs A* represents the absorbance of the well with a flavonoid concentration; *Abs B* the absorbance of the well with a flavonoid concentration without the addition of the inoculum (obtained from the actual absorbance of bacteria, without interference from the test substances); *Abs C* the absorbance of the control well.

RESULTS AND DISCUSSION

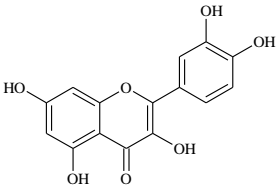
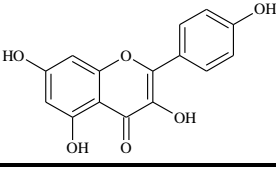
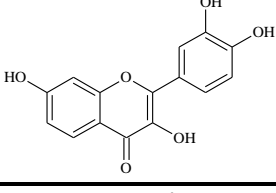
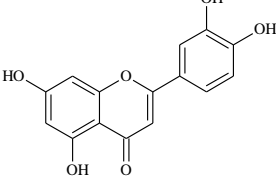
The antibacterial activity of flavonoids (quercetin, kaempferol, fisetin, luteolin, flavone, 3-hydroxyflavone, 5-hydroxyflavone, 7-

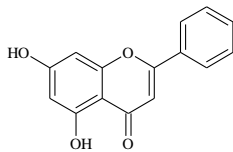
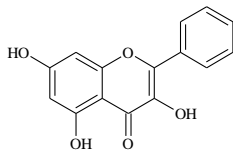
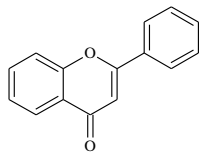
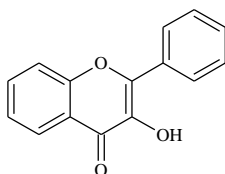
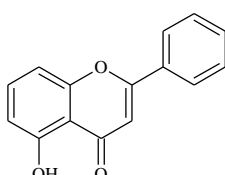
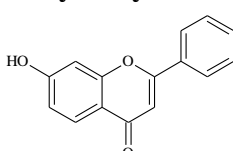
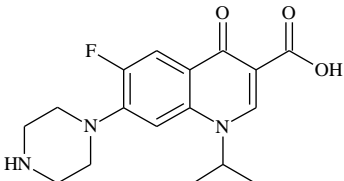
hydroxyflavone, chrysin and galangin) was determined by the percentage of the bacterial growth obtained when put against *S. aureus* and *E. coli*. From these results, the MIC for each microorganism was determined (Table 1).

The flavonoids evaluated were effective against *S. aureus* and *E. coli*, to varying degrees (Table 1), and the *S. aureus* strain was more sensitive to different flavonoids, being kaempferol the most active compound, with MIC of 62.5 µg/ mL (Table 1).

With respect to *E. coli*, the compounds evaluated did not completely inhibit bacterial growth, they only reduced the percentage of bacterial cells, under the conditions used in this study (Table 1).

Table 1 Minimum inhibitory concentration (MIC) of flavonoids (quercetin, kaempferol, luteolin, fisetin, chrysin, galangin, flavone, 3- hydroxyflavone, 5- hydroxyflavone and 7- hydroxyflavone) by dilution in microplates

Treatments	MIC (µg/ mL)	
	<i>S. aureus</i> (Gram-positive)	<i>E. coli</i> (Gram-negative)
<p>Quercetin</p> 	125	> 500
<p>Kaempferol</p> 	62,5	> 500
<p>Fisetin</p> 	125	> 500
<p>Luteolin</p> 	125	> 500

Chrysin		
	250	> 500
Galangin		
	125	> 500
Flavone		
	500	> 500
3- hydroxyflavone		
	500	> 500
5- hydroxyflavone		
	500	> 500
7- hydroxyflavone		
	500	> 500
C+: ciprofloxacin chloride		
	$2,14 \times 10^{-3}$	$3,34 \times 10^{-5}$

The classification of most bacteria, developed by the Danish physician Hans Christian Gram in 1884, is based on the fact that Gram-positive bacteria retains a crystal violet-iodine complex when put through

decolorization with alcohol or acetone, while Gram-negative bacteria do not. This behavior is due, among other aspects, because the Gram-positive bacteria possess a thick peptidoglycan layer in their cell wall

whereas Gram-negative bacteria possess a thin peptidoglycan layer and a lipopolysaccharide outer membrane. The Gram stain is important for the differentiation of Gram-positive and Gram-negative bacteria since they have different susceptibilities to a variety of antibiotics [6].

At present, the resistance to antimicrobial-agents is recognized as a major global public health problem [7]. Moreover, the use of antibiotics is often accompanied by side effects and thus the search for compounds that are active against antibiotic-resistant strains of bacteria is continuing among the flavonoids, compounds which are non-toxic or have low toxicity [6].

However, considering the varied structure-activity relationships of different series of compounds, it cannot be inferred that the biological behavior of a drug is determined by the influence of a single parameter or variable. Furthermore, in most cases, the presence or introduction of various functional groups in a compound does not permit an accurate explanation of the kind and intensity of the biological activity [8]. However, the study of the structure-activity relationship performed in this work allows us to make some general observations of the structure and antibacterial activity of the analyzed flavonoids.

In the present study, the *S. aureus* strain was more sensitive to different flavonoids. It is important to note that *S. aureus* is the cause of a number of diseases that affect humans and animals. In spite of advances in medical science, epidemiology and the discovery of new antibiotics, *S. aureus* infections still present considerable morbidity and mortality rates [8].

Gram-positive and Gram-negative bacterial species might present different sensitivity values towards phenolic compounds because of the differences in their membrane structure and the associated cell wall differences [7].

The degree of hydroxylation of flavonoid molecules evaluated in the present study appears to have a crucial role in the observed antibacterial effects. Kaempferol, the most active compound, has a hydroxyl group in the B ring, a double bond between carbons 2 and 3 in conjunction with a 4-carbonyl group and hydroxyl groups at positions 3, 5 and 7, showing that the hydrophilic / lipophilic balance

seems to be important for antibacterial activity. According to Miceli et al. [7], for a phenolic compound to function effectively as an antimicrobial it should be placed at a lipid–water interface, having to be, therefore, partially hydrophobic. The partial hydrophobicity of some phenolic compounds allows them to act efficiently at the membrane–interface of the Gram-positive bacteria. This can severely impair the plasticity of the membrane and therefore destabilize the cell by weakening the membrane integrity, which may result in the disruption of the bacterial membrane and also of critical transport processes.

Moreover, the flavonoids evaluated in this study have two aromatic rings bound by an α,β -unsaturated carbonyl group. Carbonyl compounds usually exert their action through the direct interaction with enzymes, usually through the hydrogen bonds [8], forming complexes with a cell wall or by interrupting the synthesis of bacterial envelopes [9].

Recently, in a review described by Cushnie and Lamb [3], researchers have suggested that the antibacterial activity of flavonoids may be attributable to cytoplasmic membrane damage (caused by perforation and/or a reduction in membrane fluidity), inhibition of nucleic acid synthesis (caused by topoisomerase inhibition) and to the inhibition of energy metabolisms (caused by NADH-cytochrome c reductase inhibition). Evidence has also been presented for two new mechanisms, which are: 1) inhibition of cell wall synthesis and 2) inhibition of cell membrane synthesis, caused by the inhibition of related enzymes.

Since flavonoids have an aggregation effect in bacterial cells [3], the antibacterial activity detected on Gram-positive bacteria may be due to the formation of pseudomulticellular aggregates. Probably, the flavonoids are not killing bacterial cells but merely inducing the formation of bacterial aggregates and thereby reducing the number of CFUs in viable counts [2, 7]. However, this aggregation effect decreases the surface area, which may result in a decrease on oxygen consumption by the bacteria, an observation previously thought to indicate the disruption on the electron transport chain, and in decreasing the uptake of nutrients such as uridine and

thymidine, an observation previously thought to indicate the inhibition of nucleic acid synthesis [3]. Thus, the apparent ability of flavonoids to generate cell aggregates deserves further research, since in this study, the observed reduction in the growth of *S. aureus* markedly in the different concentrations of flavonoids tested.

With respect to *E. coli*, Gram-negative bacteria, which have an external lipo-polysaccharide layer and additional minor membrane components, besides an intact plasma membrane around its cell, potentially have more buffering capacity and hydrophobicity and therefore can create an unfavorable environment for simple phenolics to exert their hyperacidification effect [7]. Moreover, according to Hendra et al. [10], Gram-negative microorganisms are typically more resistant to antimicrobial agents than Gram-positive bacteria. This has for a long time been explained by the presence of an outer-membrane permeability barrier in the Gram-negative bacteria, which limits the access of the antimicrobial agents into the bacterial cells.

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CONCLUSIONS

Considering the enormous scientific effort put in elaborating new antibacterial compounds, flavonoids, due to their extremely large amount of biological properties, are placed among the most attractive plant derivatives that enrich the current therapy options [9]. This study allows us to conclude that, besides the importance of assessing the structural change of antimicrobial flavonoids, the identification of the mechanisms of action in the flavonoids is key to the pharmacological development of these compounds. Identification of their cellular target(s) would permit anticipation of problems relating to clinical safety and drug resistance.

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