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Evaluation of antirotavirus activity of flavonoids

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Abstract
Flavonoids are dietary components and the most ubiquitous phenolic compounds found in nature, showing a range of pharmacological activities including antiviral action. This study describes the antiviral screening of 60 different flavones and flavonols against human rotavirus (Wa-1 strain) as well as their cytotoxicity in MA104 cells. Cytotoxicity was investigated by cell morphology assessment and antirotavirus activity by cytopathic effect inhibition. Results were expressed as CC50 and IC50, respectively, in order to calculate the selectivity index (SI = CC50/IC50) of each compound. Structure–activity relationships (SAR) were proposed based on antirotavirus activity.

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1. Introduction

Flavonoids are polyphenolic compounds that can be found as dietary components such as food products, beverages and herbal medicines with different health benefits shown in a large number of studies [1]. Several pharmacological activities have been described for flavonoids such as antitumor [2], antibacterial [2–4], antifungal [3,4], antiallergic, estrogenic, anti-inflammatory [2], prevention of cardiovascular diseases [5] and antiviral [3,4,6]. In addition, they are well-known antioxidants and metal ion-chelators [4,7].

Natural products have shown to be an important source of useful compounds in antiviral chemotherapy [8,9]. Compounds with promising activity can be used directly as drugs or as leads for the synthesis of new drugs. During the last few years, efforts have been made to increase the number of compounds with antiviral activity. Clinical use of the currently available antiviral drugs has use restrictions as narrow spectrum of activity, limited therapeutic usefulness and variable degrees of toxicity [10]. Furthermore, the therapeutic potency of most of the antiviral agents encountered so far is counterbalanced by their severe side effects in humans and, in some cases; the efficacy of these drugs is limited by increase of viral resistance [11]. Therefore, the search for antiviral compounds with high efficacy, low toxicity and minor side effects must continue to improve drug therapy.

Flavonoids have been investigated for antiviral activity, for instance, against human cytomegalovirus [12,13], herpes simplex virus types 1 and 2 [14–17], influenza virus, respiratory syncytial virus, adenovirus, varicella zoster virus [12], poliovirus [14,18,19], rhinovirus [19], sindbis virus [20], coronavirus, parainfluenzavirus, coxsackievirus B [8], HIV [21] and rotavirus [6].

Among enteric viruses, rotaviruses are the major cause of severe diarrhea and it is believed that they would account for about 30 to 80% of pediatric hospitalizations for acute gastroenteritis. Rotaviruses affect nearly the population
Flavones identified as 8, 10, 12, 20, 21, 28, 29, 31, 32 and 34 were purchased from Sigma and others were synthesized [26]. Flavonols identified as 49, 54, 55 and 57 were synthesized [26] and others were purchased from Sigma.

Compounds were assayed for their toxicity to MA104 cells by microscopical morphology evaluation [27,28] and for their antiviral activity (Wa-1 strain) by cytopathogenicity inhibition [29]. They were dissolved in 1% of dimethyl sulfoxide (DMSO, Merck, Darmstadt, Germany) diluted in 199 medium (Sigma Chemical Co., St. Louis, MO, USA) and filtered through 0.22 μm membranes (Millipore, Bedford, MA, USA). All stock solutions were stored at 4 °C protected from light until used. The used cell line was MA104 cells (Biological Science Institute, University of São Paulo, Brazil), grown in 199 Medium (Sigma Chemical Co., St. Louis, MO, USA) and supplemented with 10% fetal bovine serum (Gibco BRL, New York, USA), penicillin G (100 U/ml), streptomycin (100 µg/ml) and amphotericin B (0.025 µg/ml) (Gibco BRL, New York, USA). The cell cultures were maintained at 37 °C in a humidified 5% CO2 atmosphere. The human rotavirus Wa-1 (ATCC: VR2018) was used and it was propagated in MA104 cells in the presence of trypsin (Sigma, 5 µg/ml). Stock viruses were prepared as previously described by Barardi et al. [27] and the supernatant fluids were harvested, titrated and stored at −80 °C until used. Virus titers were estimated from cytopathogenicity by the limit-dilution method and expressed as 50% tissue culture infectious dose per ml (TCID50/ml).

The CC50 (cytotoxic concentration for 50% of cells) and IC50 (inhibitory concentration for 50% of infected cells) values were estimated from concentration–effect curves after linear regression analysis, and represent the mean values of three independent experiments.

The selectivity index (SI = CC50/IC50) was calculated for each tested flavonoid. According to Sidwell [30], when SI ≥ 4 promising antiviral activity must be considered.

### 3. Results

The selectivity indexes (SI) were calculated from the cytotoxic and inhibitor concentrations (Tables 1 and 2) and the values obtained were considered to propose a structure–activity relationship. In tests with non-cytotoxic concentrations, the compounds showed different degrees of antiviral activity, excepting the flavonols 1, 3, 5, 7, 8, 14–16 and 28–30, and the flavones 35, 44–46, 48 and 55. The majority of tested flavonoids and flavonones demonstrated SI ≥ 4, indicating a favorable activity against human rotavirus (Wa-1 strain).

### 4. Discussion

In the present study, the cytotoxicity and antiviral action of 60 flavonoids were evaluated on MA104 cells, which have susceptibility to human rotavirus. Taking into account these results, the selectivity index for each compound was calculated and these values were considered to describe SAR. The tested flavonoids were divided in groups according to their similar structural characteristics and some SAR were proposed considering the conformational effects exerted by the substituents.
Antiviral activity was provided for compounds with one substituent ethoxyl in A ring (1, 3 and 5) did not grant antiviral activity, but not when it was in meta position (2, 4 and 6). Considering just one methoxyl in B ring (7 and 8), antiviral activity was not observed, in contrast to the existence of two (9 and 10) or three (11 and 12) methoxyl radicals in B ring. A considerable antitrovirus action was detected in the presence of benzoxyl radical in the same ring (13, 19 and 20). In view of ethoxyl radicals in B ring (14–18), the promising antiviral activity was linked to the presence of two of this radical in meta position (18). Compounds with butoxyl radicals in B ring (22–26) also showed activity, with higher activity for orto/meta positions of this radical (25).

Antiviral activity was provided for compounds with one butoxyl group in A ring (38), one ethyl radical in B ring (21) and one benzoxyl radical simultaneously in A and B rings (39).

When A ring has one methoxyl group (29–34), the antiviral activity was detected only if the B ring also has a methoxyl at the R4’ position (31–34).

With one substituent ethoxyl in A ring (35–37), an antiviral activity was detected only in the presence of one ethoxyl radical in B ring at the R3’ or R4’ position (36 and 37).

In view of the A and B rings containing two hydroxyl groups each one (28), the antiviral activity was not observed. The same result was observed when the flavonol comprised only one hydroxyl in B ring and no substituent in A ring (3).

For the flavonols, the presence of two or more methoxyl and ethoxyl radicals, one or two butoxyl radicals, and one benzoxyl radical showed antiviral activity while compounds containing only one methoxyl or ethoxyl radical did not reduce the rotavirus infection.

In view of the role that sialic acid has in infection by rotavirus, Fazli et al. [31] evaluated the antiviral effect of flavonoids, the presence of two or more methoxyl and ethoxyl radicals, one or two butoxyl radicals, and one benzoxyl radical showed antiviral activity while compounds containing only one methoxyl or ethoxyl radical did not reduce the rotavirus infection.

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Concerning the tested flavonoids, an antiviral action was shown when the B and C rings did not have methoxyl substituents (40–42) and had one hydroxyl and hydroxyl radicals in A ring.

In the case of flavonoids without methoxyl substituents in A and B rings (43–47), the presence of O-rhamnose and O-rhamno-glucose at R3 position (44 and 45, respectively) or the absence of radical (46) in C ring did not provide antiviral activity. Bae et al. [32] tested the in vitro inhibitory action of different rhamnoglycosides on rotavirus infection in that dosmin and hesperidin had a higher activity (IC₅₀ = 10 μM); however, the presence of methoxyl groups in A ring is relevant to antiviral activity when these compounds were compared.

Considering the same A and C rings (49 and 55), the absence of methoxyl in R5 position of A ring (49) could explain the antiviral activity when these compounds were compared.

For the tested flavonoids, the SAR analysis suggests that the presence of methoxyl group in A ring is relevant to antiviral action, nevertheless, the characteristics about the other rings also need to be considered to each compound.

Table 2
Substitution patterns of tested flavonoids and their cytotoxic (CC₅₀) and viral inhibitory (EC₅₀) concentrations, as well as their selective indices (SI = CC₅₀/IC₅₀).

| Number | R3′ | R4′ | R5′ | R3 | R5 | R6 | R7 | MW | CC₅₀ | IC₅₀ | SI |
|--------|-----|-----|-----|----|----|----|----|----|------|------|----|
| 40     | H   | OH  | H   | H  | OH | OCH₃| OCH₃| 344.0| 22.67 | 7.56 | 3.0 |
| 41     | H   | OH  | H   | H  | OH | OCH₃| OCH₃| 316.0| 49.37 | 12.34| 4.0 |
| 42     | H   | OH  | H   | H  | OH | OCH₃| OCH₃| 284.6| 54.81 | 18.27| 3.0 |
| 43     | OH  | OH  | H   | OCH₃| OH | H   | OH  | 316.26| 49.33 | 12.33| 4.0 |
| 44     | OH  | OH  | H   | O-RHAMNOSE| OH | H   | OH  | 448.38| 69.58 | NA  | -   |
| 45     | OH  | OH  | H   | O-RHAMNO-GLUCOSE| OH | H   | OH  | 610.51| 51.10 | NA  | -   |
| 46     | OH  | OH  | H   | H  | OH | H   | OH  | 286.24| 27.25 | NA  | -   |
| 47     | OH  | OH  | H   | O-SO₃| OH | H   | OH  | 381.3| 40.91 | 10.23| 4.0 |
| 48     | H   | OCH₃| H   | H  | H  | OCH₃| OCH₃| 357.38| 21.83 | NA  | -   |
| 49     | H   | OCH₃| H   | H  | OCH₃| OCH₃| OCH₃| 372.37| 167.84| 28.20| 6.0 |
| 50     | H   | OCH₃| H   | H  | OH | H   | OCH₃| 298.0| 52.35 | 13.09| 4.0 |
| 51     | H   | OCH₃| H   | H  | OH | OCH₃| OCH₃| 358.0| 43.58 | 7.26 | 6.0 |
| 52     | H   | OCH₃| H   | H  | OH | OCH₃| OCH₃| 328.0| 23.78 | 15.55| 1.5 |
| 53     | H   | OCH₃| OCH₃| H  | OH | OCH₃| OCH₃| 342.4| 45.58 | 17.53| 2.6 |
| 54     | H   | OCH₃| OCH₃| H  | OCH₃| OCH₃| OCH₃| 372.37| 41.89 | 6.98 | 6.0 |
| 55     | H   | OCH₃| OCH₃| H  | OCH₃| OCH₃| OCH₃| 402.4| 38.77 | NA  | -   |
| 56     | H   | OCH₃| OCH₃| OCH₃| OH | H   | OH  | 342.4| 45.58 | 7.60 | 1.5 |
| 57     | H   | OCH₃| OCH₃| OCH₃| OCH₃| OH | OCH₃| 432.42| 36.08| 18.04| 2.0 |
| 58     | OCH₃| H   | H   | OH | OCH₃| OCH₃| OCH₃| 376.34| 124.62| 31.09| 4.0 |
| 59     | OCH₃| OH  | H   | OCH₃| OH | H   | OCH₃| 374.34| 41.67 | 6.95 | 6.0 |
| 60     | OH  | OCH₃| H   | OCH₃| OH | OCH₃| OCH₃| 404.37| 77.16 | 77.16| 1.0 |

MW = molecular weight; NA = no antiviral activity; CC₅₀ (μM); MA104 cells; IC₅₀ (μM); human rotavirus. Substances 49, 54, 55 and 57 were synthesized [26] and others were purchased from Sigma.

L.A. Savi et al. / Fitoterapia 81 (2010) 1142–1146

1145

... flavonoids can degrade to variable extents in the digestive tract.
tract. A screening of bioactive compounds, synthetic or isolated from natural sources, and the evaluation of their SAR, allied to technological advances, are required to develop more effective and safer new drugs than those already available.

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