Original Article

Lack of Activity of Rutin Isolated from *Tontelea micrantha* Leaves against Vero and BHK, Fungi, Bacteria and *Mayaro virus* and its *in silico* Activity

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Abstract

Objectives: This work aimed to evaluate the antibacterial, antifungal, and anti-*Mayaro virus* (MAYV) activity of rutin, a flavonoid isolated from the leaves of *Tontelea micrantha* (Celastraceae). **Materials and Methods:** The antibacterial and antifungal activities were evaluated by the broth microdilution method through the determination of the minimum inhibitory concentration. The anti-MAYV activity was determined by the rutin concentration required to protect 50% of the cells after viral infection. The indirect 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide colorimetric assay was used to determine the cytotoxic concentration of rutin to 50% of Vero and BHK cells. The antimicrobial activity spectrum of rutin was predicted using the online software prediction of activity spectra for substances (PASS). **Results:** Although the PASS prediction had shown that a higher probability of rutin acts as an antifungal agent, the *in vitro* assays showed no antimicrobial activity. Regarding the cytotoxicity assay, rutin was not toxic to Vero and BHK cells. **Conclusions:** Many biological activities have been described for flavonoids, but the flavone rutin did not show antimicrobial activity *in vitro*. The results obtained in this work suggest that rutin is not a promising antimicrobial agent.

Keywords: Antibacterial activity, antifungal activity, anti-*Mayaro virus* activity, rutin

Introduction

Public concern about infectious diseases has increased considerably in the past decades. It was believed that the discovery and development of antimicrobial agents had controlled these infections, but nowadays, they account for 25% of the world's deaths.[1] In addition, the development of new antimicrobials is a slow and expensive process, and such diseases are highly lethal.[2] For example, infectious diarrhea is the second leading cause of death for children under 5-year-old and is responsible for 760,000 deaths annually.[1]

Moreover, the increase in the bacterial and fungal resistance acquisition due to the exposure to subinhibitory concentrations of antimicrobials has made the development of new agents a global effort.[3] In addition to bacterial and fungal infections, viral infections have made the scenario even more adverse because there are no specific vaccines and medicines to treat the majority of these infections. An example is the *Mayaro virus* (MAYV), whose disease is treated with non-specific anti-inflammatories and analgesics.[4]

Therefore, the search for new bioactive agents, mainly of natural sources, has been highlighted. Among them, flavonoids

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have been increasingly explored.[5] Flavonoids are one of the most common secondary metabolites of plants.[6] For this class of phenolic compounds has been described antioxidant, antibacterial, antifungal, antitumoral, anti-inflammatory, anti-allergic, and antiviral activities.[7]

Rutin (3',4',5,7-tetrahydroxy-flavone-3-rutinoside), also named rutoside, sophorin, and Vitamin P, is a glycoside combining the flavonol quercetin and the disaccharide rutinose (α-L-rhamnopyran osyl-(1→6)-β-D-gluc opyranose) [Figure 1]. This flavonoid is found in a wide variety of plants, including *Tontelea micrantha*, a species of the Celastraceae family. Rutin has several pharmacological properties, such as antioxidant, cytoprotectant, vasoprotective, and neuroprotective. This flavonoid is also found in many daily consumption products, such as buckwheat, green tea, vegetables, and fruits such as apples and lemons.[8] Thus, the present study aimed to evaluate the activity of rutin isolated from *T. micrantha* leaves, against four *Candida* species, fourteen bacteria, and MAYV. Furthermore, in parallel, the antimicrobial activity of rutin was predicted using the software prediction of activity spectra for substances (PASS).

Materials and Methods

Plant material and rutin isolation

Tontelea micrantha was collected in Montes Claros, Minas Gerais, Brazil. The plant was identified by Dr. Maria Olívia Mercadante-Simões, and a voucher specimen (Number BHCB 214.463) was deposited at the Herbarium of the Department of Botany of the University of Minas Gerais. The leaves of *T. micrantha* were dried at room temperature and then were powdered and submitted to extractions with different organic solvents: hexane, ethyl acetate, and methanol. The leaf methanolic extract was fractionated on a chromatographic column using polyamide as the stationary phase. The column was eluted in reverse polarity phase, with water, methanol, ethyl acetate, and chloroform, pure or in mixtures, furnishing 52 fractions of 300 mL each. The fractions were distilled to reduce the volume. The fractions were grouped according to the profile presented in plates of silica gel G-60, revealed with NP-PEG (methanol solution with 1% of 2-aminoethyl diphenylborinate (p/v) +5% poly (ethylene glycol) ethanol solution) reagent and ultraviolet light. The fractions 11–21 eluted with H_2O/MeOH 7:3 showed a yellowish solid during rotary volume reduction. The material was filtered and the solid was separated. Then, the solid was subjected to Sephadex LH-20 column chromatography and was eluted with methanol. From fractions 5–7, the flavonoid rutin was isolated (154 mg), and its structure was confirmed from its ^{1}H and ^{13}C NMR spectral data.

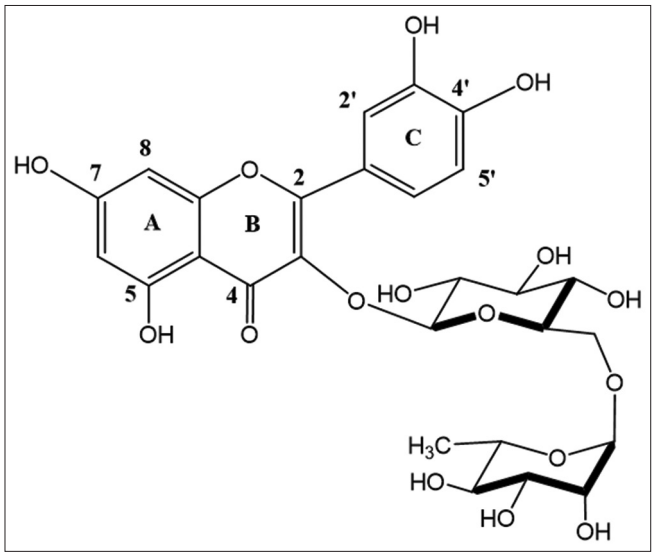

Figure 1: Chemical structure of rutin (3',4',5,7-tetrahydroxy -flavone-3-rutinoside)

Microorganisms

The microorganisms employed in the assays were obtained from the American Type Culture Collection (ATCC) and cordially provided by Fundação Oswaldo Cruz (FIOCRUZ, Rio de Janeiro, Brazil). The antifungal assay was performed using four *Candida* species (*Candida albicans* ATCC 18804, *C. albicans* ATCC 10231, *Candida krusei* ATCC 34135, *Candida glabrata* ATCC 2001, and *Candida tropicalis* ATCC 28707). For the antibacterial assay, nine Gram-negative bacteria such as *Enterobacter cloacae* ATCC 23355, *Escherichia coli* ATCC 25922, *Acinetobacter baumannii* ATCC 19606, *Klebsiella pneumoniae* ATCC 4352, *Klebsiella oxytoca* ATCC 0182, *Shigella flexneri* ATCC 12022, *Pseudomonas aeruginosa* ATCC 25619, *Salmonella enterica serovar typhimurium* ATCC 14028, and *Salmonella choleraesius* ATCC 10708 and five Gram-positive bacteria *Staphylococcus aureus* ATCC 29213, *Staphylococcus saprophyticus* ATCC 15305, *Streptococcus agalactiae* ATCC 13813, *Enterococcus faecalis* ATCC 19433, and *Bacillus cereus* ATCC 11778 were used.

Cell lineage and virus

The cytotoxicity assay was performed using Vero cells ATCC CCL-81 and BHK-21 ATCC CCL-10 cells kindly provided by FIOCRUZ, Brazil. Vero cells are continuous lineage fibroblasts from African Green Monkey kidney (*Cercopithecus aethiops*), and BHK-21 cells are fibroblasts from baby hamster kidney. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (HiMedia, India) supplemented with 5% of fetal bovine serum (Cultilab, Brazil), 100.0 µg/mL of streptomycin (Sigma-Aldrich, USA), 100 U/mL of penicillin (Sigma-Aldrich, USA), and 2.5 µg/mL amphotericin B (Sigma-Aldrich, USA). MAYV (BeAr20290 strain (GenBank accession no. KY618127) was provided by PhD. Maurício Lacerda Nogueira of the Faculty of Medicine of São José do Rio Preto (FAMERP), Brazil.

Antibacterial assay

The minimum inhibitory concentration (MIC) was determined using the broth microdilution method, as described by the Clinical and Laboratory Standards Institute (CLSI) document M07-A9, with minor modifications.[9] Briefly, rutin was diluted in Mueller-Hinton broth (HiMedia®) at concentrations ranging

from 0.24 to 500 µg/mL. Isolated bacterial colonies were suspended in saline solution 0.85%, and the turbidity of the resulting solution was adjusted according to the McFarland 0.5 scale ($OD_{625\ nm}$ = 10^8 CFU/mL). The solution was further diluted to a cell density of 10^6 CFU/mL. The microplates were incubated for 24 h at 37°C. MIC was considered the lowest concentration of compound capable of inhibiting bacterial growth. The assay was performed in triplicate in three independent experiments. Amoxicillin was used as positive control and DMSO as a negative control.

Antifungal assay

The antifungal assay was performed using the broth microdilution method according to the document M27-A3 of CLSI, with slight modifications.[10] Rutin was diluted in Sabouraud Dextrose Broth (Acumedia®) in a concentration range of 0.24–500 µg/mL. *Candida*-isolated colonies were dissolved in saline solution 0.85%, and the turbidity was adjusted according to the 0.5 McFarland scale ($OD_{530\ nm}$ = 10^6 CFU/mL). Then, the preinoculum was diluted until a cell density of 10^3 CFU/mL. The microplates were incubated for 48 h at 37°C, and the MIC was considered the lowest concentration of rutin required to inhibit the growth of the yeast. The assay was performed in triplicate in three independent experiments. Nystatin was used as positive control and DMSO as a negative control.

Minimum bactericidal concentration and minimum fungicidal concentration

After the MIC was determined, 10.0 µL of the solution of the MIC microplate was plated on Petri dishes containing Mueller-Hinton agar (Kasv, India) or Sabouraud Dextrose agar (Acumedia, USA). The dishes were incubated for 24 h (for bacteria) or 48 h (for yeast) at 37°C. Minimum bactericidal concentration/minimum fungicidal concentration was considered the lowest concentration of compound capable of inhibiting 99% of microbial growth relative to the untreated cells.

Cytotoxicity assay

The indirect 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay[11] was used to determine the cytotoxic concentration of rutin to 50% (CC_{50}) of the cells. Vero and BHK cells (5×10^4 cells/well) were cultured in 96-well microplates for 24 h at 37°C, and then, they were treated with different concentrations of the flavonoid (1–1000 µg/mL). The microplates were incubated for 48 h at 37°C in a humidified atmosphere containing 5% of CO_2. The medium of the wells was replaced by 25 µL of MTT (Sigma-Aldrich, USA) and the microplates were incubated for 2 h at 37°C in 5% of CO_2. Then, 100 µL/well of DMSO was added to dilute the formazan crystals formed by the MTT metabolization. The optical density of the wells was determined with a spectrophotometer at 492 nm, and CC_{50} represents the concentration of rutin needed to reduce 50% of the cell viability. The assay was performed in triplicate in three independent experiments.

Antiviral assay

Vero cells (ATCC CCL-81) (5×10^4 cells/well) were cultured in 96-well microplates for 24 h at 37°C. Then, the microplate medium was replaced by DMEM medium containing MAYV at a multiplicity of infection of 0.1 virus per cell. Rutin was serially diluted in DMEM and added at concentrations ranging from 1 to 500 µg/mL. The microplates were incubated for 48 h, and cell viability was measured by the MTT method, similar to the cytotoxicity assay. The effective antiviral concentration (EC_{50}) refers to the concentration of the compound that protected 50% of treated cells. Ribavirin was used as positive control, and the experiment was performed in triplicate in three independent experiments.

In silico prediction of antimicrobial activity using prediction of activity spectra for substances

The software online PASS was used to predict the antimicrobial activity of rutin. This online tool provides predictions of many biological activities of organic compounds comparing them with a known chemical molecule.[12] The prediction of antibacterial, antifungal, and antiviral activity of rutin was performed through its molecular structural analysis. PASS estimates the probability of the compound to be active (Pa) or inactive (Pi). Only values of Pa greater than Pi were considered.

Statistical analysis

The results of the cytotoxic and antiviral assays were analyzed by linear regression using the Statistical Software GraphPad Prism v. 7 (GraphPad Software, Inc. La Jolla, California, USA).

Results

MIC values showed in Table 1 suggest that the flavonoid rutin is inactive against Gram-positive and Gram-negative bacteria (MIC >500 µg/mL). In addition, *Candida* species were also insensitive to the effect of rutin (MIC >500 µg/mL) when compared with ketoconazole, which was highly active. On the other hand, amoxicillin showed antibacterial activity against all bacterial strains tested [Table 1]. This broad-spectrum β-lactam exhibited bacteriostatic and bactericidal effect, which means that this penicillin derivative was able to kill these bacterial pathogens under the experimental conditions employed. The same results were obtained for the antifungal ketoconazole [Table 1], which was highly active against the *Candida* species tested (MIC ranging from 2 to 8 µg/mL).

Regarding the antiviral assay, rutin showed no activity against MAYV (EC_{50} >500 µg/mL) [Figure 2]. The treatment with rutin [Figure 2d] and DMSO [Figure 2c] did not inhibit the viral infection process when compared to the viral control [Figure 2b]. In addition, ribavirin [Figure 2e], the positive control used, was the only compound that reduced the cytopathic effect of MAYV when compared to the cellular control [Figure 2a]. Although rutin did not present antibacterial, antifungal, and anti-MAYV effects, it exhibited low cytotoxicity. The CC_{50} value of rutin was higher than the highest concentration tested (CC_{50} >1000 µg/mL) against

Table 1: Minimum inhibitory concentration and minimum bactericidal or fungicidal concentrations of rutin against pathogenic bacteria and *Candida* species

Microorganisms tested	Rutin		Standard drug			
			Amoxicillin		Nystatin	
	MIC	MBC/MFC	MIC	MBC	MIC	MFC
Gram-negative bacteria						
Enterobacter cloacae ATCC 23355	>500	-	500	500	-	-
Escherichia coli ATCC 25922	>500	-	1.95	3.9	-	-
Acinetobacter baumannii ATCC 19606	>500	-	125	250	-	-
Klebsiella pneumoniae ATCC 4352	>500	-	0.24	15.62	-	-
Klebsiella oxytoca ATCC 0182	>500	-	62.5	62.5	-	-
Shigella flexneri ATCC 12022	>500	-	0.24	0.98	-	-
Pseudomonas aeruginosa ATCC 25619	>500	-	0.24	0.24	-	-
Salmonella enterica ATCC 14028	>500	-	0.49	62.5	-	-
Salmonella choleraesius ATCC 10708	>500	-	0.24	0.24	-	-
Gram-Positive bacteria						
Staphylococcus aureus ATCC 29213	>500	-	0.98	0.98	-	-
Streptococcus agalactiae ATCC 13813	>500	-	0.98	3.9	-	-
Staphylococcus saprophyticus ATCC 15305	>500	-	1.96	1.96	-	-
Enterococcus faecalis ATCC 19433	>500	-	0.98	0.98	-	-
Bacillus cereus ATCC 11778	>500	-	6.25	100	-	-
Candida species						
Candida albicans ATCC 18804	>500	-	-	-	2	2
Candida albicans ATCC 10231	>500	-	-	-	4	4
Candida glabrata ATCVC 2001	>500	-	-	-	4	4
Candida krusei ATCC 34135	>500	-	-	-	8	8
Candida tropicalis ATCC 28707	>500	-	-	-	2	2

Results are expressed in μg/mL. MIC: Minimum inhibitory concentration, MBC: Minimum bactericidal concentration, MFC: Minimum fungicidal concentration, -: Activity not detected, ATCC: American Type Culture Collection

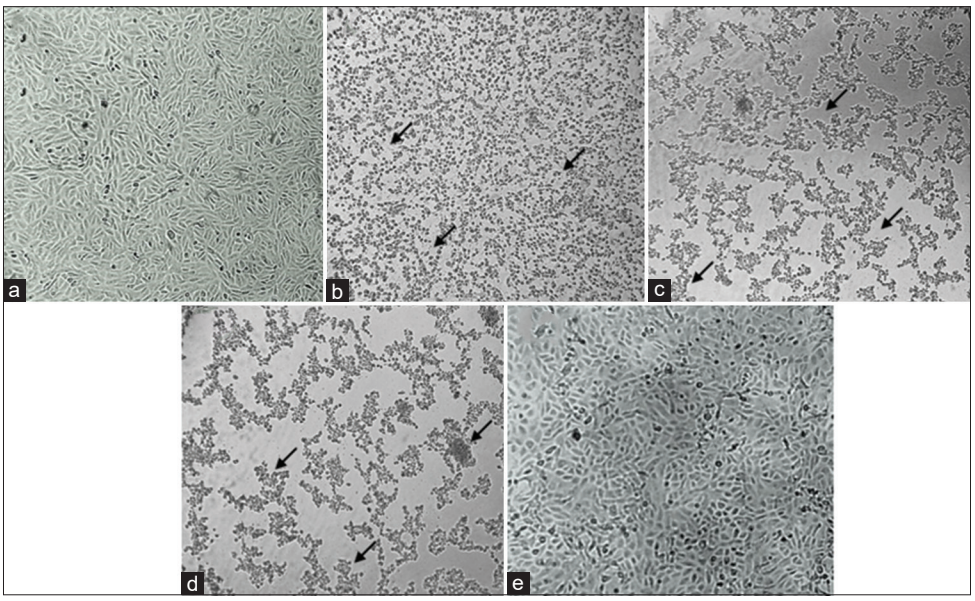

Figure 2: Absence of antiviral activity of rutin after *Mayaro virus* infection (moi 0, 1). Viral cytopathic effect in Vero cells. (a) Cellular control: untreated and uninfected cells; (b) viral control: infected and untreated cells; (c) vehicle control: Infected and treated cells with 0.2% (v/v) DMSO; (d) rutin treated (500 μg/mL) and infected cells; and (e) positive control: Ribavirin treated (200 μg/mL) and infected cells. The cytopathic effect reduction was observed only in ribavirin when compared to viral control. Black arrows indicate some clusters of cells from the cytopathic effect of the *Mayaro virus* (×100)

Table 2: Predictions of biological activities for rutin using the software online prediction of activity spectra for substances

Activity	Pa	Pi
Antibacterial	0.677	0.005
Antifungal	0.784	0.006
Antiviral	0.263	0.0053

Pa: Probability "to be active", Pi: Probability "to be inactive"

Vero and BHK-21 cells, suggesting low nephrotoxicity of this flavonoid.[13]

Using the PASS online software, the possible antimicrobial activity spectrum of rutin was determined [Table 2]. Rutin exhibited the highest Pa for antifungal activity (0.784) and the lowest Pa for antiviral activity (0.263). For the antibacterial effect, the Pa value was 0.677.

Discussion

The biological activity of many plant preparations is attributed to flavonoids and can be enhanced or reduced depending on the substituent groups present in the benzene rings of these compounds.[14,15] As an example, chalcones are more effective against methicillin-resistant *S. aureus* (MRSA) than flavones and flavonones due to the presence of hydroxyl groups at the 2' position of these compounds.[16] Furthermore, methyl groups are responsible for drastically reducing the antibacterial activity of flavonoids.[15]

According to Mbaveng *et al.*,[17] the antimicrobial activity is considered low if the MIC value is higher than 100 µg/mL. Thus, rutin had no significant action against the microorganisms tested (MIC >500 µg/mL). In agreement with the results obtained in this work, Alves *et al.*[18] showed that the rutin isolated from different mushrooms species showed no antimicrobial activity against Gram-positive and Gram-negative bacteria (MIC >1 mg/mL) and had low activity against *Listeria monocytogenes* (MIC = 1.0 mg/mL). The authors also demonstrated that the presence of OCH_3 and H groups in the rings A and C [Figure 1] is responsible for the anti-MRSA effect of the flavonoids tested. Furthermore, when the OH group is missing, no activity was detected, as in the case of rutin.[18]

Moreover, rutin is commonly found in association with other flavonoids, which can enhance its biological effects.[8] As an example, rutin and vanillin showed no activity against *B. cereus*, *E. coli*, *Salmonella enterica,* and *S. aureus*.[19] However, the combination of these compounds produced a similar or better effect than the extract, which suggests a possible synergistic effect among the compounds in the extract.[19]

The search for new bioactive agents should start with the evaluation of the cytotoxic effect of the compounds because it can ensure the safety of the molecule.[3] Then, rutin showed no toxic effect against Vero and BHK-21 cells, suggesting its safety. Furthermore, this flavonoid can be used in combination with other compounds, which can produce a synergistic effect, accentuating its biological activity.

The lack of specific antiviral agents or vaccines to treat viral infections has encouraged the search for new bioactive molecules with antiviral potential.[20] It is known that the antiviral effect of rutin is associated with its potential of affecting the viral envelope and the reverse transcriptase of enveloped RNA viruses.[21] However, there is no report about the antiviral effect of rutin against MAYV, an important emergent virus. In the antiviral assay, rutin did not affect any of the viral infection phases.

The software PASS compares the structure of the molecule with more than 205,000 known compounds and exhibits more than 3750 kinds of biological activities.[12] Pa values greater than 0.7 indicate that the probability of the molecule exhibits experimental activity is high; if 0.5< Pa < 0.7, the possibility of the compound being active experimentally is lower; and if Pa <0.5, the chance of the molecule exhibits activity empirically is small, but if this happens, it is possible that it is a new compound.[22] Increasingly, predictions using the software PASS have been used to confirm or compare the activity of organic compounds.[12] PASS calculations suggest that rutin is more likely to be an antifungal agent (Pa >0.7). However, this flavonoid shows neither antifungal activity nor antibacterial and antiviral activity. It is possible because PASS can only elucidate the effect of a compound experimentally, which highlights the importance of *in vitro* assays to confirm the results obtained by bioinformatics.[23] Although *in silico* analysis facilitates the prospection of bioactive compounds, *in silico* assay conditions are not able to simulate the *in vitro* and *in vivo* conditions, where the molecule may behave differently.

Conclusion

Through the *in vitro* results, it was demonstrated that rutin is not active against bacterial and fungal pathogens and MAYV, even though the PASS analysis showed the possibility of this flavonoid presents antifungal activity. Furthermore, this flavonoid presented low cytotoxicity, highlighting its safety.

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Conflicts of interest

There are no conflicts of interest.

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