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Potent antiviral flavone glycosides from *Ficus benjamina* leaves

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ABSTRACT

Crude ethanol extracts from *Ficus benjamina* leaves strongly inhibit Herpes Simplex Virus 1 and 2 (HSV-1/2) as well as Varicella Zoster Virus (VZV) cell infection *in vitro*. Bioassay-guided fractionation of the crude extract demonstrated that the most efficient inhibition of HSV-1 and HSV-2 was obtained with the flavonoid fraction. The present study was aimed to further isolate, purify and identify substances with potent antiviral activity from the flavonoid fraction of *F. benjamina* extracts. Flavonoids were collected from the leaf ethanol extracts through repeated purification procedure and HPLC analysis. The antiviral activity of each substance was then evaluated in cell culture. Three known flavone glycosides, (**1**) quercetin 3-O-rutinoside, (**2**) kaempferol 3-O-rutinoside and (**3**) kaempferol 3-O-robinobioside, showing highest antiviral efficiency were selected and their structure was determined by spectroscopic analyses including NMR and mass spectrometry (MS). These three flavones were highly effective against HSV-1 reaching a selectivity index (SI) of 266, 100 and 666 for compound 1, 2 and 3, respectively, while the SI of their aglycons, quercetin and kaempferol amounted only in 7.1 and 3.2, respectively. Kaempferol 3-O-robinobioside showed similar SI to that of acyclovir (ACV), the standard anti-HSV drug. Although highly effective against HSV-1 and HSV-2, these flavone glycosides did not show any significant activity against VZV.

1. Introduction

There is an ever increasing need for novel and improved antiviral agents against Herpes Simplex Virus-1 and -2 (HSV-1 and HSV-2), as alternatives to the popular drug acyclovir (ACV) and other nucleoside derivatives used worldwide [1]. This need is dictated by several drawbacks associated to the commonly used antiherpetic drugs, including development of anti-ACV resistant mutant viruses [2], major side effects, such as vomiting, and diarrhea, and the low efficiency of ACV in recurrent HSV attacks [3].

In a previous study, we have screened the antiviral activity of extracts from different plant species [4,5], including weeping fig *(Ficus benjamina*), a plant whose phytochemicals and biological properties have only been moderately investigated so far. *F. benjamina* fruit extracts showed antitumor and antibacterial activity [6], while aqueous and alcoholic leaf extracts had significant antinociceptive activity (reducing sensitivity to painful stimuli) in analgesiome test [7].The presence of polyphenols in *F. benjamina* grandular epithelium has been reported [8]. A new triterpene, named serrate-3-one, along with phytoconstituents pentacontanyl decanoate, friedelin and beta-sitosterol have been detected in *F. benjamina* (var. *comosa*) benzenoid extracts [7].

Unlike other ornamental *Ficus* species, *F. benjamina* is particularly resistant to various plant viruses [5]. We reasoned that such resistant plants might produce antiviral compounds, which in turn could also inhibit animal and human viruses. In agreement with this hypothesis, we found that ethanol extracts from *F. benjamina* leaves strongly inhibited HSV-1, HSV-2 and Varicella Zoster Virus (VZV) [5]. Fractionation of these extracts revealed that the active compounds against HSV-1 and HSV-2 belonged to the flavonoid fraction [5].

The antiviral properties of flavonoids are well established [9,10]. Recently, increased attention has been devoted to flavonoids in the context of their antiviral activity against the

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influenza virus [11]. Specifically, the flavonoids quercetin, quercetin 3-rhamnoside and quercetin 7-rhamnoside, found in a range of plant species, were reported to inhibit several viruses [12,13].

In this study we have isolated and identified three flavone glycosides from *F. benjamina* and assessed their antiviral activity against HSV-1, as compared to the aglycons of the isolated flavone glycosides, quercetin and kaempferol, and to the most common antiherpetic drug, ACV. In addition, the antiviral activity of the above-mentioned three flavone glycosides against HSV-2 and VZV was estimated.

2. Materials and methods

2.1. Materials

Quercetin, kaempferol, rutine and ACV were purchased from Sigma Israel, and HPLC solvents from MERK (USA).

2.2. Extract preparation

Ethanol extract was prepared from leaves of *F. benjamina*. Plant tissues were crushed, incubated at room temperature for 48 hours in ethanol, centrifuged at 2000 rpm for 10 min and the supernatant was evaporated by lyophilizer. Extracts were separated into different fractions by reverse phase column with rising methanol gradient: 0%, 20%, 40%, 60%, 80% and 100% (RP-C18 Sep-Pack).

2.3. HPLC

Gradient elution was performed with solution A, composed of water–acetic acid (97: 3 V/V) and solution B–methanol as described previously [14]. UV detector at 360 nm with reverse phase column (Betasil C-18, 5 μm, 250 × 0.46 mm; Thermo-Hypersil, UK) was used.

2.4. Liquid chromatography–mass spectrometry (LC–MS) instrumentation and conditions

LC–MS Agilent 1100LC series (Waldbronn, Germany), Bruker Esquire 3000plus MS (Bremen, Germany) instrument consisting of a C18 column (Betasil C18, 5 μm, 250 × 4.6 mm; Thermo-Hypersil, UK) and methanol–water as the mobile phase (see HPLC in the method above) were used. The UV detector was set at 360 nm, the flow rate at 1 mL/min, and injection volume of 10 μL. The MS conditions were optimized as follows: API electron spray interface, negative mode polarity, a drying gas flow of 10 L/min, a nebulizer gas pressure of 60 psi, a drying gas temperature of 335 °C, a fragmentor voltage of 0.4 V, a capillary voltage of 4451 V, and a scan range of *m/z* 25–1000, at 1.15 s/scan.

2.5. Compound **1** (quercetin 3-O-α-rhamnopyranosyl(1—6)-β-glucopyranoside)

^{1}H and ^{13}C NMR data in agreement with published data [15]. LC-ESI-MS/MS (ion trap) of m/z 609 $[M-H]^-$, m/z: 301.0 $[(\text{quercetin-H})]^-$; HR-MS *m/z* $[M+Na]^+$ 633.1467 (calcd for $C_{27}H_{30}O_{16}Na$, 633.1426).

2.6. Compound **2** (kaempferol 3-O-α-rhamnopyranosyl(1—6)-β-glucopyranoside)

^{1}H and ^{13}C NMR data in agreement with published data [16] LC-ESI-MS/MS (ion trap) of m/z 593 $[M-H]^-$, m/z: 447 $[(M-H)-\text{Rha}]^-$; 326.7 $[(M-H)-\text{Rha}-120]^-$; 284.7 $[(\text{kaempferol}-H)]^-$; 256.6 ; 228.6 ; 212.7. HR-MS *m/z* $[M+Na]+$ 617.1497 (calcd for $C_{27}H_{30}O_{15}Na$, 617.1478).

2.7. Compound **3** (kaempferol 3-O-a-rhamnopyranosyl(1—6)-b-galactopyranoside)

^{1}H and ^{13}C NMR data in agreement with published data [17]. LC-ESI-MS/MS (ion trap) of m/z 593 $[M-H]^-$, m/z : 447 $[(M-H)-\text{Rha}]^-$; 326.8 $[(M-H)-\text{Rha}-120]^-$; 284.7 $[(\text{kaempferol}-H)]^-$; 254.6 ; 228.7 ; 210.6. HR-MS *m/z* $[M+Na]^+$ 617.1498 (calcd for $C_{27}H_{30}O_{15}Na$, 617.1477).

2.8. Cells and viruses

African green monkey kidney (Vero) cells were purchased from the American Type Culture Collection (ATCC), Rockville, MD, USA. Cells were grown in RPMI medium supplemented with 10% new born calf serum and antibiotics penicillin, streptomycin and nystatin and incubated at 37 °C in a humidified air containing 5% CO_2.

HSV-1 was obtained from the ATCC (VR-735), HSV-2 and VZV were obtained from the virology laboratory Soroka University Medical Center, Beer-Sheva, Israel.

2.9. Cytotoxicity examination

Vero cells were treated with various concentrations of tested components. The toxicity of the components was tested by three methods. 1. Direct count: cells were counted by Neubauer hemacytometer, indicating their replication rate. 2. Morphological changes were daily observed by optical inverted microscope, and 3. MTT assay, performed as described [18]. Briefly, Vero cells were incubated with 50 μg/mL MTT solution at 37 °C for 5 h. This solution was converted by mitochondrial succinate dehydrogenase enzyme into the purple crystal formazan. Then, the MTT solution was removed and replaced with a SDS solution to dissolve the formazan. After overnight incubation at 37 °C, solution absorbance was measured by spectrophotometer at 570 nm, indicating the metabolic activity of the cells.

According to the obtained results the CC_{50} (a concentration causing 50% toxicity) of the tested compounds was determined.

2.10. Virus infection

Vero cells were seeded at 0.15×10^6 cells/well in 24-well culture plates, in RPMI with 10% NBCS and antibiotics. Following overnight incubation, medium was removed and each well was infected at a multiplicity of infection (MOI) of 0.1 PFU/cell for 2 hours at 37 °C. The unabsorbed virus was removed and cells were overlaid with either a layer of CMC (for plaque assay) or RPMI containing 2% NBCS and antibiotics. The infection development was evaluated by plaque assay and cytopathic effect (CPE) development as previously