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Mohamed AA Orabi
Faculty of Pharmacy, Al-Azhar University, Assiut 71524, Egypt.

Esam A Orabi
A) Department of Chemistry, Faculty of Science, Assiut University, Assiut 71516, Egypt.
B) Department of Chemistry and Biochemistry, Concordia University, 7141 Sherbrooke Street West, Montréal, Québec H4B 1R6, Canada

Correspondence:
Mohamed AA Orabi
Faculty of Pharmacy, Al-Azhar University, Assiut 71524, Egypt.

Antiviral and antioxidant activities of flavonoids of *Ficus virens*: Experimental and theoretical investigations

Mohamed AA Orabi and Esam A Orabi

Abstract
A phytochemical investigation of *Ficus virens* leaves led to isolation of six flavonoids (**1**–**6**). On the basis of 2D and/or 1D NMR, and mass data, together with the comparison with the literature values, the flavonoids were identified as quercetin (**1**), quercetin-3-*O*-α-D-arabinopyranoside (**2**), quercetin-3-*O*-β-D-galactopyranoside (**3**), kaempferol-3-*O*-α-D-arabinopyranoside (**4**), kaempferol-3-*O*-β-D-galactopyranoside (**5**), and vogelin J (**6**). The procedures for simultaneous isolation of the glycosides **2**–**5** are described. The antioxidant activity of the isolated flavonoids were experimentally evaluated. The structural characteristics and structure-antioxidant activity relationship of these isolates are studied by quantum mechanical calculations on the molecules and on the free radicals derived from each compound by an abstraction of single hydrogen from the various aromatic OH groups. The calculations are performed at the B3LYP/6-31+G(d,p) level of theory, and show that glycosylation of quercetin and kaempferol distorts the planar geometry of the aromatic moiety of both the molecular and radical compounds. The viral inhibitory effects of the isolated flavonoids on Coxsackie B4 (CVB4), and hepatitis A virus (HAV) are also investigated. The flavonoids **1**, **3**, and **6** exerted mild anti CVB4 inhibitory effects, while **2**, and **3** exerted mild inhibitory activities against HAV.

Keywords: *Ficus virens*, Flavonoid glycosides, DPPH, antioxidant, Quantum chemistry.

1. Introduction
The plant-derived medicines are mainly favored because of their low toxicity, rapid degradation in the environment, and limited resistance [1]. *Ficus virens* W. T. Aiton (Moraceae), synonym *Ficus cunninghamii* Miq., is a medium sized tree commonly known as white fig [2]. Its syconia are edible, and the new foliage can also be eaten as a vegetable and pickle [3]. In Egypt, many *Ficus* species are grown in streets, gardens, parks for the ornamental and shade purposes. The use of *Ficus* species as health-improving food or as therapeutic agent is known long ago. In folk medicine, many parts of *F. virens* such as bark, latex, leaves and fruits are used in the treatment of blood diseases, apoplexy, vertigo, delirium, pain, rheumatism, diabetes and also as antioxidants [4]. *F. virens* has good amount of phenolic content, and exert strong free radical scavenging activity [5–9]. In this study, six known flavonoids (**1**–**6**) were isolated from leaves of *F. virens*. Among them, four flavonoid glycosides, quercetin-3-*O*-α-D-arabinopyranoside (**2**), quercetin-3-*O*-β-D-galactopyranoside (**3**), kaempferol-3-*O*-α-D-arabinopyranoside (**4**), and kaempferol-3-*O*-β-D-galactopyranoside (**5**), which are usually reported in literatures as inseparable mixture [10], are isolated in high purity conditions. The chromatographic procedures including preparative reversed-phase (RP)-HPLC technique for simultaneous isolation of these glycosides are shown in this work. The DPPH radical scavenging activities of the isolated flavonoid in comparison with that of standard kaempferol are experimentally investigated. The results revealed that the glycosylation process lowers the radical scavenging ability of the flavonoids compared to their aglycone parts. To understand the structural-antioxidant activity of these compounds, we performed quantum mechanical calculations to determine the structural and energetic properties of these flavonoids and of their radicals.

Besides the protective effect of the flavonoids by scavenging oxidants from the biological systems, in many folk medicines, part of the therapeutic effect was ascribed for the flavonoid class of compounds, because of their broad pharmacological activities. Some flavonoids-rich plant extracts as well as single pure flavonoids showed noticeable antiviral activity by *in vivo* and *in vitro* studies [1, 11].

The CVB4 and HAV belong to Picornaviridae family of viruses; small plus-strand RNA viruses that cause a wide range of pathogenicity ranging from lethal and severe (encephalitis, myocarditis) to mild (common cold and influenza-like diseases) [12]. CVB4 is thought to cause diseases such as myocarditis, pancreatitis, hepatitis, aseptic meningitis, meningoencephalitis, gastroenteritis, necrotizing enterocolitis, and pneumonia, and even death in neonates [13]. HAV is the most common cause of acute viral hepatitis that transmitted through the fecal-oral route; it causes about 10 million infections worldwide each year [14]. To investigate the antiviral potency of the isolated compounds, we measured the inhibitory effects of the isolated flavonoids on CVB4 and HAV viruses.

2. Material and methods

2.1 Materials

The DPPH (Sigma-Aldrich Chemicals Co, Germany). Organic solvents (El-Nasr Pharmaceuticals Chemicals Co., Cairo, Egypt). Minimum essential media (MEM) (Caisson / USA). Trypsin-EDTA 1:250 U (Molekulu/UK). MTT (Biobasic/Canada). Penicillin, streptomycin, and ampicillin (Chemical industries and development company, Egypt). Doxorubicin, a standard anticancer agent, and ribavirin, a standard anti-viral agent, (Sigma-Aldrich Chemicals Co, Germany).

2.2 Plant materials

Leaves from *F. virens* tree were collected at May 2011 from the experimental station of ornamental plants, Faculty of Agriculture, Assiut, Egypt. A voucher specimen (Fv 185011) was deposited at the same Department.

2.3 Extraction and isolation procedures

The air-dried, powdered leaves (200 g) were exhaustively extracted using MeOH/H2O (7:3, v/v, 1L × 7) at room temperature over a period of 7 days. The obtained extract was filtered, and MeOH of the extract was evaporated under vacuum at 50 °C. The resulted aqueous extract (400 mL) was partitioned using *n*-hexane (4 × 400 mL), dichloromethane (DCM) (4 × 400 mL), ethyl acetate (5 × 400 mL) and *n*-butanol (4 × 400 mL) to obtain the respective, *n*-hexane (6 g), DCM (3 g), ethyl acetate (12 g), *n*-butanol (9 g) and aqueous (13 g) fractions. A part (3 g) of the ethyl acetate fraction was subjected to silica gel column chromatography with a gradient of DCM/MeOH (from 1:0 to 0:1, v/v) as an eluent, collecting 100 mL fractions. Eluates were monitored by TLC using pre-coated Silica gel G60 F254 plates (Merck, Germany), and spots were visualized under UV, and by spraying with 10% aqueous H_2SO_4 followed by heating at 110 ºC for 5 min. Similar fractions were combined together and concentrated under reduced pressure to give five fractions (A–E). Fraction B (300 mg), eluted with DCM/MeOH (9.5:0.5, v/v) was subjected to silica gel column, eluted with a gradient of *n*-hexane/ethyl acetate (1:0 – 0:1, v/v), collecting 20 mL/fraction, and yielded seven sub-fractions (B1–B7). Crystallization from DCM/MeOH (1:1, v/v) of the sub-fraction B2 (70 mg), eluted with *n*-hexane/ethyl acetate (6:4, v/v), afforded pure yellow crystals of vogelin J (11 mg, **6**). The sub-fraction B4 (100 mg), eluted with *n*-hexane/ethyl acetate (4:6, v/v), was further purified on Sephadex LH-20 column (1 i.d. × 17 cm) eluted with DCM/MeOH (1:1, v/v) to afford quercetin (9 mg, 1). The fraction D (300 mg), eluted by DCM/MeOH (8:2, v/v), was dissolved in DCM. A DCM-insoluble yellow granular powder revealed four distinct peaks upon RPHPLC analysis. A preparative RPHPLC purification of the DCM-insoluble part (57 mg) led to isolation of quercetin-3-*O*-α-D-arabinopyranoside (**2**, 4.6 mg), quercetin-3-*O*- β-D-galactopyranoside (**3**, 19 mg), kaempferol-3-*O*-α-D-arabinopyranoside (**4**, 2.8 mg), and kaempferol-3-*O*-β-D-galactopyranoside (**5**, 3.4 mg).

2.4 Spectroscopic analyses

Electrospray ionization mass (ESIMS) spectra were acquired on an API-4000 instrument (AB Sciex, Framingham, MA, USA). The solvent used was [CH_3CN/H_2O (1:1, v/v)]. The 1D (^1H and ^{13}C) and 2D (^1H–^1H COSY, HSQC and HMBC) NMR spectra were recorded on a Varian INOVA AS 600 instrument (600 MHz for ^1H and 151 MHz for ^{13}C; Agilent, Santa Clara, CA, USA). Chemical shifts are given in δ (ppm) values relative to that of the solvent signal [acetone-d_6 (δ_H 2.04; δ_C 29.8), DMSO-d_6 (δ_H 2.50; δ_C 39.5)] on the tetramethylsilane scale. Reversed-phase (RP) HPLC was performed on a YMC-Pack ODS-A A-303 (YMC, Japan) column (4.6 i.d. × 250 mm) developed using acetonitrile – water (2:8, v/v) with 0.1% acetic acid, at a flow rate of 1 mL/min in an oven set at 40 °C. Detection was performed based on UV absorption at 280 nm. Preparative RP-HPLC was performed at 40 °C on a YMC-Pack ODS-A, A-324 column (10 i.d. × 300 mm) using acetonitrile – water (2:8, v/v) with 0.1% acetic acid, at a flow rate of 2 mL/min with UV detection at 280 nm.

Quercetin (1): ESI-MS *m/z* 603 $[2M-H]^-$; ^1H NMR (600 MHz, DMSO-d_6) δ 12.48 (1H, s, C-5 OH), 10.77 (1H, s, C-7 OH), 9.57 (1H, s, C-3 OH), 9.33 (1H, s, C-4′ OH), 9.28 (1H, s, C-3′ OH), 7.67 (1H, d, *J* = 2.4 Hz, H-2′), 7.53 (1H, dd, *J* = 2.4, 8.4 Hz, H-6′), 6.88 (1H, d, *J* = 8.4 Hz, H-5′), 6.41 (1H, d, *J* = 1.8 Hz, H-8), 6.18 (1H, d, *J* = 1.8 Hz, H-6); ^{13}C NMR (151 MHz, DMSO-d_6) δ 175.8 (C-3), 163.8 (C-7), 160.7 (C-5), 156.1 (C-9), 147.7 (C-4′), 146.8 (C-2), 145.0 (C-3′), 135.7 (C-3), 121.9 (C-1'), 119.9 (C-6″), 115.6 (C-2′), 115.0 (C-5′), 103.0 (C-10).

Quercetin-3-*O*-α-D-arabinopyranoside (2): ESI-MS *m/z* 433 $[M-H]^-$; ^1H NMR [600 MHz, (acetone-d_6–D_2O, 7:3)] δ 7.85 (1H, d, *J* = 2.4 Hz, H-2′), 7.53 (1H, dd, *J* = 2.4, 8.4 Hz, H-6′), 6.90 (1H, d, *J* = 8.4 Hz, H-5′), 6.47 (1H, d, *J* = 1.8 Hz, H-8), 6.22 (1H, d, *J* = 1.8 Hz, H-6), 5.14 (1H, d, *J* = 6.6 Hz, H-1″), 3.92 (1H, dd, *J* = 6.6, 9.0 Hz, H-2″), 3.83 (1H, ddd, *J* = 1.8, 3.6, 7.2 Hz, H-4″), 3.73 (1H, dd, *J* = 3.6, 12.0 Hz, H-5″), 3.66 (1H, dd, *J* = 3.6, 8.4 Hz, H-3″), 3.39 (1H, dd, *J* = 1.8, 12.6 Hz, H-5″); ^{13}C [151 MHz, (acetone-d_6–D_2O, 7–3),] δ 178.7 (C-3), 165.2 (C-7), 162.0 (C-5), 157.8 (C-2), 157.6 (C-9), 149.3 (C-4′), 145.2 (C-3′), 134.9 (C-3), 122.3 (C-6′), 122.0 (C-1′), 117.2 (C-2′), 115.9 (C-5′), 104.9 (C-10), 103.7 (C-1″), 99.4 (C-6), 94.5 (C-8), 73.2 (C-3″), 72.0 (C-2″), 68.0 (C-4″), 66.2 (C-5″).

Quercetin-3-*O*-β-D-galactopyranoside (=hyperoside = hyperin, 3): ESI-MS *m/z* 463 $[M-H]^-$; ^1H NMR [600 MHz, (acetone-d_6–D_2O, 7:3)] δ 7.90 (1H, d, *J* = 2.4 Hz, H-2′), 7.53 (1H, dd, *J* = 2.4, 8.4 Hz, H-6′), 6.90 (1H, d, *J* = 9.0 Hz, H-5′), 6.41 (1H, d, *J* = 1.8 Hz, H-8), 6.21 (1H, d, *J* = 1.8 Hz, H-6), 5.10 (1H, d, *J* = 7.8 Hz, H-1″), 3.90 (1H, br. d, *J* = 3.6 Hz, H-4″), 3.83 (1H, dd, *J* = 7.8, 9.6 Hz, H-2″), 3.61 (1H, dd, *J* = 6.6, 9.6 Hz, H-6″), 3.60 (1H, dd, *J* = 3.6, 9.6 Hz, H-3″), 3.48 (1H, br.t, *J* = 6 Hz, H-5″), 3.44 (1H, dd, *J* = 6.6, 10.8 Hz, H-6″); ^{13}C NMR [151 MHz, (acetone-d_6–D_2O, 7–3)] δ 178.6 (C-3), 165.2 (C-7), 161.8 (C-5), 157.8 (C-2), 157.5 (C-9), 149.2 (C-4′),

145.0 (C-3′), 134.4 (C-3), 122.2 (C-6′), 121.9 (C-1′), 117.4 (C-2′), 115.8 (C-5′), 104.7 (C-10), 104.4 (C-1″), 99.5 (C-6), 94.5 (C-8), 76.0 (C-5″), 74.0 (C-3″), 72.3 (C-2″), 68.6 (C-4″), 60.7 (C-6″).

Kaempferol-3-*O*-α-D-arabinopyranoside (4): ESI-MS *m/z* 417 $[M-H]^-$; ^1H NMR [600 MHz, (acetone-d_6–D_2O, 7:3)] δ 7.8 (2H, d, J = 8.4 Hz, H-2′/6′), 6.93 (2H, d, J = 7.8 Hz, H-3′/5′), 6.47 (1H, d, J = 1.8 Hz, H-8), 6.23 (1H, d, J = 1.8 Hz H-6), 5.14 (1H, d, J = 6 Hz, H-1″), 3.90 (1H, dd, J = 6.0, 9.0 Hz, H-2″), 3.80 (1H, dd, J = 3.6, 7.8 Hz, H-4″), 3.67 (1H, dd, J = 4.8, 12.0 Hz, H-5″), 3.66 (1H, dd, J = 3.6, 7.8 Hz, H-3″), 3.32 (1H, dd, J = 2.4, 12.0 Hz, H-5″).

Kaempferol-3-*O*-β-D-galactopyranoside (= Trifolin, 5): ESI-MS *m/z* 447 $[M-H]^-$; ^1H NMR [600 MHz, (acetone-d_6–D_2O, 7:3)] δ 8.07 (2H, d, J = 8.4 Hz, H-2′/6′), 6.91 (2H, d, J = 8.4 Hz, H-3′/5′), 6.45 (1H, d, J = 1.8 Hz, H-8), 6.22 (1H, d, J = 1.8 Hz H-6), 5.1 (1H, d, J = 8.4 Hz, H-1″), 3.86 (1H, dd, J = 1.2, 3 Hz, H-4″), 3.78 (1H, dd, J = 7.8, 9.6 Hz, H-2″), 3.55 (1H, dd, J = 3, 9.6 Hz, H-3″), 3.57 (1H, dd, J = 6.6, 9.6 Hz, H-6″), 3.44 (1H, ddd, J = 1.2, 6.6, 11.4 Hz, H-5″), 3.40 (1H, dd, J = 6,6 11.4 Hz, H-6″); ^{13}C NMR [151 MHz, (acetone-d_6–D_2O, 7:3)] δ 178.8 (C-3), 165.1 (C-7), 161.9 (C-5), 160.7 (C-2), 158.3 (C-4′), 157.6 (C-9), 134.8 (C-3), 131.8 (C-2′/C-6′), 115.8 (C-3′/C-5′), 104.9 (C-10), 104.1 (C-1″), 99.5 (C-6), 94.5 (C-8), 76.0 (C-5″), 74.0 (C-3″), 72.1 (C-2″), 68.6 (C-4″), 60.7 (C-6″).

Vogeline J (6): ESI-MS M/Z 335 $[M-H]^-$; ^1H NMR [600 MHz, (acetone-d_6–D_2O, 7:3)] δ 6.63 (1H, s, H-3), 6.45 (1H, d, J = 0.6 Hz, H-8), 7.88 (2H, d, J = 9.0 Hz, H-2′/H-6′), 6.98 (2H, d, J = 9.0 Hz, H-3′/H-5′), 5.72 (1H, d, J = 9.0 Hz, H-3″), 6.6 (1H, dd, J = 0.6, 9.0 Hz, H-4″), 1.42 (6H, s, H-5′ and 6′); ^{13}C NMR [151 MHz, (acetone-d_6–D_2O, 7:3)] δ 183.12 (C-4), 165.3 (C-2), 162.2 (C-4′), 159.97 (C-7), 157.7 (C-9), 156.59 (C-5), 129.4 (C-3″), 129.14 (C-2′), 129.14 (C-6′), 122.47 (C-1′), 116.75 (C-3′), 116.75 (C-5′), 115.48 (C-4″), 105.93 (C-6), 105.61 (C-10), 103.72 (C-3), 95.7 (C-8), 78.71 (C-2″), 28.34 (C-5″), 28.34 (C-6″).

2.5 Cell lines and viruses

VERO cell line, and the viruses HAV and CVB4 were obtained from center of viral research and studies, Faculty of Medicine, Al-Azhar University, Cairo, Egypt. Vero cells were grown in minimum essential medium (MEM). The media were supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 units/mL penicillin, 100 µg/mL streptomycin and 1% HEPES (4-2-hydroxyethyl-1-piperazine ethane sulfonic acid) and incubated in a humidified 5% CO_2 atmosphere. The medium used for the cytotoxicity and antiviral assays contained 2% of fetal bovine serum. Viral stocks were prepared in Vero cells. The virus titer was estimated from cytopathogenicity of cells induced by viral infection and expressed as 50% tissue culture infectious doses (TCID50) [15].

2.6 Cytotoxicity assay

Two-fold dilutions (1000 –1.9 µg/mL) from the pure compounds were prepared in MEM (containing 2% antibiotics and 2% FBS). The cytotoxic activity of the samples was determined in Vero cells by using the colorimetric 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT) method [16]. Briefly, Vero cells were grown in 96-well microtiter plates at concentration 5×10^3 cells/well for 24 h, in 5% CO_2 incubator (Jouan CO_2 incubator, France) at 37 °C. The 24 h cell monolayers were treated with each sample dilutions (each dilution in triplicate), while cell control contained only medium. The cells were incubated for 48 h at 37 °C in a 5% CO_2 incubator. The culture medium was removed, and 20 µL of MTT solution (5 mg/mL) was added to cells in each well for 4 h at 37 °C. After removal of MTT, 200 µL DMSO was added to solubilize the formazan crystal, an MTT metabolic product, and incubated for 30 min at 37 °C. The optical densities (OD) were measured at 560 nm using an ELISA reader (MRX microplate reader, Dynex technologies, USA). The percentage of cytotoxicity is calculated as [(A−B/A) × 100], where A and B indicate the mean of three optical densities of control, and treated cells, respectively. The 50% cytotoxic concentration (CC_{50}) is defined as the concentration (µg/mL) that can reduce 50% of cell viability compared to control cells. The maximum non-toxic concentration (MNTC) of each extract was determined and was used for assessing the viral inhibitory activities.

2.7 Antiviral assay

The direct effect of the flavonoids on CVB4, and HAV infectivity was evaluated according to the protocols described previously [15] with little modification. Briefly, Vero cells grown in 96-well plates at concentration 1×10^3 cells/well were incubated at 37 °C and 5% CO_2 overnight to allow the cells to attach to the wells. The viral suspensions containing 100 TCID50 of the virus were either incubated with an equal volume of MNTC of the flavonoids or with drug-free vehicle at 4 °C for 1 h. The cells monolayers were treated with 100 µL viral/sample suspension, and the plates were placed on a shaking table (150 rpm) for 5 min. Plates were then incubated at 37 °C in 5% CO_2 atmosphere for 24 h to allow the virus to take effect. The cell monolayers were rinsed carefully with phosphate buffered solution. The veridical effect was determined by an MTT assay following the procedures described above. The antiviral activity for a given compound was calculated from the equation % antiviral activity = [(A−B)/(C−B)] × 100). Where, A is the mean optical density of the treated cells, B is the mean optical density of virus control, and C is the mean optical density of cell control.

2.8 Practical assessment of the DPPH radical scavenging activities

The DPPH radical scavenging activities was determined by a modified method by Wang *et al*., 2007 [17]. DPPH radical was prepared in MeOH as a 100 µM solution. A flavonoid was dissolved in MeOH, and two fold dilutions (final concentrations 0.78 – 100 µg/mL) were prepared. To 270 µL DPPH solution 30 µL of the different dilution (in triplicate) of the samples in MeOH was added. The mixture was shaken vigorously and kept in the dark for 30 min. The absorbance was measured on UV spectrophotometer at 517 nm and the scavenging activity was determined by the following equation: % Scavenging activity = $[A_{control} - A_{sample}]/A_{control} \times 100]$. The IC_{50} value for each sample was determined graphically by plotting the percentage disappearance of DPPH as a function of the sample concentration.

2.9 Quantum mechanical (QM) calculations

All QM calculations are performed in gas phase with Gaussian 09 software [18] using the B3LYP functional [19, 20] and the 6-31+G(d,p) basis set. The geometry of the six isolated flavonoids is optimized starting with various initial conformers

of each compound. Frequency calculations are performed on the optimized geometries to confirm that they are energy minima (no imaginary frequencies). Free radicals generated from the most stable conformer of each compound by hydrogen abstraction from the OH groups in positions 3′, 4′, 5, or 7 are also optimized at the same level of theory. A relaxed potential energy scan is performed by scanning the $\varphi_{3\text{-}2\text{-}1'\text{-}6'}$ dihedral angle in quercetin from 0° to 180° in 5° increment.

3. Results and discussion

3.1 Preparative HPLC purification and structural identification of the isolated flavonoids

An aqueous MeOH extract of leaves of *F. virens* was fractionated by partitioning in *n*-hexane, DCM, ethyl acetate, and *n*-butanol. The ethyl acetate fraction was subjected to silica gel chromatographic fractionation with DCM/MeOH gradients. Crystallization from [DCM/MeOH (1:1, v/v)] of the early DCM/MeOH (9.5:0.5, v/v) eluate afforded pure sample of **6**, while repeated purification of the later eluate on Sephadex LH-20 afforded the pure flavonoid **1**. Preparative HPLC purification of the EtOAc polar part, eluted with DCM/MeOH (8:2, v/v), led to purification of four flavonoid glycosides (**2**–5). On the basis of spectroscopic experiments including ^{1}H and ^{13}C NMR, COSY, HSQC and HMBC, together with ESI mass spectrometry and comparing of the obtained data (Experimental section) with reported values, the flavonoids (**Fig. 1**) were identified as quercetin (**1**) [21], quercetin-3-*O*-α-D-arabinopyranoside (**2**) [22, 23], quercetin-3-*O*-β-D-galactopyranoside (**3**) [24], kaempferol-3-*O*-α-D-arabinopyranoside (**4**) [22], kaempferol-3-*O*-β-D-galactopyranoside (**5**) [25], and vogelin J (**6**) [26].

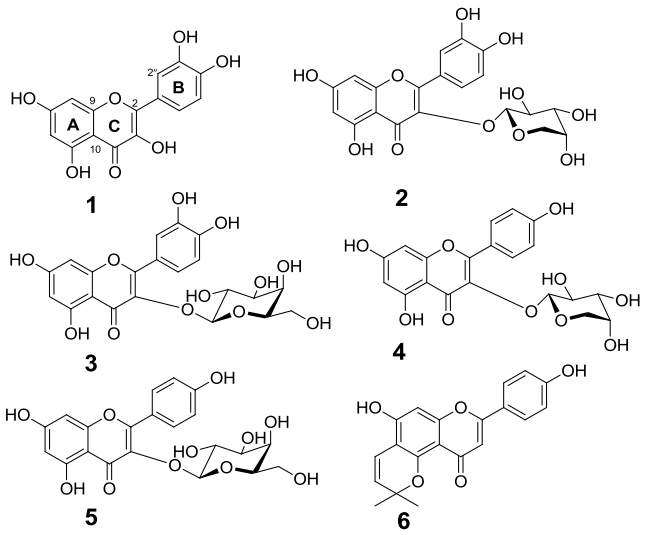

Fig 1: Structures of the compounds **1**–**6**.

3.2 Antioxidant activity

3.2.1 DPPH radical scavenging activity

The *F. virens* leaves and stem bark are rich with phenolic components [7, 9]. An aqueous MeOH extract of leaves of *F. virens*, and the different fractions, EtOAc and *n*-BuOH, obtained by successive fractionation of the MeOH extract showed high anti-DPPH radical scavenging activities [5]. In the present study, phytochemical investigation of EtOAc fraction of an aqueous MeOH extract of leaves of *F. virens* led to purification of six known flavonoids (**1**–**6**). In our knowledge, the antioxidant effect of vogelin J (**6**) was not reported previously, whereas the antiradical activities of few or more of the other molecules **1**–**5**, were studied extensively by various methods. Since the antioxidant capacity depends on the concentration ratio between antioxidants and target, the reaction conditions, and other factors [27], the anti-DPPH radical activities of the different isolated compounds, as well as an authentic sample of the flavonoid kaempferol were evaluated under the same experimental conditions. The concentration of antioxidant needed to decrease the initial DPPH concentration by 50% (IC_{50}) was determined (see experimental section). A lower IC_{50} value corresponds to a higher antioxidant power. On the basis of the obtained results, quercetin [**1**, (IC_{50} = 14 ± 1.12)] is the most active, followed by quercetin glycosides [2 (IC_{50} = 35 ± 1.21), and **3** (IC_{50} = 37 ± 1.74)], kaempferol (IC_{50} = 40 ± 0.82), kaempferol glycosides [**4**, (IC_{50} = 54 ± 1.13) and **5** (**I**C_{50} = 59 ± 1.42], and vogelin J [**6**, (IC_{50} >100)].

3.2.2 QM investigation of the structural-antioxidant activity relationship

The most commonly occurring flavonols are those with dihydroxylation in the 3′ and 4′ positions of the B ring, and to a smaller extent, those with a single hydroxylation at the 4′ position. The phenolic OHs are responsible for the antioxidant activity of flavonoids. A number of studies have been focused on understanding the structure-antioxidant activity relationship of these compounds [28–38]. Although several factors, the most important of which are lipophilicity, iron-chelation, and scavenging of free radicals, determine the antioxidant power of a compound [39], experiments are suggesting a relation between the structure and the free radical scavenging ability of flavonoids [35, 39]. Determination of the structural properties of flavonoids and of their radicals is thus an important step toward understanding their antioxidant potency. To the best of our knowledge, computational investigations on flavonoids have focused on the simple, sugar-free (aglycones) compounds [29, 39]. Previous experimental studies [38] and our herein investigation show that the flavonoid glycosides are less active as anti-DPPH radical than their non-glycosylated analogues. Studying the influence of the sugar moiety on flavonoids geometry aids in understanding the reason of reduction in antioxidant activity upon glycosylation [40].

3.2.3 Optimized geometries of molecular flavonoids

Geometry optimization of the six isolated compounds as well as of kaempferol revealed various stable conformers for each molecule. We report in **Fig. 2** the geometry of the global minimum conformer of each compound.

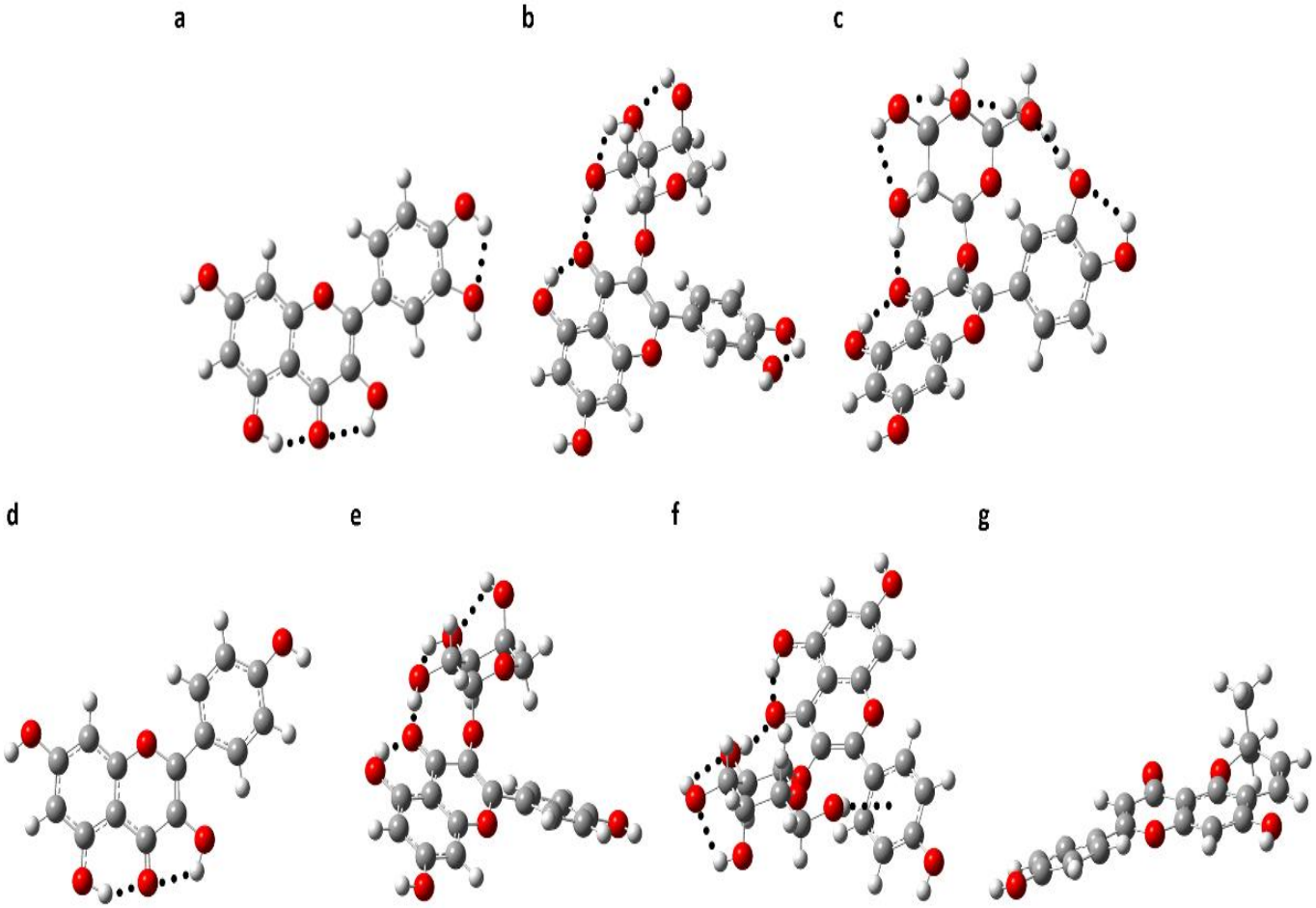

Fig 2: Optimized global minimum structures of quercetin (**a**), quercetin-3-*O*-α-D-arabinopyranoside (**b**), quercetin-3-*O*-β-D-galactopyranoside (**c**), kaempferol (**d**), kaempferol-3-*O*-α-D-arabinopyranoside (**e**), kaempferol-3-*O*-β-D-galactopyranoside (**f**), and vogelin J (**g**) calculated in gas phase at the B3LYP/6-31+G (d,p) level. Atom color code: oxygen (red), carbon (gray) and hydrogen (white). Doted lines show intramolecular O–H···O hydrogen bonding and O–H···π interactions (C–H···O hydrogen bonds are not shown).

The geometries of quercetin (**Fig. 2**, **a**) and kaempferol (**Fig. 2**, **d**) are characterized by planar geometry, the torsion angle $\varphi_{3\text{-}2\text{-}1'\text{-}6'}$ is 0°. This planarity results in full conjugation through the molecule [39]. In contrast to quercetin and kaempferol, the geometry of their glycosylation derivatives **2**–**5** is not planar with $\varphi_{3\text{-}2\text{-}1'\text{-}6'}$ = 24°–61°. The largest deviation in planarity occurs for Quercetin-3-*O*-β-D-galactopyranoside, due to the large rotation of ring B as to allow for hydrogen bonding between its OH groups and the sugar group (**Fig. 2**, **c**). The hydroxyl group of the CH_2OH sugar group in kaempferol-3-*O*-β-D-galactopyranoside interacts with ring B through O–H···π interaction (**Fig. 2**, **f**). Such interaction mode is known to provide stabilization energy of ~2.5 kcal/mol [41]. Vogalin J (**6**) is also characterized by a nonplanar geometry, $\varphi_{3\text{-}2\text{-}1'\text{-}6'}$ = 18° (**Fig. 2**, **g**). The non-planarity of **6** is a consequence of the absence of the OH group at position 3, which when present stabilizes the planar conformation through the week C–H···O hydrogen bonding. It can thus be substantiated that flavonoid aglycones possess planar geometry only when possessing an OH group at position 3 and the glycosylated flavonoids are nonplanar. Distortion in planarity results in ring B being not totally conjugated to the rest of the molecule. To calculate the energy barrier associated with rotation of the $\varphi_{3\text{-}2\text{-}1'\text{-}6'}$ dihedral angle, we performed a relaxed potential energy surface scan of the $\varphi_{3\text{-}2\text{-}1'\text{-}6'}$ dihedral angle from 0° to 180° and the results are shown in **Fig. 3**. Calculations show an energy barrier of 5.1–5.6 kcal/mol for a 180° rotation.

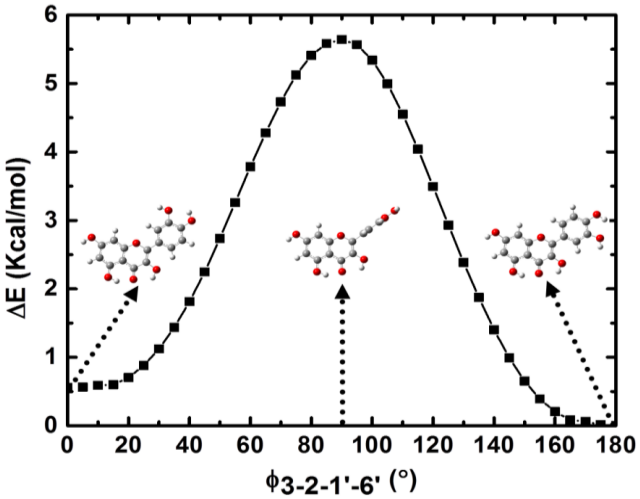

Fig. 3. Relaxed potential energy scan of quercetin calculated by scanning the dihedral angle $\varphi_{3\text{-}2\text{-}1'\text{-}6'}$ in gas phase at B3LYP/6-31+G (d,p) level. ΔE is the energy relative to the lowest energy conformer ($\varphi_{3\text{-}2\text{-}1'\text{-}6'}$ = 180°).

3.2.4 Optimized geometries of radical flavonoids

One of the proposed mechanisms for the antioxidant activity of flavonoids is via scavenging free radicals and hence preventing their oxidation. The activity of flavonoids as antioxidants thus largely depends on the stability of their free radicals. Toward understanding the experimental trend in the antioxidant activity of the six isolated flavonoids, we perform

geometry optimization on free radicals derived from these flavonoids through abstraction of a hydrogen atom from the different aromatic OH group. The optimized geometries together with the total electronic energies of these radicals are reported in **Fig. 4**.

The optimized geometries of quercetin radicals (**Fig. 4**, **a**–**e**) possess planer geometry, $\varphi_{3\text{-}2\text{-}1'\text{-}6'} = 0°$. Conformers **b**, **c**, **d**, and **e**, are 2.61, 6.99, 13.84, and 22.68 kcal/mol less stable than conformer **a**, indicating that radical stability follows the order C-4′ > C-3′ > C-3 > C-7 > C-5.

Radicals of quercetin-3-*O*-α-D-arabinopyranoside (**2**), are reported in (**Fig. 4**, **f**–i). In these four radicals, ring B is non-planar with ring C ($\varphi_{3\text{-}2\text{-}1'\text{-}6'}$ = 29°–34°). In respective, conformers **g**, **h**, and **i** are 1.30, 13.97, and 23.68 kcal/mol less stable than conformer **f**. This suggests that glycosylation does not alter the relative stability of the different free radicals (C-4′ > C-3′ > C-7 > C-5).

Free radicals derived from the most stable conformer of quercetin-3-*O*-β-D-galactopyranoside (**3**) are shown in (**Fig. 4**, **j**–**m**). Loss of H from the OH group at C-3′ or C-4′ breaks the hydrogen bonding between ring B and the sugar group. Similar to radicals of compound **2**, these four radicals have nonplanar arrangements of rings B and C ($\varphi_{3\text{-}2\text{-}1'\text{-}6'}$ = 30°–60°). Compared to the most stable radical **j**, the radicals **k**, **l**, and **m** are 0.93, 10.21, and 19.44 kcal/mol less stable, respectively.

While radicals formed at C-4′ remain the most stable in both quercetin and its glycosides **2** and **3**, results suggest that quercetin radical is more stable. This is attributed to the planar geometry of quercetin radical, resulting in full conjugation through the molecule. This indicates that compound **1** is a better radical scavenger and hence a better antioxidant than compounds **2** and **3**. This is in agreement with experimental results. It is found that the antioxidant activity of **1** is ~14 µg/mL compared to ~35 µg/mL for **2** and ~37 µg/mL for **3**.

In order to investigate the influence of the sugar groups of compounds **4** and **5** on the stability of the various possible free radicals, we optimized the geometry of four free radicals of kaempferol (**Fig. 4**, **n**–**q**). Similar to quercetin, all radicals are planar. The trend in the stability of these four radicals follows the order C-3 ≈ C-4' > C-7 > C-5 (conformers **o**, **p**, and **q** are 0.03, 6.01, and 15.00 kcal/mol less stable than conformer **n**). This indicates that the 6.99 kcal/mol higher stability of C-4′ radical relative to C-3 radical in quercetin (**1**) is due to stabilization of the C-4′ radical by hydrogen bonding with the OH group at C-3′. In another mean, the radical formed at C-4' in quercetin (**1**) is more stable than that of kaempferol. Quercetin (**1**) is hence a better radical scavenger and better antioxidant than kaempferol. This matches the experimental finding of an antioxidant activity of ~14 µg/mL for quercetin and ~40 µg/mL for kaempferol.

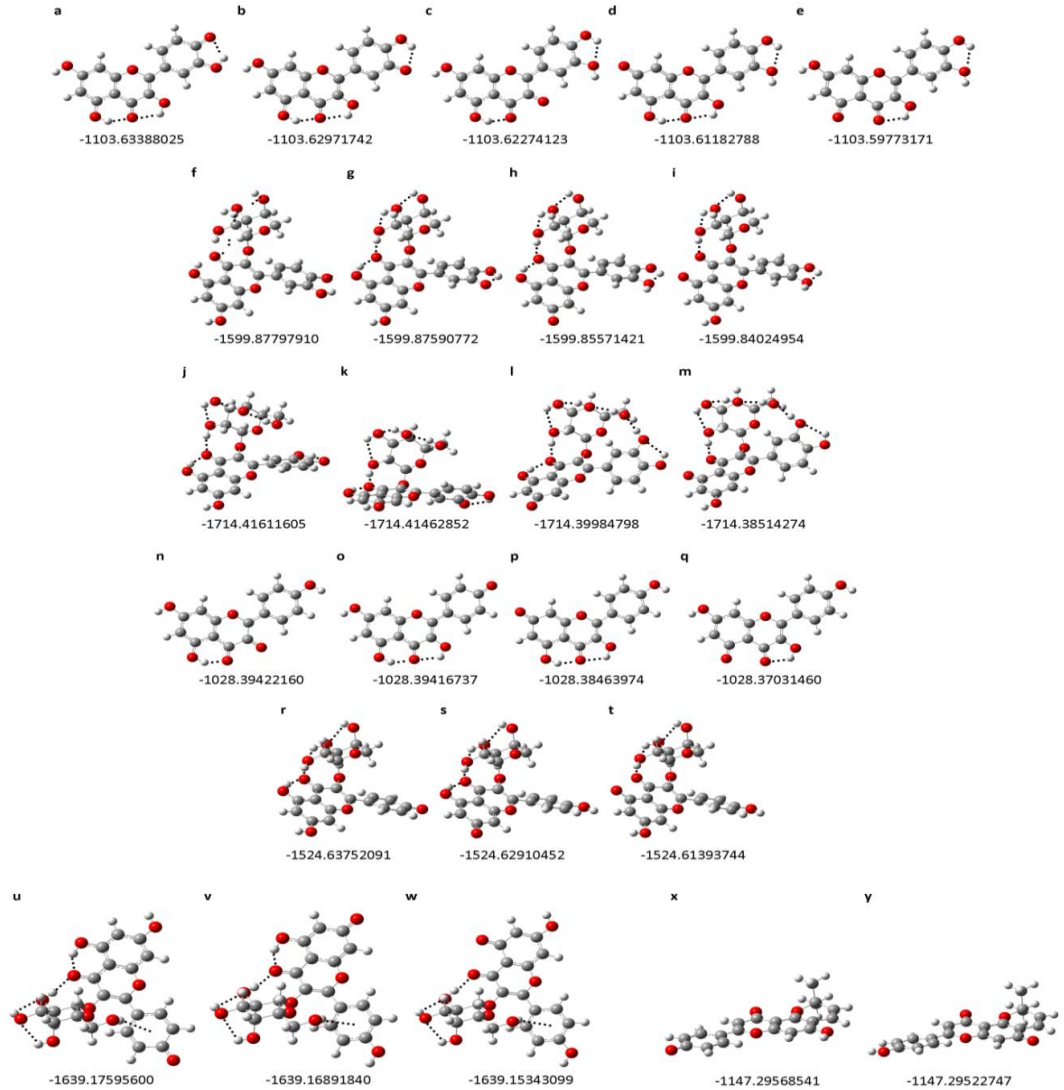

Fig 4: Optimized geometries of free radicals of compounds **1** (**a**–**e**), **2** (**f**–**i**), **3** (**j**–**m**), kaempferol (**n**–**q**), **4** (**r**–**t**), **5** (**u**–**w**), and **6** (**x**, **y**) generated by abstraction of a hydrogen atom from the aromatic OH groups. Numbers represent the total electronic energy (in a.u.) and doted lines show intramolecular O–H···O hydrogen bonds and O–H···π interactions.

Free radicals formed by the removal of hydrogen from the aromatic OH groups at C-4′, C-5, and C-7 of compound **4** are reported in **Fig. 4** (conformers **r**–**t**) and those formed of compound **5** are shown in **Fig. 4** (**u**–**w**). Free radicals of both compounds are non-planar, $\varphi_{3\text{-}2\text{-}1'\text{-}6'}$ = 27°–32° for radicals of **4** and $\varphi_{3\text{-}2\text{-}1'\text{-}6'}$ = 12°–19° for radicals of **5**. Calculations show that structures **s** and **t** are 5.28 and 14.80 kcal/mol less stable than structure **r** and that structures **v** and **w** are 4.42 and 14.13 kcal/mol less stable than structure **u**. This shows that glycosylation of kaempferol does not alter the relative stability of its free radicals. Again the planarity of kaempferol radicals allow for full conjugation and thus kaempferol is expected to possess a higher antioxidant activity than **4** and **5**. It is experimentally found that kaempferol has an antioxidant activity of ~40 µg/mL compared to ~54 µg/mL for compound **4** and ~59 µg/mL for compound **5**.

Two radicals are optimized for vogelin J (**6**) (**Fig. 4**, **n** and **o**). Radical **x** (formed at C-4′) is only 0.29 kcal/mol more stable than radical **y** (formed at C-7). A structural comparison between kaempferol and vogelin J (**6**) shows that the higher stability of the free radicals at C-4′ relative to that at C-7 in the case of kaempferol is mainly due to the OH at C-3. The absence of this hydroxyl group in **6** seems to destabilize the radical at C-4′ by ~ 6 kcal/mol. This is likely because the electronegative oxygen atom at C-3 provides stabilization of the free radical. Among the six isolated compounds and kaempferol, vogelin J (**6**) is thus expected to have the lowest antioxidant activity. In fact our experimental measurements have shown no antioxidant activity of vogelin J (**6**) up to 100 µg/mL.

3.3. Antiviral activities of the isolated flavonoids

A wide variety of active phytochemicals have been found to have therapeutic effects against different genetically and functionally diverse viruses [42]. Searching for therapeutic agents among flavonoids is of great interest being safely consumed with large quantities in fruits and vegetables [43]. Some plant-derived extracts rich with flavonoids such as quercetin glycosides showed high activity against viral infection in *in vivo* and *in vitro* studies. Quercetin and quercetin-3-*O*-glycosides showed anti-Mayaro virus activity [1]. The flavonoid dihydroquercetin (taxifolin) exhibit strong *in vitro* and *in vivo* antiviral activities against CVB4 [12] and HAV [44]. Some flavonoids have also showed antiviral activities against influenza virus [45, 46], HSV-1 and HSV-2 [22, 47], enterovirus 71 [48] and dengue virus [49]. In our efforts to explore the potential of natural products as source of low toxicity and high antiviral selectivity candidate, we examined the *in vitro* antiviral activity of six pure flavonoids (**1**–**6**) from *F. virens*. To avoid the toxic effect of the flavonoid on the virus carrier cells, Vero cells, the maximum non-toxic concentration (MNTC) and the 50% cytotoxic concentration (CC_{50}) of the flavonoids **1**–**6** were first determined. The results revealed noticeable low cytotoxic effect for the glycosylated flavonoid (**2**–**5**) than the aglycones **1** and **6**. Consequently, the inhibitory effect of each compound at its MNTC was then examined against HAV and CVB4 viruses using the MTT assay. Results of the virus inhibitory effects of the flavonoids **1**–**6** (Table 1) demonstrated that quercetin (**1**), quercetin-3-*O*-α-D-arabinopyranoside (**2**), and vogelin J (**6**) exert mild inhibitory (20.3, 10.4 and, 8.6%) activity on CVB4 at their MNTC, 15.6, 15.6, and 62.5 µg/mL, respectively. On the other hand, only the quercetin glycosides **2** and **3** exhibited mild inhibitory effects (8.0 and 12.3% at their MNTC (62.5 and 15.6) on HAV, respectively. Kaempferol-3-*O*-glycosides (**4** and **5**) didn't produce antiviral activity at the experimental conditions. Our results demonstrate that leaves of *F. virens* is a cheap source of flavonoids with mild antiviral activity against the CVB4 and HAV viruses.

Table 1: Cytotoxicity on Vero cells, and the anti-CVB4 and anti-HAV effects of the flavonoids **1**–**6**.

	Cytotoxicity (µg/mL)[a]		Virus inhibitory activities (%)[a]	
Compound	**CC_{50}**	**MNTC**	**CVB4**	**HAV**
1	199.3±2.3	15.6±1.2	20.3±0.7	1.2±0.05
2	360.3±3.7	62.5±3.2	8.6±0.11	8.0±0.04
3	313.3±2.11	15.6±2.1	0	12.3±0.8
4	329.9±5.3	62.5±2.	0	0
5	313.3±1.19	31.3±1.9	0	0
6	195.1±2.4	15.6±1.5	10.4±0.14	0.4±0.13
Doxorubicin[b]	2.5±0.14	–	–	–
Ribavirin[c] (1.25 µg/mL)	–	–	49.0±1.2	54.0±1.7

[a] The values represent the mean ± SD
[b] Doxorubicin as standard cytotoxic drug
[c] Ribavirin as standard antiviral drug

Although the low antiviral activity of quercetin and its 3-*O*-glycosides at our experimental conditions, this is the first report on the anti-CVB4 and HAV properties of these flavonoids. Our results are an addition to the known antiviral properties of flavonoids shown in recent reviews [42, 50]. However, further anti-CVB4 and HAV investigations by *in vivo* methods are required to ascertain the antiviral effects of these fairly common metabolites in plants. Exploring for naturally occurring flavonoids with antiviral activities has started since the 1940s, however, efforts are still necessary to develop these natural compounds as therapeutic antiviral agents [43].

4. Conclusion

Flavonoids in fruits and vegetables make them favorable healthy foods because of their antioxidant properties associated with protection against inflammation, cancer, and numerous other chronic diseases. The structure-antioxidant relationships of flavonoids have been discussed in various reports. However, the majority of these discussions were almost descriptive rather than elucidative [51]. An explanation attempt based on a quantum chemical study was shown for the flavonoid aglycones [39]. Our calculations shown here demonstrated that flavonoid aglycones containing OH at position 3 are planar and that glycosylation distorts this planarity. The calculations predict antioxidant activity of the

compounds that follow the trend **1** > **2** ≈ **3** > kaempferol > **4** ≈ **5** > **6**, which is in agreement with experimental findings. Results indicate that the antioxidant activity of flavonoids increases with the number of aromatic OH groups, especially at ring B, and that an OH at position 3 is important for the flavonoids. The results are also suggesting two structural requirements for efficient antioxidant flavonoids. These are planarity and number of phenolic hydroxyl groups. Planarity provides extended conjugation through the aromatic moiety and hence stabilizes the formed free radicals. Phenolic hydroxyl groups stabilize the radical through hydrogen bonding and via the electron withdrawing nature of the highly electronegative oxygen atoms. These factors should be taken in consideration when designing highly efficient antioxidants. In addition, despite the low viral inhibitory activities of the tested flavonoids, our data from the antiviral experiment are an important step in the evaluation of natural products as sources of novel drugs to be used to overcome drug resistance, or to replace currently used antivirals with serious side effects.

5. Conflict of interest

The authors declare that there is no conflict of interest.

6. Acknowledgments

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