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A NEW STEROID SAPONIN FROM THE RHIZOMES OF *Paris polyphylla* var. *yunnanensis*

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Phytochemical investigation of the ethanol extract from the rhizomes of Paris polyphylla *var.* yunnanensis *led to the isolation of three steroid saponins (**1–3**) and two triterpenoid saponins (**4**, **5**). Among them, compound **1** is a new steroid saponin, and **3–5** were obtained for the first time from the genus* Paris. *Their structures were elucidated on the basis of extensive analyses of spectroscopic data including IR, UV, MS, and 1D and 2D NMR. All of these compounds were tested for their cytotoxic activities on human nasopharyngeal cancer cells (CNE) and antiviral activities against respiratory syncytial virus (RSV). Steroidal saponins **1** and **2** showed moderate effects on CNE cells, with IC_{50} values 32.56 and 33.10 μM, respectively.*

Keywords: *Paris polyphylla* var. *yunnanensis*, Liliaceae, saponin, human nasopharyngeal cancer cells, respiratory syncytial virus.

The genus *Paris* (family Liliaceae) consists of more than 24 species worldwide, 19 of which are distributed in China, mainly in Yunnan and Sichuan Provinces of southwestern China [1]. There is a long history of the rhizomes of *Paris polyphylla* var. *yunnanensis* being used as a traditional Chinese medicine to treat of traumatic injuries, abscess, mastitis, chronic bronchitis, and hepatopathy [2, 3]. Pharmacological studies have demonstrated that *P. polyphylla* var. *yunnanensis* possessed antitumor, hemostatic, immunoregulatory, sedative, antifungal, and gastro-protective effects [4–11]. Phytochemical studies have shown the plant to be rich in steroidal saponins [12–15]. In addition, other classes of compounds, such as phytocholesterols [16, 18], ecdysteroids [16–18], flavones [18], and triterpenoid saponins [19], have also been isolated. In previous research work, we obtained some new and (or) cytotoxic steroidal saponins, sterol glycosides, and triterpenoid saponins in the 95% ethanol-eluting fraction of macroporous resin from the crude extract of the rhizomes of *P. polyphylla* var. *yunnanensis* [19–21]. As a part of our continuing studies in the isolation of interesting and biologically active compounds from the plant, three steroid saponins (**1–3**) and two triterpenoid saponins (**4**, **5**) were isolated in the 30% ethanol-eluting fraction of macroporous resin. Compound **1** is a new steroid saponin, and **3–5** are obtained for the first time from the genus *Paris*. In addition, the isolated compounds were evaluated for their cytotoxic activities on CNE cells and antiviral activities against RSV. This article reports the isolation, structural elucidation, and antiproliferative and antiviral activities of these compounds.

Ethanol (30%) of macroporous resin from the ethanol extract of the plant was subjected to silica gel, ODS silica gel, Sephadex LH-20, and preparative HPLC to afford five compounds.

Compound **1** was obtained as a white powder. The molecular formula was determined as $C_{56}H_{88}O_{30}$ based on its HR-ESI-MS at *m/z* 1263.52522 $[M + Na]^+$. The IR spectrum exhibited absorption bands at 3399 and 1644 cm^{-1}, indicating the presence of hydroxyl and double bond. Acid hydrolysis of **1** afforded D-glucose, D-galactose, D-xylose, and L-rhamnose, which were determined by HPLC analysis of their derivatives.

The ^1H NMR spectrum of **1** (Table 1) showed signals of two methyl singlet at δ 1.33 and 1.05 (each 3H, s), one methyl doublet at δ 1.69 (3H, d, J = 5.9 Hz), three olefinic protons at 5.54 (1H, d, J = 5.2 Hz), 5.06 (1H, s) and 4.97 (1H, s), and five anomeric protons at δ 6.31 (1H, s), 5.77 (1H, d, J = 8.1 Hz), 4.89 (1H, d, J = 7.6 Hz), 4.83 (1H, d, J = 7.4 Hz), and 4.70 (1H, d, J = 7.8 Hz).

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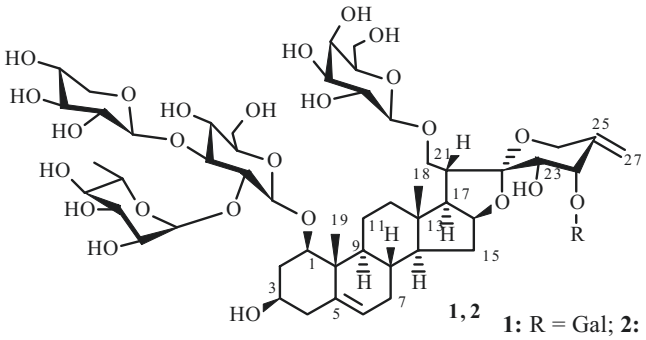
TABLE 1. ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) Spectral Data of **1** (C_5D_5N, δ, ppm, J/Hz)*

C atom	δ_C	δ_H	C atom	δ_C	δ_H
1	85.2	3.74 (overlapped)	Rha		
2	38.3	2.39 (m), 2.62 (m)	1″	101.9	6.31 (s)
3	68.4	3.74 (overlapped)	2″	72.6	4.72 (overlapped)
4	44.0	2.53 (m), 2.68 (m)	3″	72.6	4.72 (overlapped)
5	139.6	–	4″	74.4	4.26 (m)
6	125.2	5.54 (d, J = 5.2)	5″	69.8	4.77 (overlapped)
7	32.1	1.44 (m), 1.73 (m)	6″	19.5	1.69 (d, J = 5.9)
8	33.3	1.44 (m)	Gal		
9	50.6	1.56 (m)	1‴	104.3	5.77 (d, J = 8.1)
10	43.0	–	2‴	71.5	4.56 (overlapped)
11	24.3	1.46 (m), 2.78 (m)	3‴	73.5	4.74 (overlapped)
12	40.3	1.48 (m), 1.88 (m)	4‴	71.1	4.56 (overlapped)
13	41.3	–	5‴	75.5	4.66 (dd, J = 8.2, 3.0)
14	57.3	1.17 (m)	6‴	62.6	4.22 (m), 4.35 (overlapped)
15	32.6	1.44 (m), 1.84 (m)	Gal		
16	83.7	4.54 (overlapped)	1⁗	105.4	4.83 (d, J = 7.4)
17	58.6	1.94 (m)	2⁗	72.7	4.41 (overlapped)
18	17.2	1.05 (s)	3⁗	75.7	4.12 (dd, J = 9.3, 3.2)
19	15.3	1.33 (s)	4⁗	70.5	4.43 (m)
20	43.9	3.42 (q, J = 6.5)	5⁗	77.3	4.05 (overlapped)
21	70.2	3.99 (m), 4.45 (m)	6⁗	62.6	4.22 (m), 4.35 (overlapped)
22	111.7	–	Xyl		
23	71.5	4.32 (d, J = 6.3)	1⁗′	105.5	4.89 (d, J = 7.6)
24	82.4	4.68 (overlapped)	2⁗′	74.9	3.91 (overlapped)
25	143.8	–	3⁗′	78.6	4.07 (overlapped)
26	61.8	3.99 (d, J = 8.9), 4.84 (d, J = 7.5)	4⁗′	70.2	4.07 (overlapped)
27	114.2	4.97 (s), 5.06 (s)	5⁗′	67.5	3.68 (dd, J = 10.5, 10.5), 4.24 (m)
Glc					
1′	100.7	4.70 (d, J = 7.8)			
2′	76.6	4.05 (overlapped)			
3′	88.6	4.00 (m)			
4′	70.9	3.80 (m)			
5′	77.9	3.74 (overlapped)			
6′	63.5	4.22 (m), 4.44 (overlapped)			

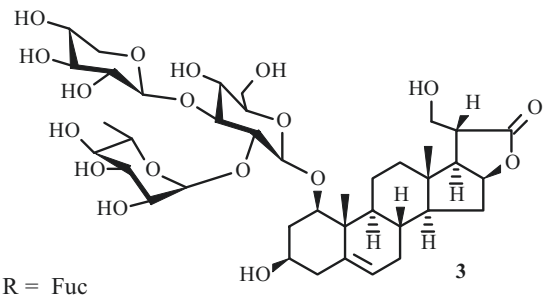
*Signals were assigned on the basis of ^{1}H–^{1}H COSY, HSQC, HMBC, and NOESY experiments.

The ^{13}C NMR (Table 1) and DEPT spectra exhibited 56 carbon signals, including five quaternary carbons, 35 methines, 13 methylenes, and three methyls. Four olefinic carbon signals at δ 143.8, 139.6, 125.2, and 114.2, a characteristic quaternary carbon signal at δ 111.7, and five anomeric carbon resonances at δ 105.5, 105.4, 104.3, 101.9, and 100.7 were observed, suggesting that **1** was a spirostanol glycoside with five sugars. Comparison of the ^{1}H and ^{13}C NMR spectroscopic data of **1** (Table 1) with those of parisyunnanoside I [22] identified the aglycone of **1** as (23*S*,24*S*)-spirost-5,25(27)-diene-1*β*,3*β*,21,23*α*,24*α*-pentol. The chemical shift values of the aglycone were in accordance with those of parisyunnanoside I, which indicated that the sugar chains were connected with C-1, 21, and 24 of compound **1**.

For the sugar moiety, the five anomeric protons at δ 6.31 (1H, br.s), 5.77 (1H, d, J = 8.1 Hz), 4.89 (1H, d, J = 7.6 Hz), 4.83 (1H, d, J = 7.4 Hz), and 4.70 (1H, d, J = 7.8 Hz) showed correlations with five carbons at δ 101.9, 104.3, 105.5, 105.4, and 100.7, respectively, in the HSQC spectrum. The J values of the anomeric proton signals indicated the presence of *β*-glucopyranosyl, *α*-rhamnopyranosyl, *β*-galactopyranosyl, and *β*-xylopyranosyl. Comparison of the spectroscopic data of the sugar part of **1** with those of parisyunnanoside I showed that they were similar, except for an additional methylene group (δ_C 62.6) in **1** instead of the methyl group (δ_C 16.9) in the sugar moiety at C-24. Moreover, the ^{1}H and ^{13}C NMR spectroscopic data of the sugar moiety at C-24 closely resembled those of 24-*O*-*β*-D-galactopyranosyl-(23*S*,24*S*)-spirost-5,25(27)-diene-1*β*,3*β*,23,24-tetraol-1-*O*-*β*-D-xylopyranosyl-(1→6)-*β*-D-glucopyranosyl-(1→3)-[*α*-L-rhamnopyranosyl-(1→2)]-*β*-D-glucopyranoside [23], which suggested the sugar moiety at C-24 to be *β*-D-galactopyranosyl.



1, 2 **1:** R = Gal; **2:** R = Fuc



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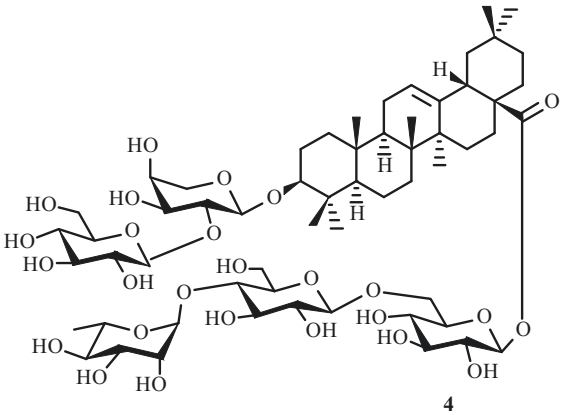

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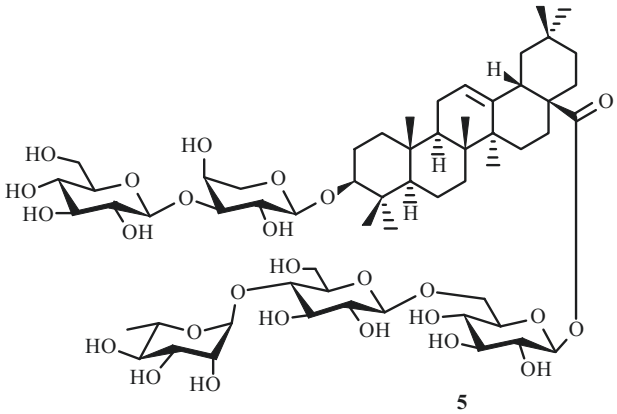

5

The HMBC correlations between the signals of C-1 (δ 85.2) of the aglycone and H-1′ [δ 4.70 (1H, d, J = 7.8 Hz)] of Glc, between C-2′ (δ 76.6) of Glc and H-1″ [δ 6.31 (1H, s)] of Rha, and between C-3′ (δ 88.6) and H-1‴′ [δ 4.89 (1H, d, J = 7.86 Hz)] of Xyl indicated that the connectivity of the sugars at C-1 was at 1-*O*-*α*-L-rhamnopyranosyl-(1→2)-[*β*-D-xylopyranosyl-(1→3)]-*β*-D-glucopyranosyl.

In addition, cross-peaks between C-21 (δ 70.2) of the aglycone and H-1⁗ of the Gal [δ 4.83 (1H, d, J = 7.4 Hz)] and between C-24 (δ 82.4) of the aglycone and H-1‴ of the Gal [δ 5.77 (1H, d, J = 8.1 Hz)] were clearly observed. Therefore, structure **1** was determined as (23*S*,24*S*)-spirost-5,25(27)-diene-1*β*,3*β*,21,23*α*,24*α*-pentol-1-*O*-{*α*-L-rhamnopyranosyl-(1→2)-[*β*-D-xylopyranosyl-(1→3)]-*β*-D-glucopyranosyl}-21-*O*-*β*-D-galactopyranosyl-24-*O*-*β*-D-galactopyranosyl.

The known compounds were identified as parisyunnanoside I (**2**) [22], 3*β*,21-dihydroxypregnan-5-en-20*S*-(22,16)-lactone-1-*O*-*α*-L-rhamnopyranosyl (1→2)-[*β*-D-xylopyranosyl-(1→3)]-*β*-D-glucopyranoside (**3**) [4], 3-*O*-*β*-D-glucopyranosyl (1→2)-*α*-L-arabinopyranosyl oleanolic acid 28-*O*-*α*-L-rhamnopyranosyl (1→4)-*β*-D-glucopyranosyl (1→6)-*β*-D-glucopyranoside (**4**) [24], and 3-*O*-*β*-D-glucopyranosyl (1→3)-*α*-L-arabinopyranosyl oleanolic acid 28-*O*-*α*-L-rhamnopyranosyl (1→4)-*β*-D-glucopyranosyl (1→6)-*β*-D-glucopyranoside (**5**) [24] by comparing their physical and spectroscopic data with the literature. To the best of our knowledge, **3**–**5** are obtained for the first time from the genus *Paris*.

The isolated compounds **1**–**5** were tested for their cytotoxicities against CNE cells *in vitro* with cisplatin as positive control. Steroidal saponins **1** and **2** exhibited moderate effects on CNE cells, with IC_{50} values 32.56 and 33.10 μM, respectively (the IC_{50} value of cisplatin was 9.35 μM). Steroidal saponins **3** did not show any cytotoxicities at the concentration of 100 μM. Our previous studies showed that diosgenin and pennogenin saponins were the most active constituents, which had a close relationship with the type and sequence of saccharides [20, 21]. Comparing the structure of the three steroidal saponins, there is an additional carbonyl group in **3** instead of the F ring in **1** and **2**, which cause the activities to disappear. The results indicated that the F ring in steroidal saponins might be the active group against CNE cells. In addition, triterpenoid saponins **4** and **5** did not show cytotoxicities at the concentration of 100 μM. We had found that triterpenoid saponins with the aglycone of 3*β*-hydroxy-olea-12-en-28-oic acid exhibited an inhibitory effect on CNE cells in our previous study [19]. However, the inhibitory effect disappeared when the 28-carboxylic acid in this aglycone was esterified by sugar chains as compound **4** and **5**. The results indicated that the cytotoxicities of triterpenoid saponins might be related to 28-carboxylic acid.

Besides, we preliminarily assessed the *in vitro* antiviral activities of the isolated compounds against RSV infection due to the traditional use of the plant for the treatment of chronic bronchitis [25]. However, all the isolated compounds were inactive against RSV infection on host cells at the high concentration of 100 μM.

EXPERIMENTAL

General Experimental Procedures. Optical rotations were determined on a Jasco P-1020 digital polarmeter (Jasco, Tokyo, Japan). UV spectra were recorded using a Jasco V-550 UV/VIS spectrophotometer (Jasco, Tokyo, Japan). An JASCO FT/IR-480 plus FT-IR spectrometer was used to determine IR spectra. ESI-MS data were obtained with a Finnigan LCQ Advantage Max mass spectrometer (Thermo Electron, USA). HR-ESI-MS data were measured on an Agilent 6210 LC/MSD TOF mass spectrometer (Waters, USA). 1D and 2D NMR spectra were taken on a Bruker AV-400 (Bruker, Faellanden, Switzerland) spectrometer using TMS as the internal standard, and chemical shifts (δ) are expressed in ppm with reference to the solvent signals. Open column chromatography (CC) was performed using macroporous resin (Diaion HP-20), silica gel (200–300 mesh; Qingdao Marine Chemical, Inc.), ODS silica gel (50 μm; YMC), and Sephadex LH-20 (Pharmacia). Thin-layer chromatography (TLC) was performed using precoated silica gel plates (GF254; Yantai Chemical Industry Research, Inc.). Analytical HPLC was carried out on a Waters chromatograph equipped with an evaporative light-scattering detector, a P680 pump, and a C_{18} reversed-phase column (Cosmosil, 5 μm, 4.6 mm × 250 mm). Preparative HPLC was performed on a Varian Prostar system equipped with UV detectors (Varian, USA) and a preparative Cosmosil C_{18} column (20 mm × 250 mm, Nacalai Tesque, Japan). All the reagents were purchased from Tianjin Damao Chemical Company (Damao, Tianjin, China).

Plant Material. The dried rhizomes of *Paris polyphylla* var. *yunnanensis* were collected in Yunnan Province, China, in February of 2009, and authenticated by Prof. Guang-Xiong Zhou (College of Pharmacy, Jinan University). A voucher specimen (No. 20090226) was deposited in the Institute of Traditional Chinese Medicine and Natural Products, Jinan University, Guangzhou, P. R. China.

Extraction and Isolation. Dried rhizomes of *Paris polyphylla* var. *yunnanensis* (10.0 kg) were pulverized and extracted with EtOH–H_2O (70:30, v/v) at room temperature, and the combined extracts were concentrated to afford the crude extract (1.5 kg). The extract was dissolved in 2 L of water and then passed through a Diaion HP-20 column eluted with EtOH–H_2O (30:70, 60:40, 95:5, v/v, successively, 15 L for each).

The fraction eluted by EtOH–H_2O (30:70) was subjected to column chromatography over silica gel (200–300 mesh) using eluent $CHCl_3$–MeOH (98:2→0:100), and the extracts were combined to give seven portions (A–G). Portion D (15.3 g) was subjected to ODS silica gel column chromatography using eluent MeOH–H_2O (15:85, 30:70, 50:50, 70:30, 90:10, v/v, respectively, 2.5 L for each) and the extracts were combined to give 13 fractions (a–m). Fraction D-b (856.4 mg) was purified by Sephadex LH-20 (MeOH–H_2O, 1:1) and then preparative HPLC to yield compound **1** (MeOH–H_2O, 70:30, 210 nm, retention time 25.3 min, 10.0 mg) and compound **2** (MeOH–H_2O, 70:30, 210 nm, retention time 30.5 min, 39.4 mg). Fraction D-c (364.7 mg) was subjected to Sephadex LH-20 CC using MeOH–H_2O (50:50) as eluent and recrystallized from MeOH to yield compound **3** (11.3 mg). Portion F (12.0 g) was subjected to Sephadex LH-20 CC using $CHCl_3$–MeOH (50:50) as the eluent and recrystallized from $CHCl_3$ to obtain compound **4** (13.4 mg). Portion G (8.9 g) was subjected to column chromatography over silica gel (200–300 mesh) using eluent $CHCl_3$–MeOH (100:0→0:100) and preparative HPLC to yield compound **5** (MeOH–H_2O, 70:30, 210 nm, retention time 33.6 min, 14.2 mg).

(23*S*,24*S*)-Spirost-5,25(27)-diene-1β,3β,21,23α,24α-pentol-1-*O*-{α-L-rhamnopyranosyl-(1→2)-[β-D-xylopyranosyl-(1→3)]-β-D-glucopyranosyl}-21-*O*-β-D-galactopyranosyl-24-*O*-β-D-galactopyranosyl (1). White amorphous powder. $[\alpha]_D^{25.4}$ –47.5° (*c* 1.0, MeOH). UV (CH_3OH, λ_{max}, nm): 203. IR (KBr, ν_{max}, cm^{-1}): 3399, 1644, 1372, 1049. For ^1H (400 MHz) and ^{13}C NMR (100 MHz) spectral data, see Table 1. HR-ESI-MS *m/z* 1263.52522 $[M + Na]^+$ (calcd for $C_{56}H_{88}O_{30}Na$, 1263.52526).

HPLC Spectrum of Sugar Derivatives of 1. Compound **1** (3 mg) was treated with 4 mol/L (10 mL) under reflux conditions at 90°C being maintained for 6 h. The reaction mixture was partitioned between $CHCl_3$ and H_2O three times, and then the H_2O layer was concentrated to dryness. The mixture was dissolved in anhydrous pyridine (2 mL) and reacted with L-cysteine methyl ester hydrochloride (2 mg) in an oven at 60°C for 1 h. Isothiocyanate (2 mL) was added and the mixture warmed to 60°C for another 1 h. The reaction products were analyzed by HPLC; flow rate, 0.8 mL/min; sample size: 10 μL; column, Cosmosil 5C_{18}-MS-II column (4.6 × 250 mm i.d., Nacalai Tesque Inc.); mobile phase, 25% CH_3CN–H_2O (0.05% CH_3COOH); measurement wavelength, 250 nm; column temperature, 35°C. The same reactions were applied to standard sugars. The derivatives of D-galactose, D-glucose, D-xylose, and L-rhamnose gave peaks with retention times of 17.6, 18.5, 21.9, and 34.2 min, respectively.

Cells and Viruses. Human larynx epidermoid carcinoma (HEp-2, ATCC CCL-23) cells, as well as RSV A2 (ATCC-VR-1540) and Long (ATCC-VR-26) strains, were purchased from Medicinal Virology Institute, Wu Han University. CNE (human nasopharyngeal carcinoma cell line) was provided by the Cell Bank of Type Culture Collection of the Chinese

Academy of Sciences. HEp-2 cells were grown in Dulbecco modified eagle medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and antimicrobials (growth medium, GM) and cultured at 37°C in a humidified atmosphere supplied with 5% CO_2. For RSV propagation, RSV stock diluted using DMEM with 2% FBS and antimicrobials (maintenance medium, MM) was added to the confluent HEp-2 cells. Virus titer was determined by plaque- reduction assay as approximately 6×10^6 plaque – forming unit (pfu) mL^{-1}. Virus was stored in 10% saturated sucrose solution at –80°C until use. CNE cells were maintained in the RPMI-1640 medium supplemented with 10% fetal bovine serum (Gibco). They were incubated at 95% relative humidity and 5% CO_2 at 37°C.

MTT Assay. MTT assay was used to assess the cytotoxicities of the compounds in the present study [26]. First at all, different concentrations of the compounds were added to the cells after incubation in a 96-well microtiter plate for 24 h; medium without the compounds was used as control. After incubation for 72 h, the medium was replaced by 30 μL of the MTT, and the cells were further incubated for another 4 h to allow MTT formazan formation. Then the medium was replaced by DMSO (200 μL) in each well to dissolve the formazan crystals. Absorbancies were detected by a microplate reader (Thermo Scientific, USA) at 570 nm. Each assay was performed three times, and the IC_{50} value was calculated as the concentration giving 50% inhibition.

Antiviral Assay. CPE reduction assay was adopted to evaluate the antiviral activities of the compounds as described in the previous report [27]. First of all, the cytotoxic activities of the tested samples on HEp-2 cells were observed under a light microscope. The maximum noncytotoxic concentration (MNCC) of the sample was defined as the maximum concentration of the sample that did not exert a toxic effect (0% CPE) under microscopic monitoring. Then, the antiviral activities of the samples were tested at the beginning concentrations of NMCC. The concentration that reduced 50% of the CPE with respect to the virus control was estimated from the plots of the data and was defined as the 50% inhibitory concentration (IC_{50}).

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