

Virucidal Effect of Guggulsterone Isolated from *Commiphora gileadensis*

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ABSTRACT

Commiphora gileadensis, locally known as becham, is a plant used in traditional Arabian medicine for treating headache, constipation, stomach, joint pain, and inflammatory disorders. Several studies have reported its antibacterial properties; however, no study has demonstrated its antiviral activity. This study aimed to evaluate the antiviral activity of *C. gileadensis* as well as to isolate its active compound and investigate its mode of action. This activity was evaluated using 4 viruses, herpes simplex virus type 2 (HSV-2), respiratory syncytial virus type B (RSV-B), coxsackie virus B type 3, and adenovirus type 5 by performing the plaque reduction assay and the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays for enveloped and nonenveloped viruses, respectively. The methanol extract of *C. gileadensis* leaves only showed antiviral activity against enveloped viruses with a selectivity index of 11.19 and 10.25 for HSV-2 and RSV-B, respectively. The study of the mechanism underlying antiviral activity demonstrated a virucidal effect by direct contact with these target viruses. The active compound, isolated using bio-guided assays involving TLC, was identified as guggulsterone by HPLC-diode array detection coupled with electrospray ionization mass spectrometry. Guggulsterone is an antagonist of the bile acid receptor and a modulator of cholesterol metabolism; however, its antimicrobial properties have been reported for the first time in this study.

Introduction

Six species of the genus *Commiphora* grow in Saudi Arabia, especially in the west and southwest regions [1]. *Commiphora gileadensis* (L.) C. Chr (syn: *Commiphora opobalsamum* [L.] Engl.), generally referred to as “balsam of Mecca” [2], belongs to the family Burseraceae. It is widely found in the Mediterranean Basin, in areas around the Red Sea, particularly on the borders of Saudi Arabia,

Yemen, Oman, and Eritrea [3]. *C. gileadensis* locally known as *ood-e-balsan*, *bechan*, or *balessan*, is a small, thorny, medicinal tree that widely grows in Mecca region of Saudi Arabia. *Bechan* is a large, strong-smelling green shrub that grows to 10–12 feet [4]. On damage to the bark, it produces an oleo-gum resin, which is fragrant. The plant is famous for producing this valuable fragrant resin, as well as for its medicinal properties [5]. In traditional Arabian medicine, *C. gileadensis* extracts are used to treat headache,

ABBREVIATIONS

ADV-5	adenovirus type 5
CC ₅₀	50% cytotoxic concentration
CVB-3	coxsackievirus B type 3
HEp-2	human epithelial type 2
HPLC-DAD-ESI-MS/MS	HPLC-diode array detection coupled with electrospray ionization mass spectrometry
HSV-2	herpes simplex virus type 2
IC ₅₀	50% inhibitory concentration
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
Rf	retention factor
RSV-B	respiratory syncytial virus type B
SI	selectivity index
Slv	virucidal selectivity index
VC ₅₀	50% virucidal concentration
Vero	African green monkey kidney

urinary retention, jaundice, constipation [6], stomach, and liver diseases, joint pain, and inflammatory disorders [7]. Conversely, the phytochemical screening of the aerial parts of this plant revealed the presence of flavonoids, sterols, triterpenes, saponins, volatile bases, and oils [8]. The plant's aqueous extract was shown to have hypotensive effects [9] and protective effects on the gastric mucosa of rats [10]. In addition, its ethanol extract demonstrated a hepatoprotective effect and strongly induced apoptosis in immortalized and transformed human epidermal cell lines [11]. Reportedly, members of the genus *Commiphora* exhibit antibacterial activity. For example, the ethyl acetate extract of *Commiphora molmol* Engl. demonstrated moderate activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Plasmodium falciparum* [12], and the essential oil obtained from this plant exhibited high activity against both methicillin- and gentamicin-resistant *S. aureus* strains [13] and biofilm formation [14]. Furthermore, the methanol extracts of *C. gileadensis* rhizomes have good activity against staphylococci and streptococci [15].

Currently, to the best of our knowledge, there are no studies assessing the antiviral activities of *C. gileadensis*. Therefore, this study aimed to evaluate the antiviral activity of this plant as well as to isolate its active compound and determine its mode of action. The study was conducted on 4 viruses that are the causative agents of several human pathologies: HSV-2, which causes genital ulcers; CVB-3, which causes several conditions such as pleurodynia, myocarditis, pericarditis, and insulin-dependent diabetes; RSV-B, which causes respiratory diseases, such as runny nose, cough, and sore throat; and ADV-5, which causes conjunctivitis and upper respiratory tract infections, such as pharyngitis, laryngitis, and sinusitis.

Results

A total of 1.66 g of methanol extract was obtained from 5 g of dried leaves. After liquid-liquid separation, the weight of hexane,

dichloromethane, and methanol fractions was 370, 138, and 926 mg, respectively.

The cytotoxicity of the methanol extract of *C. gileadensis* leaves and its fractions was evaluated using Vero and HEp-2 cells by determining the CC₅₀ (► **Table 1**). Results revealed no significant difference among the extracts with regard to the cytotoxicity against the 2 cell lines. After liquid-liquid extraction, the low-polarity fraction (dichloromethane) demonstrated high cytotoxicity as its CC₅₀ highly decreased, while the polar fraction (methanol) demonstrated less cytotoxicity as its CC₅₀ substantially increased.

The IC₅₀ and SI of the methanol extract of *C. gileadensis* leaves against the enveloped viruses (HSV-2 and RSV-B) was approximately 20 µg/mL and > 10 (► **Table 1**). This extract demonstrated no activity against the nonenveloped viruses (CVB-3 and ADV-5).

After separation by liquid-liquid extraction, only the methanol fraction demonstrated activity with reduction in IC₅₀ to 10 µg/mL. The decrease in IC₅₀ with an increase in CC₅₀ strongly improved SI by approximately 10-fold (> 100) against both HSV-2 and RSV-B (► **Table 1**).

The methanol extract of *C. gileadensis* leaves demonstrated a virucidal activity against both HSV-2 and RSV-B by complete inhibition of virus mediated by direct contact for 2 h at a concentration of 10 × IC₅₀ (► **Fig. 1**). Moreover, less activity was observed during the viral adsorption, which can be explained by direct contact with the extract during the steps involving recognition and binding to specific host cell receptors.

The virucidal effect increased with contact time as shown in ► **Fig. 2**. At a concentration of 10 × IC₅₀, the methanol extract of *C. gileadensis* leaves completely inhibited enveloped viruses after 1 h of contact. The VC₅₀, which represents the extract concentration required to induce 50% viral inhibition after 2 h of contact with the virus, was 15.12 (13.16–17.08) and 13.92 (10.85–16.23) µg/mL for HSV-2 and RSV-B, respectively. VC₅₀ decreased compared with IC₅₀, providing a Slv of 15.80 and 17.17, respectively. The increase in SI indicated that the active extract was more effective when it was in direct contact with the virus (virucidal effect). Conversely, the methanol extract of *C. gileadensis* leaves showed preventive (before virus infection) or curative (after virus infection) antiviral effects.

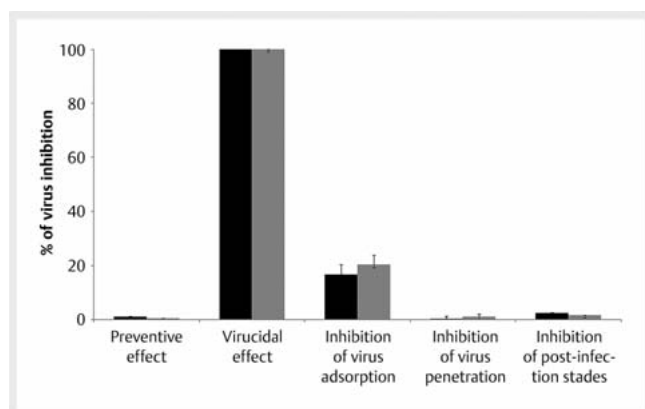
The separation of the methanol extract of *C. gileadensis* leaves using TLC and the mobile phase, as described in the Material and Methods section, showed only 1 band with the following characteristics: red color under white light, blue color under UV light, and an RF = 0.18. The dried fraction (11.6 mg) comprised 6.63% of the active fraction. A second separation of this band using TLC, using the same mobile phase system with changes in the proportion of solvents, revealed no impurities.

The dried active fraction was dissolved in 500 µL of 75% ethanol and subjected to HPLC-DAD-ESI-MS/MS. ► **Fig. 3** shows the UV profile (► **Fig. 3 a**) and total ion chromatogram registered in the positive-ionization mode (► **Fig. 3 b**). The HPLC profile showed 2 peaks: the one eluting at a retention time (Rt) of 18.62 min was the most abundant. This compound demonstrated the pseudomolecular ion at m/z 313 [M + H]⁺, which fragmented leading to a base peak at m/z 109 due to the steroid ring-A and a secondary ion at m/z 97. This fragmentation pattern led to the putative identification of guggulsterone (► **Fig. 4**) by comparing the data

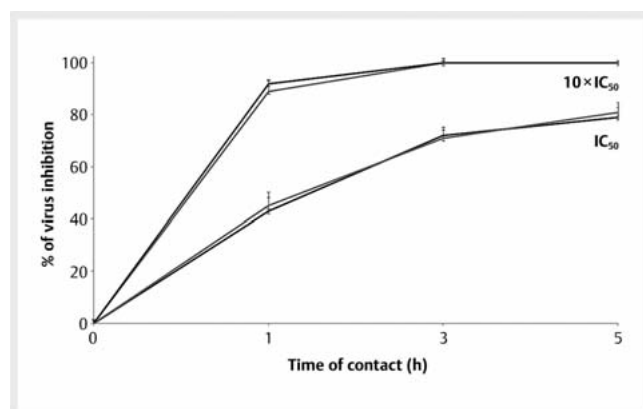
► **Table 1** Cytotoxicity and antiviral activity of *C. gileadensis* leaves methanol extract and its fractions.

Extracts	CC ₅₀ (µg/mL)		IC ₅₀ (µg/mL)				SI = CC ₅₀ /IC ₅₀			
	Vero cells	Hep-2 cells	HSV-2	RSV-B	CVB-3	ADV-5	HSV-2	RSV-B	CVB-3	ADV-5
Methanol extract	239 (261.11–216.89)	264 (290.85–234.15)	21.35 (23.12–19.58)	23.31 (25.54–21.08)	NA	NA	11.19 (11.84–10.54)	10.25 (21.47–9.57)	–	–
Hexane fraction	208.33 (225.39–190.94)	183.33 (203.02–163.64)	NA	NA	NA	NA	–	–	–	–
Dichloro-methane fraction	74.67 (82.88–66.46)	66.50 (75.58–56.92)	NA	NA	NA	NA	–	–	–	–
Methanol fraction	1320 (1463–1177)	1512 (1638–1386)	10.41 (11.37–9.45)	11.26 (11.69–10.83)	NA	NA	126.80 (131.61–121.99)	117.23 (125.67–108.79)	–	–

CC₅₀: cytotoxic concentration 50%; IC₅₀: inhibitory concentration 50%; SI: selectivity index; HSV-2 and RSV-B were grown on Vero cells, CVB-3 and ADV-5 on Hep-2 cells. CC₅₀ and IC₅₀ values are expressed as mean (95% confidence interval) of 3 independent experiments. NA: not active.



► **Fig. 1** The mechanism underlying anti-HSV-2 and RSV-B activity of the methanol extract of *C. gileadensis* leaves. Sample concentration = $10 \times IC_{50}$. Black color: HSV-2. Gray color: RSV-B. Data represent the percentage of virus inhibition by comparing with virus control and are expressed as mean (95% confidence interval) of 3 independent experiments.



► **Fig. 2** The effect of time course on HSV-2 and RSV-B inhibition by direct contact with the methanol extract of *C. gileadensis* leaves. Sample concentrations = $10 \times IC_{50}$ and IC_{50} . Black color: HSV-2. Gray color: RSV-B. Data represent the percentage of virus inhibition by comparing with virus control and are expressed as mean (95% confidence interval) of 3 independent experiments.

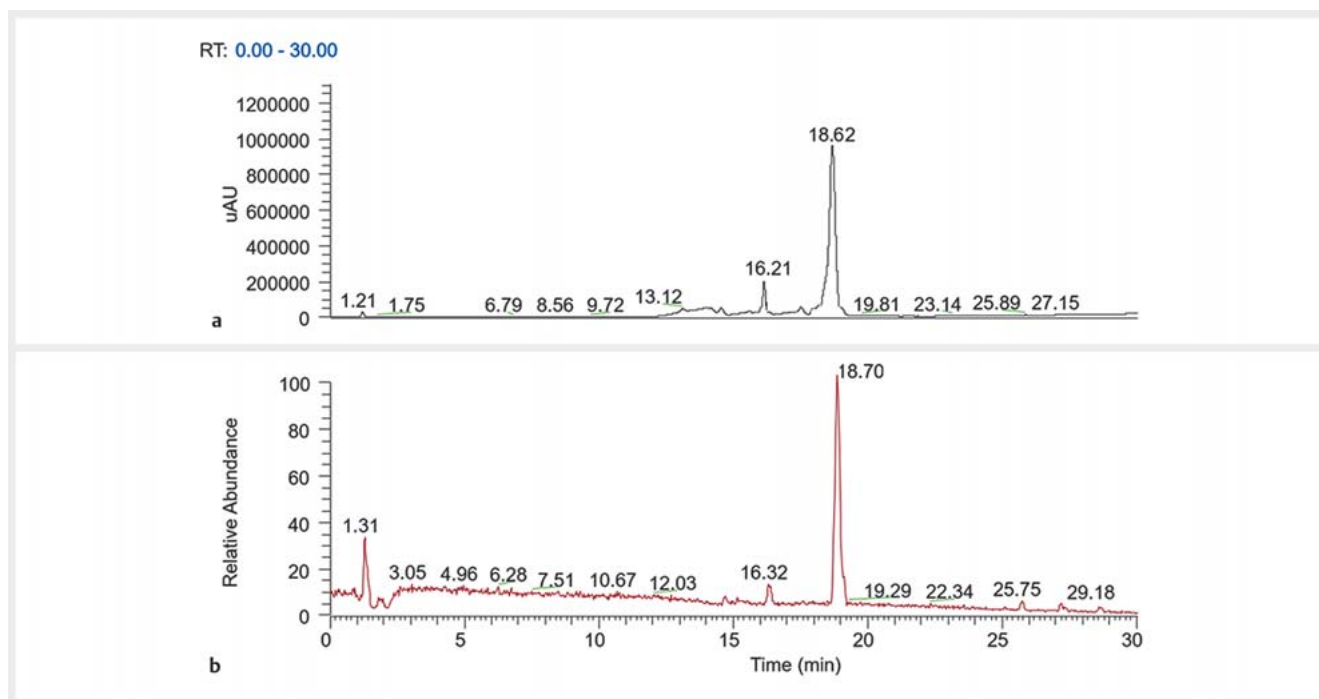
obtained in this investigation with those reported in the literature [16]. The peak eluting at a Rt of 16.21 min provided a pseudo-molecular ion at m/z 313 as well. As the registered signal in the MS spectrum was too weak for fragmentation, we hypothesized that this signal corresponded to another guggulsterone isomer. Therefore, based on the elution order, we considered that the E-isomer corresponded to the less retained compound. The chemical structure of the E- and Z-isomers of guggulsterone are illustrated in ► **Fig. 5**.

Discussion

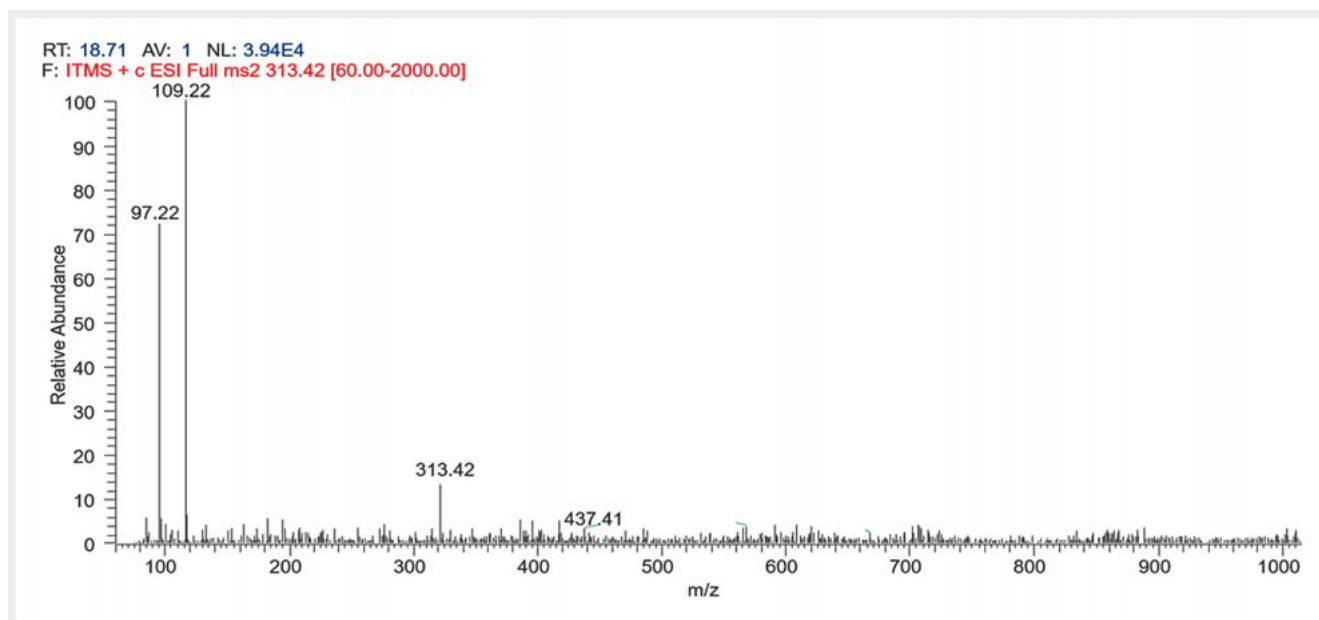
This is the first report describing the antiviral effect of *C. gileadensis*. The methanol extract of leaves demonstrated this activity and appeared to target enveloped viruses (HSV-2 and RSV-B). This ac-

tivity was clearly identified to be virucidal in nature, probably owing to the interaction of the active compound with receptor proteins present on the envelope, which could inhibit the ability of these viruses to bind to specific cell receptors, thus rendering them incapable of infecting host cells.

Studies have also reported the antiviral activity of other members of the genus *Commiphora*. For example, the crude extract of *Commiphora swynnertonii* Burt, exhibits a strong antiviral activity against Newcastle disease virus [17]. Moreover, the methanol extracts of *C. molmol*, *Commiphora parvifolia* Engl., and *Commiphora erythraea* Engl. have been reported to have activities against hepatitis C [18], herpes virus type 1 [19], and parainfluenza type 3 virus [20], respectively. The compounds responsible for these activities appear to be terpenoids as the main chemical constituents of the methanol of *C. parvifolia* are triterpenoids [19], while



► Fig. 3 UV profile registered at 254 nm (a) and total ion chromatogram (TIC) of active fraction (b)

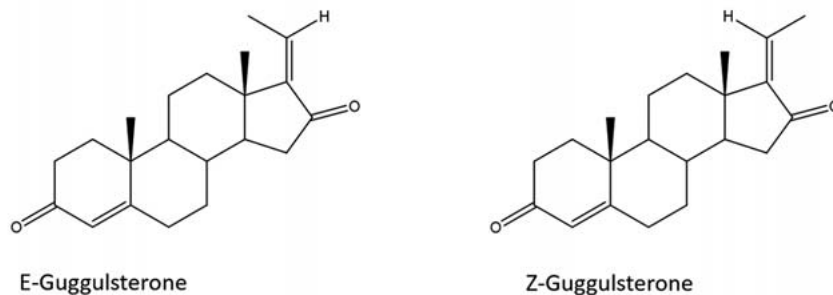


► Fig. 4 MS/MS spectrum of guggulsterone registered in positive ionization mode.

the active fractions of the methanol extract of *C. erythraea* are furanosesquiterpenoids [17].

The active compound responsible for the antiviral activity of *C. gileadensis* was isolated using bio-guided assays from the methanol fraction extracted using liquid-liquid separation from the methanol extract of leaves. The results of the HPLC-DAD-ESI-MS/MS analysis indicated the putative presence of the E- and

Z-isomers of guggulsterone [4,17(20)-pregnadiene-3,16-dione]. These compounds have been previously reported in other species of *Commiphora*, such as *Commiphora mukul* Engl. and *Commiphora wightii* (Arn.) and always in resins [21, 22]; therefore, this study is the first to report the putative identification of guggulsterone isomers in *C. gileadensis* leaves.



► **Fig. 5** Chemical structure of E- and Z-guggulsterone.

Guggulsterone modulates cholesterol and triglyceride levels in serum and inhibits cholesterol synthesis in the liver [23–26]. Moreover, it was discovered as an antagonist of the farnesoid X receptor, which is the bile acid receptor [26, 27] that is implied in the downregulation of the bile salt export pump [28, 29]. Consequently, guggulsterone has been used for treating hyperlipidemia and obesity. In addition to antagonizing farnesoid X receptor, guggulsterone modulates the activity of other steroid receptors, such as estrogen receptor alpha, progesterone receptor, and pregnane X receptor [29, 30]. Moreover, it inhibits the development of tumor cells with no considerable signs of toxicity on normal cells through the suppression of transcription factor activation, especially the NF- κ B [31] that is implicated in the proliferation and growth of malignant cells [32–34]. NF- κ B is also involved in the regulation of various factors associated with inflammation, such as cytokines, chemokines, inflammation-associated fibroblasts, and cell-adhesion molecules [35, 36]. Thus, guggulsterone was shown to exert a potent anti-inflammatory effect. Conversely, it has been found to be orally active in rats with a good pharmacokinetic profile with a bioavailability of > 40% and a half-life of approximately 10 h [37].

In summary, the present study demonstrated, for the first time, the antiviral activity of guggulsterone, which is the active compound isolated from *C. gileadensis* leaves; moreover, it highlighted the diversity of the biological activities of guggulsterone, such as anti-tumor, anti-inflammatory, and anti-lipid activities. This molecule exhibited a virucidal effect probably due to interaction with specific receptors of the enveloped viruses. However, further experiments are required to explain the mechanism of this interaction as well as to determine the nature of target receptors.

Materials and Methods

Plant material and extract preparation

C. gileadensis leaves were collected from Bader province, El Medina El Mounaoura (24°28' 07" North–39°36' 39" East), the Kingdom of Saudi Arabia. The identity of this plant was confirmed by Prof. Abderrazak Smaoui (Laboratory of Extremophile Plants, Center of Biotechnology of Borj Cedria, Tunisia); moreover, a voucher specimen was deposited at the herbarium of this Center (registra-

tion number: CG-KSA-CBBC-24/11/17). The leaves were lyophilized and ground. Subsequently, 5 g of powder was macerated with 50 mL of methanol for 24 h. The extract obtained was filtered and subjected to sequential liquid-liquid separation using hexane and dichloromethane (1 : 1, v/v). Solvents were removed under vacuum by rotary evaporation, and the dried fractions (the hexane, dichloromethane and methanol fractions) were dissolved in 75% ethanol to obtain a final concentration of 50 mg/mL each.

Cells and viruses

The Vero and HEp-2 cell line were cultured in DMEM supplemented with 5% fetal FBS and maintained in DMEM with 2% FBS. HSV-2 and RSV-B were grown on Vero cells and CVB-3 and ADV-5 on HEp-2 cells. The Vero cell line was graciously provided by Pr. Hela KALLEL (Laboratory of Laboratory of Molecular Microbiology, Vaccinology and Biotechnological Development, Pasteur Institute of Tunis, Tunisia); the clinical isolate of HSV-2 was provided by Mrs. Ahlem BEN YAHIA (Laboratory of Clinical Virology, Pasteur Institute of Tunis, Tunisia) and the HEp-2 cell line and the clinical isolates of CVB-3 and ADV-5 were provided by Dr. Hela JAIDANE and Dr. Khira ZDIRI (Laboratory of Transmissible Diseases, Faculty of Pharmacy of Monastir, Tunisia). The titration of HSV-2 and RSV-B was performed using the plaque assay [38] and that of CVB-3 and ADV-5 was performed using the endpoint dilution method [39].

Cytotoxicity assay

A 2-fold serial dilution of the sample starting from 3.33 mg/mL was added to semi-confluent cells in 96-well tissue culture plates. After 72 h of incubation, cells were trypsinized to obtain a single-cell suspension, followed by the addition of trypan blue solution (10%, v/v). Living cells were counted as compared to untreated cells (cell control). The sample concentration that was able to reduce 50% of cell viability (CC_{50}) was determined using linear regression analysis based on data obtained from a dose-response curve.

Antiviral activity assay

The anti-HSV-2 and anti-RSV-B assays were performed in 35-mm dishes using the plaque assay [40], while the anti-CVB-3 and anti-ADV-5 assays conducted in 96-well tissue culture plates using the MTT method [41] with some modifications in both the methods.

A serial 2-fold dilution of sample starting from $CC_{50}/2$ was incubated with a viral culture (200 plaque forming unit for HSV-2 and RSV-B and 50 tissue culture infective doses for CVB-3 and ADV-5) for 1 h on confluent monolayer cells. After 48 h of incubation, the percentage of virus inhibition by the samples was evaluated compared with that by the untreated viruses (virus control). IC_{50} , defined as the concentration of sample that is able to reduce 50% cell infectivity, was determined by linear regression analysis using data from a dose-response curve. Antiviral activity was evaluated by determining SI calculated by the ratio of CC_{50} to IC_{50} . The extract was estimated to have activity when SI was > 10 .

Preventive effect assay

The active fraction ($10 \times IC_{50}$) was incubated on confluent monolayer cells for 2 h at $37^{\circ}C$. Then cells were washed twice with PBS to eliminate free compounds; subsequently, the viral culture (at the same titles used in the antiviral activity assay) was inoculated on cells for 1 h. Cells were then washed with PBS to eliminate unadsorbed viruses, followed by culture in DMEM. After 48 h of incubation at $37^{\circ}C$, the percentage of viral inhibition was calculated compared with incubation in virus control, as previously described.

Virucidal assay

The active fraction ($10 \times IC_{50}$) and viral culture (100 times the title used in the antiviral activity assay) were mixed and incubated for 2 h at $37^{\circ}C$. The mixture was diluted 100-fold to eliminate the effect of the extract on virus replication and then added to confluent monolayer cells. After 48 h of incubation at $37^{\circ}C$, the percentage of viral inhibition was calculated by comparing with virus control, as previously described.

Dose-response virucidal assay

The dose-response virucidal assay was performed with a 2-fold serial dilution of active fraction starting from $10 \times IC_{50}$. After 48 h of incubation at $37^{\circ}C$, the percentage of viral inhibition following treatment with each dilution was calculated by comparing with virus control, as previously described. VC_{50} , defined as the sample concentration that reduces 50% of cell infectivity after direct contact with virus, was determined by linear regression analysis using data obtained from a dose-response curve. Virucidal activity was evaluated by determining the SI_v calculated using CC_{50}/VC_{50} .

Time-course virucidal assay

The time-course virucidal assay was performed at different contact times (0, 1, 3, and 5 h) of active fractions (concentrations, $10 \times IC_{50}$ and IC_{50}) with virus. After 48 h of incubation at $37^{\circ}C$, the percentage of viral inhibition was determined by comparing it with virus control.

Virus adsorption assay

The active fraction ($10 \times IC_{50}$) and viral culture (at same titles used in the antiviral activity assay) were incubated on confluent monolayer cells at $37^{\circ}C$. After 1 h of incubation, cells were washed with PBS to remove unadsorbed viruses and free active compounds, followed by exposure to DMEM. After 48 h of incubation at $37^{\circ}C$,

the percentage of viral inhibition was calculated by comparing with virus control, as described previously.

Virus penetration assay

Viral culture (at same titles used in the antiviral activity assay) was incubated on confluent monolayer cells at $4^{\circ}C$. After 1 h, cells were washed with PBS to remove unadsorbed viruses; subsequently, they were treated with active fraction ($10 \times IC_{50}$) for 1 h at $37^{\circ}C$. Then cells were washed with PBS to remove free compounds and treated with citrate buffer (40 mM citric acid, 10 mM KCl, and 135 mM NaCl; pH = 3) for 1 min to inactivate adsorbed viruses. After removing citrate buffer, cells were exposed to DMEM. After 48 h of incubation, the percentage of viral inhibition was calculated by comparing with virus control, as described previously.

Post-infection assay

Viral culture (at same titles used in the antiviral activity assay) was incubated on confluent monolayer cells for 1 h at $37^{\circ}C$. Subsequently, the viral culture was removed by washing of cells with PBS to remove unadsorbed viruses. Then the cells were treated with the active fraction (concentration, $10 \times IC_{50}$). After 6 h of incubation at $37^{\circ}C$, cells were washed with PBS to remove free compounds and then exposed to DMEM. After 48 h of incubation, the percentage of viral inhibition was calculated by comparing with virus control, as described previously.

Isolation of the active compound by TLC

For the isolation of the antiviral compound, the active fraction was subjected to TLC. A total of 175 mg of active extracts was placed on a pre-coated silica gel 60 F254 glass plate (20×20 cm; Glass Backed TLC Extra Hard Layer 60 Å) and was subjected to separation using hexane/ethyl acetate/methanol at a ratio of 3:3:1. After separation, the plate was allowed to dry at room temperature and observed under UV light (254 nm). The bands observed under UV light were scraped off and dissolved in 75% ethanol under agitation for 15 min. The obtained fractions were filtrated, dried, weighed, dissolved in 75% ethanol to obtain a final concentration of 10 mg/mL final, and evaluated for their antiviral activities. The active fraction was subjected again to TLC under similar conditions to verify its purity.

Identification of the active compound by HPLC-DAD-ESI-MSn

The analyses were performed using a Thermo Scientific LCQ FLEET system consisting of LCQ FLEET ion trap mass spectrometer, Surveyor MS Pump/Autosampler/PDA Detector (Thermo Fisher Scientific) through an ESI source. A Gemini C18 110A analytical column (150×2.00 mm i.d., 5 μ m) with pre-column (Phenomenex) was used for the separation. The mobile phase consisted of formic acid 0.1% in water (solvent A) and methanol (solvent B) at 0.3 mL/min (injected volume 10 μ L). A linear solvent gradient used was as follows: from 15% B to 100% B in 30 min, with a final plateau of 3 min at 100% B. The ion trap operated in data-dependent, full scan (60–2000 m/z), and MS^n mode to obtain fragment ions m/z with a collision energy of 35% and an isolation width of 3 m/z . The positive parameters of the ion mode ESI source have been op-

timized to an ionization voltage of 5.0 kV, a capillary temperature of 300°C, a capillary voltage of 33 V, a sheath gas flow rate of 30 arbitrary units (AU), and an auxiliary gas flow rate of 10 AU.

Statistical analysis

All experiments were conducted in triplicate; statistical values are expressed as mean (95% confidence interval).

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Conflict of Interest

The authors declare that they have no conflict of interest.

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