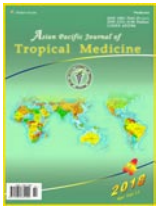

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Inhibitory activities of extracts of *Rumex dentatus*, *Commelina benghalensis*, *Ajuga bracteosa*, *Ziziphus mauritiana* as well as their compounds of gallic acid and emodin against dengue virus

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ABSTRACT

Objective: To investigate the inhibitory effects against dengue virus serotype 2 (DENV-2) by five different fractions (extracted by methanol, ethanol, benzene, chloroform and *n*-hexane) of *Rumex dentatus*, *Commelina benghalensis*, *Ajuga bracteosa* and *Ziziphus mauritiana*, as well as their constituents (gallic acid, emodin, and isovanillic acid). **Methods:** All the samples were tested for cytotoxicity on baby hamster kidney cells by MTT assay and for anti-DENV-2 activity by plaque reduction neutralization assay using two DENV-2 doses (45 and 90 plaque-forming units or PFU). **Results:** All the samples except isovanillic acid exhibited significant prophylactic effects against DENV-2 infectivity (without cytotoxicity) when administered to cells before infection, but were not effective when given 6 h post-infection. The methanol extract of *Rumex dentatus* demonstrated the highest antiviral efficacy by inhibiting DENV-2 replication, with IC_{50} of 0.154 μg/mL and 0.234 μg/mL, when added before infection with 45 and 90 PFU of virus, respectively. Gallic acid also exhibited significant antiviral effects by prophylactic treatment prior to virus adsorption on cells, with IC_{50} of 0.191 μg/mL and 0.522 μg/mL at 45 and 90 PFU of DENV-2 infection, respectively. **Conclusions:** The highly potent activities of the extracts and constituent compounds of these plants against DENV-2 infectivity highlight their potential as targets for further research to identify novel antiviral agents against dengue.

1. Introduction

Dengue is a virulent acute systemic viral disease that represents a major health, economic and social problem in tropical and sub-tropical areas of the world[1]. It is an arthropod-borne human disease that spreads through the bite of the *Aedes* mosquito which serves as the carrier of the pathogenic viruses. *Aedes aegypti* is the primary vector, while *Aedes albopictus* is the secondary mosquito vector[2]. Four genetically distinct but antigenically similar dengue virus (DENV) serotypes (DENV-1, 2, 3, 4) belonging to the Flavivirus family cause the infection[3]. Dengue viruses are prevalent in over 125 tropical and sub-tropical countries, and 390 000 000 infections occur annually[4,5]. DENV infection in humans is frequently asymptomatic, but may result in a spectrum of clinical

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manifestations that vary from undifferentiated fever to severe dengue such as fatal dengue shock syndrome[6,7]. Lifelong immunity may develop after infection in a serotype-specific manner[6], but may progress to more severe disease following secondary infection by a heterologous serotype[8]. During secondary infection by a different viral serotype, the memory B and T cells corresponding to the primary infection are preferentially reactivated, resulting in antibody-dependent enhancement of the infection that initiates mechanisms of severe dengue[9,10]. Although the four DENV serotypes produce similar types of infection, DENV-2 and DENV-3 are more frequently linked to severe and fatal dengue hemorrhagic fever or DHF[11,12]. In Pakistan, many dengue outbreaks have been documented from 1994 to 2011[13]. The first report of dengue from Pakistan was in 1982, in which 12 out of 174 patients from Punjab were victims of the infection[14]. Although a dengue vaccine is currently available, it is suboptimal[5], thus emphasizing the need for developing new antiviral drugs[15]. Medicinal plants have been used for the treatment of human diseases for centuries. These remedies remain an important source of novel bioactive chemical compounds, such as antimicrobial agents. We previously documented the antiviral activities of extracts of *Houttuynia cordata* and its constituent compounds on dengue virus and murine coronavirus infections[16,17].

Pakistan has a plethora of natural resources, varied ecological regions, and abundant flora. Out of 6 000 higher plant species found in Pakistan, 600-700 are reported to be used medicinally, and belong to different plant families[18]. One of the medicinally important plants, *Rumex dentatus* (*R. dentatus*) L. (known as toothed dock), is used locally as a vegetable in the Kashmir valley. It belongs to the family Polygonaceae, and is found throughout temperate western Himalayas from Kashmir to Kumaon at 8 000-12 000 feet. It produces a large variety of chemically complex and bioactive compounds, and is traditionally utilized for its bactericidal, anti-tumor, anti-dermatitis, anti-inflammatory, astringent, tonic, diuretic, and laxative properties[19-21]. Also known as Ber and belonging to the family Rhamnaceae, *Ziziphus mauritiana* (*Z. mauritiana*) is a spiny fruit tree that grows in tropical and sub-tropical regions of the world. Different parts of this plant are used in traditional medicine for treating diseases such as allergies, depression, ulcers and asthma. The leaves of *Z. mauritiana* are used for the treatment of liver diseases, asthma and fever, while the fruits are used as wound healing, sedative and anti-cancer agents[22]. *Commelina benghalensis* (*C. benghalensis*) Linn. (Commelinaceae), commonly known as Benghal dayflower or Dew flower, is a tropical perennial herb native to Asia and Africa. In Pakistan, it is used as animal fodder, but is also eaten by humans as a vegetable. The plant is also medicinally important, is used for its laxative effects, and to treat leprosy, psychosis and skin inflammation[23]. The hepato-protective activity of its root extract against paracetamol-induced hepatic damage in Wistar rats is also documented[24]. The plant is also well-known for its anti-cancer, anti-tumor and anti-oxidant activities[25-27]. *Ajuga bracteosa* (*A. bracteosa*) Wall. ex. Benth. (family Labiateae) is an important medicinal herb widely distributed in Kashmir and sub-Himalayan tract. Its leaves have diuretic functions, and have been effectively used as a blood purifier and cooling agent[28]. Moreover, this herb is used for treatment of gout, cancer, gastric ulcer, palsy, liver fibrosis, and protozoal diseases[29,30].

The objectives of this study are to analyze the DENV antiviral activities of these four medicinally important plants: *R. dentatus*, *C. benghalensis*, *A. bracteosa*, *Z. mauritiana*, together with three pure compounds: gallic acid, emodin and isovanillic acid.

2. Materials and methods

2.1. Plants and extracts

R. dentatus, *C. benghalensis*, *Z. mauritiana* and *A. bracteosa* were collected from Balakot, Pakistan. Plants were identified and authenticated by a taxonomist, Rizwana Aleem Qureshi, Professor of Taxonomy, Department of Plant Sciences, Quaid-i-Azam University, Islamabad, Pakistan. A voucher specimen of each plant was deposited in the herbarium of the Plant Biochemistry and Molecular Biology Laboratory, Quaid-i-Azam University, Islamabad (*Z. mauritiana* HPBMBL-16-018, *C. benghalensis* HPBMBL-16-019, *R. dentatus* HPBMBL-16-023, *A. bracteosa* HPBMBL-16-043). Five different extracts of each plant were prepared using methanol, ethanol, benzene, chloroform and *n*-hexane solvents. Plants were washed thoroughly with tap water, rinsed with distilled water, dried and ground to powder form. Each plant material (50 g) was extracted with 500 mL of each solvent by maceration. Plant powder was soaked in the respective solvent for 7 d, and then filtered through Whatman filter paper number 1. Filtrates were evaporated using a rotary evaporator, and extracts were dissolved in DMSO at 20 mg/mL, and stored at 4 ℃ for further use. Extracts are abbreviated as follows: *R. dentatus* methanol (RM), ethanol (RE), benzene (RB), chloroform (RC), *n*-hexane (RH); *A. bracteosa* (AM, AE, AB, AC, AH); *C. benghalensis* (CM, CE, CB, CC, CH); and *Z. mauritiana* (ZM, ZE, ZB, ZC, ZH).

2.2. High performance liquid chromatography (HPLC) analysis

In view of their significant antiviral activities, the RM, CM, AH and ZM extracts were selected for HPLC analysis. Pure compounds were purchased from Sigma-Aldrich, *i.e.* gallic acid, emodin, and isovanillic acid. Plant extracts were analyzed by HPLC apparatus (Agilent) using Sorbex RXC8 analytical column (Agilent) with 5 μm particle size and 25 mL capacity. Mobile phase consisted of eluent A (acetonitrile-methanol-water-acetic acid at 5:10:85:1) and eluent B (acetonitrile-methanol-acetic acid at 40:60:1). The gradient (A:B) utilized was as follows: 0-20 min (0% to 50% B), 20-25 min (50% to 100% B), and then isocratic 100% B (25-40 min) at a flow rate of 1 mL/min. The injection volume of each sample was 20 μL, with the detection wavelength set at 252 nm. Each sample was filtered through a 0.45 μm membrane before injection, and the column was reconditioned for 10 min before the next analysis. All samples

were assayed in triplicates. Quantification was carried out by the integration of the peak using the external standard method. All chromatographic operations were performed at ambient temperature.

2.3. Cell culture and virus maintenance

The New Guinea C strain of DENV-2 was propagated in the C6/36 *Aedes albopictus* mosquito cell line. Tissue culture flasks (25 or 75 cm^2) with confluent monolayers of C6/36 cells were inoculated with 0.2 or 1.0 mL of infected cell culture fluid, and incubated at 28 ℃ until complete cytopathic effect was observed in about 3-5 d. The infected cell culture fluid was then harvested, aliquoted, and stored at –80 ℃, or used to inoculate fresh monolayers of C6/36 cells. Baby hamster kidney (BHK-21) cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum at 37 ℃ under 5% CO_2.

2.4. MTT cytotoxicity assay

The MTT cell proliferation assay was performed to determine the maximum non-toxic doses of the plant extracts and compounds. Various concentrations of each fraction and compound were added (in triplicate) to wells containing cell monolayers and incubated at 37 ℃ under 5% CO_2 for 48 h. After incubation, MTT reagent was added to each well, and further incubated for 4 h or until purple precipitates were visible under an inverted microscope. Then, 100 μL of 100% DMSO was added to each well, and incubated for 15 min at room temperature. The absorbance at 570 nm was measured, and the cell inhibition rate calculated from the formula: [1–(OD of sample with cells)–(OD of sample without cells) divided by (OD of solvent with cells)–(OD of medium without cells)]×100%. The plant extract fractions and compounds were tested at the same concentrations as those for plaque reduction neutralization tests. The assay included wells containing medium only as well as untreated control cells. Each experiment was repeated, and the means and standard deviations were calculated. The inhibition rate of each extract and compound was plotted against various concentrations of the test agents to ascertain the concentration that causes 50% cytotoxicity (CC_{50}).

2.5. Antiviral activity

To test different concentrations of plant extracts and compounds, each was two-fold serially diluted, while two DENV-2 concentrations (45 and 90 plaque-forming units or PFU) were investigated. Hence, test samples were evaluated for anti-DENV-2 activity by plaque reduction neutralization test[31,32] using two investigational approaches. In both strategies, BHK-21 fibroblasts were cultured to form cell monolayers in 24-well plates with RPMI-1640 supplemented with 10% fetal bovine serum at 37 ℃ under 5% CO_2. The test samples were dissolved in 1% PBS, and two-fold serial dilutions were prepared to evaluate various concentrations. DENV-2 New Guinea C neutralizing monoclonal antibody 3H5.1 (Chemicon) served as the positive control. Negative controls included 0.5% DMSO, virus alone, and cells alone. Each experiment was performed in duplicate.

2.6. Prophylactic strategy

In the first experimental strategy, DENV-2 (45 and 90 PFU) was incubated with various concentrations of each sample together with controls for 1 h before adding to the cells. These virus-sample mixtures were incubated with the cells for another hour at 37 ℃ under 5% CO_2 with rocking at 15-min intervals. Then, cells were overlaid with 1.2% Avicel at 37 ℃ under 5% CO_2 for 5 d.

2.7. Therapeutic strategy

In the second experimental approach, cells were first infected with 45 and 90 PFU of DENV-2, and after 6 h post-infection, various concentrations of each extract and compound were added to the infected cells. The cells were also overlaid with 1.2% Avicel under the same conditions for 5 d. The cells were then fixed with 20% formaldehyde, stained with 1% crystal violet, and the number of plaques was counted. The percentage plaque reduction of each sample at each dilution was determined as follows: (mean number of plaques in virus control)–(average number of plaques in sample) ×100% divided by (mean number of plaques in virus control). The percentage plaque reduction was plotted against various concentrations of the test agents to determine the concentration that causes 50% plaque reduction (IC_{50}).

3. Results

3.1. Identification of gallic acid, emodin and isovanillic acid in plant extracts

HPLC analysis was performed on the plant extracts with potent antiviral activity to determine the presence of gallic acid, emodin and isovanillic acid in these extracts. The pure compounds of gallic acid, emodin and isovanillic acid served as standards, and their retention times were 4.83, 24.34 and 17.06 min, respectively (Figure 1). The peaks detected in the RM extract at 4.73, 24.23 and 17.31 min were comparable to those of the standards, and thus confirmed the presence of gallic acid, emodin and isovanillic acid, respectively. The chromatograms of AH (0.23 mg/dry weight extract), ZM (0.02 mg/dry weight extract) and CM (1.61 mg/dry weight extract) demonstrated the presence of gallic acid with retention times of 4.80, 4.43 and 4.52 min, respectively (Figure 2). Emodin and isovanillic acid were detected only in the RM extract. The quantities of compounds in the RM, CM, AH and ZM extracts were computed from the calibration curves of the standard solutions of pure compounds.

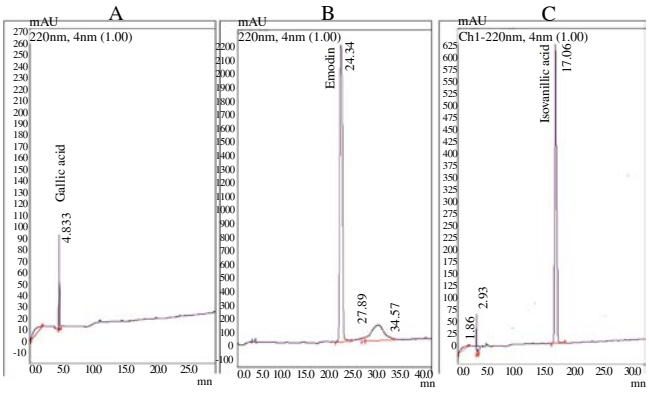

Figure 1. Chromatograms of HPLC analyses of standards of compounds.
(A) Gallic acid, (B) Emodin, (C) Isovanillic acid.

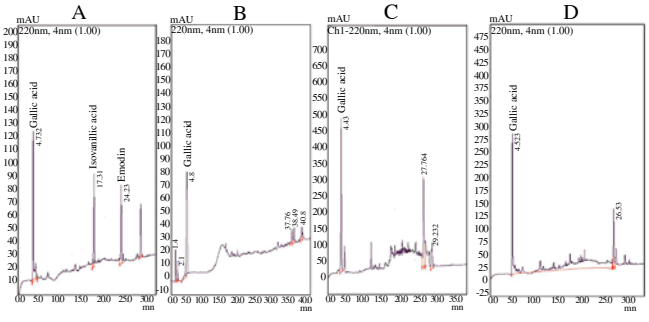

Figure 2. Chromatograms of HPLC analyses of plant extracts.
(A) *R. dentatus*, (B) *A. bracteosa*, (C) *Z. mauritiana*, (D) *C. benghalensis*.

3.2. DENV-2 antiviral activities using prophylactic strategy of treatment with plant extracts

All the five fractions (methanol, ethanol, benzene, chloroform and *n*-hexane) of the four plants (at highest concentration of 200 μg/mL and two-fold serially diluted to 0.09 μg/mL) were tested for anti-DENV-2 activity by plaque reduction neutralization test assay. Samples were evaluated for their prophylactic (pre-incubated with virus) and chemotherapeutic (6 h post-infection) effects, *i.e.* their ability to inhibit virus replication at challenge doses of 45 and 90 PFU. All the samples exhibited DENV-2 inhibition when added before virus infection at various concentrations without toxicity to BHK-21 cells. Among the fractions of *R. dentatus*, RM at 45 PFU virus dose exhibited the highest IC_{50} of 0.154 μg/mL, CC_{50} of 211.300 μg/mL, and selectivity index of 1 372.080 (Table 1); while at 90 PFU virus dose, the values were IC_{50} of 0.234 μg/mL, CC_{50} of 211.300 μg/mL, and selectivity index of 902.990. The CM fraction of *C. benghalensis* was found to be the most potent, demonstrating IC_{50} of 0.270 μg/mL, CC_{50} of 298.100 μg/mL, and selectivity index of 1 104.070 at 45 PFU (Table 2); while at 90 PFU, the values were IC_{50} of 0.516 μg/mL, CC_{50} of 298.100 μg/mL, and selectivity index of 577.490. For extracts of *A. bracteosa*, AH exhibited the highest inhibitory activity with IC_{50} of 0.340 μg/mL, CC_{50} of 290.000 μg/mL, and selectivity index of 852.940 at 45 PFU (Table 3); but at 90 PFU, the values were IC_{50} of 0.831 μg/mL, CC_{50} of 290.000 μg/mL, and selectivity index of 348.980. For extracts of *Z. mauritiana*, ZE at 45 PFU revealed the highest IC_{50} of 0.182 μg/mL, with CC_{50} of 324.100 μg/mL and selectivity index of 1 780.770 (Table 4); while at 90 PFU, the values were IC_{50} of 0.640 μg/mL, CC_{50} of 324.100 μg/mL, and selectivity index of 506.410.

Table 1

IC_{50}, CC_{50} and selectivity index of various fractions of *R. dentatus* at 45 and 90 PFU of DENV-2 infection.

Plants	At 45 PFU			At 90 PFU		
	IC_{50} μg/mL	CC_{50} μg/mL	Selectivity index	IC_{50} μg/mL	CC_{50} μg/mL	Selectivity index
RM	0.154	211.300	1 372.080	0.234	211.300	902.990
RE	0.190	234.100	1 232.110	0.462	234.100	506.710
RB	0.663	204.000	307.690	1.015	204.000	200.990
RC	1.048	189.800	181.110	3.347	189.800	56.710
RH	0.499	194.700	390.180	1.393	194.700	139.770

Table 2

IC_{50}, CC_{50} and selectivity index of various extracts of *C. benghalensis* at 45 and 90 PFU of DENV-2 infection.

Plants	At 45 PFU			At 90 PFU		
	IC_{50} μg/mL	CC_{50} μg/mL	Selectivity index	IC_{50} μg/mL	CC_{50} μg/mL	Selectivity index
CM	0.270	298.100	1 104.070	0.516	298.100	577.490
CE	1.500	229.000	152.670	1.845	229.000	124.120
CB	0.482	249.500	517.630	0.865	249.500	288.440
CC	0.502	277.000	551.790	2.005	277.000	138.130
CH	0.290	283.400	977.240	1.032	283.400	274.610

Table 3

IC_{50}, CC_{50} and selectivity index of various extracts of *A. bracteosa* at 45 and 90 PFU of DENV-2 infection.

Plants	At 45 PFU			At 90 PFU		
	IC_{50} μg/mL	CC_{50} μg/mL	Selectivity index	IC_{50} μg/mL	CC_{50} μg/mL	Selectivity index
AM	0.417	305.800	733.330	0.970	305.800	315.260
AE	0.585	334.700	572.140	1.236	334.700	270.790
AB	0.728	356.400	489.560	1.751	356.400	203.540
AC	1.681	283.000	168.350	1.932	283.000	146.480
AH	0.340	290.000	852.940	0.831	290.000	348.980

Table 4

IC_{50}, CC_{50} and selectivity index of various extracts of *Z. mauritiana* at 45 and 90 PFU of DENV-2 infection.

Plants	At 45 PFU			At 90 PFU		
	IC_{50} μg/mL	CC_{50} μg/mL	Selectivity index	IC_{50} μg/mL	CC_{50} μg/mL	Selectivity index
ZM	0.240	284.300	1 184.580	0.790	284.300	359.870
ZE	0.182	324.100	1 780.770	0.640	324.100	506.410
ZB	0.956	305.800	319.870	1.563	305.800	195.640
ZC	1.065	254.800	239.250	1.289	254.800	197.700
ZH	1.194	169.900	142.290	1.891	169.900	89.860

3.3. Inhibitory effects of gallic acid and emodin on DENV-2 virus replication

Individual pure compounds of gallic acid, emodin and isovanillic acid were also tested for inhibitory activity against DENV-2 using the same experimental strategies for plant extracts, starting with the

highest concentration of 200 μg/mL down to 0.195 μg/mL Gallic acid revealed the highest inhibitory activity with IC_{50} of 0.191μg/mL, CC_{50} of 89.765 μg/mL, and selectivity index of 469.408 at 45 PFU; while the values were IC_{50} of 0.522 μg/mL, CC_{50} of 89.765μg/mL, and selectivity index of 171.963 at 90 PFU. Emodin also exhibited antiviral activity with IC_{50} values of 2.368 μg/mL and 5.515 μg/mL at 45 and 90 PFU, respectively. However, isovanillic acid did not display any significant DENV-2 inhibition (Table 5).

Table 5

IC_{50}, CC_{50} and selectivity index of isovanillic acid, emodin and gallic acid at 45 and 90 PFU of DENV-2 infection.

Compounds	At 45 PFU			At 90 PFU		
	IC_{50} μg/mL	CC_{50} μg/mL	Selectivity index	IC_{50} μg/mL	CC_{50} μg/mL	Selectivity index
Isovanillic acid	22.067	83.254	3.772	40.186	83.254	2.071
Emodin	2.368	97.681	41.250	5.515	97.681	17.711
Gallic acid	0.191	89.765	469.408	0.522	89.765	171.963

3.4. Absence of chemotherapeutic effects of plant extracts and compounds on DENV-2 replication

All the plant samples and pure compounds were also analyzed to determine any inhibition of DENV-2 replication by treatment post-infection. However, none of the plant fractions and compounds could inhibit the virus replication significantly when treatment was administered after 6 h post-infection.

4. Discussion

Given that many modern drugs are derived from natural precursors[33,34], ethno-pharmacology and traditional medicine offer an attractive option for identifying starting material for drug discovery initiatives[35]. This is the motivation of the current study which is to explore part of the indigenous herbal bio-resource in the Indian subcontinent, and to identify plants that may possess anti-DENV inhibitory activity. Plants provide a large range of natural compounds belonging to different molecular families that possess various medicinal properties. The isolation of various bioactive compounds from a range of plants has highlighted them as sources of novel antiviral agents[36,37]. Interestingly, the antiviral properties of plant-based extracts are often higher than their synthetic analogues in several studies[38]. In this regard, the production of plant-based antiviral drugs offers a potentially promising alternative to combat viral diseases. In order to identify the step of the viral cycle where replication was prevented, DENV-2 was pretreated with the extracts or compounds prior to infection. In another strategy, the cells were first infected with virus, followed by addition of the extracts or compounds post-infection. A total of 20 extracts derived from four medicinal plants and three individual compounds were screened for prophylactic and chemotherapeutic effects against DENV-2. The range of concentrations of extracts did not cause significant toxicity to the BHK-21 cells. We observed increasing prophylactic effects of the plant extracts and compounds (except isovanillic acid) in a dose-dependent manner. The most active plant extract was RM from *R. dentatus*, with a highly substantial IC_{50}, while gallic acid was the most potent compound, when samples were allowed to interact with virus for one hour before the virus adsorption stage. It is thus likely that their antiviral activity is mediated by inactivation of the virus to prevent cellular entry. Previous ethnobotanical surveys also support our findings, as *R. dentatus* is widely used as medicinal plant-based remedies for different diseases[39-41], *e.g.* antiviral activity of fruit extracts of *Rumex cyprus*. *R. dentatus* also possesses antibacterial, antifungal, insecticidal, molluscicidal, and allelopathic activities[42-44]. In addition, the CM extract of *C. benghalensis* possessed IC_{50} of 0.270 μg/mL and 0.516 μg/mL at 45 and 90 PFU, respectively. *C. benghalensis* is used in ethnomedicine given that it possesses antimicrobial, antioxidant, anxiolytic and sedative properties[45,46]. For extracts of *A. bracteosa*, the highest inhibition of DENV-2 was observed for the AH fraction with a very low IC_{50}. Some members of the genus *Ajuga* have already been evaluated for antiviral potential. Antiviral activity of *Ajuga decumbens* against respiratory syncytial virus was reported[47], with an IC_{50} value of 131.600 μg/mL. Similarly, Luo *et al*[48] documented *in vitro* inhibition of infectious bronchitis virus by water extracts of A. decumbens at concentrations between 750 and 1 500 mg/mL. Aqueous and methanol extracts of *Ajuga integrifolia* Ham. Buch are also effective against human immunodeficiency virus types 1 and 2[49]. We also observed DENV-2 inhibitory effect of the ethanol extract of *Z. mauritiana*. Potent antioxidant activities of *Z. mauritiana* are reported for extracts from its seeds, fruits and leaves[50-52]. Furthermore, its bark and pulp[53] exhibit strong cytotoxic potential against various cell lines. This species also demonstrates antimicrobial, anti-inflammatory[54], antidiabetic[55], antimicrobial[56] and anxiolytic properties[57]. Of all the samples tested, only isovanillic acid could not inhibit DENV-2 replication even at higher concentrations. Using the chemotherapeutic approach where samples were introduced following DENV-2 infection, none could inhibit virus replication, which alludes to these plant extracts and compounds acting as viral entry inhibitors. Our study has revealed that these plant extracts contain active components responsible for anti-DENV activity, and future studies are warranted to identify and isolate these active constituents which may also aid in determining their mechanisms of action.

In conclusion, our study elucidated the *in vitro* DENV-2 inhibitory activities by extracts of *R. dentatus, A. bracteosa*, *C. benghalensis*, *Z. mauritiana*, as well as gallic acid and emodin. They were generally free from detrimental effects, and exerted their antiviral activities by prophylactic treatment, but not by treatment post-infection. More complete investigations are warranted on these plants for isolation, purification and characterization of bioactive principles responsible for anti-dengue activity, and to elucidate their underlying mechanisms of DENV inhibition.

Conflict of interest statement

The authors declare no conflict of interest.

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