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Anti-dengue viral activity of *Glycyrrhiza glabra* roots in Vero cells

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Despite being a global public health problem, there are no antiviral agents for dengue. Plants are the sources of most approved drugs and many phytochemicals have exhibited in vitro antiviral activity. We explored the antiviral potential of the aqueous extract of *Glycyrrhiza glabra* roots (GGaq) on dengue viruses. Plaque reduction antiviral assay for half maximal inhibitory concentrations (IC_{50}) was done in Vero cells infected with dengue serotypes 1–4 and exposed to varying concentrations of GGaq. Half-maximal cytotoxic concentration (CC_{50}) of the GGaq was 651.9 µg/mL. The IC_{50} of the four dengue serotypes (10–50 µg/mL) indicated moderate inhibition by GGaq. 98–100% inhibition of all dengue serotypes was seen with GGaq at the maximum nontoxic dose. Preparative thin layer chromatography of GGaq, isolated sub fractions E and F which had low to moderate antiviral activity (IC_{50} 12.65–85.95 and IC_{50} 13.14–69.27 respectively). They exhibited good therapeutic potential only for dengue serotype-4. Virus adsorption to cells was significantly inhibited by GG (50%) and sub fraction E (24.9%). Chloroquine diphosphate was used as the positive control in all assays. The aqueous extract of GG was non-toxic and had better antiviral activity than sub fractions E and F.

Keywords Dengue, Antiviral, *Glycyrrhiza glabra*, Vero cell, Fractionation

Dengue is a global public health problem with 390 million dengue infections occurring per year with 96 million symptomatic cases[1]. Prevention of dengue by control of the *Aedes* mosquitoes has been challenging due to rapid urbanization. Hopes of developing a vaccine has been confounded by the phenomenon of antibody dependent enhancement of dengue infection; as a result the live-attenuated vaccine Dengvaxia, can be recommended only for seropositive populations[2]. Since severe dengue is more likely with high viral loads[3], it is believed that an antiviral that lowers viraemia by one to two orders of magnitude, may be associated with a favourable prognosis[4]. Antiviral drugs reduce the viral load and the period of viraemia leading to reduced transmission from infected humans to mosquitoes. Despite scientific studies, identification of specific antiviral agents for dengue has been difficult and clinical trials with repurposed pharmaceuticals have so far been disappointing[5].

Plants are the direct or indirect sources of approximately 60% of approved drugs and seven out of ten synthetic drugs are modelled on a natural product[6]. A large reservoir of lead compounds potentially exists in nature, which could be used either directly or serve as lead structures on which new anti-dengue viral agents could be modelled[6]. Out of 52 medicinal plants which are used to treat fevers in Sri Lankan traditional medicine, four were shortlisted for bioassay-guided screening as, (1) they had not been investigated previously for anti-dengue activity (2) they contained previously reported anti-dengue viral phytochemicals; (3) anti-viral effect against other viruses had been reported; and (4) they have been shown to be hepatoprotective. Based on these criteria, whole plant of *Munronia pinnata* (MP), roots of *Glycyrrhiza glabra* (GG), leaves of *Psidium guajava* (PG) and dried flowers of *Aegle marmelos* (AM) were investigated by us, but only GG was selected for fractionation and further studies[7]. This study reports the inhibitory activity of the aqueous extract of GG roots and two of its sub fractions, in Vero cells infected with the four dengue serotypes.

Glycyrrhiza glabra Linn., commonly known as liquorice, belongs to the family *Leguminosae.* It is a natural medicine derived from the roots and rhizomes of *Glycyrrhiza* species and is one of the most widely used medicinal

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plants, found in traditional formulas since antiquity. The use of the plant can be traced back to ancient Assyrian, Egyptian, Chinese, Indian, Greek and Romans cultures and was used in Arabic medicine during the Middle Ages[8]. There is evidence of its use in the past for the treatment respiratory tract infections, hepatitis, burning sensation of stomach, heart palpitations, angina, bladder and kidney pain, diuresis and skin ulcers[9]. Glycyrrhizin or glycyrrhizic acid (a tetracyclic triterpenoid saponin) and its primary metabolite 18β-glycyrrhetinic acid are the most important pharmacologically active components of the licorice root, and both have been shown to have antiviral and immunomodulatory properties[10].

At the time our study was initiated there were no publications on the anti-dengue viral activity of the aqueous extract of GG roots on all four serotypes of dengue. Since then four publications have reported the potential use of glycyrrhizin for dengue virus inhibition. Chaudhary et al. demonstrated that glycyrrhizic acid suppressed autophagy (which is needed for successful viral replication) in dengue serotype 2 infected A549 cell line[11]. Another group have revealed that *n*-butanol extract of Glycyrrhizae Radix et Rhizome (a well-known traditional Chinese medicine), inhibits dengue virus through targeting envelope proteins. In mice, it significantly relieved clinical symptoms and prolonged duration of survival[12]. Hour et al. (2022) synthesised and studied a group of known and novel glycyrrhizic acid derivatives, using dengue virus 1 and 2 in Vero E6 and A549 cells. They found that conjugation of glycyrrhizic acid derivatives with amino acids and methyl/ethyl esters is a prospective way to produce new and potent dengue virus inhibitors[13,14].

GG compounds have also been effective with other flaviviruses. In Japan, glycyrrhizin has been used for over 20 years for the treatment of chronic hepatitis C patients who do not respond to interferon. A significant reduction in serum aminotransferases and an improvement in liver histology has been reported in randomized controlled trials[15]. Several pathogenic flaviviruses such as Japanese encephalitis[16], mammalian tick-borne encephalitis and yellow fever were also inhibited by glycyrrhizin[17].

In addition, glycyrrhizin antiviral action against other viruses have been reported in the past. In AIDS patients, long term intravenous glycyrrhizin produced a significant clinical improvement[18]. In vitro inhibition of HIV replication, interferon-inducing and natural killer (NK)-enhancing effects have been demonstrated in experiments[19]. In mice infected with influenza virus A2 (H2N2), antiviral effects of glycyrrhizin are due to stimulation of IFN-gamma production by T cells[20]. Survival of HSV1-infected mice was improved with glycyrrhizin treatment in another study[21]. Other herpesviruses such as Epstein Barr virus[22] and cytomegalovirus have been inhibited by glycyrrhizin in vitro[23].

Glycyrrhizin has been used intravenously for the treatment of chronic hepatitis B in Japan and improves liver function with occasional complete recovery from hepatitis[24] and it also suppresses secretion of hepatitis B surface antigen[25]. Furthermore, hepatitis A virus[26] and SARS-associated coronavirus replication have been suppressed by glycyrrhizin[27] whereas, 18β-glycyrrhetinic acid has demonstrated inhibition of human respiratory syncytial virus[28] and rotavirus[29]. Most of these studies have been performed with one or two serotypes of the dengue virus albeit it has been established that antiviral activity is dependent on the dengue serotype[30]. Considering the evidence for antiviral properties in GG we deemed it worthwhile to validate its effects on all four dengue serotypes with an aqueous extract preparation.

Methods

Preparation of aqueous extract of GG roots

Fresh roots of GG were purchased from Manning Market (an open market) in Pettah, and authenticated by the Bandaranaike Memorial Ayurveda Research Institute, Navinna, Sri Lanka (Registration no. 1603MS2017001). The GG roots were washed well in running tap water, air dried and cut into small pieces and then washed in distilled water, followed by double distilled water. Sixty grams of GG roots were heated in 1920 mL of water in a beaker at 70°-80°C until reduced to 240 mL according to the traditional method of preparation. The extract was filtered, lyophilized and stored at -20°C until required.

Cells and viruses

African green monkey kidney (Vero) cells and *Aedes albopictus* larval cells (C6/36), obtained from the Dengue Research Centre, University of Sri Jayewardenepura, Sri Lanka, were grown in complete growth medium (CGM) that consisted of 12 g/L of Dulbecco's minimum essential medium (DMEM)/F12; Sigma, USA D0547) supplemented with 5% foetal bovine serum (FBS) and 1.2 g/L $NaHCO_3$. FBS was reduced to 1.5% in the maintenance medium (MM) which otherwise had similar constituents as CGM. C6/36 cells were used for propagating dengue serotypes 1–4 in MM at 28 °C for seven days. The supernatants were harvested and aliquots were stored at -70°C.

Virus titration

Virus titration was done by plaque assay in Vero cells at 37 °C with 5% carbon dioxide (CO_2).

The protocol, kindly shared by Prof. Damonte, Laboratorio de Virología, Universidad de Buenos Aires, Argentina, was followed with a few modifications. The seeding density of cells, the volume of virus inoculum and incubation period needed to be optimised. Twenty-four well polystyrene plates (UltraCruz, Santa Cruz Biotechnology, USA, Cat No. sc-204445) were seeded with 1.75×10^5 Vero cells/well in 500 µL of CGM and incubated at 37°C in 5% CO_2 atmosphere for 24 h to obtain 80% confluent monolayers. Serial tenfold dilutions of virus suspension in MM (250 µL per well) were added, and incubated at 37 °C with 5% CO_2 for one hour with periodic agitation. An equivalent volume of virus diluent alone was added to the control wells. Thereafter, the virus suspension was replaced with 1 mL/well of overlay medium (OM) containing 1% methylcellulose (Sigma, USA) in MM and incubated under the same conditions for seven days post-infection (the incubation period for each serotype was pre-determined). Cells were fixed with 4% w/v formaldehyde (Sigma, USA) and stained with 1% w/v crystal violet (Himedia, India) in 10% ethanol (Sigma, USA). Each dilution of virus was tested in

triplicate wells and two independent titrations were performed. The average plaque count was used to calculate the virus titre and was expressed as plaque forming units per millilitre (PFU/mL).

Cell cytotoxicity assay

Vero 5×10^3 cells/well in 200 µL of CGM were seeded onto 96-well flat bottomed polystyrene plates (Corning, USA, Cat No. 3598) to obtain 80% confluent monolayers after 24 h incubation at 37ºC in 5% CO_2. Cells were exposed to GG extract at concentrations ranging from 33.3 to 1000.0 µg/ mL. Each concentration was tested in triplicate and plates were incubated for seven days (37ºC, 5% CO_2). Following this the supernatant was removed and 50 µL of 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2 H-tetrazolium bromide (MTT) solution (5 mg/mL) was added to each well and incubated at 37 °C for two hours. The supernatant was then replaced with 100 µL of acidic isopropanol to solubilize the precipitate, and the absorbance was determined at 570 nm and 630 nm (Multiscan microplate spectrophotometer, Model No. 1530, Multisky, Thermo Fisher Scientific, USA). A dose–response curve of the percentage inhibition of cell viability and log concentrations of the extract, was plotted using GraphPad Prism software (Version 9.0.0.) and the half maximal cytotoxic concentration (CC_{50}) was determined by nonlinear regression analysis. Chloroquine diphosphate (positive control; Sigma Aldrich, USA) 0.1–25.0 µg/mL and Fractions E and F of GG extract (2.5–250 µg/mL) were tested similarly.

Antiviral activity of GG extract, sub fractions E and F

Plaque reduction antiviral assay was performed on Vero cell monolayers with all four dengue virus serotypes as described by Talarico et al., except for a few modifications to seeding density of Vero cells and virus inoculum[30]. Vero cells were seeded at 1.75×10^5 cells/well in 500 µL of CGM, onto 24-well polystyrene plates (UltraCruz, Santa Cruz Biotechnology, USA, Cat No. sc-204445) and incubated at 37ºC in 5% CO_2 for 24 h, to obtain 80% confluent cell monolayers. Dengue virus (50 PFU/well) in 250 µL MM was added to each well and incubated for one hour under the same conditions, with periodic agitation. Following virus adsorption, the virus inoculum was replaced with one mL/ well of OM containing GG extract at different concentrations (10.41–166.67 µg/ mL). Positive controls with 50 PFU/well of virus were left untreated with extract while negative control wells were not infected with virus but treated with GG extract. Plates were incubated for seven days (37ºC, 5% CO_2). Thereafter, the cells were fixed and stained as described above in *Virus titration* method and the plaques were counted. Antiviral assays were performed twice and each concentration of GG extract was tested in duplicate wells. Percentage inhibition of virus was plotted against log of GG concentration using GraphPad Prism (Version 9.0.0.) software and nonlinear regression analysis was performed to calculate the half maximal inhibitory concentration (IC_{50}). Antiviral assays with sub fractions E and F (2.5–40 µg/mL) of GG extract were performed with the four dengue serotypes as well.

Plaque reduction assay for fractions 1–7 and sub fractions A-F

Residues of separated bands from preparative thin layer chromatography (PTLC) were weighed, and used to prepare the overlay medium for the plaque reduction assay for fractions 1–7. Antiviral activity of the seven fractions was tested with dengue virus-1 (DV1) and the percentage inhibition per unit weight of fraction was calculated for comparison. All fractions were tested in duplicate wells. Sub fractions A-F of fraction-2 were tested similarly.

Effect of GG extract, sub fraction E and F on dengue viral adsorption

The protocol of Talarico et al. was followed with a few modifications (i.e. seeding cell count and volume of virus inoculum) to elicit the effect on virus adsorption[30]. Confluent monolayers of Vero cells in 24-well plates were infected with 100 PFU/well of DV1 in the presence of either the GG extract (166.7 µg/mL), sub fraction E (40 µg/mL) or sub fraction F (40 µg/mL) in separate wells and incubated at 4 °C for one hour with periodic agitation. Although DV4 had the lowest $IC_{50,}$ since it causes mainly mild infections, DV1 was selected for the viral adsorption study. Severe outbreaks in Sri Lanka at the time of this study were mainly caused by DV1. Supernatants with unabsorbed virus were then removed and cells were washed twice with phosphate buffered saline (PBS) followed by the addition of OM (1mL) to each well. Virus plaques were counted after seven days of incubation at 37 °C with 5% CO_2[30]. Percentage inhibition of DV1 after treatment with sub fractions E, F or GG were compared with the positive control and analysed by Dunnett's multiple comparison test.

Gas chromatography of GG extract

Extract of GG was submitted to gas chromatography-mass spectrometry (GCMS). The GG powder was submitted to Agilent Technologies 7890B GC System with a triple-axis detector (Agilant Technologies, USA). Inlet temperature and auxiliary pressure controller temperature were maintained at 250 °C and 300 °C respectively.

Bioassay guided fractionation of GG

PTLC for fractionation was conducted with the solvent system ethyl acetate (EA: 27227, Sigma, USA): methanol (Me: 32213, Sigma, USA): distilled water (DW) at the ratio of 10:01:01 for the fractionation. The solvent system of 1-butanol (24124, Sigma Aldrich): acetic acid (33209, Sigma Aldrich): DW at the ratio of 4:1:5, was used for sub fractionation with PTLC. Aluminium coated Silica gel 60 F_{254} sheets (1.05554.0007, Supelco, Canada) were used as the stationary phase. The plates were dried and visualized using anisaldehyde spray reagent and UV light.

High performance liquid chromatography (HPLC) of GG sub fractions E and F

The HPLC analysis was carried out on Waters 2535, a quaternary gradient module system with a photodiode array detector and separated by the HPLC column, Reliant C18 5 µm (4.6 mm x 250 mm column). The mobile

CC_{50} (µg/mL) ± SEM		IC_{50} (µg/mL) ± SEM				SI (CC_{50}/ IC_{50})			
		DV1	DV2	DV3	DV4	DV1	DV2	DV3	DV4
GG	651.9 ± 25.41	49.66 ± 7.55	40.57 ± 6.08	42.27 ± 2.19	20.12 ± 1.56	13.12	16.06	15.42	32.40
CQ	17.00 ± 0.63	2.48 ± 0.12	7.98 ± 1.06	2.41 ± 0.13	NI	6.86	2.13	7.06	NI

Table 1. *Glycyrrhiza glabra* (GG) and chloroquine (CQ) cytotoxicity in Vero cells and dengue viral inhibition. CC_{50}: half maximal cytotoxic concentration; IC_{50}: half maximal inhibitory concentration; SI: selectivity index; DV1: dengue serotype-1; DV2: dengue serotype-2; DV3: dengue serotype-3; DV4: dengue serotype-4; SEM: standard error of mean; NI: not inhibited.

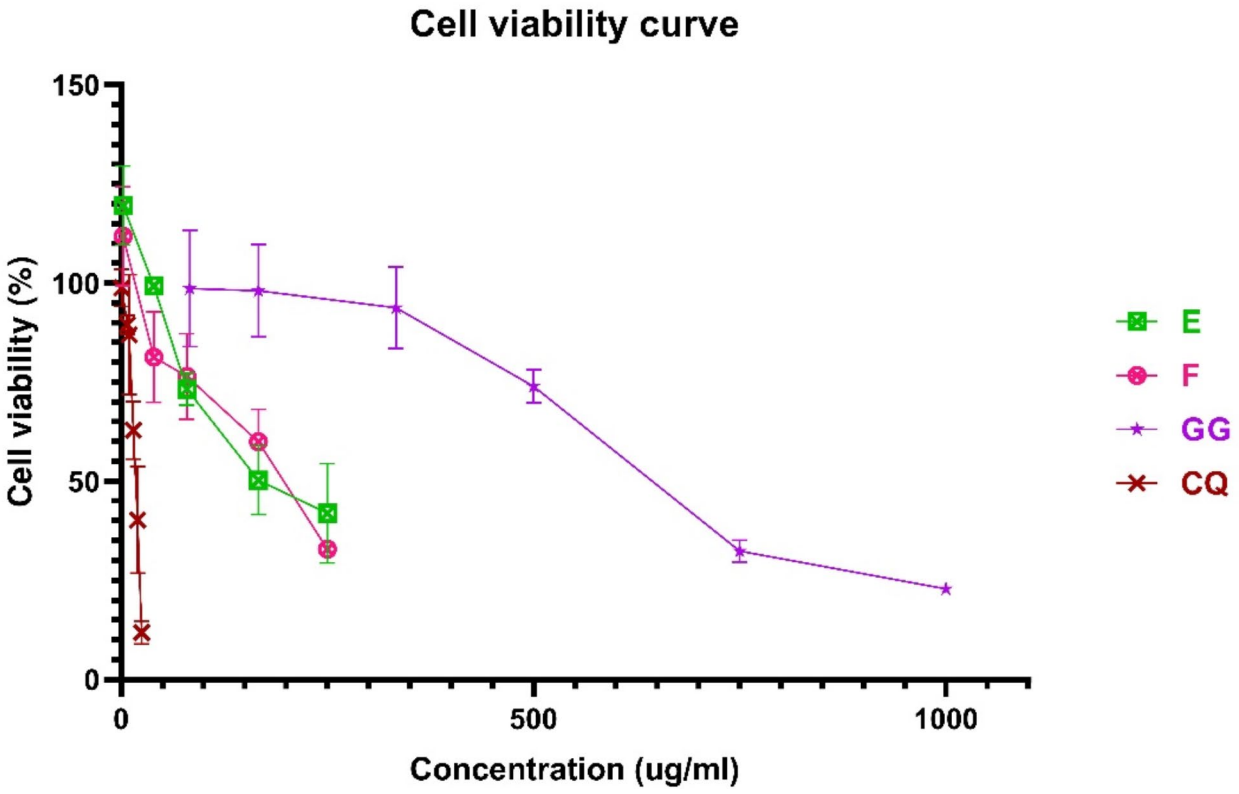

Fig. 1. Percentage of Vero cell viability following treatment with different concentrations of aqueous extract of *Glycyrrhiza glabra* (GG) (33.33–1000 µg/mL), sub fraction E (2.5–250 µg/mL), sub fraction F (2.5–250 µg/mL) and CQ (0.1–25 µg/mL) and incubation for seven days at 37 °C in 5% CO_2. Data points are the mean of triplicate wells ± SEM.

phase of 1% acetic acid and 10% methanol in distilled water was supplied in a gradient of 10–100% over 30 min at 1 mL/min with an injection volume of 10 µL. Chromatograms of 254 nm were recorded.

Statistical analysis

GraphPad Prism software (Version 9.0.0) was used for plotting dose response curves, nonlinear regression analysis and calculations of CC_{50} and IC_{50}. The ratio of CC_{50}/IC_{50} was the selectivity index (SI).

Results

GG had low cytotoxicity (CC_{50} 651.9 µg/mL) in Vero cells and the MNTD was 166.67 µg/mL, whereas CQ was strongly cytotoxic (CC_{50} 17.0 µg/mL) and the MNTD was 10.0 µg/mL (Table 1; Fig. 1). The GG extract displayed dose dependent inhibition and was 98–100% inhibitory to all four serotypes at the MNTD (Fig. 2) and displayed IC_{50} values between 20 and 50 µg/mL signifying moderate inhibitory activities (Table 1). The SI for all four dengue serotypes were > 10, suggesting that the GG extract had good potential as an antiviral agent (Table 1).

CC_{50} > 500 µg/mL: low cytotoxicity; 100–500 µg/mL: moderate cytotoxicity; 10–100 µg/mL: strong cytotoxicity; <10 µg/mL: very strong cytotoxicity; IC_{50} < 10 µg/mL: good activity; 10–50 µg/mL: moderate

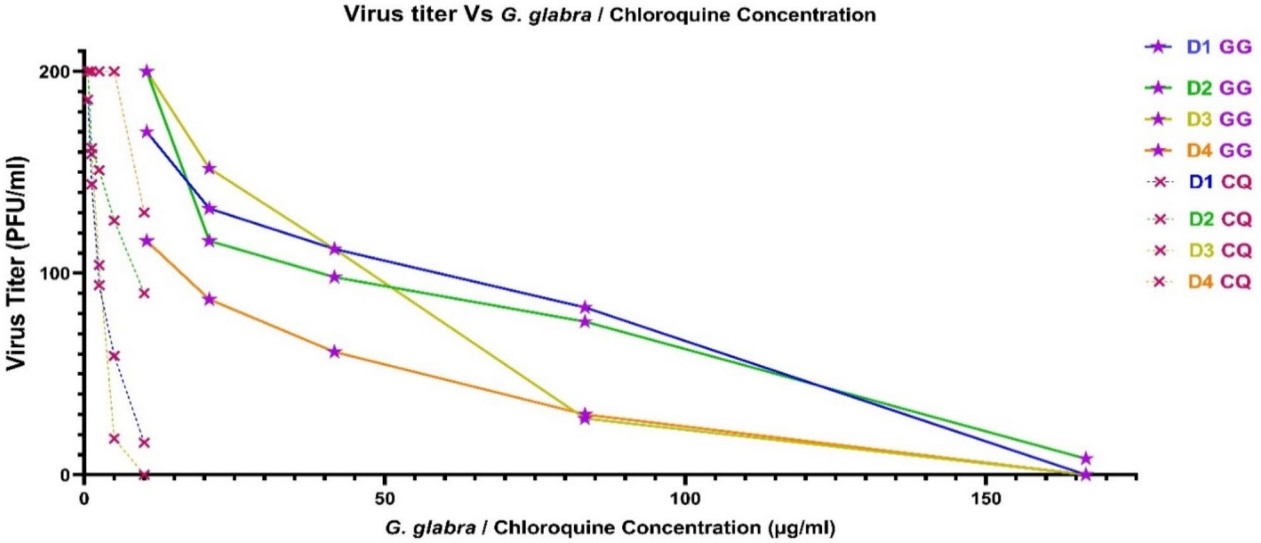

Fig. 2. Results of plaque reduction assay performed in Vero cells infected with 50 PFU/mL of dengue virus following treatment with aqueous extract of *Glycyrrhiza glabra* (GG; 10.4–166.7 µg/mL) or chloroquine diphosphate (CQ; 0.6–10 µg/mL). Independent assays were performed for dengue serotype 1 (D1), dengue serotype 2 (D2), dengue serotype 3 (D3) and dengue serotype 4 (D4) which were incubated with GG or CQ for 7 days at 37°C in 5% CO_2. Each value given is the mean of duplicate assays ± SEM (Supplementary Tables S4-S7).

activity; 50–100 µg/mL: low activity; >100 µg/mL: inactive; SI ≥ 10: recommended for further studies; 1–10: re-evaluate using other bio systems; <1: too toxic to be used[31].

Fractionation of aqueous extract of GG

Seven fractions (fractions 1–7) were isolated from the GG extract by PTLC (Supplementary Table S1). Fraction 2 had the best inhibitory activity for DV1, and yielded seven sub fractions (A-G) by PTLC (Supplementary Table S2). Further fractionation by PTLC was not possible and sub fractions E and F were therefore selected for subsequent investigations, based on their inhibitory activity. The HPLC chromatogram and the compounds identified in the GG extract via the compound library are given in Supplementary Figs. 1, 2, 3 & Table S3.

Water, methanol and ethyl acetate and their mixtures are found to be highly polar solvents which could be used to isolate hydrophilic and polar compounds[32–34]. The polar solvent system of n-butanol: acetic acid: distilled water supports the separation of polar compounds[35]. The HPLC chromatogram of sub fraction E showed six separately identifiable peaks suggesting the presence of six different molecules, whereas the chromatogram of F had one dominant peak suggesting the presence of a single compound with mild impurities. However, both E and F had a common pattern of well-defined peaks at retention times 36.673 and 36.754 respectively, suggesting the presence of closely related compounds in both sub fractions. Nonetheless, there were no standards to elucidate the identity of compounds.

The UV spectrum of peaks at 36.678 min showed two absorption bands at 228 and 277 nm which are very common in phenolic compounds[36]. However, the absence of UV absorption between 310 and 350 nm suggested that the above compound is unlikely to be a flavonoid[37]. Since the PTLC sub fractions E and F were soluble in highly polar solvents such as methanol and water, the compounds would potentially contain polar moieties as well. All this evidence shed some light on the presence of phenolic glycosides in sub fractions E and F which might contribute to antiviral properties. Since the active fractions are polar, direct injection of sample to GC might not yield informative results. Hence pre-column derivation of samples would have been the better approach.

Cytotoxicity of sub fractions E and F and inhibition of dengue serotypes 1–4 in Vero cells

Both sub fractions were moderately cytotoxic to Vero cells and the MNTDs were 40 µg/mL (Table 2). They were more toxic to cells than the GG extract (Fig. 1). Except for the low inhibitory activity for DV2, sub fractions E and F had moderate inhibitory activity for the other dengue serotypes; only DV4 had a SI > 10 (Table 2; Figs. 3 and 4).

CC_{50} > 500 µg/mL = low cytotoxicity; 100–500 µg/mL = moderate cytotoxicity; 10–100 µg/mL = strong cytotoxicity; <10 µg/mL very strong cytotoxicity; IC_{50} < 10 µg/mL = good activity; 10–50 µg/mL = moderate activity; 50–100 µg/mL = low activity; >100 µg/mL = inactive; SI ≥ 10 = recommended for further studies; 1–10 = re-evaluate using other bio systems; <1 = too toxic to be used[31].

GG Sub fraction	CC_{50} µg/mL ± SEM	IC_{50} µg/mL ± SEM				SI			
		DV1	DV2	DV3	DV4	DV1	DV2	DV3	DV4
E	184.3 ± 30.56	33.18 ± 4.65	85.95 ± 15.52	48.07 ± 6.47	12.65 ± 2.24	5.55	2.14	3.83	14.56
F	186.1 ± 34.79	29.00 ± 3.94	69.27 ± 14.10	48.08 ± 2.67	13.14 ± 2.23	6.41	2.68	3.87	14.16

Table 2. Cytotoxicity and dengue viral inhibitory activity of sub fractions E and F of GG extract. CC_{50}: half maximal cytotoxic concentration; IC_{50}: half maximal inhibitory concentration; SI: selectivity index; NI: not inhibited; SEM: standard error of mean; DV1: dengue virus-1; DV2: dengue virus-2; DV3: dengue virus-3; DV4: dengue virus-4.

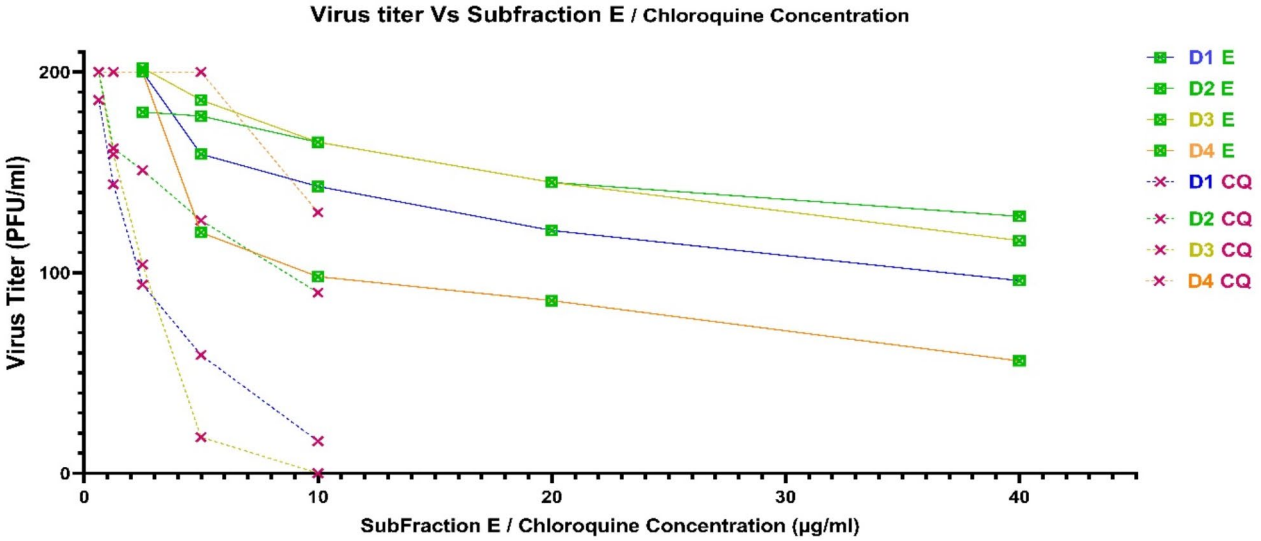

Fig. 3. Results of plaque reduction assay performed in Vero cells infected with 50 PFU/mL of dengue virus, followed by treatment with sub fraction E (2.5–40 µg/mL) or chloroquine diphosphate (CQ; 0.6–10 µg/mL). Independent assays were performed for dengue serotype-1 (D1), dengue serotype-2 (D2), dengue serotype-3 (D3) and dengue serotype-4 (D4) which were incubated with sub fraction E or CQ for 7 days at 37°C in 5% CO_2. Each value given is the mean of duplicate assays ± SEM (Supplementary Tables S8-S11).

Effect of GG extract and sub fractions E and F on dengue viral adsorption on Vero cells

The GG extract significantly ($p < 0.0001$) inhibited virus adsorption by reducing infecting virus titres from 58.65 PFU/mL (control) to 28.7 PFU/mL (average percentage inhibition 50.4 ± 3.7). Sub fraction E significantly reduced virus titres from 50 PFU/mL (control) to 37.3 PFU/mL (average percentage inhibition 24.9 ± 3.2), although the effect of sub fraction F (mean percentage inhibition 8.3 ± 5.3) was not significant (Fig. 5; Supplementary Table S16).

Discussion

This is the first report of anti-dengue viral activity of the aqueous extract of GG on all four dengue serotypes in Vero cells. Aqueous extract of GG inhibited all four dengue serotypes, whereas the positive control CQ, inhibited only DV1, DV2 and DV3. CQ was selected as the reference as it has previously been reported to have good inhibitory activity[38,39]. At its MNTD (166.67 µg/mL) GG extract inhibited all four dengue serotypes at almost a magnitude of two. It exhibited moderate inhibitory activity (IC_{50} 20.12–49.66 µg/mL) for all four dengue serotypes. Unlike CQ which was strongly toxic (CC_{50} 17 µg/mL), the aqueous extract of GG was non-toxic (CC_{50} 651.9 µg/mL) to Vero cells, increasing the therapeutic range of GG. Glycyrrhizin is a plant glycoside extracted from GG, which was found to be a potent inhibitor with DV1, DV2 and DV4[17]. In our study, the inhibition caused by the aqueous extract of GG cannot be attributed to glycyrrhizin, as it was not identified by GCMS (Supplementary Table S3).

Compared to GG extract, sub fractions E (CC_{50} 184.3 µg/mL) and F (186.1 µg/mL) were more toxic to Vero cells. They were moderately inhibitory to D1 and D3 but showed less inhibition of D2. Further evaluation with other D4 virus strains is necessary to fully explain the most pronounced antiviral effect observed with only D4. The inhibitory activity of these two sub fractions was lower compared to the GG extract, albeit not significantly different (IC_{50} values differed only by a factor of 1.1–2.1). Inhibition of dengue virus adsorption to cells, was significantly more with GG extract than with sub fractions E or F (Fig. 5.). GG extract is capable of inhibiting

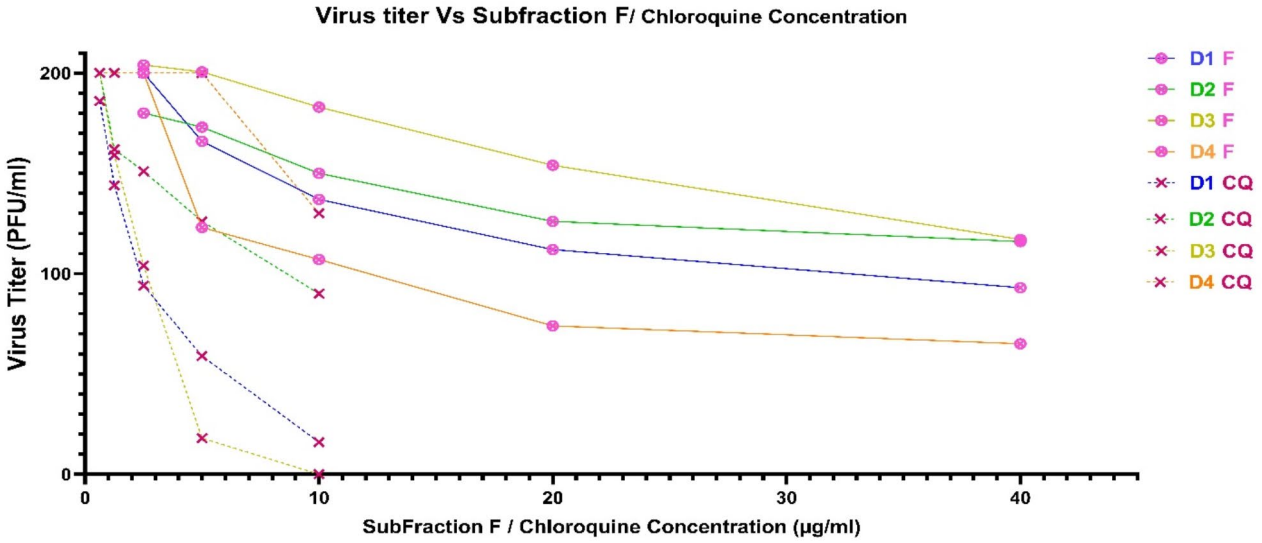

Fig. 4. Results of plaque reduction assay performed in Vero cells infected with 50 PFU/mL of dengue virus, followed by treatment with sub fraction F (2.5–40 µg/mL) or chloroquine diphosphate (CQ; 0.6–10 µg/mL). Independent assays were performed for dengue serotype-1 (D1), dengue serotype-2 (D2), dengue serotype-3 (D3) and dengue serotype-4 (D4) which were incubated with sub fraction F or CQ for 7 days at 37ºC in 5% CO_2. Each value given is the mean of duplicate assays ± SEM (Supplementary Tables S12-S15).

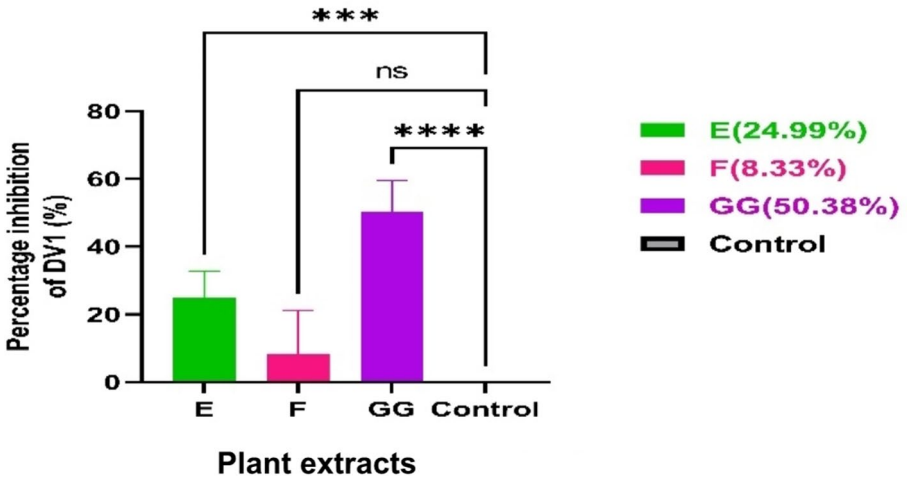

Fig. 5. Inhibition of DV1 adsorption by extract of *Glycyrrhiza glabra* (GG; 166.7 µg/mL), sub fractions E and F (40 µg/mL each) as determined by the plaque reduction assay. Each extract was tested in triplicate wells but the control wells had no extract. Percentage inhibition is given as the mean ± SEM of two independent assays. Mean percentage inhibition of sub fractions E, F and GG were compared with controls and analysed by Dunnett's method for multiple comparisons. **** $p < 0.0001$, *** $p < 0.0005$, ns = not significant.

the dengue virus before adsorption and after virus internalization, as evidenced by the higher inhibition (almost 100% at MNTD) in the antiviral assays, compared to the anti-adsorption experiment (50%). Our findings indicate that the aqueous extract of GG is a better inhibitor of the dengue virus than its sub fractions.

A popular Chinese herbal medicine *Glycyrrhizae Radix* et Rhizome (dry root and rhizome of *Glycyrrhiza uralensis* Fisch., *Glycyrrhiza inflata* Bat., and *Glycyrrhiza glabra* L.) was capable of preventing adsorption of DV2 but was incapable of inhibiting the virus cycle after internalization. Similar to our findings this herbal medicine exhibited significant inhibitory activities in the adsorption stage of the DV2 replication cycle by targeting the envelope protein. This study also reported that when glycyrrhizin, glycyrrhetnic acid, liquiritigenin, and isoliquiritigenin compounds of the herbal medicine were individually investigated, they were incapable of significant dengue virus inhibition suggesting that a combination of active molecules might be responsible for this action[12]. In our study, none of these compounds were identified in the aqueous extract of GG. Hour et al.

(2022) discovered two lead compounds of Glycyrrhizic acid which exhibited a substantial decrease in the dengue virus attachment stage[13]. To elicit the full range of activity of GG, our findings need to be confirmed in human cell lines using higher virus concentrations as well. Virucidal activity and time of addition studies to determine the site of action, are needed for a better understanding of the potential of GG extract.

Conclusion

The aqueous extract of GG was non-toxic and had better antiviral activity than sub fractions E and F. It has good potential as a therapeutic agent. These findings add to the existing knowledge of anti-dengue viral activity of GG and encourages further research of GG which could lead to the formulation of an antiviral compound.

Data availability

The datasets supporting the conclusions of this article are included within the article and its additional file and are available from the corresponding author on reasonable request.

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Author contributions

K.G.J. Investigation, original draft preparation; K.G. Funding acquisition, conceptualization, methodology, supervision of cell culture work, project administration, original draft preparation, review & editing; S.S. Supervision of fractionation, original draft preparation, review & editing; C.G. Funding acquisition, supervision of cell cytotoxicity assays, review & editingP.S. supervision of cell cytotoxicity assays, review & editing N.P. performed HPLC.

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