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Antiviral activity of *Withania somnifera* and *Curcuma longa* against Foot and Mouth Disease Virus

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ABSTRACT

Foot and mouth disease (FMD) is a highly contagious viral disease of cloven-hooved animals that has devastating outcomes. Apart from vaccination, there is no antiviral therapy available to treat the on-going FMD. The current trial was carried out to investigate the antiviral activity of *Withania somnifera* (*W. somnifera*) and *Curcuma longa* (*C. longa*) plants traditionally used against Foot and mouth disease virus (FMDV). Tissue culture technique along with 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay was used to evaluate the antiviral properties of two medicinal plants using the FMDV. The ethanol and ethyl acetate extracts of *W. somnifera* and *C. longa* were used to determine in-vitro antiviral activity against the FMDV serotype-O. The maximum non-toxic dose (MNTD) of ethanolic and ethyl acetate extract of *W. somnifera* roots and *C. longa* were 0.625 mg/ml, 0.312 mg/ml, 5 mg/ml, and 0.625 mg/ml, respectively. For antiviral activity test, 10 tissue culture infective dose 50 (TCID-50) of FMDV type-O was incubated with MNTD of each of the plant extracts for one hour prior to inoculation on cells. The preliminary antiviral extracts were tested for 3 days, and the cytopathic effects were assessed by MTT dye uptake assay. The percentage protection of baby hamster kidney-21 (BHK-21) cells against FMDV by using MNTD of ethanolic and ethyl acetate extracts of *W. somnifera* and *C. longa* were found 77.4, 76.3, 55.4, and 67.1%, respectively. Results indicated that the extract of *W. somnifera* possesses more antiviral effect as compared to *C. longa,* although the ethanolic extracts of both plants showed highest activity as compared to their ethyl acetate extracts. As these two medicinal plants possess good antiviral activities in-vitro against the FMDV Serotype O, therefore, further research is needed to be carried out by performing in-vivo trials, so that their antiviral properties may be assessed, and their use may be recommended in the field.

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Introduction

Foot and mouth disease (FMD) is among the utmost extremely contagious diseases of cloven-footed animals which accounts for huge economic losses in terms of lowered productivity, including reduction or complete loss of milk production and draught ability, as well as emaciation (Ajmal et al. 1989). Foot and mouth disease virus (FMDV) is belonging to Picornaviridae family and its genus as Aptho-virus (Grubman and Baxt 2004). This virus has distinct serotypes (A, O, Asia-1, SAT-1, SAT-2, and SAT-3) and within each serotype: there is no cross-immunity due to serotype variation. Subsequently, vaccinal strain selection varies with the prevalence of FMDV serotype and subtype. A great level of investigation is needed to determine the antigenic shift and drift in the prevalent virus strains and

careful vaccine matching studies are needed to be conducted (Ma et al., 2011).

Creation of drug therapies having a potent antiviral effect which targets particular viral protein has been attempted numerous times. However, approved drugs to treat FMD virus infection is not yet present (Abeer and Boseil 2011).

Recent success in phytomedicine and nutraceutical use of medicinal plants crude extract as the antiviral agent has raised optimism about phyto-antiviral agents(Jassim and Naji 2003). The antiviral activity of medicinal plants such as the crude extracts of *Acacia icatechu* Wild, *Cassia fistula* Linn, *Tamarindus indicus* Linn, and *Imperata cylindrica* Linn against Newcastle Disease and vaccinia virus, as well as the water crude extract of *Punica granatum* Linn against Red virus and Coxsackie virus, have been investigated (Chungsamarnyart et al. 2007). The leaves of *Melia azedarach* Linn, *Withania somnifera* (Ashwagandha) roots, and its leaves, *Ocimum sanctum* (Tulsi) leaves, *Curcuma longa* (Turmeric) had significant antiviral activity against many serotypes of FMDV (Trivikram et al. 2013).

Curcumin (diferuloylmethane), the most bioactive yellow portion of *C. longa*, has been shown to have a wide range of biological effects, including anti-inflammatory, antioxidant, anticarcinogenic, antimutagenic, anticoagulant, antifertility, antidiabetic, antibacterial, antifungal, antiprotozoal, antiviral, antifibrotic, antivenom, antiulcer, hypotensive, and hypocholesteremic (Chattopadhyay et al., 2004).

W. somnifera is a plant with a wide range of medicinal properties. Because of its antiviral properties, it is also used to treat genital disease caused by the herpes simplex virus (Fani et al. 2015). The hydro-alcoholic extract of *W. somnifera* roots inhibited the infectious bursal disease (IBD) virus by 99.9% in its maximum non-toxic dose, indicating that the extract of *W. somnifera* roots possesses antiviral properties against IBD virus (Pant et al., 2012).

The aim of our study was to determine the antiviral activity of *W. somnifera* and *C. longa* extracts against FMDV using baby hamster kidney cells (BHK-21 cells) as these plants are traditionally used to treat the FMD infected animals in the fields.

Materials and Methods

The current study was performed in-vitro on the cell line therefore no ethical approval was taken before the trail.

Study area

The in-vitro antiviral study was conducted at Abasyn University, Peshawar, Pakistan in collaboration with Foot and Mouth Disease Research Centre, Veterinary Research Institute, Peshawar, Pakistan.

Sample collection of medicinal plants and its identification

The roots of *W. somnifera* and *C. longa* were purchased from a local market. Plant identification and authentication were carried out at the Department of Botany, University of Peshawar, Pakistan.

Medicinal plants drying and its grinding

The roots of *W. somnifera* and *C. longa* were diced into smaller parts and dried for 3-4 days at 40°C under hot circulating air. To prepare and obtain crude extract in ethanol and ethyl acetate solvent, the dried parts were ground into a fine powder.

Preparation of medicinal plants extract

Pant et al. (2012) published a procedure for extract preparation. Ten grams of powdered *W. somnifera* roots and *C. longa* were first immersed in 500ml each of ethanol and ethyl acetate, then incubated at 40°C in a shaking incubator for 48 hours under continuous agitation. The mixtures were purified first through muslin fabric, then through filter paper to extract the supernatant after 48 hours of incubation. The filtrates were dried in a water bath at 50°C and stored at 4°C.

Viral isolates and cell line

To determine the antiviral activity of plant extracts against FMDV serotype-O, the virus isolate, and BHK-21 cells maintained in Glasgow Minimum Essential Medium (GMEM) were obtained from Foot and Mouth Disease Research Centre, Veterinary Research Institute Peshawar, Pakistan.

FMDV antigen detection ELISA (Serotyping of FMDV-O)

Morioka et al. (2014) explained how the serotype of FMDV was determined using a sandwich ELISA. The FMDV sample was confirmed through sandwich ELISA, before that cell-culture sample was prepared and about 1ml sample was taken in a sterile 1.5ml centrifuge tube. The tube was then centrifuged for 10 minutes at 2000X g. Then the 250ul off the supernatant was taken in another sterile tube and equal volume of the sample diluent (supplied with kit) was added. Then the precoated 96 well plate, was taken and 50ul of the sample diluent was added to all the wells of 11th and 12th column as a positive and negative control. The 50ul of the sample (cell cultured) was added to all the wells of first column. The plate was then incubated for one hour at room temperature. After incubation, the content of the plate was discarded, and washed with 1x wash buffer three times with 300ul to each well with 3 minutes incubation between each wash. After each washing the plate was tapped on the absorbent paper. The conjugate A and B was prepared as mentioned in the procedure. About 50ul of the conjugate A was added from well A to F and conjugate B to G and H. The plate was then incubated for another hour at room temperature. After second incubation, the plate was again washed as previously described but in the last wash a 5 minute incubation was given to the plate.

Then the 50ul of the conjugate was added to all wells in dark and plate was then placed for another 20 minutes incubation at dark chamber. After incubation 50ul of the stop solution was added to each well of the plate and then the plate was read on

an ELISA reader at 450nm primary wavelength and 630nm as a secondary wavelength. The optical density (OD) value was then calculated as mentioned in the provided protocol with the kit.

Antiviral effect determination of extracts against FMDV Serotype –O

Using the procedures of Gupta et al. (2010), the antiviral effect of each extract against FMDV type-O was evaluated.

Formulation and formation of different concentrations of extract

About 100mg of plant extract was dissolved in 5ml PBS and transferred through a 0.2m membrane filter to assess antiviral efficacy. To prepare 2-fold dilutions, a diluted extract of 0.5ml filtered PBS was applied to ten Eppendorf tubes. Later, 0.5ml plant extract was transferred to the first Eppendorf tube, mixed thoroughly, and then 0.5ml of this mixture was moved to the second Eppendorf tube, and so on until the tenth Eppendorf tube was reached. This method yielded 100ul of extract diluted twofold at concentrations of 20, 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, and 0.039 mg/ml.

Evaluation of maximum non-toxic dose

Two techniques were used to determine the toxicity of *W. somnifera* roots and *C. longa* extracts on BHK-21 cells. Visual examination of the extract's cytopathic effect on cells. Optical density (OD) values were used to assess toxicity using the MTT dye system in 96 well microtiter plates. About 2×10^5 cells of BHK-21 were seeded and incubated with 100µl of two-fold dilution of each extract prepared at concentration of 20, 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078 and 0.039 mg/ml in triplicates in 96-well plates and incubated at 37°C in a humid environment with 5% CO2 for at least 3 days.

MTT dye assay

The quantity of viable cells was determined visibly as well as by using the MTT dye uptake assay. Each well of the plates received a 10ul volume of MTT dye, which was incubated for 4 hours at 37°C. The supernatant was decanted after incubation, and 100µl of DMSO was transferred to each well, which was then held in a shaking incubator at room temperature for 15 minutes. The dye that the viable cells used was removed by adding DMSO. A microplate reader was used to calculate the OD value at 560nm, which was documented. A maximum nontoxic dose of an extract was established as a concentration at which the number of viable cells did not reduce as compared to control.

Biological titration

Biological titer (Tissue culture infective dose (TCID50) of FMDV serotype-O was determined as referred by Reed and Muench (1938).

Antiviral assay

The antiviral efficacy of non-toxic concentrations of plant extracts against FMDV serotype-O was tested in BHK-21 cell lines using a cytopathic inhibition assay. The MTT dye uptake approach was used to test the reduction in cytotoxicity caused by the virus in extract-treated BHK-21 cells infected with 10 TCID50 viruses. For the antiviral assay, all the wells of the microtiter plate were seeded with $2x10^5$ BHK-21 cells. Then a challenge dose of 10TCID50 was mixed separately in tubes with a non-toxic concentration of the plant extracts and then incubated for one hour at 37°C in a CO2 incubator. After the incubation, each mixture (virus and extract) was supplied to the plate having the culture cells and containing only maintenance media with 5% fetal calf serum. The plate was then incubated for 3 days at 37°C using a carbon dioxide incubator. The control plates only contain media without extracts and viruses. After 24 hours of the incubation, the plates were examined for cytopathic effect on the cells and the data was recorded accordingly. After three days of incubation, cell viability was determined by transferring the 10µl MTT dye (5mg/ml) and incubating the plate for another 4 hours at 37°C. The supernatant was discarded and the MTT dye was removed with the help of 100µl DMSO per well by shaking the plate for at least 15 minutes. Then the OD of the plate was taken at 560nm with the help of a Microplate reader. The percent safety of the extract was calculated as below.

100 percent (ODT)V-(ODC)V/(ODC)M-(ODC)V

Where,

(ODT)V denotes the absorbances of the virus infected cells and plant extract

(ODC)V represents the absorbances of the virus infected cells without plant extract

(ODC)M stands for the absorbances of the cells without virus infection

Analytical statistics

The data was compiled in Microsoft Excel and the standard error of mean for OD was calculated by using descriptive statistics through SPSS version 16.

Results

W. somnifera and *C. longa* extracts were tested for antiviral activity against an FMD virus isolate in the current trial. In the BHK-21 cell line, the MNTD of plant extracts was measured.

MNTD of W. somnifera Roots in the BHK-21 cell line

In BHK21 cell line, the ethanolic and ethyl acetate extracts of *W. somnifera* roots showed significant cytotoxicity. As calculated and compared by OD values with control, toxic concentration cells were circular and pH of medium often became acidic, and nontoxic concentration cells were similar to control. The nontoxic concentrations for ethanolic and ethyl acetate extracts of *W. somnifera* roots were 0.625 mg/ml (OD Value = 0.260) and 0.312 mg/ml (OD Value = 0.289), respectively, according to the MTT dye assay (Fig. 1 and 2; Table 1).

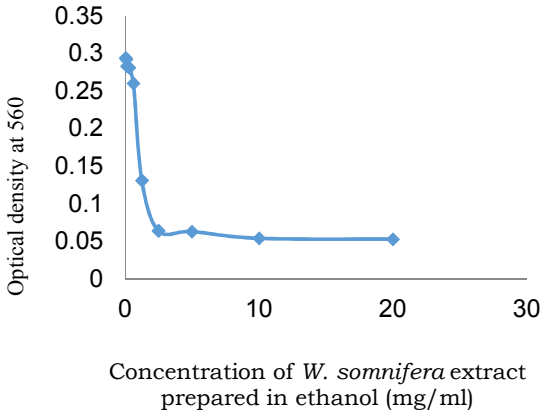

Fig. 1: Determination of MNTD of ethanol extract of *W. somnifera* in BHK-21 cell line based on optical density values

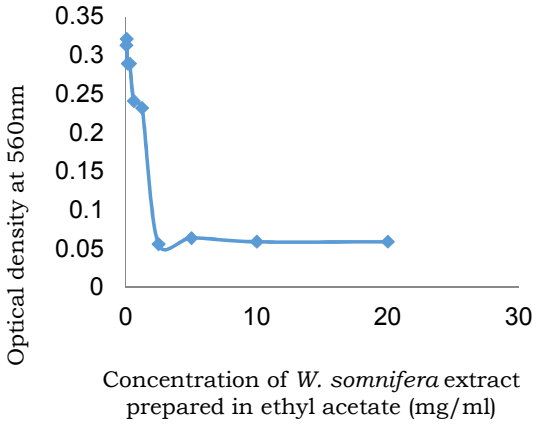

Fig. 2: Determination of MNTD of ethyl acetate extract of *W. somnifera* in BHK-21 cell line based on optical density values.

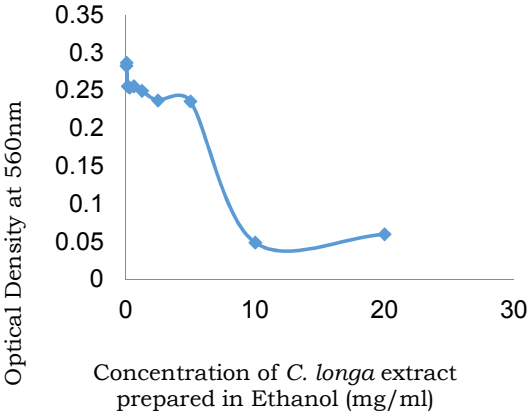

Fig. 3: Determination of MNTD of ethanol extract of *C. longa* in BHK-21 cell line based on optical density values

Table 1: Determination of MNTD of *W. somnifera* roots extracts in BHK-21 cell line

Different concentration of plant extracts in mg/ml	*W. somnifera* roots extract		Cell control (Mean±S.E.)
	Prepared in Ethanol	Prepared in Ethyl Acetate	
20.0	0.053	0.059	0.235 ± 0.0157 (for ethanolic extract) 0.265 ± 0.01 (for ethyl acetate extract)
10.0	0.054	0.059	
5.0	0.063	0.064	
2.5	0.064	0.056	
1.25	0.131	0.232	
0.625	0.260	0.241	
0.312	0.281	0.289	
0.156	0.283	0.289	
0.078	0.292	0.321	
0.039	0.294	0.313	

On the BHK-21 cell line, the ethanolic extract of *C. longa* had a mild cytotoxic effect, while the ethyl acetate extract of *C. longa* had a high cytotoxic effect. Ethanolic and ethyl acetate extracts of *C. longa* had non-toxic concentrations of 5 mg/ml (OD Value = 0.236) and 0.625 mg/ml (OD Value = 0.220), respectively. (Fig. 3 and 4; Table 2).

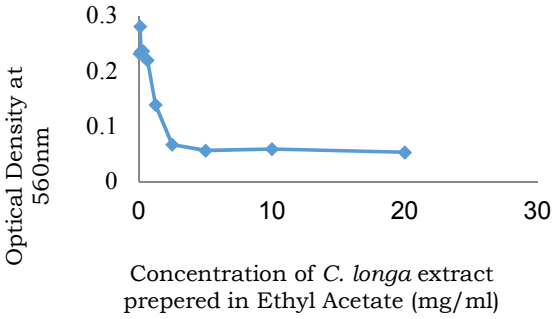

Fig. 4: Determination of MNTD of ethyl acetate extract of *C. longa* in BHK-21 cell line based on optical density values

Table 2: Determination of MNTD of *C. longa* extracts in BHK-21 cell line

Different concentration of plant extracts in mg/ml	*C. longa* Extract		Cell control (Mean±S.E.)
	Prepared in Ethanol	Prepared in Ethyl Acetate	
20.0	0.060	0.053	0.235 ± 0.0157
10.0	0.049	0.059	
5.0	0.236	0.056	
2.5	0.237	0.067	
1.25	0.250	0.139	
0.625	0.256	0.220	
0.312	0.254	0.236	
0.156	0.256	0.236	
0.078	0.287	0.281	
0.039	0.283	0.232	

Determination of TCID 50 of FMDV using a BHK-21 cell-line

In the BHK-21 cell line, the FMDV titration (TCID 50) was found to be 10-5.8 TCID 50 per ml.

Antiviral Assay

The antiviral effects of non-toxic concentrations of ethanolic and ethyl acetate extracts of *W. somnifera* and *C. longa* were measured against FMDV using an inverted microscope and the MTT dye assay (Table 3 and 4).

Table 3: Antiviral effect of *W. somnifera* and *C. longa* against FMDV (Optical density value)

Row Status	Ethanol Extract			Ethyl Acetate Extract		
W. somnifera	0.223	0.227	0.246	0.217	0.243	0.229
C. longa	0.185	0.191	0.177	0.212	0.201	0.216
Control						
Cell control	0.291		0.249	0.292		0.293
Virus control	0.066		0.058	0.060		0.073

Table 4: Mean SE values of *W. somnifera* and *C. longa* antiviral activity against FMDV

Row Status		Mean	SEM	N
W. somnifera	Ethanol Extract	0.232	0.0071	3
	Ethyl Acetate Extract	0.229	0.0075	3
C. longa	Ethanol Extract	0.184	0.0041	3
	Ethyl Acetate Extract	0.209	0.0044	3
Cell control		0.281	0.0108	4
Virus control		0.064	0.0034	4

Table 5: *W. somnifera* and *C. longa* provide a high percentage of protection against FMDV

Plant Extracts	Ethanol Extract	Ethyl Acetate Extract
W. somnifera	77.4%	76.3%
C. longa	55.4%	67.1%

The percent protection of *W. somnifera* and *C. longa* ethanolic and ethyl acetate extracts (at MNTD) were detected 77.4, 76.3, 55.4, and 67.1% respectively, hence, showing their antiviral activity against FMDV Serotype-O (Fig. 5; Table 5).

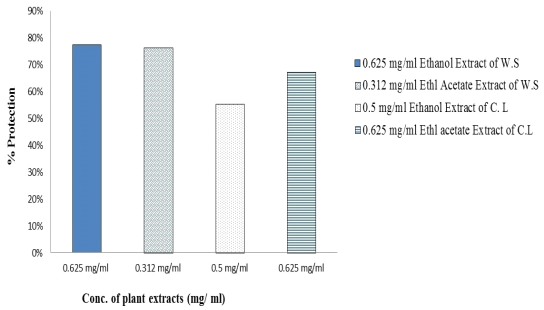

Fig. 5: Percent defense of *W. somnifera* and *C. longa* against Virus

Discussion

Foot and mouth disease (FMD) is an economically significant disease that is found all over the world and is prevalent in Pakistani cattle and buffaloes. The only preventive mechanisms for FMD are stamping out sick animals and prophylactic measures, since there is currently no FDA-approved medication for FMDV. Though stamping out of infected animals is the best strategy, Pakistan is a developing country whose economy cannot support the high cost of this practice, so vaccination is used instead. However, due to the emergence of new strains and mutation, FMD vaccines usually face protection issues (Rahman et al. 2018). In addition, FMD vaccines do not have protection until seven days after vaccination.

There have been many attempts to create an antiviral drug therapy that attacks particular viral targets. Several plants and herbs have been discovered to have the ability to act as new antiviral agents (Jassim and Naji, 2003). The antiviral properties of *W. somnifera* and *C. longa* were studied in-vitro in this research.

The ethanolic and ethyl acetate extracts of *W. somnifera* exhibited the potent antiviral activity of 77.4 and 76.3% respectively, against FMDV. These results agree with study previously conducted by Kambizi et al. (2007) who tested *W. somnifera* extract for antiviral activity against HSV-1, HIV, and the Infectious Bursal Disease Virus. Grover et al. (2011) found that Withaferin A, a steroidal compound found in *W. somnifera*, has antiviral activity against the Herpes Simplex Virus. Sahoo et al. (2021), findings also support our results, according to their report the *Curcuma longa* (Turmeric) contains curcumin, demethoxycurcumin, and bisdemethoxycurcumin. Curcumin has long been used as a common home remedy for coughs, sore throats, and respiratory illnesses, and may be a useful immune booster for SARS-CoV-2 therapy in the current pandemic scenario. Both turmeric and curcuman were easily tolerated at extremely large doses without causing any adverse effects. Parida et al. (2021) found that traditional medicine has utilized turmeric (*Curcuma longa* L.) for diabetes, hepatitis, hemorrhoids, hysteria, skin disease, inflammation, anorexia, hepatic difficulties, cough, and sinusitis. The ethanolic and ethyl acetate extracts of *W. somnifera* and *C. longa* were shown to have antiviral properties against FMDV in this research. These results are

confirmed by Trivikram et al. (2013), who discovered that aqueous extracts of *W. somnifera* and *C. longa* have significant antiviral effects against FMDV. Another antiviral study (Dhawan et al., 2021) has been conducted recently to evaluate the antiviral properties of the *W. somnifera* on corona virus, which stated that four steroidal lactones in *W. somnifera*: withanone, withanolide D, withanolide A, and withaferin A. WFA is an anti-tumorigenic, anti-inflammatory, pro-apoptotic, anti-angiogenic, and anti-invasive molecule. Antiviral protein SARS-S CoV-2 may be changed and inhibit the SARS-main CoV-2 protease (Mpro). Withanolides have been found to decrease cytokine production in the lungs during infection. Another research tested withanone as a strong inhibitor of SARS-CoV-2 coronavirus entrance into host cells (Balkrishna et al., 2021).

The ethanolic and ethyl acetate extracts of *W. somnifera* roots, as well as the ethyl acetate extract of *C. longa*, were found to have significant cytotoxic effects in the BHK-21 cell line, but the concentrations of 0.625, 0.312, and 0.625 mg/ml were found to be non-toxic to the BHK21 cells. The ethanolic extracts of *C. longa* had only minor cytotoxic effects in the BHK-21 cell line, but a concentration of 5 mg/ml was determined to be non-toxic to the cells. This highlights the importance of exercising strict care before using it in-vivo.

Curcumin and its counterparts, gallium-curcumin and Cu-curcumin have been shown to have similar in-vitro antiviral efficacy against herpes simplex virus type 1 (HSV-1) in cell culture with IC50 values of 33.0, 13.9 and 23.1 g/mL, respectively. According to another review, curcumin has potent antiviral properties against the Epstein-Barr virus (Zorofchian et al., 2014) . Another research revealed that *Curcumin longa* hydroalcoholic extract has a strong inhibitory impact on HSV-1 replication (Fani et al., 2015).

Singh et al. (2010) investigated various bio-conjugates of curcumin against a number of viruses, including FMD, parainfluenza virus type 3 (PIV-3), respiratory syncytial virus (RSV), and others. MTT testing revealed curcumin's remarkable antiviral properties against Vesicular stomatitis virus, and Flock House Virus, with EC50 values of 0.011 and 0.029 M, respectively.

Curcumin is also anti-HIV (human immunodeficiency virus) since it inhibits the HIV-1 integrase enzyme, which is needed for viral replication (Mazumder et al. ,1995). According to our findings, *C. longa* extract offers only a small percentage of cell defense against FMDV serotype-O, ranging from 55.4 to 67.1% for both solvents using MNTD of 5 and 0.625 mg/ml, respectively. It is likely that the dilution solution, phosphate buffer saline solution, used to prepare various amounts of extracts for assessing MNTD does not dissolve curcumin properly, an active part of *C. longa*, and hence does not move through the filter during filtration. This assessment agrees with (Liu et al., 2008), who suggested that *C. longa* contains a naturally occurring phenolic compound called curcumin, which has low water solubility due to its lipophilic origin, limiting its medicinal applications in aqueous systems. So far, the research suggests that *W. somnifera* and *C. longa* may be useful natural medicinal plants for treating FMD.

Conclusion

The current research found that both *W. somnifera* roots and *C. longa* had potent anti-FMDV properties. In the BHK-21 cell line, ethanolic and ethyl acetate extracts of *W. somnifera* roots, as well as ethyl acetate extracts of *C. longa*, exhibited significant cytotoxic effects. It implies that strict care should be exercised prior to its in-vivo use. *C. longa* ethanolic extracts had only a minor cytotoxic effect. When compared to *C. longa* extracts with MNTD of 0.625, 0.312, 5 and 0.625 mg/ml, *W. Somnifera* ethanolic and ethyl acetate extracts showed the best protection of BHK-21 cells against FMDV. Our results showed that both solvents extract of *W. somnifera* possesses high antiviral activity.

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Authors contribution

The experiment was developed and designed by FA and AS. The research was conducted by BK, SU, and HUR, with laboratory analysis conducted by SK. MA was responsible for supervising and coordinating the studies, as well as providing scientific information. QU and R analyzed the experimental data statistically. FA and HUR drafted the manuscript. All authors contributed to the manuscript's critical revision and approved the final version.

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