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Original Article

Inhibition of Foot-and-Mouth Disease Virus Replication by Hydro-alcoholic and Aqueous-Acetic Acid Extracts of *Alhagi maurorum*

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Abstract

Foot-and-mouth disease (FMD) is a major infectious disease of cloven-hoofed animals that is caused by the FMD virus (FMDV). This disease has significantly adverse economic impacts; therefore, rapid control measures are urgent. Hydro-alcoholic and aqueous-acetic acid extracts of *A. maurorum* were prepared and their anti-FMDV activity was evaluated. Gas Chromatography–Mass Spectrometry (GC-MS) analysis of methanolic and ethanolic extracts was performed to find the likely active compounds of *A. maurorum*. The cytotoxicity of the extracts was assayed and the antiviral activity of them was evaluated by measuring the percentage of viable FMDV infected-cells via the MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) assay at different stages of the virus replication cycle. The results indicated that the plant extracts exhibit antiviral activity against FMDV at all stages of the experiment, although the most significant effects were observed in virucidal and pre-treatment assays. GC-MS of the extracts resulted in the separation of 3 and 2 main peaks for the methanolic and ethanolic extracts respectively. The major compound was found to be 1, 2-Benzenedicarboxylic acid, diisooctyl ester. These findings represent the anti-FMDV activities of *A. maurorum* extracts at several stages of the virus replication cycle; therefore, it could be considered for the potential development of anti-FMDV therapeutics.

Keywords: 1,2-Benzenedicarboxylic acid, diisooctyl ester, *Alhagi maurorum*, Antiviral activity, Cytotoxicity, Foot-and-mouth disease vius, Gas Chromatography–Mass Spectrometry analysis

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1. Introduction

Foot-and-mouth disease (FMD) affects cloven-hoofed animals, including wildlife and livestock. This disease is caused by the FMD virus (FMDV) which belongs to the *Aphtovirus* genus of the *Picornaviridae*

family and characterized by lameness, fever, and vesicular lesions particularly on the tongue, hooves, feet, snout, and teats. It is rarely fatal but has significantly adverse economic impacts because it leads to a decrease in the quantity of produce obtained from cattle [1]. The World Organization for Animal Health put FMD on its "A list" of infectious diseases affecting animals, indicating it to be a transmissible, serious, and rapidly spreading disease of socioeconomic importance [2].

Current control measures for FMDV include culling and vaccination; however, there are a number of concerns such as serotype dependence, "immunity-gap," and maternal antibody response [3]. Although vaccination is an important tool for controlling FMDV, complete clinical protection is only achieved 7 days after the immunization [4]. To overcome these concerns, alternative methods need to be investigated and developed. Prophylactic antiviral compounds with low cost, high potency, and specificity that can be easily stored may be used as an alternative to emergency FMD vaccinations [3, 4].

Plants have had an important role in folk medicine since ancient times pertaining to the treatment of various human and animal diseases. Interest pertaining to plants being a significant source of new pharmaceuticals is now growing [5]. Researchers have studied several hundred plant species with a potential of being a source of novel antiviral agents [6]. The *Leguminosae* family comprises various medicinal plants that provide important raw materials used in the pharmaceutical industry. *Alhagi maurorum* Medik. a plant belonging to this family, is commonly known as camel thorn and has widely been used in traditional medicine [7]. Taranjebin, one of the most important compounds in traditional Iranian medicine, is obtained from *A. maurorum* and is used in Iranian ethnomedicine for the treatment of some viral diseases such as rubella, measles, and smallpox [8]. A few studies have been conducted on this plant's medicinal properties.

Numerous secondary metabolites have been isolated from the methanolic and ethanolic extracts of *A. maurorum.* Chemical investigation on this species revealed the presence of antioxidant compounds, essential oils, terpenoids, ketones acid derivatives, hydrocarbons, alkaloids [9], flavonoids [10], fatty acids, sterols [11], coumarins [12], vitamins [7], and phenolic constituents [13]. Various studies have tested this plant's medicinal properties; however, to date, no study has reported its potential antiviral benefits although traditional ranchers in Iran use this plant to treat FMD. Therefore, in this study, *A. maurorum* was collected and effects of its hydroalcoholic and aqueous-based extracts on FMDV were evaluated.

2. Materials and Methods

2.1. Extracts Preparation

The camel thorn plant was collected from Ahvaz, Iran in spring 2014; and was identified as *Alhagi maurorum* Medik. by Dr. Vaezi, Division of Botany, Shahid Chamran University of Ahvaz. A voucher specimen

was deposited in the Herbarium of SCUA, Voucher Number 452. The aerial parts of plant were cleaned and dried in shade for 14 days and were then powderized for maceration extraction. To prepare ethanolic, methanolic, and aqueous-acetic acid extracts, 25 g of powder was extracted using 100 ml of solvents containing 90% ethanol, 80% methanol, and 2% aqueous acetic acid respectively. The solutions were shaken for 48 h, filtered and incubated at 40°C in order to remove the solvents.

2.2. Cell Culture and Virus

Baby hamster kidney-21 (BHK-21) cells were cultured in growth medium containing RPMI-1640, 10% fetal bovine serum (FBS), and antibiotics (100 U/mL penicillin-streptomycin) followed by incubation at 37°C in 5% CO_2 atmosphere. FMDV/O/2010/IRAN was received from the Razi Vaccine and Serum Research Institute.

2.3. Cytotoxicity Assay

The cytotoxicity of extracts was assayed by the MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide)(Sigma-Aldrich chemical; Germany) method as described by Zandi et al [14]. BHK cells were seeded in a 96-well plate and incubated for 48 h at 37°C in a 5% CO_2 atmosphere until a monolayer was formed. The cells were then treated with 0.003, 0.03, 0.3, 3, 30, and 300 mg/ml of each extract using 3 wells for each concentration and incubated for 72 h at 37°C. The percentage of non-viable cells was determined by the following formula: [1−(At/As)] × 100, where At is the absorbance of the test sample and As is the absorbance of the control sample (i.e., cells with no added extracts).

2.4. Antiviral Activity

The antiviral activity of the extracts was evaluated by measuring the percentage of viable cells via MTT assay, because FMDV induced death in the infected cells. Microscopic observations were performed throughout each experiment to identify normal cells and CPEs. The antiviral activity of tested extracts was investigated at different stages of the virus replication cycle.

Virucidal assay (extracellular phase):

For this assay, two-fold serial dilutions of non-toxic extract concentrations (0.3, 0.15, 0.075, 0.0375, 0.0187, and 0.0094 mg/ml of ethanolic and aqueous extracts and also, 3, 1.5, 0.75, 0.375, 0.187, and 0.094 mg/ml of methanolic extract) were prepared. For each extract, 100 µl of each concentration was incubated with a fixed concentration of FMDV (one multiplicity of infection (MOI)) at 4°C for 1 h. The mixtures were then added to the 96-well plate containing a BHK cell monolayer and incubated for 1 h. Later, the cells were washed with PBS; media with 2% FBS was added, and the wells were incubated for 72 h until CPE appeared in untreated wells.

2.5. Pre-treatment Assay

100 µl of each non-cytotoxic concentration of extracts described above was added to BHK cells on a 96-well plate and incubated

for 2 h. The cells were washed with PBS, and wells were inoculated with 1 MOI of virus and incubated for 1 h. After washing with PBS, the media was added, and the plates were incubated for 72 h.

2.6. Co-treatment Assay

One MOI of virus and 100 µl of serial diluted extracts were inoculated to the plates contemporaneously and *incubated* for 1 h. After washing with PBS, they were incubated for 72 h at 37°C in 5% CO_2 atmosphere.

2.7. Adsorption Assay

For this stage, 1 MOI of virus was added to each well of BHK monolayers and incubated for 1 h. During this time, the virus would probably attach to the cells' receptors. Further, the medium was removed, the extract concentrations were added, and the plates were incubated for an additional hour. Subsequently, the wells were washed, the medium was added, and the plates were further incubated for 72 h.

2.8. *Post-treatment Assay and monitoring of the Intracellular Replication Phase of FMD Virus*

1 MOI of virus was inoculated to the cells for 1 h, and the cell monolayers were washed and incubated for another 2 h. Within this time the virus would likely enter the BHK cells; 100 µl of each extract at different concentrations was then added for an additional 1 h. To evaluate the effects of extracts on the intracellular replication phase of FMDV, serial dilutions of each extract were added at 4, 6, 8, 10, and 12 h after viral penetration. In all the steps, cells infected with untreated virus were used as controls.

2.9. Gas Chromatography–Mass Spectrometry (GC–MS) Analysis

GC–MS analysis of the ethanolic and methanolic extracts of *A. maurorum* was performed using an Agilent chromatograph (Series 7890A) interfaced with a mass spectrometer (Series 5975C; Agilent) equipped with an HP-5 MS capillary column (Agilent) (length = 30 mm, i.d. = 25 µm, thickness = 0.25 µm). An electron ionization system with an ionizing energy of 70 eV was employed for detection. Helium was used as the carrier gas at a flow rate of 1 ml/min. The analysis was conducted using an oven temperature of 40°C for 1 min (initial column temperature) that was gradually increased to 280°C at a rate of 5°C/min and was maintained at 280°C for 1 min, for a total analysis time of about 50 min. The injector temperature was set at 280°C. The mass spectrometry scan was obtained from 50 to 550 m/z. The injection volume was 1 µl, and the split ratio was 10:1. The identification of components was performed by correlating the obtained mass spectra data with NIST MS Search version on Wiley-8 library.

2.10. Statistical Analysis

Each test was repeated 3 times, and the data are represented as mean ± SD. The percent inhibition rate of viral infection by extracts in each test was calculated using the following formula: $[(A_{tv} - A_{vc})/ (A_{cc} - A_{vc})]$

×100. A_{tv} indicates the absorbance of FMDV-infected cells treated with extract. A_{vc} and A_{cc} indicate the absorbances of virus-infected cells and control cells, respectively. A concentration achieving 50% inhibition was defined as the $IC_{50,}$ which was determined by linear regression based on dose-response curves. Finally, the *in vitro* anti-FMDV activity of each extract was expressed as a selectivity index, a marker of antiviral activity, which was calculated as CC_{50}/IC_{50} [14]. Data analysis using Duncan tests was performed with SAS software.

3. Results and Discussion

3.1. GC-MS Analysis

The ethanolic and methanolic extracts of *A. maurorum* have more antiviral activity than the aqueous-acetic acid extract; therefore, GC-MS analysis was performed to identify the chemical compounds present in these extracts. Gas chromatography of the extracts resulted in the separation of 3 main peaks for the methanolic extract and 2 main peaks for the ethanolic extract. Constituents present in these extracts detected by GC-MS are shown in table 1. The major compound was found to be 1, 2-Benzenedicarboxylic acid, diisooctyl ester (Table 1).

3.2. Cytotoxicity of Extracts

To determine the optimal concentrations of extracts that have no cytotoxic effect on the BHK cell line, the MTT assay was performed and CC_{50} was calculated. The methanolic extract at concentration of 3 mg/ml or less had no cytotoxic impacts on cells, while concentration of 0.3 mg/ml was gained for ethanolic and aqueous-acetic acid extracts. The methanolic extract with a CC_{50} of 30.5 mg/ml showed the least cytotoxic effects against the BHK cell line (Figure 1).

3.3. Antiviral Assay

To determine whether the direct action of extracts with FMDV have virucidal activity, extracts were pre-incubated with virus prior to infection and then inoculated into the cells. The MTT assay showed that all the extracts had significant virucidal activity. At this stage, the methanolic extract had the highest SI (Table 2). In the pre-treatment experiment, cells were exposed to extracts for 2 h prior to viral infection. This assay showed that the non-cytotoxic concentrations of extracts have anti-FMDV properties and may prevent FMDV-induced cell death. At this stage, the methanolic extract had the highest SI and the ethanolic extract had the lowest IC_{50}. Inoculation of the virus simultaneously with extracts into the cells (co-treatment assay) revealed the least inhibitory impacts. The adsorption assay showed that ethanolic, aqueous-acetic acid and methanolic extracts had inhibitory impacts on the virus. At this step, the ethanolic extract had the lowest IC_{50} and the methanolic extract had the highest SI. The addition of extracts after virus adsorption or during the intracellular replication period slightly reduced virus CPEs and infected-cell death. Extracts exhibited inhibitory effects until 4 h after virus penetration; however, the most prominent activity was until 2 h (Figure 2) (Table 2).

Table1. Chemical constituents of *Alhagi maurorum* ethanolic and methanolic extracts, detected by GC-MS.

S. No	Retention time (min)	Name of the compound	Area%	
			Ethanolic extract	Methanolic extract
1	4.786	Octane	----------	0.74
2	41.544	9-Octadecenamide, (Z)-Erucylamide	----------	0.94
3	42.362	Ethanol, 2-butoxy-, phosphate	15.92	7.87
4	44.062	Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-ethyl	----------	2.47
5	44.445	1,2-Benzenedicarboxylic acid, diisooctyl ester	55.37	----------
6	44.497	1,2-Benzenedicarboxylic acid, diisooctyl ester	----------	63.9
7	45.893	Methoxyacetic acid, 3-tridecyl ester	----------	0.7
8	47.146	Phosphine, tridodecyl	----------	3.54
9	47.878	2-Ethyl-2-imidazoline	----------	3.94
10	48.056	Phosphine, tridodecyl	----------	0.55
11	48.668	Phosphine, tridodecyl	9.23	----------
12	49.274	Phosphine, tridodecyl	19.71	----------
13	49.429	Phosphine, tridodecyl	----------	14.5
14	49.458	Phosphine, tridodecyl	5.9	----------
15	49.561	Phosphine, tridodecyl	----------	0.85
16	49.612	Phosphine, tridodecyl	0.71	----------
17	49.835	Phosphine, tridodecyl	1.22	----------

Table 2. Anti-FMDV activity of ethanolic, methanolic, and aqueous-acetic acid extracts**.**

Extract		Virucidal assay		Pre-treatment assay		Co-treatment assay		Adsorption assay		Post-treatment assay		Intracellular assay	
	CC_{50}	SI	IC_{50} (mg/ml)	SI	IC_{50} (mg/ml)	SI	IC_{50} (mg/ml)	SI	IC_{50} (mg/ml)	SI	IC_{50} (mg/ml)	SI	IC_{50} (mg/ml)
Ethanoli	2.71	135.5	0.02	67.75	0.04	3.19	0.85	24.63	0.11	13.55	0.08	33.87	0.12
Methanolic	30.5	508.3	0.06	72.6	0.42	15.72	1.92	80.26	0.38	50	0.17	20.07	1.52
Aqueous-acetic acid	2.61	65.25	0.04	29	0.09	7.46	0.35	15.35	0.17	13.05	0.2	8.7	0.3

CC_{50} cytotoxicity concentration 50; SI selectivity index; IC_{50} inhibition concentration 50

To date, only a few antiviral compounds have been introduced against FMDV. The use of anti-FMDV compounds can be a stop-gap measure in the face of outbreaks [4].

Some secondary products of plants have been reported to exhibit anti-FMDV activity. Apigenin, a naturally existing flavonoid in plants, inhibits FMDV-mediated CPE. This

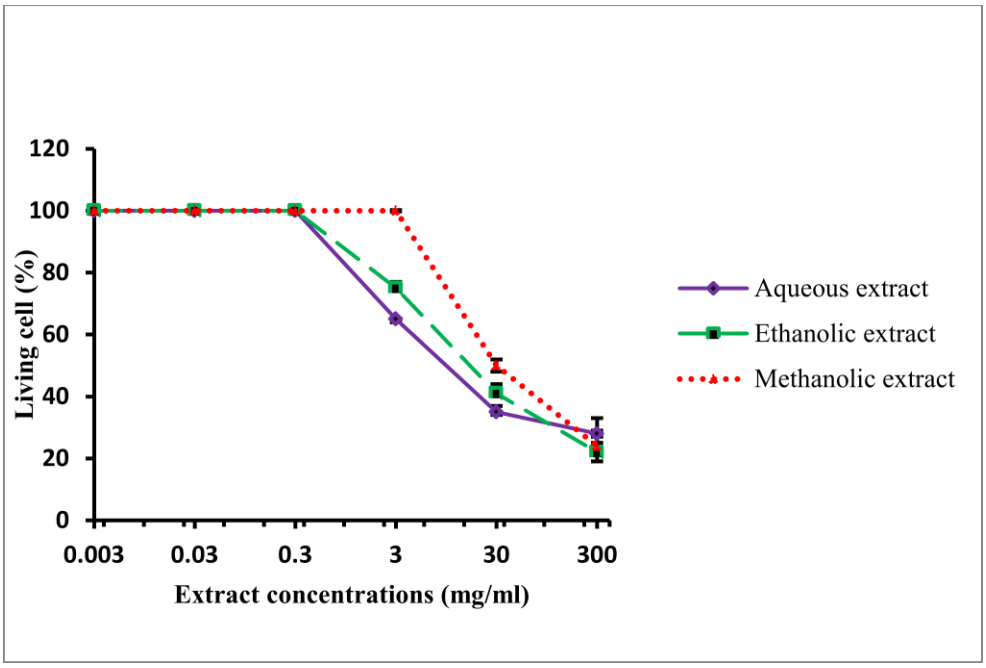


Figure 1. Cytotoxicity of aqueous-acetic acid, ethanolic, and methanolic extracts using MTT assay on BHK -21 cell line.

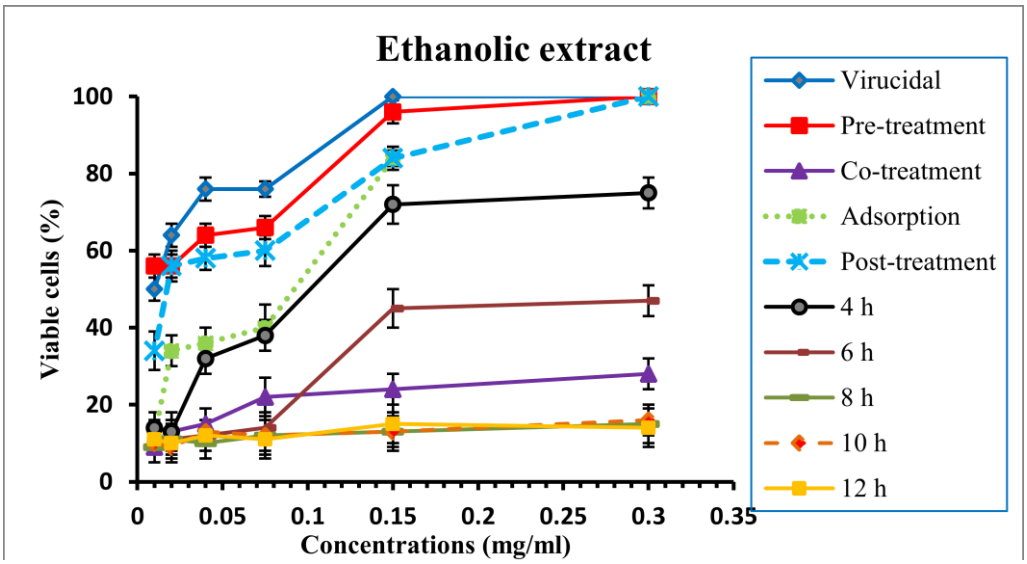
flavonoid inhibits the virus after entry into the cell by interfering with its translational activity driven by internal ribosome entry sites [15]. In addition, meliacine, a peptide isolated from the leaves of *Melia azedarach* L. blocks FMDV penetration by preventing the uncoating step. It also interferes with the release of viral particles. Apigenin and meliacine do not show extracellular virucidal activity [6, 15, 16].

FMDV is an endemic viral disease in Iran. In folk medicine, *A. murorum* is used by traditional farmers to treat and decrease the signs of FMD. In our study the *in vitro* effects of the plant on FMDV were investigated. MTT assay showed that the methanolic extract has lower cytotoxicity than the ethanolic and aqueous extracts; however, in general, this plant assessed a low toxic behavior in BHK cells. The effects of the extracts against FMDV were evaluated in different sections as follow: i) direct virucidal activity, ii) protective prophylactic effect, iii) co-treatment and adsorption assay, and iv) mode of intracellular antiviral activity. All the extracts exhibited a dose-dependent inhibition effect against FMDV in BHK cells with the IC_{50} values presented in table 2. Results indicated that the plant extracts exhibit antiviral activity against FMDV at all stages of the experiment, although the most significant effects were observed in virucidal and pre-treatment assays (Table 2) (Figure 2). The extracts exhibited a potent extracellular anti-FMDV effect when preincubated with the virus. The extracts presumably have antiviral compounds that free virions are very sensitive to them. Our results suggest that a possible mechanism whereby the *A. marorum* extracts inhibit FMDV infection could be attributed to their ability to bind and/or inactivate structural and/or non-structural viral protein(s) of

FMDV. This direct virucidal activity on extracellular free FMDV particles could introduce the plant as a candidate to neutralize free virions at viremic stages. Good prophylactic effects were observed when the cells were pre-treated with extracts prior to infection. These observations could be because of the interference *A. maurorum* extracts with virion structures or masking viral receptors, which are necessary for virus adsorption or entry into host cells. It is also likely that the uptake of the plant extracts by the cells during pretreatment could inactivate the virus's intracellular replication phase. Nonetheless, the anti-FMDV activity of extracts added into BHK cells simultaneously with viruses was relatively less significant as compared with the other treatments (Table 2). In this stage, there has presumably not been enough time for the extracts to affect the virus or mask viral receptors on the cell surface. The investigation of adsorption and post-treatment assays showed the moderate effect of extracts on virus infectivity. Following the addition of extracts until 4 h after adsorption of FMDV into BHK cells, we noticed an inhibition of FMDV replication; however, after this, there was no significant antiviral activity. This may be because of the effects of compounds' extracts on non-structural proteins of virus or enzymes for virus replication. One replication cycle of FMDV takes 4–6 h, and the extracts are likely more effective in the first cycle of replication; therefore, subsequent increasing of the number of viruses decreases the antiviral activity of the extracts.

Plants synthesize compounds for defense against microorganisms, insects, and herbivores. The main advantage of natural products is that the crude extracts contain a mixture of substances such as phenols, alcohols, acids, esters, aldehydes, etc. Therefore, it is difficult to develop resistance by pathogen agents unlike the synthetic antibiotics that contain a single substance [17].

A



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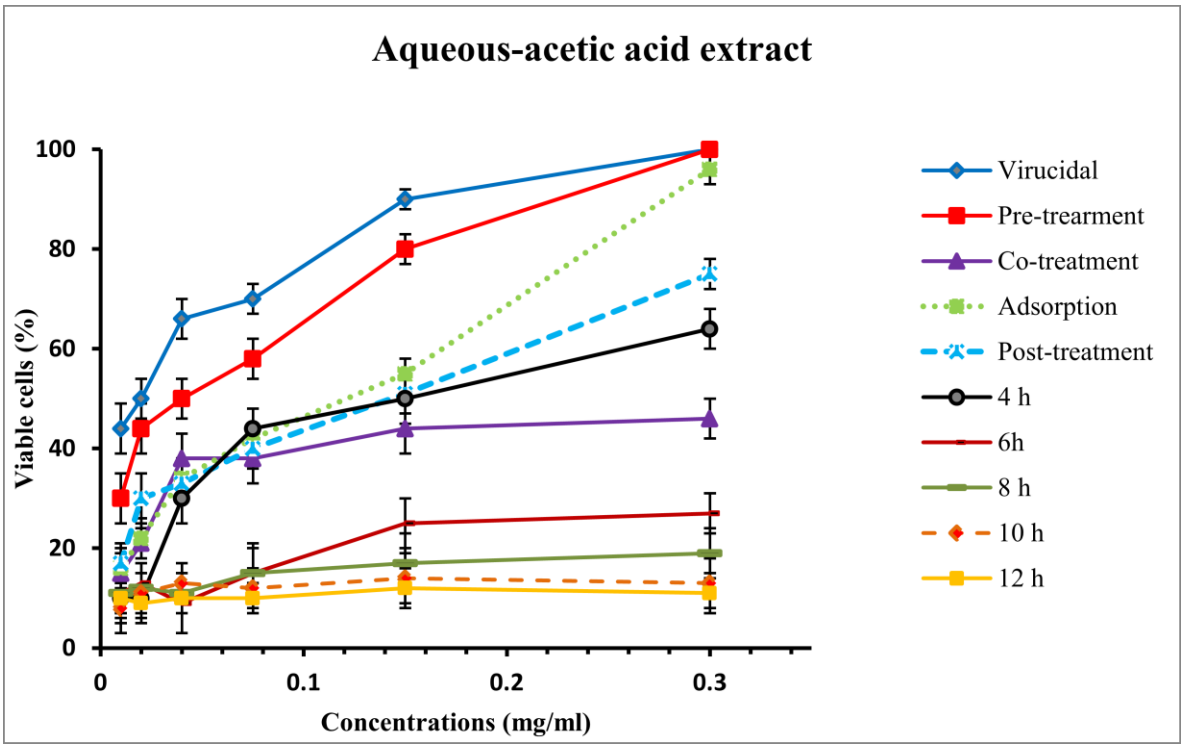

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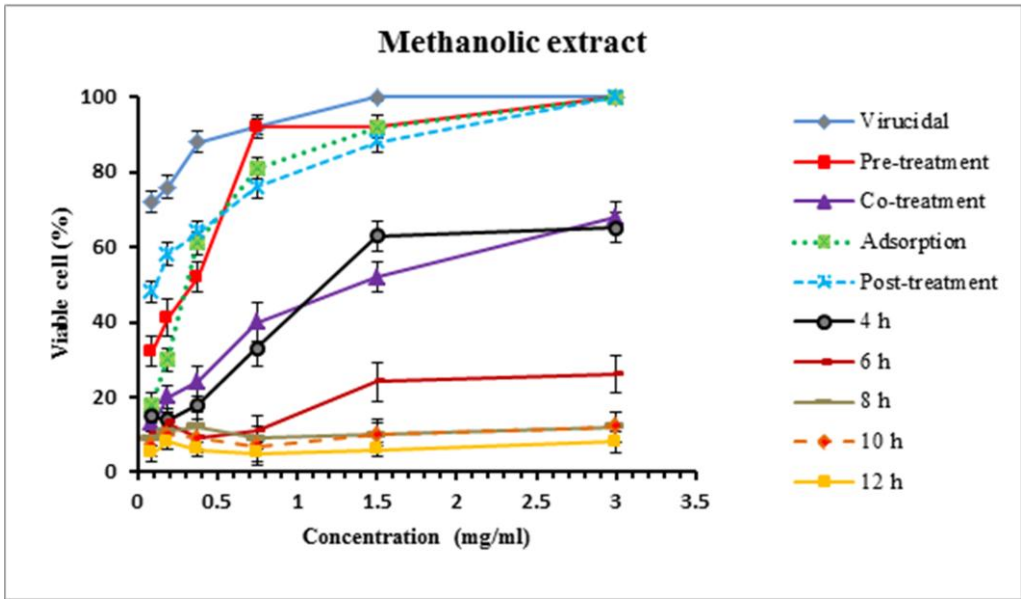

Figure 2. Antiviral activities of *Alhagi maurorum* aqueous-acetic acid (A), ethanolic (B), and methanolic (C) extracts against FMDV on BHK-21 cell line by the MTT method at virucidal, pre-treatment, co-treatment, adsorption, post-treatment and intracellular (4, 6, 8, 10, and 12 h after virus penetration) assays.

For detection of *A. murorum* constituent(s) that led to antiviral activity, GC-MS analysis was performed. Methanolic and ethanolic extracts were more active against FMDV;

therefore, these extracts were analyzed. The GC-MS results showed that different classes of medicinally valued phytochemicals are present in these plant extracts. These compounds responsible for possible antiviral activities are not yet reported to be present in *A. maurorum*. Earlier findings on secondary metabolites of *A. maurorum* are different from our study. It is may be because of differences in extraction methods, solvents, part of plant that was used, plant genetics, climate conditions, and geographical origins. The GC-MS analysis showed that 1, 2-Benzenedicarboxylic acid, diisooctyl ester also known as diisooctyl phthalate, has the highest concentration among detected secondary metabolites. This compound has been detected in various sources from plant extracts, endophytic fungi, and microbes [18]. This compound is well known as an antimicrobial agent. It also has anti-inflammatory and antifouling properties [19].Velmurugan et al. reported that active compounds of *Psidium guajava* Linn root bark have activity against white spot syndrome virus (WSSV) [20]. GC-MS analysis demonstrated that one of main compounds of this plant is 1,2-benzenedicarboxylic acid [20]. Bindhu et al. reported that 1, 2-Benzenedicarboxylic acid, diisooctyl ester in the *Agathi grandiflora* extract helps to control the WSSV and boosts the immune system against WSSV [19]. Krishnan et al. showed that 1,2-benzene dicarboxylic acid extracted from *Streptomyces* sp. has cytotoxic effects on some cancer cell lines such as HepG2 and MCF7 [21]. Presumably, this compound is one of the active components of extracts against FMDV.

Phenol, 2, 2′-methylenebis [6-(1, 1-dimethylethyl)-4-methyl- or Antioxidant 425 was the phenolic compound identified from the extracts. This compound was detected in *Streptomyces cavouresis* KUV39 isolated from vermicompost samples in India and *Streptomyces* sp. MUM256. It was demonstrated that this compound exhibited potent antioxidant properties and cytotoxicity against Hela cells; therefore, it could be used as a preventive agent for oxidative-stress related diseases [18]. FMDV induces free oxygen radicals, so it may be possible to reduce oxidative stresses of this virus by phenolic compounds that are known as potent antioxidant agents.

4. Conclusion

The anti-FMDV activities of *A. maurorum* extracts were noticeable at more stages of the experiment including pre-treatment, adsorption, post-treatment, and especially virucidal assays. The potent, direct virucidal activity of *A. maurorum* extracts was revealed; therefore, it can be used as an important criterion for anti-FMDV drug development to neutralize extracellular FMDV circulating in viremic cases. Our findings also highlight the presence of different substances in *A. maurorum* methanolic and ethanolic extracts that perhaps are the naturally active anti-FMDV constituents. The mechanism of how the extract precisely inhibits FMDV replication

remains ambiguous; future studies may determine whether the inhibitory effect is because of binding and/or inactivation of structural and/or non-structural viral protein(s) of FMDV by some constituents of the extracts and/or is due to effects of extracts on virus receptors on the cell surface.

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