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ANTIVIRAL ACTIVITY OF PLANT EXTRACTS AGAINST FMDV IN VITRO A PRELIMINARY REPORT

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Plant extracts, antiviral, FMDV, BHK-21 cell line

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ABSTRACT

This study aims at determining the antiviral effects of herbal extracts on the Foot and Mouth Disease Virus (FMDV) of livestock. The FMDV suspention was inoculated on BHK-21 cell line and the cytopathic effects were determined after introduction of herbal extracts of eight different plant samples to check the antiviral activity and these herbal extracts are: *Withania somnifera* (Ashwagandha) Roots, *Withania somnifera* (Ashwagandha) Leaves, *Azadirachta indica* (Neem) Bark, *Azadirachta indica* (Neem) Leaves, *Acacia catechu* (Khair) bark, *Ficus bengalensis* Vad stems, *Ocimum sanctum* (Tulsi) leaves, *Curcuma longa* (Turmeric) in suitable concentrations. In this preliminary study, the antiviral activity was observed in vitro in the four different plant extracts such as Ashwagandha Roots, Ashwagandha Leaves, Tulsi leaves and Turmeric. The Ashwagandha Roots and Tulsi leaves extracts are effective up to 1:2 dilutions, while Ashwagandha Leaves and Turmeric extracts are effective up to 1:1 dilution for the antiviral activity.

INTRODUCTION

Foot-and-mouth disease (FMD) is a transboundary, economically devastating and highly contagious viral disease of livestock, most importantly cattle, buffalo and pig. The disease also affects goats, sheeps, wild ruminant species and elephants (Pattnaik et al 2012). It can rapidly spread through a region if control and eradication practices are not implemented upon its detection. Weight loss, poor growth, permanent hoof damage, and chronic mastitis are just some of the sequelae of infection. The detection of FMD in a country impacts international trade and embargoes could cause significant economic losses. (http://www.vet-zone.com/Livestock/Foot-and-Mouth-Disease.html). The causative FMD virus is antigenically diverse having seven distinct serotypes and many variants within them. Being a single stranded RNA virus, it confirms the quasispecies nature with emergences and reemergences of different genetic lineages with altered antigenicity within the serotypes, making vaccination based control programme a highly cost effective, time consuming and difficult to achieve. As per the OIE and FAO, the disease is a major threat to food security of the world, and particularly the countries having the disease are more prone to food insecurity (Pattnaik et. Al. 2012) FMD is the most feared infectious animal disease owing to nearly 100% morbidity, rapid spread, severe decrease in livestock production, and mortality in young animals. The causative agent, FMD virus (FMDV) belongs to genus Aphthovirus in the family Picornaviridae. The virus exists as seven distinct serotypes (O, A, C, Asia1 and Southern African Territories (SAT) 1-3 and multiple subtypes in each serotype (Domingo et al 2003). Vaccination or recovery from infection, with one serotype does not protect against infection from other serotypes and sometimes against another subtype within the same serotype. Wide host range, ability of the virus to infect animals with a small dose, rapid rate of virus replication, high level of viral excretion and multiple modes of transmission aggravate the FMD outbreak scenario. The disease situation in India is complicated due to the plurality of the circulating virus strains in serotypes O, A and Asia 1, unrestricted movement of animals from infected areas and in apparent infection in small ruminants. Among the serotypes, type O is the most prevalent one and accounts for 83–93% of the outbreaks followed by Asia 1 (3–10%) and A (3–6.5%) (Pattnaik et. Al. 2012). FMD vaccines, whether used prophylactically or for control of an outbreak must closely match the type and subtype of the prevalent FMDV strain. With seven sero-types, and more than 60 subtypes of FMDV, this task is one of the biggest challenges in FMD vaccination (http://www.vet-zone.com/Livestock/Foot-and-Mouth-Disease.html).

FMDV has a single stranded, positive sense RNA genome, approximately about 8,500 nucleotides in length, and enclosed within icosahedral protein capsid made up of sixty copies of each structural proteins (VP1–VP4). The genome has a single open reading frame (ORF), flanked by two untranslated regions (UTRs). A small viral protein, VPg, is covalently linked to the 50 end of viral RNA (Sangar et.al.1977). Chungsamarnyart et. al. 2007 also studied the antiviral effect of the forty-seven ethanol crude-extracts of 42 plants against the Foot and Mouth Disease virus (FMDV) type O, local strain KPS/005/2545. The distribution of FMD virus types in the Maharshtra State since inception indicate that the disease occurs mostly because of "O" subtype followed by "Asia-1" and "A" type. There is no occurance of disease due to Type"C"since1992 (http://www.indg.in/agriculture/animalhusbandary/Foot%20and%20Mouth%20Disease.pdf).

Survey of the Foot & Mouth Disease

Survey of the FMD was done in Shindewadi village (Tal. Malshiras, Dist. Solapur) as it had the outbreak of the FMD in the month January and February in 2008.

Animal	Cattle	Goat	Total
Total Population	884	560	1444
No. of animals affected/died	80/0	5/0	85/0
Morbidity	9.04 %	0.89 %	5.88 %
Mortality	0 %	0 %	0 %
Loss In Milk Production	30%	20%	25%
Vaccination Status	0	0	0
Clinical symptoms	- Foot & Mouth lesion, lesion on udder. - Salivation, fever, Weight loss, lameness. - Reduction in milk production.		
Sources of Disease	-Breath, saliva, urine, faces of affected animals. -Migrant animals.		
Treatment	-Antibiotics and Ointments		
Recovery	-100% -Longer time (arvg. 40 days)		
Economic loss / animal	-Rs. 10,000		
Pattern of the Disease	-Sub-acute		
Type of Disease	-Endemic		

Infection of susceptible animals with FMD virus leads to the appearance of vesicles (blisters) on the feet, in and around the mouth and tongue, and on the mammary glands of females. Vesicles can also occur at other sites, such as inside the nostrils and at pressure points on the limbs - especially in pigs (http://www.gov.mb.ca/agriculture/livestock/anhealth/jaa01s00.html) and in our preliminary study, similar symptoms in infected animals were observed from outbreak area.

MATERIALS AND METHODS

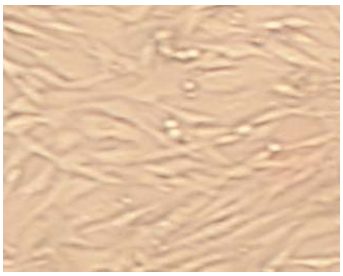
Plant materials and reagents : The medicinally important plants were collected from 'Nakshatra Udyan' (a botanical garden based on its ayurvedic medicinal applications) of VSBT. Eight different plant samples were used to check the antiviral activity such as *Withania somnifera* (Ashwagandha) Roots, *Withania somnifera* (Ashwagandha) Leaves, *Azadirachta indica* (Neem) Bark, *Azadirachta indica* (Neem) Leaves, Acacia species (Khair) bark, *Ficus bengalensis* (Vad) stems, *Ocimum sanctum* (Tulsi) leaves, *Curcuma longa* (Turmeric). Aqueous extracts of the above different plant parts were made by heating that plant part fine powder in boiling water. These extracts were then filtered by blotting paper and then by Whatman filter paper. The plant extracts were then sterilized by autoclaving at 121^0C for 15 minutes at 15 psi pressure. Reagents such as Dulbecco's Modified Eagle's Medium (DMEM), Trypsin Phosphate Versene Glucose (TPVG), antibiotic antimycotic solution (10,000 U/ml penicillin, and 10 mg/ml streptomycin and amphotericin B 100 µg/ml), Trypan Blue, Fetal Calf Serum (FCS) were purchased from Himedia, Mumbai, India.

Maintenance of BHK-21 cell line : The cell line BHK 21 (clone 13) procured from National Centre for Cell Science (NCCS), Pune was used for the in vitro propagation of the virus. The cell line was split in the ratio 1:4 initially and was allowed to get acclimatized to DMEM. After sufficient acclimatization, the cell line was split in the ratio of 1:2 to obtain confluent monolayer of cells. The entire medium was removed from culture flasks under the aseptic conditions in laminar air flow cabinet. 10 ml fresh DMEM culture medium containing 10% FBS and 1 ml antibiotic antimycotic solution was added. The culture flasks were then incubated at 37^0C and 5% CO_2 concentration. After the cells had grown to 90% confluence in culture flasks containing DMEM supplemented with 10% FBS, 10,000 U/ml penicillin, and 100 µg/ml streptomycin was added and the cells were incubated at 37^0C and 5% CO_2 concentration. Then, the culture medium was removed and cells were washed once with PBS. The cells were treated with 500µl of TPVG solution for 2-5 minutes to detach the monolayer of cells from the culture flasks. 4.5 mL of above culture medium was then added to inactivate the trypsin. The cell suspension was centrifuged at 5000 rpm for 5 minutes at room temperature. The cell pellet was then redissolved in 2 ml of same culture medium and the cell count was taken by using Neubauer chamber. The cell viability was checked by using Trypan Blue dye exclusion technique in which 0.4 % Trypan Blue dye solution was mixed with equal volume of cell suspension. Cells were then seeded in new culture flasks at the final seeding cell density of 1 x 10^6 cells/ml.

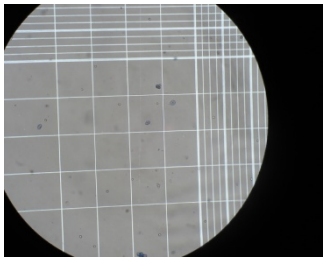
The exhaustion of the culture medium for growth by the BHK-21 monolayer of cells results in change in color of the medium from red to orange and then to yellow. So the cells were fed after every 72 hours interval and so the BHK-21 cells were growing normally to the confluence. BHK-21 cell line was successfully fed and maintained by using DMEM supplemented with 10% FBS, 10,000 U/ml penicillin, and 100 µg/ml streptomycin and amphotericin B. The BHK-21 cell line was adapted to the DMEM culture medium.

Passaging of BHK-21 cell line: BHK-21 cell line was passaged successfully at ~90% confluence by TPVG treatment for 2-5 minutes at room temperature. TPVG treatment for 2-5 minutes at room temperature is sufficient for detachment of monolayer of adhered BHK-21 cells without disturbing the cells integrity.

a) BHK-21 cell line at ~90% confluence before Passaging (10X)



b) Cell count by using Neubauer chamber (10X)



c) BHK-21 cell line after Passaging getting adhered (10X- Phase contrast)

Specimen collection for FMDV isolation:

Foot and mouth lesion specimens were collected in the solution containing equal volume of phosphate buffer (pH 7.2) and glycerol with antibiotic antimycotic solution in the sterile screw capped falcon tubes. The collected tissue samples were transported to the laboratory in ice box at 0-4^{0}C. These specimens were then stored at -80^{0}C until further processing. The specimens were collected from infected animals from outbreak area with symptoms of FMDV infection.

Diagnosis of the Foot and Mouth Disease:

Diagnosis of FMD was done by in-vitro virus isolation on BHK-21 line. The tissue specimen collected from the FMD affected cattle suspected to be having FMD virus were thawed to room temperature. These tissue samples were crushed in phosphate buffer (pH 7.2) by using sterile mortar and pestle and glass beads. Then, this suspension was centrifuged at 8000 rpm for 10 minutes at 4^{0}C. Supernatant was then collected and filtered by using syringe filter assembly with sterile 0.22 µm

membrane filter to remove all the other contaminations. 2 ml of this filtrate was then added to the culture flasks having 90% confluent BHK-21 cell line after removal of all the culture medium. These flasks were then incubated for 60 minutes. Then, FMDV suspension was removed completely and 10 ml of DMEM medium supplemented with 10% FBS, 10,000 U/ml penicillin, and 100 µg/mL streptomycin was provided to cell line. These culture flasks were incubated in CO_2 incubator at 37^0C, 5% CO_2 concentration, observed for cytopathic effects (CPE) of virus on BHK-21 cell line.

Screening the in-vitro antiviral effect of plant extracts on FMDV:

BHK-21 cells were seeded into each well of the 96 well plate at the initial cell density of $1x10^4$. After the cells reached to 90% confluence, culture medium was removed from each well. Then, cells were incubated with 50µl of FMDV suspension for 1 hour. Virus suspension was removed and 50µl of four dilutions of plant extract were added to the wells. 100µl culture medium was added to the wells and the plate was incubated in CO_2 incubator at 5% CO_2 concentration for 48 hours. The addition of the virus suspension and plant extracts was done in the following manner:

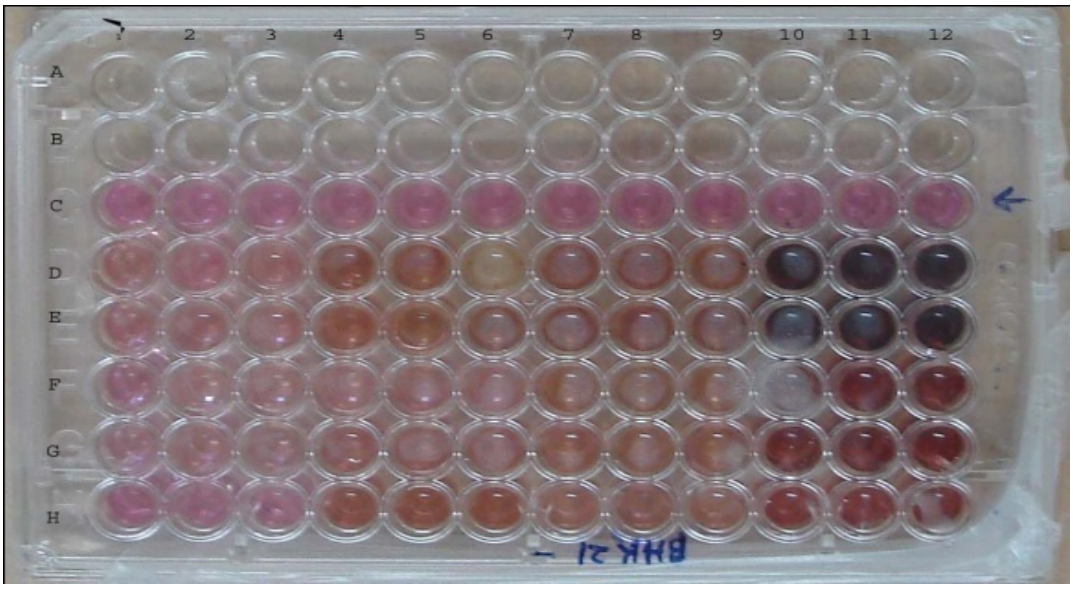


Plate 1

Wells A1-B12: Empty
Wells C1-C6: Monolayer of BHK-21 cells
Wells C7-C12: Cells+ FMDV
Wells D1-D12: Cells + 4 plant extracts in triplicate
Wells E1-E12: Cells + FMDV + 1st 4 plant extracts in triplicate (neat)
Wells F1-F12: Cells + FMDV + 1:1dilution of 1st 4 plant extracts in triplicate
Wells G1-G12: Cells + FMDV + 1:2 dilution of 1st 4 plant extracts in triplicate
Wells H1-H12: Cells + FMDV+ 1:4 dilution of 1st 4 plant extracts in triplicate

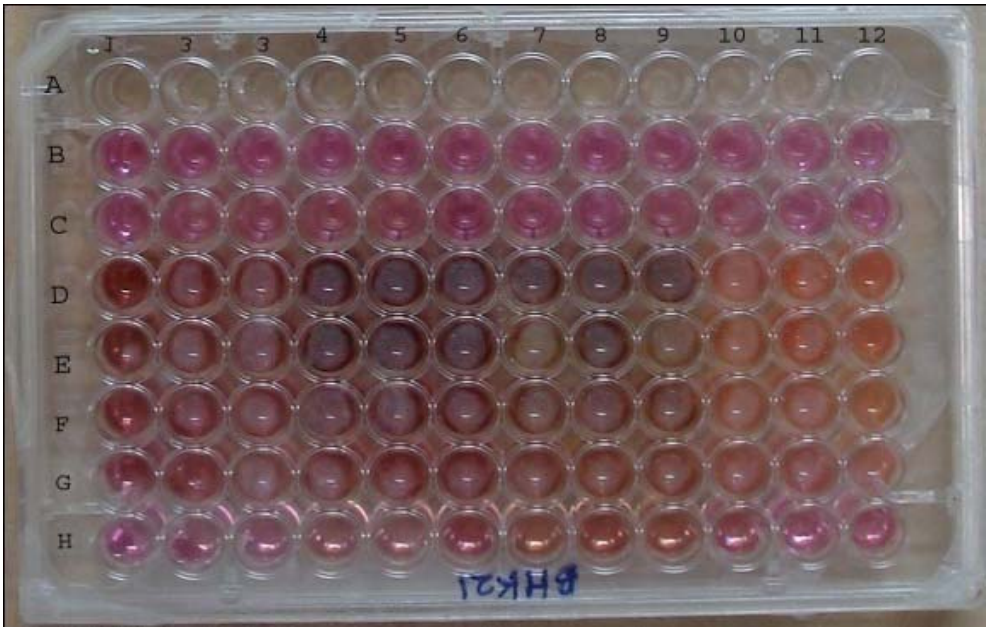


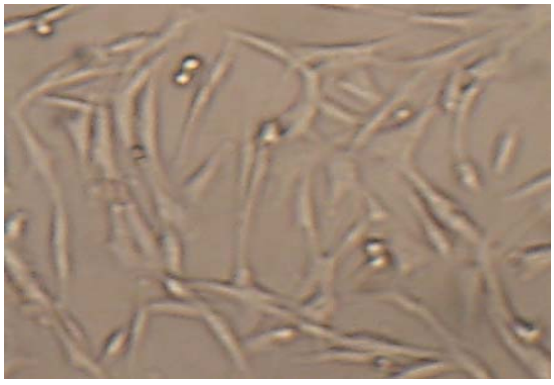
Plate 2

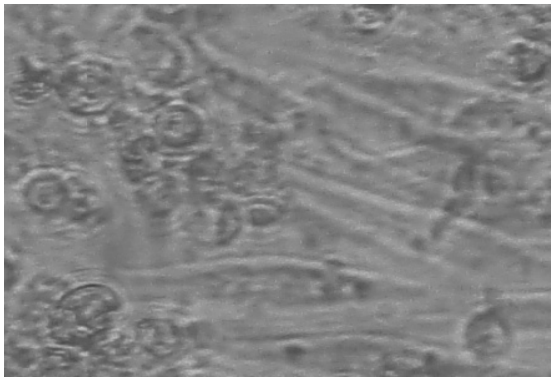
Wells A1-A12: Empty
Wells B1-B12: Monolayer of BHK-21 cells
Wells C1-C12: Cells + FMDV
Wells D1-D12: Cells + 4 plant extracts in triplicate
Wells E1-E12: Cells + FMDV + last 4 plant extracts in triplicate (Neat)
Wells F1-F12: Cells + FMDV + 1:1 dilution of last 4 plant extracts in triplicate
Wells G1-G12: Cells + FMDV + 1:2 dilution of last 4 plant extracts in triplicate
Wells H1-H12: Cells + FMDV + 1:4 dilution of last 4 plant extracts in triplicate

Observations:

a)BHK-21 cell line before incubation with FMD sample (10X)

b) BHK-21 cell line after incubation with FMD sample (40X)





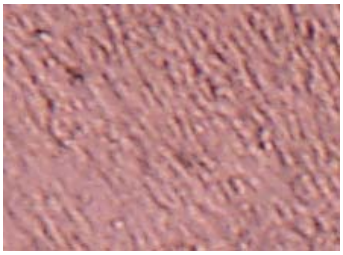
In Fig. (b) the cytopathic effects were observed like rounding of cells, detachment of cell monolayer, granulation while in Fig. (a) these cytopathic effects are not observed.

As the cytopathic effects like rounding of cells, detachment of cell monolayer, granulation were observed, it confirms the presence of FMD virus in the specimen collected from cattle showing the clinical symptoms of FMD. The Foot and Mouth Disease in cattle was diagnosed by using the technique of virus isolation in-vitro on BHK-21 cell line.

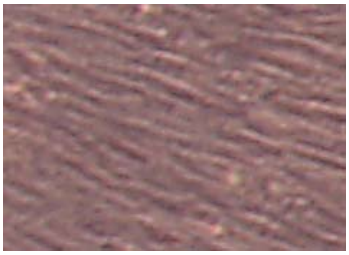
Screening the in-vitro antiviral effect of plant extracts on FMDV:

Observations:

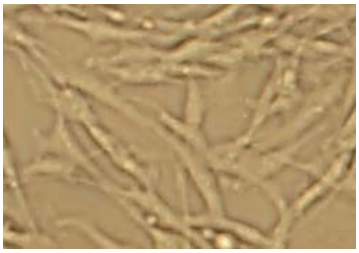
a) BHK-21 cell line with Ashwagandha root extracts and FMDV suspension (10X) **(1:2 dilution)**



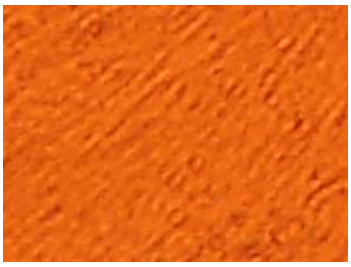
b) BHK-21 cell line with Ashwagandha leaves extract and FMDV suspension (10X) **(1:2 dilution)**



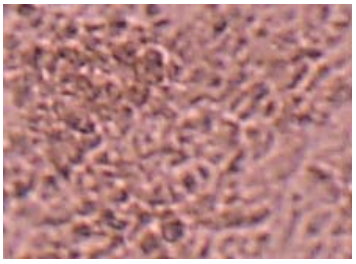
g) BHK-21 cell line with Tulsi leaves extract+ FMDV Suspension (10X) **(1:1 dilution)**



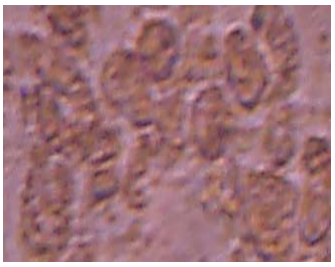
h) BHK-21 cell line with Turmeric extract+ FMDV Suspension (10X) **(1:1 dilution)**



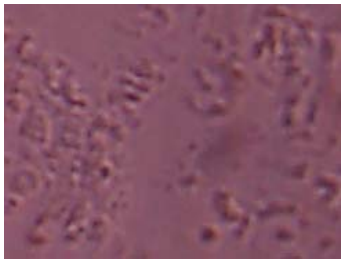
e) BHK-21 cell line with Vad stem extact+ FMDV Suspension (10X)



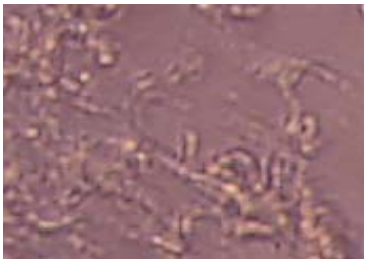
f) BHK-21 cell line with Acacia bark extracts+ FMDV suspension (40X)



c) BHK-21 cell line with Neem bark extract+ FMDV Suspension (10X)



d) BHK-21 cell line with Neem leves extract+ FMDV Suspension (10X)



OBSERVATION TABLE

Note- each cell in observation table represents single well in 96 well plate.
[ ] = well number. CPE= cells showing cytopathic effect
E= Empty well. + = Plant extract showing antiviral effect
N= Normal monolayer of cells - = Plant extract not showing antiviral effect and CPE were observed.

Plate 1

[A1] E	[A2] E	[A3] E	[A4] E	[A5] E	[A6] E	[A7] E	[A8] E	[A9] E	[A10] E	[A11] E	[A12] E
[B1] E	[B2] E	[B3] E	[B4] E	[B5] E	[B6] E	[B7] E	[B8] E	[B9] E	[B10] E	[B11] E	[B12] E
[C1] N	[C2] N	[C3] N	[C4] N	[C5] N	[C6] N	[C7] CPE	[C8] CPE	[C9] CPE	[C10] CPE	[C11] CPE	[C12] CPE
[D1] N	[D2] N	[D3] N	[D4] N	[D5] N	[D6] N	[D7] N	[D8] N	[D9] N	[D10] N	[D11] N	[D12] N
[E1] +	[E2] +	[E3] +	[E4] +	[E5] +	[E6] +	[E7] -	[E8] -	[E9] -	[E10] -	[E11] -	[E12] -
[F1] +	[F2] +	[F3] +	[F4] +	[F5] +	[F6] +	[F7] -	[F8] -	[F9] -	[F10] -	[F11] -	[F12] -
[G1] +	[G2] +	[G3] +	[G4] -	[G5] -	[G6] -	[G7] -	[G8] -	[G9] -	[G10] -	[G11] -	[G12] -
[H1] -	[H2] -	[H3] -	[H4] -	[H5] -	[H6] -	[H7] -	[H8] -	[H9] -	[H10] -	[H11] -	[H12] -

Plate 1:
Wells A1-B12: Empty
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Wells D1-D12: Cells+4 plant extracts in triplicate
Wells E1-E12: Cells+FMDV+1st 4 plant extracts in triplicate (neat)
Wells F1-F12: Cells+FMDV+1:1dilution of 1st 4 plant extracts in triplicate
Wells G1-G12: Cells+FMDV+1:2dilution of 1st 4 plant extracts in triplicate
Wells H1-H12: Cells+FMDV+1:4dilution of 1st 4 plant extracts in triplicate

Plate 2

[A1] E	[A2] E	[A3] E	[A4] E	[A5] E	[A6] E	[A7] E	[A8] E	[A9] E	[A10] E	[A11] E	[A12] E
[B1] E	[B2] E	[B3] E	[B4] E	[B5] E	[B6] E	[B7] E	[B8] E	[B9] E	[B10] E	[B11] E	[B12] E
[C1] N	[C2] N	[C3] N	[C4] N	[C5] N	[C6] N	[C7] CPE	[C8] CPE	[C9] CPE	[C10] CPE	[C11] CPE	[C12] CPE
[D1] N	[D2] N	[D3] N	[D4] N	[D5] N	[D6] N	[D7] N	[D8] N	[D9] N	[D10] N	[D11] N	[D12] N
[E1] -	[E2] -	[E3] -	[E4] -	[E5] -	[E6] -	[E7] +	[E8] +	[E9] +	[E10] +	[E11] +	[E12] +
[F1] -	[F2] -	[F3] -	[F4] -	[F5] -	[F6] -	[F7] +	[F8] +	[F9] +	[F10] +	[F11] +	[F12] +
[G1] -	[G2] -	[G3] -	[G4] -	[G5] -	[G6] -	[G7] +	[G8] +	[G9] +	[G10] -	[G11] -	[G12] -
[H1] -	[H2] -	[H3] -	[H4] -	[H5] -	[H6] -	[H7] -	[H8] -	[H9] -	[H10] -	[H11] -	[H12] -

Plate 2:
Wells A1-A12: Empty
Wells B1-B12: Monolayer of BHK-21 cells
Wells C1-C12: Cells+ FMDV
Wells D1-D12: Cells+4 plant extracts in triplicate
Wells E1-E12: Cells+FMDV+last 4 plant extracts in triplicate(Neat)
Wells F1-F12: Cells+FMDV+1:1dilution of last 4 plant extracts in triplicate.
Wells G1-G12: Cells+FMDV+1:2dilution of last 4 plant extracts in triplicate.
Wells H1-H12: Cells+FMDV+1:4dilution of last 4 plant extracts in triplicate.

RESULTS

Ashwagandha Roots and Tulsi leaves extracts are effective up to 1:2 dilutions, while Ashwagandha Leaves and Turmeric extracts are effective up to 1:1 dilution for their antiviral activity.

CONCLUSION

The antiviral activity was shown by the four different plant extracts these are as follows: Ashwagandha Roots, Ashwagandha Leaves, Tulsi leaves, Turmeric.

DISCUSSION

Foot & Mouth Disease is the disease is widespread globally with outbreaks in regions and hence it should be included in disease surveillance programme worldwide to diagnose and prevent the spread of the disease in farm animals and preventing the economic loss to farmers. It will help for the development of new disease diagnostic techniques, initializing the basic research required for it. It will also be useful for the development of new therapies for treating the disease. The antiviral activity of Ashwagandha Roots and Tulsi leaves extracts against FMD virus was observed up to maximum of 1:2 dilutions and of Ashwagandha leaves and Turmeric extracts may be due to some of the phytochemicals or secondary metabolites in these plant parts. As per the review of the antiviral activity of neem (Kausik 2002), aqueous leaf extract offers antiviral activity against Vaccinia virus (Rao et. al. 1969), Chikungunya and measles virus in vitro (Gogate and Marathe 1989). The antiviral and virucidal effects of the methanolic extract of neem leaves (NCL-11) have recently been demonstrated against group-B Coxsackie viruses (Badam et al 1999). NCL-11 inhibits plaque formation in different antigenic types of Coxsackie virus B at a concentration of 1 mg/ml at 96 h in vitro. Further studies indicated that NCL-11 is most effective in Coxsackie virus B-4 as a virusidal agent, in addition to its interference at the early events of its replication (Kausik 2002). Antiviral activity of *Withania somnifera* was observed as per Pant et. al. (2012) on Infectious Bursal Disease Virus Replication. Potent antiviral flavone glycosides activity from *Ficus benjamina* leaves was observed as per Yarmolinsky et al (2011). Extracts of *Acacia arabica* inhibited the replication of Goat Pox Virus in vitro as per Bhanuprakash et. al. (2008). As per Jayati et. al. (2013), there is antiviral activity of *Ocimum sanctum* against Newcastledisease Virus of poultry. Mazumber et al. (1995) demonstrated that curcumin has an antiviral activity, being a HIV-1 integrase inhibitor (IC50 = 40 μM) and suggested that curcumin analogs may be developed as anti-AIDS drugs. The n butanol–methanolic extracts of *A. confusa* stem possess a strong inhibitory effect on HCV replication (Lee et. al. 2011). Thus, the Ashwagandha, Tulsi and Turmeric extracts included in this

prelimiary study also possess antiviral activity and thus exerted anti FMDV activity and further mechanism of the antiFMDV inhibitory activity is proposed to be studied.

As per Chungsamarnyart et. al. 2007, after mixing the FMDV with plant extracts for 1 hour of *Azadirachta indica* J.v.s.v. (Sa-dao) leaves, CPE negative of two replications; Anti-FMDV type O activity was observed at these dilutions 1024(0.195 µg/ml), 2048(0.097 µg/ml), 4096,(0.048 µg/ml), 8192(0.024 µg/ml) with FMDV concentrations at $10^{3.35}$ $TCID_{50}$(2242 particles/100µl), $10^{3.05}$ $TCID_{50}$ (1,121 particles/100µl), $10^{2.74}$ $TCID_{50}$ (560 particles/100µl), $10^{2.44}$ $TCID_{50}$ (280 particles/100µl) and for *Curcuma zedoaria* R. rhizomes CPE negative of two replications; Anti-FMDV type O activity was observed at 8192 dilution(0.024 µg/ml) at $10^{2.44}$ $TCID_{50}$ (280 particles/100µl). Similarly in our study, we observed the antiviral effect of *Curcuma longa* at 1:1 dilution. The secondary metabolites or the phytochemicals from these plants with antiFMDV activity may be at antiviral proteins as observed against tobacco mosaic virus (Kwon et al., 1997). The cyclic peptide from the leaves of *Melia azedarach* Linn. had anti-replication activity against FMDV (Andrei et. al., 1994) and anti-viral reproducing cycle within 1 h against several serotypes of FMDV (Wachsman and Coto, 1995).

Other studies evaluated the effect of plant extracts from traditional medicine using various assays, which found extracts with anti-NS3 protease activity (Calland et. al. 2012, Ravikumar et. al. 2011), anti-NS5B activity Kong et. al. 2007 and with anti-replicative effect in the replicon model (Lee et al 2011, Lee et al 2011). Ashwagandha, turmeric and tulsi plant extracts may be inhibiting the FMDV at various stages of entry into the susceptible cells, replication and inhibiting the viral assembly proteins and exit from the cells. Thus, further study pertaining to the mechanism of their phytochemicals inhibiting the FMDV be done and antiviral herbal drugs based on the phytochemicals from these plants prepared and further inhibition of the FMDV virus in affected and non affected animals be studied for the efficacy of these herbal drugs. The antiviral activity of these plants may be due to inhibitory effect of their phytochemicals at these steps of viral attachment, entry, replication, assembly and transmission from cell to cells. Many molecules that selectively inhibit picornavirus replication and that target either structural or non-structural picornaviral proteins have been identified. The most well characterized targets are the viral capsid protein VP1 and the 3C (and 2A) protease. Targets that have been shown to be valuable but of which the exact mechanism of action still remains to be elucidated, include the viral non-structural proteins 3A and 2C. The development of highly potent and safe inhibitors of picornavirus replication remains of utmost importance (Palma et al www.interscience.wiley. com).Thus, Ashwagandha, Tulsi and Turmeric

phytochemicals also may be FMDV inhibitors and further studies based on this preliminary study on the types of phytohemicals involved and their mechanism in inhibition of FMDV can be done. Some natural medicines have been shown to possess antiviral activities against herpes simplex virus (Amoros et. al. 1987, Cavallaro et. al. 1995), influenza virus, human immunodeficiency virus (Chang et. al.1988, Tabba et. al. 1989, Yao et. al. 1992), hepatitis B and C viruses (Kitazato et. al. 2007, Hudson 1989). The screening of natural products has led to the discovery of potent inhibitors of in vitro viral growth (Baker et al 1995). Similar screening of natural products from Ashwagandha, Tulsi and Turmeric must be done for their inhibitory activity on FMDV.Even if traditional medicine represents an attractive source of new natural antivirals, studies with herbs need to be standardized in order to clearly evaluate the effects due to the plant extracts on HCV infection and should provide all methodological details. Compounds isolated from these herbs could be used for designing and developing drugs for treatment of hepatitis C (Calland et. al. 2012). Further study with combined effects of this polyherbal drug phytochemicals against FMDV and individual plant extracts against FMDV should be done. Similarly, designing and development of herbal drugs comprising Ashwagandha, Tulsi, Turmeric as polyherbal formulation for treatment of FMDV can be done.

Durk et. al. (2010) identified several non-competitive inhibitors of FMDV 3D pol that target a novel binding pocket, which can be used for future structure-based drug design studies. Such studies can lead to the discovery of even more potent antivirals that could provide alternative or supplementary options to contain future outbreaks of FMD. It can be also studied amongst the different plant extracts with antiFMDV activity from other traditional medicinal plants globally to discover those plants which may possess even more potent antiFMDV activity such as Ashwagandha, Tulsi and Turmeric. Clearly various families of viruses with different structures and replication schemes, and consequently bearing different potential molecular targets, are involved in respiratory symptoms and many of them are susceptible to *Echinacea* extracts. Among the possible viral targets are: (i) the virion itself (membrane components); (ii) cellular attachment or entry; (iii) one or more of the many stages in virus replication and development, particularly those that involve virus-specific enzymes; (iv) egress of progeny virus from infected cells (Hudson 2012). However, a logical alternative approach is the use of a noncytotoxic agent that has the capacity to inhibit many different respiratory viruses simultaneously and recent evidence indicates that certain herbal extracts could fulfill this requirement (Fedson 2009, Roxas and Jurenka 2007, Hudson 2009). Thus, effect of Ashwagandha,

Tulsi and Turmeric as noncytotoxic extracts on inhibiting the FMDV and other members of Picornaviridae can also be done .

Aqueous extracts from 151 traditionally used medicinal plants were studied (Guo et. al. 2006) to detect the activities against Coxsackie virus B3, including effect on viral replication; effect on viral adsorption and subsequent replication and *in vitro* viral inactivation. The results showed that aqueous extracts of 16 used medicinal plants in China exhibited *in vitro* anti-Coxsackie virus B3 during the three different antiviral assays. Among the 16 medicinal plants, all exhibited strong anti-Coxsackie virus B3 activity on *in vitro* viral inactivation. But the extracts from *Sargentodoxa Cuneata* (Oliv.) *Rehd.et Wils*., *Sophora tonkinensis Gapnep.*, *Paeonia veitchii Lynch.*, *Paeonia lactiftora Pall., Ephedra Sinica stapf*, *Spatholobus suberectus Dunn*. and *Cyrtomium fortunei J. sm*. appeared to possess the strongest anti-Coxsackie virus B3 activity on viral replication; and the extract from *Cyrtomium fortunei J. sm*. *Sargentodoxa Cuneata* (Oliv.) *Rehd.et Wils*., and *Spatholobus suberectus Dunn*. strongly inhibited the adsorption of Coxsackie virus B3 to the cell (Guo et al 2006). Thus, comparison amongst Ashwagandha, Tulsi and Turmeric for their increased inhibitory activity at different steps from viral entry to exit from the cell can be studied and which plant extracts are more effective at different steps should also be studied. As per Watanabe et al (2011) a triterpenoid, toosendanin (TSN**)** is also effective at suppressing HCV infection and replication. The mechanisms of antiviral action of samarangenin B (Sam B) isolated from *Limonium sinense* seem to be mediated, at least in part, by inhibiting HSV-1 α gene expression, including expression of the ICP_0 and ICP4 genes, by blocking β transcripts such as DNA polymerase mRNA, and by arresting HSV-1 DNA synthesis and structural protein expression in Vero cells. These results show that Sam B is an antiviral agent against HSV-1 replication (Kuo et al 2002). Two extracts, one from *Euphorbiaceae*, *Trigonostema xyphophylloides* (TXE) and one from *Dipterocarpaceae*, *Vatica astrotricha* (VAD) inhibited HIV-1 replication and syncytia formation in CD4+ Jurkat cells and had little adverse effects on host cell proliferation and survival. Treatment of these two extracts during the infection significantly blocked infection of the reporter virus. These results demonstrate that TXE and VAD inhibit HIV-1 replication likely by blocking HIV-1 interaction with target cells, i.e., the interaction between gp120 and CD4/CCR5 or gp120 and CD4/CXCR4 and point to the potential of developing these two extracts to be HIV-1 entry inhibitors (Park et al 2009). Similarly, the mechanism of inhibition of FMDV with Ashwagandha, Tulsi and Turmeric should be studied further.

The activities of 13 sesquiterpenes isolated from *Tripterygium wilfordii* Hook fil. var. *regelii* Makino were studied against herpes simplex virus type 1 (HSV-1) *in vitro*. Among these compounds, only triptofordin C-2 showed a selectivity index of more than 10. The compound, which could also inhibit the replication of human cytomegalovirus (HCMV) did not affect either adsorption or penetration of HSV-1 to host cells but showed moderate virucidal activity against several enveloped viruses including HSV-1, HCMV, measles virus and influenza A virus. Triptofordin C-2 suppressed viral protein synthesis of infected cells when added at early steps of HSV-1 replication and exerted inhibition of translation of the transcripts of the immediate early genes. When acyclovir and triptofordin C-2 were evaluated in combination for antiviral activity against HSV-1 replication, additive antiviral effects were observed for this virus (Hayashi et. al. 1996). One of the major target sites of inhibitory action of eugeniin is viral DNA synthesis; the inhibitory action for viral DNA polymerase activity was novel compared with anti-HSV nucleoside analogs (Kurokawa et. al. 1998). The effect of polyherbal formulation comprising groups of different phytochemicals of same type as anti FMDV should be studied and whether some groups of phytochemicals from this polyherbal formuation possess increased antiFMDV activity and also whether these groups exert antiFMDV activity at different steps of FMDV infection should be studied. In addition, the effects of Ashwagandha, Tulsi and Turmeric against FMDV in combination with other chemicals for their additive effect can also be studied and whether these plant extracts possess novel and enhanced antiFMDV activity compared to Ribavirin (Torre et. al. 1987) can be studied. Pure flavonoids and aqueous extracts of *Caesalpinia pulcherrima* Swartz were used in experiments to test their influence on a series of viruses namely herpesviruses (HSV-1, HSV-2) and adenoviruses (ADV-3, ADV-8, ADV-11) (Chiang et. al. 2003). The EC50 was defined as the concentration required to achieve 50% protection against virus-induced cytopathic effects and the selectivity index (SI) was determined as the ratio of CC50 (concentration of 50% cellular cytotoxicity) to EC50. Results showed that aqueous extracts of *C. pulcherrima* and its related quercetin possessed a broad-spectrum antiviral activity. Among them the strongest activities against ADV-8 were fruit and seed (EC50 = 41.2 mg/L, SI = 83.2), stem and leaf (EC50 = 61.8 mg/L, SI = 52.1) and flower (EC50 = 177.9 mg/L, SI = 15.5), whereas quercetin possessed the strongest anti-ADV-3 activity (EC50 = 24.3 mg/L, SI = 20.4). In conclusion, some compounds of *C. pulcherrima* which possess antiviral activities may be derived from the flavonoid of quercetin. The mode of action of quercetin against HSV-1 and ADV-3 was found to be at the early stage of multiplication and with SI values greater than 20 suggesting the

potential use of this compound for treatment of the infection caused by these two viruses (Chiang et. al. 2003). The aim of this preliminary study was to search for new antiFMDV plant extracts from Nakshatra Udyan at VSBT in vitro and further study on the enhanced antiFMDV activity from different parts of the same plants of Ashwagandha, Tulsi and Turmeric should be done (Chiang et. al. 2003). Thus, based on their activity, mechanism, the phytochemicals from Ashwagandha, Tulsi and Turmeric can be studied further to determine as novel inhibitors of FMDV replication and novel inhibitors of FMDV proteases in developing novel herbal drugs against FMDV.

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