Evaluation of antiviral activity of plant extracts against foot and mouth disease virus \textit{in vitro}

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\textbf{Abstract:} The aim of this study was to evaluate antiviral activity of chloroformic leaves extracts of three plants: \textit{Azadirachta indica}, \textit{Moringa oleifera} and \textit{Morus alba} against Foot and Mouth disease virus using MTT assay (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide). Antiviral and cytotoxic activity of each extract was evaluated as cell survival percentage and results were expressed as Means ± S.D. The concentrations which resulted in cell survival percentages of greater than 50\% are considered to be effective antiviral concentrations. From the tested plant extracts, \textit{Moringa oleifera} showed potent antiviral activity (p<0.05) while \textit{Azadirachta indica} showed significant antiviral activity in the range of 1-50µg/ml & 12-100µg/ml respectively. In contrast no antiviral activity was observed by \textit{Morus alba} as all the tested concentration resulted in significant reduction (p<0.05) in cell survival percentage.

\textbf{Keywords:} Foot and mouth disease virus, plant extract, antiviral activity.

\section{INTRODUCTION}
Viral infections are global health problem and so far no considerable antiviral agents are available (Esimone et al., 2007). In addition emerging resistance to antiviral agents is also a great problem. Thus development of new and effective antiviral agents is essential in this regard. Various plants are reported to have antiviral properties against RNA and DNA viruses (Naithani et al., 2008). \textit{Azadirachta indica} (AI) commonly known as Neem is used for the treatment of several types of diseases includes inflammation, infections, fever, skin diseases and viral infections (Subapriya and Nagini, 2005). Its antiviral activity has been reported for different viruses (Faccin-Galhardi et al., 2012, Parida et al., 2002, Saha et al., 2010). \textit{Moringa oleifera} (MO) commonly known as Sonjna act as diuretic, antipyretic, anticancer, anti-inflammatory, antibacterial and antiviral (Mahmood et al., 2010). MO has proved antiviral activity against various viruses (Lipipun et al., 2003, Virmani and Garg, 2005), Commonly known as White mulberry. Plant is used as antioxidant, antibacterial, anti-diabetic, anti-hypertensive and antiviral (Butt et al., 2008). Its antiviral potential has been reported against different viruses (Du et al., 2003, Jacob et al., 2007).

FMDV, a picornavirus is positive sense single stranded RNA virus (+ ss RNA virus) and has seven serotypes (A, O, C, Asia 1, SAT 1, SAT2 and SAT3). It causes Foot and mouth disease (FMD) which is economically devastating, transmissible viral infection (Aftosa, 2007). It is endemic in much of Africa and Asia. In case of FMDV, antiviral drugs should be arranged as soon as an outbreak is detected and thereby provide a potent adjunct to vaccines for faster control of FMDV (Birtley et al., 2005).

MTT assay is a fast and reliable technique. MTT assay is an accurate, safe and sensitive cell viability assay and allow samples to be measured directly in the plate by using a micro titer plate reader or ELISA plate reader (Weyermann et al., 2005).

AI, MO and MA have reported to possess antiviral activity. Moreover they are inexpensive, easily available and quite abundant in Pakistan. This study is an effort to search effective common local plants for antiviral activity against FMDV.

\section{MATERIALS AND METHODS}
This project was designed to evaluate antiviral activity of chloroformic leaves extracts of \textit{Azadirachta indica} (AI), \textit{Moringa oleifera} (MO) and \textit{Morus alba} (MA) against FMDV. For this purpose cytotoxic activity of each plant was also evaluated by utilizing \textit{in vitro} cell culture technique on Baby hamster kidney (BHK) -21 cell line by MTT colorimetric assay. Cytotoxic and antiviral activity were evaluated as cell survival percentage (CSP\%) and results were expressed as Means ± S.D.

\textbf{Plant collection}
The leaves of plants were collected from district Lahore, Pakistan, air dried under shade and authenticated from herbarium, The University of Punjab Lahore, Pakistan.

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Preparation of extracts and dilutions
Chloroformic extract of each plant was obtained by Soxhlet apparatus (CG-1368) (Davey and Anthony, 2010). A stock solution of 40µg/ml was prepared in cell culture media (M-199) and sterilized by filtration. Twofold serial dilutions of 200µg/ml, 100µg/ml, 50µg/ml, 25µg/ml, 12µg/ml, 6µg/ml and 1µg/ml of each plant was prepared in M-199 cell culture media.

Cell line and virus
BHK-21 cell line and identified, purified, characterized FMDV were taken from Quality operation lab (WTO), University of Veterinary and Animal Sciences Lahore. BHK-21 cells were cryopreserved (Day and Stacey, 2007) for further research. The 50% tissue culture infective dose (TCID₅₀) for FMDV in BHK-21 cell cultures was calculated by Reed and Munch method (1938) and the virus titre was 10⁶ TCID₅₀ (Reed and Munch, 1938).

Cytotoxicity assay
BHK-21 confluent monolayers were grown in 96-well cell culture plates. Each dilution of extract was added in triplicate wells. Each plate was covered and incubated at 37°C with 5% CO₂. BHK-21 cells and cell culture media was used as “negative control or cell control” whereas BHK-21 cells, cell culture media and DMSO (20%) were taken as “positive control”.

Antiviral assay
80-90% confluent BHK-21 cells were grown in 96 well cell culture plates. FMDV was added to each concentration of extracts at the titre of 10⁶ TCID₅₀ and then added to each well in triplicate manner. Plates were incubated at 37°C for 48 hours with 5% CO₂. “Negative control” was kept as BHK-21 cells and cell culture media while “Positive control” was used as BHK-21 cells, cell culture media and FMDV.

Cytotoxic activity and cells viability were determined by MTT assay (Twenty man and Luscombe, 1987) for cytotoxic and antiviral assay respectively. Each concentration was tested in triplicate wells as described in fig. 1.

STATISTICAL ANALYSIS
Cytotoxic and antiviral activity of each extract was evaluated as cell survival percentage (CSP) and results were expressed as means ± S.D. Statistical analysis was done using Statistical Packages for Social Sciences (SPSS) 19. Results were analyzed by Two way Analysis of Variance (ANOVA) (Jerrold 2007) with post hoc Scheffe test between plants and different concentrations to determine the effective concentration. Significant cut off value was P<0.05.

RESULTS
Eight different concentrations (1, 6, 12, 25, 50, 100, 200 & 400) in µg/ml were investigated for cytotoxic evaluation of each plant extract. In addition the same concentrations were also evaluated for antiviral activity against FMDV. For AI, 12-100µg/ml and for MO the concentrations of 1-50µg/ml with CSP >50% showed significant reduction (p<0.05) in cytotoxic activity. On contrary, all the tested concentrations of MA showed significant (p<0.05) reduction in CSP. Two-way ANOVA showed significant difference between plant-groups (F=2163.82, 2,72, p<0.001). Post-hoc analysis showed significant difference between all the three plants (P<0.001). Comparison of estimated marginal means of cytotoxic and antiviral activity of each plant is graphically represented in figs. 2 and 3 respectively.

DISCUSSION
From the tested extracts, MO extract was found to be most active against FMDV as compared to AI and MA extracts. Antiviral potential for MO leaves have been reported for Equine herpes virus (Double stranded DNA

Fig. 1: 96 well cell culture plates showing concentrations in triplicate.
virus), Herpes Simplex virus (Double stranded DNA virus), Epstein Bar virus (Double stranded DNA virus), Hepatitis virus (ds DNA virus), Rhinovirus (+ sense ss RNA virus), HIV (Retro RNA virus) Newcastle Disease Virus (Chollom et al., 2012, Virmani and Garg, 2005). Lipipun et al. (2003) reported in vitro and in vivo anti-HSV-1 activity of MO ethanolic extract indicating EC₅₀ (Effective concentration for 50% plaque reduction) of 100µg/ml (Lipipun et al., 2003). However in the present study antiviral concentration is found to be 1-50µg/ml on BHK-21 cells indicating more potent antiviral activity against FMDV might be due to difference in type of extract, assay method and virus. Glycosides, flavonoids and caffeoyl acids are found in MO as described by Bennett et al. (2003)(Bennett et al., 2003). Quercetin and kaempferol are major compounds of phenolics present in MO (Siddhuraju and Becker, 2003). In addition, isothiocyanate type compounds are also reported in MO (Eilert et al., 1981, Faizi et al., 1994) and these isothiocyanates as well as their different modified constituents have been shown to inhibit different viruses (Karakuş et al., 2009, Tajima et al., 2007) during initial stages of viral replication cycle. Kaseem and co workers isolated kemperol glycosides from Diplotaxis harra and investigated their antiviral activity by inhibition of FMDV-mediated cytopathic effect (Kassem et al., 2013). For kaempferol different antiviral mechanistic actions have been suggested that includes inhibition of: internal ribosome entry site (IRES) activity (Tsai et al., 2011), virus attachment to the cell membrane, viral entry and viral polymerase (Behbahani et al., 2013), viral ion channels (Schwarz et al., 2014).

Quercetin has been investigated as antiviral compound against equine herpes virus type 1 (EHV-1), adenoviruses (AdV-3, AdV-8, and AdV-11) and bluetongue virus (BTV) (Tharanath et al., 2013). It has been reported that quercetin targets the initial stages of virus replication cycle of porcine epidemic diarrhea virus (PEDV)(Choi et al., 2009). The occurrence of isothiocynates, kaempferol and quercetin in MO leaves could be associated with observed antiviral activity in the present study.

![Fig. 2: Comparison of Estimated marginal means of Cytotoxic activity of Azadirachta indica, Moringa oleifera & Morus alba against FMDV ANOVA, Post hoc Scheffe test; p<0.05.](image)

![Fig. 3: Comparison of Estimated marginal means of Antiviral activity of Azadirachta indica, Moringa oleifera & Morus alba against FMDV ANOVA, Post hoc Scheffe test; p<0.05.](image)

AI leaves have been reported for antiviral activity against chikungunya virus (Gogate and Marathe, 1986) chicken pox (DNA), poxvirus (DNA)(Kaij-a-Kamb et al., 1991), coxsackie B type of viruses (Badam et al., 1999), dengue virus (Parida et al., 2002) smallpox (DNA), infectious bursal disease virus(Virmani et al., 2009), herpes simplex type 1 virus (Amer et al., 2010, Bharitkar et al., 2014) bovine herpes virus type-1 (Saha et al., 2010) and duck plague virus (Xu et al., 2012). In a study, (Faccin-Galhardi et al., 2012) evaluated antiviral activity of polysaccharides present in AI leaves against poliovirus type 1. A peptide named Meliacine (isolated from Melia azedarach L leaves) inhibited the process of uncoating of FMDV in BHK-21 cells by preventing vacuolar acidification (Wachsman et al., 1998). Like FMDV, poliovirus also belongs to family Picornaviridae and observed antiviral activity of AI in the present study could be associated to Meliacine or any other peptide. Additionally, this plant contains general class of natural products called triterpenes; more specifically, limonoids which had been reported as antiviral against influenza A and herpes simplex type 1 virus (Khan et al., 2005). One
of the limonoid, 1-cinnamoyl-3,11-dihydroxymeliacarpin, derived from leaves of *Melia azedarach* L inhibited vesicular stomatitis (VSV) and herpes simplex (HSV-1) viruses, through induction of IFN-γ and TNF-α (Alché et al., 2003). Thus presence of triterpenes in AI could be linked to anti-FMDV activity.

Although antiviral activity of *Morus alba* (MA) has been reported against various viruses like HSV-1, murine norovirus-1 (MNV-1) and feline calicivirus-F9 (Du et al., 2003, Lee et al., 2014). Kayo et al. (2001) also reported antiviral flavonoids like Moralinone, flavane and isoquercetin in MA leaves. On contrary, MA in the present study did not exhibit antiviral activity rather all tested concentrations were observed to be cytotoxic. This discrepancy might be due to difference in solvent, virus, cell line, or assay technique (Asano et al., 2001). Moreover phenolic acids are reported to exhibit cytotoxicity by affecting DNA synthesis, RNA reductase and microsomal mixed-function oxidase (Picardo et al., 1987) and the presence of these compounds in MA leaves could be associated with its observed cytotoxicity.

At present few therapeutic drugs are available against FMDV. Different studies have been conducted and still going on in search of effective anti-FMDV agents. In this regard, different steps and particular protein/enzymes of viral replication cycle could be possible targets. An inhibitor of viral polymerase enzyme has been indicated to overcome FMD virus. Different Silencing RNA (siRNA) techniques have been widely investigated to combat FMDV(Kim et al., 2008). Nevertheless more advancement is required for effective delivery and to prevent viral escape.

Comparison of chloroformic extract of *Azadirachta indica*, *Moringa oleifera* and *Morus alba* showed that *Moringa oleifera* extract had potent antiviral activity while *Azadirachta indica* indicated considerable antiviral activity in the range of 1-50µg/ml & 12-100µg/ml respectively. At higher concentrations the extracts were found to be cytotoxic.

**CONCLUSION**

In accordance, these plants could be helpful in the development of effective and economical antiviral agents. This is a preliminary *in vitro* study reporting antiviral potential of plant extracts. Further research including *in vivo* studies is required to elucidate the antiviral phytochemicals with their mechanism of action.

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