

Evaluation of cytotoxic and antiviral activities of aqueous leaves extracts of different plants against foot and mouth disease virus infection in farming animals

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Abstract: Cytotoxic and antiviral activity of aqueous leaves extracts of three plants: *Azadirachta indica*, *Moringa oleifera* and *Morus alba* against Foot and Mouth disease virus (FMDV) were determined using MTT assay (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide). Eight different concentrations of each plant were evaluated. Cytotoxic and antiviral activity of each extract was evaluated as cell survival percentage and results were expressed as Means $\pm$ S.D. From the tested plant extracts, *Azadirachta indica* & *Moringa oleifera* exhibited cytotoxicity at 200 & 100 μ g/ml respectively. In case of antiviral assay, *Moringa oleifera* showed potent antiviral activity ($p < 0.05$) while *Azadirachta indica* showed significant antiviral activity in the range of 12.5-50 μ g/ml & 50-100 μ g/ml respectively. In contrast no anti-FMDV activity in the present study was observed with *Morus alba*, although all the tested concentrations were found to be safe.

Keywords: *Azadirachta indica*, *Moringa oleifera*, *Morus alba*, foot and mouth disease virus, MTT assay (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide).

INTRODUCTION

Foot-and-mouth disease virus (FMDV) belongs to genus *Aphthovirus*, family *Picornaviridae* is a positive sense single stranded RNA virus (+ ss RNA virus) and has seven serotypes. Foot-and-mouth disease is a fulminating infection of farming animals (Mettenleiter and Sobrino, 2008; Brito *et al.*, 2015). It is the etiological agent of economically most important animal disease threatening the cattle industry since sixteenth century (Mahy, 2005). The mortality rate is high specially in young animals (Wang *et al.*, 2015).

Symptoms of the disease are abrupt manifestation of sores on the mouth, nose and feet. These symptoms can appear within 2 to 3 days post exposure and can take up to 7 to about 10 days (Leforban, 1999).

This viral problem occurs around the whole globe including Pakistan, India, Bangladesh (Subramaniam *et al.*, 2015; Nandi *et al.*, 2015). Abubakar *et al.* (2015) reported that outbreaks of Foot and mouth disease during the period of January 2010 to December 2011 in Pakistan were 65.52 % of serotype O, 24.14 % serotype A and 10.35 % of serotype Asia-1.

Naithani *et al.* (2008) revealed that various plants have antiviral properties against different type of RNA as well

as DNA viruses. Plants have enriched profile of antiviral constituents for example flavonoids, alkaloids, polysaccharides, coumarins, glycosides, lignans, saponins, polyines, thiophenes, proteins and polyphenolics etc (Jassim and Naji, 2003). Hence herbal drugs may be expanded and progressed to be used as effective drugs with broad spectrum of antiviral activity, increased safety margin, good quality, less resistance and fewer side effects.

There are different methods for evaluation of antiviral activities of plants which include animal models, animal protection studies, egg inoculation and cell culture methods (Abonyl *et al.*, 2010). In this experiment we have utilized MTT assay to evaluate cytotoxic and antiviral activity of aqueous leaves extracts of three plants:

Azadirachta indica, *Moringa oleifera* and *Morus alba* against FMDV.

Azadirachta indica (*A. indica*) commonly known as Neem has been evaluated for antiviral activity against coxsackie - B type of viruses (Badam *et al.*, 1999), dengue virus, herpes simplex type 1 virus (Tiwari *et al.*, 2010), bovine herpes virus type-1 and polio virus type-I (Galhardi *et al.*,

2012). *Moringa oleifera* (*M. oleifera*) commonly known as Sonjha has been reported to possess antiviral potential against human immunodeficiency virus (Abram *et al.*, 1993), epstein bar virus, rhinovirus, herpes simplex virus

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type I and equine herpes virus type 1 (Meenakshi and Garg, 2005). *Morus alba* (*M. alba*) commonly known as White mulberry has shown antiviral activity against herpes simplex type I (Du *et al.*, 2003) and bovine viral diarrhea virus (Jacob *et al.*, 2007).

These plants have shown antiviral activity. Moreover they are inexpensive, easily available and quite abundant in Pakistan so these plants are chosen for antiviral evaluation against FMDV.

MATERIALS AND METHODS

The project was conducted to evaluate cytotoxic and antiviral activity of aqueous leaves extracts of *Azadirachta indica*, *Moringa oleifera* and *Morus alba* against FMDV. In this regard, in -vitro cell culture technique was utilized on Baby hamster kidney (BHK) - 21 cell line by MTT colorimetric assay. Cytotoxic and antiviral activity were evaluated in term of cell survival percentage (CSP%) and results were shown as Means $\pm$ S.D.

Plant collection

The plant leaves were collected from Lahore, Pakistan, air dried under shade and identified from herbarium, The University of Punjab Lahore, Pakistan.

Preparation of extracts and dilutions

Each plant was subjected to maceration to obtain aqueous extract. Eight concentrations 400 μ g/ml, 200 μ g/ml, 100 μ g/ml, 50 μ g/ml, 25 μ g/ml, 12 μ g/ml, 6 μ g/ml and 1 μ g/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. Reed and Munch method was applied to calculate Tissue culture infective dose (TCID₅₀) for FMDV (Purchase and Lowrence, 1989). The virus was used at 10⁶ TCID₅₀.

Cytotoxicity assay

96-well cell culture plates were used to obtain confluent monolayer of BHK-21 cells. Each dilution of aqueous extract was added in triplicate wells with incubation temperature of 37° C, homogenized with 5% CO₂. Following controls were used in the study:

Negative control = BHK-21 cells + cell culture media
Positive control = BHK-21 cells + cell culture media + DMSO (20%).

Antiviral assay

After obtaining 80-90% confluency of BHK-21 cells in 96 well cell culture plates. FMDV was added to each dilution

at the titre of 10⁶ TCID₅₀. Again triplicate sampling was used for results confirmation. Sample plates of each extract dilutions along with BHK cells and virus were incubated at 37°C with 5% CO₂ for 48 hours. Following controls were used in the antiviral study:

Negative control = BHK-21 cells + cell culture media
Positive control = BHK-21 cells + cell culture media + FMDV.

Cytotoxic activity and cells viability were determined by MTT assay (SGS Pakistan (Private) Limited) (Twentyman and Luscombe, 1987) for cytotoxic and antiviral assay respectively. Each concentration was tested in triplicate wells and all the experiments were carried out as replicates.

MTT assay for Cytotoxicity and Antiviral evaluation

After an incubation of 5-6 days, growth media was removed from each well. 100 μ l of 0.5% MTT solution was poured in each test and control well of the plates and incubated at 37°C for 4 hours. MTT dye was removed, 100 μ l of DMSO (10%) was poured in each well of the plates and then plates were incubated at 37°C for 2 hours. Optical density (OD) value of each plate was determined by multi well ELISA reader at a wave length of 570 nm. Cell survival percentages were calculated for both cytotoxic and antiviral assay (table and 2).

STATISTICAL ANALYSIS

All concentrations were tested in triplicate and all the experiments were carried out as replicates. Results were expressed as Mean $\pm$ SD. Statistical analysis was applied on the obtained data using Statistical Packages for Social Sciences (SPSS) - 20. Results were analyzed by Two – way Analysis of Variance (ANOVA) (Jerrold, 2007) with post hoc Scheffe test. Values were considered significant at P < 0.05.

RESULTS

Each plant extract was evaluated for cytotoxic as well as antiviral potential by in vitro cell culture technique. Results for cytotoxic and antiviral activity of each plant are represented in table 1 for cytotoxicity assay and table 2 for antiviral assay.

The cytotoxicity results of *A. indica* revealed that concentrations up to 100 μ g/ml were safe and showed significant reduction in cytotoxic activity for BHK-21 cells as indicated by CSP >50%. On contrary, higher concentration (400 μ g/ml) of *A. indica* resulted in significant (p<0.05) reduction in CSP (<50%). With increased concentrations there is decrease in CSP in cytotoxicity assay.

Cytotoxic assay of *M. oleifera* leaves showed that 100, 200 and 400 µg/ml were cytotoxic with CSP < 100% while at low concentrations, no cytotoxicity for BHK-21 cells (CSP >50%) were resulted which indicated its safety. Regarding cytotoxicity assay of *M. alba* leaves, all concentrations exhibited non-cytotoxicity and safety for BHK-21 cells (CSP <50%).

Antiviral activity

In the present study antiviral assay showed that 50 and 100 µg/ml concentrations of *A. indica* resulted in potent antiviral activity against FMDV as virus growth was retarded which is indicated by CSP of 83 & 92% respectively. Below 50 µg/ml no remarkable anti-FMDV activity was observed although these concentrations were non cytotoxic in cytotoxicity assay (CSP>50%). High concentrations of (200 and 400 µg/ml) did not exhibit antiviral activity which is evident by CSP of only 2 to 3% in cytotoxicity assay.

Antiviral evaluation of *M. oleifera* leaves indicated three concentrations: 12, 25, 50 µg/ml showed significant antiviral activity against FMDV without damaging the cell culture in MTT assay. Nevertheless low (1, 6 µg/ml) and high (100, 200 and 400 µg/ml) concentrations showed no anti-FMDV potential. However higher doses were cytotoxic for BHK-21 cell culture. While *M. alba* aqueous extract in the present study showed no remarkable antiviral activity against FMDV as cell survival percentages at all concentrations were below 50% besides that none of the concentration was cytotoxic in cytotoxicity assay.

Two-way ANOVA showed significant difference between plant-groups ($P < 0.001$). Post-hoc analysis showed significant difference between all the three plants ($P < 0.001$).

DISCUSSION

Foot and mouth disease (FMD) is life threatening veterinary viral infection (Aftosa, 2007) which is caused by Foot and Mouth disease virus (FMDV), a picornavirus. Disease symptoms include fever, weight loss, vesicular lesions on feet and mouth including the tongue and palate. FMD is not only endemic in Africa, Asia and parts of South America but also endemic in Pakistan and causes huge economic losses to commercial cattle and buffalos. About 1286 FMD outbreaks were estimated during the years 2002 and 2005 in Pakistan. There is a relatively high annually FMD prevalence between 41% and 50% in the Southern Sindh region around Karachi (Zahur et al., 2006). A loss of Rs. 4 million was recorded in only one Tehsil of District Lahore during one year and Rs. 27.449 million was assessed in District Sahiwal in the year 1998. Many plants with antiviral activities against diverse group of viruses have been reported (Naithani et al., 2008) such

as Hepatitis B, C viruses, human immunodeficiency virus, polio, pox viruses, measles, herpes simplex, epstein bar virus and rhinoviruses etc. The present experiment was designed with the same concept that plants possess antiviral activities and this study is an effort to search effective common local plants for antiviral activity against FMDV.

The present research was conducted to evaluate cytotoxic and antiviral potential of leaves of three plants:

Azadirachta indica, *Moringa oleifera* and *Morus alba* against FMDV at different concentrations.

From the tested plant extracts, in cytotoxicity assay *A. indica* and *M. oleifera* were found to be cytotoxic at higher concentrations. However *M. alba* was non-cytotoxic at all the tested concentrations.

In the present study *A. indica* exhibited cytotoxicity at higher concentration (200 µg /ml) which is in contrast to another study where methanolic extract of *A. indica* leaves exhibited cytotoxic concentration 10,000 µg /ml against coxsackie -B group of viruses by using plaque inhibition assay (Badam et al., 1999). This variability may be due to variability in analytical assay used, extract type, virus and cell line. In another study, Qureshi et al. (2015) evaluated cytotoxic potential of *A. indica* leaf and seed by chromosomal aberration assay in Chickpea (*Cicer arietinum* L.) root tip cells. Alchei et al. (2003) isolated one of cytotoxic limonoid from leaves of *Melia azedarach*. Different studies had reported that *A. indica* contains terpenoid which had shown cytotoxic activity (Chatterjee and Pakrshi, 1994; Podolak et al., 2010). The presence of terpenoids in *A. indica* leaves could be linked to its cytotoxicity.

Regarding *M. oleifera*, aqueous extract was toxic to the cells at and above 100 µg/ml. The results are in accordance with another study in Thailand where ethanolic and water extracts of *Moringa oleifera* showed cytotoxicity at concentration above than 100 µg/ml for cancer cells COR L-23 and PC3 and normal cells 10FS (Saetung et al., 2005). Cytotoxicity of *M. oleifera* aqueous leaves extracts on Hella cells causes extremely high death rate of cells at 100 µg/ml (Nair and Varalakshmi, 2011). Different studies had reported that *M. oleifera* contains phenolic compounds which possess cytotoxic activity at higher concentration (Makkar and Becker, 2007; Anwar et al., 2005). The cytotoxic activity of extract in the present study could be related to the presence of phenolic compounds.

In the present study aqueous *A. indica* leaves extract showed antiviral activity against FMDV at 100 µg/ml. Results are in line with another study in which Tiwari et al., (2010) evaluated effective antiviral concentration of

Table 1: Cytotoxic assay of *Azadirachta indica*, *Moringa oleifera* & *Morus alba*

Concentrations (µg/ml)	<i>Azadirachta indica</i>		<i>Moringa oleifera</i>		<i>Morus alba</i>	
	Mean OD ± SD	Cell survival %	Mean OD ± SD	Cell survival %	Mean OD ± SD	Cell survival %
1	1.188±0.072	93	0.923±0.015	70	1.038±0.017	80
6	1.199±0.16	94	1.004±0.008	77	1.246±0.016	82
12	1.142±0.054*	89	1.188±0.092	93	1.188±0.026	93
25	1.119±0.011*	87	1.211±0.319	95	1.107±0.083	86
50	1.015±0.159	78	1.211±0.048	95	0.866±0.038	65
100	0.958±0.116	73	0.566±0.021	39	1.015±0.013	78
200	0.147±0.027*	3	0.244±0.023*	11	0.877±0.011	66
400	0.141±0.003*	2	0.221±0.006*	9	0.831±0.041	62

Table 2: Antiviral assay of *Azadirachta indica*, *Moringa oleifera* & *Morus alba*

Concentrations (µg/ml)	<i>Azadirachta indica</i>		<i>Moringa oleifera</i>		<i>Morus alba</i>	
	Mean OD ± SD	Cell survival %	Mean OD ± SD	Cell survival %	Mean OD ± SD	Cell survival %
1	0.312±0.010	17	0.256±0.003	12	0.256±0.012	12
6	0.336±0.016	19	0.463±0.009*	30	0.348±0.089	20
12	0.451±0.049	29	1.142±0.168*	89*	0.497±0.032	33
25	0.382±0.03	23	1.119±0.060	87*	1.157±0.005	39
50	1.073±0.011*	83	1.083±0.120	84*	0.293±0.037	25
100	1.176±0.009*	92	0.302±0.004*	16	0.194±0.004	6
200	0.256±0.010	12	0.233±0.017	10	0.234±0.017	10
400	0.175±0.019	5	0.244±0.009*	11	0.247±0.009	12

Two- way ANOVA, post hoc Scheffe test by SPSS-20. * = significant difference (p<0.05)

A. *indica* aqueous bark extract at 100 µg/ml against Herpes simplex type 1 (HSV-1). Amer *et al.* (2010) also reported that aqueous leaves extract had virucidal potential against HSV-1. Pectic arabinogalactan is a component derivative present in Neem plant showed antiviral potential against bovine herpes type-1 virus at 110µg/ml. The compound was cytotoxic at higher concentration (Saha *et al.*, 2010). It might be pectic arabinogalactan compound in *A. indica* leaves which had shown antiviral activity against FMDV in the present study at 100µg/ml and cytotoxicity at higher concentrations. In another in vitro study aqueous *A. indica* leaves extract exhibited antiviral potential against Dengue virus type-2 using virus inhibition assay in dose dependent manner (Parida *et al.*, 2002). Virmani *et al.* (2009) reported that alcoholic extract of *A. indica* seed had significant antiviral activity against Infectious bursal disease virus in-vitro on Vero cell line at 20µg/ml. In another study two polysaccharides present in AI leaves along with their sulfated derivatives were found to be effective antiviral against poliovirus type -1 at 12 µg/ml to 80 µg/ml (Galhardi *et al.*, 2012).

In a study, the ethanolic extract of leaves of *M. oleifera* had shown antiviral activity against HSV at 100±5.3 ug/ml and cytotoxic activity at 875±35 µg/ml (Lipipun *et al.*, 2003). In the present study aqueous *M. oleifera* leaves

extract was also effective against FMDV at 100 µg/ml. In case of antiviral assay our results are in accordance with Lipipun *et al.* (2003) but present cytotoxicity results are in contrast with the study of Lipipun *et al.* (2003). Niaziminin is one of the thiocarbamate compounds present in *M. oleifera* leaves that had considerable antiviral activity against Epstein bar virus (RNA virus) (Murakami and Kitazono, 1998). In the present study, it might be Niaziminin or any other member of thiocarbamate group that could be associated with its antiviral activity.

M. alba in the present study exhibited no anti-FMDV activity though all tested concentrations were found to be non-cytotoxic and safe. Nevertheless, in the previous studies, antiviral activity of *M. alba* has also been reported against different viruses like HSV-1, murine norovirus-1 and feline calicivirus-F9 (Kayo *et al.*, 2001; Lee *et al.*, 2014). The differences in the studies could be attributed to variation in the type of extract, cell line or evaluation technique.

CONCLUSION

Comparison of aqueous extract of *Azadirachta indica*, *Moringa oleifera* and *Morus alba* revealed that *Moringa oleifera* exhibited remarkable antiviral effect while

Azadirachta indica also resulted in significant antiviral activity. The plant extracts exhibited cytotoxicity at higher concentrations. In contrast no anti-FMDV activity in the present study was exhibited by *Morus alba*, beside that all the tested concentrations were found to be safe in cytotoxicity assay. Further research is essential to fraction the extracts and elucidate the exact cytotoxic and antiviral phytochemicals of these plants with their mechanism of action.

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