

In vitro evaluation of antiviral activity of leaf extracts of *Azadirachta indica*, *Moringa oleifera*, and *Morus alba* against the foot and mouth disease virus on BHK-21 cell line

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ABSTRACT: Plants are widely used for the treatment of different ailments including viral infections. There is a great need to explore antiviral agents of herbal origin to combat the viral resistance. Foot and mouth disease (FMD) is highly infectious viral disease of cloven-footed animals. Three local Pakistani plants *Azadirachta indica* (AI), *Moringa oleifera* (MO) and *Morus alba* (MA) are reported to possess antiviral activity against DNA and RNA viruses. In the present study ethanolic leaf extracts of these plants were evaluated for their cytotoxicity against BHK-21 (baby hamster kidney) cell line and antiviral activity against FMD virus by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colourimetric assay. Ethanolic extracts of each plant were obtained and stock solution of 40 mg/ml was prepared in cell culture media (M-199) and sterilized by filtration. Two-fold serial dilutions of 200 µg/ml, 100 µg/ml, 50 µg/ml, 25 µg/ml, 12 µg/ml, 6 µg/ml, and 1 µg/ml of each plant were prepared in M-199 cell culture media. The BHK-21 cell line was identified, purified, and characterized. Tissue culture infective dose (TCID₅₀) for FMD virus was calculated by ten-fold dilutions of FMD virus. The virus was used at the rate of 10⁶ TCID₅₀. Ethanolic extract of AI exhibited stronger anti-FMD virus activity than MO and MA at a concentration nontoxic to the BHK-21 cell line. These plants may be helpful to elucidate effective antiviral agents against FMD virus.

KEYWORDS: MMT assay, herbal plants

INTRODUCTION

Plants are extensively used to treat different types of diseases¹. Viral infections are global health problem and so far few antiviral agents are available² along with emerging resistance problems. There is a great need of identifying antiviral drugs from herbal origin to overcome these problems. Various plants have been reported to have antiviral properties against RNA and DNA viruses³. *Azadirachta indica* (AI) commonly known as neem is used for the treatment of several types of diseases including inflammation, infections, fever, skin diseases, and viral infections⁴. Its antiviral activity has been reported for different viruses^{5–8}. *Moringa oleifera* (MO) commonly known as sonjina acts as antiviral, diuretic, antipyretic, anticancer, anti-inflammatory, and antibacterial plant⁹. MO has proved to have antiviral activity against various viruses¹⁰. *Morus alba*

(MA) commonly known as white mulberry is used as antioxidant, antibacterial, antiviral, anti-diabetic, and anti-hypertensive¹¹. Its antiviral potential has been reported against different viruses¹².

Foot and mouth disease virus (FMDV), a picornavirus, is a negative sense RNA virus which causes foot and mouth disease (FMD). FMD is an economically devastating and transmissible viral infection. Disease symptoms include fever, weight loss, vesicular lesions on feet and mouth including the tongue and palate¹³. FMD is endemic in much of Africa and Asia¹⁴ including Pakistan and causes huge economic losses to commercial cattle and buffalo¹⁵. About 1286 FMD outbreaks were estimated during the years 2002 and 2005 in Pakistan. In Pakistan, it is reported that during the months of December 2001 and January 2002, an outbreak of FMDV type-A was recorded in a crossbred dairy herd at Livestock Experiment Station, Qadirabad¹⁶.

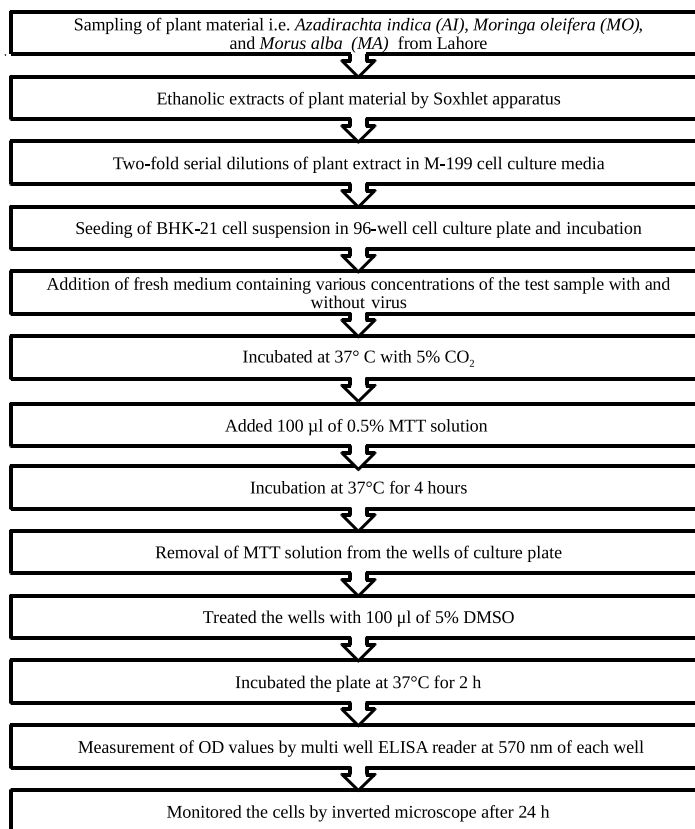


Fig. 1 MTT assay for antiviral and cytotoxic activity.

Because of associated economic loss and difficulty in control, FMD ranks first in the A list of infectious diseases of animals published by the Office International des Epizooties (OIE). In the 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 FMDV¹⁷.

AI, MO, and MA have shown 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.

MATERIALS AND METHODS

In vitro cell culture technique has been used to evaluate antiviral and cytotoxic activity of plant leaves against FMDV on BHK-21 (baby hamster kidney) cell line using MTT assay. The experiment was laid out in randomized complete design.

The plant materials were collected from district Lahore, Pakistan. Ethanollic extract of each plant was obtained by Soxhlet apparatus (CG-1368)¹⁸. Two-fold serial dilutions of 200 µg/ml, 100 µg/ml,

50 µg/ml, 25 µg/ml, 12 µg/ml, 6 µg/ml, and 1 µg/ml of each plant were prepared from stock extract in M-199 cell culture media.

In this study, BHK-21 cell suspension (100 µl) was seeded in each well of 96-well cell culture and was incubated. Fresh medium containing different concentrations of the test sample was added after 24 h of seeding with and without virus for antiviral and cytotoxic study. Each concentration was tested in triplicate wells. Each plate was covered and incubated at 37 °C with 5% CO₂. Positive and negative control was also run along the experiments. After incubation, 100 µl of 0.5% MTT solution was added. The cell culture plates were incubated at 37 °C for 4 h. After incubation, MTT solution was removed from the wells and each well was treated with 100 µl of 5% DMSO. The plates were again incubated at 37 °C for 2 h. Optical density values of each well were measured by multi well ELISA reader at 570 nm. Cells were monitored daily for cytopathic effects by using inverted microscope. Cells viability and cytotoxic activity were determined by MTT assay for antiviral and cytotoxic assay, respectively, described in Fig. 1.

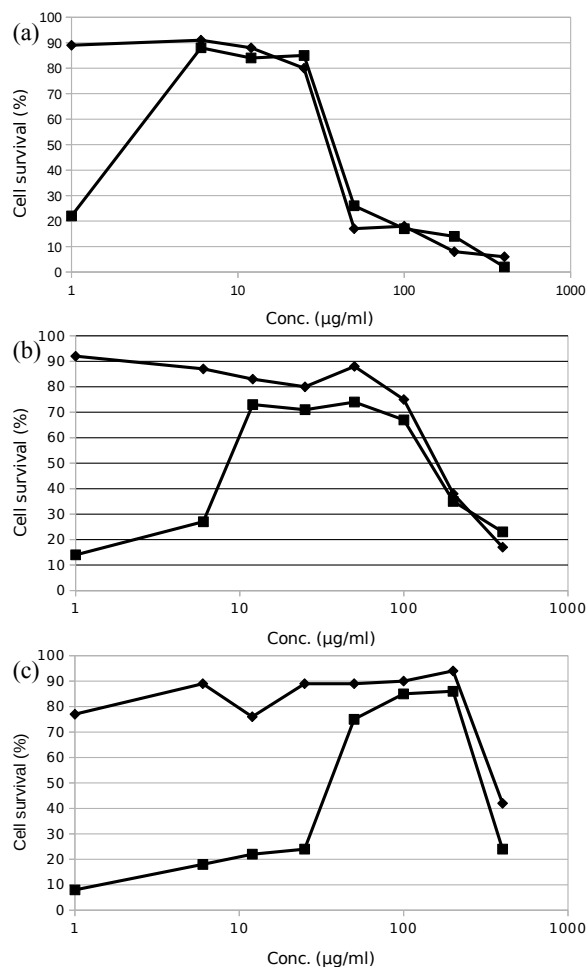


Fig. 2 Antiviral (diamonds) and cytotoxic (squares) activity of (a) AI, (b) MO, and (c) MA ethanolic leaf extracts against FMDV.

RESULTS

Ethanolic extract of AI resulted in potent antiviral activity against FMDV at concentrations 6–25 µg/ml, as the cell survival percentage (CSP) was above 50% at this range (Fig. 2a). MA and MO showed anti-FMDV activity at concentration ranges of 12–100 µg/ml and 50–300 µg/ml, respectively (Fig. 2b, Fig. 2c). Leaves of the AI plant were more effective against FMDV as compared to MO and MA leaves. The antiviral activity was in the order of AI > MO > MA. AI leaves were found to be cytotoxic at the concentration range of 50–400 µg/ml as the CSP was lower than 50% at that range. In the case of the cytotoxic assay, it was noticed that with the increase in concentration there was a decrease in CSP. AI leaves were found to be more cytotoxic as compared to MO and MA leaves. The cytotoxic activity

was in the order of AI > MO > MA. MA leaves extract was found to be least cytotoxic. Results were analysed by one way ANOVA which were found to be significant.

DISCUSSION

Natural products and their derivatives provide an excellent source for antiviral drugs⁸ due to fewer side-effects, low cost, more bioavailability, and easy availability along with less potential to cause resistance¹⁹. Hence utilization of herbal drugs in viral infections is an important future concern. Medicinal plants contain numerous phytochemicals responsible to exhibit antiviral potential. Flavonoids, saponins, lignans, polyphenolics, furyl-compounds, terpenoids, alkaloids, polyines, thiophenes, coumarins sulphides, peptides, and proteins are of great significance²⁰.

The present study was designed to evaluate antiviral and cytotoxic activities of local medicinal plants against FMDV by using MTT assay. Ethanolic AI extract was found to exhibit potent anti-FMDV activity. AI has been reported to possess antiviral activity against dengue virus type-2⁵ and HSV-1⁶. Pectic arabinogalactan, a component derivative present in neem plant, showed antiviral potential against bovine herpes type-1 virus at 110 µg/ml⁷. It might be pectic arabinogalactan compound in AI leaves which showed antiviral activity against FMDV in the present study. The ethanolic AI leaf extracts contain polysaccharides which showed antiviral activity against poliovirus type 1⁸. Thus polysaccharides present in alcoholic extract of AI leaf extracts might be responsible for antiviral activity against FMDV. Methanolic extract of neem leaves exhibited cytotoxic concentration of 10 000 µg/ml against Coxsackie B group of viruses by using plaque inhibition assay. This variability may be due to variability in analytical assay used, extract type, virus, cell line, difference in extraction method or plant constituents. Different studies reported that AI contains terpenoid which showed cytotoxic activity.

MO alcoholic leaf extract had significant antiviral activity against equine herpes virus type 1¹⁰. Ethanolic extract of *Moringa oleifera* leaves showed antiviral activity against HSV at 100 ± 5 µg/ml and cytotoxic activity at 875 ± 35 µg/ml²¹. Niaziminin is one of the thiocarbamate compounds present in MO leaves that had considerable antiviral activity against Epstein-Barr virus (RNA virus)²². In the present study, it might be niaziminin or any other member of thiocarbamate group that exerted its

antiviral activity against FMDV. In the cytotoxic assay, the ethanolic extracts of MO were toxic to the cells at higher concentration (100 µg/ml).

The results are in accordance with another study in Thailand where ethanolic extracts of MO showed cytotoxicity at concentration above 100 µg/ml for cancer cells COR L-23 and PC3 and normal cells 10FS²³. Other studies reported that MO contains phenolic compounds which possess cytotoxic activity at higher concentration²⁴.

MA exhibited antiviral activity against HSV-1¹². MA leaves have antiviral flavonoids like morabalone, flavane, and isoquercetin against HIV²⁵. In the present study ethanolic MA leave extracts were found to be cytotoxic at high concentrations (400 µg/ml). MA leaves contain flavonoids²⁶ and some flavonoids are cytotoxic at high concentrations. It is reported that some flavonoids constituents present in ethanolic MA leave extracts showed cytotoxic activity against A549, be17402, BGC823, HCT-8, and A2780 cell lines in vitro by the MTT method²⁷. Phenolic acids such as gallic, protocatechuic, *p*-hydroxybenzoic, vanillic, chlorogenic, syringic, *p*-coumaric, ferulic, and *m*-coumaric acids were reported in MA leaves²⁷. Phenolic agents exhibit cytotoxicity by affecting DNA synthesis, RNA reductase and microsomal mixed-function oxidase. In cell cultures some phenols readily oxidized and cause cytotoxic effect by generation of toxic oxygen species. It might be some flavonoid constituents or phenolic acids due to which MA ethanolic extracts had exerted cytotoxicity in the present study.

CONCLUSIONS

Ethanolic leave extracts of AI, MO, and MA showed in vitro antiviral activities against FMDV and may have compounds responsible for antiviral potential. Further research is required to elucidate the phytochemicals of the plants which may be helpful in the development of effective, economical and less resistant antiviral agents.

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