

Original Research Article

Evaluation of the cytotoxic and antiviral effects of ethanol extract of three *Opuntia* species of Peste des Petits ruminant virus

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Abstract

Purpose: To assess *in vitro* the virocidal effects of different species of cactus plant on the lethal action of Peste des petits virus (PPRV).

Method: Ethanol extracts of different cactus plants were obtained. A serial twofold dilution of the extracts was prepared. Cytotoxic and antiviral activities were examined through MTT assay at various concentrations. Vero cell lines were grown in 96 well plates up to an 80 % confluent monolayer. The plates were divided into two groups, one for antiviral and the other for cytotoxicity activity. The cells were exposed to various concentrations of the ethanol extracts to assess the cytotoxicity, whereas to assess the antiviral activity, PPRV was re-incubated with the extracts and then exposed to cells. MMT dye was added and the results were evaluated as cell survival (%).

Results: At higher concentrations, i.e., 500 - 1000 µg/mL, ethanol extracts from all the *Opuntia* species displayed cytotoxic effects. The ethanol extract of OM exhibited the greatest antiviral potential of all the extracts, while the extract of *Opuntia stricta* (OS) was the least effective against PPRV in the cultured cells. There were significant differences ($p < 0.05$) in the concentration of *Opuntia manocantha* (OM), *Opuntia delinii* OD and *Opuntia stricta* (OS) with reference to antiviral activity. OM showed antiviral activity against PPRV from 3.25 to 125 µg/mL, OD antiviral activity from 31.25 to 62.5 µg/ml whereas OS showed antiviral activity at 2.5 µg/mL.

Conclusion: The ethanol extract of *Opuntia* species reduces the infection of PPRV in Caprine.

Keywords: Cactus, *Opuntia* spp., Peste des petits ruminants virus (PPRV), Vero cell line

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INTRODUCTION

Virus, being intracellular pathogen, is difficult to control and eliminate. Viral control and elimination is the main obstacle in the treatment of viral diseases. Modern therapeutic agents are

derived directly or indirectly from medicinal plants [1,2]. Still, 25 % of the therapeutic agents are derived from plant sources. Medicinal plants have a natural tendency to exhibit antiviral effect against DNA and RNA viruses [3]. The high mutation rate of viruses is closely associated with

outbreaks of new viral diseases, which lead to scientists exploring new therapeutic agents [4].

Medicinal plants have flavonoids, alkaloids and tannins as active ingredients [5]. These ingredients execute their specific actions through different mechanisms, such as interference with viral transcription or translation, inhibition of virus attachment and interruption of virus replication. [6]. *Opuntia species* has been previously reported to be affective in the treatment of different diseases such as spasmodic and whooping cough. [7]. Its leaf extract has been reported to reduce glucose concentration, anti-inflammatory and pain-relieving potential [8,9]. Its antibacterial activity against *Bacillus subtilis*, *E. coli*, *Proteus vulgaris*, *Bacillus cereus* and *Staphylococcus aureus* has also been documented [10]. *PPRV* is an enveloped virus with RNA as its genetic material; it belongs to genus *Morbillivirus* of the family *Paramyxoviridae* and commonly infects *small ruminants* [11]. Signs of the disease include fibrino-necrotic tracheitis, erosive-ulcerative stomatitis, diarrhea and fever [12]. High economic losses are reported due to this disease. The only measure to control it is the use of live attenuated vaccines [13]. A number of cell lines including, MRC-5, CHO-K1 and BHK-21 are susceptible to *PPRV* infection but its productivity is higher in Vero cells [14].

This study has been designed to evaluate the antiviral activity of ethanol extracts of *Opuntia species* against *PPRV* in Vero cultured cells.

EXPERIMENTAL

These *Opuntia* plant species (*Opuntia delinii* (OD), *Opuntia manocantha* (OM), and *Opuntia stricta* (OS),) were collected from herbarium of Government College University Lahore, Pakistan and taxonomically authenticated by Prof. Dr. Zaheer-ud-Din Khan (Botany Department) in 2010, with voucher nos. 873/2010, 874/2010 and 875/2010, respectively.

MTT assay

Eight different concentrations of each extract, i.e., 1000, 500, 250, 125, 62.5, 31.25, 15.625 and 7.8125 µg/mL of OD, OM, and OS, were explored for their antiviral and cytotoxic potential against *PPRV* on Vero cell lines by MTT assay. The extracts were obtained using a soxhlet apparatus, and then dried in a rotatory evaporator [15]. These extracts were dissolved in cell culture media to check their antiviral and cytotoxic activity. A confluent monolayer of Vero cells were infected with strains of *PPRV*. In this

study, uninfected cells with media was used as negative control whereas *PPRV* infected cells with media were used as positive control. The viability of Vero cells was determined by applying 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Same procedure was used to check cytotoxicity of these plants. In this case, cells with dimethyl sulfoxide and the media were used as negative control. Cell survival percentage was calculated to check both antiviral and cytotoxic activities of the extracts.

Extraction from medicinal plants

Fresh leaves of the plants were washed with distilled water, chopped, and subjected to the extraction procedure described in this subsection without drying. Ethanol extracts were obtained from the chopped leaves with the help of a soxhlet apparatus (CG-1368) [15].

Briefly, ethanol extract was obtained from 100 g of the chopped plant material in a Soxhlet apparatus. The extracts were dried and kept in a refrigerator till further use.

Preparation of stock solutions

A stock solution of each of ethanol extracts was prepared by dissolving 0.02 g of the dried extract in 2 mL of cell culture medium. Syringe filters (0.2 µm) were used to filter the stock solutions. A serial two-fold dilution of each of the extracts was prepared in the cell culture medium to achieve concentrations of 1000, 500, 250, 125, 62.5, 31.25, 15.63, and 7.81 µg/mL. The protocol was carried out in a biosafety level 2 (BSL-2) laboratory.

Virus and cell line

The *PPRV* and Vero cells were procured from the culture bank of the Quality Operations Laboratory, University of Veterinary and Animal Sciences, Lahore. The cells were grown in a cell culture medium (Dulbecco modified essential medium (DMEM) supplemented with 10% fetal bovine serum. The cells were propagated in a 75cm² cell culture flasks to attain a confluent monolayer according to the procedure described by OIE [16]. After collection of the virus stock solution, the TCID₅₀ (median tissue culture infectious dose) values were calculated [17].

Antiviral assay

The antiviral assay was performed in 96-well cell culture plates. The Vero cells were cultured in the plates to attain 80 - 90 % confluency in the

growth medium. Cell culture media were replaced with fresh maintenance media (DMEM supplemented with 2 % fetal bovine serum). All the concentrations were repeated in triplicate. PPRV was inoculated at a concentration of 10^6 TCID₅₀/mL. Filtration was performed using 0.2 µm syringe filter and incubated at 4 °C for 15 - 30 min. Assay for positive and negative controls was also performed. The plates were then incubated at 37 °C in the presence of 5 % CO₂.

After 5-6 days of incubation cell culture media were removed and plate were washed with phosphate buffer saline solution and then loaded with fresh media. One hundred microliter of 0.5 % MTT solution was poured in both the experimental and control wells. The 96 wells plates, wells were then placed in an incubator for 3 - 5 h. The media in all the wells were each replaced with 100 µL of 5 % DMSO. The 96-well plates were read out at 570 nm using an ELISA reader to obtain their optical density (OD). The MMT dye in the live cells were biochemically converted to formazine which remained inside the biochemical active cells. The formazine was then solubilized in DMSO as described above which resulted in purple solution and the intensity of purple solution directly proportional to the cell survival percentage.

Cytotoxicity assay

Two plates each with a 80–90% confluent monolayer of cell line were selected for cytotoxic assay. The different concentrations of plant extracts were inoculated in the cultured cells. The plates were incubated at 37 °C and in the presence of 5 % CO₂ for 5-6 days. The procedure for performing MTT assay was the same as described above for the antiviral assay.

Assessment of cell survival (CSP)

Cell survival was calculated as in Eq 1.

$$CSP (\%) = \{(At - An)/Ap - An) \dots\dots\dots (1)$$

where At, An, and Ap are the absorbance of test, negative control and positive control samples, respectively.

Statistical analysis

One-way analysis of variance (ANOVA) was used to determine the statistical significance of the differences between the control and experimental groups [18]. Data is presented as the mean ± SD. P < 0.05 was considered statistically significant.

RESULTS

At higher concentrations, i.e. 500 – 1000 µg/mL, all extracts displayed cytotoxic effects while at lower concentrations, i.e. 3.25 – 125 µg/mL, the extracts were non-cytotoxic (Table 1).

The ethanol extract of OM displayed the highest antiviral activity, while the ethanol extract of OS proved the least effective against PPRV in the cultured cells. OM showed antiviral activity at 125 µg/mL whereas OD and OS showing antiviral activity at the concentrations less than 62.5 µg/mL.

Comparison of antiviral and cytotoxic activities of OD, OM, and OS are graphically presented in figures 1, 2, and 3, respectively.

Table 1: Antiviral and cytotoxic concentrations of ethanol extract of three *Opuntia* species

Plant used	Antiviral conc.* (µg/mL)**	Cytotoxic conc.* (µg/mL)
<i>Opuntia delinii</i>	31.25-62.5	125-1000
<i>Opuntia manocantha</i>	3.25-125	250-1000
<i>Opuntia stricta</i>	62.5	125-1000

*Conc. = concentration, ** µg/mL

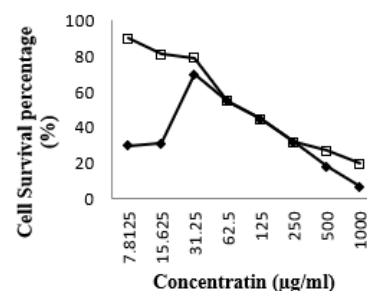


Figure 1: Comparison of antiviral (◆) and cytotoxic (□) activities of *Opuntia delinii* ethanol extract

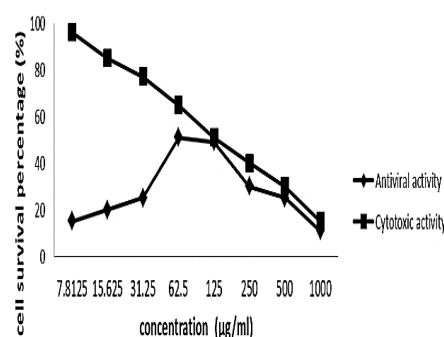


Figure 2: Comparison of antiviral (◆) and cytotoxic (□) activities of *Opuntia manocantha* ethanol extract

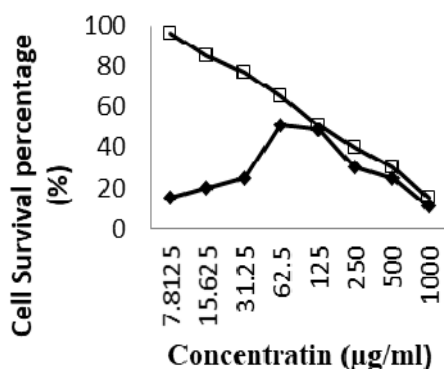


Figure 3: Comparison of antiviral (◆) and cytotoxic (□) activities of *Opuntia stricta* ethanol extracts *Opuntia manocantha*

DISCUSSION

A wide range of medicinal plants have been reported to show antiviral activities and therefore used as therapeutic agents against viral diseases [19]. Three cactus plants, namely OD, OM, and OS were selected for the present study as reports are available on their antiviral activity [20]. The results of the present study revealed that, the concentrations of 31.25 - 62.5 µg/mL of the *Opuntia* species exhibited antiviral action against PPRV. And this finding is supported by another study which found that *Opuntia delinii* serve as an antiviral agent at .40-.80mg/mL against H7N3 avian influenza virus [21].

In the present study, *Opuntia* spp. showed cytotoxicity at higher doses than those previously reported. The possible reason may be *in vitro* system used for the study. In this study, the experiments were carried out *in vitro* using cell culture which has been reported to be much more sensitive than all other *in vitro* techniques, as a known and definite concentration is available to the target cells. Similar findings were reported in which *Opuntia* extracts at concentrations of 1 mg/mL were effective against equine herpes, herpes simplex, influenza, pseudo rabies and respiratory syncytial virus [22]. In the present study, different concentrations of *Opuntia* species were evaluated for their cytotoxic and antiviral potential. OD showed antiviral activity at concentrations of 31.25 and 62.5 µg/mL and cytotoxicity at 250 to 1000 µg/mL. *Opuntia* species are effective against both DNA and RNA viruses. Regarding the active moieties present in *Opuntia* species, chemical studies have shown the presence of the three glycosides of isorhamnetin and quercetin, namely 3-O-methyl quercetin, isoquercetin, kaempferide, kaempferol, isorhamnetin and quercetin, all

which are actually responsible for the therapeutic potential of *Opuntia* species [23]. Some researchers have also reported that the active ingredient in *Opuntia* is protein in nature [22]. In the present study, there may also be quercetin or its derivatives responsible for the antiviral activity of *Opuntia* species, since. *Opuntia streptacantha* resulted in the inhibition of both intracellular and extracellular viruses. OS showed that this inhibition action on both the DNA and RNA viruses. Bosulama *et al* reported the antiviral activity of the ethanol extract of *Opuntia ficus-indica*. They evaluated this activity using two enveloped virus, namely *herpes simplex* and *influenza A*, and one non-envelope virus namely *polio virus*. Two active compounds contained in the plants were identified, which showed their activities against enveloped viruses [24]. PPR virus is also an enveloped virus, and therefore, it can be interpreted that *Opuntia* species might be more active against enveloped viruses.

Opuntia species were subjected to a cell proliferation assay in order to exclude the probability that the reported inhibition of nitric oxide by these species was false positive and occurred due to the cytotoxic effects of the plant species. 3-(4, 5- dimethylthiazole-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay was performed to assess the cell proliferation [25].

There are some limitations when we compare *in vitro* studies with *in vivo* ones. For this reason, the results of the present study cannot be compared with those of *in vivo* studies because the pharmacodynamics of drug has a great tendency to change its *in vitro* results. Other factors that can influence a drug's *in vitro* action are absorption, clearance, excretion, and time of drug exposure drug metabolism. *In vitro* studies help in the identification of novel molecules from different origins, but such molecule should be subsequently if they must be used in *In vivo* conditions.

CONCLUSION

It has been demonstrated that the ethanol extract of *Opuntia* plant has strong antiviral potential, as it successfully inhibits the replication of PPR virus in Vero cultured cells, i.e., it ensured that the virus did not exhibit cytopathic effects

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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