EVALUATION OF ANTIVIRAL AND CYTOTOXIC ACTIVITY OF *CALOTROPIS PROCERA* AGAINST FOOT AND MOUTH DISEASE VIRUS

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ABSTRACT

Foot and mouth disease virus is a single-stranded, non-segmented positive sense RNA virus that causes disease in cloven hoofed animals and results in huge economic loss. *Calotropis procera*, a native of Indian sub-continent, is an erect, branched plant which contains medicinally important chemical constituents having antiviral and antibacterial activity. This study was designed to evaluate the antiviral and cytotoxic activity of aqueous and methanolic extracts of flowers, leaves and root bark of *C. procera* against FMDV. Antiviral and cytotoxic activity of aqueous and methanolic extracts of flowers, leaves and root bark of *C. procera* in BHK21 cell lines were studied using MTT colorimetric assay. Aqueous extract of leaves showed no antiviral activity, while methanolic extract of leaves and aqueous extract of root at the concentration of 0.15 to 0.625 mg/ml were effective against FMDV without being toxic to the cells. The aqueous extract from flowers was effective and non-toxic at the concentrations from 0.075 to 0.15 mg/ml. The maximum antiviral activity against FMDV was exhibited by methanolic extract of leaves.

KEY WORDS: Foot-and-mouth disease virus, *Calotropis procera*, MTT

Running title: Pharmacological properties of *Calotropis procera*

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INTRODUCTION

Foot-and-mouth disease (FMD) is an acute vesicular disease of cloven-hoofed animals caused by foot-and-mouth disease virus (FMDV). The virus is a single-stranded non segmented positive sense RNA virus that is the member of the genus *Apthovirus* and family *Picornaviridae* (Domingo et al., 2002). Animals that can be affected are cattle, goats, sheep and swine (Alexandersen and Mowat 2005). The disease proceeds by the development of vesicles on the nose, tongue, lips, inter-digital space and coronary band above the hoof (Bachrach, 1978). Most common mode of transmission of disease is contaminated droplets of air (Alexandersen et al. 2003). The virus has seven serotypes; Southern African Territories (SAT) 1, SAT 2, SAT 3, O, A, C and Asia 1. Pakistan is endemic region for FMD specially serotypes O, A, C and Asia 1are responsible for spread of disease among animals (Klein et al. 2008). According to the economic survey of Pakistan (2013-2014) population of cattle, buffalo, sheep and goat is 39.7, 34.6, 29.1 and 66.6 million respectively so this large population is always on risk. Due to FMD, annually direct and indirect economic loss of US$6.5 and 21 billion is estimated (Knight-Jones and Rushton, 2013). In advance countries FMD is usually controlled by vaccination and slaughtering policy (Davies, 2002) but in Pakistan due to economical reasons slaughtering is not practised. Only way to control this disease is by active and passive immunization (Anonymous, 2001) but vaccination is not successful sometimes because there is no cross protection among different serotypes of FMDV (Bachrach, 1968; Domingo et al., 2003) Therefore it is important to find other strategies to treat and control FMD.

Plant extracts and the components present in them, have been known to exhibit various biological activities like, antimicrobial (Stermitz et al., 2000), antibacterial (Kanatt et al., 2008) antifungal (Sokovic et al., 2009) and antioxidant activities (Kanatt et al., 2010). Especially in viral infection it is important to find the antiviral agents which are harmless for the host (Harvey,

2000) and plant extracts could be the potential candidates. Previously ethanol extract of *Cassia fistula* fruit has shown its effects against FMDV (Rizvi et al., 2009).

Different parts of the *Calotropis procera* (*C. procera*) have been used all over the world due to its antitumor, antihelmintic, antioxidant, antimicrobial and antimalarial activity (Sharma et al., 2011). *C. procera* is an erect, much branched, 2 to 3 m high plant, having purple spotted white flowers. Plant grows throughout the year in unfarmed soils, mostly in arid and dry zones of Indian sub-continent (Silva et al., 2010). This plant is a semi woody weed having different common names as arka, madar, mudar etc. It is a spreading shrub, contains resin, cardenolids, steroid glucosides, uscharin, calotoxin, and calactin. Its leaves and stalks contain biologically active chemical constituents of sterols, resins, cardenolides, calotropin, calotropagenin etc (Shaker et al., 2010). Composition of flowers shows the presence of high amount of ash and proteins with varying quantities of anthocyanins and alkaloids. Root bark and root contain bitter yellow resin but no alkaloid. Whole plant contains flavone glycosides and cardiac glycoside (Magalhaes et al., 2010). Classical methods for evaluating antiviral properties of plants and their extracts for In vivo studies includes use of animal models and for Invitro studies includes animal protection studies, egg inoculation studies and cell culture methods. Cell culture model is new, more precise and economical technique for antiviral evaluation (Abonyi et al., 2009). In this research activity we have used invitro cell culture technique for evaluation of antiviral and cytotoxic activity of flowers, leaves and root of *C. procera*.

MATERIALS AND METHODS

Preparation of aqueous and methanolic extracts

Flowers, leaves and root bark of *C. procera* were collected from Bahawal nager, Pakistan. The plant was botanically identified and confirmed by the Department of Herbarium, Government College University, Lahore, Pakistan. In order to obtain aqueous extracts 100 grams of the dried leaves powder was left in distilled water for 6 hours in oven at slow heat (35ºC) then it was left at room temperature for 12 hours. Extract was filtered through Whatman No.1 filter paper and centrifuged at 5000 *g* for 15 minutes. Then supernatant was collected and concentrated by rotary evaporator (Stuart RE-300) to make the final volume (Rakshit et al. 2010). Same procedure was followed for obtaining aqueous extracts of other parts of plant.

Methanolic extracts of all parts were obtained by using soxhelet apparatus (CG-1368). Extraction was done by the method described previously (Joshi 2013). Briefly, 50 grams fine powder of leaves was placed inside a thimble made from filter paper and 500 ml of methanol was taken in distillation flask of soxhelet apparatus. Extracts were concentrated by rotary evaporator (Stuart RE-300) and finally converted to semisolid mass by drying oven.

For antiviral and cytotoxic assay, the dried extracts were weighed and dissolved in respective solvents for making 2X solution (10 mg/ml) and further diluted in cell culture media to make two fold dilutions of 5, 2.5, 1.25, 0.6, 0.3, 0.15, 0.075 and 0.032 mg/ml starting from 10 mg/ml of extract. All test dilutions were made in duplicate way, one set for checking antiviral activity and the other for cytotoxicity.

Virus stock and BHK-21 cell line

Purified FMDV and BHK-21 cell line were obtained from Quality Operation Laboratory (QOL), University of Veterinary and Animal Sciences (UVAS), Lahore, Pakistan. Tissue culture infective dose ($TCID_{50}$) of FMDV was calculated by following the method described by Reed and Munch, (1938). Percentage viability of BHK-21 cells was determined with the help of dye exclusion method (Freshney, 2010). Formula for determining viability of cells is as follows.

$$\% \text{ Viable cells} = \frac{\text{Number of viable cells/ml}}{\text{Total number of cells/ml}} \times 100$$

Seeding of BHK-21 cells in 96-well cell culture plates

M199 media having 10% fetal bovine serum was used for obtaining cell suspension. The volume of 100 µl of the cell suspension was seeded in each well of all six 96-well cell culture plates. Three plates were selected for cytotoxicity assay and three for antiviral assay. All plates were incubated in 5% CO_2 at 37°C for 48 hours. Plates were monitored under inverted microscope (Olympus CK40, Japan) until they reached to a confluency of 80–90% (Freshney, 2010).

Cytotoxicity assay

Growth medium of plates, having confluency of 80–90%, was removed and wells were washed with sterilized PBS (phosphate buffer saline). Media of each well of the plate was replaced with 100 µl of freshly prepared M199 media. Cytotoxicity of test material was assayed by adding 100 µl of the each dilution of the extract (as described above) in triplicate to separate wells and plates were incubated at 37◦C with 5% CO2 for 4 days. The wells having BHK-21 cells and M199 medium only, were considered as positive control, while the wells having DMSO (20%) and M199 media were considered as negative control (Freshney, 2010).

Anti viral assay

Similar procedure of washing and replacement of media was adopted for antiviral assay as was used for cytotoxicity assay. Each dilution of the extracts from all test parts were mixed with 10^6 $TCID_{50}$ FMDV and incubated at 4°C for 30 minutes. A volume of 100 µl of the mixture of FMDV and respective dilution was poured in triplicate manner in their respective well of 96 well cell culture plate. One column of plate was kept as negative control, having virus and M199 media with no extract while one column was used as positive control having M199 media without FMDV. Plates were incubated for 4 days at 37°C, cell viability was examined by MTT colorimetric assay (Twentyman and Luscombe 1987) for the evaluation of antiviral activity.

MTT assay

After 4 days (96 hours) of incubation of plates, media was removed from each plate. The volume of 100µl of 0.5% (0.5 mg/100ml M199 media) MTT [3-(4,5-dimethylthiazol-2-yl)-2,diphenyltetrazolium bromide] solution was poured in each well including control wells and incubated them at 37°C for 4 hours. MTT dye was replaced from each well with 100 µl of 20% DMSO (Dimethyl sulfoxide) and incubated again for 2 hours at 37°C. Optical density of each plate was calculated by multi well ELISA reader at wave length of 570 nm (Harne et al., 2012). Cell survival percentage (CSP) was calculated by following formula.

$$CSP = \frac{\text{Mean OD of test} - \text{Mean OD of negative control}}{\text{Mean OD of positive control}} \times 100$$

Statistical analysis

The data was analyzed by statistic package for social sciences (SPSS for Windows version12, SPSS inc., Chicago, IL, USA). The results were evaluated as CSP and expressed in terms of means ± S.D. Results for both cytotoxic and antiviral activities were compared using Chi-Square test and $P < 0.05$ was considered as significant.

RESULTS

Percentage yield of plant extracts

Methanolic and aqueous leaves extracts of flowers, leaves and root bark were obtained with the help of soxhelet apparatus and phenomena of maceration respectively. Percentage yield of each extract is represented in Table 1 and Table 2.

Cytotoxicity assay

The results of cytotoxicity assay were shown as cell survival percentage (CSP). The CSP ≥ 50% obtained by any dilution represents non-cytotoxicity of the respective dilution. The aqueous extracts of *C. procera* leaves, flowers and root bark were non cytotoxic to cells at concentration ranging from 0.032 to 0.625mg/ml because at these concentrations CSP was above 50% (Table 3). While methanolic extracts of all three parts showed CSP above 50% at concentrations ranging from 0.032 to 0.15 mg/ml. In addition, extract from leaves and root showed CSP more than 50% on 0.31 to 0.625 mg/ml and 0.31 mg/ml respectively (Table 4). All the extracts were cytotoxic at 5 mg/ml because CSP was less than 50% for all extracts on this concentration (Table 3 and 4).

3.3. Antiviral assay

Antiviral assay revealed that there was no antiviral activity by aqueous extract of *C. procera* leaves (Table 5 and Figure 1). However, methanolic extract of *C. procera* leaves was effective against FMDV on the concentration ranging from 0.15 to 0.625 mg/ml (Table 6) because CSP was higher than 50%, extract was also non cytotoxic on these concentrations (Figure 2).

Aqueous extract from flowers was effective against virus on 0.075 mg/ml and 0.15 mg/ml and extract was also non cytotoxic on these concentrations (Figure 3). All the other concentrations showed CSP less than 50% (Table 5). Methanolic extracts from flowers revealed

antiviral effect on 0.15 mg/ml (Table 6) and extracts were non cytotoxic on antiviral concentration (Figure 4).

Aqueous extracts from root bark were antiviral at concentrations 0.15 mg/ml to 0.625 mg/ml (Table 5) and these concentrations were non-cytotoxic too (Figure 5). Methanolic extracts from root bark revealed antiviral effect on 0.075 mg/ml and extract was non cytotoxic on this concentration.

Table 1 - Percentage yield of aqueous extracts of leaves, flower and root

Part used	Quantity used for extraction	Dried extract obtained	% w/w yield
Leaves	100 gm	18.5 gm	18.5
Flowers	100 gm	14 gm	14
R. Bark	100 gm	6.8 gm	6.8

Table 2 - Percentage yield of methanol extracts of leaves, flower and root

Part used	Quantity used for extraction	Dried extract obtained	% w/w yield
Leaves	50 gm	3 gm	3
Flowers	50 gm	5 gm	5
R. Bark	50 gm	4.8 gm	4.8

Table 3 - Cytotoxic activities of aqueous extracts from leaves, flowers and root bark

No.	Conc. used (mg/ml)	Mean cell survival percentage ± Standard deviation		
		Leaves	**Flowers**	**Root**
1	5	5±0.03	25±0.01	5±0.05
2	2.5	39±0.13	27±0.01	0±0.001

3	1.25	60±0.06	45±0.30	30±0.004
4	0.625	65±0.101	54±0.21	63±0.05
5	0.31	66±0.051	64±0.1	75±0.001
6	0.15	71±0.051	80±0.177	68±0.044
7	0.075	76±0.027	84±0.1	91±0.029
8	0.032	76±0.030	96±0.01	80±0.01

Table 4 - Cytotoxic activities of methanolic extracts from leaves, flowers and root bark

No.	**Conc. used (mg/ml)**	**Mean cell survival percentage ± Standard deviation**		
		Leaves	**Flowers**	**Root**
1	5	2±0.009	0± 0.03	8±0.001
2	2.5	2±0.011	0± 0.002	13±0.01
3	1.25	41±0.185	0± 0.05	48±0.07
4	0.625	70±0.032	42± 0.04	17±0.001
5	0.31	77±0.050	47± 0.002	65±0.006
6	0.15	61±0.150	58± 0.05	69±0.02
7	0.075	68±0.260	58± 0.008	75±0.01
8	0.032	82±0.142	91± 0.01	78±0.02

Table 5 - Antiviral activities of aqueous extracts from leaves, root bark and flowers

No.	**Conc. used (mg/ml)**	**Mean cell survival percentage ± Standard deviation**		
		Leaves	**Flowers**	**Root**

1	5	0±0.01	5±0.005	2±0.005
2	2.5	2±0.002	4±0.007	0±0.005
3	1.25	6±0.01	6±0.005	22±0.09
4	0.625	13±0.03	4±0.015	52±0.013
5	0.31	37±0.03	44±0.030	61±0
6	0.15	42±0.022	69±0.012	62±0.001
7	0.075	39±0.07	51±0.006	32±0.005
8	0.032	1±0.006	9±0.023	41±0.02

Table 6 - Antiviral activities of methanolic extracts from leaves, root bark and flowers

No.	**Conc. used (mg/ml)**	**Mean cell survival percentage ± Standard deviation**		
		Leaves	**Flowers**	**Root**
1	5	0±0.001	3±0.02	0±0.008
2	2.5	0±0.001	0±0.005	6±0.05
3	1.25	3±0.046	0±0.050	4±0.019
4	0.625	50±0.033	15±0.03	0±0.012
5	0.31	79±0.023	45±0.006	49±0
6	0.15	70±0.010	52±0.3	38±0.09
7	0.075	47±0.011	20±0.05	76±0.06
8	0.032	0±0.001	0±0.011	38±0.09

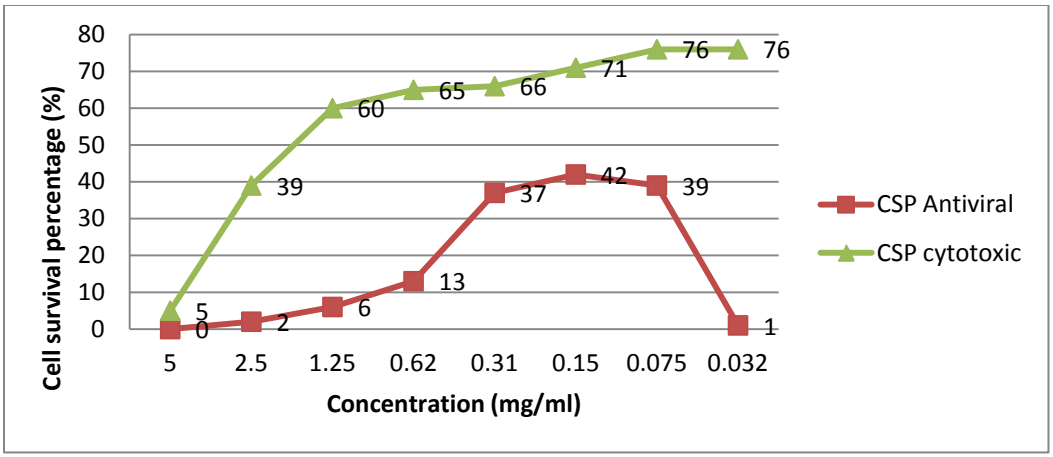

Figure 1 – Graphical presentation of the comparison between anti-viral and cytotoxic potential of aqueous extract from the leaves of *Calotropis procera*

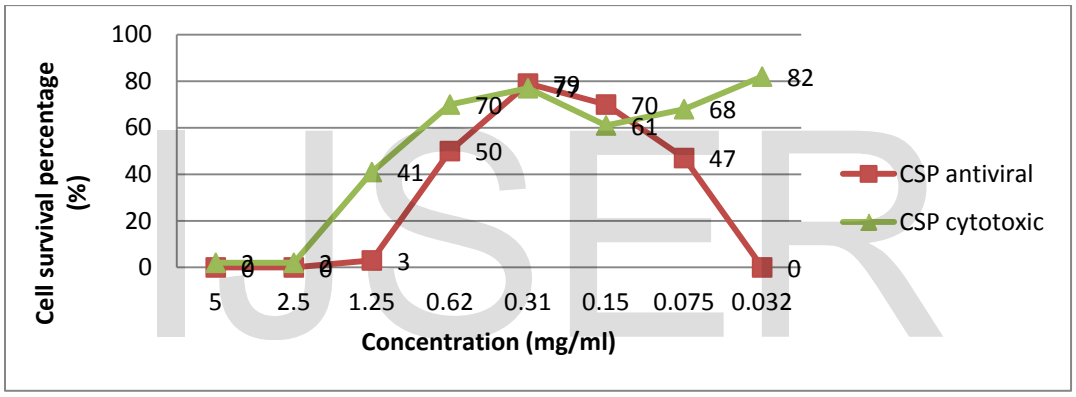

Figure 2 – Graphical presentation of the comparison between anti-viral and cytotoxic potential of methanolic extract from the leaves of *Calotropis procera*.

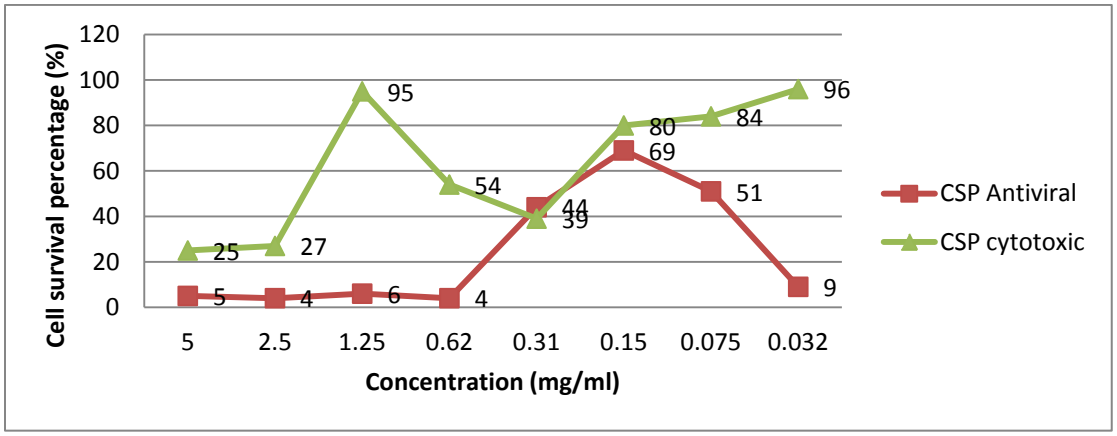

Figure 3 – Graphical presentation of the comparison between anti-viral and cytotoxic potential of aqueous extract from the flowers of *Calotropis procera.*

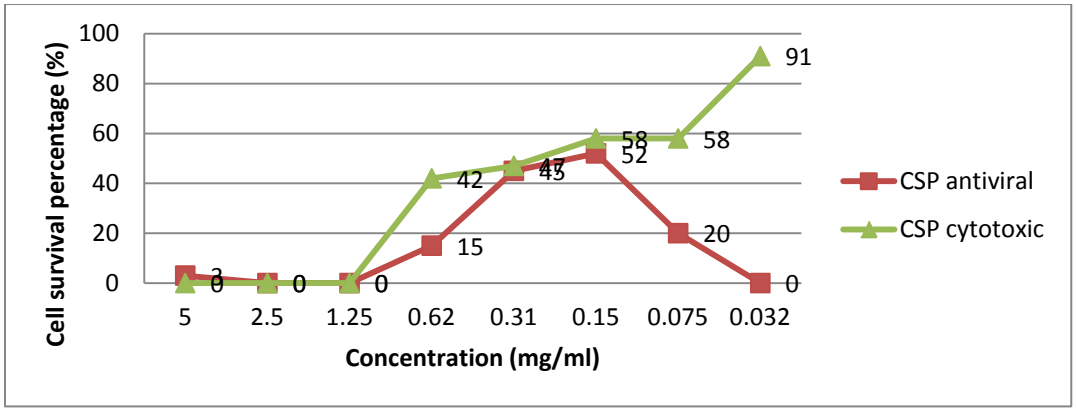

Figure 4 – Graphical presentation of the comparison between anti-viral and cytotoxic potential of methanolic extract from the flowers of *Calotropis procera.*

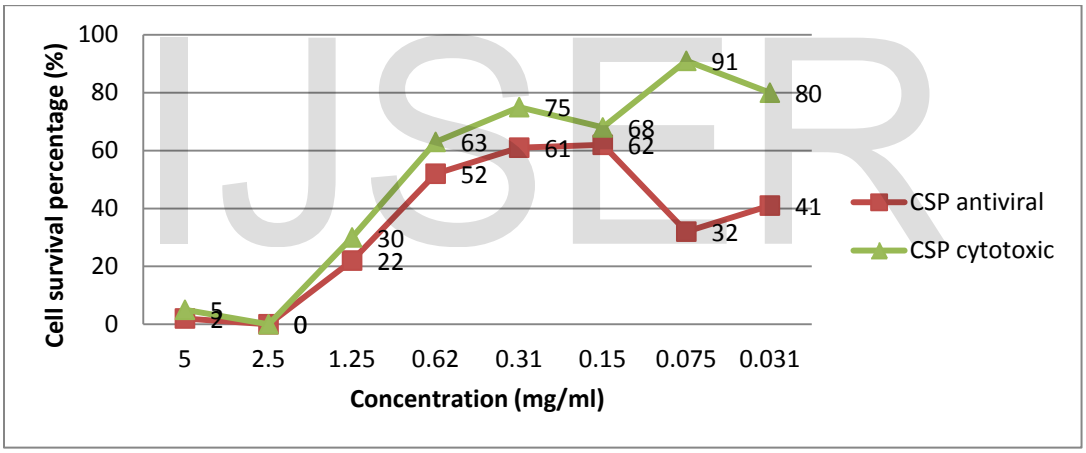

Figure 5 - Graphical presentation of the comparison between anti-viral and cytotoxic potential of aqueous extract from the root bark of *Calotropis procera.*

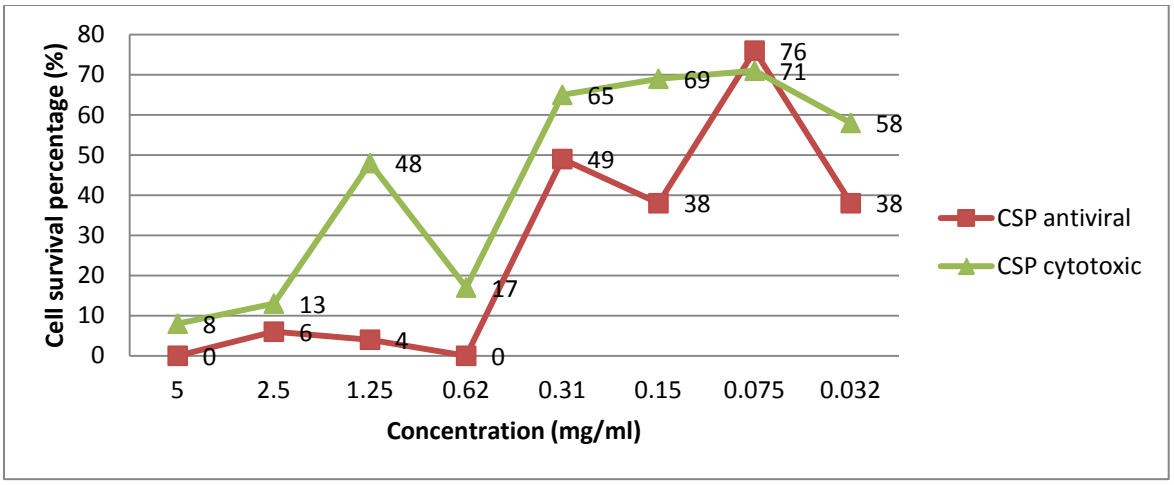

Figure 6 – Graphical presentation of the comparison between anti-viral and cytotoxic potential of methanolic extract from the root bark of *Calotropis procera.*

4. Discussion

Treatment of viral infections is very difficult because viruses grow inside the cells of the host and uses cellular machinery. Any antiviral agent should selectively kill the virus without being toxic to the host. Previous studies show that plant extracts contain some components which are antiviral in nature, e.g phyto-polysaccharides extracted from plants act as strong inhibitors against viruses (Abad Martinez et al., 2005). Extracts obtained from leaves of *Melia azedarach* manifests antiviral activity against vesicular stomatitis virus (VSV) by uncoating of nucleocapsids of VSV (Barquero et al., 2004). The aqueous extract of *Cynodon dactylon* showed antiviral activity against white spot syndrome virus (WSSV) (Balasubramanian et al., 2007). Extracts of leaves of *Artocarpus integrifolia* and *Spondias lutea* were able to inhibit the growth of simian human viruses (SA-11) and rotaviruses (HCR3) in vitro (Goncalves et al., 2005). Ethanol, acetone and methanolic extracts of *Phyllanthus urinaria* were able to inhibit infection of HSV-2 by affecting the initial stage of infection of virus and by diminishing the infectivity of virus (Yang et al., 2005). In a recent study extracts of six species of plants in different solvents exhibited antiviral activities when tested against various viral infections including canine distemper virus, lumpy skin disease virus, feline herpesvirus-1 and canine parainfluenza virus-2 (Bagla et al., 2012).

In this study methanolic extract of *C. procera* leaves showed antiviral activity against FMDV while aqueous extracts showed no antiviral activity (Table 5 and Table 6; Figure 1 and Figure 2). Previously, leaf extract of *C. procera* prepared only in organic solvents *(*ethyl acetate, hexane and methanol) showed an antiviral activity against white spot syndrome virus (WSSV). The activity was due to alkaloids, tannin, cardiac glycosides, and phenols present in extracts. This suggests that *C. procera* contain organic compounds which are less toxic to the cells but interfere in the growth of FMDV. Antiviral activity of *C. procera* has also been investigated against human immunodeficiency virus (HIV-1). Antiviral activity was tested by inhibition of p24 antigen's expression on various concentration of plant extract ranging from 2 to 5 mg/ml. It was revealed that inhibition of the p24 antigen expression was in dose dependent manner and plant extract was proved effective against HIV-1 (Rakshit et al., 2010). Similarly in the current research work there was dose dependent increase in antiviral effect of leaves extract from concentration ranging from 0.15 to 0.625 mg/ml. *C. procera* is also effective against TMV (Mahmoud et al., 2010), plant virus that is positive sense single stranded RNA virus like FMDV. In addition to antiviral activity, leaves of this plant have properties of being anthelmintic, antispasmotic, anlgesic, antipyretic, antiinflamatory, antimalarial and hepatoprotectant (Magalhaes et al. 2010).

Aqueous extract of *C. procera* flowers had shown significant antiviral activity against FMDV (Table 5 and Figure 4). Antiviral activity of flowers of *C. procera* was not studied before and assay predicts that antiviral activity of flowers against FMDV was in such order: aqueous extract > methanolic extract. Previous studies on flower revealed that aqueous extracts have better potential against *Haemonchus contortus* than that of methanolic extracts (Iqbal et al., 2005). According to previous research on flowers, composition of flowers shows the presence of high amount of ash and proteins with varying quantities of anthocyanins and alkaloids (Akhtar et al., 1992) and these components have the ability to kill virus (Jassim and Naji 2003).

In the present study aqueous extract of root bark of *C.procera* was effective against FMDV from 0.15 to 0.625 mg/ml. Similar results were observed when root bark extract of *Calotropis gigantea,* (quite similar to *C. procera*), was tested against vesicular stomatitis (VSV) viruses and herpes simplex type-1 (HSV-1) on doses ranging from 0.002 to 0.1mg/ml. *Calotropis gigantea* exhibited antiviral activity against both viruses (0.002-0.1 mg/ml) and were

non cytotoxic to Hela cell lines on test concentrations (Ali et al., 1996). The difference in dose concentration in both studies could be owing to the composition of the plant or may be due to use of different cell lines. The root bark of *C. procera* is medicinally important due to the presence of resin, cardenolids, steroid glucosides etc. Cardenolide has been isolated from *C. procera* showed antibacterial activity against both gram positive and gram negative bacteria (Akhtar et al. 1992). Antitumor potential of aqueous, hexane, methanolic and ethylacetate extract were tested for evaluating invitro growth inhibitory activity against cancer cells. Tetrazolium bromide (MTT) colorimetry assay was used to find cellular proliferation activities. Data revealed that hexane, methanolic and ethanolic extracts has cytotoxicity, while aqueous extracts did not posses cytotoxic effect. Root extracts arrest cells growth in S phase and cells entry in G2/M phase get prevented and prevent the proliferation of cells by following mechanism of apoptotis and cell cycle disruption (Mathur et al. 2009).

In the current study, active constituents responsible for antiviral and cytotoxic activity were not determined but on the basis of previous work on antiviral potential of *C. procera*, hypothesis could be made that as *C.procera* has antiviral potential against other RNA viruses (Rakshit et al., 2010; Mahmoud, Gad-Rab et al., 2010; Ali et al., 1996) it must have chemical constituents responsible for its antiviral effect against FMDV.

5. Conclusion

The results showed that only methanolic leaves and aqueous root extracts were having significant anti-viral effect against FMDV. Aqueous extract of flowers showed better antiviral potential as compared to methanolic extract of flowers. The extract were also non cytotoxic on the concentrations having antiviral potential. The results of this research activity will be helpful for treatment against foot-and-mouth disease virus.

Conflict of interest

The authors declare that they have no conflict of interest.

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