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In-vitro Antiviral Activity of *Eugenia jambolana* Plant Extract on Buffalopox Virus: Conventional and qPCR Methods

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Abstract: Buffalopox is one of the zoonotic infections having a significant public health impact. The virus is closely related to Vaccinia virus. There is no prophylactic available to combat the buffalopox infection. Ample number of reports is available indicating the use of herbal preparations for veterinary use in India. In this resesrch, four plants having known medicinal importance were screened for buffalopox virus inhibition *in vitro*. Of the four plant tested, extract from *Eugenia jambolana* leaves had an inhibition of 98.52% at its maximum non toxic concentration (1999.73±0.50 μg mL^{-1}) in all cytopathic effect inhibition assays. The inhibition of buffalopox virus replication was further confirmed using PCR (ATI and C18Lgenes) and Real Time-PCR (C18L gene) assays specific for buffalopox virus. Our results indicate that the extract from *Eugenia jambolana* leaves inhibit the buffalopox virus *in vitro*.

Key words: Antiviral activity, buffalopox virus, *eugenia jambolana*, *in vitro*

INTRODUCTION

Buffalopox is an important zoonotic infection of domestic buffaloes (*Bubalus bubalis*), cows and human beings. In buffaloes, it is associated with high morbidity and productivity losses with reduced working capacity of animals and in humans pock like lesions are seen usually on hands. People born after small pox vaccination cessation in 1978 are potentially susceptible to Buffalo-pox Virus (BPXV) infection, a close Variant of Vaccinia Virus (VACV) (Dumbell and Richardson, 1993). Buffalopox zoonotic outbreaks have been reported from many parts of the country namely Namakkal district (Tamil nadu) and various districts of Maharashtra infecting human beings, cattle and buffaloes (Nedunchelliyan *et al*., 1992; Kolhapure *et al*., 1997; Singh *et al*., 2006). The disease is prevalent throughout the major buffalo rearing-areas and outbreaks have been reported in many countries viz., Indonesia, Egypt and Pakistan including India. Combating such disease can contribute prompting buffalo and their byproducts production and a rapid decline in zoonotic infection. The disease is caused by Buffalopox Virus (BPXV), a prototype member of the *Orthopoxvirus* genus in the family *Poxviridae*. BPXV infection although zoonotic, self-limiting and confined to local skin, no commercial vaccine is available as a prophylactic yet. Further, application of Cost-Benefit-Analysis (CBA) criteria has tremendously limited the use of veterinary vaccines and treatments (Lamien *et al*., 2005). Several reports do indicate that many indigenous people make use of their own herbal preparations for veterinary use (Thyagarajan *et al*., 1990). Selection of BPXV inhibitors could also be of value in obtaining antiviral agents against pox viruses (Clercq and Neyts, 2004) and fowl pox virus (Lamien *et al*., 2005) in general and small pox virus in particular, which has re-emerged as a terror for use as a biowarfare agent (Berman and Handerson, 1998).

In India, the traditional medicine is accosted through the tradipractioners and herbalists in the treatment of infectious diseases in human beings and animals. Among the remedies used, plant drugs constitute a significant part. Scientific investigations have spotlit the importance of many plants. Some of the notable examples include *Ocimum sanctum* (Tulsi) inhibits vaccinia and Ranikhet disease viruses (Dhar *et al*., 1968) the *Azadirachta indica* leaf extract found active against pox and herpes viruses, an extract of *Opuntia streptacantha* (Cactus) inhibits equine herpes and pseudorabies viruses, a peptide from *Melia azedarach* leaves (Argentinean plant) found to inhibit FMDV *in vitro*, while theaflavin from black tea neutralizes the viral activity of bovine rota and corona virus infections as reviewed (Jassim and Naji, 2003).

Various preparations of *Acacia arabica* (Babul), *Ocimum sanctum* (Tulsi), *Eugenia jambolana* (Jamun) and *Perscia vulgaris* (Peach) are commonly used in the treatment of several ailments of animals and human

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beings traditionally (Jayvir *et al*., 2002; Sagrawat *et al*., 2006). However, a very scanty report is available on antiviral activity of *Ocimum sanctum* as mentioned above but not on the rest. Therefore, in the present investigation, the *in vitro* antiviral activity of the above extracts was studied against BPXV replication, a zoonotic infectious viral agent. Primarily, various cytotoxicity assays of each extract were carried out in Vero cells and their protective efficacy was monitored by the inhibition of the BPXV as indicated by reduction or absence of Cytopathic Effect (CPE) *in vitro* and also by PCR and quantitative real time-polymerase chain reaction (qRT-PCR).

MATERIALS AND METHODS

Extracts and their preparation: The leaf extracts of *Acacia arabica*, *Eugenia Jambolana*, *Ocimum sanctum* and *Perscia vulgaris* were tested for their antiviral activity. *Aqueous preparation*: The leaves of above plants were cut in to pieces, washed thoroughly, air dried and powdered in a warring blender. The required quantity of the extract was dissolved in Earle's Minimum Essential Medium (EMEM) and stirred. Then, the suspension was centrifuged at 500×g for 10 min. and the supernatant was filtered through 0.22 μm filter and kept at -20°C until further use. *Ethanolic preparation:* The ethanolic extract of *Ocimum sanctum* was prepared as per the method described (Berghe *et al*., 1978). The leaves were dissolved in absolute alcohol in the ratio of 1:3, prefiltered and concentrated into a thick paste. The rest of the steps were followed as afore mentioned.

Cells and virus: The Vero cell lines obtained from ATCC were maintained in the laboratory with regular passage and used in the present study. The Buffalopox Virus (BPXV, Vijayawada-96) isolate has been adapted in Vero cell and maintained in the Division was utilized.

Cytotoxicity testing

Determination of Maximum Non Toxic Concentration/Dose (MNTC/MNTD): The cytotoxicity assay of each extract was performed according to Goncalves *et al*. (2005) with modifications. This method is based on cellular morphologic changes. In brief, different concentrations of each extract were prepared in Maintenance Media (EMEM). Vero cells were grown to confluence for 48 h in 24-well plates. Then, the monolayers were washed with media containing antibiotics. Later, several concentrations of each extract were placed in contact with confluent Vero cell monolayers in quadruplicates and incubated in a 5% CO_2 atmosphere at 37°C for 4 days. The appropriate cell controls without any extract were also included in the test. The cells were observed at 24 h intervals for visible morphological changes in an inverted optical microscope (Leitz), where we compared the treated and untreated controls. The highest concentrations of the extracts showing no cellular morphologic changes were considered as its MNTC. The concentrations exhibiting toxicity to cells were excluded and concentrations below MNTC were employed for antiviral screening.

Determination of Cell Cytotoxicity $_{50}$ (CC_{50}/ CyC_{50})

Dye exclusion method: The concentration of the extracts inducing 50% of cell lysis and death (CC50 or CyC50) was calculated as per Tolo *et al*. (2006). Briefly, Vero cells were seeded at a concentration of $3x10^4$ cells/well in 24-well tissue culture plates (TPP, Germany) and grown at 37°C for 2 days under 5% CO_2. The culture medium was replaced with the maintenance medium containing 1% bovine calf serum with various concentrations of extract and cells were further grown for 72 h at 37°C under CO_2.Then, the medium was removed and the cells were treated with trypsin and the total cell count and viable cells were determined by trypan blue (0.4%) exclusion test. The total, viable and % of viable cells were calculated using haemocytometer as follows; Total Cells mL^{-1}= (Total Cell Count/5)×(Dilution factor)×$10^{4,}$ Viable cells mL^{-1}= (Viable Cell Count/5)×(Dilution factor)×10^4 and Percentage of Viable Cells = 100×(Viable Cell Count/Total Cell Count). The concentration of the extract reducing the cell viability by 50% (CC_{50}/CyC_{50}) was determined from a curve relating percent cell viability to the concentration of the extract.

Optical density method: The cytotoxicity of the extracts was studied as per the method of Gebre-Mariam *et al*. (2006). The 48 h old Vero cell monolayers in a 24-well plate were treated with different concentrations of extracts in test medium after removing the growth medium. Then the cells were incubated at 37°C in 5% CO_2 atmosphere for 72 h. Cells without extract in six wells per plate were used as controls. At the end of the incubation period, the test medium containing the extract was removed from the plate and washed gently with normal saline. Then the wells were stained with 1% crystal violet in 10% formalin for 15 min. The cell viability was evaluated as the percentage of the mean value of the optical density resulting from the six cell controls, which was set at 100%. The 50% Cytotoxic Concentrations (CC_{50}) were calculated from the mean dose response of three independent assays.

Virus inhibition assays

Cytopathic Effect (CPE) reduction assay: The antiviral activity was determined by the reduction of virus titres using $TCID_{50}$ determinations with modifications (Goncalves *et al*., 2005). The Vero cells were grown in 96-well cell culture plates over a period of 48 h. After decanting the growth medium, the cells were treated with extracts at their respective MNTCs (50 μL $well^{-1}$). Afterwards, logarithmic dilutions of BPXV were added at 50 μL $well^{-1}$ in treated and untreated cell cultures and incubated in a 5% CO_2 atmosphere at 37°C. The virus titres ($TCID_{50}$ mL^{-1}) were calculated after 3 days of incubation (Reed and Muench, 1938). The experiment was carried out in quadruplicates. The antiviral activity was expressed in terms of Percentage of Inhibition (PI) using antilogarithm values of $TCID_{50}$, as follows: PI= [1-(T antilogarithm/C antilogarithm)]×100. An extract is considered active when the percentage of inhibition was higher than 80%, at its MNTC.

Effective Concentration$_{50}$ (EC_{50}): The end point assay as modified (Bergh *et al*., 1978) and adapted by Lamien *et al*. (2005) was followed. Vero cells were seeded at 2×10^4 cells/well on 96-well cell culture plates. After 48 h, the growth medium was removed and replaced with two-fold dilution (log2) of plant extract in maintenance medium(EMEM + 1% Bovine calf serum with antibiotics) at 50 μL quantities. BPXV (100$TCID_{50}$/50 μL) was then used to infect each well. Each experiment was run in triplicates with infected-untreated and uninfected-treated as controls. Virus specific CPE was recorded after an incubation period of 72 h as follows: 0% CPE = score 0; 1-25% CPE = score 1; 26-50% CPE = score 2; 51-75% CPE = score 3 and 76-100% CPE=score 4. The effective concentration reducing CPE by 50% (EC_{50}) in comparison with virus controls was estimated from data plots (Serkedjieva and Alan, 1998) and expressed as the mean of three independent experiments. The Selectivity Index (SI)/ Therapeutic Index (TI) was calculated as the ratio of CC_{50}/EC_{50}.

One step growth curve: To determine stage at which the extract has effect on the replication of BPXV, a one step growth curve was performed according to Yamasaki and Tagaya (1980). Vero cell monolayer in a 96-well plate aged 48 h were washed with maintenance medium and then infected with BPXV @0.01 m.o.i and extract at its MNTC was added at various time intervals (0, 18, 24, 42, 60 h) including before or at the time of infection. After, 72 h of infection the virus was harvested with three cycles of freezing and thawing. The harvested virus was titrated in Vero cells and expressed as $TCID_{50}$ mL^{-1}.

Dose response study: Forty eight hours old Vero cell monolayers were washed with maintenance medium and infected with 10-fold dilutions ($log_{10,}$ 10^{-1} to 10^{-8}) of BPXV at 50 μL $well^{-1}$ of each dilution in triplicate. Then various concentrations of extract in 50 μL $well^{-1}$ were added. Each concentration was added in triplicate wells of virus dilutions from 10^{-1} to10^{-8}. Appropriate virus and cell controls were also maintained. The reduction in titre in the treated and untreated virus controls was noted and titre was determined according to Reed and Muench (1938). The experiment was performed in accordance with Yamasaki and Tagaya (1980).

Polymerase Chain Reaction (PCR) and Real Time PCR (RT-PCR): For conventional PCR, the 96-well cell culture plates which were used for the determination of CPE reduction assay were frozen and thawed three times at -20°C. The respective dilutions of the virus infected, infected and treated, uninfected and treated and cell controls were pooled separately. Such pooled samples were clarified at 2000×g in a micro centrifuge for 10 min; the virus containing supernatant was used for total DNA extraction using an AuPrep commercial DNA extraction kit (Life Tech. India Pvt Ltd., New Delhi). Similarly for RT-PCR, 48 h old Vero cells in 25cm^2 tissue culture flasks were infected with BPXV at 0.01 multiplicity of infection (m.o.i) and treated with extract at MNTC (1999.73±0.50 μg mL^{-1}) simultaneously and after 1 h virus adsorption and incubated at 37°C under 5% CO_2 for 72 h. Controls included were Vero cells and Vero cells treated with extract. After 72 h, all the flasks were freeze thawed for 3 times and DNA was extracted as afore mentioned.

The primers designed in-house/published were used to amplify BPXV partial gene segments viz., A Type Inclusion (ATI) and C18L. Cowpox Virus (CoPV) ATI gene specific published primers, CoPV03-GGGATATCAAGGAATGCGA and CoPV04-TCCATATCAGCATTGCTTTC (Meyer and Rziha, 1993) were used to amplify a segment of 552bp in the BPXV genome. Similarly, C18L gene-specific primers were designed based on VACV genome to amplify the C18L region in the BPXV genome, so as to generate a 369bp fragment (un published data). The amplified products were analyzed in 1% agarose gel and the results were documented.

To assess the quantity of viral genome template in the harvests, Real Time-PCR using C18L gene-specific primers of BPXV was also performed for the total DNA extracted from BPXV infected-treated Vero cells with jamun @ 1999.73±0.50 μg mL^{-1} or without extract. Subsequent RT-PCR was carried out in 25 μL volume using 10pMole concentration of primers and QuantiTect™

SYBR® Green PCR Master Mix containing HotStarTaq® DNA polymerase (QIAGEN Inc, Valencia, USA) in Mx 3000p™ machine (Stratagene Inc, LaJolla, CA, USA). PCR was carried out for 30 cycles: denaturation at 94°C for 30 sec, primer annealing at 55°C for 60 sec and extension at 72° C for 60 sec with an initial activation of enzyme at 95°C for 15 min. Mean Cycle Threshold (CT) values of duplicate samples were used for analysis. Melting curves of the PCR products were deduced for calculation of dissociation curves. The entire analysis was done using inbuilt M×3000P software program of the M×3000p™ machine.

RESULTS AND DISCUSSION

Buffalopox is one of the most important emerging and reemerging zoonotic infections. The BPXV is very closely related to strains used for small pox vaccine in human beings. The present generation is highly susceptible to the infection as the vaccination against small pox was surceased in 1978. Moreover, no vaccine is available against buffalopox. Therefore, the present investigation was aimed to identify an antiviral agent against BPXV. This is not only value in preventing/treating buffalopox but also of great prophylactic grandness in obtaining antiviral agents especially against small pox virus, a biowarfare agent (Berman and Handerson, 1998). Therefore, few medicinal plants having known ethnoveterinary applications were tested for their *in vitro* antiviral activities against BPXV.

Before assessing the antiviral activities of the extracts, the cytotoxicity of the extracts on the host cell (Vero cell) was studied. An antiviral drug should be active against the virus without inducing significant toxicity on the host cell. Therefore, the maximum concentration of the drug at which there is no visible toxicity to the cell was determined. *Acacia arabica, Ocimum sanctum* (aqueous), *Ocimum sanctum* (ethanolic), *Eugenia jambolana* and *Perscia vulgaris* leaves extracts above 99.93±0.38, 999.95±0.50, 799.03±0.56, 1999.73±0.50 and 3000.43±0.60 μg mL^{-1} concentrations, respectively were toxic to Vero cell lines (Table 1). The toxicity was evidenced in the form of rounding, clumping and finally detachment of the cells from the surface as the incubation was prolonged. But, the MNTCs were well tolerated by the Vero cells and therefore, these concentrations were used to test the antiviral efficacy. The 50% Cytotoxic Concentration (CC_{50}) of each extract based on dye exclusion and optical density methods are indicated in Table 1. It is clear from the table that the *Acasia arabica* is more toxic, whereas, *Perscia vulgaris* least toxic to Vero cells than other extracts.

The EC_{50} of *Eugenia jambolana* against BPXV was found to be 134±0.2 μg mL^{-1} with a selective/therapeutic index of 56.47. In all the assays used, EC_{50} value was considerably less than the CyC50 values (thus higher SI/TI values) suggesting wider safety and good potency of the extract. *Acacia arabica, Ocimum sanctum* (aqueous and alcoholic) and *Perscia vulgaris* were found non effective against BPXV in their respective MNTC levels, whereas, *Eugenia jambolana* at a concentration of 1999.73±0.50 μg mL^{-1} had potentially inhibited the replication of BPXV. The titre of BPXV at this concentration was found $10^{5.1}$ $TCID_{50}$ mL^{-1}, when compared to the virus control of $10^{6.93}$ $TCID_{50}$ mL^{-1}. An inhibition of virus titre of 1.83 (68-fold, amounts to 98.52%) was observed with *Eugenia jambolana* at its MNTC (Table 1). Therefore, only this extract was further investigated.

In one step growth, maximum inhibition (99.89%, 1000-fold) of BPXV was observed, when the extract of *Eugenia jambolana* was treated at zero hours post infection (hpi). Treatment of extract at different intervals viz.18, 24, 42 and 60 hpi was still effective (89.99%, 10-fold), but the percentage of inhibition was uniform (Fig. 1). Once the virus was adsorbed to cell, the inhibitory effect remained constant throughout. The maximum PI of BPXV was observed at 0 hour of infection. This clearly indicates that the extract acts at the adsorption/entry level of the virus. There was a graded increase in the Percentage of Inhibition (PI) of virus replication as the concentration of the extract was increased within and around MNTC. The PI was 36.9% at 125 μg mL^{-1} followed by 71.8% at 250 μg,

Table 1: Cytotoxicity and inhibitory effects of plant extracts on Vero cells and BPXV

Name of the plant with family (Indian name)	Type of extract	MNTC/MNTD (μg mL^{-1})	CC_{50} mL^{-1} Mean±STDEV (Dye Exclusion)	CC_{50} mL^{-1} Mean±STDEV ($OD_{540/630}$)	Infectivity titre of virus ($TCID_{50}$ mL^{-1})		Percentage Inhibition (PI)
					Virus control	Virus+Extract	
Acacia arabica (Babul)					*Buffalopox virus(BPXV)*		
(*F. Mimosaceae*)	Leaves, Aqueous	99.93±0.38	476.5 ±4.4	467.7±0.5	6.93	6.93	0
Eugenia jambolana(Jamun), (*F. Myrtaceae*)	Leaves, Aqueous	1999.73±0.50	7567.7 ±2.5	7893.7±1.5	6.93	5.10*	98.52
Ocimum sanctum(Tulsi) (*F.Labiatae*)	Leaves, Aqueous	999.95±0.50	3749.7 ±1.2	3849.3±0.5	6.93	6.93	0
Ocimum sanctum (Tulsi)	Leaves, Ethanolic	799.03±0.56	1881.7 ±3.5	1886.7±1.5	6.93	6.93	0
Perscia vulgaris (Peach) (*F. Rosaceae*)	Leaves, Aqueous	3000.43±0.60	8335 ±2.3	8337.3±1.1	6.93	6.93	0

*Inhibition

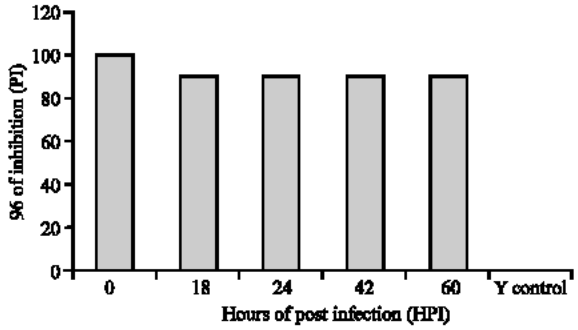

Fig. 1: Growth curve of BPXV in the presence of *Eugenia jambolana* at MNTC

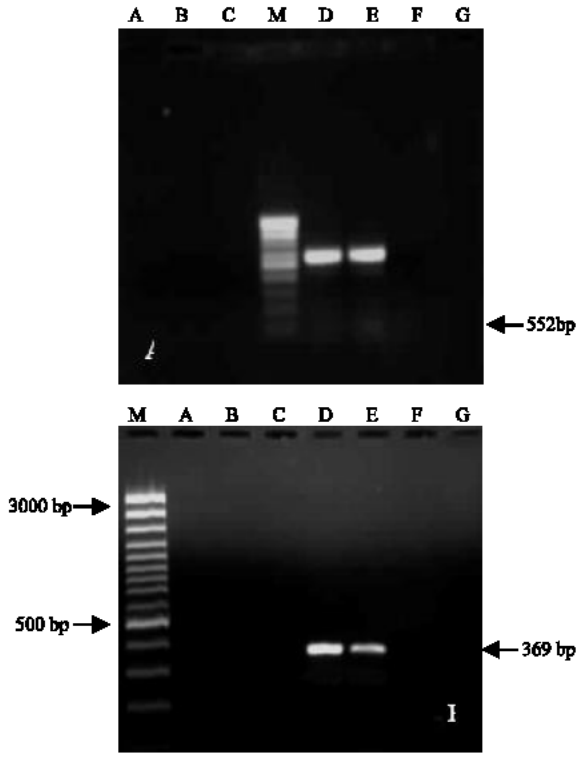

Fig. 2: Agarose gel electrophoresis of PCR products from infected Vero cells with or without *Eugenia jambolana* using BPXV (A) ATI and (B) C18L genes specific primers Lane A: DNA isolated from well with extract+BPXV at 10^{-5} dilution, Lane B: DNA isolated from well with extract+BPXV at 10^{-5} dilution, Lane C: DNA isolated from well with extract+BPXV at 10^{-6} dilution, Lane M: 100bp ladder marker, Lane D: BPV control at 10^{-5} dilution, Lane E: BPV control at 10^{-6} dilution, Lane F: Vero Cell+ Extract Control and Lane G: Blank Control

90% at 500 and 1000 μg mL^{-1}, 94.4% at 1500 μg mL^{-1} and 99% at 2000 μg mL^{-1}. All the CPE assays vindicated the potential inhibition of BPXV replication in the presence of extract. The dose response study, as anticipated, indicated that the lower concentrations have less inhibitory effect than the higher ones.

To ascertain the inhibitory effect of the extract; PCR was performed using CoPV A-Type Inclusion (ATI) gene primer (CoPV-3/CoPV-4) and C18L gene specific primers of BPXV. The amplification of the specific products 552bp and 369bp were obtained in virus control up to 10^{-6} dilution, whereas such amplification was not observed at 10^{-5} dilution and onwards of virus which were treated with the extract at MNTC (Fig. 2A and B). Further, the infected as well as infected and treated with the extract (both preadsorbed and simultaneously infected) samples from which the DNA was extracted were subjected to real time PCR to quantify the load of virus template and also monitor the differential amplification pattern of BPXV 'C18L' gene at MNTC of extract., 1999.73±0.50 μg mL^{-1}. The real-time PCR amplification plot indicated that, ΔCt between the virus control and extract treated cells was 2.47 and 2.17 cycles, which was approximately equivalent to 5.54 and 6.54 fold reduction in the virus yield in the presence of *Eugenia jambolana* extract at MNTC (Fig. 3A and B). The dissociation curve obtained using PCR products from treated and virus control cells confirmed the presence of specific product of T_m 79.57 to 79.62°C and the absence of primer-dimers (Fig. 3B). However, the healthy control cells/Non Template Control (NTC) yielded little primer-dimers of T_m 72.41 (Fig. 3B). Further, on checking final PCR products (10 μL) in 1.5% agarose gel, 369bp BPXV 'C18L' gene-specific PCR products were also confirmed in treated and virus control cells. The healthy control cells did not yield such amplification (Fig. 3C). The control curves fluorescence (dR) or threshold (Ct) were much below the amplification plots of the test samples throughout the run. Further, antiviral property was confirmed in PCR, using ATI and C18L genes specific primers. In conventional PCR, there was no amplification of the desired fragments, but it could be seen in RT-PCR as the later is reported to be more sensitive than the conventional PCR. Though amplification was observed in RT-PCR, there was a significant-fold decrease in the amplification in the treated wells compared to the virus control. These studies clearly signal that the extract from *Eugenia jambolana* significantly *inhibits* the BPXV replication.

Phytochemically, the leaves of *Eugenia jambolana* are reported to contain sitosterol, betulinic acid, crategolic acid, n-hepatcosane, n-nonacosane, n-hentriacontane, n-octacosanol, n-triacontanol, n-dotricontnol and sugars-glucose, fructose, acids-oxalic, citric, glycolic acids and aminoacids-glycine, alanine, tyrosine and leucine; 15 polyphenols and two acetylated flavonol glycosides and quercetin, myricetin and myricitrin (Sagrawat *et al*., 2006).

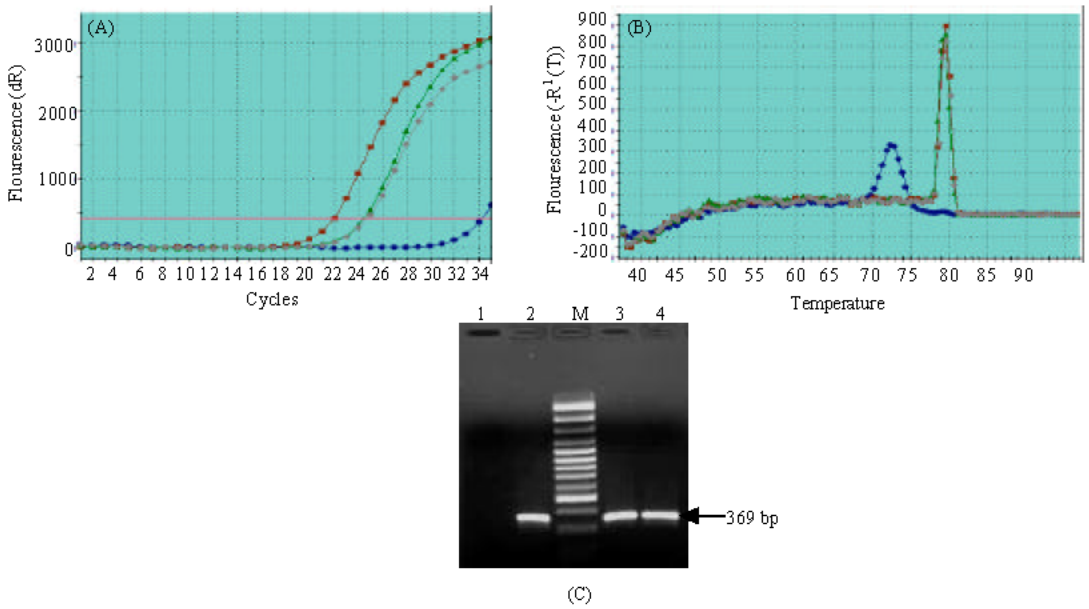

Fig. 3: Real time PCR on infected Vero cells with or without *Eugenia jambolana* using BXPV specific C18L gene primers [A]. PCR amplification plot: Red Line: BPXV grown without *Eugenia jambolana* as virus control; Green Line: *Eugenia jambolana* (1999.73±0.50 μg mL^{-1}) treated post BPXV adsorption; Grey Line: *Eugenia jambolana* (1999.73±0.50 μg m^{-1}) treated simultaneously with BPXV. Blue line; uninfected Vero cell as negative control. [B]. Dissociation curve: Analysis of amplicons derived from melting temperature. [C]. Agarose gel electrophoresis of real time PCR products in 1.5 % Agarose gel. Lane 1, uninfected Vero cell as negative cell control; Lane 2: Virus control; Lane M: 100bp plus DNA ladder (MBI, Fermentas, USA); Lane 3, 4: *Eugenia jambolana* (1999.73±0.50 μg m^{-1}) treated post BPXV adsorption and *Eugenia jambolana* (1999.73±0.50 μg m^{-1}) treated simultaneously with BPXV, respectively

The antiviral activity against several DNA and RNA viruses of plant derived flavonoids has also been reported elsewhere (Narayana *et al*., 2001) including the inhibition of ectromelia pox virus in mice by quercetin (Berghe *et al*., 1978). The active principle though not identified in our study, as the leaves *of Eugenia jambolana* contain several polyphenolic and flavonoids, the antiviral activity could probably be attributed to these compounds. The extracts form seed, leaves and bark of *Eugenia jambolana* are long-familiar for their broad antibacterial activities as well (Sagrawat *et al*., 2006).

CONCLUSION

The present study provided the scientific basis to the ethnoveterinary use of *Eugenia jambolana* to treat the infections of animals by quantifying the cytotoxic and effective antiviral concentrations *in vitro*. The qPCR is more sensitive than the conventional PCR in determining the antiviral effect of active principle of plant origin. Further, studies on isolation and characterization of active principle responsible for the observed antiviral activity would be of paramount significance.

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REFERENCES

Breman, J.G. and D.A. Henderson, 1998. Poxvirus dilemmas-monkey pox, small pox and biologic terrorism. The New England J. Med., 339: 556-559.

De Clercq, E. and J. Neyts, 2004. Therapeutic potential of nucleoside/nucleotide analogues against poxvirus infections. Rev. Med. Virol., 4: 289-300.

Dhar, M.L., M.M. Dhar, B.N. Dhawan, B.N. Mehrotra and G. Roy, 1968. Screening of Indian Medicinal plants for biological activity. Indian J. Exp. Biol., 6: 232.

Dumbell, K. and M. Richardson, 1993. Virological investigations of specimens from buffaloes affected by buffalopox in Maharashtra State, India between 1985 and 1987. Arch. Virol., 128: 257-267.

Gebre-Mariam, T., R. Neubert, P.C. Schmidt, P. Wutzler and M. Schmidtke, 2006. Antiviral activities of some Ethopian medicinal plants used for the treatment of dermatological disorders. J. Ethnopharmacli., 104: 182-187.

Goncalves, J.L.S., R.C. Lopes, D.B. Oliveira, S.S. Costa, M.M.F.S. Miranda, M.T.V. Romanos, N.S.O. Santos and M.D. Wigg, 2005. *In vitro* anti-rotavirus activity of some medicinal plants used in Brazil against diarrhea. J. Ethnopharmacol., 99: 403-407.

Jassim, S.A.A. and M.A. Naji, 2003. A Review, Novel antiviral agents: a medicinal plant perspective. J. Applied MIcrobiol., 95: 412- 427.

Jayvir, A., M. Parabia, G. Bhatt and R. Khamar, 2002. Nature heals, A Glossary f selected Indigenous medicinal plants of India. (2nd Edn.), Sristi Innovations, Ahemedabad, India.

Kolhapure, R.M., R.P. Deolankar, C.D. Tupe, C.G. Raut, A. Basu, B.M. Dama, S.D. Pawar, M.V. Joshi, V.S. Padbidri, M.K. Goverdhan and K. Banerjee, 1997. Investigation of buffalopox outbreaks in Maharashtra State during 1992-1996. Indian J. Med. Res., 106: 441-446.

Lamien, C.E., A. Meda, J. Mans, M. Romito, O.G. Nacoulma and G.J. Viljoen, 2005. Inhibition of fowl pox virus by an aqueous acetone extract from galls of *Guiera senegalensis* J. F. Gmel (Combretaceae). J. Ethnopharmacol., 96: 249-253.

Meyer, H. and H.J. Rziha, 1993. Characterization of the gene encoding the A-type inclusion protein of camel pox virus and sequence comparison with other orthopoxviruses. J. Gen. Virol., 74: 1679- 1684.

Narayana, K.R., M.S. Reddy, M.R. Chaluvady and D.R. Krishna, 2001. Bioflavonoids classification, pharmacological, biochemical effects and therapeutic potential. Indian J. Pharmacol., 33: 2-16.

Nedunchelliyan, S., D.S. Reddy and K.S. Venkataraman, 1992. Buffalopox infection in man. Indian J. Pub. Hlth., 36: 57.

Reed, L.J. and H. Muench, 1938. A simple method of estimation of 50% end points. Am. J. Hyg., 27: 493-497.

Sagrawat, H., A.S. Mann and M.D. Kharya, 2006. Pharmacological potential of *Eugenia jambolana*: A review. Pharmacognosy Magazine, 2: 96-105.

Serkedjieva, J. and J.H. Alan, 1998. *In vitro* antiinfluenza virus activity of plant preparation from *Geranium sanguineum* L. Antiviral Res., 37: 121-130.

Singh, R.K., M. Hosamani, V. Balamurugan, C.C. Satheesh, K.R. Shingal, S.B. Tatwarti, R. Bambal, G.V. Ramteke and M.P. Yadav, 2006. An outbreak of Buffalopox in buffalo (*Bubalus bubalis*) dairy herds in Aurangabad, India. Rev. Sci. Tech. Off. Int. Epiz., (In Press).

Thyagarajan, S.P., S. Jayaram and T. Valliammai, 1990. *Phyllanthus amarus* and Hepatitis B (Letter), Lancet, 336: 49-50.

Tolo, F.M., G.M. Rukunga, F.W. Muli, E.N.M. Njagi, W. Njue, K. Kumon, G.M. Mungai, C.N. Muthaura, J.M. Muli, L.K. Keter, E. Oishi and M.W. Kofi-Tsekpo, 2006. Antiviral activity of the extracts of a Kenyan medicinal plant *Carissa edulis* against herpes simplex virus. J. Ethnopharmacol., 104: 92-99.

Van Den Berghe, D.A., M. Leven, F. Mertens and A.J. Veletinck, 1978. Screening of higher plants for biological activities ii. Antiviral activity, Lloydia, 41: 463-471.

Yamazaki, Z. and I. Tagaya, 1980. Antiviral effects of atropine and caffeine. J. Gen. Virol., 50: 429-430.