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Research Article

Antiviral activity of *Sphaeranthus indicus* against *Bluetongue virus* (BTV) in BHK 21 cell lines

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Abstract

Medicinal plants have been widely used for treatment of variety of infectious and non-infectious diseases. Current theories of reciprocal interactions suggest counter-resistance, genetic adaptability, polymorphic immune capacity, and pleomorphism among microbial agents shows immune diversity of species and endless biochemical possibilities. In order to adapt to environmental conditions plants produce a number of natural products that have antimicrobial activities. These natural products include isoflavonoides, indoles, phytosterols, polysaccharides, sesquiterpenes, furanocoumarians, alkaloides,glucans, tannins, and a variety of vitamins, trace metals (antioxidants), co-enzymes and other photochemical substances. Viruses are obligate intracellular parasites, which contain little more than bundles of gene strands of either RNA or DNA, and may be surrounded by a lipid-containing envelope most of the steps in their replication involve normal cellular metabolic pathways, and this makes it difficult to design a treatment to attack the virion directly, or its replication, without accompanying adverse effects on the infected cells. Viral enzymes play a key role in triggering disease. If viral enzymes could be neutralized, viral replication would not take place. The proteolytic processing of viral polyprotein precursors by a viral proteinase is essential for maturation of the virus. Designing specific inhibitors for each of viral protease is thus a desirable objective.

Keywords: *Sphaeranthus indicus;* antiviral activity; BHK 21 cell lines; Bluetongue virus

Introduction

Antiviral drug is defined as an agent which suppresses the activity of virus by inhibiting its capability to multiply/replicate effectively. Virus causes variety of life threatening diseases in humans and animals, thus the discovery of antiviral drugs became prominent in order to treat viral infections. Vast number of biologically active anti viral drugs has been discovered so far based on their diverse therapeutic functions against various infectious diseases. Therefore screening of different plant extracts for anti viral property has been progressed and noticed that virucidal activity present in different plant extracts and herbs forced plants to be used as folklore medicine (such as Neem, Garlic, Aloevera , Ginger, Calendula, Poke weed etc). This natural treatment against viruses had gain wide importance due to their novel approach in hosts system. Number of antiviral drugs identified to date and their further implementation is under progress.

Almost 40% of currently available drugs are direct or indirect derivatives of plants. Plants are rich source of phytochemicals like alkaloids, anthocyanins, carotenoids, flavonoids, isoflavones, lignans, monoterpenes, organosulfides**,** phenolic acids, saponins and many more (Al-Yahya, 2005; Hassan *et al.,* 2006; Anitha and RanjithaKumari, 2006; Akomo*et al.,* 2009; Rahman*et al.,* 2009; Amabeoku and Kinyua, 2010; Ndjonka*et al.,* 2010). These phytochemicals have been proved to be responsible for their antimicrobial (Sampathkumar*et al,*, 2008; Krishnan *et al.,* 2010), antihypertensive (Amalia*et al.,* 2008), anti-diabetic (Qureshi*et al.,* 2009), antioxidant (Momtaz and Abdollahi, 2010), hepatoprotective (Mahalakshmi *et al.*, 2010; Ansari *et al.*, 2011), cardioprotective

(Ojha *et al.,* 2008; Fard*et al.,* 2008) and other therapeutic activities. As these phyto chemicals are effective against virus, bacteria, and fungi, commercial usage of this antiviral agent against various infections has become challenging area in research.

Bluetongue disease is a haemorrhagic and non-contagious viral disease that causes serious illness in livestock mainly sheep, and less frequently cattle, goat, buffalo and deer. Major symptoms of affected ruminants include vascular endothelial damage which result in oedema (lips, nose, face, submandibulum, eyelids and sometimes ears), congestion (mouth, nose, conjunctiva, skin and coronary bands), haemorrhage, inflammation and necrosis. The mortality rate and the severity of the clinical signs vary with the breed and age of animal infected, the type and strain of the virus and certain interactions with the environment (Spreull 1905, Thomas and Neitz 1947, Luedke*et al.,*1964). Even though vaccination is most effective preventive measure during outbreak, protection of an animal from the threat may not occur more than two weeks of initial vaccination (LinlinGu*et al.,* 2012). Hence, there is a pressing need to develop an antiviral drug which can be offered as a therapeutic agent during outbreaks.

Sphaeranthus indicus Linn.is a medicinal plant widely used in Indian traditional system of medicine for curing various ailments. It grows in rice fields, dry waste places and cultivated lands in tropical parts of India. It is distributed throughout India, Sri Lanka, Africa and Australia from sea level to 1200 m altitude. (Chatterjee and Pakrashi, 2003). All the parts of the S. indicus have medicinal uses. In Ayurvedic system of medicine, the whole herb is used in insanity, tuberculosis, indigestion, bronchitis, spleen diseases, elephantiasis, anaemia, pain in the uterus and vagina, piles, biliousness, epileptic convulsions, asthma, leukoderma, dysentery (Chopra *et al.,* 1956) vomiting, urinary discharges, pain in the rectum, looseness of the breasts, hemicranias (Kirtikar and Basu, 1981). The whole herb is used in Ayurvedic preparations to treat epilepsy, mental disorders and hemicranins (Ambavade*et al.,* 2006 and Jha*et al.,* 2010). Leaves dried in the shade and powdered are used in doses of 20 grains twice a day in chronic skin diseases as an antisyphilitic and a nervine tonic (Prajapati*et al.,* 2003 and Nadkarni, 2007). Hot water extract of the herb is used as an antihelmintic, as a diuretic, as a fish poison (Paranjape, 2001) and as an aphrodisiac (Kapoor and Kapoor, 1980). Flowers are tonic, cooling, alterative and used in conjunctivitis (Chopra *et al.,* 1996) and give strength to weak eyes (Agarwal, 1997). The oil prepared using the plant root is reportedly useful in treating scrofula and as an aphrodisiac. The external application of a paste of this herb is beneficial in treating pruritus and edema, arthritis, filariasis, gout and cervical adenopathy (Sahu, 1984). Pulverized seeds have antimicrobial property. It is also stuffed into holes of crabs to kill them. Aqueous extract is poisonous to American cockroaches (Chopra *et al.,* 1958). In unani, the herb is used as a tonic, laxative, emmenagogue, and also it increases the appetite, enriches the blood, lessens inflammation, cools the brain and gives luster to the eye, is good for sore eyes, jaundice, scalding of urine, gleet, biliousness, boils, scabies, ringworm in the waist, diseases of the chest. The plant is traditionally used for diarrhoea (Girach*et al.,* 1994). The entire plant is used as an emmenogogue (Saha*et al.,* 1961). Hot water extract of the entire plant is used for glandular swelling of the neck and for jaundice (Ikram, 1981).

Materials and Methods

Glassware and chemicals were used in this work was purchased from Himedia, glass are sterilized at 15Lbs pressure for 15min.

Cell lines

Entire work was carried out in Department of Virology, S.V. University, Tirupati. BHK 21 cell lines were grown in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (Hi-Media Co., Mumbai, India) and antibiotics Penicillin (100 IU/mL), Streptomycin (100 µg/mL). BTV was propagated in BHK21 cell lines and used at a concentration of $2.6x10^5$ $TCID_{50}$ in all *In vitro* experiments.

Media

Glasgow's Modified Eagles Medium (M/s Himedia, Bombay,cat No.AT007) was used for maintenance and propagation of BHK21 cells.

Basal Medium

The basal medium was prepared by dissolving 13.54g powder in 950ml of TGDW. The pH of the medium was adjusted to 7.2 by adding sterile 8% sodium bi

carbonate and volume made up to 1 litre with TGDW and sterilized by filtering through 0.22µm membrane filter. Benzyl pencilin and streptomycin were added to the medium at a concentration of 100IU/ml and 100µg/ml respectively. The medium was checked for sterility and stored at 4oc until used.

Fetal bovine serum

Fetal calf serum obtained from Hi-media, Mumbai, India) was used for preparation of medium for BHK21 cell lines.

Growth medium

Growth medium was prepared by adding 10% fetal bovine serum to the basal medium which promote rapid cell growth

: Maintaince medium was prepared by supplementing the basal medium with 2% fetal calf serum, are intended to keep cell culture in a steady state

Trypsin-Versene Glucose solution (TVG)

TVG was prepared and sterilized by passing through o.22µm membrane filter subjected to sterility check and stored at -20ºc.

Trizol reagent containing phenol, guanidine and isothiocynate are used for RNA isolation. Reverse transcriptase Ribonuclease inhibitor,Taq polymerase, deoxyribonucleotide triphosphate (dNTP) mix containing dATP, dGTP, dCTP, dTTP at final concentration of 10mm are enzymes used for this work. Oligonucleotide primer sequence were obtained from Sigma –Aldrich, Hyderabad (NSP3 F-5'-ATGCTATCCGGGCTGAT-3'; NSP3 R-5'-CCCCGTTAGACAGCAGT-3'). Working solution was prepared from original stock (100 p.mol/µl concentration .20p.mol of each primer was used for synthesis of cDNA and also for PCR amplification.

Propagation of BHK21

Confluent monolayer's were washed with Ca^{+2} and Mg^{+2} free PBS(CMF-PBS),pH7.2 ,1ml of pre warmed (37ºC) TVG was added to each monolayer in 25cm2 tissue culture flask and spread over the monolayer. After 1min, the TVG solution was discarded. And to above culture flask, 5ml of growth medium containing 8% fetal calf serum was added till single suspension was obtained. The cell suspension was diluted with growth medium and seeded in fresh flasks at a split ratio of 1:3 for BHK21 cell lines .Thee flasks were incubated at 37ºc and observed at 24hr interval s for growth of cells. The monolayer's when confluent were used for virus infection.

Propagation of BTV-9 in BHK21 cell lines

Roux flasks were seeded with approximately2x10 ^{5}cells/ml in 65ml of growth medium and incubated at 37ºC in 5% CO_2,and cells were allowed to become 90% confluent before being infected with BTV. Cell monolayer was washed once with CMF-PBS pH7.2. Virus was diluted in MEM with antibiotics to establish a multiplicity of infection of 0.01.Following a 60min viral adsorption, maintain medium was added to cells. Cells were incubated until 90-100% CPE developed. The sequential changes in infected monolayer were observed at different time intervals under inverted microscope. Uninfected culture serves as controls.

The virus was harvested by subjecting the virus suspension to 3 cycles of alternate freezing and rapid thawing.The frozen and thawed cellular suspension was centrifuged at 3000rpm for 10min at 4ºc to remove the cell debris. The resultant supernatant was served as stock virus for next passage and was stored at 4ºC till use.

Titration of BTV in BHK21 cell lines

Serial tenfold dilution of the virus stock were made in maintaince medium .To the confluent monolayer's in 24 well tissue culture plates,50µlof virus inoculums for each dilution was added to 5 wells.After 1hr of adsorption at 37ºC, the virus inoculums was removed and 100µl of maintaince medium was added to all the wells including controls.Later the plates were sealed and incubated at 37ºC in CO_2chamber .The infected wells along with the control wells were observed for CPE every day.

The plates were unsealed and the medium in the wells was discarded carefully. The monolayers were washed with PBS and the PBS is discarded. The wells were filled with 0.1% crystal violet in 10% normal saline and left to stain for 30min at room temperature. The stain was aspirated off, monolayer washed with tap

water and left to dry.Later the results were read macroscopically.

Cytopathic effect

Monolayer's of BHK21 cells were grown and incubated with virus in tissue culture bottles .The cytopathic effect in the infected culture were observed for 5 days after post inoculation and compared with suitable uninfected control cultures.

Cytotoxicity assay

Selected medicinal plant aerial parts were air dried, grounded to fine powder. This fine powder was dissolved in methanol+ water and made up to a final concentration of 10mg/10ml followed by filtration through 0.2µm filters (Sartorius). Then it was serially diluted (two fold dilution) to evaluate cytotoxicity induced by *Sphaeranthus indicus*plant extract.

Confluent monolayer of BHK 21 cell lines were prepared as cell suspension by trypsinization and seeded at a concentration of 5000 cells/well in a 96 well tissue culture plate. Plates were incubated at 37°C in a CO_2 incubator for 24-48hrs. After observing the monolayer, growth medium was removed and cells were washed twice with FCS free MEM. Quadruplicate wells of confluent monolayers of BHK 21 cells were incubated with different concentration of the medicinal plant extract up to 72hrs and cell viability was examined by ability of the cells to cleave the tetrazolium salt MTT [3-(4,5-dimethyl thiazol-2ol)-2,5 diphenyltetrazoliumbromide), Sigma-Aldrich, USA], by the mitochondrial succinate dehydrogenase which develops a formazan blue colour product. Intensity of colour was directly proportional to the concentration of test compound. The 50% inhibitory concentration (IC_{50}) was calculated by regression analysis at which IC_{50} of the test compound was minimum. It was further checked by plating efficiency of the cells with the subtoxic dose of selected plant extract.

Screening the plant extract for antiviral activity

BHK 21 cells seeded at a concentration of 5000 cells/well in 24 well tissue culture plates along with inhibitory concentration of plant extract and BTV ($2.6x10^5$ $TCID_{50}$). Cells without extract and cells with BTV were used as cell control and virus control respectively. Each assay was tested in triplicates. Plates were incubated at 37ºC at 5% CO_2 atmosphere and observed up to 72hrs. Specific cytopathic changes induced by BTV were observed at a time intervals of 24, 48 and 72hour of post inoculation. The cell viability was evaluated by trypan blue exclusion method.

Amplification of test compound treated BTV infected cell lines by RT-PCR

RNA Extraction

RNA extraction was carried out using the single step method described by Chomczynski and Sacchi (1987) with Trizol reagent (Sigma Aldrich, USA). Viral RNA was extracted from the infected cell culture fluid.
NS3 RT-PCR

ComplementaryDNA (cDNA) synthesis

Reverse transcription of the BTV NS3 gene was carried out by using the Meloney Murine Leukemia virus (M-MuLV) reverse transcription enzyme (Bangalore Geneipvt.Ltd.,)as per the method described by Byregodwa(2000).

To 7µl of total RNA,2µl of FQW and 1µl of DMSO were added and the RNA was denatured by heating at 95ºc for 5min before snap cooling on ice. 20p.mole of each primer (primers NS3F and NS3R) were added to the denatured RNA , on ice along with 4µl of 5x 1st strand synthesis buffer (280mM TrisHCl, pH8.3 373mM Kcl,15mM $Mgcl_2$) and 1µl of 10mM of dNTP mix, primers were allowed to anneal by keeping at room temperature for 5min and cDNA synthesis was carried out by the addition of 5 units of MuLV reverse transcriptase at 42ºC for 90min.The RT was stopped by heating the mixture at 75ºC for 10 min and the cDNA thus prepared was stored at -20ºC.

Polymerase Chain Reaction of NS3 gene

The synthesized cDNA was used to amplify the NS3 gene of BTV by polymerase chain reaction. The reaction was carried out in a final volume of 50µl in 200µl PCR tubes .The following were added carefully
10X Taq DNA polymerase buffer -5µl

10mM dNTP mix -2µl
$MgCl_2$ 25mM -3µl
Forward primer -1µl

Reverse primer -1µl
cDNA -2µl
Taq DNA polymerase -0.5µl

The total volume of the reaction mixture was made up to 50µl with nuclease free water.The tubes were then spinned for the 10seconds and PCR carried out in an eppendof master cycle.The reaction conditions was standardized for the amplification of 250bp of NS3 gene.The amplified product was analyzed by Agarose gel electrophoresis.

Agarose gel electrophoresis

1% Agarose was prepared in 0.5% TBE containing EtBr at a final concentration of 0.5µg/ml. The final PCR product was mixed with 1µl of 6X gel loading dye and electrophoresis at 60 volts for 90min.After the electrophoresis, the amplified PCR product was visualized in a UV transilluminator and compared with 1Kb DNA ladder.

Results

Virus Titration

BTV- 9 serotype was passaged in BHK21 cell line and infectivity titres were calculated at different passage levels. The infectivity titre of BTV-9 between 32^{nd} and 36^{th} passage calculated as $2.6x10^5$ $TCID_{50}$ by Read and Munech method.

Cytopathogenecity of BTV

Confluent monolayer's of BHK21 cells were infected with 0.1ml of BTV. The infected and uninfected cultures were observed after 0,24,48,72 and 96hrs post infection(PI) for the presence of any changes induced by the virus. The initiation of cytopathic effect (CPE) was observed as early as 24hrs PI, with initial rounding and detachment of cells. Characteristic CPE of BTV with ballooning and clumping of cells was observed .Swollen spindle shaped cells and long cytoplasmic bridges appeared by 48hrs.Complete detachment of the monolayer take place by 72 hrs and the detached cells appeared as clumps. The uninfected monolayer did not show any changes and were normal until 120hrs of incubation except for a few detached cells due to overcrowding or over growth of cells.

Cytotoxicity assay

The 50% inhibitory concentration (IC_{50}) was calculated by regression analysis by plotting different concentrations of medicinal plant extractand percent viability of cell respectively. IC_{50} was calculated accordingly as 3.9µg/ml (Fig: 1 & 2). It was further checked by plating efficiency of the cells with the subtoxic dose of medicinal plant extract, no effect on cell growth was observed.

Screening the compounds for antiviral activity

After 72hrs of PI the cells were stained with trypan blue and counted for viable cells. The virus induced cytopathic changes also observed in time. It was found that there was no significant cytopathic changes were observed in cells treated with medicinal plant extract at an effective concentration of 3.9µg/ml (Fig: 3).

Amplification of test compound treated BTV infected cell lines by RT-PCR

The virus progress in medicinal plant extracted cells was further confirmed by performing the NS3 RT-PCR (Fig: 4). Viral RNA was isolated at different time intervals and subjected to PCR amplification. On progress there was no amplification of BTV was observed after agarose gel electrophoresis. It was further checked with positive control which given 250bp amplification product in gel electrophoresis.

Discussion

Medicinal plants play an important role in health care. The demand for medicinal plants in health care is about 70-80%. Medicinal plants are the local heritage with global importance. Traditional medicines are used by about 60 per cent of the world's population. These are not only used for primary health care not just in rural areas in developing countries, but also in developed countries as well where modern medicines are predominantly used. While the traditional medicines are derived from medicinal plants, minerals, and organic matter, the herbal drugs are prepared from medicinal plants only. There is an increasing need for search of new compounds with antiviral activity as the treatment of viral infections with the available antiviral drugs is often unsatisfactory due to the

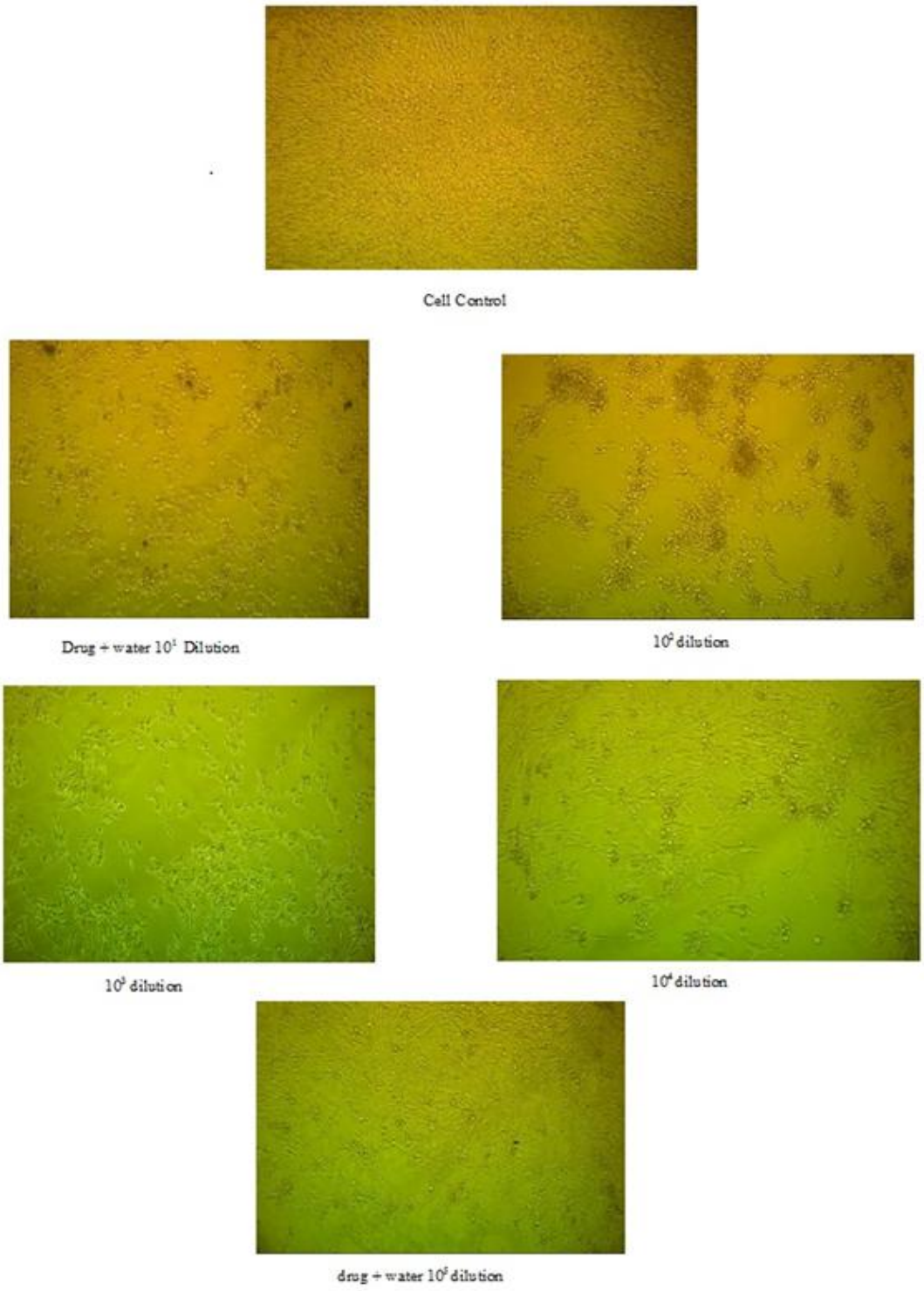


Figure1: Cytotoxity of cells observed under inverted microscope. cellular changes like roundening, syncytia formation and detachment of cells were observed. Remaining figure are same to cell control.

Cell control

Drug + Water + Methanol 10^{1} dilution

10^{2} dilution

10^{3} dilution

10^{4} dilution

10^{5} dilution

10^{6} dilution

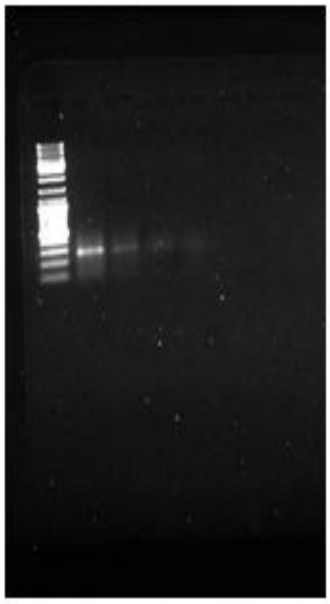
Figure2:Cyto toxicity of compound with solvent and water at different dilutions observed under inverted microscope. Cellular changes like roundening, syncytia formation and detachment of cells. Compounds.have no effect on cellular changes.

Virus Control

Virostatic efficacy of Drug+water at 10^5 dilution

Virostatic efficacy of Drug+water at 10^6 dilution

Figure3: Anti viral property of compound againist BTV in cell lines. No cyto pathic change was observed.



Lane 1 : 1Kb DNA ladder

Lane 2 to 5: Identification of BTV in infected Vero controls at different time intervals by detection of 250bp amplification (24, 48 and 72hrs of PI)

Lane 6 : Cell control

Lane 7 to 8 : Cells + Virus + Plant extract inhibited the virus progress

in cell lines

Figure4:Amplification of test compound treated BTV infected cell lines by RT-PCR, with positive control which given 250bp amplification product in gel electrophoresis.

problem of viral resistance (Field AK, Biron KK, 1994) with the problem of viral latency and conflicting efficacy in recurrent infection in immunocompromised patients (Vlietinck AJ, VandenBerghe DA, 1991). The Indian subcontinent is endowed with rich and diverse local health tradition, which is equally matched with rich and diverse plant genetic source (Hudson JB. 1990).

In 1940's it was thought that BTV was confined only to Southern Africa, but emergence of this disease throughout world was reported during early 1940's this brought global recognization to BTV. Molecular studies of BTV are under progress for last three decades and it is one of the best understood viruses at the molecular and structural levels.There was number of commercially available vaccines are there, but still the disease was endemic in most of the areas. This is mainly due to constant mutations in virus leads to highly pathogenic stain development and change in vector habitats also another important cause. This makes difficult to eradicate this disease from world. Hence, there was an urgent need to develop a suitable antiviral agent where vaccination fails to protect the animal from disease.

Recent studies are carrying out in order to recognize the suitable antiviral agent either synthetic or from a natural source. The present study was one of the attempts to recognize virostatic efficacy of medicinal plant extact against BTV in vitro. The results obtained in this study were given promising evidence that medicinal plant extract have efficacy to minimize the BTV in vitro in BHK cell lines. Further it was necessary to recognize the effective compound present in extract that giving virucidal property to medicinal plant *Sphaeranthus indicus* extract. It was further also need to evaluate the mechanistic action of the above extract on BTV in vivo by using suitable animal models.

Conclusion

In conclusion this is only a preliminary report that medicinal plant extract have efficacy to prevent the virus progress in cell cultures. Further identification of compound responsible for this activity is needed to develop good antiviral agent for bluetongue virus. It also required evaluating the mechanistic action of extract on virus using suitable animal model.

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