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Antiviral Activity of Dasyscyphin C Extracted from *Eclipta Prostrata* Against Fish Nodavirus

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Abstract

Antiviral activity of dasyscyphin C ($C_{28}H_{40}O_8$) extracted from leaves of *Eclipta prostrata* was evaluated against fish nodavirus, grouper nervous necrosis virus (GNNV) infected SIGE (Sahul Indian Grouper Eye) cell lines under *in vitro* conditions. Nodavirus infection to SIGE cells was confirmed by cytopathic effect (CPE) and reverse transcriptase-polymerase chain reaction. The susceptibility of the virus to the isolated compound was confirmed by measuring the virus titre ($TCID_{50}$/ ml) at every 24 hrs. The isolated compound was found to be effective in inhibiting the proliferation of fish nodavirus (FNV) infected in the SIGE cell lines at 20µg/ mL and the cell viability was decreased to 22% as determined by MTT assay. The virus titre ($TCID_{50}$ / ml) was 3.7 at the end of 7th day in dasyscyphin C treated SIGE cells post inoculated with nodavirus. Based on the results it can be concluded that dasyscyphin C may be used as antiviral agent against fish nodavirus.

Keywords: Dasyscyphin C; Fish nodavirus: Grouper nervous necrosis virus; Sahul indian grouper eye; Cytopathic effect

Introduction

The tradition of use of medicinal plants for the treatment of a spectrum of diseases is not uncommon in worldwide. Natural products from plant are the significant source for discovering effective antiviral drugs (Hudson, 1990). Although there were many antiviral drugs being synthesized in the recent past, still the search for potent antiviral agents are continued to get ride of viral diseases (De Clercq, 1993). Animal cell culture is the most valuable and significant technique for analyzing the effectiveness of particular antiviral agent. A number of fish cell lines are recently developed for viral diagnostics. They can be easily cultured in the laboratory which is effortless process for screening of effective antiviral drugs (Parameswaran et al., 2007).

Aquaculture is an important economic activity in many countries (Edward, 2002). In India cultivation of marine fish is of great economic value. One of the major threats to aquaculture industry is the occurrence of infectious disease caused by viruses, which affect many high value fish species that leads to huge economic loss. The growth rate of aquaculture is increasing every year and the fish disease is the most common to the pond keepers which spread easily results in unbarrable loss and closure of industry. Viral infection caused by nodaviruses belongs to nodaviridae family are common which causes viral nervous necrosis (VNN) also referred to as viral encephalopathy and retinopathy (VER) and fish encephalitis (Munday et al., 2002). Because of VNN, high mortalities occur in several marine fish species of economic importance to the aquaculture industry.

There are several antiviral compounds are commercially available, even though the search for novel product from plant source which has no side-effects is still on trial. Saponins are a diverse group of compounds widely distributed in the plant kingdom, which are usually characterized by their structure containing a steroidal or triterpenoid aglycone and one or more sugar chains. Studies on antiviral activity of plant derived saponins were not extensively studied.

The *E. prostrata* plant is a small, branched annual herb commonly called as 'Karichalai' belongs to a family Asteraceae, with white flower heads, is native to the tropical and subtropical regions of the world. The leaf paste is applied on the affected area of teeth to control the tooth ache and the leaves decoction was long been used orally for control of jaundice (Sandhya et al., 2006). Hence a study was planed to evaluate the antiviral potential of active principle isolated from *E. prostrata* leaves.

Materials and Methods

Plant material

The leaves of *E. prostrata* were collected from medicinal garden, VIT University. A voucher specimen was prepared and deposited in the herbarium section of the VIT University. The leaves of *E. prostrata* were washed with distilled water, shade dried, powdered and stored in an air- tight container until further use.

Extraction and purification of active saponin

The powdered sample was defatted by petroleum ether for 3 h at 40°C. After filtering the petroleum ether, the sample was extracted with methanol for 3 h with mild heating. The methanol extract was concentrated and re-extracted with methanol and acetone (1:5v/v) (Yan et al., 1996).The precipitate obtained was dried under vacuum, which turns to a whitish amorphous powder after complete drying. It was loaded on Merck silica gel-60 (230-400 mesh) column and eluted with chloroform-methanol-water (70:30:10) (Favel et al., 2005). The first fraction collected was air dried under room temperature (28°C) and the residue obtained was treated as pure saponin *E. prostrata*. The purity of the saponin isolated was analyzed by thin layer chromatography using chloroform and methanol (7:3) as solvent system.

Structural elucidation

The purified compound was subjected to structural elucidation by UV (TECHCOMP), FT-IR (THERMO NICOLET -330),

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^{1}H-NMR (JEOL GSX-500), ^{13}C-NMR (JEOL GSX-500) and MS (FINNIGAN MAT-8230). All chemicals used for extraction and purification were of analytical grade obtained from SRL, Mumbai, India.

Cell line and culture media

The continuous cell line, SIGE consists predominantly of epithelial cells was applied to study the cytotoxic and antiviral effect of dasyscyphin C. Cells were cultured routinely in 75cm^2 culture flasks at 28°C in Leibovitz's L-15 culture medium, with 10% fetal bovine serum (FBS) and 2% penicillin-streptomycin (100 mg/ml streptomycin, 100 IU/ml penicillin) for seeding of cells to tissue culture plates.

RNA extraction

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) reagent according to the protocol of the manufacturer. Briefly, the virus infected cells were frozen and thawed thrice and centrifuged at 5000 x g for 7 min at 4°C. 200µl of clarified cell culture supernatant was mixed with 1 ml of total RNA isolation reagent TRIzol and incubated for 5 min at room temperature. After incubation, the sample was transferred to 1.5 ml centrifuge tube to which 0.2 ml of chloroform was added. The sample was vigorously shaken for 2 to 3 min at room temperature and then centrifuged at 12000 x g for 15 min at room temperature. The aqueous phase was removed and transferred to a new 1.5ml centrifuge tube and to this 500µl of isopropanol was added and incubated for 15 minutes in ice. Then it was centrifuged at 12,000 rpm at 4°C for 15 minutes. The supernatant was discarded and the RNA pellet was washed with 1 ml of 75% ethanol. The pellet was air-dried and resuspended in DEPC (Diethyl pyrocarboanate)-water and the amount of nucleic acid in the sample were quantified by measuring the absorbance at 260 nm in spectrophotometer. The purity of the preparation was checked by measuring the ratio of OD_{260nm}/OD_{280nm}.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA from nodavirus was extracted by using Trizol reagent (Gibco-BRL). The first strand cDNA was synthesized using reverse transcriptase (Promega) and oligo (dT) primer according to the manufacturer's instructions. The cDNA reaction products were subjected to PCR with the primer set (5'-GTTCCCTGTACAACGATTCC-3'/5' TGAGCAGCCCCGTC AAATCC-3' for nodavirus. Uninfected cells were served as control. The PCR products were analyzed by electrophoresis in 1% agarose gel electrophoresis.

RT-PCR was carried out using the Reverse-IT TM 1-step RT-PCR kit (ABgene), allowing reverse transcription (RT) and amplification to be performed in a single reaction tube. An aliquot of 10 µg RNA was treated with 200 U of RNase-free DNase I (Bangalore Genei, India) at 37°C for 30 minutes to remove any viral genomic DNA contamination and then re-extracted with phenol-chloroform. The DNase-treated total RNA was denatured by heating at 85°C for 10 min in 10 µl DEPC-water containing 100-pmol oligo-dT primer (Bangalore Genei, India). The first strand cDNA was synthesized by the addition of 3 µl 5x M-MuLV buffer, 1 µl 100mM DTT, 1 µl 10 mM dNTPs, 10 U rRNasin (Bangalore Genei, India), and 100 U M-MuLV reverse transcriptase (New England Biolabs, MA, USA). The reaction proceeded at 37°C for 1 h. The cDNA reaction product was subjected to PCR with specific primers and different annealing temperatures for the respective viruses. The RT-PCR products (10µl) were then analyzed by electrophoresis on agarose gel stained with ethidium bromide and visualized by ultraviolet transillumination.

Observation of morphological alterations of cells

Cells treated with dasyscyphin C were observed under microscope for morphological alterations. The cell morphology was observed and photographed under an inverted microscope (Carl Zeiss - Germany, Nikon - Japan).

Antiviral activity by MTT assay

The effect of different concentrations (0 to 20µg/ml) of dasyscyphin C on the proliferation of nodavirus infected SIGE cells was tested for period of 6 days under *in vitro* conditions by MTT assay as described by earlier. Borenfreund et al. (1985). It is based on the reduction of soluble yellow MTT tetrazolium salt to a blue insoluble MTT formazan product by mitochondrial succinic dehydrogenase. After a 24hrs of exposure, the test medium was replaced by 20 µl of 5 mg/ml MTT (Sigma, St Louis) in PBS. MTT stock solution was prepared by filtering through a 0.22 µm filter to sterilize and remove the small amount of insoluble residue. After staining for 4 h at 20°C, the staining solution was carefully removed by aspiration and the cells were rinsed twice with PBS rapidly, and then 150 µl/well of DMSO or acidified isoproponal (100µl of HCl in 100ml of Isoproponal) was added to solubilize the blue formazan crystals produced. The absorbance of each well was measured at 570 nm (test wavelength) and at 630 nm (reference wavelength) in the microplate reader.

Cytopathic effects of fish nodavirus

Cytopathic effects of fish nodavirus (NNV) post-infected with normal and dasyscyphin C treated SIGE cells were observed and photographed under an inverted microscope (Carl Zeiss - Germany, Nikon - Japan).

Titration of virus (TCID50)

Viral supernatants of 4 different time-interval groups were diluted from 10^{-1} to 10^{-8} and used to infect SIGE cells with 6 repetitions per dilution to perform $TCID_{50}$ assay. Viral titers were calculated as described earlier by Reed - Muench (1938) and by the Spearman-Karber method (Hamilton et al., 1977). Cytopathic effect (CPE) was observed for each day and the titre was determined on day 7. Virus titre was expressed as $TCID_{50}$/ml.

Results and Discussion

The active principle extracted from *E.prostrata* and its chemical structure was arrived from the following spectral data. It is a Jelly, H NMR (500 MHz in $CDCl_3$), (OH) - 2.032 (m, 2H) , 1-H -0.992 (t,2H), 2-H -0.889 (m,2H), 3-H -0.910 (t,2H), 5-H -1.316 (t,1H), 6-H -0.859 (t,2H), 7-H -0.876 (dd,2H), 9-H -1.301 (d,1H), 11-H -1.704 (t,2H), 12-H -5.197 (m,1H), 16-H -6.158 (s,1H), 18-H -1.629 (t,3H), 19,20-H -1.270 (s,6H), 21-H -2.310 (s,3H), 22-H -5.411 (m,2H), 22-H -5.411 (m,2H), 2'-H -3.683 (s,2H), 4'-H -4.069 (s,2H), 6'-H -1.149 (s,3H) and ESI-m/z : 503 [M]-.

Dasyscyphin C (Figure 1) identity was proved by TLC, UV, FT-IR, ^{1}H NMR and MS analysis. The presence of triterpenes in TLC plate was confirmed by Libermann-Burchard reaction and Carr-Price reagent. UV spectrum showed maximum absorption bands at 234, 238, 302 nm for dasyscyphin C and the IR spectrum showed at 3435.80, 2921.82, 1635.05, 1245.75, 1050.66 cm^{-1} for dasyscyphin C. The FINNIGAN MAT 8230MS showed the [M] - ion at m/z 503 with the base peak at m/z 208 for dasyscyphin C. The chemical shift assignments obtained for dasyscyphin C from 1H-NMR corresponding to the molecular formula C_{28} H_{40} O_8. The chemical shift assignments obtained for Dasyscyphin C from ^{1}H-NMR corresponding to the molecular formula C_{28} H_{40} O_8 was similar to as previously been reported (Parra et al., 2006).

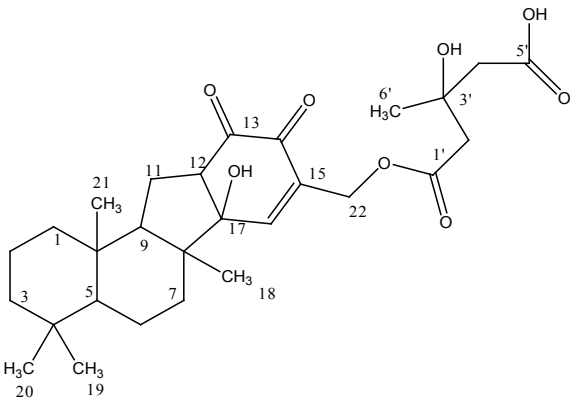

Figure 1: Structure of Dasyscyphin C ($C_{28}H_{40}O_8$).

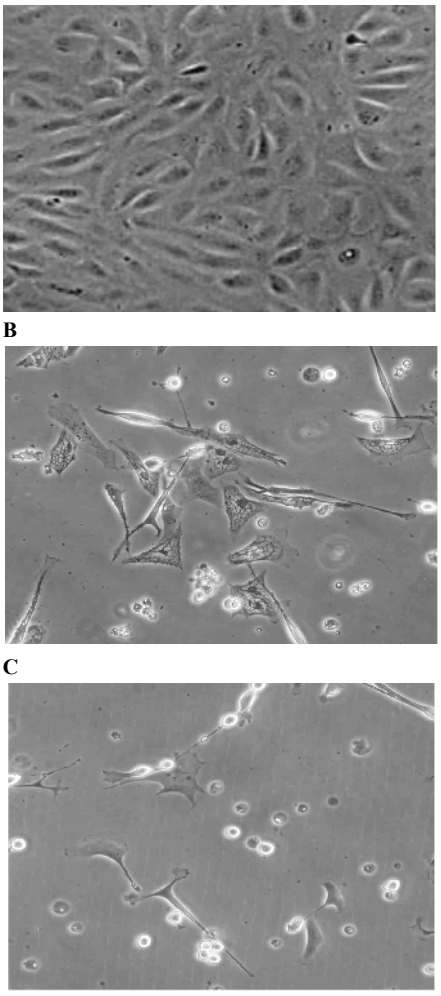

Figure 2: Susceptibility of SIGE cells to nodavirus (A) Confluent uninfected SIGE cells. (B) Extensive CPE with multiple vacuolation (arrows) in SIGE cells infected with nodavirus. (C) FNV infected SIGE cells treated with dasyscyphin C.

The susceptibility of grouper eye cell line to fish nodavirus was evaluated by observation of cytopathic effect in the cell line and further confirmation by RT-PCR. No CPE was observed in uninfected grouper eye cells (Figure 2A). Significant CPE was observed in the cells at 32 h post infection with nodavirus. Initially, the specific CPE developed as localized areas of rounded and refractile cells that later spread over the monolayer in 48 h post infection to form a network of degenerating cells. The monolayer was completely disintegrated after 3 days. The CPE with typical multiple vacuolation was observed in cells infected with nodavirus (Figure 2B). The CPE due to these viruses observed in the grouper eye cell line was further confirmed by RT-PCR. The CPE was also observed in five successive passages after reinfection using infected tissue culture fluid. FNV infected SIGE cells treated with dasyscyphin C is shown in (Figure 2C). dasyscyphin C treatment inhibits the viral growth as evidenced by inhibition of proliferation of SIGE cells. The results of RT-PCR analysis on cell lines infected with nodavirus is shown in Figure 3 and these results showed the appearance of prominent band of RT-PCR amplified product of nodavirus (294 bp) (Figure 3).

The results of the viral replication efficiency test carried out on this cell line are shown in Figure 4. The viral titre increased with time demonstrating that viral replication took place. In the grouper eye cell line, the viral titre increased significantly from 2.5 log $TCID_{50}$ml-1 to 4.1 log $TCID_{50}$ml-1 for nodavirus at 5 days post inoculation. These observations have been further confirmed by RT-PCR using specific primers for this virus. In the nodaviral infection, CPE was more apparent and consisted mainly of cells rounding up, possessing multiple vacuolations, and detaching from the monolayer. Dasyscyphin C exhibited concentration dependent inhibition of nodavirus infected SIGE cells in the culture medium. At 20µg/ml of dasyscyphin C inhibits 50% cell viability at the end of 3 days and at the end of 6 days only 22% cells are viable. It indicates that dasyscyphin C

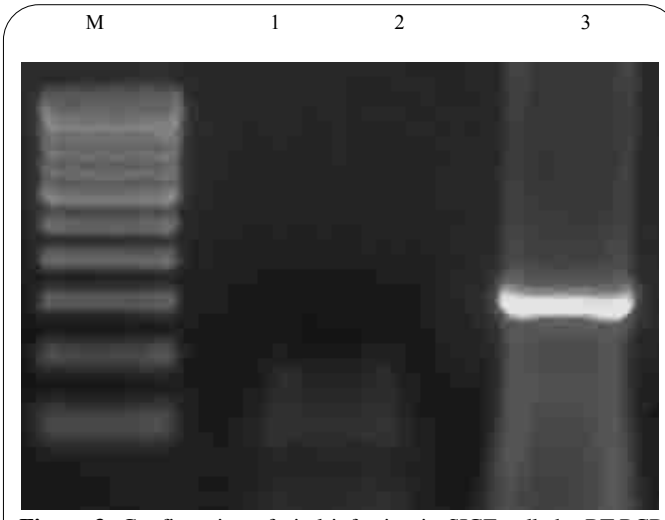

Figure 3: Confirmation of viral infection in SIGE cells by RT-PCR [M-100 bp marker. Lane 1, uninfected cells; Lane 2, uninfected cells; Lane 3, nodavirus Infected cells (294 bp).

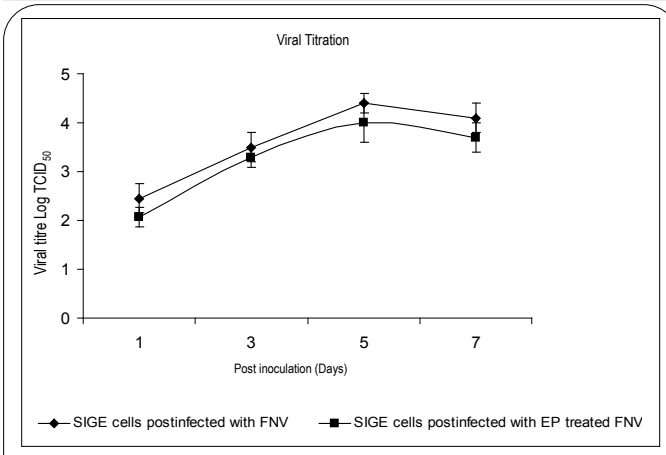

Figure 4: Viral titre for nodavirus at 1, 3, 5 and 7 days postinoculation in SIGE cell line.

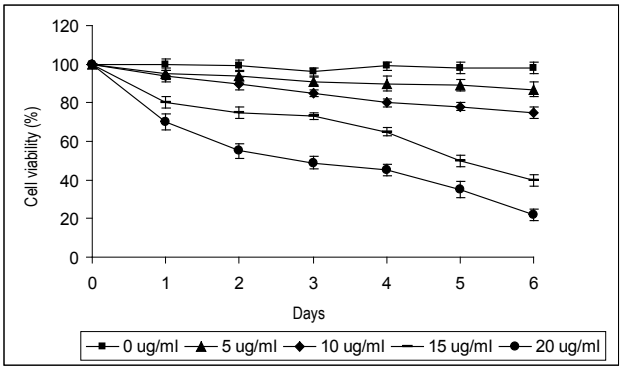

Figure 5: Antiviral activity of dasyscyphin C (5- 20 µg/ml) on the SIGE cell viability post infected with FNV.

was very effective in inhibiting the proliferation of FNV infected SIGE cells.

Dasyscyphin C (50 µg/ml) exhibits mild cytotoxic activity (7%) on Vero cells after 48 hrs of incubation. However, Dasyscyphin C inhibits the viability of HeLa cells under *in vitro* conditions and the extent of cytotoxic activity was 52% on the growth of He La cells after 48 hrs of incubation with the IC_{50} value of 56.6µg/ml (khanna and Kannabiran, 2009). The antimicrobial activity of Dasyscyphin C against *P.aeruginosa, E.coli, S. typhi, K. pneumoniae, P. mirablis, S.aureus* and fungal pathogens *A. fumigatus, A. niger* and *A. flavus* was reported (khanna and Kannabiran, 2008). The other study in our laboratory also observed the leishmanicidal activity on *Leishmania major* under in vitro conditions (khanna and Kannabiran, 2008). Dasyscyphin C also exhibits larvicidal activity against *Culex qinquifaciatus* mosquito larvae (unpublished data). However further studies are needed to establish its activity under *in vivo* conditions. A similar compound like dasyscyphin C was isolated from the fermentation broth of the ascomycete *Dasayscyphus niveus* was reported to be a potent cytototoxic to several human cell lines with the IC_{50} value of 0.5 to 3 µg/ml (Parra et al., 2006). The presence of α,β-unsaturated 1,2 dione moiety of dasyscyphin may be responsible for the observed activities.

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