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Research Article

ANTIVIRAL EFFECTS OF L-FUCOSE FROM *PADINA GYMNOSPORA* AGAINST VARIOUS STRAINS OF DENGUE VIRUS

R.Swaminathan[1], M. Syed Ali*[1], V. Anuradha[2], N. Yogananth[1], G.K.Saravanan[1]

[1]*PG and Research Department of Biotechnology, Mohamed Sathak College of arts and science, Sholiganallur, Chennai, Tamil Nadu, India*

[2]*PG and Research Department of Biochemistry, Mohamed Sathak College of arts and science, Sholiganallur, Chennai, Tamil Nadu, India*

**Corresponding author: syedmicro555@gmail.com*

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ABSTRACT

Now a days, dengue is a very serious communal and economic public health crisis which impose continuous search of natural bioactive compounds to manage its control. The main focus of the present study is to explore L-Fucose from marine algae as phytotherapeutics for dengue treatment. Dengue is a major mosquito-borne disease andtill date no efficient antiviral drug or vaccine is available on the market for control of it. The L-Fucose is reported to exhibit antiviral properties but its mechanism on inhibition of dengue virus DENV1-4 was not studied in detail. In this study, the nontoxic concentration of the drug to the normal cell was calculated and its potential antiviral activity against dengue virus DENV1-4 was evaluated using Vero cell line. Experiments were conducted *in vitro* to determine the potency of polysaccharide against dengue virus by propagating them in Vero cell lines. Viral titer was determined and cytotoxicity assay were performed by $CC_{50.}$ The antiviral potential of the medicine was detected by using MTT assay and Plaque Assay. The co-treatment technique used in antiviral assay with concentration 40µg/ml showed better antiviral activity.

Keywords: Dengue, Antiviral, L-Fucose, DEN-V, CC50.

1. INTRODUCTION

Dengue, one amongst the mosquito-borne viral infections affecting humans causing a dreadful flu-like illness and sometimes provoking a rigorous fatal obstacle termed as severe dengue. It is a rapidly mounting pandemic-vulnerable viral disease across several zones of the globe, including the Western Pacific Region [1]. Dengue affirms a significant socio-economic public health problem in the 21st century [2]. Dengue virus belongs to the family of Flaviviridae, *Flavivirus* genus and it carries four antigenically distinct serotypes, i.e., DENV-1, DENV-2, DENV-3 and DENV-4. This virus causes different kind of illness in humans i.e., dengue hemorrhagic fever (DHF), acute fevered dengue fever (DF) and dengue shock syndrome (DSS) etc. [3].

In humans, Dengue is transmitted by *Aedes aegypti* and *Aedes albopictus* mosquitoes [4]. The female mosquito carries the serotypes of DENV from a disease carrier of animal or human, consequently the viral strains upon infection causes viral multiplication in mosquito tissues and eventually the salivary gland. Besides that, the viral particles can also be transmitted to eggs by vertical transmission [5]. Mammalian receptor proteins generally mediate cell to cell communication and elicit biochemical events such as DC-SIGN [6]. Nevertheless, the task of this protein and its expression as result of viral transformation, initiation of signaling cascade and the use of these receptors by DENV to enter cultured cells remains unclear.

Marine mangroves consituting seaweeds, sea grasses and lichens have been extensively studied and reported over long period of time and recognized as producers of biologically active substances in India. Many synthetic antioxidants have been found to exert toxic and mutagenic side-effects in contrast to the naturally occurring antioxidants. Seaweeds have been recognized to have potent antioxidant property [7, 8]. Free radicals are reactive chemical species arises as independent molecules that contains one or more lone pair of electrons. These radicals comprise of oxy-radicals,

oxygen free radicals, hydroxy and peroxy radicals and exist alone and in different combination and these are collectively termed as the reactive oxygen species (ROS). Owing to their paramagnetic nature, these radicals initiate rapid and high chemical reactivity upon excitation. Thus, it can allow mobility of electrons from other atoms or molecules, leading to oxidation of reacting molecules and this is a nautal phenomenon exhibited by biological system as a consequence of inflammation and illness [9]. The range of bio active metabolites from seaweeds can be employed for managing the lethal illnesses like cancer, Acquired Immuno Deficiency Syndrome (AIDS), arthiritis etc., [10,11] while some metabolites have been widely applied to treat inflammation etc [12]. Brown macro-algae possess a broad range of acid polysaccharides like the alginic acids, uronic acid, the homo fucans (sulfated fucan) and the heterofucans [13]. In brown algae, these polymers occupies the intercellular tissues or mucilaginous matrix. The biochemical composition and structural constituents of algal fucans may differs with the algal species and also among different components of the seaweed [14]. Therefore, each new refined sulfated L-fucose is a sole compound, thus acting as a prospective novel drug. Previously, the anti-inflammatory potential of a fucoidan from the alga *Fucus* vesiculosus, sulfated fucans from the Fucales and Laminariales has been investigated [15,16]. Furthermore, these compounds are a powerful inhibitor of leukocyte migration during the inflammatory response, due to their interaction with glycoproteins P and L-selectin of the platelets [17,18]. The present investigation aims to evaluate the antiviral activity of L-fucose from the algae *Padina gymnospora* against anti - dengue activity tested on DENV.

2. MATERIAL AND METHODS

2.1. Algae collection and sample preparation

The algae, *Padina gymnospora* was collected from the coastal waters around Rameshwaram, Tamilnadu. The samples were cleaned with normal water, sun dried and again dried them at 60°C in a hot air oven. Then, the samples were crushed into fine powder form by using electronic blender and stored in a refrigerator at 4°C until further analysis.

2.2. Extraction and ısolation of L-Fucose from seaweed

The isolation and extraction of fucoidan from *Padina gymnospora*, brown macroalgae was evaluated by following the method of Yang et al., 2008 [19] with slight modifications. 8.0 g of algal powder was added to 100 mL of absolute alcohol (85% ethanol) and kept in rotary shaker for 12 hrs at room temperature. After 12hrs of reflux, the lipids and pigments from the sample was removed by centrifuging the mixture at 3273rpm for 10 minutes and the supernatent was transfered. The pellet was further washed with acetone and kept at ambient temperature overnight. For extraction, 5.0 g of the pellet was dissolved in 250mL of deionized water with occasional stirring and incubated at 65°C for 1 hour. Next, 1% $CaCl_2$ was added gently to the supernatant for the alginate to precipitate out of the solution and the solution was centrifuged again at same rpm for 10 min, and the successive supernatants were collected and subsequently mixed to 85% ethanol to obtain a final concentration of 30% (v/v). The resulting precipitate obtained was incubated overnight at 4°C in a refrigerator. Finally, the fucoidan was recovered from the pooled supernatants by centrifugation at 3273rpm for 10 min and stored at 4°C after being lyophilized. The commercial pure Fucoidan from *Padina gymnospora* (Sigma, USA) was used as a reference.

2.2.1. Purification of L-Fucose

A 200 mg of extracted fucoidan was mixed with 20 ml of distilled water and boiled at reflux for 3Hrs with 0.75 ml of 3.0 M HCl and cooled. Then the aliquote was centrifuged at 3000 rpm for 15mins and 1M NaOH was added to the supernatant for neutralisation and gently poured over 100ml of ethanol taken in a conical flask. The conical flask aid in the settling down of the precipitated fucoidan. The precipitate obtained was washed and dissolved in distilled water and freeze dried.

2.2.2. Identification of L-Fucose

For the analysis of L-fucose content in the *P. gymnospora* HPLC was used, the system comprising a pump and injection valve with a 20-µL sample loop, PL Hi-Plex H column and refractive index detector. 5mg of the sample was mixed with 2 ml of 2 M trifluoroacetic acid for sample injection and maintained at temperature of 121°C for 60 minutes. The reaction mixture was dried by using vacuum concentrator. Distilled water was added to re-dissolve the sample and pH of the sample was adjusted to 7. The sample at a concentration of 0.1 mg/ml was injected into the HPLC system. The column was maintained in a 65°C column oven (COLBOX), and double distilled water was used as the mobile phase with a flow rate of 0.5 mL/min. The pure

monosaccharide, L-fucose was used as standard. The post-run chromatographic data was analysed by the software Chromera of perkin-elmer system.

2.2.3. Preparation of medium

A 0.1 N sodium bicarbonate ($NaHCO_3$) was prepared with double distilled water and the pH was adjusted to 7.0. Then, the medium was sterilized by polyether-sulfone membrane filter (0.22μm pore size) under vacuum condition.

2.2.4. Cell lines and virus strains

Vero cell lines were purchased from NCCS (National Centre for Cell Science), Pune, India. The MEM medium was used for the proliferation of the serotypes DENV I-IV. The cell lines were subcultured and maintained in MEM medium fortified with 10% fetal calf serum (FCS) and incubated at 37°C. DENV I - IV stocks were seeded into vero cells. The seeded virus were added to 80%-100% confluent cells at a multiplicity of infection in MEM media to generate the stock. After one week, dengue virus causing a cytopathic effect (CPE) in the monolayers of vero cell culture was captured as evidence of infection and the cells were inturn harvested from media and seggregated by centrifugation at 14,000 rpm for 30 min. 20% FCS was added to the supernatant and stored at -80°C.

2.2.5. Cytotoxicity assay

The vero cells were loaded at a concentration of 1 lakh cells/well onto a 96-well microtiter plates and incubated at 36°C in 5% CO_2 incubator for 48 hour. The growth medium was decanted and medium containing fucoidan (100-1000μg) was added in tetrads to test the efficacy of fucoidan. The extract free medium was used as control. The plates were again incubated in 5% CO_2 environment at 37°C and subsequently 3rd, 5th and 7th day absorbance were noted microscopically for cytotoxicity. Microscopic reading was confirmed by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay.

2.2.6. Estimation of $TCID_{50}$ of dengue virus pool

The set of nine sterile test tubes was taken and about 1.8 ml of MEM without FCS was added to each of nine test tubes labelled as 10^{-1}-10^{-9}. 200 μl of the dengue virus pool was added slowly to the first dilution using sterile pipette and mixed thoroughly. Next, 200 μl of sample from first tube was added in to the next series of tube and mixed. The dilution continued sequentially till the sample reachedthe tube for 10^{-9} dilution. 100 μl from each dilution was added in a row of 10 wells from A to H labelled from -1 to -8. 5 wells each of 11th and 12th rows were used as cell control, to which 100μl of MEM without FCS was added. 100 μl of vero cell suspension (10^5cells/ml) was poured on to all the wells and the plates were incubated at 37°C in an incubator maintaining 5% CO_2 environment. The plates were observed on day 3, 5 and 7. The CPE was recorded and $TCID_{50}$ was estimated by Karber's method. The proportion of wells with CPE in each serially diluted DEN I-IV was calculated and the $TCID_{50}$ was estimated using the formula 'Log $TCID_{50}$ = L - d (s - 0.5)', whereby L = lowest dilution factor; d = difference between dilution steps; s = sum of proportion. The value of $TCID_{50}$ determined was applied in the antiviral assay.

2.3. Antiviral assay

The antiviral assay was analysed in tetrads. The varying concentrations of algal extracts were prepared in MEM without FCS and filtered. Simultaneously, 1, 10, and 100 $TCID_{50}$ of dengue 1-4 virus pool was prepared in MEM without FCS based on the initial $TCID_{50}$ of the virus pool. The antiviral assay was performed with algal extracts by direct pre-infection incubation assay or viral inactivation assay [20]. The test was repeated thrice at different periods for reproducibility, before use of the extracts in the drug assay. The control samples of cell, virus and algal extract were also simultaneously incubated and observed for antiviral activity.

2.4. Statistical analysis

Data was analysed and interpreted as mean of triplicate and standard deviations. All statistical analysis were performed by Student's *t*-test and *P* value less than 0.05 was considered to be statistically significant.

3. RESULTS AND DISCUSSION

3.1. Estimation of Antiviral activity of poly-saccharide against dengue virus (DENV)

The vero cells cultured in the tissue culture flasks containing MEM fortified with 10% FCS formed a monolayer sheet of cells. The morphology of the uninfected cells, the DENV- infected vero cells and the various CPEc cells were examined under inverted light microscope at 40× magnifications. Upon incubation, the vero cells uninfected with viral strain showed well-

defined polygonal shape and black nuclei in the centre when visualised under 40× magnification. From this result, it was observed that the cells infected with dengue exhibited CPE and the morphological alterations of the infected vero cells were the evidents of viral infection. The maximum inhibition (75%) of L-Fucose extract of *Padina gymnospora* was registered aganist the tested dengue strain. The L-Fucose extracted from of *P. gymnospora* has the ability to maintain the integrity of majority of the normal uninfected vero cell structures without increasing CPE. The monolayer sheet of cells still observed to be normal showing decreased cell death and less percentage of CPE. The effectiveness of the L-Fucose extract of *P. gymnospora* in preventing the dengue viral replication was further confirmed by MTT experiment, which showed that viability of cells not significantly affected upon infection with dengue. These findings, based on CPE and MTT eperiments revealed that the L-Fucose had the potential to decrease cell death in vero cells without decreasing viral replication. Thus, in a preliminary study for identifying an anti-dengue active molecule, L-Fucose from *P. gymnospora* was discovered to have the therapeutic potential as dengue medication, with a total inhibition of 75%. (Fig.1a & b).

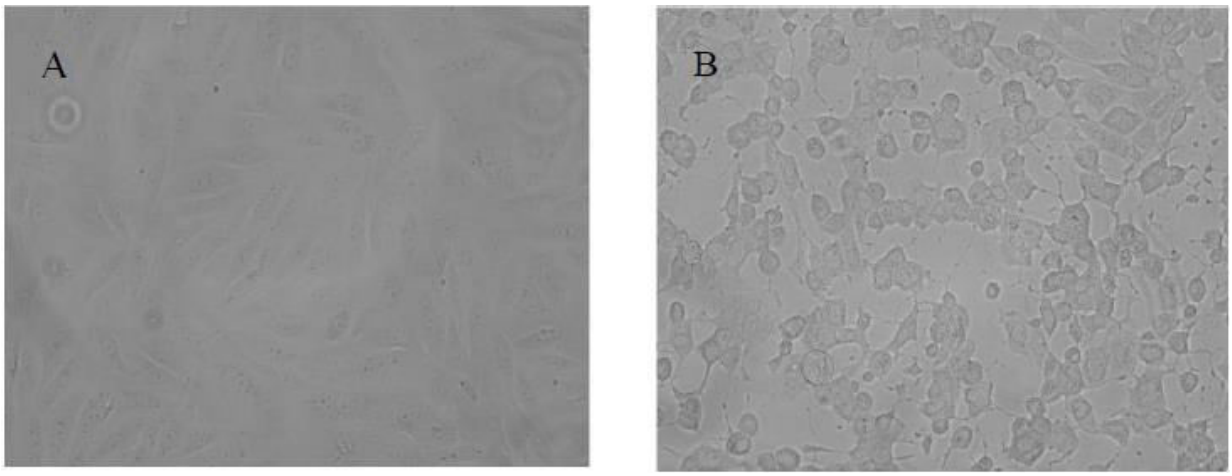


Fig. 1: Represents the A) Cell Control and B) Cytopathic Effect Caused By DEN in Vero Cell

3.2. Confirmation of Virus

The dengue viral infection was confirmed by performing haemagglutination test. The inoculated flasks which produced CPE were examined with goose RBCs at various pH with varying dilution. Agglutination reaction was observed in well-marked as 1:8 dilutions at pH of 6.4. The titre of the virus present in the inoculated flask was confirmed as 1:8. Further the virus is used for $TCID_{50}$ to detect the concentration of virus.

3.2.1. $TCID_{50}$ Assay

The anti-dengue property of L-Fucose from *Padina gymnospora* was conducted by *invitro* antiviral assay. The antiviral activity was initiated using fucoidan against $TCID_{50}$. The grading system was developed as given by Kudi and Myint (1999) [21], to determine the qualitative analysis of antiviral assay. $TCID_{50}$ was performed using 8 different dilutions of virus for the identification of median infective dose at a level of *invitro* cell culture. At 10^{-2} dilution of DENV, half of the cell cultures showed CPE. This dilution can be defined as the end point where 50% of cell culture replicates show cytopathic effect. The table represents the 96-well tissue culture plate where presence or absence of CPE is illustrated. Plaque assay was performed using $TCID_{50}$ value obtained from the previous experiment. We arrived at 10^{-2} dilution of DENV, and 180 plaque forming units per ml PFU/ml) were observed (Table 1).

3.2.2. Cytopathic Assay

The studies on cytotoxicity of Polysaccharide at the concentration of 25-150 µg/mL in Vero cell line showed that the drug treated cell lines developed confluence up to 150 µg/mL concentrations at 48 h as comparable to that of untreated controls. However, we observed cytotoxic features of polysaccharidein Vero cells when treated at higher concentrations (>75 µg/ml) as visualised by microscopic studies and determined by MTT assay (Fig.2). The cytotoxic effects

were observed to be concentration dependent and the CC_{50} values for polysaccharidewas found to be about 75µg/mL as concentrations above the CC_{50} values led to a reduction of more than 50% cell viability. The cytotoxic features such as changes in morphology of cells, necrosis, disruption or loss of monolayer, loss of cell adherence, granulation of inclusion bodies and vacuolization in the cytoplasm, and cell damage were observed in cells treated at higher concentrations against untreated controls as well as cells treated at concentrations lower than the CC_{50} values.

Table 1: $TCID_{50}$ Value for the DEN Virus

Conc.	Well	1	2	3	4	5	6	7	8	9	10	11 (cc)	12 (cc)	Sum of proportions
10^{-1}	A	+	+	-	+	+	-	+	-	+	+	-	-	7/10=0.7
10^{-2}	B	+	-	+	+	-	-	-	+	+	-	-	-	5/10=0.5
10^{-3}	C	+	-	-	+	-	+	+	-	-	-	-	-	4/10=0.4
10^{-4}	D	+	-	+	-	-	-	+	-	-	-	-	-	3/10=0.3
10^{-5}	E	-	+	-	-	-	-	-	+	-	-	-	-	2/10=0.2
10^{-6}	F	-	-	-	+	-	-	-	-	-	-	-	-	1/10=0.1
10^{-7}	G	-	-	-	-	-	-	-	-	-	-	-	-	0/10=0
10^{-8}	H	-	-	-	-	-	-	-	-	-	-	-	-	0/10=0

(+ CPE Present, - CPE Absent, CC - Cell control)

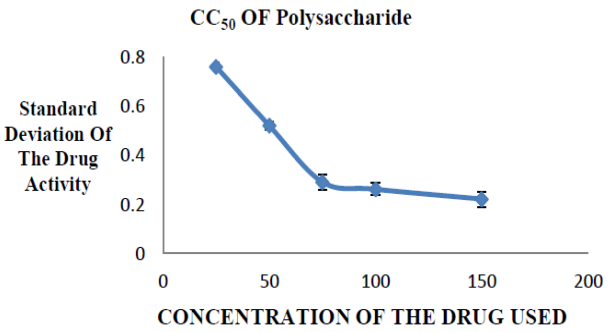
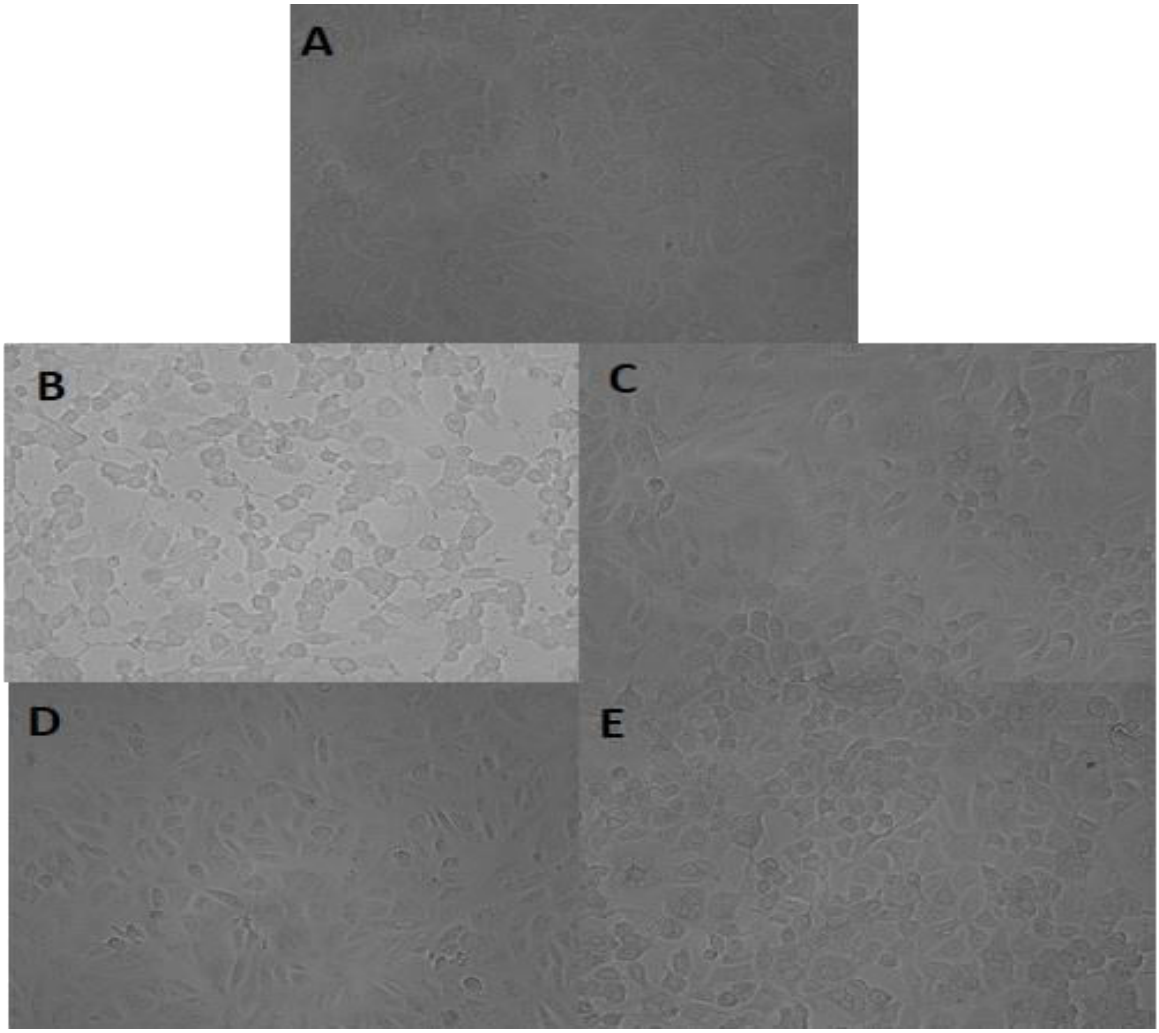


Fig. 2: CC_{50} concentration of Polysaccharide

3.3. Antiviral activity against DEN Virus

The antiviral activity of the drug was performed with 10^{-2} concentration of the DENV (1-4) virus pool. Viral titer of the infected vero cell line was reduced up to 82% by treatment with polysaccharide compared to the control samples in pre-treatment assay. Decrease in dengue viral titre in post-treatment was observed up to 78%.The antiviral activity of the polysaccharide was observed to be better when they were treated as co-infection on to the cells. The viral infection on vero cells were reduced up to 66.8%. It indicates that polysaccharide is comparatively more active against dengue, which explores its medicinal importance (Fig.3). A great variety of marine algae species have been described in Indian medicine and have been used to cure infectious disorders for ages. *Padina gymnospora*, a brown algae, is one of the treatments for dengue fever. Fucoidan from *Padina gymnospora* was tested for antiviral activity against dengue 1-4 viruses, despite the fact that it is said to be effective in the treatment of infectious disorders. Fucoidans from various brown algae have been shown to possess antiviral action against DNA-containing [22] and RNA-containing [23] viruses, making the fucoidans interesting drug molecules for the development of fucoidan-based medicinal products. Meanwhile, standardising and optimization of extraction of fucoidan is time-consuming [24] mainly due to the structural diversity of fucoidans found across the species [25] and also because of analytical approaches. Therefore, the difficult task is the identification of the structurally bioactive fragments by comparing the chemical composition of fucoidans amongst various sources responsible for their biological activity as well as the validation of standardized fucoidan-based drug products with a more pronounced antiviral effect. Our study aimed to determine the effect of the native fucoidan and its regular derivative at the different stages of the viral life cycle, including all the four serotype of significant human pathogens DENV1, DENV2, DENV3 and DENV4. The results showed the potential of fucoidans to increase the resistance towards viral infection (preventive effect) by either directly affecting the viral multiplication (virucidal effect), or inhibit the early stage of virus replication (virus-inhibiting effect). The multifaceted mechanisms of action of tested fucoidans are similar to the mode of action of other sulfated polysaccharides observed from different algae [25]. The natural substance that is going to be promoted as therapeutic agent needs to be effective and safe. Selectivity index is a ratio between the cytotoxic and the

inhibitory capacity of a substance (CC_{50}/IC_{50}). The determination of selectivity index may thus help researchers to screen a substance that is appropriate for further development and to measure the effectiveness and safety of a product. Hence, in this study, virus titer was detected up to 48hours in fetal calf serum that was inoculated with DEN V 1-4 cells.



The antiviral activity of Polysaccharide seemed to be better in co-treatment when compared with the neat virus. The drug control observed doesn't show any cytotoxicity when compared with the cell control.A) Pre Treatment At 30µg/ml; B) Post treatment at 30µg/ml; C) Co Treatment at 30µg/ml; D) Drug Control; E) DENV virus (10^{-2})

Fig. 3: Antiviral activity of polysaccharide

4. CONCLUSION

Even though bio prospecting has recently been replaced by more targeted tools for identifying lead compounds with pharmacological benefits, our results showed it is still useful for resolving new drugs to combat dengue. Additionally, combining *in vitro* evaluations allowed us to identify promising antivirals as well as their possible mechanism of action.In near future, a thorough understanding on experimental problems concerning the extraction, quality standards, and optimising the bioactivity of fucoidan and studies related to exlore the possible mechanism at molecular level will be carried out to fully utilize the therapeutic nature of marine fucoidans.

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Conflict of interest

None declared

Source of funding

None declared

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