



Anti-Dengue Activity of ZnO Nanoparticles of Crude Fucoidan from Brown Seaweed *S.marginatum*

Ramalingam Kothai¹ · Balasubramanian Arul¹ · Venkattappan Anbazhagan²

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Abstract

Dengue fever is a rapidly spreading infection that affects people all over the tropics and subtropics, posing a significant public health threat. The brown seaweed *Stoechospermum marginatum* was found all over the world, from South Africa (Indian Ocean) to Australia (Pacific Ocean), among other places. In India, it is only available along the coast of the Bay of Bengal, which is a small region. Various metal oxides were proved to be successful in the formation of nanoparticles and zinc is one among them. In this present study, an attempt was made to study the anti-dengue activity of green synthesized zinc oxide nanoparticles of crude fucoidan isolated from brown seaweed *S. marginatum*. The fucoidan was isolated from the seaweed by acid extraction method and then characterized by UV, HPLC, and Fourier Transform Infra-Red (FT-IR) Spectroscopy. Then it was biosynthesized into ZnO nanoparticles and characterized by SEM-EDAX analysis. The results showed the formation of fucoidans and SEM studies showed the crystalline nature of the synthesized nanoparticles. The size of nanoparticles was in the range of 80–126 nm. The synthesized nanoparticles were tested with the C6/36 cell line and it was shown 99.09% of anti-dengue activity against the tested cell line. As an antiviral agent, the ZnO nanoparticles of fucoidans have been shown to be an excellent lead molecule for the treatment of dengue fever.

Keywords Zinc oxide nanoparticles · Seaweeds · Fucoidan · Anti-dengue

Introduction

Green nanoparticles are being synthesized using plant extracts, which is an exciting new development in the field of nanotechnology. When compared to chemical and physical methods of synthesis, the biosynthesis of green nanoparticles using plant extracts has both

✉ Balasubramanian Arul
arul1971@yahoo.com

¹ Department of Pharmacology, Vinayaka Mission's College of Pharmacy, Vinayaka Mission's Research Foundation (Deemed to be University), Tamilnadu 636008 Salem, India

² Department of Chemistry, Vinayaka Mission's Kirupananda Variyar Arts and Science College, Vinayaka Missions Research Foundation (Deemed to be University), Tamilnadu 636308 Salem, India

economic and environmental advantages, according to the researchers [1]. Nanoparticles (NPs) are typically less than 100 nm in size, and it has been hypothesized that their biocidal efficacy is due to a combination of their small size and high surface-to-volume ratio, which allows them to form intimate interactions with bacterial membranes [2–5]. The greater stability of inorganic antibacterial agents such as metals and metal oxides over organic antibacterial agents gives them a distinct advantage over organic antibacterial agents [6, 7]. Zinc oxide (ZnO), one of these metal oxides, has gotten a lot of attention because it acts as an antibacterial agent.

There are at least 72 viruses in the Flaviviridae family, including Japanese encephalitis, Yellow fever, Zika, and West Nile virus. Dengue is the most well-known mosquito-borne viral infection. Dengue virus is an enveloped, single-stranded, positive-sense RNA virus that is spread by the *Aedes aegypti* and *Aedes albopictus* mosquitoes. Dengue viral infections are one of the most important mosquito borne diseases in the world [8, 9]. It is found in 4 different serotypes in different regions of the world. Dengue fever has been extremely dangerous from its inception, but its prevalence has increased significantly in recent decades. Despite the fact that there is no particular surveillance, the WHO estimates that the dengue virus is present in more than 100 countries, including the United States [10]. According to WHO, there was a six fold increase in the number of cases reported (from 0.5 million in 2010 to over 3.34 million in 2016) and also there was a sharp increase in 2019 [11]. At this time, there is no appropriate treatment available for dengue fever. The use of medicinal plants has been around since the dawn of civilization, and they have been used to treat a wide range of illnesses, from the common to the life-threatening. When used in conjunction with conventional antiviral medications, herbal medications can be used to treat or increase the therapeutic effect of the drugs [12]. Deliberate use of many herbal remedies against dengue fever infection is becoming more common as an alternative approach to alleviating disease symptoms [13].

Brown seaweeds belonging to the Dictyocoeae family are a rich source of a heteropolymer of sulfated L-fucose, known as sulfated polysaccharides. It was called as fucoidans because they were the first sulfated polysaccharides isolated from the Fucales of the Phaeophyceae, which are algae-derived polysaccharides. Fucoidans are found embedded in the intercellular thallus tissues. These, sulfated polysaccharides, also known as fucans, have been shown to be the source of potent bioactive principles that have shown promise in the treatment of viral infections, ulcer, and adhesion problems and also they possess anticoagulant, anti-inflammatory, anti-proliferative, and anti-tumor properties [14].

It is revealed in the report that fucoidans have an inhibitory effect on vascular smooth muscle cells and that it inhibits the proliferation and binding of sperm-ova binding in various species [15]. Different seaweed species produce polysaccharides that are structurally distinct from one another, and the composition of these polysaccharides varies with different parts of the thallus. Fucoidans, which are polysaccharides found in the cell wall surface of the brown algae, are a valuable source of nutrition [16]. It was discovered that fucoidans (sulfated polysaccharides composed of L-fucose and sulfuric acid esters) have numerous and diverse pharmaceutical and biomedical applications [17] and can be used in a variety of therapeutic and diagnostic procedures [18]. This is due to the presence of the sulfate moiety in its chemical structures, which makes it a powerful antimicrobial agent [19].

The brown seaweed *S.marginatum* was distributed from South Africa (Indian Ocean) to Australia (Pacific Ocean). In India, it is only available along the coast of the Bay of Bengal, which is a small region. *S.marginatum* has a thick and dichotomously branched thallus with a flat surface, but it does not have a midrib. The margin was complete, the apex was bifid, or flatly truncated. The apex of the branches has a notch in it. It is a species of forking

plant that can grow to be 40 cm in length and 8–11 mm breadth. Fertile plants are easily distinguished from nonfertile plants by the dark lines that run along the margins of densely packed sporangia. *S. marginatum* has a thallus that is flat and isolateral in shape. It contains a sporangium with four tetraspores, which is unique to this species. Each sporangium has many elongated, cylindrical spores, which are arranged in a spiral pattern. The spores have echinate surfaces and stain darkly when exposed to light.

The epidermal layers of *S. marginatum* are cutinized and distinct. The epidermal cell is tiny, square-shaped, with thick walls and a dark stain on its surface. Between the epidermal layers, there were approximately 8 to 9 layers of relatively homogenous compact parenchyma cells present. Between the epidermal layer and the dermal layer, there seem to be approximately five layers of huge laterally elongated cells with move up and down walls arranged in a layering pattern. The stalk's marginal part is thin, thick, and rounded, with a slight incline [20].

High sulfate content in the various species demonstrates significant antiviral and antimicrobial properties, indicating that they are beneficial [21]. So the isolation of novel fucoidans from brown seaweed will serve as potent biomolecules with diverse pharmacological activities. Fucoidan extraction, isolation, and characterization from seaweeds are still in the early stages in India, despite the large number of seaweeds that occur in the 7200 km of Indian coastline that can be harvested. When it comes to being a source of fucoidan, Indian brown macroalgae are not subjected to a comprehensive examination. The Gulf of Mannar, located on India's southeast coast, has been designated as a biosphere reserve, and the seaweeds that grow abundantly in the Gulf region's 21 islands are being harvested for commercial purposes [20].

To explore the anti-dengue properties of green synthesized ZnO nanoparticles of crude fucoidan isolated from brown seaweed *S. marginatum*, as well as their characterization, the current study is being conducted.

Materials and Methods

Materials

Cysteine hydrochloride, sorbitol, phenol, HCl, H₂SO₄, potassium bromide, and barium chloride were of analytical grade and procured from LobaChemi India. HPLC grade methanol and water were purchased from Rankem, India and precoated TLC Plates for chromatographic separation were obtained from Anchrom Enterprises India Private Limited from Mumbai, Maharashtra, India. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), phosphate buffer saline (PBS), minimum essential medium (MEM), and trypsin, were obtained from Hi-Media Laboratories in Mumbai, India. The foetal calf serum (FCS) was obtained from Gibco Laboratories (NV, USA). Geno-Dengue Sen's S1-S4 PCR kit, contained commercially available chemicals such as buffer, enzymes, dNTPs, a dengue-specific primer and probes was used in this study.

Collection of Seaweed *S. marginatum*

Thorough washing in seawater followed by thorough washing in tap water was performed to remove epiphytes and other superfluous matters from freshly harvested, matured, free of disease, and robust brown algae *S. marginatum* (C.Ag.) Kuetz. collected along the coastline

of Tuticorin, South India during July 2020. For future reference, the voucher specimens (SMA-1) of *S.marginatum* were kept in our museum.

Extraction of Crude Fucoidan

100 g of fresh *S.marginatum* was soaked in 100 mL of 0.1 N HCl at 95°C for 12 h before being used. The extraction procedure was carried out thrice and the extracts were combined and filtered using Whatman No. 1 filter paper to remove any impurities and contaminants. The filtrate was dialyzed against water and then lyophilized to remove any remaining water. The determination of true fucoidans was carried out using the gravimetric method, and the findings have been represented as a percentage of the algae weight [22].

Characterization of Fucoidans

The biochemical composition of crude fucoidan was derived by analyzing the total carbohydrates, L-fucose, and sulfate contents.

Estimation of Total Carbohydrate

Using the methods described by Dubois and Rezzonico, the total carbohydrates present in a sample of water were estimated [23]. To obtain a homogenate solution, 100 mg dried crude fucoidan was ground into a fine powder with 10 mL of distilled water in a mortar and pestle for 30 min with intermittent grinding. An aliquot of 200 µL of the sample was placed in a test tube, 200 µL of 5% phenol, and 1 mL of the con. H₂SO₄ was added to the sample. The addition of concentrated H₂SO₄ in a short span of time aided in the mixing and heat development required for the test. In this experiment, the samples were left to stand at room temperature before being measured for absorbance at 485 nm, the results were recorded in comparison to a blank. Different concentrations of D-galactose were used to prepare a standard graph, and the amount of total carbohydrates could be calculated from the graph.

Estimation of L-fucose

Using the method developed by Dishe and Shettles, L-fucose concentrations can be estimated with high accuracy [24]. 1 mL of the sample solution was poured into the test tubes, which were then placed in an ice-water bath to cool until they were completely chilled. Next, the tube was filled with 4.5 mL of H₂SO₄ reagent, which was then thoroughly mixed together. The tubes were brought to room temperature by placed in a water bath (25°C) for 3–4 min and then placed in hot boiling water for 3 min. Each tube was treated with 5% cysteine hydrochloride solution and mixed after the tubes were allowed to cool under running tap water for a short period. At 396 and 427 nm, the absorbance was measured in comparison to a blank prepared with reagents without a sample [25]. To calculate the absorbance values, the following equation was used:

$$\text{Absorbance} = (A_{396} - A_{427})$$

Whereas A₃₉₆ = Absorbance at 396 nm.

A₄₂₇ = Absorbance at 427 nm.

Estimation of Sulfate by the Turbidometric Method

According to Czerwinska and colleagues, the accuracy of the estimated sulfate contents was confirmed through comparison with reference compounds [26]. *S. marginatum* fucoidan extract (10 mg) was homogenized with 6 mL of 1 N HCl and transferred to a hydrolyzing tube with proper sealing. Boiling the sample in a water bath for 4 h results in hydrolysis of the sample. After that, the sample was allowed to cool down to room temperature. A Whatman filter paper No. 1 was used to filter the solution, and the volume was increased to 25 mL by adding double distilled water to the filter paper after it had been filtered. 1 mL of the filtrate solution was mixed with 1 mL of 6 M HCl and 5 mL of 70% sorbitol. It was stirred with a magnetic stirrer and 1 g of barium chloride crystals was added while stirring and the absorbance at 470 nm was measured immediately.

Chromatographic Techniques

TLC Analysis

On 10×20 cm silica gel-G plates (Merck, Germany), a Thin Layer Chromatography experiment was carried out. It was determined that *S. marginatum* contains polysaccharides that were qualitatively evaluated and identified. A 9:1 mixture of methanol and water was used to separate the polysaccharides, and when Barfode's reagent was sprayed on developed plates, the appearance of a red-colored spot confirmed the presence of polysaccharides.

HPLC Analysis

After being centrifuged at 3000 rpm for 10 min, the extracts of *S. marginatum* were filtered through Whatman filter paper No. 1 with the help of a high-pressure vacuum pump [27]. Using a Shimadzu LC-10AT-VP HPLC system equipped with an LC-10AT pump, SPD-10AT UV-Vis detector, Rheodyne injector with a 20 µL injection loop, and manual injector, the separation of monosaccharides was accomplished. The column used was a Hypersil ODS C-18 column (4.6×250 mm, 5 micron size) with a C-18 guard column. In order to achieve the best elution results, gradient solvent systems with a flow rate of 1 mL/min were used at room temperature (25–28 °C). In this experiment, the mobile phase was 0.1% v/v of methanol (solvent A) and water (solvent B). During the experiment, the mobile phase was freshly prepared every day, passed through a 0.45 µm filter, and sonicated prior to use. The entire performance lasted 15 min. The sample injection volume was 20 µL, and the detector wavelength was UV-254 nm, which was used in this experiment. A Whatman filter paper No. 1 was used to filter the sample, and it was then preserved. 2 mol/L of trifluoroacetic acid was used to hydrolyze the fucoidans and convert them into their respective monosaccharides. The monosaccharide was isolated by reverse-phase HPLC using a gradient elution technique, and the concentration was monitored using ultraviolet detection at 254 nm [28]. Table 1 depicts the chromatographic conditions that existed at the time of the experiment.

Table 1 Chromatographic conditions for the separation of fucoidans from *s.marginatum*

Chromatographic Parameters	Specifications
Instrument	Shimadzu LC-10AT VP HPLC system
Flow rate	1 mL min ⁻¹
Column	Hypersil ODS C-18 column with a C-18 guard column
Mobile phase	0.1% v/v methanol (solvent A) and water (solvent B)
Injection volume	20 μL
Elution	Gradient Elution
Detector	SPD-10AT UV-Vis detector
Detector wavelength	254 nm
Retention time	4.430 min

Fourier Transform Infrared Spectrometry (FTIR) Analysis

The polysaccharide samples (10 mg) were mixed with 90 mg of potassium bromide (KBr) powder of spectroscopic grade and ground before being compressed into 1 mm pellets for FT-IR analysis (Perkin Elmer USA) recorded in the frequency range of 400 to 4000 cm⁻¹ [29].

Synthesis of Zinc Oxide Nanoparticles of Crude Fucoidan

Zinc nitrate hexahydrate (Zn(NO₃)₂·6H₂O) solution 0.1 M was prepared by dissolving 6.58 g zinc nitrate hexahydrate in 300 mL of double-distilled water. To obtain complex formation, 10 mL of the fucoidan solution were added slowly drop by drop while magnetic stirring at 60° C for approximately 2 h. After stirring, the complex was collected and centrifuged at 10,000 rpm for 10 min. The collected pellets were dried for 8 h at 80°C and then stored in airtight glass bottles for further investigation.

Green synthesis of ZnO nanoparticles was carried out using crude fucoidan isolated from *S.marginatum* and zinc nitrate hexahydrate as a precursor. The addition of a dark brown crude fucoidan solution to a colourless zinc nitrate hexahydrate solution resulted in the formation of a yellowish-white precipitate, which indicated the formation of zinc oxide nanoparticles. When greenish *Andrographis paniculata* leaf extract was added to the zinc nitrate precursor, it resulted a similar colour change was observed by Rajakumar et al. [30]. Additionally, Bhuyan et al., reported the formation of a whitish precipitate [31]. However, Umar et al. discovered that when *Albizia lebbek* leaf extract was added to the precursor zinc acetate, the colour changed from brown to dark brown [32].

Characterization of Zinc Oxide Nanoparticles

UV- Visible Spectrophotometry Analysis

The preliminary investigation includes the use of ultraviolet spectroscopy (Model: Shimadzu UV 1601), which confirms the formation of ZnO nanoparticles in the first place. Standard procedures were used to evaluate the compounds in ultraviolet light (UV) in the range of 300-800 nm.

SEM with EDX Analysis

A sample of ZnO nanoparticles was prepared on carbon-coated tape by placing a small quantity of dried sample on a grid and allowing the film to dry under a mercury lamp for 5 min, followed by a second drying step. The morphology of the biosynthesized ZnO nanoparticles was determined using scanning electron microscopy (SEM). The TESCAN VEGA3 SBH was used to carry out SEM and EDX analysis on the samples under investigation.

Antidengue assays

C6/36 Cells Culture

It was necessary to maintain the Aedes cell line (ATCC® CRL-1660 TM) C6/36 in a minimal essential medium (MEM) containing 10% foetal calf serum (FCS), 2 mL of glutamine, penicillin (100 U/mL), and streptomycin (100 µg/ml). A humidified environment containing 5% CO₂ was used to incubate the cells in culture at 28° C [33, 34]. The medium was changed 14 days once.

Preparation of Stock Solution of Fucoidan ZnO Nanoparticles for Cell Viability and Antiviral Assays

Approximately 3000 µg of crude fucoidan ZnO nanoparticles were weighed and dissolved in 1 mL MEM based on their solubility. The pH of the medium was kept at 7.0 throughout the experiment. In the 96 well plates, the solution was diluted to various serial dilution concentrations (1500 µg/mL to 23.44 µg/mL) and then tested. In order to remove impurities from the extract, it was filtered through a 0.22 µmsyringe filter (MILLEX® GV). Extracted stocks were stored at a temperature of -20°C for future use.

In-vitro Anti-Dengue Assay

Antiviral testing was performed in a laboratory setting using monolayers of the C6/36 cell line which were cultured in a 96-well plate. It consists of a dengue-2 virus (TR-1751) control that contained 10, 100, and 1000 viral copies/mL in duplicate copies as a positive control, and just the cells alone (negative control). The experiment was done by mixing 100 copies of the virus suspension, with the results being recorded. Replicated plates of ZnO nanoparticles were used to pretreat virus suspension for 55 min with gentle shaking every 10 min. After being pretreated, the virus was redistributed to individual wells of C6/36 cell lines and incubated for 55 min, with gentle shaking every 10 min, until the virus was completely destroyed. After the inoculums had adsorption, the medium was sucked out of each well and flushed off.

In the following step, 100 µL of virus growth medium was added to the cell layer without disturbing the layer. A CO₂ incubator was used to maintain a constant temperature of 28° C for 7 days without the cells disturbing the culture plates. Following the completion of the incubation period, the plate was frozen at -70° C, and the lysates were collected and

stocked into a 2 mL glass vial for further investigation. Each vial was then subjected to RNA extraction procedure [35, 36]. Furthermore, the antiviral activity of the plant extract was performed by using real-time RT-PCR against the dengue-2 serotype cells with positive control in a subsequent study.

RNA Extraction

Qiagen's QIAmp Viral RNA kit (Qiagen, Germany) was used to isolate viral RNA from a total of 140 μL of culture supernatant, which was then processed as per the manufacturer's instructions. The final elution was performed in 50 μL of buffer before being stored at -80°C until used in the anti-viral testing.

Quantitative Anti-Viral Assay

To determine the inhibitory effect of ZnO nanoparticles of crude fucoidan on the DENV-2 serotype, real-time RT-PCR was used. The experiment was carried out with the aid of a commercially available quantitative kit (Geno-Dengue Sen's 1–4 PCR kit). Extracted samples were also treated in the same way as quantitation standards for dengue 1–4 serotypes (10^1 – 10^5 copies/ μL), and a similar volume (15 μL) was used in each case as well. The kit was used to specifically amplify and quantify dengue viruses. Each lysate had previously been subjected to RNA extraction (sample). It was carried out in a real-time PCR instrument, the ABI 7500, for this experiment. All of the PCR reagents were thawed and thoroughly mixed before the experiment began. The master mix was created in accordance with the instructions provided by the kit manufacturer. Following that, the required number of PCR tubes were prepared by adding 10 μL of master mix and 15 μL of extracted RNA to each lysate tube, along with 15 μL of the standards (Dengue 1–4, S 1–5) as a positive control and 15 μL of water (PCR grade) as a negative control. Following that, all of the reagents in the PCR tubes were thoroughly mixed and transferred into the ABI 7500 in near real-time. Conditions of thermocycler amplification include reverse transcription at 50°C for 15 min, denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 30 s and a final extension step at 72°C for 15 s. While the annealing process was taking place, the fluorescence emission data was collected. The standard curve was used for quantitation in subsequent runs, provided that at least one/two standards are used in the current run with back titration.

Data Analysis

Tukey's test was used to determine the percentages of cell viability of the ZnO nanoparticles of crude fucoidan. The data were analyzed using Microsoft Excel 2007 and a total of three samples were used in the analysis. The results were expressed as the mean of $\pm\text{SD}$ of all wells, and the cell viability of the plant and the test compound were calculated using the following formula;

$$\text{Cell viability(\%)} = \frac{\text{Absorbance of treated cell} - \text{Absorbance of blank}}{\text{Absorbance of cells control} - \text{Absorbance of blank}} \times 100$$

Results

The present investigation was carried out on the Zinc oxide nanoparticles of crude fucoidan isolated from the brown seaweed *S.marginatum* in order to determine its potential *in-vitro* anti-dengue activity. In this present study, fucoidans were extracted from *S.marginatum* using an acid extraction process, and the yield was calculated in terms of its weight% (%w/w). The acid extraction of fucoidan at 65 °C for 3 h resulted in a yield of $3.81 \pm 0.73\%$ after the extraction process.

Thin Layer Chromatography Analysis

It was determined that *S. marginatum* contains polysaccharides that were qualitatively evaluated and identified. Upon spraying the TLC plates with Barfode's reagent, the plates developed the appearance of red-colored spots, which confirmed the presence of polysaccharides in the solution.

RP-HPLC Analysis

The optimal wavelength for the qualitative HPLC fingerprint profile of *S. marginatum* was selected at 254 nm due to the sharpness of the peaks and the proper baseline of the profile (Fig. 1). The *S. marginatum* was subjected to HPLC in order to isolate and identify the constituents present in the alcoholic extract prepared by acid extraction. This is confirmed by the presence of a sharp peak with a retention time (Rt) of 4.430 min, which indicates the presence of sulfated polysaccharides.

FT-IR Spectroscopy Analysis of Fucoidan Fractions

When fucoidans were extracted from *S.marginatum*, the FT-IR spectra revealed the characteristic absorption bands of fucoidans (Fig. 2). The major functional groups of fucoidan were the hydroxyl group, sulphate group, and methyl group (Fig. 3). The

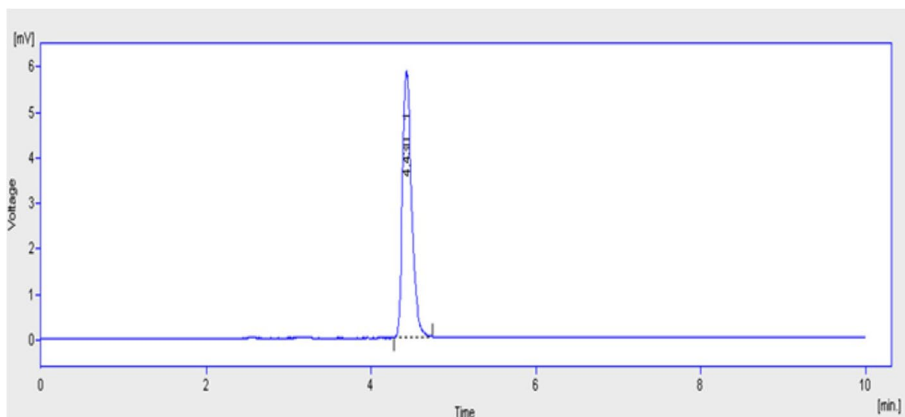


Fig. 1 RP-HPLC Chromatogram of *S.marginatum*

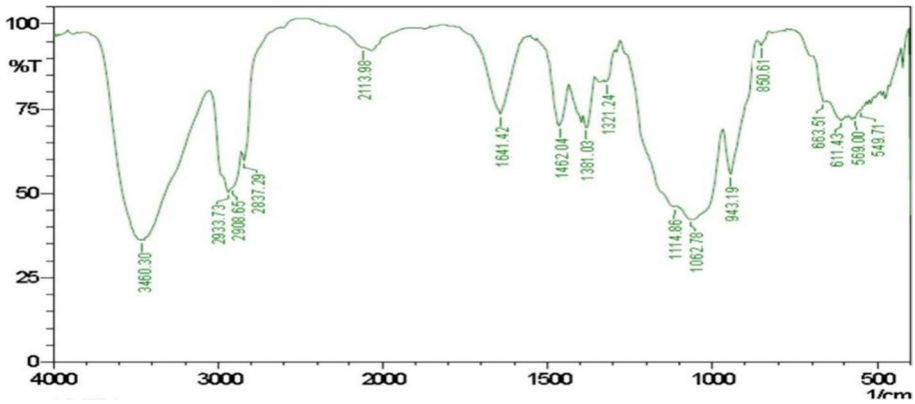
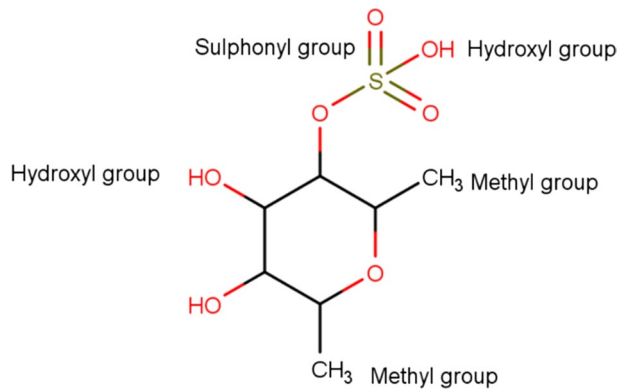


Fig. 2 FTIR Spectrum of fucoidans from *S.marginatum*

Fig. 3 Functional groups of Fucoidan



preliminary identification of fucoidan functional groups is always performed by FTIR analysis in the range of 400–4000 cm^{-1} . The typical IR characteristic of fucoidans can be evaluated by interpreting the O–H group of monomeric monosaccharides at 3400–3450 cm^{-1} , C–H asymmetric stretching of methyl group at 2900–2950 cm^{-1} , and S=O asymmetric stretching of sulfate group at 1114–1062 cm^{-1} . The FTIR spectrum showed the peaks at 3460.30, 2933.73 and 850.51 cm^{-1} , which confirms the hydroxyl, methyl and sulfate groups of fucoidans which suggested a complex pattern of substitution in the sample [28].

SEM and EDX Analysis

The surface morphology of the biosynthesized ZnO nanoparticles of crude fucoidan was shown in Fig. 4 and the Energy-dispersive X-ray spectroscopic analysis was shown in Fig. 5. It was observed that the synthesized nanoparticles were crystal in nature with the size range of 400–700 nm. Elemental analysis of synthesized ZnO nanoparticles characterized by SEM-EDX showed the presence of Zn in the sample ZnO nanoparticles. A

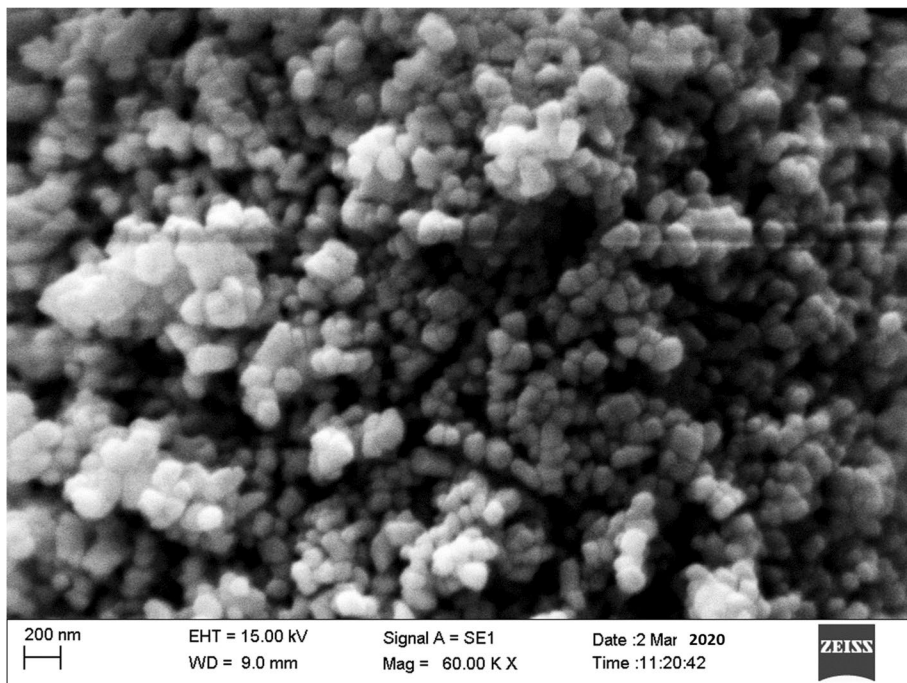


Fig. 4 SEM photograph of ZnO nanoparticles of crude fucoidans from *S.marginatum*

strong peak signal was observed in EDX, which confirms the purity of synthesized ZnO nanoparticles.

Maximum Non-toxic Dose of ZnO Nanoparticles

The cell viability and toxicity of the C6/36 cell line were assessed using the MTT assay, and the maximum non-toxic dose of crude fucoidan was calculated to be 46.87 $\mu\text{g}/\text{ml}$ (Fig. 6). Similarly, the IC_{50} was determined using the MTT assay in the Vero cell line using ZnO nanoparticles of crude fucoidan of *S. marginatum* and was found to be 149.33 $\mu\text{g}/\text{ml}$.

Quantitative Anti-Viral Assay by RT-PCR

According to the findings of this study, the ZnO nanoparticles of crude fucoidan isolated from *S. marginatum* were 99.09% effective against the dengue 2 viruses when treated with a concentration of 46.88 $\mu\text{g}/\text{ml}$ of the virus. As shown in Table 2, the anti-dengue activity of ZnO nanoparticles derived from crude fucoidan isolated from *S. marginatum* as determined by real-time RT-PCR was superior to that of crude fucoidan isolated from *S. marginatum*.

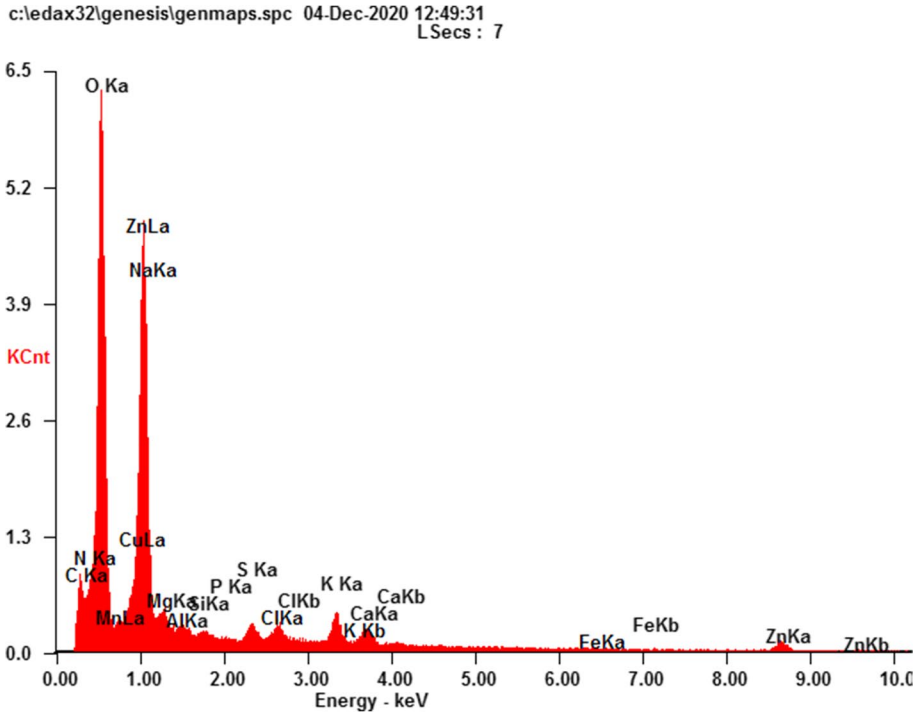


Fig. 5 EDAX Spectrum of ZnO nanoparticles of crude fucidans from *S. marginatum*

Discussion

Dengue and other viruses continue to pose a threat to the world and have emerged as a major public health concern on a global scale. The World Health Organization is extremely concerned about dengue because it affects a large proportion of the world's population.

Conventional antivirals used for the ailments of viral diseases were subjected to a high incidence of resistance. As a result, the determination of the antiviral effect of seaweeds as potent agents against clinical pathogens becomes extremely important in the treatment of dreadful viral infections, which are becoming increasingly common.

The anti-dengue studies of the seaweed *S. marginatum* and its isolated fucoidan were not performed. It was attempted in the present work to investigate the anti-dengue activity of ZnO nanoparticles of crude fucoidan isolated from *S. marginatum* by green biosynthesis in relation to dengue transmission.

The presence of the components of fucidans was confirmed through chemical analysis. The presence of fucidans is demonstrated by the appearance of distinct spots with characteristics that are distinct from carbohydrates in the TLC analysis, which is further supported by the appearance of an individual peak in the HPLC studies. FTIR studies provide conclusive evidence of the presence of functional groups in fucidans, including the presence of prominent hydroxyl and carbonyl groups.

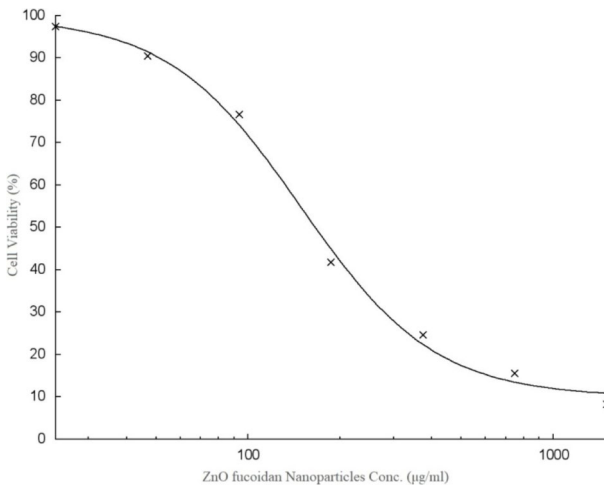
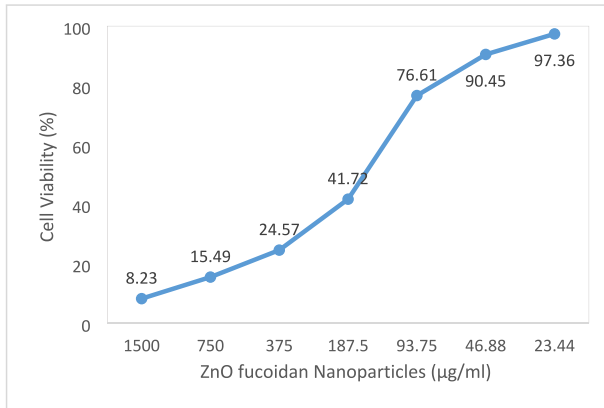


Fig. 6 Maximum non-toxic dose (MNTD) and IC₅₀ of crude fucoidan loaded ZnO nanoparticles

Table 2 The anti-dengue activity of ZnO nanoparticles of crude fucoidan from *S.marginatum*

S. No.	Compound	Quantity Mean ± SD (Virus copies/ml)	CT Mean ± SD	% inhibition
1	crude fucoidan	1,053,841.26 ± 58562.94	31.26 ± 0.71	62.48%
2	ZnO nanoparticles of crude fucoidan	25,428.35 ± 2386.78	25.81 ± 0.18	99.09%
3	Positive control (100 copies/ml)	2,809,004.0 ± 101,590.52	17.59 ± 0.06	-

n = 3, Values are expressed as mean ± SD

Some antiviral compounds are derived from natural sources, and microalgae are one of the most promising candidates for the production of these compounds. According to the researchers [37, 38], algae are responsible for 9% of biomedical compounds and some of the compounds are unable to replicate through chemical methods [39–41]. Furthermore, microalgae are able to produce huge amounts of biomass, and as a result,

extremely high levels of compounds, without incurring any excess energy or costs [42–44].

Umezawa et al. conducted a study on the antiviral property of microalgae. They discovered that an extract of *Chlorella pyrenoidosa*, containing acid polysaccharides, had an inhibitory effect in mice against the vesicular stomatitis virus (VSV) [45].

Anti-HSV1 and HSV2 activity of seaweed *Arthospirafusiforme*, which produces a sulfate polysaccharide were proved [46]. *Porphyridium* is a red microalgaethathas received a great deal of attention in recent years because of its potential biotechnological applications in the biomedical field. It is covered by a sulfate polysaccharide envelope that has antitumor, antibacterial, and antiviral activity, among other things [47].

High antiviral activity of sulfate polysaccharides was produced by *Porphyridium-cruentum*. Both Varicella zoster (HH3), a double-stranded DNA virus belonging to the Herpesvirus family, and vaccinia virus are susceptible to their effects in some cases [48, 49]. This species' methanol extracts, which contained sulfated polysaccharides, were found to have high *in-vitro*antiviral activity against Piscine novirhabdovirus (VHSV) and African swine fever virus (ASFV) [50].

The present study discovered that the biosynthesized ZnO nanoparticles of crude fucoidan isolated from the brown seaweed *S. marginatum* had 99.09% activity against the dengue-2 virus. This high level of activity is likely due to the crude nature of fucoidan, but the formation of ZnO nanoparticles is responsible for the reduction in the percentage of activity seen so far. It has been shown that fucoidan in its purified and sulfated forms may have 100% activity against the dengue2 virus in cell lines that have been studied.

Conclusion

Seaweeds have been discovered to be a rich and viable source of biomolecules with therapeutic potential. A study on biosynthesized ZnO nanoparticles of crude fucoidan from brown seaweed *S.marginatum* was conducted to determine whether they had anti-dengue activity against C6/36 cell lines *in vitro*. The crude extract was analyzed by TLC, HPLC, and FTIR studies, and the results showed that fucoidans were present in significant amounts. Isolated fucoidans were synthesized into ZnO nanoparticles and tested for anti-dengue activity against a variety of cell lines. The results showed that the nanoparticles had 99.09% activity against the cell lines tested. As an antiviral agent, the ZnO nanoparticles of fucoidans have been shown to be an excellent lead molecule for the treatment of dengue fever.

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Declarations

Conflict of Interest No potential conflict of interest was reported by the authors.

References

- Azizi, S., Ahmad, M. B., Namvar, F., & Mohamad, R. (2014). Green biosynthesis and characterization of zinc oxide nanoparticles using brown marine macroalga *Sargassum muticum* aqueous extract. *Materials Letters*, *116*, 275–277
- Allaker, R. P. (2010). The use of nanoparticles to control oral biofilm formation. *Journal of Dental Research*, *89*(11), 1175–86
- Morones, J. R., Elechiguerra, J. L., Camacho, A., Holt, K., Kouri, J. B., Ramírez, J. T., et al. (2005). The bactericidal effect of silver nanoparticles. *Nanotechnology*, *16*(10), 2346–2353
- Kaviyarasu, K., Kanimozhi, K., Matinise, N., Maria Magdalane, C., Mola, G. T., Kennedy, J., et al. (2017). Antiproliferative effects on human lung cell lines A549 activity of cadmium selenide nanoparticles extracted from cytotoxic effects: Investigation of bio-electronic application. *Materials Science and Engineering: C [Internet]*, *76*, 1012–25. Available from: <https://www.sciencedirect.com/science/article/pii/S0928493117300127>
- Kaviyarasu, K., Geetha, N., Kanimozhi, K., Maria Magdalane, C., Sivaranjani, S., Ayeshamariam, A., et al. (2017). In vitro cytotoxicity effect and antibacterial performance of human lung epithelial cells A549 activity of Zinc oxide doped TiO₂ nanocrystals: Investigation of bio-medical application by chemical method. *Materials Science and Engineering: C [Internet]*, *74*, 325–33. Available from: <https://www.sciencedirect.com/science/article/pii/S0928493116316940>
- Sawai, J. (2003). Quantitative evaluation of antibacterial activities of metallic oxide powders (ZnO, MgO and CaO) by conductimetric assay. *Journal of Microbiol Methods*, *54*(2), 177–182
- Sondi, I., & Salopek-Sondi, B. (2004). Silver nanoparticles as antimicrobial agent: A case study on *E. Coli* as a Model for Gram-Negative Bacteria. *Journal of Colloid and Interface Science*, *275*, 177–182
- Gurugama, P., Garg, P., Perera, J., Wijewickrama, A., & Seneviratne, S. L. (2010). Dengue viral infections. *Indian Journal of Dermatology [Internet]*, *55*(1), 68–78. Available from: <https://pubmed.ncbi.nlm.nih.gov/20418983>
- Malavige, G. N., Fernando, S., Fernando, D. J., & Seneviratne, S. L. (2004). Dengue viral infections. *Postgraduate Medical Journal [Internet]*, *80*(948), 588 LP – 601. Available from: <http://pmj.bmj.com/content/80/948/588.abstract>
- Ramalingam, K., Varghese, C. S., Elias, C., Mathew, G. M., & Balasubramanian, A. (2019). A retrospective study on the effect of vitamin C in the management of dengue fever in three different states of India. *International Journal of Research in Pharmaceutical Sciences*, *10*(4), 2670–2673
- Brady, O. J., Gething, P. W., Bhatt, S., Messina, J. P., Brownstein, J. S., Hoen, A. G., et al. (2012). Refining the global spatial limits of dengue virus transmission by evidence-based consensus. *Plos Neglected Tropical Diseases*, *6*(8), e1760
- Hozumi, T., Matsumoto, T., Ooyama, H., Namba, T., Shiraki, K., Hattori, M., et al. (1995). *Antiviral agent containing crude drug*. Google Patents
- Handel, A. S., Ayala, E. B., Borbor-Cordova, M. J., Fessler, A. G., Finkelstein, J. L., Espinoza, R. X. R., et al. (2016). Knowledge, attitudes, and practices regarding dengue infection among public sector healthcare providers in Machala, Ecuador. *Tropical Diseases, Travel Medicine and Vaccines*, *2*, 8
- Eluvakkal, T., Sivakumar, S. R., & Arunkumar, K. (2010). Fucoidan in some Indian brown seaweeds found along the coast gulf of Mannar. *International Journal of Botany*, *Vol. 6*, 176–181
- Berteau, O., & Mulloy, B. (2003). Sulfated fucans, fresh perspectives: structures, functions, and biological properties of sulfated fucans and an overview of enzymes active toward this class of polysaccharide. *Glycobiology*, *13*(6), 29R–40R
- Zhang, J., Peng, J., Chen, X., Gong, Y., Wan, L., Gao, F., et al. (2016). Rapid identification of bile acids in snake bile using ultrahigh-performance liquid chromatography with electrospray ionization quadrupole time-of-flight tandem mass spectrometry. *Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences*, *1036–1037*, 157–169
- Bilan, M. I., Grachev, A. A., Shashkov, A. S., Nifantiev, N. E., & Usov, A. I. (2006). Structure of a fucoidan from the brown seaweed *Fucus serratus* L. *Carbohydrate Research*, *341*(2), 238–245
- Guvener, K. C. B., Guvener, & Guler, E. (1999). In I. Akat (Ed.), *Pharmacological activities of marine algae*. *Introduction Applied Phycolgy* (pp. 67–92). SPB Academic Publishing by The Hague,
- Adhikari, U., Mateu, C. G., Chattopadhyay, K., Pujol, C. A., Damonte, E. B., & Ray, B. (2006). Structure and antiviral activity of sulfated fucans from *Stoechospermum marginatum*. *Phytochemistry*, *67*(22), 2474–2482
- Kumaresan, S., Margret, R. J., & Mohan, V. (2008). Microscopic and preliminary Phytochemical studies on marine algae *Padinatra stromatica* and *Stoechospermum marginatum* from Tuticorin coast, Tamil Nadu. *Journal of Medicinal and Aromatic Plant Sciences*, *30*, 375–380

21. Saravanan, R., Sadiq, I. S., & Jawahar, P. (2017). *Sea urchin diversity and its resources from the Gulf of Mannar*, 4th Indian Biodiversity Congress 180–182
22. Xing, R., Liu, S., Yu, H., Chen, X., Qin, Y., Li, K., et al. (2013). Extraction and separation of fucoidan from *Laminaria japonica* with chitosan as extractant. *Biomed Research International*, 2013, 193689
23. Grignon-Dubois, M., & Rezzonico, B. (2018). Phenolic chemistry of the seagrass *Zostera noltei* Hornem. Part 1: First evidence of three infraspecific flavonoid chemotypes in three distinctive geographical regions. *Phytochemistry*, 146, 91–101
24. Dische, Z., & Shettles, L. B. (1948). A specific color reaction of methylpentoses and a spectrophotometric micromethod for their determination. *Journal Of Biological Chemistry*, 175(2), 595–603
25. Chale-Dzul, J., Pérez-Cabeza de Vaca, R., Quintal-Novelo, C., Olivera-Castillo, L., & Moo-Puc, R. (2020). Hepatoprotective effect of a fucoidan extract from *Sargassum fluitans* Borgesen against CCl₄(4)-induced toxicity in rats. *International Journal of Biological Macromolecules*, 145, 500–509
26. Czerwinska, J., Parkin, M. C., Dargan, P. I., George, C., Kicman, A. T., & Abbate, V. (2019). Stability of mephedrone and five of its phase I metabolites in human whole blood. *Drug Testing and Analysis*, 11(4), 586–94
27. Marimuthu, J., @Antoniamy, Essakimuthu, P., Narayanan, J., Anantham, B., Tharmaraj, R. J. J. M., et al. (2012). Phytochemical characterization of brown seaweed *Sargassum wightii*. *Asian Pacific Journal of Tropical Disease [Internet]*, 2, S109–13. Available from: <https://www.sciencedirect.com/science/article/pii/S222180812601340>
28. Liu, S., Wang, Q., Song, Y., He, Y., Ren, D., Cong, H., et al. (2018). Studies on the hepatoprotective effect of fucoidans from brown algae *Kjellmaniella crassifolia*. *Carbohydrate Polymers [Internet]*, 193, 298–306. Available from: <https://www.sciencedirect.com/science/article/pii/S0144861718303412>
29. Bilan, M. I., Grachev, A. A., Ustuzhanina, N. E., Shashkov, A. S., Nifantiev, N. E., & Usov, A. I. (2002). Structure of a fucoidan from the brown seaweed *Fucus evanescens* C.Ag. *Carbohydrate Research*, 337(8), 719–730
30. Rajakumar, G., Thiruvengadam, M., Mydhili, G., Gomathi, T., & Chung, I. M. (2018). Green approach for synthesis of zinc oxide nanoparticles from *Andrographis paniculata* leaf extract and evaluation of their antioxidant, anti-diabetic, and anti-inflammatory activities. *Bioprocess and Biosystems Engineering*, 41(1), 21–30
31. Bhuyan, T., Mishra, K., Khanuja, M., Prasad, R., & Varma, A. (2015). Biosynthesis of zinc oxide nanoparticles from *Azadirachta indica* for antibacterial and photocatalytic applications. *Materials Science in Semiconductor Processing*, 32, 55–61
32. Umar, H., Kavaz, D., & Rizaner, N. (2019). Biosynthesis of zinc oxide nanoparticles using *Albizia lebbek* stem bark, and evaluation of its antimicrobial, antioxidant, and cytotoxic activities on human breast cancer cell lines. *International Journal of Nanomedicine*, 14, 87–100
33. Medina, F., Medina, J. F., Colón, C., Vergne, E., Santiago, G. A., & Muñoz-Jordán, J. L. (2012). Dengue virus: isolation, propagation, quantification, and storage. *Current Protocols in Microbiology*, Chap. 15, Unit 15D.2
34. Acosta, E. G., Castilla, V., & Damonte, E. B. (2011). Infectious dengue-1 virus entry into mosquito C6/36 cells. *Virus Research*, 160(1–2), 173–179
35. Rothan, H. A., Zulqarnain, M., Ammar, Y. A., Tan, E. C., Rahman, N. A., & Yusof, R. (2014). Screening of antiviral activities in medicinal plants extracts against dengue virus using dengue NS2B-NS3 protease assay. *Tropical Biomedicine*, 31(2), 286–296
36. Ramalingam, S., Karupannan, S., Padmanaban, P., Vijayan, S., Sheriff, K., Palani, G., et al. (2018). Anti-dengue activity of *Andrographis paniculata* extracts and quantification of dengue viral inhibition by SYBR green reverse transcription polymerase chain reaction. *Ayu*, 39(2), 87–91
37. Ahmadi, A., Zorofchian Moghadamtousi, S., Abubakar, S., & Zandi, K. (2015). Antiviral potential of algae polysaccharides isolated from marine sources: A review. *Biomed Research International*, 2015, 825203
38. Yasuhara-Bell, J., & Lu, Y. (2010). Marine compounds and their antiviral activities. *Antiviral Research*, 86(3), 231–240
39. Carbone, D. A., Olivieri, G., Pollio, A., & Melkonian, M. (2020). Biomass and phycobiliprotein production of *Galdieria sulphuraria*, immobilized on a twin-layer porous substrate photobioreactor. *Applied Microbiology and Biotechnology*, 104(7), 3109–3119
40. Priyadarshani, I., & Rath, B. (2012). Commercial and industrial applications of micro algae-a review. *Journal of Algal Biomass Utilization*, 3(4), 89–100
41. Yamaguchi, K. (1996). Recent advances in microalgal bioscience in Japan, with special reference to utilization of biomass and metabolites: a review. *Journal of Applied Phycology*, 8(6), 487–502

42. Borowitzka, M. A. (1999). Commercial production of microalgae: ponds, tanks, tubes and fermenters. *Journal of Biotechnology*, 70(1-3), 313–321
43. Pulz, O., & Gross, W. (2004). Valuable products from biotechnology of microalgae. *Applied Microbiology and Biotechnology*, 65(6), 635–648
44. Carbone, D. A., Olivieri, G., Pollio, A., & Gabriele, Melkonian, M. (2017). Growth and biomass productivity of *Scenedesmus vacuolatus* on a twin layer system and a comparison with other types of cultivations. *Applied Microbiology And Biotechnology*, 101(23–24), 8321–8329
45. Umezawa, I., & Komiyama, K. (1985). Acidic polysaccharide CH-I isolated from *Chlorella pyrenoidosa* and the use thereof. US Patent (4533548A)
46. Sharaf, M., Amara, A., Aboul-Enein, A., Helmi, S., Ballot, A., Astani, A., et al. (2010). Molecular authentication and characterization of the antiherpetic activity of the cyanobacterium *Arthrospira fusiformis*. *Die Pharmazie*, 65(2), 132–136
47. Kavitha, M. D., Kathiresan, S., Bhattacharya, S., & Sarada, R. (2016). Culture media optimization of *Porphyridium purpureum*: production potential of biomass, total lipids, arachidonic and eicosapentaenoic acid. *Journal Of Food Science And Technology*, 53(5), 2270–2278
48. Huleihel, M., Ishanu, V., Tal, J., & Arad, S. M. (2001). Antiviral effect of red microalgal polysaccharides on Herpes simplex and Varicella zoster viruses. *Journal of Applied Phycology*, 13(2), 127–134
49. Radonic, L. M., Zimmermann, J. M., Zavallo, D., López, N., & López Bilbao, M. (2006). Rooting in Km selective media as efficient in vitro selection method for sunflower genetic transformation. *Electronic Journal of Biotechnology*, 9(3), 0
50. Fabregas, J., García, D., Fernandez-Alonso, M., Rocha, A. I., Gómez-Puertas, P., Escribano, J. M., et al. (1999). In vitro inhibition of the replication of haemorrhagic septicaemia virus (VHSV) and African swine fever virus (ASFV) by extracts from marine microalgae. *Antiviral Research*, 44(1), 67–73

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