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Protective effects of Marine Sponge Extract MS01 on Neuronal Cell Lines

G.B. Priyadharshini[1], C. Jaynthy[2]

[1] Ph.D. scholar, Department of Bioinformatics, Sathyabama Institute of Science and Technology, Chennai, India, dharthi.dharshini@gmail.com
[2]Associate professor, Department of Bioinformatics, Sathyabama Institute of Science and Technology, Chennai, India,
[2] chellamjaynthy@gmail.com

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Abstract

Background: Reactive oxygen species (ROS), byproducts of aerobic respiration, play a crucial role in cellular signal transduction. However, excessive ROS production can lead to cell death by damaging DNA, proteins, and lipids. Neuroprotection, a defensive mechanism that mitigates cell degeneration and initiates cell regeneration, can be facilitated by bioactive proteins and other substances abundant in marine sponges. In this study, we employed flow cytometry to investigate the protective effects of the sponge extract MS01 against ROS in fibroblast cell lines (L929) as well as human neuronal cell lines (HMC3 and U251).
Results: Our findings indicate that MS01 exhibits notable AChE inhibitory activity, with an IC50 value of 265.13 µg/ml. Furthermore, assessments conducted on the human malignant glioma cell line and human microglial clone 3 revealed no potential for cell death, demonstrating the non-toxic nature of the extract. Flow cytometry analysis using FITC antibody in L929 cells demonstrated a reduction in iNOS activity with a mean fluorescent intensity of 62.12 MFI, indicating therapeutic potential. Moreover, MS01 significantly decreased H2DCFDA fluorescence expression in H2O2-treated cells, indicating its protective properties.
Conclusion: The results of this study show that MS01, an extract obtained from marine sponges, appears promising for the treatment for neurodegeneration. The findings demonstrate that MS01 has characteristics that may reduce acetylcholinesterase activity, scavenge nitric oxide radicals, and efficiently suppress reactive oxygen species. The test compound additionally demonstrated no evidence of causing apoptosis or toxicity in healthy brain cells, confirming its potential therapeutic utility.

Keywords: *Reactive oxygen species, Marine sponges, Flow cytometry, cell degradation, Neuroprotection, and cell lines.*

1. BACKGROUND

Reactive oxygen species (ROS) are reactive molecules or free radicals that are by-products of aerobic respiration and perform an important role in accelerating signal transduction pathways within the cell [1,2]. Under normal physiological conditions, ROS are primarily produced by mitochondria and nicotinamide adenine dinucleotide phosphate (NADPH) oxidases and are rapidly detoxified by several enzymes and proteins such as superoxide dismutase, and glutathione, which also includes the superoxide anion, peroxide, hydrogen peroxide, hydroxyl radical, and hydroxyl ions, among others [3]. Excess ROS production or a deficiency in ROS removal can result in oxidative stress, in which reactive oxygen species damage proteins, lipids, and DNA, eventually leading to cellular stress and cell death, as well as pathological diseases such as Alzheimer's (AD), Parkinson's (PD), and Huntington's (HD). Neuroprotection is a well-established defense mechanism in the fight against degenerative brain disorders [4]. It is critical to develop reliable, accurate assays to monitor ROS levels in distinct tissue compartments in order to understand the origin of harm and measure the efficacy of antioxidants. There are several methods available for measuring reactive oxygen species (ROS), such as lipid peroxidation (LPO) assay, electron spin resonance (ESR), and fluorescence microscopy using ROS-specific fluorescent dyes

for image analysis. These techniques provide diverse approaches to quantify and study ROS levels. Flow cytometry is a technique for studying many cells quickly that combines the advantages of microscopy and biochemical analysis [5]. Flow cytometry, in particular, has proven a reliable and rapid technique for recognizing intracellular ROS in recent years [6].

The ocean is still an open source and plays an important role in the hunt for new therapeutic targets that are responsible for several diseases [7]. Sponges, which are primitive creatures found on the ocean floor, are a treasure trove of secondary metabolites and proteins [37]. The goal of the study is to demonstrate the protective nature of the Callyspongia sp. sponge extract MS01 in L929 (Normal Fibroblast Cell Line from Mouse), HMC3 (Human Microglial Clone 3 Cell Line), and U251 (Human Malignant Glioblastoma Cell Line) cell lines using flow cytometry.

2. METHODS

Callyspongia sp, a marine sponge, was obtained from the waters of Palk Bay near Rameswaram, Tamil Nadu. The sponge was then extracted using the protocol provided by Purushottama [20]. The MS01 column fraction, determined to have a high protein content using the Barford method, was chosen for further analysis. It was subsequently lyophilized and stored at -80°C for future investigations. The MS01 extract was administered at a concentration of 2mg/ml throughout the entire study [35].

2.1 Apoptosis analysis using HMC3 cells

To investigate the apoptotic mechanism of the sponge extract, HMC3 and U251 cell lines were utilized. The cells were seeded onto 6-well plates and allowed to reach 60% confluency. Subsequently, they were treated with various concentrations of the sponge extract for 24 hours, under conditions of 5% CO2 and 37°C. The apoptotic analysis was conducted using FITC Annexin V and Propidium Iodide (PI). After a 24-hour incubation period, the supernatant was removed and the cells were collected for apoptosis analysis. The staining protocol for Annexin V and Propidium iodide (PI) was followed according to the manufacturer's kit instructions. Briefly, after a wash with PBS (Phosphate Buffer Saline), the cells were suspended in 1X binding buffer. Then, 5µL of Annexin V was added and the cells were incubated for 10 minutes. Following this, 2mL of binding buffer was added and the supernatant was discarded. The cells were then resuspended in 200µL of binding buffer, and 5µL of PI was added, followed by a 15-minute incubation. After incubation, the cells were washed with 2mL of binding buffer, resuspended in 200µL of binding buffer, and analyzed using FACS (Fluorescence-Activated Cell Sorting) [9,10].

2.2. Microscopy imaging of U251 cells

U251 cells were seeded on glass bottom plates until they reached 60% confluency. Subsequently, the cells were stained with Hoechst for nuclear visualization. Following the staining process, the cells were treated with different concentrations of drugs MSS and MSE (1:10, 1:20, 1:40 dilutions) for 24 hours under conditions of 5% CO2 and 37°C. Images were captured at 0 and 24 hours using a 20X objective, Andor EMCCD Camera, and analyzed using NIS element software. Hoechst staining was performed with an excitation wavelength of 405 nm, and the emitted light was collected at 420 nm [38].

2.3. Reactive oxygen species (ROS) detection using flow cytometry

HMC3 cells were cultured in a 96-well plate at a density of 1x10^4 cells/200µl and incubated overnight in a CO2 incubator at 37°C for 24 hours. The spent medium was removed, and the cells were washed with 100µl of 1X PBS. Subsequently, the cells were treated with H2O2 at a concentration of 100µM [42], along with the test compounds MS01 at their respective IC50 concentrations. Wells containing only cell culture served as the control and were incubated for 24 hours. After the treatment period, the media was discarded, and the cells were washed with PBS. Next, the cells were stained with H2DCFDA solution and incubated at 37°C for 30 minutes in the dark. Images were captured using confocal microscopy (Opera Phenix High Content Screening system) with excitation at 488nm and emission at 535nm for the FL1 channel. The images were then analyzed using Image J software [1,23].

2.4. iNOS Expression Study

L929 cells were seeded in a 6-well culture plate at a density of 3 x 10^5 cells and incubated in a CO2 incubator at 37°C for 24 hours. The cells were then washed with PBS and treated with 200µl of trypsin-EDTA solution to detach them from the plate. Subsequently, the cells were fixed using 1ml of 4% paraformaldehyde solution and kept undisturbed on ice for 20 minutes. After washing with PBS, the cells were treated with 5µL of iNOS FITC antibody and incubated in the dark at 25°C for an additional 30 minutes. Following another wash with 0.1% sodium azide, 0.5 mL of PBS was added to the cells, thoroughly mixed, and analyzed using Flow Cytometry [11,12].

2.5. Acetylcholinesterase (AChE) Assay

To prepare the acetylthiocholine reaction mixture, 4.5 ml of assay buffer (Component B), 250µl of 20X DTNB stock solution, and 250µl of 20X acetylthiocholine stock solution were combined and the volume was adjusted

to a total of 50ml. The AChE assay kit utilized for measuring AChE inhibition in cell culture employs a colorimetric one-step assay, capable of detecting as little as 0.1 mU AChE in a 100 µL assay volume (1 mU/ml). The resulting signal can be conveniently measured using an absorbance microplate reader at approximately 410 nm [13,19]. The assay was performed as per Benfeito's protocol [13], with suitable modifications. In a 96-well plate containing seeded HMC3 cells, 5µl of 10X (1µM) Donepezil hydrochloride was added to the sample wells intended for testing. Subsequently, 45µl of the sample with varying concentrations was added and incubated for 10 minutes. Then, 50µl of the prepared acetylthiocholine reaction mixture was added to each well, including the acetylcholinesterase standard, blank, control, and test samples, resulting in a total volume of 100µl per well. The reaction mixture was incubated in the dark at room temperature for a duration of 10 to 30 minutes. The absorbance was measured at OD 410±5 nm using a microplate reader [43,44]. The acetylcholinesterase standard solution was prepared by combining the acetylcholinesterase stock solution with Assay Buffer (Component B) in a ratio of 0.2:9.8.

3. RESULTS

3.1 Apoptotic analysis of sponge extract on HMC3 cells

The extract MS01 derived from the marine sponge Callyspongia sp. was prepared as previously described and subjected to analysis in various cell lines. For the apoptosis expression study on the HMC3 cell line, BD FACS Caliber and Cell Quest Pro Software (Version: 6.0) were utilized. Flow cytometry was employed to examine the cells treated with FITC Annexin V and propidium iodide (PI) solution. The majority of untreated cells exhibited negative staining for FITC Annexin V and PI, indicating their viability and absence of apoptosis. Following a 4-hour treatment, two distinct cell populations were observed (bottom panels). The early apoptotic cells were positively stained with FITC Annexin V and negatively stained with PI. Additionally, cells that stained positive for both FITC Annexin V and PI were either in the advanced stage of apoptosis progressing towards necrosis or already deceased.

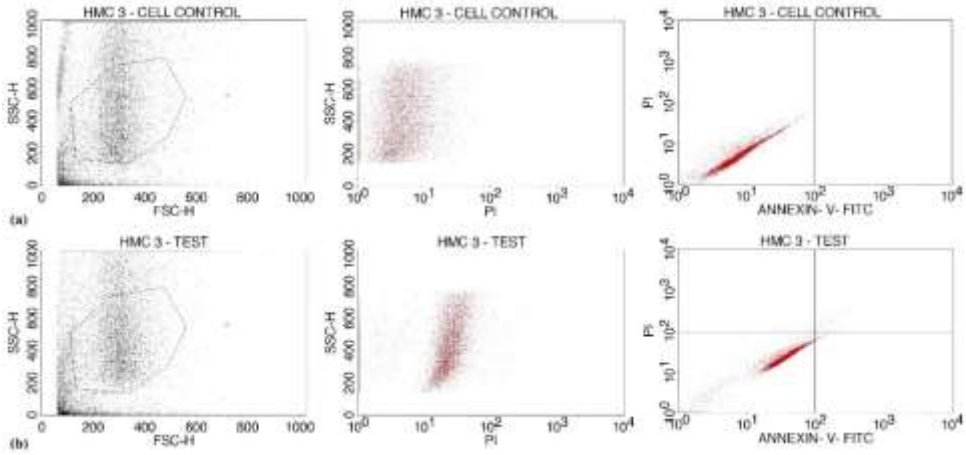


Figure 1: The apoptotic potential of sponge protein in HMC3 cell lines. (a) Annexin V-PI expression study of cell control against HMC3 cells and (b) Annexin V-PI expression study of MS01 against HMC3 cells.

In Figure 1, the lower left quadrant population corresponds to viable cells, with 99.99% and 98.79% of cells found in the control and MS01 groups, respectively. The upper left quadrant represents debris or necrotic cells, accounting for 0% and 0.11% of cells. Additionally, the upper right quadrant, indicative of late apoptotic cells, contains 0.01% and 0.49% of cells. The lower right quadrant represents early apoptotic cells, with 0% and 0.61% of cells identified, respectively.

3.2 Apoptotic assay in U251 cell line

Flow cytometry was employed to conduct an apoptosis assay on U251 cells, a cell line derived from Human Malignant Glioblastoma clone 3. The results, as shown in Figure 2, reveal the percentages of live cells stained with Annexin V and PI at various dilutions (1:10, 1:20, 1:40). In the presence of galanthamine (MSC), the live cell populations were measured at 91.4%, 96.8%, and 95% for the respective dilutions. Similarly, when treated with MS01 (MSS), the live cell percentages were recorded at 83.1%, 90.4%, and 90.9% for the corresponding dilutions.

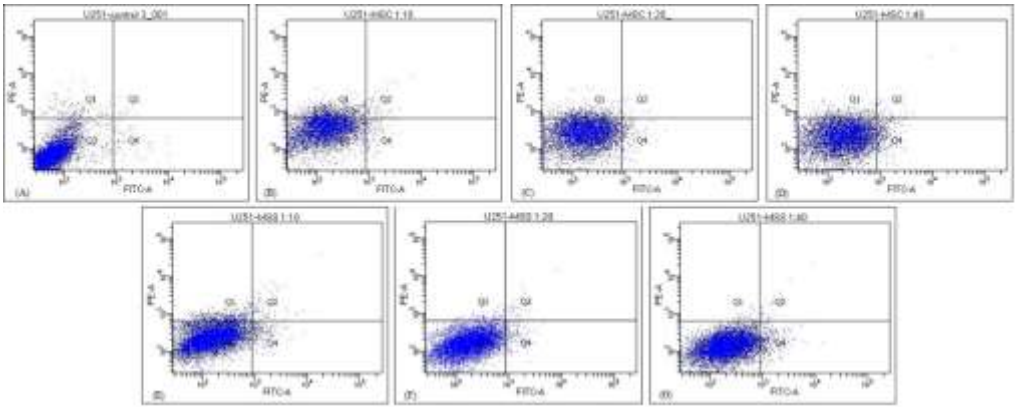


Figure 2: FACS flow cytometry images of U251 cells in which >80% of cells in the Quadrant Q3 reveal the non-apoptotic nature of the extract MS01.

Furthermore, microscopic analysis (Figure 3) was performed on cells treated with Hoechst for nuclear staining at different concentrations of galanthamine (MSC) and MS01 (MSS) (1:10, 1:20, 1:40 dilutions). After incubation for 24 hours in a 5% CO2 environment at 37°C, the blue staining indicated the presence of round or oval-shaped cells, suggesting the absence of DNA damage. These observations demonstrate that the cells remained healthy in all three concentrations, providing evidence for the non-apoptotic nature of MS01.

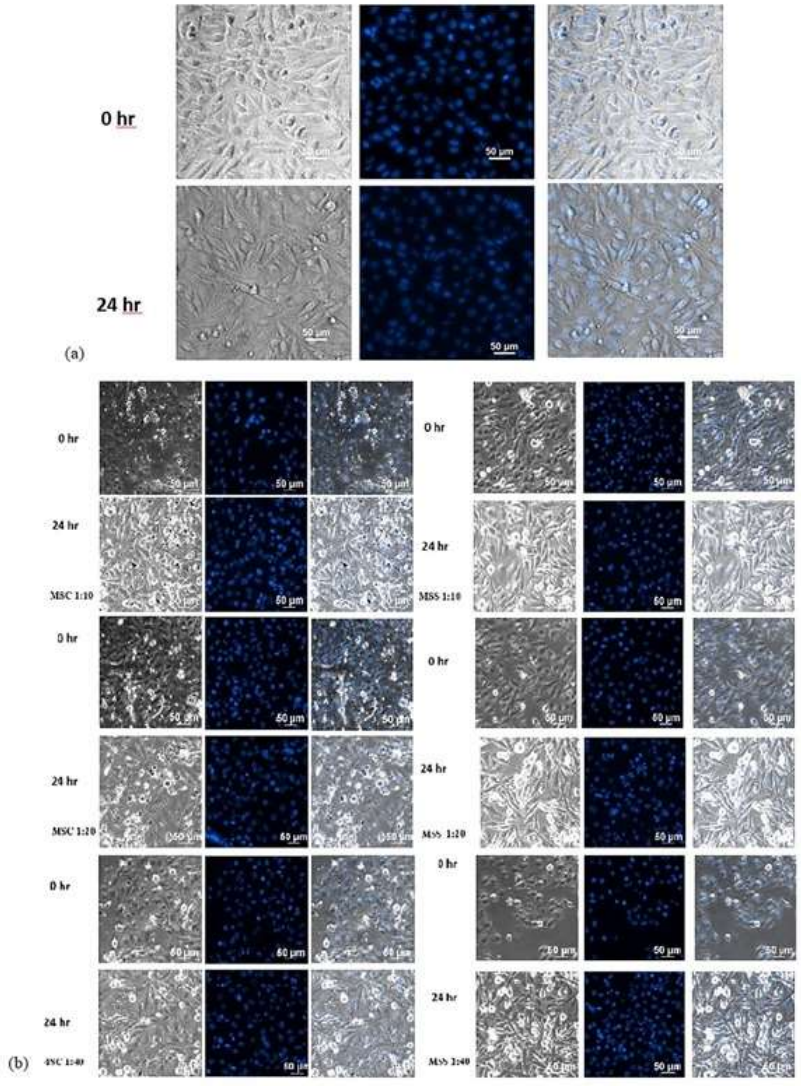


Figure 3: Apoptotic potential of sponge protein in U251 cell lines (a) cell control – DMSO (b) left side represents cells treated with galantamine and the right side represents the cells treated with MS01.

3.3 ROS activity in HMC3 cells

The microscopic image, presented as Figure 4, highlights the intensified fluorescence intensity of the H2DCFDA stain in cells treated with H2O2. However, when cells were treated with MS01 in conjunction with H2O2, the fluorescence intensity diminished. This reduction in fluorescence intensity indicates the scavenging of reactive oxygen species (H2O2) by the MS01 extract, thereby demonstrating its protective nature on HMC3 cells.

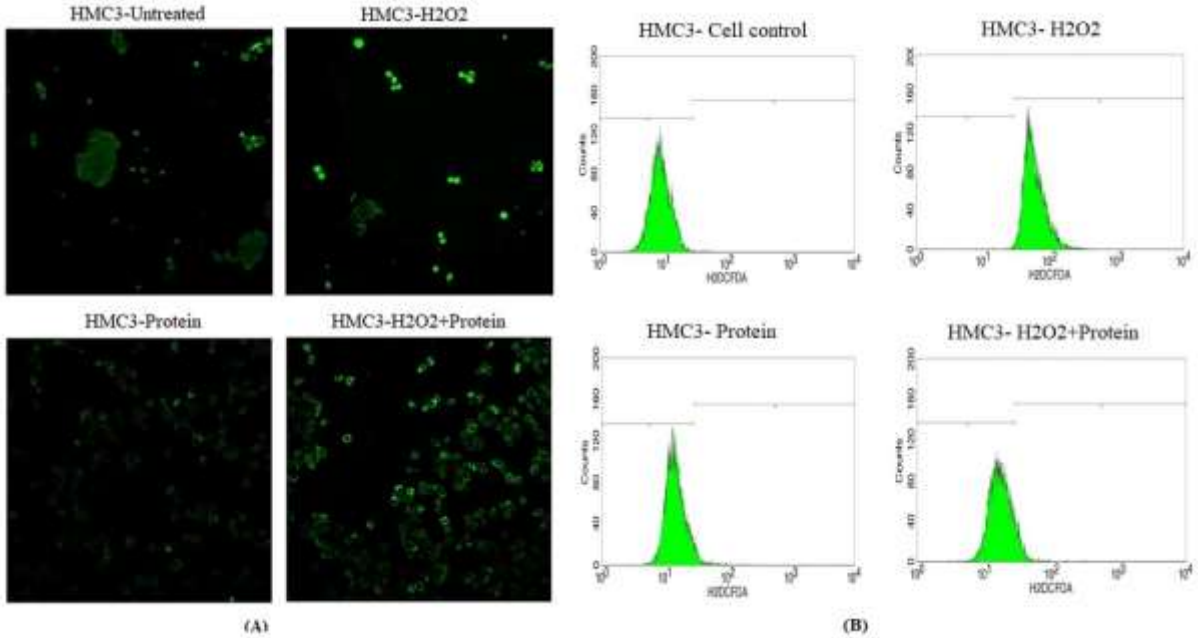


Figure 4: (A) Microscopic view of H2DCFDA fluorescence expression of the sponge extract MS01 against HMC3 cells treated with H2O2. (B) Flow cytometry data show the migration of peaks from M1 (negative expression) to M2 (positive expression) in cells treated with H2O2 and vice versa in cells treated with MS01.

In Figure 4, the flow cytometric analysis of H2DCFDA histograms in gated HMC3 singlets allowed for the distinction of cells in the M1 and M2 phases. In this context, M1 represents cells with negative ROS expression, while M2 represents cells with positive ROS expression. This distinction was achieved through software analysis using Cell Quest Software (Version 6.0). Cells treated with H2O2 exhibited an elevated H2DCFDA expression, leading them to transition to the M2 phase, which signifies a state of stress and eventual cell death. Conversely, treatment with MS01 resulted in decreased H2DCFDA expression, illustrating the protective properties of the sponge protein.

3.4 Anti- iNOS activity

The iNOS antagonist FITC antibody is a combination of an anti-iNOS antibody and FITC (Fluorescein isothiocyanate), a fluorescein molecule utilized in flow cytometry with an emission range of 525nm. This antibody is employed to detect inducible nitric oxide synthase (iNOS) and NOS (nitric oxide synthase). iNOS is an enzyme that, when stimulated by pathogen exposure, triggers the production of nitric oxide (NO), a second messenger molecule, in immune cells like macrophages.

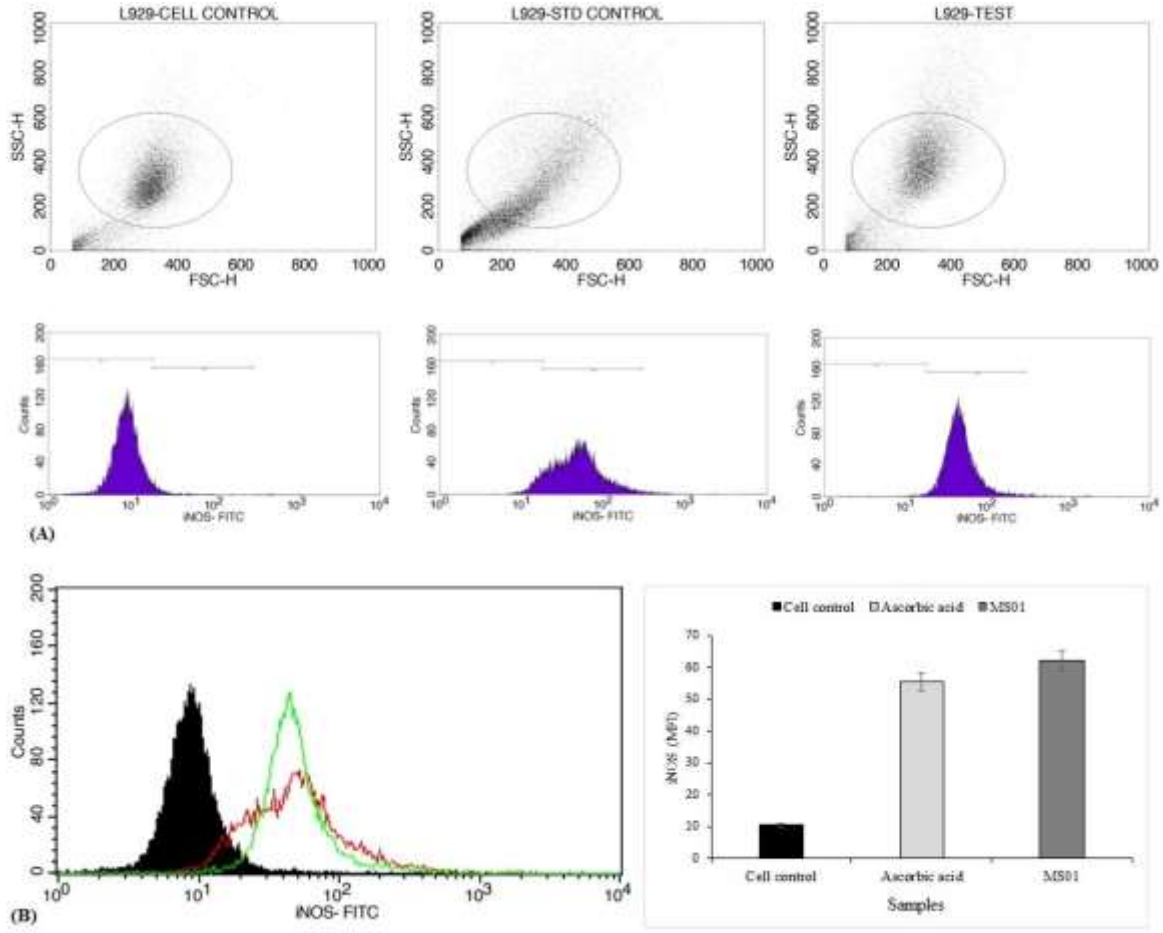
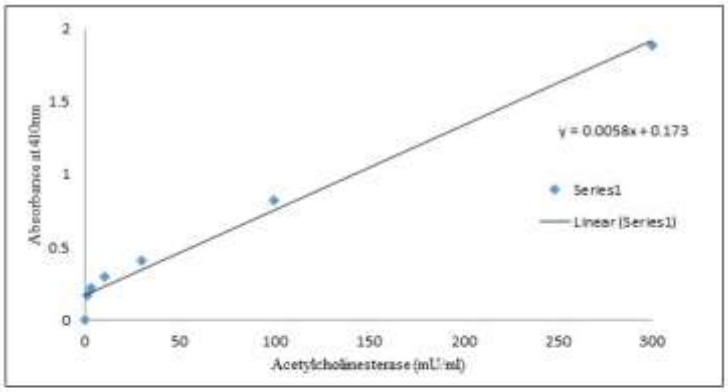


Figure 5: (A) represents the anti-INOS expression study of the ascorbic acid and MS01 treated against pathogenic L929 Cell line (top) and Histogram (bottom) showing the mean fluorescence intensity of iNOS against the cell control, Ascorbic acid (std) and MS01 treated L929 cells. (B) represents the overlay histogram (left) and graph using mean fluorescent intensity (right) of cell control, ascorbic acid and MS01.

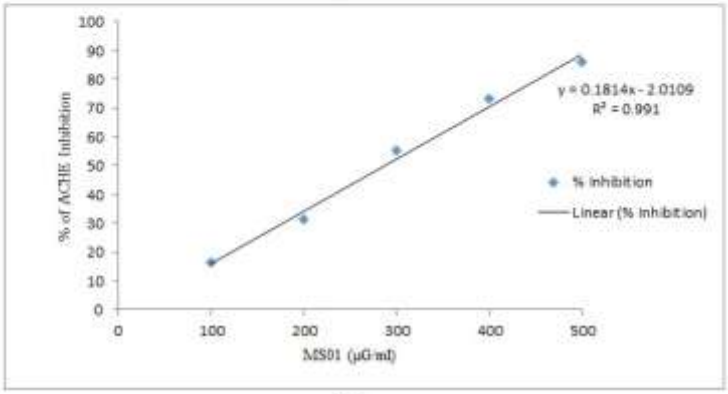
Figure 5 (a) and (b) display the results of pathogenic L929 cells in suspension treated with the standard drug ascorbic acid and the test compound MS01. The mean fluorescence intensity (MFI) of iNOS expression in the ascorbic acid-treated cells was measured at 55.44, while the MFI of anti-iNOS-FITC expression in the MS01-treated cells was recorded at 62.12. These findings suggest that the sponge protein may possess nitric oxide radical scavenging capabilities, as evidenced by the enhanced expression of anti-iNOS-FITC.

3.5 AChE inhibition assay

AChE (acetylcholinesterase) plays a significant role in tissue remodeling during cell development by reducing cell proliferation. Consequently, changes in AChE expression can contribute to apoptosis. To investigate the expression of AChE during the enzymatic cleavage of acetylthiocholine to acetate and thiocholine by AChE, an assay was conducted. Since AChE activity is primarily detected in apoptotic cells, the sponge protein extract was assessed for its inhibitory effect on acetylcholinesterase using ELISA. The experiment involved treating HMC3 cells with various concentrations of donepezil, followed by analysis and plotting the results on a regression graph, as depicted in Figure 6. The inhibitory concentration of MS01 against AChE, denoted as IC50, was determined to be 265.13µG/ml.



(A)



(B)

Figure 6: (A) represents the standard plot for Acetylcholinesterase (AChE) (B) scatter plot depicting % inhibition of AChE in cell control and MS01-treated HMC3 Cells. Were the IC50 value was obtained from the linear regression equation for y=50, and the concentration is determined as (µg/ml).

4. DISCUSSION

Marine sponges are known to be abundant in compounds with significant biological potential, particularly in terms of neuroactivity. These compounds can be readily extracted, as demonstrated by the extraction from the red sea sponge *Xestospongia testudinaria* [36]. In a specific case, the extract from this sponge species was investigated for its potential in preventing diabetic neuropathy [35]. PD-induced zebrafish models were investigated for neuroprotection by the octameric peptide xenin, which was obtained from *Callyspongia sp*.

Annexin V FITC has been employed to accurately assess the proportion of cells in a population that are actively undergoing apoptosis. This detection method relies on the characteristic loss of membrane asymmetry observed during the early stages of apoptosis. In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) undergoes translocation from the inner region of the plasma membrane to the outer, exposing PS to the external environment [28]. Annexin V, a calcium-dependent phospholipid-binding protein that has an excellent attraction for PS, was useful for the identification of apoptotic cells with exposed PS [26,27]. Propidium Iodide (PI) is a standard flow cytometric viability probe and was used to distinguish viable from dead cells. From the results, viable cells with intact membranes exclude PI, whereas the membranes of dead and damaged cells are permeable to PI [14,15,16]. The initial change in light scattering characteristics of cells in apoptotic systems is a reduction in forward scatter (FSC). Typically, this is followed by an immediate rise in (side scatter) SSC. Secondary necrosis reduces the intensity of SSC in later phases [39]. The results revealed that three distinct stages of plasma membrane alterations could be observed during the early phases of apoptosis. These stages are associated with the loss of plasma membrane asymmetry, leading to the positive staining of Annexin V. The first stage involves focal membrane changes, leading to localized exposure of phosphatidylserine (PS) and resulting in a modest or undetectable increase in Annexin V positivity (R1). This is followed by a complete loss of membrane asymmetry, characterized by strong Annexin V staining, even though the plasma membrane's barrier function remains intact (R2). Finally, the membrane's integrity becomes compromised, leading to further alterations (R3) [40]. Thus >80% of live cells in the Q3 quadrant determine the sample's apoptotic nature.

Reactive oxygen species (ROS) are molecular entities that consist of hydroxyl radicals or peroxides with unpaired electrons. These species are naturally generated within healthy aerobic cells as byproducts of processes

such as oxidative phosphorylation, oxidoreductase enzymes, or controlled metal-catalyzed oxidation [22]. However, under certain stress conditions, such as exposure to environmental oxidants and certain drugs, the production of ROS can be induced, leading to oxidative stress. Excessive ROS production can cause damage to important cellular components, including DNA, proteins, and lipids, ultimately resulting in cell death. To detect the presence of ROS, a commonly used indicator is 2', 7'-dichlorodihydrofluorescein diacetate (H2DCFDA), which can permeate the cell membrane. Once inside the cell, H2DCFDA is deacetylated by cellular esterase's, converting it into the non-fluorescent molecule H2DCF. In the presence of ROS, H2DCF is oxidized, resulting in the formation of the fluorescent product DCF [17,22,25]. The H2DCFDA probe exhibits high specificity for H2O2, and the fluorescence signal generated by the probe can be measured using flow cytometry. It has been established that the fluorescence of the DCF product is solely mediated by H2O2 [24]. It was demonstrated that enhanced ROS production increases the fluorescence expression of H2DCFDA [41]. The treatment of cells with elevated ROS generation using the MS01 sponge extract resulted in a notable decrease in fluorescence intensity, indicating the effectiveness of the extract.

The messenger molecule nitric oxide (NO), which is produced by the mouse iNOS protein and is also known as nitric oxide synthase 2 (NOS2), is capable of mediating bactericidal and tumoricidal activities [28,29]. Furthermore, iNOS plays a major role in inflammation and increases the synthesis of proinflammatory interleukins [30,31]. Upregulation of iNOS in glial cells or invading macrophages, or both, is thought to be a source of widely used radical generation, which is important in Alzheimer's disease, Parkinson's disease, and ischemia or inflammatory situations [45]. Cultured primary glial cells have been widely employed by researchers to explore the involvement of nitric oxide, peroxynitrite, or both in various processes. Upon stimulation with bacterial lipopolysaccharide (LPS) and a variety of cytokines, cultured cells demonstrate an upregulation of iNOS expression [46]. Monitoring the presence of the breakdown product nitrite (and/or nitrate) in the culture medium is a commonly employed method to assess the upregulation of iNOS and the generation of nitric oxide. The expression of iNOS and the production of NO by microglial cells have been linked to the process of dopaminergic neurodegeneration. Inhibition of iNOS expression has been shown to reduce neuronal cell death in this context [32]. According to statistical data from iNOS expression flow cytometry research, untreated/ normal L929 cells had very low iNOS expression (10.69MFU) (black), indicating no microglial expression of iNOS, when compared to the standard control value of ascorbic acid (200uM), which had a sudden increase in the MFU to 62.12 (green), indicating up regulation of iNOS production. When the cells treated with MS01 (TEST) at Concentration (2mg/mL) showed a lower intensity and down regulation of iNOS expression at 55.44 MFU (red). The observations suggest that a reduction in the mean fluorescent intensity indicates the extract MS01 exhibits Nitric Oxide Radical Scavenging Properties [18].

AChE inhibitors are important drugs that have been approved for the treatment of Alzheimer's disease (AD) and myasthenia gravis [44]. The assay of acetylcholinesterase offers a convenient way to measure AChE activity, utilizing DTNB to quantify the production of thiocholine resulting from the hydrolysis of acetylthiocholine by AChE in cell extracts [21]. The amount of thiocholine formed, which is directly related to AChE activity, was measured by assessing the absorption intensity of the DTNB adduct. AChE expression is commonly observed in the early stages of apoptosis in cells. Zhang's research findings provide the first evidence that AChE is activated and plays a significant role in apoptosis. AChE is expressed in various cell lines during apoptosis induced by different triggers. Aberrant megakaryocytopoiesis may occur as a result of cholinesterase overexpression. AChE inhibitors have shown promise in slowing down the progression of Alzheimer's disease [47]. This attributes the therapeutic effect to the role of AChE in fibril production. AChE generated from apoptotic neurons may aid in the prevention and treatment of Alzheimer's disease and other conditions characterized by uncontrolled cell death [21]. The graph illustrates a direct relationship between the concentration of MS01 and the percentage inhibition of AChE, leading to a decrease in cell death. These findings demonstrate the potent inhibitory effect of MS01 on acetylcholinesterase in HMC3 cell lines.

5. CONCLUSION

In conclusion, this study demonstrates the potential of MS01, extracted from marine sponges, as a promising candidate for combating neurodegeneration. The findings reveal that MS01 exhibits properties that may scavenge nitric oxide radicals, inhibit acetylcholinesterase activity, and effectively inhibit reactive oxygen species (ROS). Moreover, the test compound showed no signs of inducing apoptosis or toxicity in normal brain cells, further supporting its potential therapeutic value. However, it is important to note that these results alone are insufficient to establish the protective nature of MS01. Future investigations should include testing MS01 in a zebrafish model and conducting comprehensive pharmacological characterization to enhance our understanding of its potential benefits.

LIST OF ABBREVIATIONS
ROS- Reactive Oxygen Species
PS- Phosphatidylserine
H2DCFDA- 2', 7'-dichlorodihydrofluorescein diacetate
DCF- 2', 7'- dichlorofluorescein
PBS- Phosphate Buffer Saline
PI- Propidium Iodide
AChE- Acetylcholinesterase
iNOS- Anit- Nitric Oxide Scavenging
DTNB- 5,5′-dithiobis-(2-nitrobenzoic acid)
DMSO- Dimethylsulfoxide
L929- Normal Fibroblast Cell Line from Mouse
U251- Human Malignant Glioblastoma Cell Line
HMC3- Human Microglial Clone 3 Cell Line
TCA- trichloroacetic acid
NOS2- Nitric Oxide Synthase 2
FITC- Fluorescein isothiocyanate

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CONFLICT OF INTEREST
We have no conflicts of interest associated with this publication, and there has been no financial support for this work that could have influenced its outcome. As the Corresponding Author, I confirm that the manuscript has been read and approved for submission by all the named authors.

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AUTHORS CONTRIBUTION:
The work was performed by Priyadharshini, who also wrote the paper, and was led by Dr. Jaynthy, who also gave the manuscript some final editing.

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