RESEARCH ARTICLE

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Red Sea Sponge Extract Callyspongia siphonella and its Metabolites Induced Anticancer Activity in 2D and 3D Culture of Colon Cancer Cells

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Abstract

Colorectal Cancer (CRC) significantly contributes to global cancer-related mortality and morbidity. *Callyspongia siphonella* (*Callyspongia sp*.), a Red Sea sponge, has shown promising activity as an anticancer extract and a source of anticancer-active compounds. This study sought to determine the effects of *Callyspongia siphonella* and its metabolites on HCT-116 colon cancer cells. Cell viability assays showed that *Callyspongia sp*. inhibited in a dose-dependent manner, the growth of HCT-116 cell lines with IC_{50} values of 64.8±17 ug/ml on 2D culture and 141.1±6.8 ug/ml on 3D culture. The purified compounds Sipholenol-A and Sipholenone-A have an IC_{50} of 48.9±2.2 uM and 47.1±1.2 uM respectively. Following *Callyspongia sp*. treatment of HCT-116, cell cycle analysis showed arrest at G2/M.flow cytometry analysis showed an increase in total apoptosis due to *Callyspongia sp* treatment. Moreover, mitochondria membrane potential has been reported to be depolarized due to *Callyspongia sp*. which is an extra sign of apoptosis. Further investigations are needed to explain the particular underlying mechanisms of *Callyspongia sp*. extract and its metabolites Sipholenol-A and Sipholenone-A to explore their therapeutic potential in treating colon cancer.

Keywords: Red Sea sponge- *Callyspongia siphonella*- colon cancer cell lines- cytotoxic activity

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Introduction

The incidence of colorectal cancer (CRC) has significantly increased in recent years and is now the third most common cancer type. This type of cancer became the second leading cause of cancer-related death worldwide because there was a lack of therapeutic treatment and evaluation assessment [1]. CRC accounted for 10% of cancer incidence worldwide and 9.4% of cancer deaths in 2020. By 2040, it is anticipated that there will be about 3.2 million new cases of CRC worldwide based on projections for rising populations, advancing age, and advancement in society [2]. The primary explanation for the rising incidence of CRC is the increased exposure to environmental risk factors brought on by dietary and lifestyle changes toward Westernization [3]. Even though they are frequently ineffective and have many fatal side effects, conventional cancer treatments are still the most widely used form of therapy [4].

An area of cancer research that shows great promise is the exploration of novel pharmaceuticals derived from natural sources, which exhibit a reduced incidence of adverse effects. It is well known that the marine environment is a significant source of novel bioactive natural products. These products have structural and chemical characteristics that are frequently missing from the natural products found on land [5]. The vast majority of marine natural products have been extracted from invertebrates, with Porifera being cited as the primary source [6]. Natural compounds derived from marine organisms have attracted increasing attention as potential innovative cancer treatments in recent decades, as they are the source of the majority of bioactive compounds [7]. The Red Sea contains a diverse array of microorganisms and macroorganisms, a substantial portion of which have yet to be thoroughly investigated. Over the past two decades, researchers have identified a total of 58 previously unknown species that exhibit exclusive distribution within the Red Sea [8].

Marine sponges, which are classified under the Phylum Porifera, demonstrate a notable prevalence and variety owing to their soft-bodied characteristics and stationary behavior. The estimated global count of documented sponge species is approximately 8,500 [9]. Sponge metabolites exhibit significant therapeutic potential for treating human diseases as a result of their structural diversity, which makes them well-suited for the studies of marine natural product chemists [10]. Multiple cellular and

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molecular mechanisms, such as cell cycle arrest, apoptosis, and anti-inflammatory activities, have been reported as being responsible for the potential anticancer activities of metabolites derived from marine sponges [11, 12]. The family Callyspongiidae is part of the Haplosclerida order of animals and is home to the Callyspongia genus. *Callyspongia siphonella* (Callyspongia sp.) (Levi, 1965) is abundantly distributed in significant quantities throughout the Gulfs of Aqaba and the coastal regions of Saudi Arabia [13].

This study aims to assess the growth inhibition, viability, and apoptotic effects of an organic extract derived from Callyspongia sp., sourced from the coastal regions of the Saudi Red Sea. Moreover, this study involved the evaluation of Callyspongia sp. extract on HCT116 cancer cell lines using both 2D and 3D cell culture models. This investigation represents the initial documentation of the effectiveness of Callyspongia sp. extract on multi-cellular spheres. This research, with its robust and compelling dataset, represents a noteworthy contribution to the burgeoning field of marine natural products and their potential applications in colon cancer therapeutics. The comprehensive understanding garnered from this study sets the stage for future research avenues and enhances the prospects of harnessing Red Sea sponge extracts as a promising arsenal in the fight against colon cancer.

Materials and Methods

Sponge Material

In June 2022, the Red Sea sponge Callyspongia sp. (graphical abstract) was obtained through SCUBA diving from Shark Reef (Qita'Al Qursh) (N020o07'57.8", E040o05'57.8") located near Al-Lith, Saudi Arabia. A voucher specimen has been deposited at the Zoological Museum of the University of Amsterdam, bearing the identification number ZMAPOR19765. In addition, a specimen of an identification document bearing the code number DY-KSA-22 was deposited within the Red Sea Invertebrates collection at King Abdulaziz University. The sponge exhibited a tubular structure characterized by bifurcating divisions, presenting a sleek outer surface with a pink hue and slender walls. The compressibility of the sponge was observed, as it displayed a relatively soft texture while also demonstrating resistance to tearing. After the collection process, the sponge material was promptly placed on ice and subsequently frozen for further processing. Callyspongia sp. were subjected to isolation to get the purified compounds Sipholenol A and Sipholenone A as described previously [14] .

Cell Viability Assay

The Callyspongia sp.'s extract impact on the viability of HCT-116 cells was measured using the MTT assay (M1020, Solarbio, Beijing, China). In this study, $3x10^3$ HCT-116 cells were cultured per well in a 96-well plate and allowed overnight to adhere. Subsequently, the cells were treated with varying concentrations (7.8-15.5-31.25-62.5-125 ug/mL) of Callyspongia sp. extract, along with a vehicle control consisting of 0.5% DMSO or (5-10-20-40-80 uM) of Sipholenol A and Sipholenone A. The treatment duration was 48 hours. Following incubation at a temperature of 37°C for a duration of 4 hours, a volume of 100 µL of the MTT solution with a concentration of 5 mg/mL was introduced into each individual well. Subsequently, the liquid portion above the sedimented material was removed, and a volume of 100 µL of dimethyl sulfoxide (DMSO) was introduced into each well, followed by an incubation period of 10 minutes at a temperature of 37 °C. Cell viability was determined by measuring the absorbance at 490 nm using a microplate reader (BioTek, Winooski, VT, USA) and converting the obtained optical density (OD) values. The half-maximal inhibitory concentration (IC_{50}) was determined using GraphPad 9.0 software (GraphPad Software Inc., California, United States).

Cell apoptosis assay

The assessment of apoptosis was performed following the manufacturer's recommended protocols. The apoptosis detection kit (PI/Hoechst 33342) manufactured by Solarbio, a company based in China, was employed for this specific objective. Following this, a FACSAria III flow cytometer, produced by BD Bioscience and based in San Jose, CA, USA, was utilized to gather a total of 10,000 events. The HCT116 cells were exposed to a concentration of IC_{50} (64.8±17 µg/mL) for a duration of 48 hours prior to performing the apoptosis assay. The determination of the apoptotic percentage involved the combination of counts for early apoptotic cells (Hoechst+PI-) and late apoptotic cells (Hoechst+PI+).

Cell cycle assay

Following a 48-hour exposure to *Callyspongia siphonella* extract, HCT116 cells were subsequently washed with phosphate-buffered saline (PBS). Following that, the cells were turned immobile through exposure to 70% ethanol that had been pre-chilled for an extended duration. Subsequently, the cells were subjected to a rinse with phosphate-buffered saline (PBS) and subsequently treated with 100 µL of RNase A for a period of 30 minutes at a temperature of 37°C. Subsequently, a 400 µL volume of propidium iodide (PI) was added to the cells and incubated for 30 minutes in the absence of light at a temperature of 4°C. The experimental procedures were conducted following the protocol provided by the DNA Content Quantitation kit (Solarbio, Beijing, China). A sum of 10,000 events was obtained through the utilization of the FACSAria III flow cytometer.

Cell scratch-wound healing assay

A total of 25 x 10^3 HCT116 cells were evenly distributed in each well of the six-well plates, and a period of overnight incubation was provided to facilitate cell adhesion. The cells were subjected to treatment with Mitomycin C (5 g/mL; Roche, Germany) for a period of two hours with the aim of suppressing their proliferation. A vertical incision was created in the central area of the well, subsequently capturing images of the monolayer of injured cells at 0 hours, 24 hours, and 48 hours for both experimental groups. The assessment of the wound

healing gap was performed utilizing ImageJ, a software application created by the Laboratory for Optical and Computational Instrumentation (LOCI) at the University of Wisconsin.

Evaluation of mitochondrial membrane potential (JC-1 Assay)

After the trypsinization procedure, the cells were quantified prior to dilution using a culture medium that consisted of serum (DMEM, 10% FBS that had been heat-inactivated, 1% antibiotics, and 1% L-glutamine). The cells were introduced into the 24-well plate at a concentration of 1.5 x 105 cells per mL, with the addition of 1 mL of the cell suspension. The plate was subsequently exposed to a 24-hour incubation period at a temperature of 37 °C, within an environment consisting of 5% CO2 and 95% relative humidity. After the designated period of incubation, the aqueous component of the specimen was carefully extracted, and a treatment was applied at a concentration that effectively inhibits 50% of the intended target. Every experimental trial included wells for both negative and positive controls. The wells were first emptied of any substances and then washed two times with phosphate-buffered saline (PBS). Following this, 500 µL of serum-free culture medium and 500 µL of JC-1 dyeing working solution were added to each well. Subsequently, the wells were subjected to incubation at a temperature of 37 °C for a period of 20 minutes. JC-1 selectively accumulates in fully functional mitochondria, leading to the formation of J-aggregates that display a red hue. Nevertheless, in the event of depolarization of the mitochondrial membrane potential, a noticeable change occurs in the dye's color, shifting from red to green. The liquid component was discarded and subsequently underwent two washes using a JC-1 dyeing buffer. Following that, a 1 mL volume of serum-free culture medium was added to each well, and images were captured using a Leica fluorescence microscope. The acquired images were subjected to processing using the ImageJ software.

3D Culture

3.0×10^3 cells of HCT116 cells were seeded in 50 µl complete medium in a 96-well ULA round-bottomed plate (CLS7007, corning) and incubated to allow the formation of 3D spheres culture. The incubation was overnight at 37 °C in a cell culture incubator with 5% CO2 levels. For Extract treatments, 5 different concentrations of Callyspongia sp. extract was added in 50 ul to make up the volume to 100 ul. MTT Assay (M1020, solarbio, China) to measure cell viability was conducted and analysis of data was conducted according to the manufacturer's protocol. The cell viability reading was measured after 7 days for 3D culture and the treated cells luminescence reading was normalized to that of vehicle (DMSO) treated cells.

Statistical analysis

The data is displayed using the mean value along with the standard deviation (SD). The current investigation utilized the Student's t-test to assess the differences between the control group (DMSO) and the organic extract of Callyspongia sp. within the framework of statistical analysis. The calculations were conducted utilizing the GraphPad Prism 9.0 software (GraphPad, San Diego, CA, USA). The critical threshold for determining statistical significance was established as a p-value of 0.05. In the field of statistical analysis, it is customary to represent the level of statistical significance using asterisks. Specifically, an asterisk (*) is used to indicate a significance level of $P<0.05$, two asterisks (**) denote a significance level of $P<0.01$, three asterisks (***) signify a significance level of $P<0.001$, and four asterisks (****) indicate a significance level of $P<0.0001$. Three separate experiments were conducted for each possible outcome.

Results

The effect of Callyspongia siphonella extract on HCT116 cell morphology and cell proliferation

In a 2D cell culture, the application of Red Sea sponge extracts resulted in a remarkable dose-dependent reduction in HCT116 cell viability but not normal cell HDF. Specifically, the viability exhibited a substantial decrease from an initial baseline generating an IC_{50} of 64.8±17 µg/mL in HCT116 while it was 218.8 ± 3.2 µg/mL for the HDF cells (Figure 1A). Microscopic examination corroborated these findings, revealing distinct morphological changes indicative of apoptosis, including cell shrinkage and nuclear condensation (Figure 1B). These visual cues align seamlessly with the quantitative assessments, offering a comprehensive perspective on the extract's potential to induce apoptosis in HCT116 cells (Figure 1A, B). The identification of an IC_{50} concentration, such as 64.8±17 µg/mL, serves as a pivotal foundation for subsequent investigations in the following assays.

The effect of Callyspongia siphonella extract on apoptosis and cell cycle

In our investigation of the potential therapeutic effects of Callyspongia sp. extract on HCT116 colon cancer cells, we conducted a comprehensive analysis that involved multiple experimental approaches, including cell cycle analysis and apoptosis by flow cytometry. A significant increase was observed in apoptotic cells, reaching approximately 9.1% compared to .6% with control (Figure 2A,2B). The subsequent flow image,

Table 1. The IC_{50} of Callyspongia Sp. on 2D and 3D Cultures

	Type of culture	Antiproliferative Activity of Callyspongia Sp IC50 ± SEM (ug/ml or uM)
Callyspongia sp.	2D	64.8±17 ug/ml
Callyspongia sp.	3D	141.1±6.8 ug/ml
Sipholenol A	2D	48.9±2.2uM
Sipholenone A	2D	47.1±1.2uM

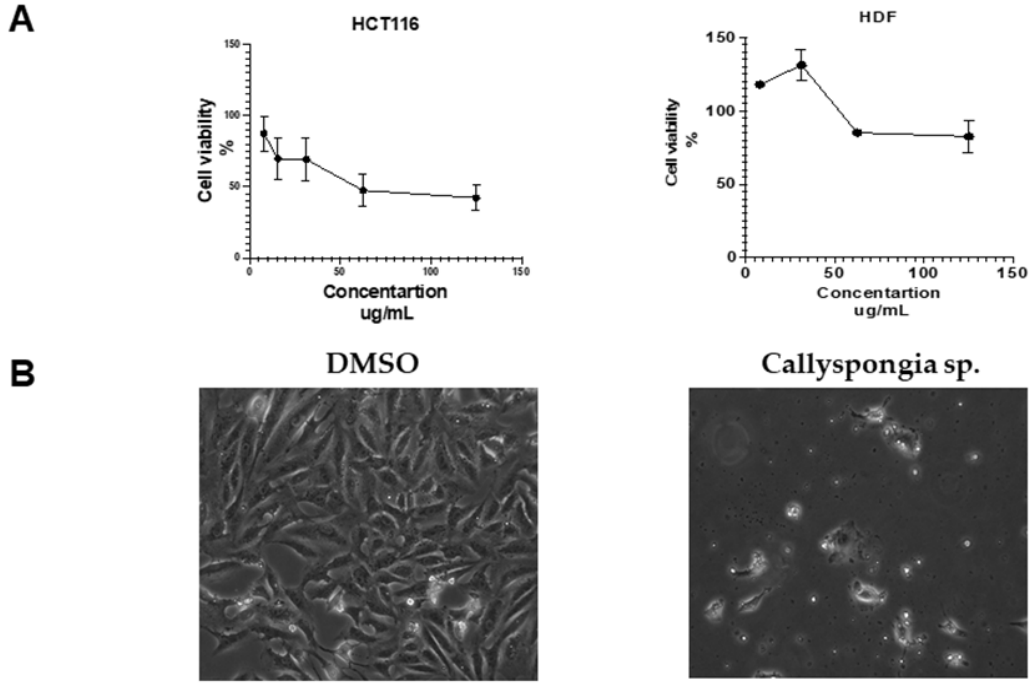

Figure 1. Cytotoxic Effect of *Callyspongia sp*. Extract on HCT116 Cells. (A) Dose response curve for Callyspongia sp. from HCT116 and HDF cells. (B) Representative images show HCT116 cellular morphology treated with DMSO and Callyspongia sp. extract.

corresponding to twice the IC_{50}, displayed a lower level of total apoptosis, however, further elevation in necrosis compared to DMSO control and IC_{50} concentration, reinforcing the extract's concentration-dependent pro-necrotic effects (Figure 2B). In the DMSO control group, the flow cytometry analysis revealed a minimal apoptotic response, indicating the normal growth and survival of HCT116 cells under control conditions (Figure 2A,2B). Furthermore, our investigation extended to the cell cycle dynamics, a critical aspect of cellular regulation frequently dysregulated in cancer [15]. In the DMSO control, we observed the typical distribution of cells

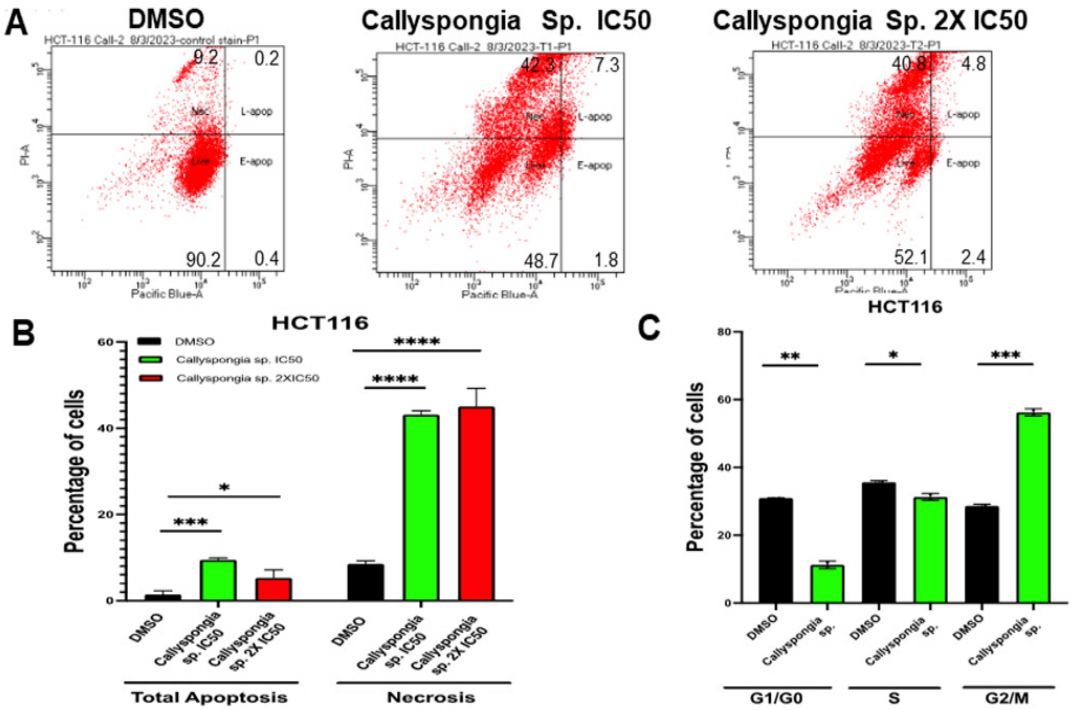

Figure 2. The Effect of *Callyspongia sp*. Extract on Apoptosis and Cell Cycle Analysis of HCT116 Cells Using Flow Cytometric Analysis. (A) The percentage of HCT116 cells represented in graphical presentation. (B) Quantification of cells in total apoptosis and necrosis. (C)Quantification of cells in different stages of cell cycle. Data are represented as mean ± SD. *Significant difference at $p < 0.05$. **Significant difference at $p < 0.01$. ***Significant difference at $p < 0.001$. ****Significant difference at $p < 0.0001$

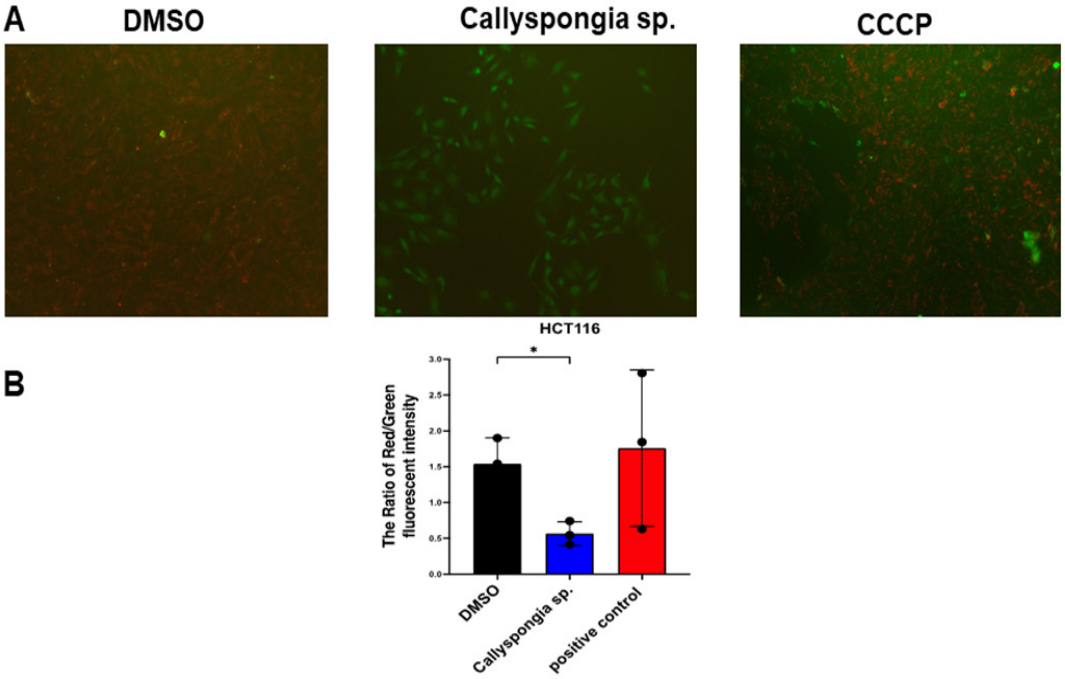

Figure 3. The Effect of *Callyspongia sp*. on Mitochondrial Membrane Potential (MMP). (A) HCT116 cells were stained with JC-1 dye after being treated with Callyspongia sp. extractor DMSO and. (B) Quantification of the ratio of red to green fluorescent intensity in HCT116 cells. Data are displayed as mean ± SD. *Significant difference at $p < 0.05$. **Significant difference at $p < 0.01$.

across the cell cycle phases, with 32% in G1/G0, 38% in S phase, and 30% in G2/M phase. However, treatment with Callyspongia sp. extract at the IC_{50} concentration hinted at potential disruptions in cell cycle progression, suggesting a propensity to induce cell cycle arrest with 55% at G2/M phase (Figure 2C).

The effect of Callyspongia siphonella on mitochondrial membrane potential

Next investigation was into the impact of Callyspongia sp. on mitochondrial membrane potential (Δψm) to provide valuable insights into the potential influence of the extract on cellular energetics. In the DMSO control condition, cells exhibited a typical mitochondrial membrane

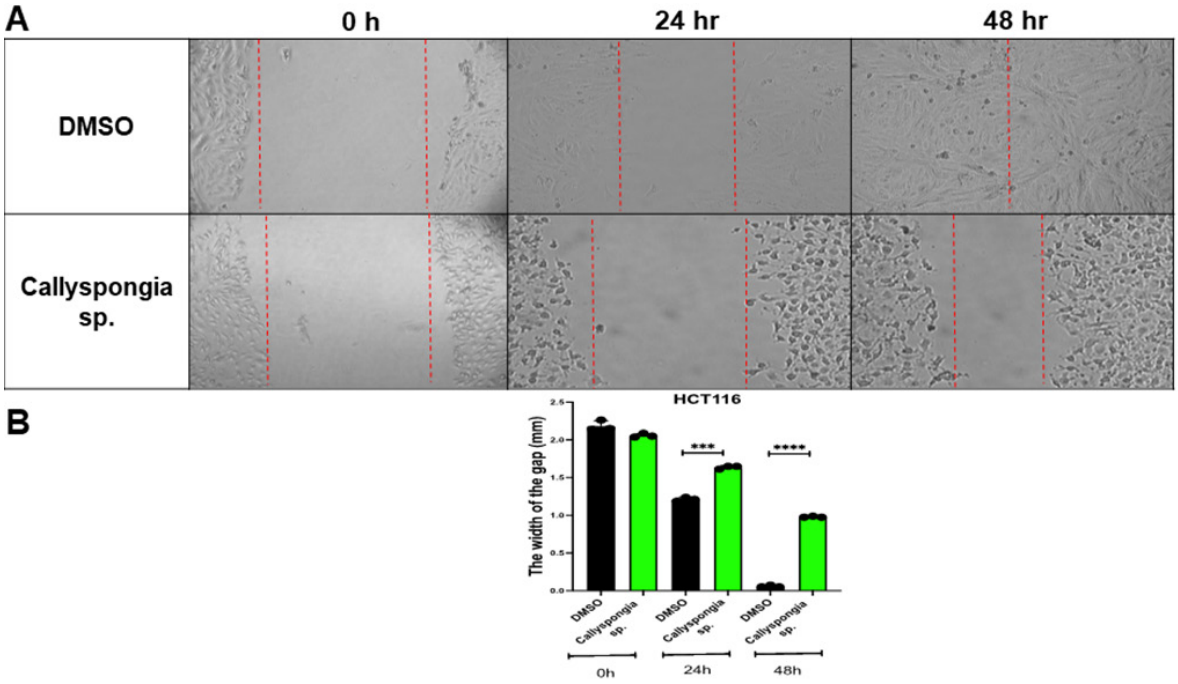

Figure 4. The Effect of *Callyspongia siphonella* on HCT116 Migrations. (A) Representative images show migration of HCT116 cells treated withCallyspongia sp. extract or DMSO at 0,24 hrs and 48 hrs time points. (B) Quantification of gap's width after treatment with Callyspongia sp. or DMSO. Data are displayed as mean ± SD. **Significant difference at $p < 0.01$. ***Significant difference at $p < 0.001$.

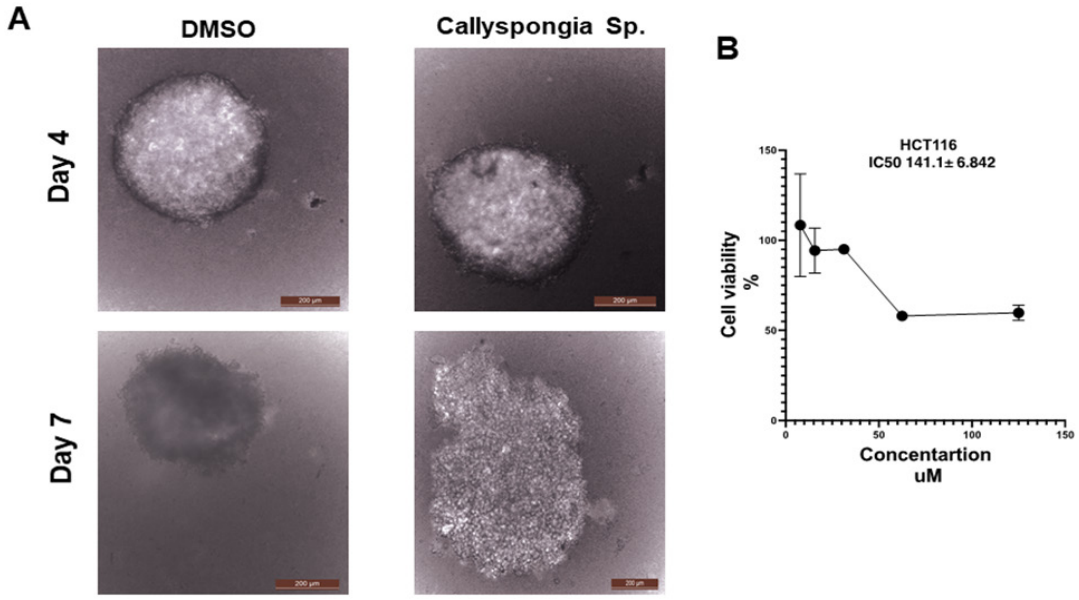

Figure 5. Cytotoxic Effect of *Callyspongia sp.* Extract on HCT116 Multicellular Spheres (MCS). (A) Representative images of spheres show HCT116 cellular morphology before being treated (Day4) and after being treated with DMSO and Callyspongia sp. extract (Day7). (B) Dose response curve for Callyspongia sp. from HCT116 cells.

potential, reflective of the baseline physiological state. The ratio of red to green fluorescence intensity was quantified and set as the reference level.

Upon treatment with Callyspongia sp. extract, a notable alteration in mitochondrial membrane potential was observed. The fluorescence intensity ratio exhibited a reduction to .5 with Callyspongia sp. compared to 1.5 with DMSO control ($P<0.05$, Figure 3A,3B). This reduction suggests a potential impact of the extract on mitochondrial function, emphasizing its ability to modulate the electrochemical gradient across the inner mitochondrial membrane. CCCP, a known mitochondrial uncoupler, was included as a positive control.

The effect of Callyspongia siphonella on 3D culture

The effect of Callyspongia sp. on HCT116 cells viability using a 3D culture system at 7 days revealed compelling insights. This technique was adopted because of the superior ability of the 3D model to replicate the complex microenvironment of tumors and highlights its relevance in assessing the effects of potential therapeutic agents [16]. At the 4-day mark, the Callyspongia sp. was added and a concentration-dependent response was observed, where the treated samples exhibited a distinct decrease in viability and the IC_{50} was calculated to be 141.1±6.8 ug/ml (Figure 5A,5B). Importantly, the 3D culture model, with its intricate mimicry of *in vivo* conditions, demonstrated even more pronounced alterations in spheroid structure and integrity (Figure 5B).

Extract of Callyspongia siphonella inhibits HCT116 migration

Next investigation was on the potential anti-migratory effects of Callyspongia sp. extract on HCT116 colon

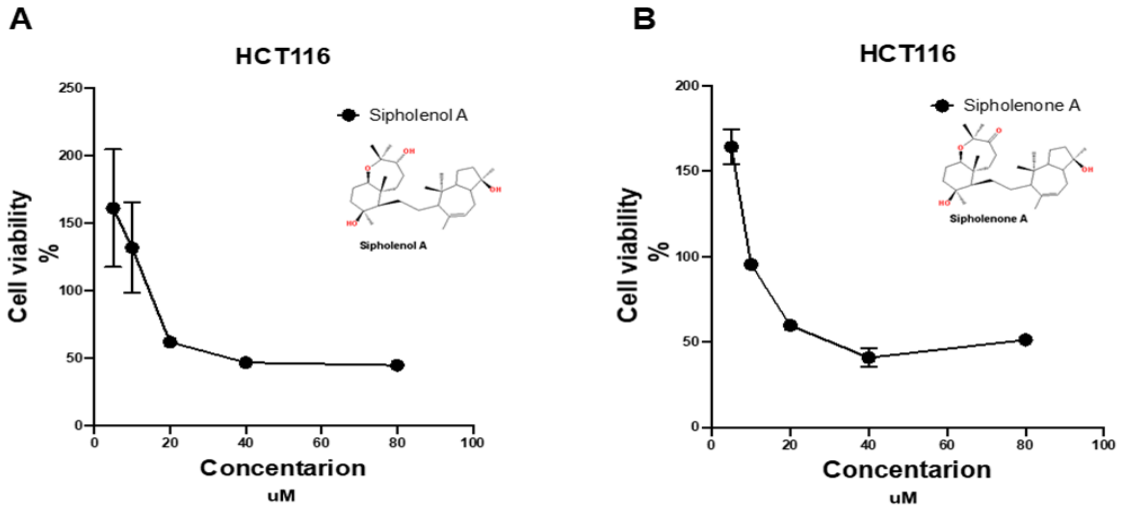

Figure 6. Cytotoxic Effect of Sipholenol A and Sipholenone A on HCT116 Cells. (A) Dose response curve for Sipholenol A from HCT116 cells. (B) Dose response curve for Sipholenone A from HCT116 cells.

cancer cells. In the DMSO control group, HCT116 cells exhibited a characteristic migratory pattern, covering the full gap within 48h (Figure 4A). This baseline observation allowed us to discern the inherent migratory capabilities of the cells. Upon treatment with Callyspongia sp. extract, a remarkable inhibitory effect on cell migration was evident across various time points 24h and 48h (Figure 4A,4B). Remarkably, at 48 hours post-treatment, the inhibitory effects reached their peak (Figure 4A,4B). The cumulative inhibitory effects observed at 48 hours are suggestive of potential downstream effects on cellular mechanisms governing metastatic potential. These findings, supported by quantitative assessments, contribute to our understanding of the extract's multifaceted influence on cancer cell behavior (Figure 4B).

The effect of purified compounds Sipholenol A and Sipholenone A on cell viability

Next investigation was conducted using purified compounds Sipholenol A and Sipholenone A from Callyspongia sp. extract on HCT116 cell's viability and elucidating the potential molecular interactions within the cellular environment. A dose response curve was generated to calculate the IC_{50} which was for Sipholenol A 48.9±2.2uM and 47.1±1.2uM for Sipholenone A (Table 1, Figure 6A, 6B).

Discussion

Colorectal cancer (CRC) is ranked second as the most fatal cancer and the third most frequent malignancy, affecting 1 in 25 women and 1 in 23 men leading to over 608000 deaths, or 8% of all cancer-related deaths [17]. Recently, Red Sea marine sponges such as *Callyspongia sp*. have gained interest among researchers as a source of bioactive compounds that have potential to be used against cancer [8]. The investigation into the anticancer activity of Red Sea sponge extracts derived from *Callyspongia sp*. on colon cancer cells HCT116 in both two-dimensional (2D) and three-dimensional (3D) culture systems have yielded a wealth of compelling findings that shed light on the potential therapeutic applications of these marine natural products. For 2D cells a reduction in cell viability generating an IC_{50} of 64.8±17 ug/ml, indicative of a potent cytotoxic effect, aligns synergistically with the outcomes observed in the 3D culture model with IC_{50} of 141.1±6.8 µg/mL. This congruence between the two distinct culture systems underscores the consistent and robust impact of Red Sea sponge extracts on inhibiting the proliferation of HCT116 colon cancer cells. A concentration-dependent apoptotic response suggests that the extract has a notable impact on triggering programmed cell death, a crucial aspect in combating cancer [18]. Notably on the cell cycle, the proportion of cells in G2/M phase increased, indicating a potential G2/M arrest, while S phase and G1 phase decreased compared to the control. This observed cell cycle dysregulation aligns cohesively with the reduction in cell viability, apoptotic response emphasizing the multifaceted impact of *Callyspongia sp*. extracts on the fate of HCT116 cells.

As mitochondrial dysfunction is often associated with various diseases, including cancer, understanding the extract's impact on these vital cellular structures provides valuable insights into its broader therapeutic potential [19]. This exploration into the effect of *Callyspongia sp*. on mitochondrial membrane potential lays the groundwork for further investigations into the underlying molecular mechanisms. The observed depolarization of mitochondrial membrane potential (MMP) due to the effect of *Callyspongia sp*. was detected indicating a mitochondria function distribution underscore the extract's potential implications for cellular bioenergetics and warrant in-depth studies to elucidate the specific pathways involved.

An anti-migration capability of the extract was detected by curbing the migratory capabilities of HCT116 cells over an extended duration at two time points. The consistency in inhibitory effects suggests a potential disruption of critical signaling pathways essential for cancer cell migration and holds promise for advancing anti-metastatic interventions [20]. Two major compounds Sipholenol A and Sipholenone A have been isolated from *Callyspongia sp*. previously [14]. Therefore, there were chosen to be tested against HCT116 and the IC_{50} was 48.9±2.2uM and 47.1±1.2uM respectively.

These findings collectively highlight the promising potential of both compounds as candidates for *in vivo* experiments.

To unravel the full therapeutic implications, it is imperative to conduct docking, molecular dynamics simulations, validation experiments and in-vivo studies, assessing factors such as bioavailability, pharmacokinetics, and overall effectiveness within a living organism.

In conclusions, these findings provide a solid foundation based on the AMIRDA standard reporting recommendations for future investigations on *Callyspongia sp*., paving the way for more in-depth mechanistic studies, *in vivo* experiments, and the isolation of specific bioactive compounds following [22]. The compelling dataset obtained from this research contributes significantly to our understanding of *Callyspongia sp*.extract's intricate effects on cellular processes, positioning it as a promising avenue for the development of targeted interventions against colon cancer.

Author Contribution Statement

Conceptualization, F.A.A.; methodology, F.A.A; software, F.A.A., validation, F.A.A., ;formal analysis, F.A.A; investigation, F.A.A. ; resources, F.A.A.; data curation, F.A.A., writing-original draft preparation, F.A.A; writing-review and editing, F.A.A.,; visualization, F.A.A; supervision, F.A.A; project administration, F.A.A.; funding acquisition, F.A.A

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Conflicts of Interest

The author declares that he has no conflicts of interest in this work.

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