





Red Sea Sponge *Callyspongia siphonella* Extract Induced Growth Inhibition and Apoptosis in Breast MCF-7 and Hepatic HepG-2 Cancer Cell Lines in 2D and 3D Cell Cultures

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Introduction: The increasing incidence of cancer diseases necessitates the urgent exploration of new bioactive compounds. One of the trends in drug discovery is marine sponges which is gaining significant support due to the abundant production of natural pharmaceutical compounds obtained from marine ecosystems. This study evaluates the anticancer properties of an organic extract from the Red Sea sponge *Callyspongia siphonella* (*C. siphonella*) on HepG-2 and MCF-7 cancer cell lines.

Methods: *C. siphonella* was collected, freeze-dried, and extracted using a methanol-dichloromethane mixture. The extract was analyzed via Liquid Chromatography-Mass Spectrometry. Cytotoxic effects were assessed through cell viability assays, apoptosis detection, cell cycle analysis, mitochondrial membrane potential assays, scratch-wound healing assays, and 3D cell culture assays.

Results: Fifteen compounds were identified in the *C. siphonella* extract. The extract showed moderate cytotoxicity against MCF-7 and HepG-2 cells, with IC₅₀ values of 35.6 ± 6.9 µg/mL and 64.4 ± 8 µg/mL, respectively, after 48 hours of treatment. It induced cell cycle arrest at the G2/M phase in MCF-7 cells and the S phase in HepG-2 cells. Apoptosis increased significantly in both cell lines, accompanied by reduced mitochondrial membrane potential. The extract inhibited cell migration, with notable reductions after 24 and 48 hours. In 3D cell cultures, the extract had IC₅₀ values of 5.1 ± 2 µg/mL for MCF-7 and 166.4 ± 27 µg/mL for HepG-2 after 7 days of treatment, showing greater potency in MCF-7 spheres compared to HepG-2 spheres.

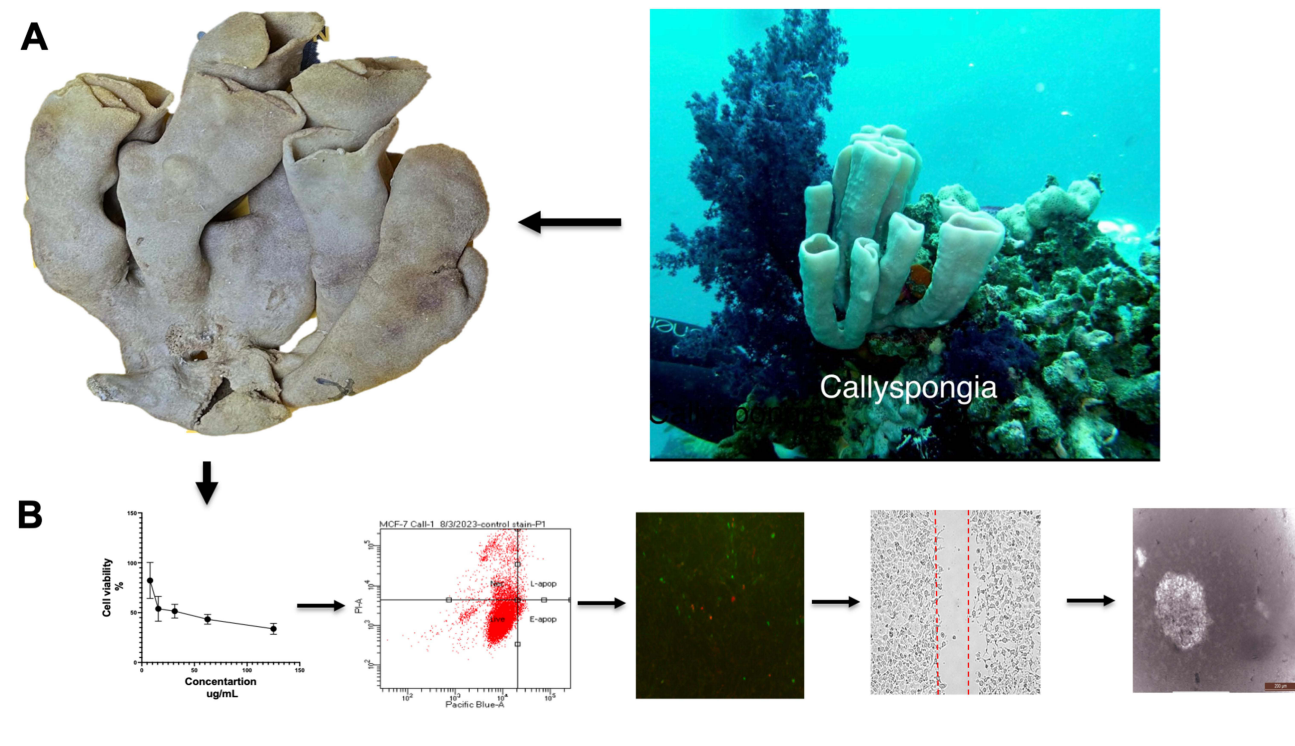
Discussion and Conclusion: The anticancer activity is attributed to the bioactive compounds. The *C. siphonella* extract's ability to induce apoptosis, disrupt mitochondrial membrane potential, and arrest the cell cycle highlights its potential as a novel anticancer agent. Additional research is required to investigate the underlying mechanism by which this extract functions as a highly effective anticancer agent.

Keywords: Red Sea sponge, *Callyspongia siphonella*, breast cancer cell lines, hepatic cancer cell line, 2D and 3D cultures, cytotoxic activity

Introduction

Marine organisms have demonstrated significant effectiveness as a valuable source of novel biologically active substances for the progress of pharmaceutical research and development, a number of which are currently marketed, or in different phases of clinical trials (<https://www.marinepharmacology.org/>). Considering this, numerous scientists have

Graphical Abstract



worked hard since the mid-20th century to ascertain unique bioactive compounds derived from marine organisms.¹ The Red Sea harbors a significant variety of micro and macroorganisms, many of which have remained largely unexplored until the present time. In the last 20 years, scientists have discovered 58 new species that are exclusive to the Red Sea.² The coral reefs situated across the center of the Red Sea, particularly along the Saudi Arabian coastline, represent a distinct and insufficiently researched ecosystem in comparison to other coral reef systems of similar biodiversity. This particular environment holds the potential for the discovery of novel bioactive compounds that possess unexplored pharmacophores.³

Due to the alarming rise in the prevalence of cancer illnesses, there is an imperative necessity to discover novel bioactive compounds. Extensive resources and substantial funding have been allocated toward the progression of cancer treatments, as indicated by the numerous research initiatives undertaken since the latter portion of the 19th century.⁴ Resistance to anticancer drugs is widely acknowledged and represents a significant issue within the domain of cancer research, accounting for approximately 90% of chemotherapy failures.⁵ The abundance of naturally derived pharmaceutical products from the oceans is a major factor supporting the current trend in natural drug discovery theory.⁶ This is because of the fact that the marine environment possesses the highest levels of biodiversity and chemical heterogeneity among all ecosystems on Earth.⁷ Marine-derived bioactive compounds have demonstrated significant potential in the therapeutic management of diverse human diseases, such as cancer.⁸ In recent times, a substantial number of marine active components, totaling at least 3000, have undergone rigorous testing to evaluate their potential as anticancer agents.⁹ Multiple cellular and molecular mechanisms have been reported to underlie the potential anticancer activities of metabolites derived from marine sponges. These mechanisms include cell cycle arrest, apoptosis, anti-inflammatory activities, and chemo-sensitization to radiotherapy.¹⁰⁻¹³ In addition, marine bioactive chemicals derived from marine organisms have been shown to possess various beneficial properties, including antioxidant, anti-inflammatory, antimicrobial, anticoagulant content, immune-stimulatory, anti-hypertensive, and wound recovery effects.¹⁴

Marine sponges, belonging to the Phylum Porifera, exhibit a high degree of abundance and diversity due to their soft-bodied nature and sedentary lifestyle. Globally, the total number of documented sponge species is estimated to be around 8500.¹⁵ Members of the genus *Callyspongia* (family Callyspongiidae) are prominent examples of this phylum. A considerable number of over 60 species exhibit a broad distribution within tropical marine environments.¹⁶ In this study, we evaluate the growth inhibition, viability, and primary apoptotic effects of an organic extract obtained from *Callyspongia siphonella* (*C. siphonella*), which was obtained from the Saudi Red Sea coasts. These assessments were conducted on HepG2 and MCF-7 cancer cell lines on 2D and 3D cell cultures, marking it as the first report of the efficacy of *C. siphonella* extract on multi-cellular spheres.

Materials and Methods

Sponge Material

The Red Sea sponge *C. siphonella* was collected from Shark Reef (*Qita 'Al Qursh*) (N020°07'57.8", E040°05'57.8") off Al-Lith, Saudi Arabia, via SCUBA diving in June 2022. A voucher specimen had been placed at the Zoological Museum of the University of Amsterdam under number ZMAPOR19765. Furthermore, an identification document specimen with code number DY-KSA-22 was placed in King Abdulaziz University's Red Sea Invertebrates collection. The sponge displayed a tubular morphology with dichotomous divisions, featuring a smooth external surface with a pink coloration and thin walls. The consistency of the sponge exhibited compressibility, being relatively soft in nature, yet proving resistant to tearing. Following the collection process, the sponge material was placed on ice and frozen directly until further processing.

Extraction and Isolation

The sponge was freeze-dried before extraction. At room temperature, three separate 150 mL extractions of the freeze-dried sponge material (20 g) were made using a methanol-dichloromethane (1:1) mixture. After filtering the combined extracts, the filtrate was vacuum-concentrated, and the resultant extract was used for biological evaluation.

Liquid Chromatography-Mass Spectrometry Analysis

The examination of the organic extract of *C. siphonella* was conducted using the ExionLC instrument (2.0 series, AB Sciex, Toronto, Canada) using the LC-MS/MS methodology. The LC instrument used in this study is connected to a time-of-flight (TOF) mass spectrometer triple (TripleTOF 5600+, AB Sciex, Toronto, Canada). The mass spectrometer is equipped with a Duo Spray™ source and operates in the electrospray ionization (ESI) mode interface. The LC system utilizes a C18 bonded-phase column with dimensions of 2.1×150 mm and particle size of 2.5 μm. The flow rate of the LC system is set at 0.3 mL/min. The organic extract of *C. siphonella* was subjected to centrifugation at a speed of 10,000 revolutions per minute for a duration of 10 minutes prior to analysis. The volume of the injection was 10 μL, while the column temperature was maintained at 40 °C. Three different mobile phases were used in this study. The mobile phase utilized for positive mode consisted of a 5 mM ammonium formate buffer with a pH of 3, which also included 1% methanol. The mobile phase (B) used in negative mode consists of a 5 mM ammonium formate buffer with a pH of 8, which includes 1% methanol. The mobile phase (C) for both samples consist of 100% acetonitrile. The acquired data were then analyzed using the data processing program MS-DIAL 3.70 software (Yokohama, Kanagawa, Japan) for the purposes of peak picking, deconvolution, formula prediction, deisotoping, and alignment. An investigation was conducted at the Proteomics and Metabolomics Center, located at the Children's Cancer Hospital in Cairo, Egypt, using high-resolution mass spectrometry.

Cell Culture

The MCF-7 human breast cancer cell line and HepG-2 human hepatocellular carcinoma, sourced from the American Type Culture Collection (Manassas, USA), were employed in this investigation. The cells were preserved and cultivated at KFMRC (King Fahd Medical research Center). The RPMI-1640 medium was supplemented with various components, including 10% fetal bovine serum, penicillin/streptomycin (100 U/mL), and Amphotericin B (0.25 μg/mL). The cell

cultures were placed in a warm and moist environment with 5% carbon dioxide and incubated at a temperature of 37 degrees Celsius. Each treatment group was cultivated using identical conditions. Nevertheless, the untreated cells were used as controls as they displayed 100% survival and were not subjected to any medications.

Cell Viability Assay

The viability of MCF-7 and HepG-2 cells was evaluated by employing the MTT assay, which was facilitated by the organic extract obtained from *C. siphonella*. The MTT assay was provided by Solarbio, a company based in Beijing, China. The present study involved the cultivation of MCF-7 and HepG-2 cells in a 96-well plate, with a cell density of 3×10^3 cells per well. The cells were then allowed to adhere overnight. Subsequently, the cells were treated with varying concentrations (7.8–15.5–31.25–62.5–125 $\mu\text{g/mL}$) of *C. siphonella* extract, along with a vehicle control consisting of 0.5% DMSO. The treatment duration was 48 hours. After being incubated at a temperature of 37°C for a period of 4 hours, a volume of 100 μL of the MTT solution, which had a concentration of 5 mg/mL, was added to each 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 assessed by quantifying the absorbance at a wavelength of 490 nm using a microplate reader manufactured by BioTek, located in Winooski, VT, USA. The resulting optical density (OD) values were subsequently converted for analysis. The IC_{50} , representing the concentration at which inhibition is half-maximal, was determined through the utilization of GraphPad 9.0 software, developed by GraphPad Software Inc. in California, United States.

Cell Apoptosis Assay

The assessment of apoptosis was performed following the protocols outlined by the manufacturer. The PI/Hoechst 33,342 apoptosis detection kit from Solarbio, China, was utilized for this purpose. Subsequently, a FACSAria III flow cytometer manufactured by BD Bioscience, located in San Jose, CA, USA, was employed to collect a total of 10,000 events. The MCF-7 and HepG-2 cell lines were subjected to treatment with a concentration of IC_{50} (35.61 and 64.40 $\mu\text{g/mL}$) for a duration of 48 h prior to conducting the apoptosis assay. The calculation of the apoptotic percentage involved the summation of the counts of early apoptotic cells (Hoechst+PI-) and late apoptotic cells (Hoechst+PI+).

Cell Cycle Assay

After subjecting MCF-7 and HepG-2 cells to a 48-hour treatment with *C. siphonella* extract, the cells were rinsed with phosphate-buffered saline (PBS). Subsequently, the cells were immobilized by treating them with 70% pre-chilled ethanol for an extended period of time. Subsequently, the cells were subjected to a rinse with phosphate-buffered saline (PBS) and subsequently treated with 100 μL of RNase A for a duration of 30 minutes at a temperature of 37°C. Following that, a volume of 400 μL of propidium iodide (PI) was introduced to the cells and allowed to incubate for a duration of 30 min under dark conditions at a temperature of 4°C. The experimental procedures were carried out in accordance with the protocol provided by the DNA Content Quantitation kit (Solarbio, Beijing, China). A total of 10,000 events were acquired using the FACSAria III flow cytometer.

Cell Scratch-Wound Healing Assay

A total of 25×10^3 MCF-7 and HepG-2 cells were evenly distributed into the individual wells of six-well plates, and subsequently left undisturbed for an overnight period to facilitate cell adhesion. The cells were treated with Mitomycin C (5 $\mu\text{g/mL}$; Roche, Germany) for a duration of two hours in order to inhibit their proliferation. A vertical scratch was made at the central region of the well, following which the monolayer of wounded cells was captured through photographs at 0, 24, and 48 h 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)

Following the trypsinization process, the cells were enumerated before being diluted using a culture medium containing serum (DMEM, 10% heat-inactivated FBS, 1% antibiotics, and 1% L-glutamine). The cells have been added into the 24-well plate at a concentration of 1.5×10^5 cells per mL, whereby 1 mL of the cell suspension was administered. 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 a humidity level of 95%. Following the incubation period, the liquid portion of the sample was cautiously removed, and a treatment at the concentration that inhibits 50% of the target was administered. Every experimental trial included wells designated for both negative and positive controls. The wells were first cleansed 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. The wells were then incubated at 37 °C for a duration of 20 minutes. JC-1 preferentially accumulates within mitochondria that are fully operational, resulting in the formation of J-aggregates that exhibit a red color. However, if the mitochondrial membrane potential undergoes depolarization, the dye's color transitions from red to green. The liquid portion was discarded and subsequently subjected to two washes using a JC-1 dyeing buffer. Subsequently, a volume of 1 mL of culture medium devoid of serum was introduced into each well, followed by capturing images utilizing a Leica fluorescence microscope. The captured images underwent processing using the ImageJ software.

Cell Viability Assay for 3D Culture

HepG2 or MCF7 cells were seeded in a 96-well ULA round-bottomed plate (CLS7007, Corning) for 3D sphere culture. The seeding density was 3.0×10^3 cells per well in 100 µL of complete medium. The cells that were initially introduced were subjected to incubation at a temperature of 37 °C within a specialized incubator designed for cell culture. This incubation period lasted for the duration of one night, during which the carbon dioxide (CO₂) levels were maintained at 5%. For drug culture treatments, 5 different concentrations of *C. siphonella* extract was added in 50 µL to make up the volume to 100 µL. The assessment of cell viability was performed utilizing the MTT Assay (M1020, solarbio, China), and subsequent data analysis was carried out in accordance with the guidelines provided by the manufacturer. The measurement of cell viability was conducted on the seventh day for the 3D culture, and the luminescence reading of the treated cells was normalized relative to the luminescence reading of the cells treated with the vehicle (DMSO).

Statistical Analysis

The data is presented in the form of the mean accompanied by the standard deviation (SD). The present study employed the Student's *t*-test to compare the distinctions between the control group (DMSO) and the organic extract of *C. siphonella* in the context of statistical analyses. The calculations were performed using the GraphPad Prism 9.0 software (GraphPad, San Diego, CA, USA). A p-value of 0.05 was deemed as the critical threshold for determining statistical significance. In statistical analysis, the level of statistical significance is commonly denoted by asterisks, with * representing a significance level of $P < 0.05$, ** indicating $P < 0.01$, *** indicating $P < 0.001$, and **** indicating $P < 0.0001$. A series of three distinct experiments were conducted for every potential outcome.

Results

Identified Compounds from *C. Siphonella* Extract According to Liquid Chromatography-Mass Spectrometry

A total of fifteen compounds (Figure 1) from different chemical classes were detected by analyzing high resolution mass spectroscopy data (HRMS) (Table 1) (Supplementary Figures 1-4). The chemical compositions for these compounds of both *Callyspongia/Siphonochalina* extracts were determined by comparison of mass spectroscopy data with previously published data in the literature. The analysis of the HRMS data reveals that fourteen of these compounds share three similar chemical formulas suggesting isomer compounds. Eleven compounds were detected in the negative ionization mode only while the rest of four compounds were detected in both negative and positive ionization mode. In addition to the aforementioned activity, (Table 2), lists the pharmacological activity of other compounds that can also be isolated from *Callyspongia/Siphonochalina siphonella*.

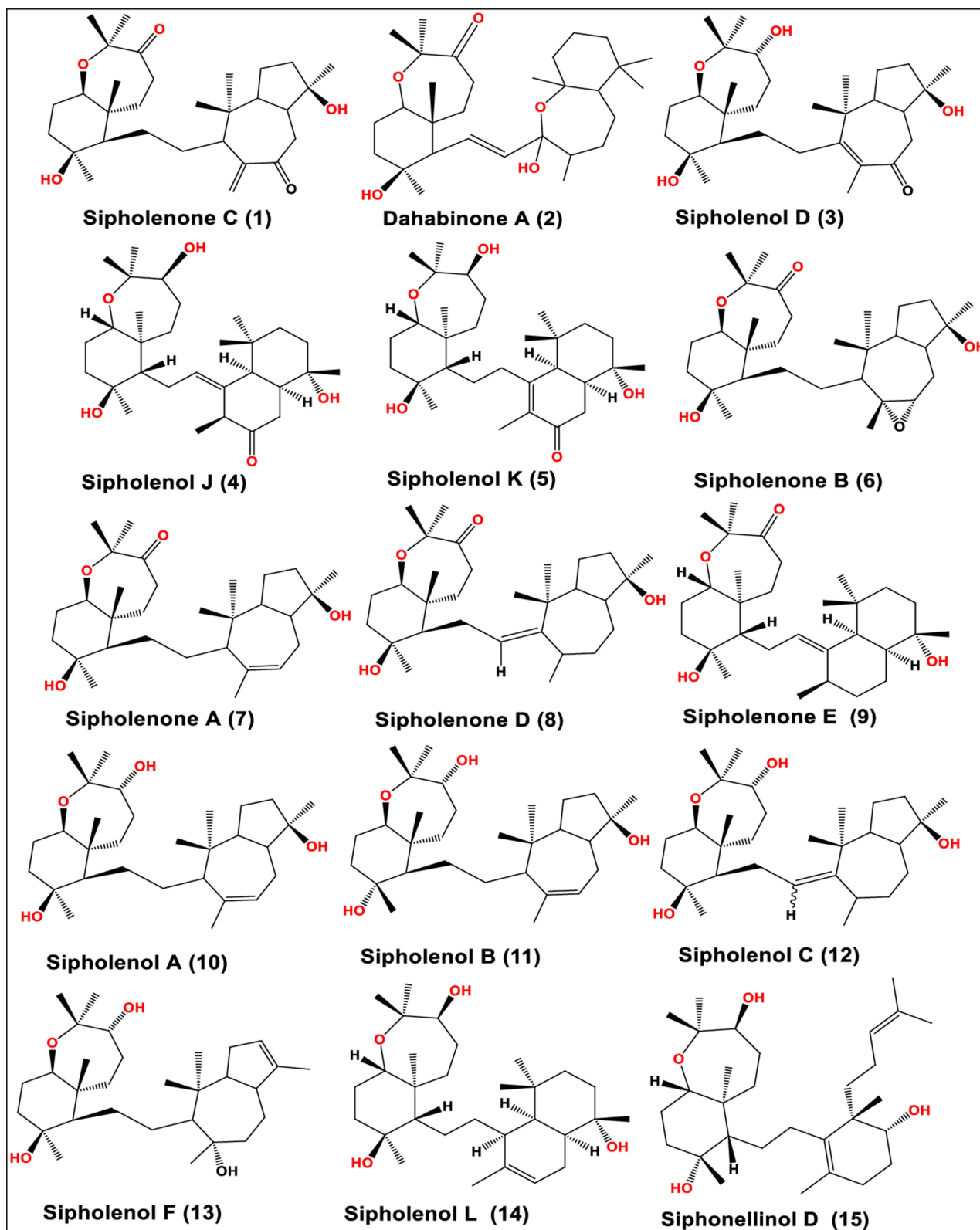


Figure 1 Chemical structures of the listed compounds.

Table 1 List of Triterpenoids/ Triterpenes Compounds Detected in *Callyspongia Siphonella* Extract. (Rt: Retention Time, M±H: Molecular ion±Hydrogen, PPM: Part per Million)

Compound	Name	Suggested formula	R _t	Exact mass M±H	Calc. mass M±H	Error (PPM)
1	Sipholenone C	C ₃₀ H ₄₈ O ₅	17.24427	489.3573 (+ve)	489.35743 (+ve)	-0.3
2	Dahabinone A	C ₃₀ H ₅₀ O ₅	13.86477	489.359 (-ve)	489.35853 (-ve)	1
3	Sipholenol D	C ₃₀ H ₅₀ O ₅	-	-	-	-
4	Sipholenol J	C ₃₀ H ₅₀ O ₅	-	-	-	-
5	Sipholenol K	C ₃₀ H ₅₀ O ₅	-	-	-	-
6	Sipholenone B	C ₃₀ H ₅₀ O ₅	-	-	-	-
7	Sipholenone A	C ₃₀ H ₅₀ O ₄	21.05748	475.3789 (+ve)	475.37817 (+ve)	1.5
8	Sipholenone D	C ₃₀ H ₅₀ O ₄	-	-	-	-
9	Sipholenone E	C ₃₀ H ₅₀ O ₄	-	-	-	-
10	Sipholenol A	C ₃₀ H ₅₂ O ₄	18.57752	475.3776 (-ve)	475.37926 (-ve)	-3.5
11	Sipholenol B	C ₃₀ H ₅₂ O ₄	-	-	-	-
12	Sipholenol C	C ₃₀ H ₅₂ O ₄	-	-	-	-
13	Sipholenol F	C ₃₀ H ₅₂ O ₄	-	-	-	-
14	Sipholenol L	C ₃₀ H ₅₂ O ₄	-	-	-	-
15	Siphonellinol D	C ₃₀ H ₅₂ O ₄	-	-	-	-

Table 2 List of Other Compounds Detected in *Callyspongia Siphonella* Extract

Name	Activity	Reference
Callyspongenol-D	Toxic towards the human mammary carcinoma cell line MCF-7	24
Neviotine-C Neviotine-A	Significant cytotoxic activities and anti-proliferative activity selectively against PC-3 and A549 cell lines	25

The Effect of *C. Siphonella* Extract on MCF-7 and HepG-2 Cells Morphology and Proliferation

Phase contrast microscopy was employed to examine the MCF-7 and HepG-2 cells subsequent to their exposure to either dimethyl sulfoxide (DMSO) or diverse concentrations of the extract derived from *C. siphonella*. The cells were cultured for a duration of 24 hours. The Results indicated a notable reduction in cellular quantities compared to the control group, achieved by decreasing the number of cells and driving changes in cell morphology. The observed cellular characteristics included stress-induced phenotypes such as cell shrinkage and disruptions in the cell membrane, ultimately leading to cell death (Figure 2A and B). The application of *C. siphonella* extract (7.8–15.5–31.25–62.5–125 µg/mL) for 48 hours led to a decrease in cell proliferation in MCF-7 and HepG-2 cells, with the extent of reduction being dependent on the dosage administered (Figure 2A and B). The IC₅₀ values for MCF-7 and HepG-2 at 48 hours were determined to be 35.6±6.9 µg/mL and 64.4±8 µg/mL, respectively (Table 3). Furthermore, the analysis of *Callyspongia* extract revealed no substantial inhibitory impact on normal HDF cells, even when exposed to the highest concentration (Supplementary Figure 5).

The Effect of *C. Siphonella* Extract on Cell Cycle

In order to gain a deeper understanding of the process by which the extract of *C. siphonella* increases cytotoxicity in the MCF-7 and HepG-2 cell line, an examination of apoptosis and cell cycle analysis was conducted using flow cytometry. For the assays, a dose of 35 µg/mL of extract that corresponds to the IC₅₀ value was chosen for MCF-7 cells and 64.4 µg/mL was chosen for HepG-2 cells. The experimental findings are illustrated in Figure 3A, and B, revealing a distinct association between the concentration of extract administered to MCF-7 cells and the observed enhancement in the G1/G0 phase from 16% to the G2/M phase at 58%. This increase is significantly higher compared to the control group. These

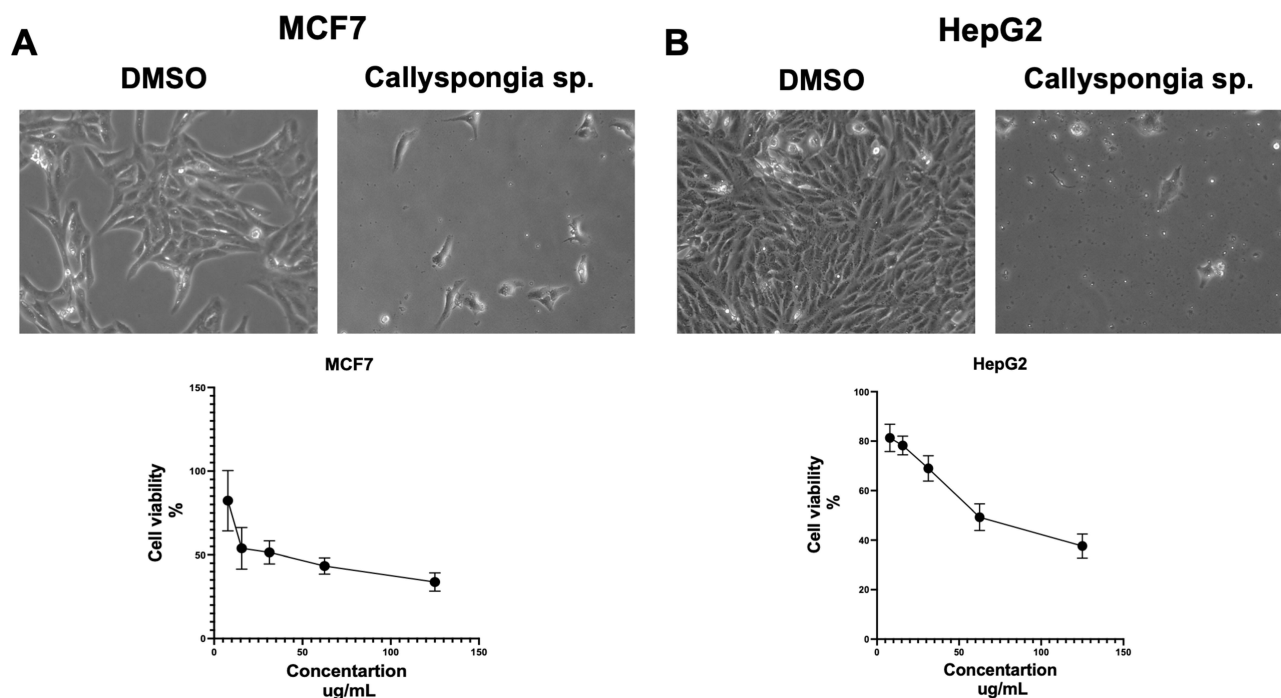


Figure 2 Cytotoxic effect of *C. siphonella* extract on MCF-7 and HepG-2 cells. Representative images show (A) MCF-7 cellular morphology treated with DMSO and *C. siphonella* extract (B) HepG-2 cellular morphology treated with DMSO and *C. siphonella* extract. Graphical presentation represents the percentage of cell viability on both cells.

findings suggest that the extract induces cell cycle arrest at the G2/M phase and inhibits cell proliferation. For HepG-2 cells, the extract induced cell cycle arrest at S phase compared to the control. Treatment with *C. siphonella* extract augments cells in the S phase from 19% (G1/G0) to 58% in (S) phase.

The Effect of *C. Siphonella* Extract on Apoptosis

The experimental results depicted in (Figure 4A, and B) demonstrate that the administration of the extract led to an elevation in necrotic cell death in both MCF-7 and HepG-2 cell lines. This increase was assessed through the implementation of Annexin V-FITC/PI double staining, which enabled a quantitative evaluation of apoptosis. The MCF-7 cells were treated for 48 h with *C. siphonella* extract at doses of 35.6 and 71.2 $\mu\text{g/mL}$. The control cells exhibited a lower percentage of cells in total apoptosis and necrosis (2% and 19%, respectively). The percentages of total apoptosis and necrosis for 35.6 $\mu\text{g/mL}$ dose on MCF7 were 9.7% and 50% and those for 71.2 $\mu\text{g/mL}$ were 2% and 62%, respectively. Also, the extract was added to HepG-2 cells for 48 h at a dose of 64.4 and 128.8 $\mu\text{g/mL}$. The control cells demonstrated a reduced proportion of cells undergoing apoptosis and necrosis, with percentages of 10% and 11% respectively. The percentage of total apoptosis was 38% while necrosis was 45% at 64.4 $\mu\text{g/mL}$ dose, and the proportion of overall apoptosis was 18%, whereas necrosis accounted for 63% at a dosage of 128.8 $\mu\text{g/mL}$ (Figure 4A and B).

Table 3 The Values of IC₅₀ on 2D and 3D Cultures

Antiproliferative activity of the extract of <i>C. siphonella</i> IC ₅₀ \pm SEM ($\mu\text{g/mL}$)		
	MCF-7	HepG2
2D	35.6 \pm 6.9 $\mu\text{g/mL}$	64.4 \pm 8 $\mu\text{g/mL}$
3D	5.1 \pm 2 $\mu\text{g/mL}$	166.4 \pm 27 $\mu\text{g/mL}$

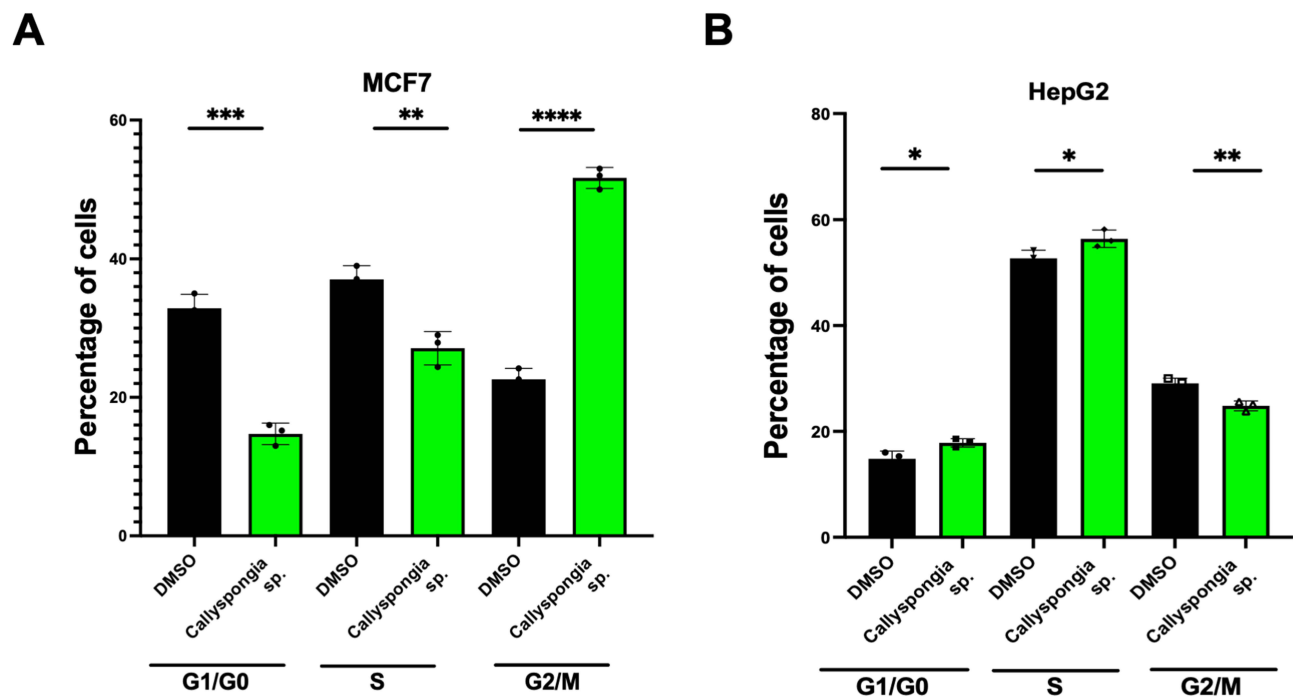


Figure 3 The effect of *C. siphonella* extract on cell-cycle stages of MCF-7 and HepG-2 cells using flow cytometric analysis. Graphical presentation represents the percentage of (A) MCF-7 cells and (B) HepG-2 cells. Data are represented as mean ± SD. *Significant difference at $p < 0.05$. **Significant difference at $p < 0.01$. ***Significant difference at $p < 0.001$. ****Significant difference at $p < 0.0001$.

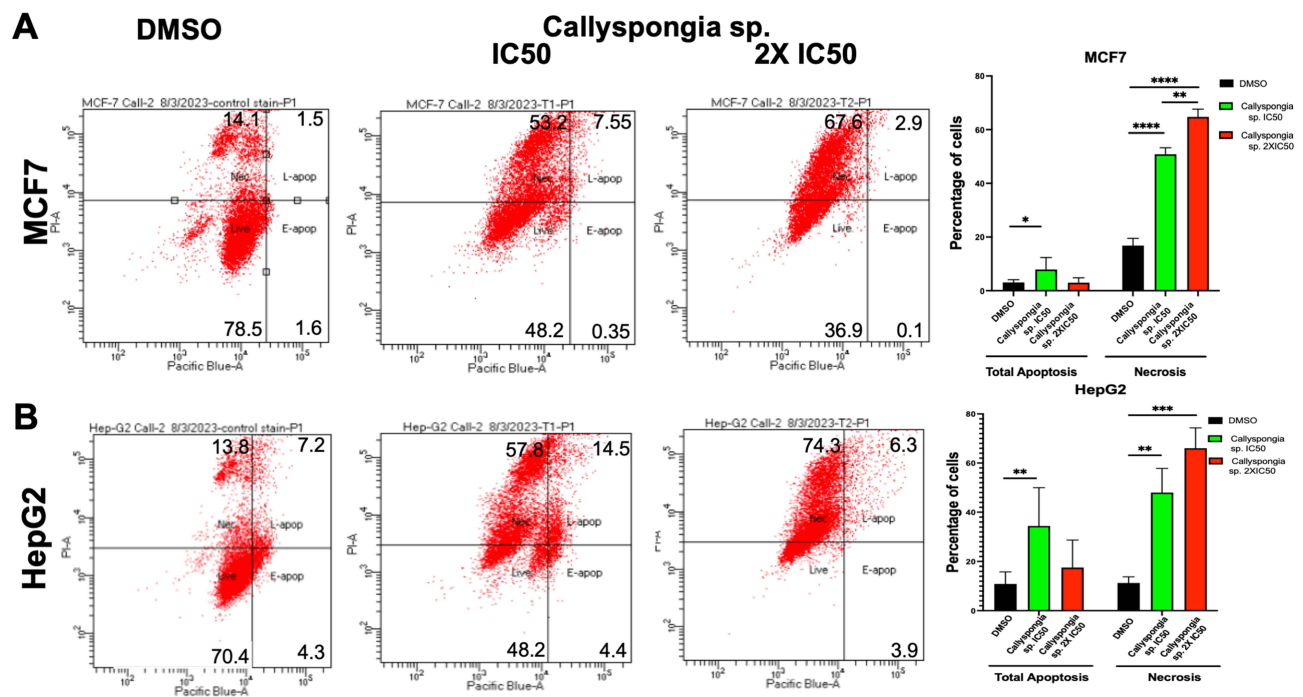


Figure 4 The effect of *C. siphonella* extract on apoptosis and necrosis using flow cytometric analysis. Representative flow cytometric dot plots of (A) MCF-7 and (B) HepG-2 cells were treated with DMSO, IC₅₀, and 2X IC₅₀ of *C. siphonella* extract. Graphical presentation shows the percentage of MCF-7 cells and HepG-2 cells. Data are displayed as mean ± SD. *Significant difference at $p < 0.05$. **Significant difference at $p < 0.01$. ***Significant difference at $p < 0.001$. ****Significant difference at $p < 0.0001$.

The Effect of *C. Siphonella* Extract on Mitochondrial Membrane Potential

The fluorescence microscope and JC-1 staining technique were employed to assess the potential mitochondrial membrane potential in order to validate the cytotoxicity data. The cells were subjected to staining using JC-1 dye, which has the ability to aggregate specifically within active mitochondria and produce red fluorescence. The cells that were subjected to the treatment with *C. siphonella* extract at IC₅₀ concentration demonstrated a notable reduction in the intensity of red/green fluorescence when compared to the negative control ($p < 0.05$ for HepG-2 and $p < 0.005$ for MCF-7). The image demonstrates that *C. siphonella* extract resulted in the depolarization of the mitochondrial membrane, as evidenced by the emission of green fluorescence (Figure 5A and B).

The Effect of *C. Siphonella* Extract on MCF-7 and HepG-2 Migrations

The MCF-7 and HepG-2 cells were subjected to treatment with an extract derived from *C. siphonella* at a concentration of 35.6 $\mu\text{g/mL}$ and 64.4 $\mu\text{g/mL}$ respectively. Comparing this treatment to the control group, MCF-7 and HepG-2 cell migration were significantly reduced at 24 h ($P < 0.001$), ($P < 0.01$), and 48 hours ($P < 0.001$), ($P < 0.01$), as shown in Figure 6A and B.

The Effect of *C. Siphonella* Extract on 3D Cell Culture Spheres

Figures 7 and 8 depict the multicellular spheres derived from MCF-7 and HepG-2 cell lines that were cultivated for a duration of 7 days under conventional culture conditions. The size of MCF-7 and HepG-2 spheroids reached 200 μm at day 4 and treatments started using 5 concentrations of *C. siphonella* extract. The IC₅₀ value was calculated to be $5.1 \pm 2 \mu\text{g/mL}$ for MCF-7 and for HepG-2 to be $166.4 \pm 27 \mu\text{g/mL}$.

Discussion

Numerous compounds have currently been under investigation for their potential as anticancer agents, with the objective of enhancing cancer therapy. Nevertheless, several treatments have proven ineffective due to the presence of intolerance

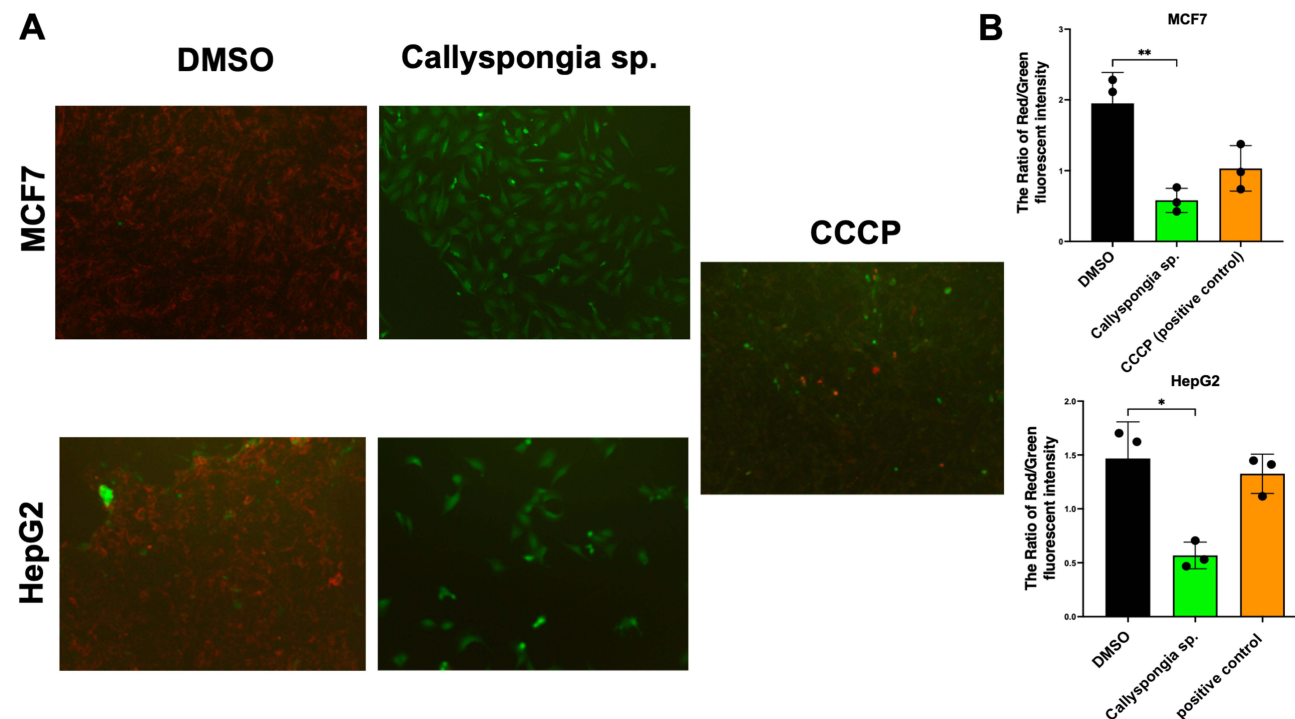


Figure 5 The effect of *C. siphonella* on mitochondrial membrane potential. (A) MCF-7 and HepG-2 cells were stained with JC-1 dye after being treated with DMSO and *C. siphonella* extract at IC₅₀ concentrations. (B) graphical data shows the ratio of red to green fluorescent intensity in both cells. Data are displayed as mean \pm SD. *Significant difference at $p < 0.05$. **Significant difference at $p < 0.01$.

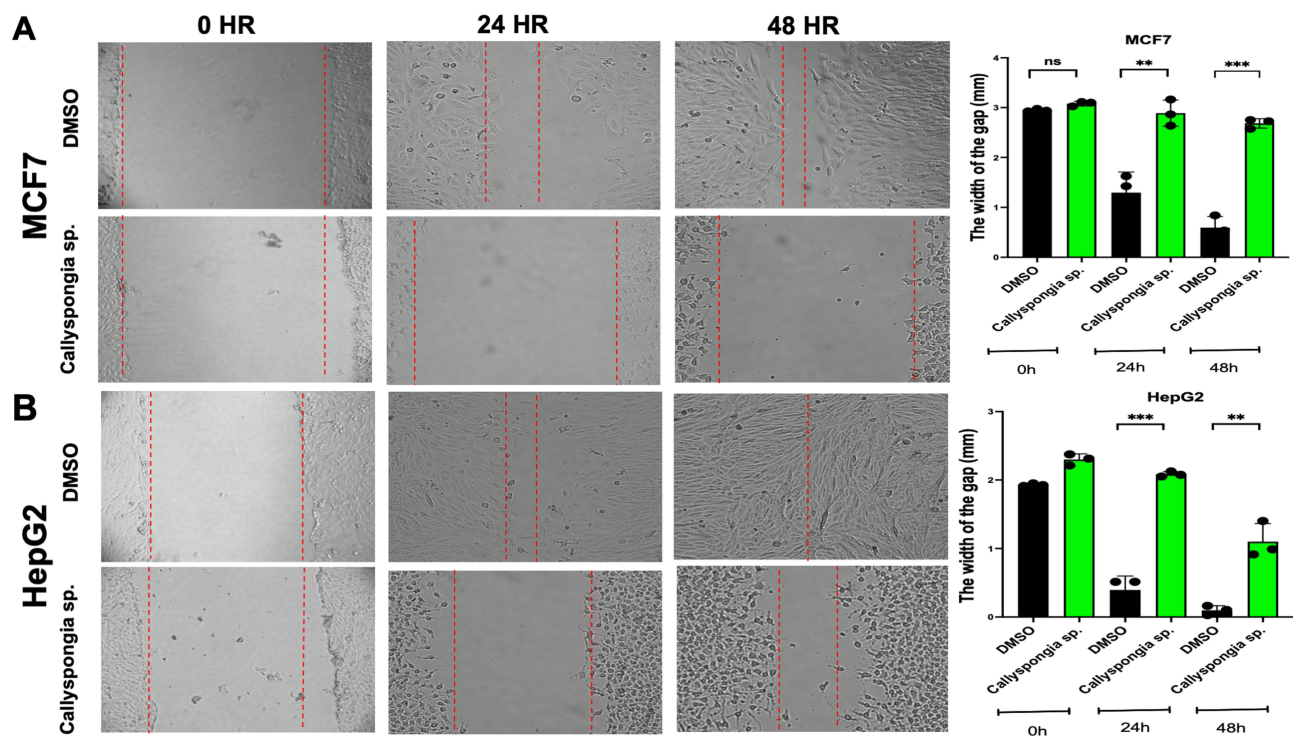


Figure 6 The effect of *C. siphonella* on MCF-7 and HepG-2 migrations. Representative images show migration of (A) MCF-7 cells and (B) HepG-2 cells treated with DMSO and *C. siphonella* extract at different time points 0, 24 and 48 hrs. Graphical data shows the width of the gap between migrated cells. Data are displayed as mean \pm SD. **Significant difference at $p < 0.01$. ***Significant difference at $p < 0.001$.

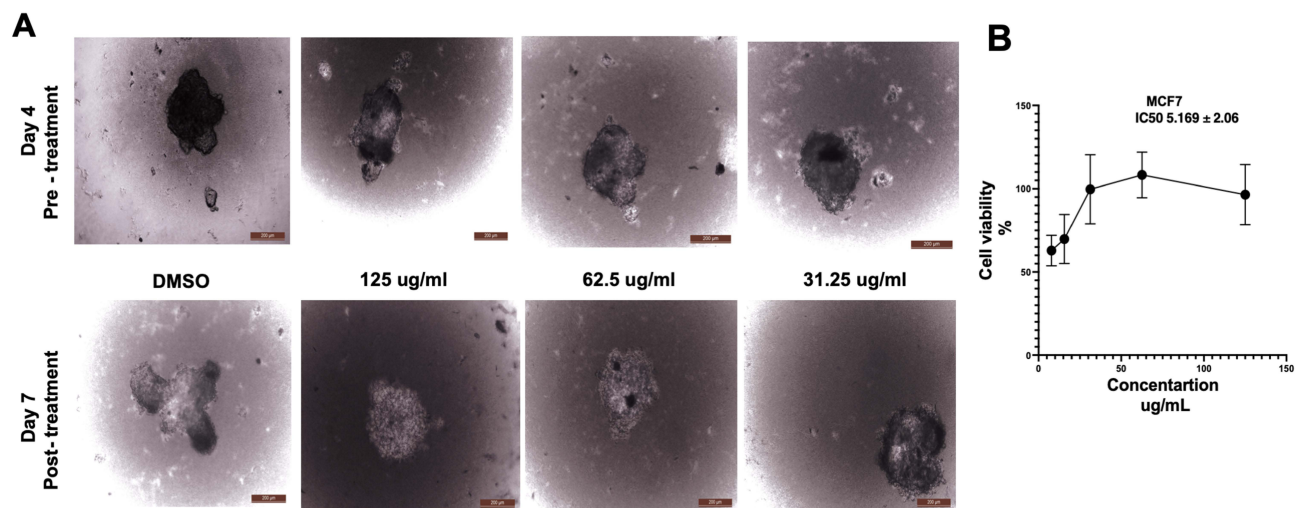


Figure 7 The effect of *C. siphonella* on the 3D culture of MCF-7. (A) Representative images show the spheroid shape of MCF-7 before and after being treated with *C. siphonella* extract at different doses 125 ug/mL, 62.5 ug/mL and 31.25 ug/mL. Scale bar 200mm. (B) Graphical presentation represents the percentage of cell viability. Data are displayed as mean \pm SD.

or resistance, leading to the progression of the disease. Over the past few years, a wide range of academic research has shed light on the significance of marine-derived natural products in the context of therapeutic interventions for various human ailments, notably cancer. Marine natural products are widely recognized as a valuable reservoir of chemical compounds with promising anticancer properties.

Among all marine organisms, sponges, which are ancient spineless multicellular organisms that are members of the Phylum “the pore bearers” (Porifera), are an extremely plentiful source for naturally occurring marine molecules,

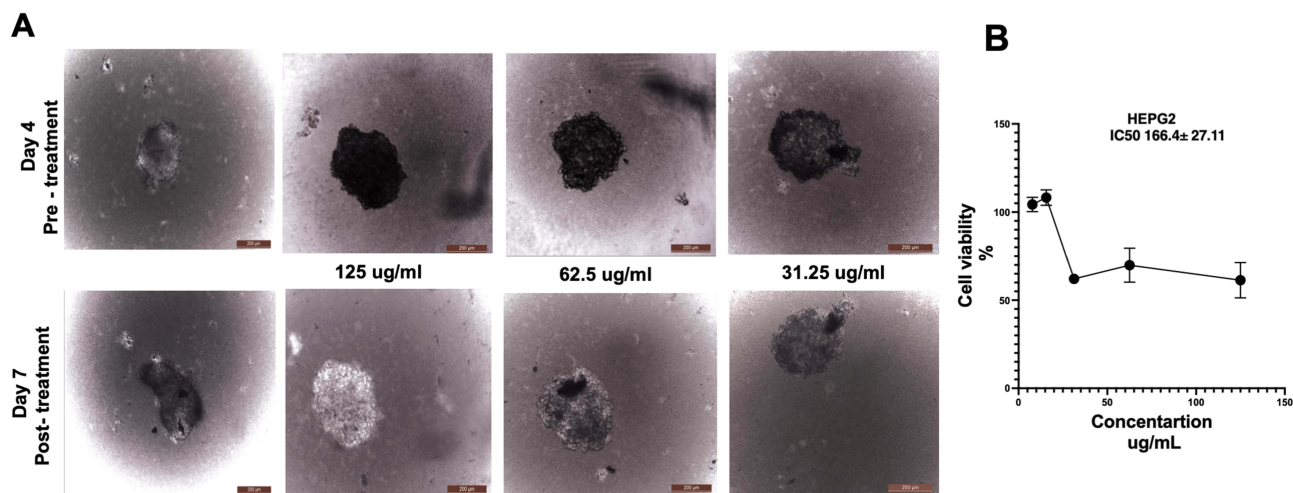


Figure 8 The effect of *C. siphonella* on the 3D culture of HepG-2. **(A)** Representative images show the spheroid shape of MCF-7 before and after being treated with *C. siphonella* extract at different doses 125 ug/mL, 62.5 ug/mL and 31.25 ug/mL. Scale bar 200mm. **(B)** Graphical presentation represents the percentage of cell viability. Data are displayed as mean \pm SD.

accounting for 30% of the entirely natural marine-based substances discovered thus far.^{13,14} Marine sponges are recognized as a significant reservoir of natural compounds that possess strong biological properties and exhibit distinctive structural attributes. Sponges also possess the ability to impede the proliferation of neighboring organisms by reducing their cellular division. Consequently, they have the potential to hinder or suppress the proliferation of cancer cells, thereby offering opportunities for the development of novel pharmaceuticals.¹⁵

The observed potent anticancer activity of *C. siphonella* extract can potentially be attributed to its diverse array of anticancer compounds (refer to Table 1). Based on the HR-LCMS profile, the organic extract of *C. siphonella* contains several terpenoid compounds, specifically triterpenoids, which have shown a wide range of anticancer properties.^{16,17} The presence of triterpenoids sipholenone A, sipholenol A, and sipholenol L in *C. siphonella* could be responsible for its cytotoxic effect. Sipholenone A has previously exhibited significant cytotoxicity against MCF-7 and HepG-2 cancer cell lines, with IC₅₀ values of 3.0 and 2.8 μ M, respectively. Sipholenol A and sipholenol L exhibited a moderate level of cytotoxicity against a human colorectal cancer cell line.^{18,19} Also, several compounds that have been identified, such as Sipholenol A, Sipholenol L, sipholenone A, Sipholenone E, and Siphonellinol D, have exhibited cytotoxic and antiproliferative effects, particularly against cancer cells that exhibit multidrug resistance (MDR) due to overexpression of P-glycoprotein (P-gp/ABCB1).^{19–29}

These compounds have been found to induce apoptosis and inhibit proliferation in the cancer cell lines MCF-7 and HepG-2. Based on the current research findings, it was observed that *C. siphonella* extract demonstrated a dose-dependent inhibition of the proliferation of MCF-7 and HepG-2 cancer cells, with IC₅₀ values of 35.6 μ g/mL and 64.4 μ g/mL respectively (Figure 1). It is noteworthy that the IC₅₀ value of the extract exhibits greater potency compared to the IC₅₀ value reported in the referenced paper from 2022,³⁰ which states an IC₅₀ value of 467.19 μ g/mL for MCF-7. This difference can be attributed to the presence of fewer anticancer compounds as determined by the GC-Mass study. In a similar manner, El-Hawary and colleagues³¹ observed that the crude ethanol extract derived from *C. siphonella* exhibits antiproliferative properties against MM.1S, OVCAR-3, and HT-29 cancer cell lines. Compared to the current methanol-dichloromethane extract of *C. siphonella*, Ibrahim et al³² discovered that the ethanolic extract was more effective as an anticancer agent against MCF-7 and Caco-2 cancer cells.

Subsequently, we conducted an assessment on the impact of *C. siphonella* extract on the advancement of the cell cycle. This can provide valuable insights into the precise mechanism of action exhibited by cytotoxic drugs. Numerous techniques have been employed for quantifying the DNA content of cellular entities. All methodologies employ dyes that exhibit specific affinity towards nucleic acids, and their fluorescence is intensified upon binding. As evidenced by prior research, a crucial aspect of initiating terminal differentiation is the termination of the cell cycle.³³ While G1 arrest has

traditionally been recognized as a key step in the process of cellular differentiation, additional research has indicated that G2 and S phase arrest may also play a role in this phenomenon.³⁴ The flow cytometric analysis conducted in this study revealed a notable augmentation in the percentage of cells in the G2/M phase in MCF-7 cells, while an increase in the S phase was observed in HepG-2 cells following treatment. Specifically, the percentage of cells in the S phase was found to be 39% for MCF-7 and 54% for HepG-2 in the control group, whereas it decreased to 30% in the MCF-7 cells and increased to 58% in the HepG-2 treated with *C. siphonella* extract after a 48 h period (Figure 2). Therefore, the observed reduction in cell proliferation was found to be correlated with a state of S-phase arrest in the aforementioned cells. Undoubtedly, there was a correlation observed between the rise in the proportion of cells in the S-phase and a decline in the number of cells in the G0/G1 and/or G2/M phases in HepG-2 cells.

In addition, this study demonstrates that *C. siphonella* extract significantly inhibits the proliferation of HepG-2 and MCF-7 cancer cell lines and induces apoptosis. Flow cytometry showed that 35.6 µg/mL of the extract had the highest apoptosis rate at 9.7%, aligning with the pro-apoptotic effects noted in marine extracts on cancer cells.^{35,36} Interestingly, at higher concentrations, the extract led to increased necrosis. At 71.2 µg/mL, apoptotic cells decreased while necrotic cell death exceeded 62%, indicating a concentration-dependent cytotoxic effect observed in both cell lines. This suggests that higher doses of the extract exceed the IC₅₀ value, shifting cell death from apoptosis to necrosis.

Moreover, the extract significantly reduced the migration of MCF-7 and HepG-2 cells at concentrations of 35.6 µg/mL and 64.4 µg/mL, respectively, with significant reductions observed at 24 and 48 hours. This highlights the extract's potential to not only inhibit proliferation but also impede cancer cell migration, corroborating findings from similar studies on anti-migratory effects of marine compounds in cancer cells.^{37,38}

The analysis of mitochondrial membrane potential in MCF-7 and HepG-2 cells following treatment with *C. siphonella* extract was conducted using the fluorescent dye JC-1, which aggregates within intact mitochondria. The reduction in red fluorescence indicates JC-1 redistribution due to mitochondrial collapse during apoptosis, as shown by the decrease in the red/green fluorescence ratio from 2 to 0.5 in MCF-7 and from 1.5 to 0.5 in HepG-2 cells (Figure 4B). Assessing mitochondrial membrane potential is crucial for understanding apoptosis mechanisms induced by *C. siphonella* extract, as its disruption is an early apoptosis hallmark leading to pro-apoptotic factor release. Monitoring these changes provides insights into the extract's cytotoxic effects on cancer cells. This study's significant decrease in membrane potential corroborates the pro-apoptotic activity of *C. siphonella*, aligning with recent studies on marine compounds targeting mitochondrial pathways in apoptosis.³⁹

It has been established and accepted that 3D culturing systems of cells do mimic the in vivo environment more than the 2D culturing system; therefore, lots of interest has been gained in 3D culture for drug development.⁴⁰ We tested the extract *C. siphonella* on both cell lines MCF-7 and HepG-2 (Figures 6 and 7) and both were sensitive to *C. siphonella* extract with IC₅₀ of 5.1 ± 2 µg/mL and 166.4 ± 2 µg/mL respectively. This gives us more confidence in the efficacy of *C. siphonella* extract against these cancer cells.

The differences in the efficacy of *C. siphonella* extract between MCF-7 and HepG-2 cancer cell lines can be partly attributed to their inherent biological characteristics. MCF-7 cells are derived from hormone-dependent breast cancer, influenced by estrogen receptors. This hormone dependence can impact the cellular response to cytotoxic agents, potentially enhancing sensitivity to certain compounds that disrupt hormonal signalling pathways.⁴¹ In contrast, HepG-2 cells are derived from liver cancer, which is not hormone-dependent. The liver's diverse metabolic functions may contribute to different reactivity and resistance profiles when exposed to anticancer agents.⁴² These findings highlight the importance of considering the specific biological context of cancer cells when evaluating anticancer compounds, emphasizing the need for tailored therapeutic strategies to maximize treatment efficacy.

Conclusions

The current study clarifies how *C. siphonella* sponge can protect itself chemically in its harsh marine environment and highlights the potential use of its protective metabolites in the future creation of novel anti-cancer drugs. Additionally, *C. siphonella* extract elicits programmed cell death and induces cell cycle arrest specifically in the S phase of these cells. It appears that *C. siphonella* extract exerts its effects via distinct and exclusive mechanisms to trigger programmed cell

death in cancerous cells. Due to the limited understanding of the mechanisms of action associated with this extract, it is imperative to acquire a more comprehensive comprehension of its properties. This endeavor would serve to elucidate its potential applications in cancer prevention, cancer treatment, and various other medical conditions. Further investigation of the in vivo biological properties of this intriguing extract would be advantageous in the advancement of novel pharmaceuticals.

Data Sharing Statement

The data presented in this study are available on request from the corresponding author.

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Author Contributions

Conceptualization, S.A.F., D.T.A.Y., A.J.A., F.A.A., L.A.S. and A.Y.; methodology, S.A.F., F.A.A., A.Y., F.A., A.J.A., M. A., N.S.B., and H.H.A; software, F.A.A., A.J.A. and A.Y.; validation, S.A.F., F.A.A., A.Y., F.A. and A.J.A.; formal analysis, F.A.A., and A.J.A.; investigation, S.A.F., D.T.A.Y., F.A.A. and L.A.S.; resources, F.A.A. and A.A.; data curation, F.A.A., A.J.A. and A.Y.; writing—original draft preparation, S.A.F; writing—review and editing, D.T.A.Y., F.A.A., and A.Y; visualization, M.A., N.S.B., and H.H.A; supervision, S.A.F. and F.A.A; project administration, S.A. F. and F.A.A.; funding acquisition, S.A.F. All authors have read and agreed to the published version of the manuscript. All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no conflicts of interest in this work.

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