Anticancer Potential of Colonial Ascidians Didemnum perlucidum and Lissoclinum bistratum Against MCF-7 Human Breast Cancer Cell Line

Mrs. Amudhanila Padmanaban[1], Dr. Abdul Jaffar Ali Hajamohideen[2*], Mrs. Subashini Neelavannan[3], Dr. Tamilselvi Madasamy[4]

[1]Research Scholar, Department of Biotechnology, Islamiah College (Autonomous), Vaniyambadi, (Affiliated to Thiruvalluvar University, Vellore, India.)
[2]Associate Professor, Department of Biotechnology, Islamiah College (Autonomous), Vaniyambadi, (Affiliated to Thiruvalluvar University, Vellore, Tirupattur, India)*
[3]Research scholar, Department of Zoology, V.V.Vanniaperumal College for Women, Virudhunagar 626 001 (Affiliated to Madurai Kamaraj University)
[4]Associate Professor of Zoology, Department of Zoology, V.V.Vanniaperumal College for Women, Virudhunagar 626 001 (Affiliated to Madurai Kamaraj University)

***Correspondence:** Dr. Abdul Jaffar Ali Hajamohideen,
*Associate Professor,PG & Research Department of Biotechnology, Islamiah College (Autonomous), Vaniyambadi. Mail ID: jaffar.ascidian@gmail.com

KEYWORDS
Anticancer activity, Ascidians, *Didemnum perlucidum*, *Lissoclinum bistratum*, MCF – 7, MTT assay

ABSTRACT
Background: Globally, breast cancer is a leading cause of morbidity and mortality, especially among women. Although the results of current treatments have improved, there remains an urgent need to develop new therapeutic agents. A potential source of bioactive substances with potent anticancer effects is marine biodiversity. Marine tunicates are believed to contain a variety of bioactive compounds with the most important anticancer properties. The family Didemnidae of the class Ascidiacea is known to be a highly adaptable source of natural marine chemicals with a wide range of biomedical applications.
Materials and Method: This study investigates the cytotoxic effects of crude and fractionated extracts of the marine ascidians *Lissoclinum bistratum* and *Didemnum perlucidum* on MCF-7 breast cancer cell lines. The cytotoxic activity was assessed using the MTT assay at different concentrations from 5 to 20 µg/ml.
Results and Discussion: The F2 fraction of both the species exhibited the highest activity, and the results showed significant dose-dependent cytotoxicity. F2 of *D. perlucidum* and *L. bistratum* had IC50 values of 10.22 µg/mL and 11.80 µg/mL, respectively, which were much lower than those of the crude extracts.
Conclusion: These results highlight the potential of *D. perlucidum* as a source of anticancer drugs, calling for further investigation into its bioactive components for potential therapeutic uses.

Introduction

In 2020, more than 19 million new cases of cancer were reported globally, resulting in nearly 10 million deaths. Breast and lung cancers are the most common in terms of prevalence and mortality, each being the reason for more than 2 million diagnoses in 2020. Despite advances in the treatment and diagnosis of malignant carcinoma, breast cancer remains one of the leading causes of death around the world. Among women, breast cancer is the leading cause of death, with approximately 600,000 deaths. In addition, by 2040, the number of new cancer cases worldwide is expected to exceed 28 million, with deaths surpassing 16 million.[1] Worldwide, breast cancer is one of the most common causes of female morbidity and mortality, accounting for 23% of breast cancer diagnoses annually (1.38 million women) and 14% of breast cancer deaths (458,000 women).[2] If the cancerous tumor is situated exclusively in the breast cells, the survival rate is expected to be 99%. If the tumor spreads to nearby lymph nodes, the spread rate would be 85%, if the cancerous tumor spread to distant parts, the survival rate would drop to 27%. Breast cancer is associated with decreased health-related quality of life and high medical costs due to high risk factor.[3,4] The oceans are home to approximately 250,000

species and are therefore a great treasure trove of life and biodiversity.[5] Keeping this in mind that until recently there were only certain types of microorganisms (bacteria, actinobacteria, cyanobacteria and fungi), microalgae, macroalgae (seaweeds), invertebrates, sponges, soft corals, sea fans, sea hares, nudi branches, bryozoans and tunicates have been investigated for cancer treatment,[6,7,8,9]
marine organisms have been shown to be important bioactive compounds, the origin of which is unk nown, leading to increased research and studies on these compounds.
Scientists suggest that marine compounds are more versatile and have greater biological activity than terrestrial counterpart.[10] Because marine organisms, especially resident organisms, are physically and biologically diverse, they have a wide range of secondary metabolites with biological effects.[11,12] This clearly demonstrates a 10- fold increase in the number of persistent organisms competing for food and space, leading to the production of secondary metabolites as part of life's defense mechanism.[13]
Over the years, many antibiotics obtained from the marine environment have been tested in human clinical trials.[14,15,16,17,18]Various marine compounds have attracted attention due to their activity against various types of cancer.[19,20,21,22] Many marine organisms such as ascidians, mollusks, and sponges have been shown to be sources of anti-inflammatory, antibacterial, and medicinal properties.[23,24,25] Drugs derived from ascidians, such as Didemnin B, Ecteinascidin 743, and aplidin, have anti-inflammatory properties and are in clinical trials.[26,13,27]
Marine tunicates are considered to possess the most important anticancer components, and various compounds with antitumor activity.[28,29,30] The tunicates of the Didemnidae family are recognized as a widely versatile source of natural marine compounds with many biomedical applications.
Many pharmaceutical properties of bioactive compounds are also dermined from natural products pr oduced by ascidians, including anti-inflammatory drugs.[31,32] A comprehensive report on 580 ascidian compounds isolated during 1994 to 2014, describing their structures and reporting biological activities (antibacterial, anti-inflammatory, antiviral, anti-diabetic, anti-proliferative, anti-parasitic).[33]
The Didemnidae family of the Tunicata class includes many genera such as Diplosoma, Lissoclinum, Polysyncraton and Tridemnum that are fertile and well-known natural biologically active producers. Among the tunicates of the Didemnidae family, the genus Didemnum stands out, with more species described than any other tunicate. The genus Didemnum is home to many different symbiotic bacteria, which may also be involved in the production of secondary bioactive metabolites isolated from whole animals. Didemnum, the most abundant species in the genus, is also very rich in bioactive secondary metabolites.[34] Many chemical and biological studies have investigated the genus Didemnum, but most of these studies have not identified animals to species. Studies have shown that the Didemnum genus is rich in many types of natural products, including peptides, alkaloids, indole/alkaloids, β-alkaloid carbolines, spiroketals, polyketides, halogenated compounds, steroids, etc. Biological investigations of these entities have shown that some of these compounds have anticancer, antibacterial and antimalarial properties.[33]
The discovery of a new cyclic hexapeptide, cycloxazoline, from the marine ascidian *Lissoclinum bistratum* has been reported. Two new cyclic hexapeptides, bistratamide A and bistratamide B, and two new macrocyclic ethers isolated from *L. bistratum* showed cytotoxic activity against human fibroblasts and tumor cell lines.[35] Bistramide D and K extracted from the sea squirt *L. bistratum* had low toxicity, antitumor activity, and could induce the differentiation (G1DT) of small cell bronchial carcinoma (NSCLCN6) in vitro, but not the activity of others.[36] Marine compounds, bistratene A and cycloxazoline isolated from *L. bistratum* accumulated HL-60 leukemia cells in G2/M phase and inhibited cytokinesis.[37] Lissoclibadin 2 (2) isolated from *Lissoclinum cf. badium* was the most interesting compound possessing potent inhibitory activity against colon (DLD-1 and HCT116), breast (MDA-MB-231), renal (ACHN), and non-small-cell lung (NCI-H460) cancer cell lines and showed no toxicity to mice, and preferable stability in rat plasma.[38] Lissoclibadins 1(1), 3(2), 4(3), 7(4), 8(5) and 14(6) from the Indonesian ascidian *Lissoclinum cf. badium* which inhibited the growth of HCT-15, HeLa-S3, MCF-7 and NCI-H28. Lysocrivasin 1 (1) is the most potent cytotoxic agent and induces apoptosis mainly through an internal pathway that activates the caspase-dependent pathway in HCT-15 cells.[39]

Natural products 6-bromotryptamine derivatives were first isolated as from *Didemnum candidum.*[40] The compound eusynstyelamide B (142), a bisindole alkaloid isolated from *D. candidum* has shown potent anti-cancer activity against MDA-MB-231.[41] Additionally, the authors found that 142 induced MDA-MB-231 cell death via apoptosis. Therefore, the genera Didemnum and Lissoclinum present surprising biological and chemical properties.

In Indian sea waters, 263 species including 41 genera, 12 families, 3 subclasses and 2 orders of the Ascidaceae family have been reported.[42] However, there are very few studies on cytotoxicity of ascidian compounds in India. Therefore, we selected the less studied group of ascidians *Didemnum perlucidum* and *Lissoclinum bistratum*, distributed throughout the Gulf of Mannar in southern India, for their anticancer properties in MCF-7 cells.

Materials and Methods

Ascidian species

For the evaluation of the anticancer activity of ascidians, the most common and abundantly available colonial ascidian species such as *Didemnum perlucidum and Lissoclinum bistratum* were chosen.

Preparation of Crude Extracts:

Preparation of animal material

Sufficient colonies of each student animal were collected by the scuba hand-picking method from various pillars of Jetty on the Mandapam coast of southern India at a depth of 1-2 meters. Freshly collected samples were washed separately with fresh seawater to remove all contaminants and other epibionts. The samples were then dried in the shade and further dried in a hot air oven at room temperature. The dried samples were ground and sieved to remove hull particles and used to prepare crude methanol extracts.

One specimen of each species is preserved at the Museum of Islamiah College (Autonomous) in Vaniyambadi, India. The species has already been described in a previous publication.[43]

Crude methanol extracts

To extract the biological compositions of the collected species, the dry material was separately immersed in 1.20 w/v methanol (100% A.R grade). The extract was filtered and then concentrated using a rotary evaporator (Buchi type). The dry extracts were resuspended in methanol for 24 hours, and the combined extracts were filtered by Whatman paper No. 1, and again forced into the rotary evaporator. The extraction residue was resuspended in 20 ml of 100% A.R. Methanol grade was used and transferred to a new beaker to remove the precipitated salt. The methanol extract dissolved in deionized water was dried and dissolved. Extracts were prepared at different concentrations dissolved in dimethylsulfoxide and stored at 4°C for further use.

Fractionation

Thin Layer Chromatography and Silica Gel Column Chromatography

The proper solvent was selected and the sample's solubility was pre-evaluated using thin-layer chromatography (TLC Silica gel 60 F_{254}) for the subsequent silica gel G-60 column chromatography.

Solvent Selection

The selection of solvents for this study was primarily based on their polarity characteristics to ensure optimal separation during chromatographic analysis. Initially, four different solvents such as chloroform, ethanol, glacial acetic acid, and DMSO were chosen, and their performance was evaluated through Thin Layer Chromatography (TLC) with various concentrations. This step aimed to identify the most effective solvent ratio for subsequent column chromatography.

The determination of the final solvent ratio was guided by the quality of separation observed in the TLC, as indicated by distinct separation bands and retention factor (Rf) values. After careful analysis and optimization, the following solvent system was selected for column chromatography: chloroform, ethanol, glacial acetic acid, and DMSO in the ratio of 2:1:5:4. This combination was found to provide

the most efficient separation of the target compounds, ensuring reliable and reproducible chromatographic outcomes.

Column Chromatography
The glass column measuring 60 x 2.54cm was used for the silica gel G-60 column chromatography. Solvent systems were selected based on the polarity such as Chloroform, ethanol, DMSO and Glacial acetic acid as mobile phase. Then 2.5ml fractions were collected every 10 minutes. The recovered fractions were concentrated, dried and utilized for the further analysis.

Cell Viability Assay
The effect of the organic extract of *D. perlucidum* and *L. bistratum* on cell viability of MCF-7 was evaluated by MTT assay.

Breast Cancer Cell Line – MCF-7
Breast Cancer Cell Line – MCF-7 was used to determine the cell Cytotoxicity activity obtained from National Centre for Cell Science, Pune, India (NCSS).

Experiment
Cells were maintained in minimal essential medium supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml). These were grown as monolayers to 70–80% confluency at 37°C, 5% CO2. Cells were seeded in 96-well plates at 5000 cells/well. After 24 hours of incubation and treatment, cells were treated with 6-fold dilutions of crude extracts of the two ascidians at concentrations of 5, 10, 15 and 20 µg/ml. Dilutions of the stock solutions were performed in medium to obtain a final extract concentration with a DMSO concentration of 0.1%. This concentration of DMSO does not affect cell viability. Control cells were incubated with medium only. This experiment was performed in triplicate on the same cell mass.
After 48 h of incubation, 15 µl of MTT (5 mg/ml) in phosphate-buffered saline (PBS) was added to each sample as tetrazolium salt (Sigma) was added as an indicator of cytotoxicity for cell viability. Wells were incubated at 30°C for 4 hours. The medium containing MTT was then shaken, the formed formazan crystals were dissolved in 100 µl of DMSO, and the absorbance at 570 nm was measured using a microreader. Tetrazolium salts are bound to formazan dyes by cellular enzymes (only in living cells). % cell viability was determined using the following formula:
% cell viability = 100- Abs (sample)/Abs (control) x100.
After removing the medium, the phosphate solution was washed away. The samples were then placed in fresh medium containing 50 µl of MTT solution (5 mg/ml) and each well was incubated for 4 hours. After incubation, DMSO was added. Viable cells were determined by absorbance at 570 nm using a microplate reader.
The inhibitory concentration required for 50% cytotoxicity (IC_{50}) values were analyzed with sigmaplot software.

Data interpretation
Absorbance values that are lower than the control cells indicate a reduction in the rate of cell proliferation. Conversely a higher absorbance rate indicates an increase in cell proliferation. Rarely, an increase in proliferation may be offset by cell death; evidence of cell death may be inferred from morphological changes.
% inhibition= (C-T/C)*100

Determination of IC_{50}
IC_{50}, the concentration of compounds required to inhibit cell proliferation by 50%, it is proposed to plot a graph of Log (concentration of extract) versus % cell inhibition. A line drawn from the 50% value on the Y axis will intersect the curve and interpolated to the X axis. The values on the X-axis are Log (concentration of the mixture). The antilogarithm of that value gives the IC_{50} value.

Results

The 48-hour in vitro cytotoxic activity of crude and fractionated extracts of *Didemum pelucidum* and *Lissoclinum bistratum* against MCF-7 cancer cells at different concentrations (5, 10, 15 and 20 µg/ml) was determined by MTT assay. MCF-7 cells were analyzed using phase contrast microscopy after exposure to different concentrations of dimethyl sulfoxide (DMSO) as negative control and extracts of *D. perlucidum* and *L. bistratum* and untreated cells as positive control. The untreated MCF-7 cells were epithelial and reached 90-100% confluence in approximately 7 days.

The results of anti-cancer effect of ascidian crude and fractions on MCF-7 cells showed that the viability of the cells was significantly reduced compared to the control group (Fig 1). The toxic effects of *D. perlucidum* extract were clearly observed by both microscopy and MTT assay, but these effects were less than *L. bistratum* (Fig 2).

Fig 1 and 2 demonstrated that the percentage viability of the cells was significantly decreased with increasing concentration of the F2 fractions as compared to the other fractions of both the species. The maximum percentage viability of the cells was 58.78%, 46.39%, 32.72%, and 15.94%, when treated with F2 fraction of *D. perlucidum* at concentrations of 5, 10, 15 and 20 µg/ml respectively. This indicated that there was a dose-dependent relationship of death rate of MCF-7 cells. Then the percentage of cell density has been decreased evident the cell death.

The IC_{50} value represents the need to inhibit half of the biological or biochemical activity. The IC_{50} values of *D. perlucidum* and *L. bistratum* extracts obtained against MCF-7 cancer cell line are depicted in the Fig 3. IC_{50} values for the F2 of both species are 10.22 and 11.80 µg/ml respectively which are lesser than crude extract (16.24 µg/ml).

Control cells had a regular ovoid or spindle shape, and the cell surface was relatively smooth and intact. Observed cell properties include stress-induced phenotypes such as cell shrinkage and membrane rupture, ultimately leading to cell death (Fig 4 and 5). ANOVA analysis showed a significant difference ($p < 0.05$) among the different fractions and crude extracts.

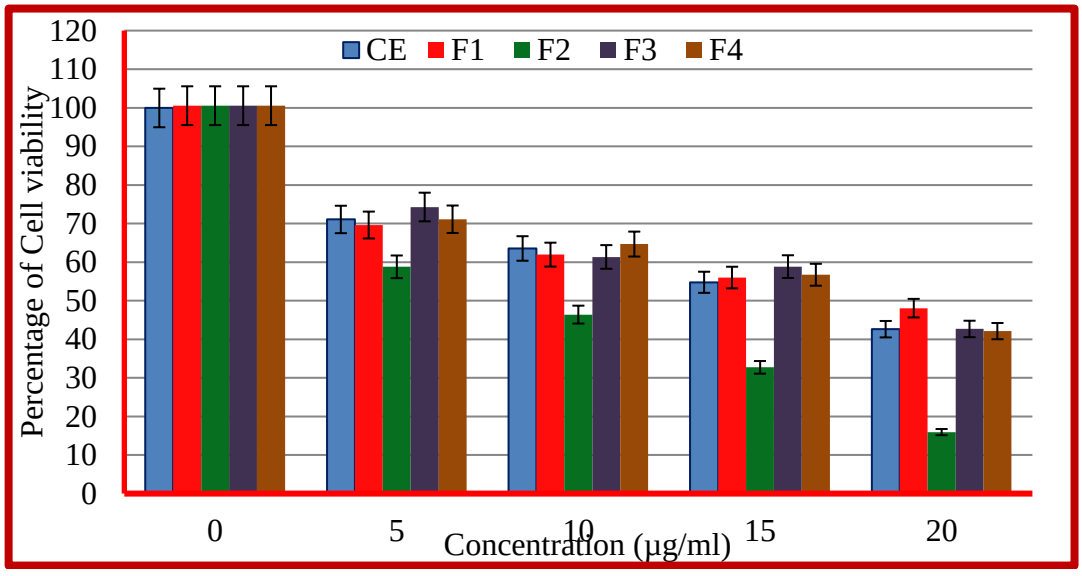

Figure 1:

Cytotoxic activity of *Didemnum perlucidum* (Crude and fractionated extracts) on human breast cancer cell line (MCF – 7). Bars represent the mean ± standard error of replicates experiments (significant as compared to control, $P < 0.005$). Cells viabilities were assessed by mitochondrial tetrazolium test assay. Cells were incubated for 72 h.

CE: Crude Extract, F1: Fraction 1, F2: Fraction 2, F3: Fraction 3 F4: Fraction 4

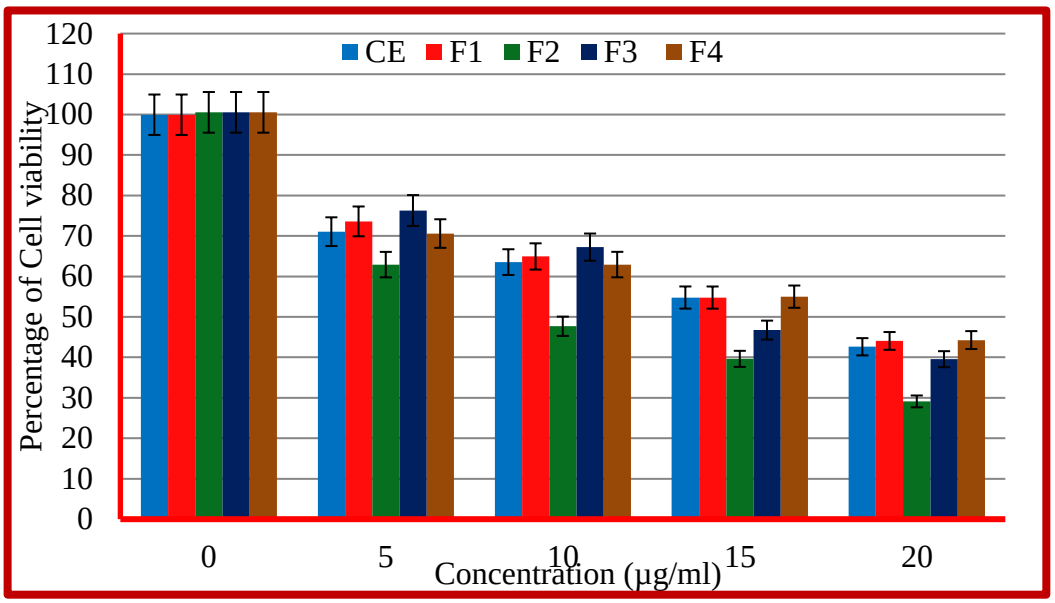

Figure 2:

Cytotoxic activity of *Lissoclinum bistratum* (Crude and fractionated extracts) on human breast cancer cell line (MCF – 7). Data represent the mean ± standard error of the mean separate experiments (significant as compared to control, $P < 0.005$). Cells viabilities were assessed by mitochondrial tetrazolium test assay. Cells were incubated for 72 h.
CE: Crude Extract, F1: Fraction 1, F2: Fraction 2, F3: Fraction 3 F4: Fraction 4

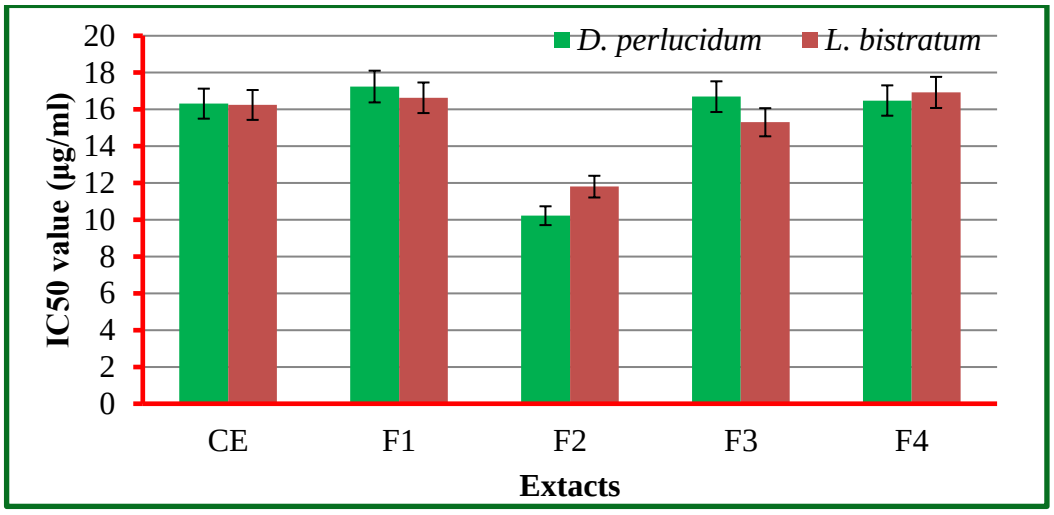

Figure 3:

The median growth inhibitory concentration values (µg/ml) of crude and partition fractions of extract of *Didemnum perlucidum* and *Lissoclinum bistratum.*
Results are expressed as mean ± SD. SD: Standard deviation, CE: Crude Extract F1: Fraction 1, F2: Fraction 2, F3: Fraction 3, F4: Fraction 4 IC_{50}: The median growth inhibitory concentration

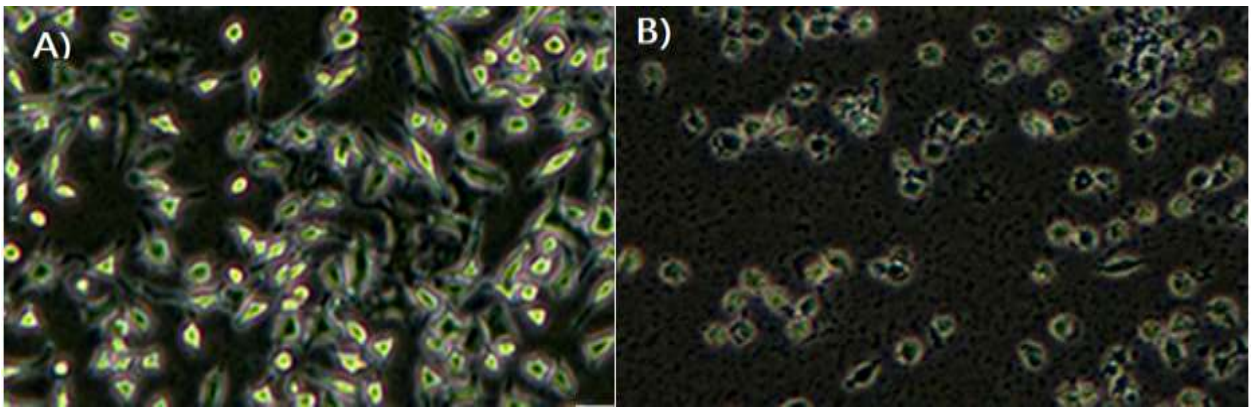

Figure 4:

Anticancer Potential of Colonial Ascidians Didemnum perlucidum and Lissoclinum bistratum Against MCF-7 Human Breast Cancer Cell Line

Inverted phase contrast images of MCF-7 in culture medium (A) and toxic effect of *D. perlucidum* at IC_{50} concentration of F2 (B). MCF-7 cells in culture reproduce in epithelioid form and they become confluent by 7 days. Visualized with a 100-fold magnification.

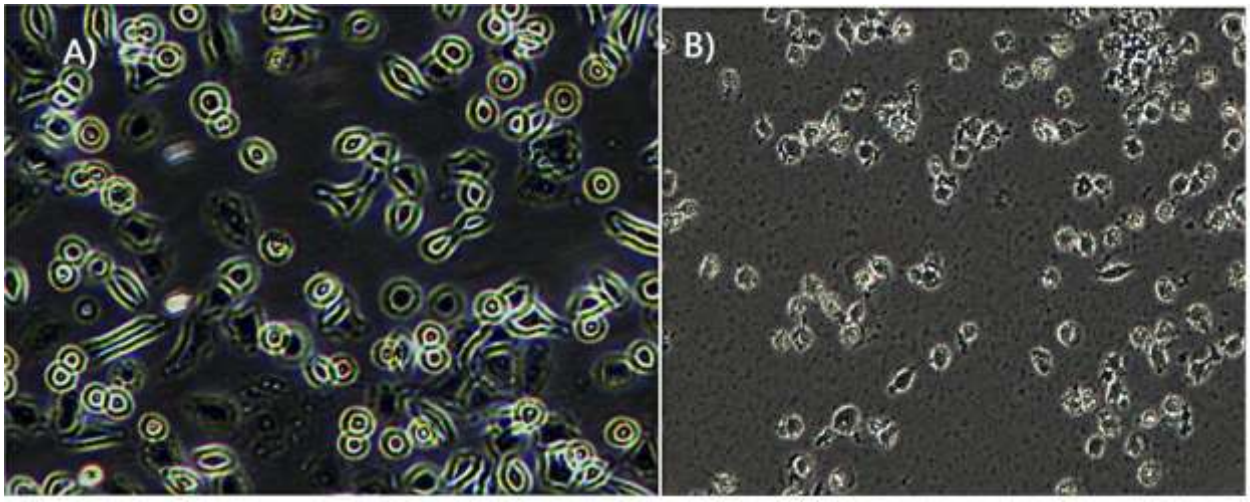

Figure 5:

Inverted phase contrast images of MCF-7 in culture medium (A) and toxic effect of *L. bistratum* at IC_{50} concentration of F2 (B). MCF-7 cells in culture reproduce in epithelioid form and they become confluent by 7 days. Visualized with a 100-fold magnification.

Discussion

We investigated the cytotoxic effects of *D. perlucidum* and *L. bistratum* extracts on MCF-7 breast cancer cells. We observed that *D. perlucidum* was more harmful than *L. bistratum* extracts. The MTT assay showed that *D. perlucidum* inhibited the proliferation and growth of breast cancer cells in a dose-dependent manner.

Several studies have been conducted to determine the bioactive compounds produced by marine ascidians.[33,44] Several metabolites were isolated and purified from several ascidians and their activity has been demonstrated against several cancer cell lines. Marine ascidians of the family Didemnidae have been reported to possess antitumor activity and exhibit cytotoxic activity against several cell lines in culture. Compounds 68, 71, 75 and 76 isolated from Australian *D. obscurum*, and 20-sulfate derivatives of lamellars B, C and L (63-65), demonstrated good inhibition of cell viability against colorectal cancer cells (COLO-205) with IC50 value of 0.0056, 0.0002, 0.00025 and 0.009 μM respectively.[45] Mollamide B (123) isolated from *D. molle* tested against non-small cell lung cancer

cell line (H460), breast cancer cells (MCF7) and CNS at 100 μM, which showed significant growth inhibition up to 29%, 44%, 42 % respectively in the cancer cell line of SF-268. However, when a National Cancer Institute (NCI) evaluated a panel of 60 cell lines, none of the tested cell lines showed above-average sensitivity to molamide B.[46]

Dehydrodidemmine B, a promising marine compound, is currently being used in many clinical trials. Another interesting group of depsipeptides is tamandarins. They also belong to the family of ascidians of Didemnidae which is found in Brazilian waters. Tamandarin showed strong cytotoxic activity against human pancreatic cancer cells BX-PC3, prostate cancer cells DU-145, and head and neck carcinoma cells UMSCC10b.[47] Lamellarins are derived from amino acids phenylalanine or tyrosine and are produced in the mollusk *Lamellaria* sp., *Didemnum* species and sponges. They have cytotoxic activity, with IC50 values ranging from nanomolar to micromolar range.[48]

Our study documents the potential influence of fractions by causing cell damage, foaming of cell membranes, and the formation of apoptotic bodies, which indicates the presence of cytotoxic effects and the presence of bioactive compounds that may have antitumor significance.

The intensity of MCF-7 cell density were decreased by increasing the concentration of extracts from 5 μg/ml to 20 μg/ml. This infers the existence of dose-dependent properties of extracts against cancer cell lines which was found effective.

Partitioning pattern of compounds into different solvents depends mainly on their structure. From these results, metabolites in Fraction 2 of *D. perlucidum* happen to be the most interesting compounds. Further studies need to be conducted to investigate the bioactive compounds of the Didmemnidae family and their efficacy as effective therapeutic tools against cancer.

Funding
The authors received no financial support from any funding agencies.

Ethical Statement
There were no use of animal or human participants in this experiment.

Conflict of Interest
The authors have no conflict of interest.

References

1. World Health Organization (WHO). One-dose human papillomavirus (HPV) vaccine offers solid protection against cervical cancer. WHO; 2022. Accessed December 5, 2024.
2. Belarbi El Hassan P, Contreras Gómez A, Chisti Y, García Camacho F, Molina Grima E. Producing drugs from marine sponges. *Biotechnology Advances*. 2003;21(7):585-598.
3. Daferner M, Anke T, Sterner O. Zopfiellamides A and B: Antimicrobial pyrrolidinone derivatives from the marine fungus *Zopfiella latipes*. *Tetrahedron*. 2002;58:7781-7784.
4. Salma Y, Lafont E, Therville N, Carpentier S, Bonnafé MJ, Levade T, Génisson Y, Andrieu-Abadie N. The natural marine anhydrophytosphingosine, Jaspine B, induces apoptosis in melanoma cells by interfering with ceramide metabolism. *Biochem Pharmacol*. 2009;78:477–485.
5. Bouchet P. The magnitude of marine biodiversity. In: Duarte CM, ed. The exploration of marine biodiversity: scientific and technological challenges. Bilbao: Fundación BBVA; 2006:33-64.
6. Faulkner DJ. Marine pharmacology. *Antonie Van Leeuwenhoek*. 2000;77:135–145.
7. Khalifa Shaden AM, Elias N, Farag MA, Chen L., Saeed A, Hegazy MF, Moustafa SM, Abd El-Wahed A, Al-Mousawi SM, Musharraf SG, Chang FR, Iwasaki A., Suenaga K., Alajlani M, Göransson U., El-Seedi HR. Marine natural products: A source of novel anticancer drugs. *Mar Drugs*. 2019;17(8):491.
8. Shinde P, Banerjee P, Mandhare A. Marine natural products as source of new drugs: A patent review (2015-2018). *Expert Opin Ther Patents*. 2019;29(8):1744-7674.

9. Wali VB, Patwardhan GA, Pelekanou V, Karn T, Cao J, Ocana A, Yan Q, Nelson B, Hatzis C, Pusztai L. Identification and validation of a novel biologics target in triple negative breast cancer. *Sci Rep*. 2019;9:14934. doi: 10.1038/s41598-019-51410-3.
10. Gribble GW. Biological activity of recently discovered halogenated marine natural products. *Mar Drugs*. 2015;13:4044-4136.
11. Frederick Grassle J. Marine Ecosystems, in: Encyclopedia of Biodiversity. Elsevier, pp. 2013;45–55. https://doi.org/10.1016/B978-0-12-384719-5.00290-2
12. Jensen PR, Moore BS, Fenical W. The marine actinomycete genus Salinispora: A model organism for secondary metabolite discovery.*Nat Prod Rep*. 2015;32:738–751.
13. Cragg GM, Newman DJ. Plants as a source of anti-cancer agents. J Ethnopharmacol. 2005;100(1–2):72-79. doi: 10.1016/j.jep.2005.05.011, PMID: 16009521.
14. da Rocha A. Natural products in anticancer therapy. *Curr Opin Pharmacol*. 2001;1:364–369.
15. Arif JM, Al-Hazzani AA, Kunhi M, Al-Khodairy F. Novel marine compounds: Anticancer or genotoxic. *J Biomed Biotechnol*. 2004;2004(2):93–98.
16. Rawat D, Joshi M, Joshi P, Atheaya H. Marine peptides and related compounds in clinical trial. Anticancer Agents Med Chem. 2006;6:33-40. doi: 10.2174/187152006774755519.
17. Gordaliza M. Natural products as leads to anticancer drugs. *Clin Transl Oncol*. 2007;9:767–776.
18. Molinski TF, Dalisay DS, Lievens SL, Saludes JP. Drug development from marine natural products. *Nat Rev Drug Discov*. 2009;8(1):69–85.
19. Mayer AMS, Gustafson KR. Marine pharmacology in 2000: Antitumor and cytotoxic compounds. *Int J Cancer*. 2003;105(3):291–299.
20. Newman DJ, Cragg GM. Marine natural products and related compounds in clinical and advanced preclinical trials. *J Nat Prod*. 2004;67(8):1216–1238.
21. Olano C, Mendez C, Salas JA. Antitumor compound from marine actinomycetes. *Mar Drugs*. 2009;7(2):210-248.
22. Rocha J, Peixe L, Gomes NCM, Calado R. Cnidarians as a source of new marine bioactive compounds—An overview of the last decade and future steps for bioprospecting. *Mar Drugs*. 2011;9(10):1860-1886.
23. Hughes Chambers C, Fenical W. Antibacterials from the sea: *Chem Eur J*. 2010;16:12512 – 12525.
24. Gupta AP, Pandotra P, Sharma R, Kushwaha M, Gupta S. Marine Resource. Studies in Natural Products Chemistry, (2013);229–325.
25. Datta D, Nath Talapatra S, Swarnakar S. Bioactive compounds from marine invertebrates for potential medicines – An overview. *Int Lett Nat Sci*. 2015;7:42-61.
26. Rinehart KL. Antitumor compounds from tunicates. *Med Res Rev*. 2000;20(1):1–27.
27. Singh R, Sharma M, Joshi P, Rawat DS. Clinical status of anti-cancer agents derived from marine sources. *Anticancer Agents Med Chem*. 2008;8:603–617.
28. Pisut DP, Pawlik JR. Anti-predatory chemical defenses of ascidians: Secondary metabolites or inorganic acids? *J Exp Mar Biol Ecol*. 2002;270:203–214.
29. Joullie MM, Leonard MS, Portonovo P, Liang B, Ding X, La Clair JJ. Chemical defense in ascidians of the Didemnidae family.*Bioconjug Chem*. 2003;14(1):30–37.
30. Wyche TP, Standiford M, Hou Y, Braun D, Johnson DA, Johnson JA, Bugni TS. Activation of the nuclear factor E2-related factor 2 pathway by novel natural products halomadurones A-D and a synthetic analogue. *Mar Drugs*. 2013;11:5089–5099. doi: 10.3390/md11125089.
31. Kang HK, Choi MC, Seo CH., Park Y. Therapeutic properties and biological benefits of marine-derived anticancer peptides. Int J Mol Sci.*2018;19:919.
32. Fang WY, Dahiya R, Qin HL, Mourya R, Maharaj S. Natural proline-rich cyclopolypeptides from marine organisms: Chemistry, synthetic methodologies and biological status. *Mar Drugs*. 2016;14(11):194.
33. Palanisamy SK, Rajendran NM, Marino A. Natural products diversity of marine ascidians (tunicates; ascidiacea) and successful drugs in clinical development. *Nat Prod Bioprospect*. 2017;7(1):1–111.

34. Shenkar N, Swalla BJ. Global diversity of Ascidiacea. *PLoS ONE*. 2011;6(6):e20657.
35. Degnan BM, Hawkins CJ, Lavin MF, McCaffrey EJ, Parry DL, Watters DJ. Novel cytotoxic compounds from the ascidian *Lissoclinum bistratum*. *J Med Chem*. 1989;32(6):1354-9.
36. Riou D, Roussakis C, Biard JF, Verbist JF. Comparative study of the antitumor activity of bistramides A, D and K against a non-small cell broncho-pulmonary carcinoma. Anticancer Res. 1993 Nov-Dec;13(6A):2331-4.
37. Watters D, Parsons P. Role of protein kinase C isoforms in the differentiation of melanoma cells. J Cell Biochem Suppl. 1994;18D:95.
38. Oda T, Kamoshita K, Maruyama S, Masuda K, Nishimoto M, Xu J, Namikoshi M. Cytotoxicity of lissoclibadins and lissoclinotoxins, isolated from a tropical ascidian Lissoclinum cf. badium, against human solid-tumor-derived cell lines. Biol Pharm Bull. 2007;30(2):385-387. doi:10.1248/bpb.30.385, PMID 17268087.
39. Tatsuta T, Hosono M, Rotinsulu H, Wewengkang DS, Sumilat DA, Namikoshi M, Yamazaki H. Lissoclibadin 1, a Polysulfur Aromatic Alkaloid from the Indonesian Ascidian Lissoclinum cf. badium, Induces Caspase-Dependent Apoptosis in Human Colon Cancer Cells and Suppresses Tumor Growth in Nude Mice. J Nat Prod. 2017;80(2):499-502. doi:10.1021/acs.orglett.3c01118,
40. Fahy E, Potts BCM, Faulkner DJ, Smith K. 6-Bromotryptamine derivatives from the Gulf of California tunicate *Didemnum candidum*. J Nat Prod. 1991;54(2):564-9. doi: 10.1021/np50074a032.
41. Liberio M, Sadowski M, Nelson C, Davis R. Identification of eusynstyelamide B as a potent cell cycle inhibitor following the generation and screening of an ascidian-derived extract library using a real time cell analyzer. Mar Drugs. 2014;12(10):5222-39. doi: 10.3390/md12105222.
42. Meenakshi VK, Gomathy S. Indian ascidians over the hundred years - A checklist. RJLBPCS. 2018 Nov-Dec;4(6):134. doi: 10.26479/2018.0406.11.
43. Abdul Jaffarali H, Akram S, Arshan K. New distributional data on ascidian fauna (Tunicata: Ascidiacea) from Mandapam coast, Gulf of Mannar, India. Biodivers. Data J. 2016;4: e7855.
44. Youssef DTA, Almagthali H, Shaala LA, Schmidt EW. Secondary metabolites of the genus Didemnum: A comprehensive review of chemical diversity and pharmacological properties. Mar Drugs. 2020;18(6):307. doi: 10.3390/md18060307.
45. Malla Reddy S, Srinivasulu M, Satyanarayana N, Kondapi AK, Venkateswarlu Y. New potent cytotoxic lamellarin alkaloids from Indian ascidian *Didemnum obscurum*. Tetrahedron. 2005;61:9242-7. doi: 10.1016/j.tet.2005.05.052.
46. Donia ABM, El-Masry EM, Abdel-Rahman AA, McLendon RE, Schiffman SS. Splenda alters gut microflora and increases intestinal P-glycoprotein and cytochrome P-450 in male rats. J Toxicol Environ Health A. 2008;71(21):1415-29. doi: 10.1080/15287390802328630.
47. Edler D, Glimelius B, Hallström M, Jakobsen A, Johnston PG, Magnusson I, Blomgren H. Thymidylate synthase expression in colorectal cancer: a prognostic and predictive marker of benefit from adjuvant fluorouracil-based chemotherapy. J Clin Oncol. 2002;20(7):1721-8. doi: 10.1200/JCO.2002.07.039.
48. Chittchang M, Batsomboon P, Ruchirawat S, Ploypradith P. Inside cover: Cytotoxicities and structure-activity relationship s of natural and unnatural lamellarins toward cancer cell lines. Chem Med Chem. 2009;4(3):298. doi: 10.1002/cmdc.200800339.