In-Vitro Anticancer and Antioxidant Properties of Aplidium Multiplicatum Ascidian on Liver Cancer

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**Abstract:** Ascidians the marine vertebrates are of immense biomedical and nutritive value. As for us, the marine natural product mining several bioactive metabolites in terms of polypeptides, amines and cycloterpenoids were isolated from several ascidian species which possess anticancer, antiviral, antimicrobial and antituberculosis effects. In the present study an ascidian species Aplidium multiplicatum methanolic extract showed significant anticancer effect against HepG2 (Liver cancer cell line), and THLE-2 (Normal Human lung tissue cell lines). The antioxidant effect of Aplidium multiplicatum revealed that ascidian extract shows 80-85% antioxidant effect through DPPH scavenging when compared to the standard quercetin drug. Future prospect of secondary metabolite isolation from the Aplidium multiplicatum may lead to candidate drug isolation for specific anticancer therapy.

**Keywords:** Novel drug, Anticancer, Ascidian, Aplidium multiplicatum, Antioxidant.

# Introduction

Hepatocellular carcinoma (HCC) is the most common kind of liver cancer globally and is associated with a significant health burden [(Aparna et al., 2021; Ganapathy & Professor and Head of Department of Prosthodontics, 2021; Verma & Muthuswamy Pandian, 2021)](https://paperpile.com/c/wr9Yj3/frYo+ee4W+Bwoa). The prognosis for liver cancer is still poor despite improvements in therapeutic modalities, which emphasizes the urgent need for new approaches to therapy [(Aparna et al., 2021; Ganapathy & Professor and Head of Department of Prosthodontics, 2021; Verma & Muthuswamy Pandian, 2021)](https://paperpile.com/c/wr9Yj3/frYo+ee4W+Bwoa). The many pharmacological effects of natural compounds, such as their antioxidant and anticancer capabilities, have attracted a lot of interest in recent years. Ascidians are among the wide variety of marine species that show promise as a source of bioactive chemicals with potential use in medicine [(Ganapathy & Professor and Head of Department of Prosthodontics, 2021; Pandiyan et al., 2022; Poornima et al., 2021)](https://paperpile.com/c/wr9Yj3/2tZFj+UjzTs+e49cW). The ascidian species Aplidium multiplicatum, which is prevalent in marine environments, has drawn attention from scientists because of its rich chemical makeup [(Leal et al., 2020)](https://paperpile.com/c/wr9Yj3/PuV0O) and proven biological activity. Based on preliminary studies, A. multiplicatum appears to contain chemicals with potential anticancer properties, which makes it a promising option for more research in cancer therapy[(Merchant et al., 2022)](https://paperpile.com/c/wr9Yj3/DciPm).

The purpose of this work is to clarify the antioxidant and anticancer effects of A. multiplicatum extract in vitro on liver cancer [(Choudhary et al., 2017)](https://paperpile.com/c/wr9Yj3/OfX8T). We want to investigate the possible mechanisms of action of A. multiplicatum extract and to thoroughly assess its cytotoxic effects on liver cancer cells using a range of experimental assays and approaches. Furthermore, the extract & antioxidant capacity will be evaluated to clarify its capacity to reduce oxidative stress, a marker of cancer development [(Merchant et al., 2025; Shenoy et al., 2022, 2023)](https://paperpile.com/c/wr9Yj3/Yk3B+ZF2O+LZna). Comprehending the anticancer and antioxidant characteristics of A. multiplicatum extract [(Donia et al., 2008)](https://paperpile.com/c/wr9Yj3/UgVtu) may facilitate the creation of innovative therapeutic agents for the treatment of liver cancer. Moreover, deciphering the fundamental processes of its bioactivity might provide valuable understanding of the complex interactions between oxidative stress and carcinogenesis, which would aid in the development of focused treatment therapies [(Ghasemi et al., 2021)](https://paperpile.com/c/wr9Yj3/TPJ1m). In the end, this research has the potential to strengthen our defenses against liver cancer and enhance patient outcomes. The present study aims to focus on the anticancer, antimicrobial and anti-inflammatory effect of the Ascidian Aplidium multiplicatum through its methanol extract [(Chokkattu et al., 2022; Marya et al., 2022; Ramamurthy et al., 2022)](https://paperpile.com/c/wr9Yj3/VYvM4+KE4Qs+YmDbY).

# Materials and methods

## Sample collection

Aplidium multiplicatum ascidian samples (Figure.1) were gathered from the coastal regions of Thiruchendur (Lat’ E: 8.495920838311422, Long’ N: 78.13086270300187). Following a thorough fresh water wash, the collected samples were extracted using methanol (weight/volume). After drying, the methanol extract was ground into a powder. Biological screening was then conducted using the dried extract.



Figure.1: Ascidian samples of Aplidium multiplicatum

## In-vitro anticancer effect of A.mutiplicatum

### Cell lines and cell culture

HepG2 (liver cancer cell line), and THLE-2 (Normal hepatocytes cells) were obtained from NCCS Pune. HepG2, and THLE-2 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) added with 10% FBS, 1% L-Glutamine, 0.1 mM (milli Molar) nonessential amino acid, and 100 U/mL penicillin/streptomycin. Cells were incubated in a humidified incubator at 37 °C with 5% CO2.

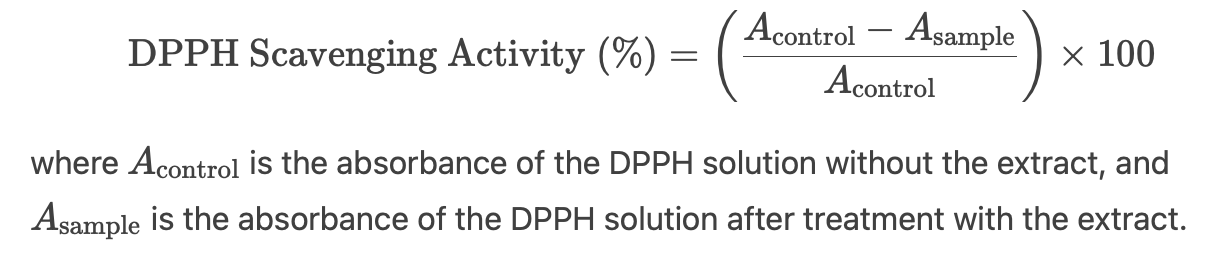
### Cell viability assay

The cells were seeded in 96-well plates containing the appropriate growth medium and allowed to proliferate until they reached approximately 70% confluency. Different doses of ascidian extract, along with a DMSO control, were administered to the cells for 24 hours. Following treatment, the medium was carefully removed, and the cells were gently washed with phosphate-buffered saline (PBS). Next, 0.5 mg/mL MTT solution was added to each well, and the plate was incubated at 37°C in the dark for 4 hours. After incubation, the MTT solution was discarded and replaced with 200 µL of DMSO to dissolve the formazan crystals. Idarubicin, a well-established chemotherapeutic agent, was included as a positive control. The plate was then shaken at 150 rpm for 5 minutes to ensure homogeneity, and the absorbance was recorded at 490 nm using a microplate reader. To ensure reproducibility, the experiment was performed in triplicate, and the mean values were used for data analysis and graph preparation. [(Adel et al., 2023; Ghasemi et al., 2021; Subramanian & Harikrishnan, 2023)](https://paperpile.com/c/wr9Yj3/TPJ1m+WNhoE+U67Fk)

## Antioxidant assay

### DPPH Assay

To evaluate the antioxidant activity, 1.0 mg of Vitex altissima leaf extract was combined with 0.25 mL of 0.5 mM DPPH (2,2-diphenyl-1-picrylhydrazyl) solution prepared in ethanol. The mixture was incubated at room temperature for 20 minutes in the dark to allow the reaction between the extract and free radicals. Absorbance was then measured at 517 nm using a spectrophotometer. The percentage of DPPH radical scavenging activity was determined by comparing the reduction in absorbance of the sample against the control (ethanol with DPPH alone), using the following formula [(Kedare & Singh, 2011)](https://paperpile.com/c/wr9Yj3/Dx0jf):

 (1)

### Nitric oxide scavenging activity

The nitric oxide (NO) scavenging activity was evaluated using a modified method adapted from Naresh et al. (2015). In this assay, sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions. The nitrite concentration was determined using Griess reagent.

Briefly, 1.0 mg of Vitex altissima leaf extract was added to 3 mL of reaction mixture containing 10 mM sodium nitroprusside in phosphate-buffered saline (PBS, pH 7.4). The mixture was incubated at 25°C for 150 minutes. At 30-minute intervals, 0.5 mL aliquots were withdrawn and mixed with an equal volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride). After 10 minutes of incubation at room temperature, the absorbance of the chromophore formed was measured at 546 nm using a spectrophotometer. Quercetin, a known antioxidant compound, served as the positive control. [(Naresh et al., 2015)](https://paperpile.com/c/wr9Yj3/Rm4Zp)

### Ferrous ion chelating activity

The ferrous ion (Fe²⁺) chelating activity of Vitex altissima leaf extract was evaluated according to a modified method based on [(Dinis et al., 1994)](https://paperpile.com/c/wr9Yj3/zYq0Q) . In this assay, 1 mg of the extract was mixed with 0.05 mL of 2 mM FeCl₂ solution. The reaction was initiated by adding 0.2 mL of 5 mM ferrozine solution. The mixture was vortexed thoroughly and allowed to stand at room temperature for 10 minutes to enable complex formation between ferrous ions and ferrozine.

The absorbance of the resulting solution was measured at 562 nm using a spectrophotometer. A control sample containing only FeCl₂ and ferrozine was processed simultaneously. All measurements were performed in triplicate, and results were expressed as mean values.

The percentage inhibition of ferrozine-Fe²⁺ complex formation was calculated using the following equation:

% Inhibition = [(A₀ - A₁)/A₀] × 100 (2)

where:  
A₀ = absorbance of the control (Fe²⁺ + ferrozine)  
A₁ = absorbance of the test sample (Fe²⁺ + ferrozine + extract)

### Reducing power assay

Based on the methodology of [(Bhalodia et al., 2013)](https://paperpile.com/c/wr9Yj3/KJQmJ) the reductive ability of sponge extracts was quantified. In this assay, 1 mg of sponge extract dissolved in 1 mL distilled water was combined with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide (K₃[Fe(CN)₆]). The reaction mixture was incubated at 50°C for 20 minutes to allow redox reactions to occur.

Following incubation, 2.5 mL of 10% trichloroacetic acid (TCA) was added to terminate the reaction. The mixture was then centrifuged at 1000 × g for 10 minutes to precipitate proteins and other interfering compounds. From the resulting supernatant, 2.5 mL was carefully collected and mixed with an equal volume of distilled water (2.5 mL) and 0.5 mL of 0.1% ferric chloride (FeCl₃) solution. The absorbance of the final solution was measured at 700 nm using a spectrophotometer.

The reducing power of the extracts was determined by their ability to reduce ferricyanide (Fe³⁺) to ferrocyanide (Fe²⁺), which then reacts with ferric chloride to form the ferric-ferrous complex. Higher absorbance values at 700 nm indicate greater reducing capacity of the tested extracts.

### Statistical analysis

The experiments were done in triplicate assay to obtain standard error mean ± values. One-Way ANOVA was performed to validate the p value of significance where p>0.5 was considered significant. SPSS package was used for One way ANOVA.

# Results

## Anticancer effect

The extract of Aplidium multiplicatum demonstrated potent anticancer activity against HepG2 liver cancer cells, showing significant cytotoxicity with an IC50 value of 136.2 μg/mL. This activity was comparable to the standard anticancer drug idarubicin used as a positive control. Importantly, the extract exhibited no cytotoxic effects on normal THLE-2 hepatocytes even at high concentrations up to 500 μg/mL, indicating its selective toxicity toward cancer cells while sparing normal cells. These findings, presented in Figures 2-4, suggest that A. multiplicatum contains bioactive compounds with specific anticancer properties and a favorable safety profile toward normal liver cells (Chehelgerdi et al., 2023). The marked difference in response between cancerous and normal cell lines highlights the potential therapeutic value of this marine extract for liver cancer treatment, warranting further investigation of its active components and mechanisms of action.

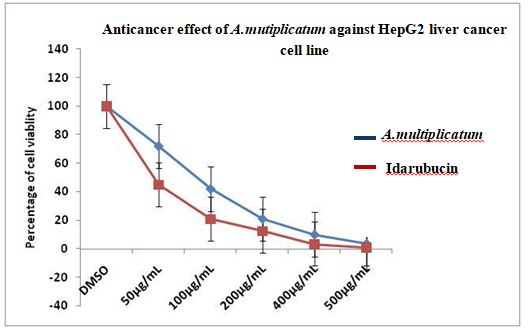
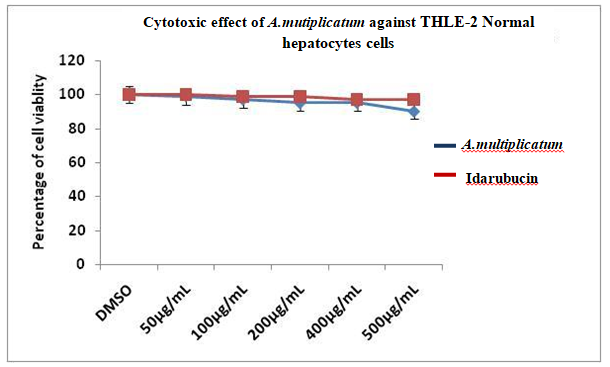


Figure.2: Anticancer effect of A.multiplicatum against HepG2 cells.

 Figure.3: Cytotoxic effect of A.multiplicatum against THLE-2 cells.

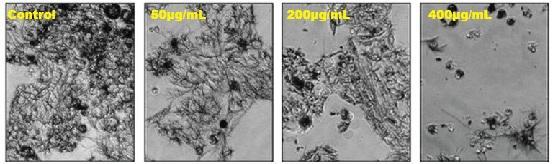


Figure.4: Anticancer effect of A.multiplicatum at different concentrations

## Antioxidant effect

A.multiplicatum extract exhibited a strong in-vitro antioxidant effect in terms of scavenging free radicals and reducing agents. The extract showed an 86.38% scavenging effect against DPPH radicals alongside a 78.67% scavenging effect against nitric oxide and a 95.39% ferrous ion chelating effect. Almost 88.63% of the reduction percentage of radicals is observed in the reducing power assay. Quercetin was used as a positive control antioxidant standard (Figure.3).

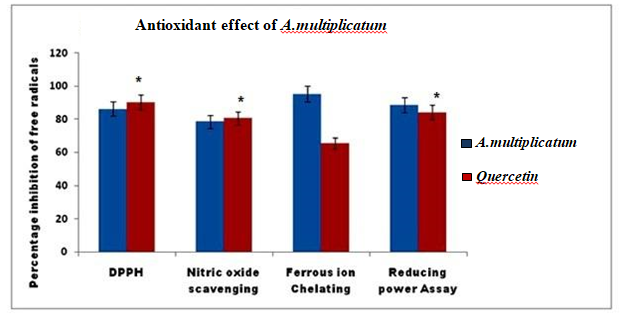


Figure.3: Antioxidant effect of A.multiplicatum

# Discussion

The current investigation revealed that Aplidium multiplicatum extract possesses significant pharmacological potential, demonstrating both anticancer and antioxidant properties. The extract showed notable cytotoxic effects against HepG2 hepatocellular carcinoma cells, with an IC50 value of 136.2 μg/mL, comparable to the standard chemotherapeutic agent idarubicin. Importantly, cytotoxicity assessments revealed the extract's selective action, showing no adverse effects on normal THLE-2 hepatocytes at concentrations up to 500 μg/mL. These findings suggest a favorable therapeutic window for potential anticancer applications. [(Amrutha Shenoy, Vinay Sivaswamy, Subhabrata Maiti, Deepak Nallaswamy, n.d.)](https://paperpile.com/c/wr9Yj3/QLe9r).

The extract also exhibited robust antioxidant capacity, demonstrating 83% DPPH radical scavenging activity and showing comparable efficacy to quercetin in ferrous ion chelation (92%), reducing power assays, and nitric oxide scavenging tests. These results align with previous findings on marine organism bioactivity [(Jain & Verma, 2022; Solanki et al., 2023; Sreevarun et al., 2023; Wadhwani et al., 2022)](https://paperpile.com/c/wr9Yj3/FfoMg+deJ5j+w373o+iW6tm). Previous research on ascidian extracts has documented similar bioactive potential (Saadh et al., 2024). Studies have reported potent antiproliferative effects (IC50 5 μg/mL) of C. dellechiajei extracts against various cancer cell lines, including A-549 lung carcinoma, H-116 colon adenocarcinoma, PSN-1 pancreatic adenocarcinoma, and SlCBR3 breast carcinoma cells [(*Anti-Inflammatory Potential Mouthwash Formulated Using Clove Ginger Mediated Zinc Oxide Nanoparticles: Vitro Study*, n.d.; Chokkattu et al., 2023; Martinez-García et al., 2007; Muthuswamy Pandian et al., 2022)](https://paperpile.com/c/wr9Yj3/1DS1V+pCdsT+WWO94+WrR01). Notably, the highest bioactivity was consistently observed in the tunic tissue of these marine organisms, supporting our findings on tissue-specific compound distribution [(Liberio et al., 2014)](https://paperpile.com/c/wr9Yj3/Lri8n). Further supporting evidence comes from studies on ascidian-derived compounds. Eusynstyelamide B from Eusynstyela latericius demonstrated specific cytotoxicity against MDA-MB-231 breast cancer cells through apoptosis induction [(Torres et al., 2002)](https://paperpile.com/c/wr9Yj3/EeFEv). Similarly, pyridoacridine alkaloids (sebastianines A and B) isolated from Brazilian Cystodytes dellechiajei showed selective activity against colon cancer cells. These collective findings underscore the therapeutic potential of marine-derived compounds in oncology research.

# Conclusion

In the present study the ascidian Aplidium multiplicatum extract has a higher anticancer and anti-oxidant effect compared to standard drugs. Further in depth research should be carried out to have a drug discovery pipeline strategy.

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