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Research Article

Preliminary Cytotoxic Screening of *Turbinaria ornata*–Derived Endophytic Fungi Using Brine Shrimp Lethality Assay

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ABSTRACT

Turbinaria ornata, a chemically rich brown seaweed, is known to possess cytotoxic potential, yet the biological activities of its associated endophytic fungi remain largely unexplored. This study aimed to evaluate the preliminary cytotoxicity of ethyl acetate extracts obtained from endophytic fungi isolated from *T. ornata* using the Brine Shrimp Lethality Test (BSLT). Four isolates (TO1–TO4) were cultured on rice media, extracted through maceration, and assessed for toxicity. Mortality data were analyzed using probit analysis to determine the median lethal concentration (LC_{50}), and toxicity categories were interpreted following Meyer's criteria. Thin-Layer Chromatography (TLC) was performed to provide preliminary phytochemical insights. Only isolate TO4 exhibited measurable toxicity, with an LC_{50} value of 807.20 ppm, whereas the other extracts were classified as non-toxic. TLC profiling revealed alkaloid and terpenoid/sterol constituents, suggesting the presence of bioactive secondary metabolites commonly associated with cytotoxic effects in marine-derived fungi. *T. ornata* harbors endophytic fungi with modest cytotoxic potential, with TO4 representing the most promising isolate. Further bioassay-guided fractionation, metabolite characterization using LC–MS and NMR, and cytotoxic evaluation against human cancer cell lines are recommended to identify potential marine-derived anticancer lead compounds.

Keywords:
Brown seaweed; BSLT; endophytic fungi; LC_{50}; *Turbinaria ornata*

INTRODUCTION

Cancer remains one of the foremost global health challenges, with incidence and mortality increasing steadily due to aging populations and shifts in lifestyle and environmental exposures (Bray et al., 2024). Current chemotherapeutic modalities, although effective, are often limited by toxicity, poor selectivity, and multidrug resistance, necessitating the continuous search for safer and more selective anticancer lead compounds (Xiao et al., 2016). Cancer-related mortality continues to rise rapidly in many developing countries, driving significant global efforts to discover and develop new anticancer agents. The process of producing a single new drug is estimated to cost around US$2.6 billion, while global spending on anticancer medications has already exceeded US$100 billion and is projected to reach US$150 billion by 2020. Despite this substantial economic investment, many currently available chemotherapeutic agents still exhibit considerable toxicity toward healthy proliferating cells, cause severe adverse effects, and show limited effectiveness against several cancer types. These issues emphasize the need to explore novel bioactive compounds from natural sources that may provide safer, more selective, and more effective alternatives for cancer treatment (Uzma et al., 2018). Natural products continue to play a central role in anticancer drug development, with an estimated more than 60% of approved chemotherapeutic agents being either natural molecules or their structural

derivatives. The marine environment, in particular, represents one of the largest and most chemically diverse reservoirs of bioactive compounds (Ghosh et al., 2024).

Marine organisms have emerged as a rich source of structurally diverse natural products, offering unique scaffolds for anticancer drug discovery (El-Seedi et al., 2025). Among marine macroalgae, brown seaweeds (Phaeophyceae) are particularly noteworthy for their chemical diversity and broad repertoire of bioactivities (Remya et al., 2022). Within this ecosystem, marine macroalgae (seaweeds) have attracted increasing scientific interest due to their capacity to produce secondary metabolites with notable pharmacological properties. Many of these algal-derived compounds exhibit mechanisms relevant to cancer therapy, including inhibition of tumor cell proliferation, induction of apoptosis, suppression of angiogenesis, and interference with metastatic processes (Malhão et al., 2021). Brown seaweeds have long been investigated because they are rich in key bioactive constituents such as fucoidan and fucoxanthin, compounds known to contribute to a wide spectrum of pharmacological activities. Numerous brown macroalgae species have demonstrated notable antioxidant, antidiabetic, anti-inflammatory, antiviral, antiproliferative, and anticoagulant properties, supporting their relevance in drug discovery. The cytotoxic and antiangiogenic activities reported in brown algae are likewise believed to be associated with their phenolic content and the presence of fucoxanthin and fucoidan, as documented in previous studies. Phenolic compounds, in particular, play an essential role in mitigating oxidative stress–mediated pathways involved in carcinogenesis through their strong antioxidant mechanisms. Consistently, the cytotoxic potential of brown algae is closely linked to these metabolically active constituents, emphasizing their importance as promising marine-derived sources of anticancer agents (Canoy & Bitacura, 2018).

Species of the genus Turbinaria exhibit multiple pharmacological properties, including antioxidant, antimicrobial, and anti-inflammatory effects (Rushdi et al., 2021). Phytochemical investigations of *Turbinaria ornata* have revealed a chemically rich profile dominated by phenolics, flavonoids, terpenoids, tannins, and saponins, supporting the species' diverse therapeutic potential (Remya et al., 2022). Recent studies further strengthen this potential; for instance, Bharath et al. (2021) successfully isolated hexadecanoic acid from *T. ornata*, demonstrating significant antiproliferative activity against HT-29 colon cancer cells, marking one of the few pure compounds from this species with validated anticancer activity. Although the bioactivity of *T. ornata* extracts has been increasingly documented, current research remains heavily focused on the macroalgal metabolites. At the same time, investigations into its associated microbiome, particularly endophytic fungi, are minimal. This underexploitation is notable given that marine macroalgae often harbor endophytes capable of producing metabolites either complementary to or more potent than their hosts (Debbab et al., 2011; El-Seedi et al., 2025). Several studies on other brown seaweeds, such as Sargassum, Padina, and Dictyota, have revealed endophytic fungi with strong cytotoxic, antimicrobial, and enzyme-inhibitory activities (Evidente, 2024; Noor et al., 2025). Moreover, recent investigations into the microbial community associated with this species are still limited, particularly regarding endophytic fungi that may contribute unique or complementary bioactive compounds.

Marine-endophyte studies emphasize that the metabolic repertoire of fungal symbionts often includes novel alkaloids, terpenoids, polyketides, and sterols with potent anticancer effects, compounds that may not be present in the host organism itself (Fu et al., 2025; Handayani et al., 2022). For example, indole-diterpenoid analogs known as shearinines, produced by Penicillium strains, have been reported to exert cytotoxic activity against several cancer cell lines and were recently expanded by the discovery of new shearinine congeners from marine-derived Penicillium with demonstrated bioactivity (Fu et al., 2025). Chaetomium species yield chaetoglobosins (a class of cytochalasans) that inhibit tumor cell proliferation, induce apoptosis, and can block migration/invasion in aggressive cancer models; specific chaetoglobosins (e.g., chaetoglobosin E) have shown tumor-suppressive effects and synergism with chemotherapeutics in preclinical studies (Duan et al., 2022). Another well-documented fungal mycotoxin with promising anticancer profiles is beauvericin, produced by Fusarium and *Beauveria* spp.; beauvericin exerts cytotoxicity via mitochondrial dysfunction, increased intracellular Ca^{2+}, oxidative stress, and apoptotic pathways, and has been investigated in both in vitro and in vivo tumor models (Wu et al., 2018). Epipolythiodiketopiperazines (e.g., gliotoxin and related derivatives) isolated from marine Aspergillus and other genera have also shown potent cytotoxicity and modulatory effects on signaling pathways relevant to cancer, though their therapeutic window requires careful evaluation due to toxicity (Nguyen et al., 2013). Historically notable is the early report of paclitaxel (Taxol) associated with an

endophytic fungus (Taxomyces andreanae), which stimulated interest in fungal production of plant anticancer agents (Kasaei et al., 2017).

The limitations of such explorations in *T. ornata* represent a significant knowledge gap, particularly considering that other Turbinaria species have been shown to host diverse fungal communities with biotechnological promise. Thus, the cytotoxic potential of *T. ornata*-derived endophytic fungi remains an unexplored but highly plausible source of novel marine-based anticancer leads, warranting systematic investigation. Marine-derived endophytic fungi are increasingly recognized as prolific producers of structurally novel and biologically potent secondary metabolites, often capable of generating compounds distinct from or complementary to their host algae (Debbab et al., 2011). Considering the demonstrated anticancer activity of metabolites derived directly from *T. ornata* and the absence of research on its fungal endophytes, targeted exploration of these microorganisms may provide a new avenue for anticancer lead discovery. Therefore, this study aims to evaluate the preliminary cytotoxicity of ethyl acetate extracts of endophytic fungi isolated from *T. ornata* using the Brine Shrimp Lethality Test (BSLT), thereby establishing foundational evidence for the identification of novel marine-derived anticancer candidates.

METHODS

Collection of samples

The brown macroalga sample was collected from the coastal waters of Nagari Air Haji Tengah, Linggo Sari Baganti District, Pesisir Selatan Regency, West Sumatra, Indonesia. Taxonomic verification was conducted at the Ecology Laboratory, Department of Biology, Universitas Andalas, Padang, where the specimen was identified as *Turbinaria ornata* (family Sargassaceae). A reference voucher was prepared under the accession number 008/EKL-H/Alga/IX/2024.

Surface sterilization and isolation of endophytic fungi

The collected brown macroalga was first surface-sterilized by washing under running water to remove debris, then immersed in 70% ethanol for 1 minute and rinsed with sterile distilled water; the final rinse was plated onto SDA medium as a negative control. Endophytic fungi were isolated using two approaches. In the direct planting method, sterilized algal tissues were cut into 1 × 1 cm fragments and placed on SDA plates, which were then incubated at 20–25 °C for 2–14 days. In the dilution–plating method, 10 g of sterilized algal material was macerated with 10 mL sterile distilled water, filtered, and serially diluted to 10^{-6}; aliquots of the final dilution were spread onto SDA and incubated at 27–29 °C for 7–8 days under aseptic conditions in a laminar air flow hood. Fungal colonies that emerged were purified by successive subculturing onto fresh SDA plates, selecting based on macroscopic traits such as colony colour and morphology, until distinct pure isolates were obtained, which were then incubated at 20–25 °C for 7–10 days (Kjer et al., 2010) .

Cultivation and extraction of endophytic fungal isolates

Pure rejuvenated endophytic fungal isolates were transferred onto rice-based solid media by placing small agar plugs onto the substrate, followed by incubation at 20–25 °C for 4–6 weeks to allow optimal mycelial colonization. After incubation, each fully colonized culture was subjected to extraction by maceration in 100 mL of ethyl acetate for three days with intermittent agitation. The resulting solvent phase was separated and filtered to remove culture debris, while the remaining residue was remacerated with fresh ethyl acetate to ensure exhaustive extraction. Combined filtrates were then concentrated using a rotary evaporator to yield the crude ethyl acetate extract of the respective endophytic fungi (Handayani et al., 2022).

Preliminary Cytotoxic assay using Brine Shrimp Lethality Test (BSLT)

A partitioned hatching chamber was prepared, with one side illuminated to provide warmth and the opposite side filled with sterile seawater. Approximately 2.5 mg of Artemia salina eggs were introduced into 250 mL of steril seawater and incubated under continuous light for 48 hours to obtain active nauplii. Fungal extracts were serially diluted in DMSO, and ten nauplii were transferred into test tubes containing 5 mL of steril seawater supplemented with extract concentrations of 1000, 500, 250, 125, and 62.5 ppm. Each concentration was tested in triplicate, and mortality was recorded after 24 hours of exposure. The median lethal concentration (LC_{50}) was subsequently determined using probit analysis, and toxicity levels were interpreted according to Meyer et al., (1982), categorizing samples as highly toxic (LC_{50} < 30 ppm), toxic (30–1000 ppm), or non-toxic (> 1000 ppm) (Kjer et al., 2010).

Thin-Layer Chromatography (TLC) and Phytochemical Profiling

The secondary metabolite composition of the ethyl acetate extract from isolate TO4 was examined using thin-layer chromatography (TLC). Analyses were carried out on silica gel 60 F254 plates, which were developed in a solvent mixture of n-hexane, ethyl acetate, and dichloromethane (1:2:3, v/v/v). Following chromatographic separation, the plates were allowed to dry and then observed under UV illumination at wavelengths of 254 and 366 nm to visualize resolved spots. To obtain preliminary phytochemical information, the chromatograms were subsequently treated with several diagnostic spray reagents, including Dragendorff's reagent for alkaloid detection, ferric chloride for phenolic constituents, and the Liebermann–Burchard reagent to indicate the presence of steroids or triterpenoids. The formation of characteristic color reactions was taken as evidence of the respective compound classes (Fathima & George, 2024).

RESULTS AND DISCUSSION

Four endophytic fungal strains, labeled TO1 through TO4, were obtained from the brown macroalga *T. ornata*. Each isolate displayed distinctive colony characteristics observable at the macroscopic level (Figure 1). Isolate TO1 presented as a dense, cottony mycelial mass with a white–cream hue and uneven colony margins. Isolate TO2 also produced a cotton-like texture but showed a combination of greenish-yellow and white pigmentation with irregular edges. In comparison, isolate TO3 exhibited a soft, cottony colony appearance characterized by green to pale yellow-cream tones accompanied by subtle white borders. Isolate TO4 differed markedly from the others, forming compact cottony colonies with a contrasting black-and-cream coloration and nonuniform margins.

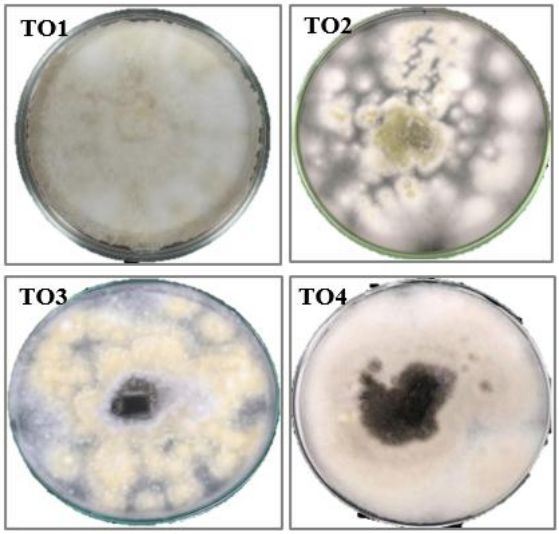

Figure 1. Macroscopic morphological features of the four endophytic fungi isolated from the brown macroalga *T. ornata* (TO1–TO4)

The brine shrimp lethality assay revealed clear variability in cytotoxic potential among the four endophytic fungal extracts obtained from *T. ornata*. Only one extract demonstrated appreciable lethality toward *Artemia salina* nauplii and was therefore classified as toxic, indicating a relatively strong bioactive response. In contrast, the remaining three extracts produced minimal mortality and were categorized as non-toxic. Cytotoxic metabolites are not uniformly distributed across the fungal endophytes associated with the host alga, with isolate TO4 emerging as the most promising candidate for further bioactivity-guided investigation. The LC_{50} values for each extract are presented in Table 1.

Table 1. Cytotoxicity Potential of Endophytic Fungal Extracts based on BSLT

Extract	LC_{50} (ppm)	Category
TO1	>1000	Non-toxic
TO2	>1000	Non-toxic
TO3	>1000	Non-toxic
TO4	807.20	Toxic

When compared with previously reported cytotoxic profiles of *T. ornata* itself, the pattern observed here is consistent with the relatively moderate lethality commonly associated with this species. A prior study reported that crude extracts of *T. ornata* displayed a median lethal concentration (LC_{50}) of approximately 320.4 µg/mL, indicating moderate bioactivity rather than high potency. This aligns with the present findings, where the majority of isolates were non-toxic and only one isolate produced noticeable lethality, suggesting that the endophytic community associated with *T. ornata* may similarly express metabolites of modest cytotoxic strength (Kurniatanty et al., 2015). A more pronounced contrast emerges when these findings are positioned against the cytotoxic profiles of fungal endophytes isolated from other brown and red seaweeds. In a recent investigation of endophytic fungi from *Gracilaria* sp. and *Sargassum* sp. from the Bay of Bengal, Bangladesh, six fungal isolates belonging to *Aspergillus*, *Cladosporium*, *Chaetomium*, and *Curvularia* displayed remarkably high lethality, with all extracts exhibiting LC_{50} values below 20.39 µg/mL. The most active isolates, *Curvularia perotidis* and *Cladosporium halotolerans*, demonstrated LC_{50} values near 9–10 µg/mL, indicating strong cytotoxic capacity. Such potency is substantially greater than the toxicity observed in the present study, underscoring that endophytic cytotoxicity can vary widely not only between isolates but also across different algal hosts and environmental conditions (Noor et al., 2025).

Several resolved spots were observed under UV illumination at 254 & 366 nm wavelengths, indicating the presence of multiple secondary metabolite components with varying polarity when developed in the mobile phase consisting of chloroform, ethyl acetate, and methanol (1:1:1) (Figure 2).

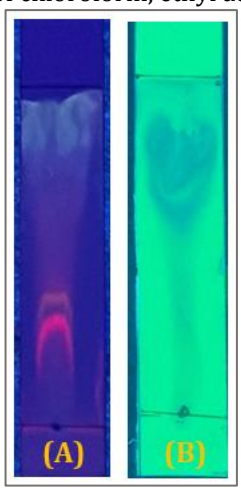

Figure 2. TLC profile of the ethyl acetate fungal extract under UV 366 nm (A), and UV 254 nm (B)

Combined observations indicate that the extract contains alkaloids (orange spot) and terpenoid/sterol (brown-green spot) constituents. At the same time, flavonoids and phenolics appear absent or below detection levels under the applied conditions (Figure 3). These chemical classes are related to secondary metabolites commonly reported from marine-derived endophytic fungi and are well known for their cytotoxic potential. For example, marine Penicillium strains have yielded indole diterpenoids, such as shearinines, which are representative alkaloid-type compounds exhibiting moderate to strong cytotoxicity across several cancer cell lines (Fu et al., 2025). Likewise, terpenoid and sterol derivatives, particularly ergosta-type metabolites, are repeatedly identified from marine endophytic Aspergillus and Penicillium, with several reports documenting strong cytotoxic effects in mammalian cell-based assays (Evidente, 2024). Collectively, the presence of these metabolite classes in the active extract supports the likelihood that the observed toxicity in the BSLT assay is driven by bioactive fungal secondary metabolites, consistent with chemotypes previously recognized for anticancer potential in marine-derived fungi.

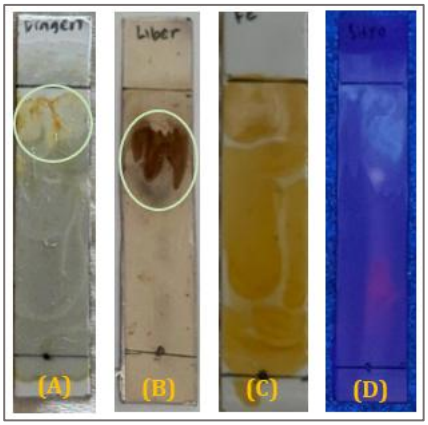

Figure 3. TLC detection of secondary metabolite classes using specific spray reagents: Dragendorff-positive alkaloids (A), Liebermann–Burchard–positive terpenoid/steroid compounds (B), $FeCl_3$-negative phenolics (C), and Citroborate-negative flavonoid-like metabolites (D).

The identification of isolate TO4 as the only endophytic fungus exhibiting measurable cytotoxicity highlights its potential as a promising source of marine-derived bioactive metabolites. However, the moderate toxicity level observed in this preliminary screening suggests the need for more in-depth investigation. Future research should include bioassay-guided fractionation, followed by comprehensive structural elucidation using LC–MS/MS, NMR, and molecular networking approaches to identify the specific metabolites responsible for the observed activity. In addition, cytotoxic evaluation on mammalian cancer cell lines (e.g., MCF-7, HeLa, HT-29, A549) is warranted to validate its anticancer relevance beyond invertebrate-based assays.

CONCLUSIONS

This study demonstrated that endophytic fungi isolated from *Turbinaria ornata* possess varying degrees of cytotoxic potential, with only one isolate (TO4) exhibiting toxicity in the brine shrimp lethality assay. TLC profiling indicated that the active extract contains alkaloid and terpenoid/sterol-type metabolites, showing the presence of bioactive secondary metabolites commonly associated with cytotoxic properties in marine-derived fungi. This study suggests that TO4 is a promising candidate for further investigation. Comprehensive bioactivity-guided fractionation, metabolite elucidation through LC–MS and NMR, and subsequent evaluation on human cancer cell lines are recommended to determine its potential as a source of novel cytotoxic lead compounds.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest related to the conduct of this research, the preparation of the manuscript, or its publication.

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