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Antiproliferative Activity of Philippine Marine Sediment-Derived Actinomycetes

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ABSTRACT

The Philippine archipelago is rich in marine biodiversity and resources that are widely unexplored. Its marine sediments harbor marine microbes that possess secondary metabolites with potent bioactivities. This study aims to determine the antiproliferative activity of the crude extracts of selected *Actinomycete* isolates (DSD011, DSD017, and DSD042) from Islas de Gigantes, Carles, Iloilo. The antiproliferative screening was done using *Saccharomyces cerevisiae* as a model organism. Crude extracts of isolates that are active in inhibiting the growth of *S. cerevisiae* were determined using the broth microdilution method. Afterward, the active extract was tested using antiproliferative and budding yeast assays. With the antiproliferative model, only DSD011 was found to inhibit the growth of *S. cerevisiae*. The percentage of live and dead cells in DSD011 was comparable to those treated with Triton X (positive control). Further, the budding yeast analysis showed that DSD011 induced G1 cell cycle arrest of nearly 50% of *S. cerevisiae* cells. Thus, DSD011, a marine sediment-derived *Actinomycete*, serves as a potential source of naturally occurring bioactive compounds with antiproliferative properties.

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INTRODUCTION

Cancer remains as one of the most lethal and widespread diseases worldwide. In 2020, cancer incidence reached 19.3 million new cases and 10.0 million new deaths, with lung cancer as the most prevalent (Sung et al., 2021). The ability of metastatic cancer cells to develop ways of resisting or avoiding the action of chemotherapeutic agents aggravate the problem posed by cancer to global health. Most treatment outcomes remain inadequate despite the progress made in developing various treatment strategies involving chemotherapeutic agents (Asati et al., 2014). Due to this, new classes of chemotherapeutic substances with much more selective action against cancer cells are urgently needed (Trotsko et al., 2018).

An intensive search for anticancer drugs in the last 50 years pointed to soil bacteria as a reliable source of novel compounds with pharmaceutical potential (Mohr, 2016). Forty-five percent of currently known microbial secondary metabolites come from *Actinomycetes*, particularly in the genus *Streptomyces* (Dalisay et al., 2013). Many of these metabolites possess potent anticancer activity against various cancer cell lines (Chen et al., 2018). However, recent discoveries of novel pharmaceuticals from *Streptomyces* have drastically decreased (Kemung et al., 2018). The steady decrease in the number of developed and approved pharmaceutical substances poses a serious problem as there are increasing incidences of anticancer resistance. Alternative approaches for developing drugs from microbes are urgently needed due to the worsening problem of anticancer resistance. One such approach is the isolation of microbes from underexplored ecosystems such as desert biomes and marine ecosystems.

Marine microorganisms are a prolific source of novel compounds with varying chemical structures, many of which exhibit bioactivities such as anticancer or antitumor activity (Butler et al., 2013). Among these microorganisms are marine *Actinomycetes* that produce secondary metabolites of varying structural classes. Besides being widely abundant in soil, *Actinomycetes* also have a wide distribution in the ocean, from shallow sea bodies to deep-sea sediments (Chen et al., 2016). In the recent years, bacteria dwelling in marine sediments have become the focus of many for the investigation of possible sources of novel bioactive compounds (Dalisay et al., 2013). Exploring this new source of microorganisms resulted in the discovery of *Salinispora*, a new taxon of obligate marine *Actinomycetes* (Kim et al., 2020). Members of this genus possess extraordinary biosynthetic diversity, as evident in their secondary metabolite biosynthetic gene clusters (SMBGC) (Xu et

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al., 2019). The existence of these remarkable SMBGCs in the genome of marine *Actinomycetes* was linked to their functional adaptation in their distinct niche and habitat (Penn et al., 2009). These SMBGCs provide specialized metabolism to marine *Actinomycetes,* positing them as a potential source of novel bioactive compounds (Letzel et al., 2017). Salinosporamide A, an irreversible proteasome inhibitor isolated from the actinomycete *Salinospora tropica,* accomplished a series of phase I clinical trials to treat multiple myeloma and other hematologic malignancies (Kim et al., 2020). Furthermore, the current investigation of secondary metabolites from actinomycetes of the genus *Nocardiopsis*, *Streptomyces*, *and Micromonospora* yielded promising bioactive compounds (Kim et al., 2018).

In response to the health crisis posed by cancer worldwide, marine sediments were collected from selected locations of Islas de Gigantes, Iloilo, as a source of marine-derived *Actinomycetes*. Marine *Actinomycetes* were isolated from the sediment samples and their crude extracts were screened against *Saccharomyces cerevisiae*. Budding yeast bioassay using *S. cerevisiae* is an established method useful for the preliminary screening for potential anticancer compounds (Sanchez-Pico et al., 2014). Budding yeast possesses cellular processes that are remarkably homologous to mammalian cells (Matuo et al., 2012). Eukaryotic cellular components such as actin (Akram et al., 2020) and telomerase (Armstrong & Tomita, 2017) are remained relatively unchanged between yeasts and human cells. Furthermore, antiproliferative compounds, such as tamoxifen (Yao et al. 2020), stevioside (Boonkaewwan et al., 2013), and lyc (Bertoli et al., 2013) were successfully identified using yeast-based screening assays. Thus, the current study used budding yeast bioassay to explore the antiproliferative activity of selected marine sediment-derived *Actinomycetes* isolated from Islas de Gigantes, Iloilo.

METHODS

Collection of marine sediments and culture isolation of Actinomycetes

Marine sediment samples were collected from the vicinity of Islas de Gigantes Carles, Iloilo through SCUBA diving at depths of 20 to 30 m from the sea surface. Sample collection was done by digging at least around 30 cm below the seafloor. Processing and inoculation of the marine sediment sample were performed as described by the methods of Sabido et al. (2020). The marine sediments were air-dried inside a biosafety cabinet and then processed using dry stamp and heat shock methods. Processed samples were inoculated in a solid marine medium (ISP4, Difco dissolved in filtered seawater) and incubated at room temperature (25°C- 28°C) for 15-30 days. The growth of *Actinomycetes* was confirmed by the presence of filamentous hyphae and/or the formation of tough, leathery colonies on the agar surface. Single colonies of *Actinomycetes* were isolated to pure cultures on enriched marine media (Dalisay et al. 2013).

Preparation of Actinomycetes crude extracts

Three (3) isolates from the marine sediment samples were investigated for antiproliferative activity using *S. cerevisiae*. These *Actinomycete* isolates were designated as DSD011, DSD017, and DSD042. The isolates were inoculated and allowed to grow in marine medium 1 (MM1) agar (Dalisay et al., 2013). The isolates inoculated in the MM1 agar were incubated for 14 days at 25-28°C. After the incubation period, the biomass of each isolate was harvested. The agar with the biomass was then extracted thrice with ethyl acetate. The extracts were then concentrated using a rotary evaporator and dried in a vacuum. The dried extracts were reconstituted in 100% dimethyl sulfoxide (DMSO) (100 mg/ml) and stored at -80°C freezer until tested for antiproliferative activity.

Preliminary screening for the antiproliferative activity of the crude extracts

A 24 h broth culture of *S. cerevisiae* ATCC 20784 (0.5 OD cell density at 620 nm) was prepared and exposed to different treatments as follows: dimethyl sulfoxide (negative control), Triton X-100 (positive control), and the reconstituted crude extracts of DSD011, DSD017, and DSD042. Five (5) µL of each extract (stock solution of 100 mg/ml) and controls were dispensed in separate wells in the 96-wells microtiter plate and added with 195 µL of the seed culture. The prepared plates were then incubated for 24 hours at 37 °C. After incubation, each plate was read using a Multiskan FC Microplate Photometer (Thermo Fisher Scientific, USA) at 620 nm. The color-corrected Optical Density (OD) and percentage growth inhibition (%GI) of the treatments and controls were computed (Myers et al., 2013). Crude extracts with %GI values of more than 50% were considered active and subjected to further testing.

Antiproliferation assay

A 24 h broth culture of *S. cerevisiae* was harvested through centrifugation at 8,000 rpm, washed with phosphate-buffered saline (PBS) pH 7.0 twice, and resuspended with 195 µl PBS. The seed suspension was adjusted to 0.5 OD cell density at 620 nm. The seed suspension was exposed to 5 µl of each extract with a final concentration of 2500 µg/ml. Triton X (positive control) and DMSO (negative control) were prepared with 2.5% v/v final concentration. Two (2) sets of untreated controls for compensation were also prepared accordingly and were incubated at 37 °C for 24 hours. After incubation, 5 µL of propidium iodide (5 µM) and 5 µL of SYTO 9 were added

to each treatment. For the compensation controls, one set of the untreated cells was stained with 5 µL of propidium iodide (5 µM), while another set of untreated cells was stained with 5 µL of SYTO 9 and incubated at 37 °C for 30 minutes in the dark. Afterward, the treated cells were harvested and washed twice by centrifugation (8,000 rpm for 5 minutes) and resuspended in 200 µL of PBS. Antiproliferative activity of the crude extracts was determined through intensiometric measurement using an Amnis Flow Sight imaging flow cytometer (AF-IFC) (Merck, USA). The AF-IFC system was calibrated, cleaned, and sterilized (sample lines). Each prepared seed suspension was then run in the AF-IFC and data acquisition was made using the INSPIRE ImageStreamX MKII software. The object count was set at 5000. The gating strategy for *S. cerevisiae* was adopted from the protocol of Patterson et al. (2015). All data acquired using the AF-IFC INSPIRE software were subjected to a gating (selecting) strategy, which mainly involved the following parameters: brightfield gradient root mean squared feature (Gradient RMS_M01_Ch01), brightfield area feature (Area_M01), brightfield aspect ratio feature (Aspect Ratio_M01), and fluorescence channel 5 (Intensity_MC_Ch05). Area values of 50-600 and Aspect ratio values of 0.4-1.0 were used to select *S. cerevisiae* cells. Crude extracts yielding >50% antiproliferative activity or more were considered active. The gated cell population was then graphed using a histogram with the Y-axis designating the cell count and the X-axis designating the fluorescence intensity reading in channel 5 (642-740 nm). Channel 5 fluorescence reading of more than 100 was considered characteristic of dead cells (Kamaruddin et al., 2019).

Budding yeast analysis

The effects of the crude extracts on the cell cycle of the yeast cells were determined through the budding yeast analysis. This is a post-hoc method from the images acquired by the imaging flow cytometer from the antiproliferative assay. Unbudded cells are in the G1 phase, small-budded cells are in the S phase, and large-budded cells are in the G2/M phase of the cell cycle (Sanchez-pico et al., 2014). Cell counts for the respective morphologies were analyzed using Image Data Exploration and Analysis Software (IDEAS). The different budding morphologies of the yeast were differentially grouped using the Area_M01 and Aspect Ratio_M01 features of the AF-IFC.

Statistical analysis

One-way analysis of variance was used to determine the significant difference between treatments in antiproliferative activity and live/dead assay. Post-hoc analysis Tukey HSD was used to determine pairwise differences between treatments. Analysis was considered significant if the p-value <0.05.

RESULTS AND DISCUSSION

Currently, the most extensively investigated microbial source of marine natural products involves bacteria isolated from marine sediments (Perez & Fenical, 2017). These marine sediment-derived microbes, particularly *Actinomycetes*, have been thoroughly studied, yielding numerous novel and unprecedented new chemical structures and potentiating the discovery of new drugs with a novel mechanism of action (Blunt et al., 2013). *Actinomycetes* are widely known to produce antiproliferative compounds in several structural classes, such as anthracyclines, enediynes, indocarbazoles, isoprenoides, macrolides, non-ribosomal peptides, and others (Sabido et al. 2020). The antiproliferative activity of these metabolites involves the induction of apoptosis through topoisomerase I or II inhibition, mitochondrial permeabilization, and inhibition of key enzymes involved in cell signal transduction (Karthikeyan et al., 2022).

Interestingly, our findings in the current study corroborate the previous findings regarding the antiproliferative activity of crude extract from a marine sediment-derived

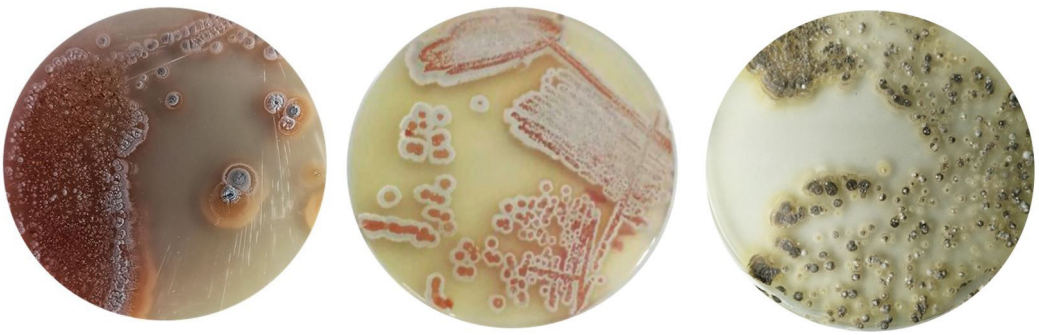

Figure 1. Streptomyces sp. Strain DSD011, DSD017, and DSD042 (left to right) grown in marine media (Sabido et al. 2021)

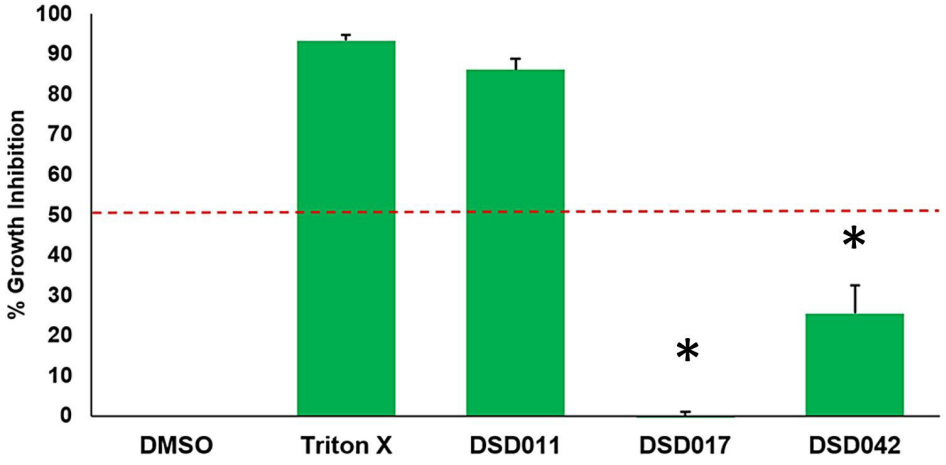

Figure 2. Percentage (%) growth inhibition of S. cerevisiae in the selected treatments and control. Concentration of Triton x is at 2.5% v/v while the rest of the treatments (isolates) are at 2500 µg/ml. The broken red line designates the 50% level for growth inhibition that is used as indicator if the isolate is bioactive as an antiproliferative agent or not. Values of % growth inhibition indicated are mean ± standard deviation (SD), n=3. (*) = significant difference with positive control (Triton-X) ($p<0.05$) using Tukey HSD. Negative control (DMSO) was excluded in the comparison.

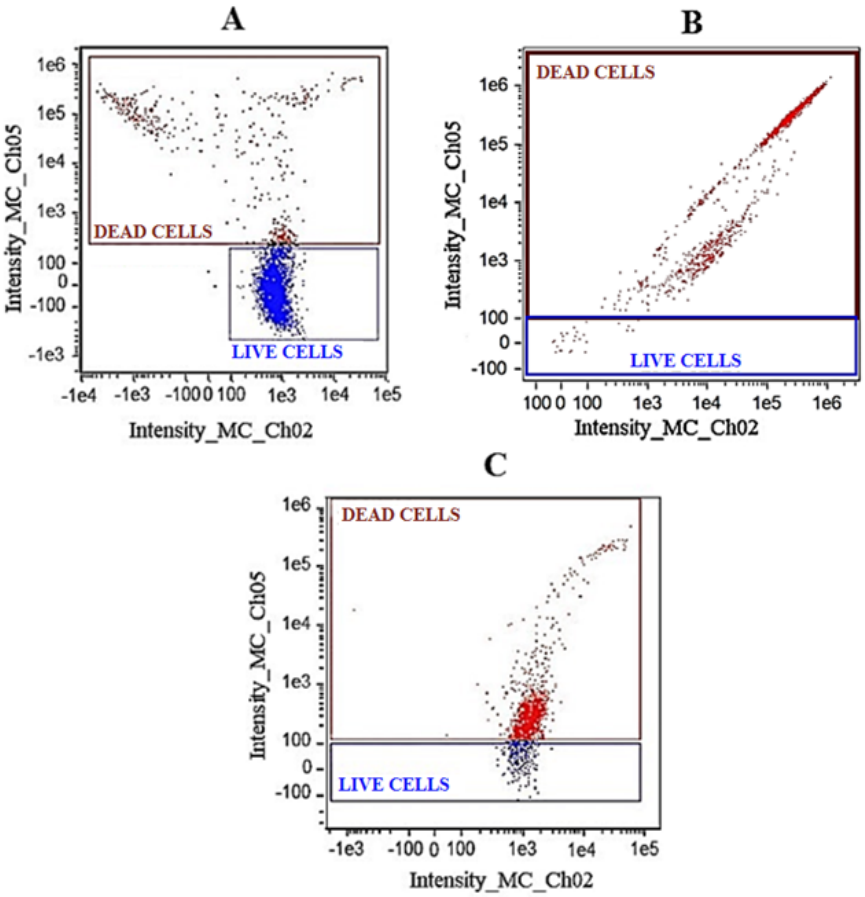

Figure 3. Representative scatterplot graphs for the fluorescence intensity emitted by the treated yeast cells in Channel 2 (505-560 nm) and Channel 5 (642-740 nm). The X-axis and Y-axis plot the fluorescence intensity of cells stained with SYTO 9 and propidium iodide, respectively. Population regions labelled in red and blue represent dead and live cells, respectively. The treatments in the experiment are as follows: (A) DMSO (negative control), (B) Triton X (final concentration at 2.5% v/v), and (C) DSD011 (2500 µg/ml).

Actinomycete. The antiproliferative activity of the crude extracts of select marine sediment-derived *Actinomycetes* (Figure 1) was determined using the budding yeast *S. cerevisiae* as a model organism (Patterson et al., 2015). *S. cerevisiae* is widely used in investigating cell cycle regulation, which is dysfunctional in cancer cells (Menacho-Marquez & Murguia, 2007). This makes *S. cerevisiae* an invaluable tool for the early stages of cancer drug discovery. Using a modified broth microdilution assay, the results showed that out of the three crude extracts (DSD011, DSD017, and DSD042), only DSD011 could inhibit more than 50% of the growth of *S. cerevisiae* (Figure 2). This average percentage inhibition of DSD011 at the concentration 2500 µg/ml (86.1% ± 2.7%) was comparable to that of the positive control Triton X at the concentration 2.5% v/v (93.3% ±1.5%. The high inhibitory activity of the DSD011 extract against *S. cerevisiae* warrants further investigation regarding its antiproliferative action mechanism and its target component of the cell cycle regulation.

Since the antiproliferative activity of DSD011 was comparable to Triton-X, we suggest that this compound might target the yeast's cell membrane or molecular systems responsible for cell cycle regulation. Therefore, further assessment through live/dead assay using imaging flow cytometry was performed utilizing a mixture of two fluorescent dyes: SYTO 9 and propidium iodide. SYTO 9 is a membrane-permeable dye that stains cells with intact membranes (viable cells) and emits green fluorescence at around 505-515 nm. On the other hand, propidium iodide is a membrane-impermeable dye that can only pass through the perforated or lysed membrane and fluorescing red at around 600-610 nm as it intercalates with DNA (Robertson et al., 2019). The uptake of propidium iodide and SYTO 9 by treated *S. cerevisiae* cells were visualized using Amnis Flow Sight imaging flow cytometer (AF-IFC) through detection channels 2 and 5 for emissions of SYTO 9 and propidium iodide, respectively. Live and dead cells were initially analyzed (Figure 3), with the majority (>50%) of *S. cerevisiae* cells treated with DMSO (negative control) being found to be alive (fluorescing at 505-560 nm), indicating living cells. In contrast, more than 50% of *S. cerevisiae* cells fluoresced at channel 5 (642-740 nm) when treated with Triton X and DSD011 extract, indicating dead cells caused by a damaged cell membrane.

Results of the live/dead assay of the treatments against *S. cerevisiae* are shown in Figure 4, and the percentage

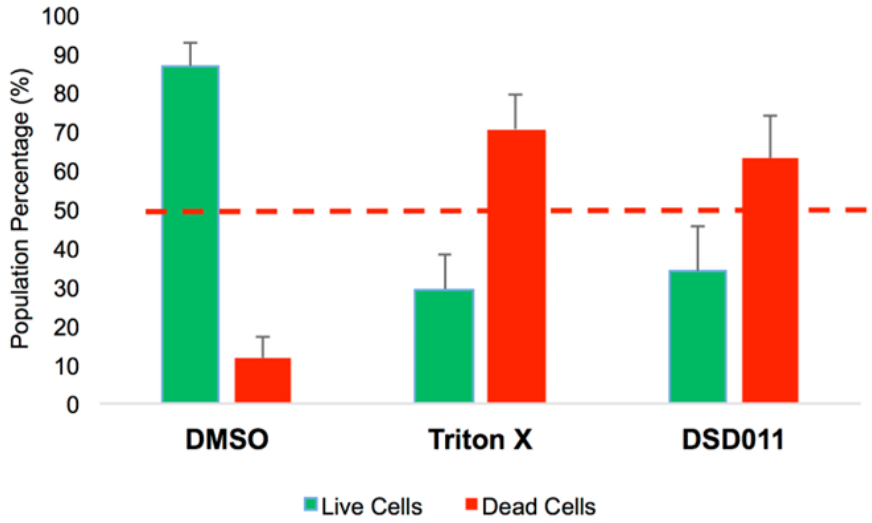

Figure 4. Live/dead assay shows % population of necrotic (dead) cell and live cells. The broken red line indicates 50% of the population percentage. Treatments with 50% dead *S. cerevisiae* population are considered antiproliferative. Values indicated are mean±standard deviation. n=3

Table 1. Percentage live/dead cell assay to the treatment groups

	DMSO	Triton-X	DSD011	*p*
Live	86.73%±5.84%	29.43%±9.13%	33.87%±11.48%	<0.001*
Dead	11.75%±5.21%	70.53%±9.17%	62.90%±10.93%	<0.001*

Data presented in mean±SD. Treatments for live and dead cells were separately analysed using One-way ANOVA. (*) = significant difference with positive control (Triton-X) (p<0.001) using Tukey HSD

Table 2. One-way Analysis of Variance (ANOVA) table for percentage live/dead cell assay

		df	Mean Square	F	Sig.
Live Cells	Between Groups	2	3048.914	24.470	0.001
	Within Groups	6	124.600		
Dead Cells	Between Groups	2	3065.035	26.554	0.001
	Within Groups	6	115.429		

Groups: Negative control (DMSO), Positive control (Triton-X), and DSD011 extract. Difference between groups for both live and dead cells were significant (p<0.001)

of comparison between crude extract DSD011 and the negative control (DMSO) and positive control (Triton X) is shown in Table 1. The percentage of live/dead cells in the treatments was normally distributed as assessed by Shapiro-Wilk's test (Live p = 0.128, Dead p = 0.086). There were no significant differences in the variance among treatment and controls with Levene statistic in terms of live cells (2, 6) = 0.816, p = 0.486, and dead cells with (2, 6)=1.184, p = 0.369. With homogenous variances, the results were analyzed using parametric one-way analysis of variance (ANOVA). In Table 2, live and dead cells among the treatment group and controls were found to be significantly different from F(2, 6)=24.470, p < 0.001, and F(2, 6)=26.554, p < 0.001, respectively. Post-hoc analysis Tukey showed that for both the live and dead cell populations, DMSO was significantly different from Triton X and DSD011. Remarkably, the mean percentage of live and dead cell populations in Triton X and DSD011 was not significantly different from the mean difference (-4.433), p = 0.0880, and mean difference (7.633), p = 0.677, respectively. The comparable live/dead population of *S. cerevisiae* cells treated with DSD011 extract to that of Triton X showed that compounds present in the extract directly kill cells instead of putting their growth into stasis.

From what we found, marine sediment of crude extract DSD011 was found to possess a measurable antiproliferative effect against *S. cerevisiae*. Taken together, this result confirms the antiproliferative activity of crude extracts of other marine sediment-derived *Actinomycete* strains, which were also reported by other researchers using different cancer cell lines (Chen et al., 2018). Diverse secondary metabolites were responsible for the potent anticancer and antiproliferative activities of extracts from marine sediment-derived *Actinomycetes* (Sabido et al., 2020). The presence of secondary metabolite biosynthetic gene clusters (SMBGCs) in marine actinobacteria allows them to synthesize various natural products, including alkaloids (Chen et al., 2019), polyketides (Lu et al., 2020), peptides (Joseph et al. 2021), isoprenoids (McKinnie et al., 2018), phenazines (Sletta et al., 2014), and their derivatives. Moreover, the vast array of extractable bioactive compounds from marine *Actinomycetes* potentiates it as a new source of drugs against different types of cancers. Therefore, it is inferred that the crude extract of DSD011 contains potential anticancer compounds, which warrants further tests for antiproliferative activity.

The antiproliferative activity of the bioactive crude extract (DSD011) was further assessed using budding yeast analysis. *S. cerevisiae* divides by budding, with each budding phase reflecting different cell cycle stages. Budding yeast possesses cellular processes that are remarkably homologous to mammalian cells (Matuo et al., 2012). Eukaryotic cellular components such as actin (Akram et al., 2020) and telomerases (Armstrong & Tomita, 2017) are highly conserved between yeasts and human cells. Furthermore, *S. cerevisiae* is cost-effective to grow and maintain, making it a spectacular model for the preliminary screening compounds with antiproliferative activity. This is in contrast to the high cost and maintenance of using human and mammalian cell lines. With respect to the cell cycle, unbudded cells are in the G1 phase, small-budded cells are in the S phase, and large-budded cells are in the G2/M phase (Figure 5). The *S. cerevisiae* cells treated with the crude extract of DSD011 exhibited a disrupted cellular growth cycle, especially in the G1 phase, as seen in the increased number of unbudded cells. This further supports the hypothesis that the anticancer compounds in the crude extract of DSD011, which caused dysregulation of the cell cycle of *S. cerevisiae*, are highly specific to proteins and molecular signals responsible for the initiation of the cell cycle. It is hypothesized that antiproliferative compounds in the extracts target proteins and molecular signals responsible for the initiation of DNA replication (Lee & Jeong, 2020). Furthermore, the number of abnormal or multibudded cells that were treated by DMSO and DSD011 were almost equal, unlike that of the positive control (Triton X), suggesting the mechanism of action of the compounds present in the crude extracts of DSD011 targets specific checkpoints or molecules in the cell cycle of *S. cerevisiae*.

The antiproliferation mechanism of DSD011 extract might be explained through various anticancer mechanisms observed from previously discovered compounds present in marine sediment-derived

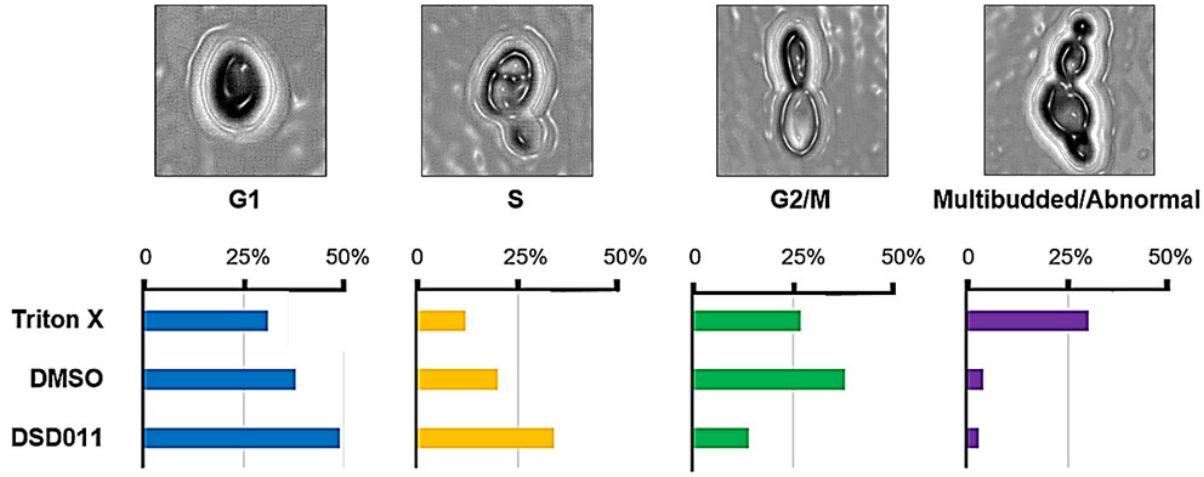

Figure 5. Percent count summary of the different budding phases of *S. cerevisiae* treated with the crude extract of DSD011, DMSO (negative control), and Triton X (positive control) (n=3). Representative image from the imaging flow cytometer are provided in each panel

Actinomycetes, such as salinosporamide A (Serrano-Aparicio et al., 2003), fijolides A (Rudolf et al., 2016), and proximicin C (Brucoli et al., 2012). Salinosporamide A, isolated from the marine sediment actinomycete *Salinispora tropica*, is widely reported as a novel 20S proteasome inhibitor. Salinosporamide A possesses a densely functionalized γ-lactam-β-lactone bicyclic core that allows it to irreversibly bind to the β subunit of the 20S proteasome (Serrano-Aparicio et al., 2003). Recently, salinosporamide A in experimentally determined concentrations was found to regulate the proliferation and entry of Jurkat T cells to the G2/M phase of the cell cycle (Lee & Jeong, 2020). Meanwhile, fijiolides A, a potent antitumor compound isolated from *Nocardiopsis sp.* (Nam et al., 2010), is a profound inhibitor of tumor necrosis factor (TNF-α)-induced activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). Inactivated NF-κB results in cell proliferation arrest by inhibiting the transcription of genes needed for cell proliferation and survival (Chen et al., 2019). Proximicin C, another antitumor compound isolated from a marine sediment *Actinomycete*, *Verrucosispora* sp. (Brucoli et al., 2012), is known to inhibit cell proliferation of different cancer cell lines through the upregulation of the tumor protein p53 and cyclin kinase inhibitor p21, which plays a critical role in the G1/S checkpoint (Sharma & Nandi et al., 2022). The arrest of *S. cerevisiae* cells in the G1 phase suggests that the crude extract of DSD011 may possess secondary metabolites with similar structures and mechanisms of action to that of the previously discussed, which were also isolated from marine sediment-derived *Actinomycetes.*

This study demonstrates that marine sediments in the Philippines harbor *Actinomycetes* that produce antiproliferative compounds. Nevertheless, further works, including *in vitro* testing of the DSD011 extract against human cancer cell lines as well as metabolomics and genomics profiling to elucidate the secondary metabolites and SMBGCs present in the extract, are required for a comprehensive understanding of the potential anticancer activity.

CONCLUSION

The challenge of discovering new chemotherapeutic compounds was taken through the screening of crude extracts of select marine sediment-derived *Actinomycetes* from Islas de Gigantes, Iloilo. One marine sediment-derived *Actinomycete* crude extract (DSD011) was found to possess antiproliferative activity by inhibiting the growth of *S. cerevisiae*. The antiproliferative activity of DSD011 was further demonstrated when *S. cerevisiae* cells were treated with its extract lysed, as seen in live/dead imaging flow cytometry results. Furthermore, the budding yeast analysis results suggest that the crude extracts of DSD011 possess secondary metabolites that target the initiation checkpoints for the G1/S phase in the cell cycle.

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CONFLICT OF INTEREST

The authors declare no conflict of interest. The funding agency had no involvement in the designing of the

research, collection of data, interpretation of results, and publication of the manuscript.

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