



Anticancer activity and metabolomic profiling of culturable marine fungi from the South Coast of Jember, East Java, Indonesia

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ABSTRACT

Our previous study revealed 17 stably re-culturable marine fungi strains isolated from the South Coast of Jember, East Java, Indonesia. This research was conducted to determine the fungi's cancer chemoprevention activity and the potential candidate's metabolite profile. First, their secondary metabolites were extracted using ethyl acetate, and then the extracts were evaluated by their cytotoxicity and selectivity on several cancer cell lines (T47D breast cancer, HeLa cervical cancer, and WiDr colon cancer) and normal cell lines (Vero) using the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The selective cytotoxic marine fungi were then analyzed for cell cycle and apoptosis induction using flow cytometry and their metabolite profile using untargeted ultra-high performance liquid chromatography—high-resolution mass spectrometry (UHPLC-HRMS). Among the extracted fungi, the *Aspergillus stromatoides* strain SaH 1 demonstrated potential as a source of cancer chemopreventive compounds on T47D breast cancer cells based on cytotoxicity and selectivity assays, warranting further bioactivity-guided isolation and characterization studies. The cell cycle and apoptosis analysis demonstrated that it induced G0-G1 and G2/M cell cycle arrest and induced apoptosis, not necrosis, suggesting that this strain could be developed as a cancer chemoprevention agent. The metabolite profile of *A. stromatoides* strain SaH 1 using UHPLC-HRMS analysis revealed 606 molecular features, 76% without reported matches, indicating potential novel metabolites that require further structural confirmation. Among them, two compounds—flavoglucin and 7 β -hydroxy-DHEA—have been reported in *Aspergillus* sp. with documented anticancer properties; these metabolites may have contributed to, but do not solely account for, the anticancer activity observed in SaH 1. This study highlights *A. stromatoides* strain SaH 1 as a preliminary candidate for breast cancer chemoprevention, with further isolation and *in vivo* validation needed to confirm its potential.

1. INTRODUCTION

Marine and coastal fungi have emerged as promising sources of cancer chemopreventive agents due to their

diverse bioactive metabolites [1–3]. These microorganisms inhabit unique ecosystems and have demonstrated cytotoxic, antiproliferative, antiangiogenic, and antitumor activities in previous studies [4]. Marine fungi from Indonesia, particularly those found along the South Coast of Jember, remain largely unexplored, despite their potential therapeutic value [5,6].

Our previous study revealed that only 17 out of 47 marine fungi isolated from the South Coast of Jember, Indonesia, are stably re-culturable [7]. In this study, we

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evaluated their cancer chemopreventive potential by assessing cytotoxicity, selectivity, effects on cell cycle progression, and apoptosis induction in T47D (breast cancer), HeLa (cervical cancer), and WiDr (colon cancer) cell lines, alongside Vero normal cells. Additionally, metabolite profiling of promising candidates was performed to support compound screening and mechanistic understanding.

Unlike previous studies that primarily investigated terrestrial *Aspergillus* strains or used targeted chemical analyses [8], or studies on marine-derived *Aspergillus* that did not integrate metabolomic profiling with functional anticancer assays [9], the present study focuses on *Aspergillus stromatoides* strain SaH 1. The primary contribution is the demonstration of its anticancer activity and comprehensive untargeted ultra-high-performance liquid chromatography–high-resolution mass spectrometry (UHPLC–HRMS) metabolomic profiling. Secondary contributions include the identification of bioactive metabolites, flavoglucan and 7 β -hydroxy-dehydroepiandrosterone (DHEA), and the establishment of a preliminary molecular landscape to facilitate further exploration of marine-derived fungi.

2. MATERIALS AND METHODS

Cancer cell lines (T47D breast cancer, HeLa cervical cancer, and WiDr colon cancer) and normal cell lines (Vero) are collected by the Department of Parasitology, Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada. RPMI 1640 (Gibco) was used for T47D, HeLa, and WiDr media culture, while M199 (Gibco) was used for Vero media culture. The culturable marine fungi isolated from the South Coast of Jember, East Java, Indonesia, were obtained from our previous study [7]. All other chemicals were purchased from local suppliers and were of analytical grade. To ensure reproducibility, the marine fungi were cultured in two independent batches. Metabolite profiles and subsequent bioactivity assays were consistent across both batches, confirming the reliability of the experimental results.

2.1. Extraction of bioactive compounds

Bioactive compounds were extracted based on the method described by Al-Saleem *et al.* [10] with a slight modification. The culturable fungi obtained from the previous study were cultured in broth media corresponding to the previous study. The strains SaH 1, WoH 6, and WoH 6.2 were cultured using Hastings media; WoHS 3.1 and WoHS 3.2 were cultured using Hastings media supplemented with streptomycin, while WoP 2 and RaP 2 were cultured using potato dextrose broth [7]. At first, a loopful of fungal cultures was introduced into 6 ml of culture media and incubated in a shaker incubator at room temperature. After 7 days of incubation, the culture was transferred into 100 ml of culture media and incubated further for 13 days. The culture was done in duplicates. On day 14, the amount of biomass formed was considered sufficient. The culture (100 ml) was homogenized using an ultrasonicator at 40% amplitude for three 30-second pulses, with 30-second intervals on ice to prevent overheating. The extraction was done using an equivalent volume of ethyl acetate by liquid-liquid extraction and then transferred to a

separating funnel. The ethyl acetate phase was collected, while the aqueous layer was extracted twice with an equivalent volume of ethyl acetate. The combined ethyl acetate phase was concentrated using a rotary evaporator [11,12]. The extract was recovered using methanol and transferred into a vial. The methanol was air dried, preparing the extract for further analysis [10]. The extract was then referred to as the ethyl acetate extract.

2.2. Modulation of cancer cells' proliferation analysis

The analysis was done using the ethyl acetate extract. The ethyl-acetate extracts were resolved in dimethyl sulfoxide (DMSO) and then diluted using the culture media appropriate for each cell line. The concentration used for DMSO was maintained at less than 1% to minimize any potential cytotoxic effects. This concentration was selected based on previous cytotoxicity studies with T47D, HeLa, WiDr, and Vero cells, which showed that 1% DMSO does not induce significant cytotoxicity in vehicle-control cells [13–15]. Doxorubicin was used as a positive control due to its broad-spectrum anticancer activity across multiple cancer cell lines, allowing appropriate benchmarking of cytotoxic effects, while 1% DMSO (vehicle control) served as the negative control. All assays were performed in triplicate as technical replicates for each treatment condition, following standard protocols for cytotoxicity, cell cycle, and apoptosis analyses. Owing to the limited quantity of fungal extracts and the large number of experimental conditions, biological replicates could not be performed, as extract yields were insufficient to generate multiple independent batches. The series of analyses comprised the following assays:

2.3. Cytotoxicity analysis using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

The cytotoxicity testing was done on various cancer cell lines, i.e., T47D breast cancer cell line, HeLa cervical cancer cell line, WiDr colon cancer cell line. Each cell density of 10^4 in 100 μ l of the suitable culture media was transferred into a 96-well microplate and incubated for 24 hours prior to treatment. A series of concentrations of the ethyl acetate extracts was added, and a further incubation for 24 hours was performed [13]. At the end of the incubation, MTT (0.5 mg/ml) was added to each well, and incubated for 4 hours until the formazan was formed. The stopper reagent containing 10% sodium dodecyl sulfate in HCl 0.1 N was added to dissolve the formazan crystal. Using an enzyme-linked immunosorbent assay reader, the microplate should be kept in the dark overnight before the absorbance measurement at λ 597 nm. The dehydrogenases in active mitochondria of living cells will break down the tetrazolium ring of MTT and form a formazan crystal [16]. The IC_{50} value of the extract was calculated from the concentration versus viable cells plot by fitting the data using multiple regression approaches, including linear regression, probit analysis, and nonlinear curve fitting; the model yielding the highest correlation coefficient was selected for IC_{50} determination [13]. Based on the IC_{50} value, an extract was considered as potentially cytotoxic with $IC_{50} < 100$ μ g/ml, moderately cytotoxic with 100 μ g/ml $< IC_{50} < 1,000$ μ g/ml, and non-toxic with $IC_{50} > 1,000$ μ g/ml [17].

2.4. Selectivity analysis based on the selectivity index

Selectivity analysis was done by comparing cytotoxicity test results against several cancer cell lines with normal cells. Vero cell lines were used as a standard cell comparator. Vero cells were selected as a general normal cell comparator to provide a baseline for assessing non-specific cytotoxicity across all cancer cell lines tested. We acknowledge that Vero cells are not tissue-specific for breast cancer; therefore, the calculated selectivity indices (SIs) reflect general cytotoxic selectivity rather than breast cancer-specific selectivity. Future studies using tissue-relevant normal mammary cells would provide a more precise assessment of breast cancer selectivity. A selectivity test was conducted using the MTT assay with the SI value as the parameter. An SI value < 1 was interpreted as unselective against cancer cells, indicating that the sample also affected normal cells [18], while the value of $1.00 \leq SI \leq 3$ indicated that the test extract exhibited higher selectivity toward cancer cell cultures than toward normal cells [19], and an SI value > 3 was interpreted as selective against cancer cells, suggesting that the sample was unlikely to affect normal cells [18]. The selective cytotoxic fungi were considered for further development as cancer chemoprevention agents; they were then evaluated further for cell cycle and apoptosis analysis on the respective cancer cell lines.

2.5. Cell cycle analysis using flow cytometry

Cell cycle analysis was carried out using the flow cytometry-propidium iodide method. Cells (3×10^5 cells/ml) in the suitable culture medium were transferred into a 12-well microplate and incubated for 24 hours before treatment. Then, they were treated with ethyl acetate extracts of potent fungi based on the cytotoxicity and selectivity assay at a concentration around IC_{50} . After 24 hours, cells were harvested and washed with phosphate buffer saline. The cells were stained with propidium iodide solution (100 μ g/ml) containing RNase A (40 μ g/ml) and Triton[®] X-100 (0.2%) (BD Cycle Test Plus DNA Reagent Kit) for 30 minutes. The cell suspension was filtered through a cell strainer cap and collected in a tube. The flow cytometer (BD FACSCanto II Flowcytometer, 8-color 3-laser) sorted the cells in citrate buffer and captured the targeted fluorescence signal given by propidium iodide. Propidium iodide intercalates into double-stranded DNA, resulting in a fluorescent signal. The signal corresponded to the DNA content of the cells. The cells were monitored for cell cycle profile using a flow cytometer and analyzed to compare the distribution of cells with the untreated group. The G1 phase was marked by 2n DNA content, and the S phase was determined by 2n-4n DNA content, while 4n DNA content represented G2/M phase of the cells [20].

2.6. Apoptosis analysis using flow cytometry

An apoptosis assay was performed using Annexin V-FITC/propidium iodide staining [21] flow cytometry under selected extract treatment. Briefly, 3×10^5 cells/ml cell density of cancer cell line in the suitable culture media was transferred into a 12-well microplate and incubated for 24 hours before treatment. Then, they were treated with ethyl acetate extracts based on the cytotoxicity and selectivity assay

at a concentration around IC_{50} . After 24 hours of incubation, the cells were harvested. The harvested cells were stained with an Annexin-V staining kit (BD Pharmingen[™] (fluorescein isothiocyanate) (FITC) Annexin V Apoptosis Detection Kit I) composed of 100 ml binding buffer, 2 ml Annexin V, and 2 ml propidium iodide for 10 minutes at room temperature in a dark place. The cells were then measured using a flow cytometer to measure fluorescence intensity using FITC detection. The annexin V targeted the phosphatidylserine flip-flop during the early apoptosis, while propidium iodide colored the DNA when the cells underwent membrane disruption during apoptotic body formation or necrosis. Further analysis to calculate the apoptotic cells was done using the software from the flow cytometry instrument [22].

2.7. Metabolite profile analysis

The ethyl acetate extract of all selected fungi was analyzed for their compounds using UHPLC-HRMS. UHPLC-HRMS analyses were conducted with regular internal calibration to ensure consistent instrument performance. Metabolite peaks were identified and verified based on exact mass comparison against the built-in database. An ACQUITY UPLC[®] H-Class (quaternary) system was tandem-connected to a Xevo[®] G2-S QToF (Waters) via an electrospray interface. Instrument control and data acquisition were performed in MassLynx (v4.2). The reverse-phase separations were performed using a mobile phase consisting of a gradient of methanol, aqueous, and formic acid. The peaks and spectra were processed and tentatively identified by comparing the reported data's retention time (Rt) and mass signals. The ethyl acetate extract was dissolved in methanol (100 mg/ml) and filtered through a 0.45 μ m filter, followed by centrifugation at 15,000 rpm. Sample (3 μ l) was injected into ACQUITY UPLC[®] BEH C18 (1.7 μ m 2.1 \times 100 mm, Waters, USA) with gradient development method starting from 5% to 90% solvent B within 16 minutes followed by an isocratic development for 4 minutes before return to 5% solvent B at 25 minutes (solvent A: MS-grade water containing 0.1% formic acid, solvent B: MS-grade methanol containing 0.1% formic acid). The flow rate used was 0.3 mL/min. Mass spectra was obtained with experiment details as follow: electrospray ionization (ESI), mode: positive, sheath gas: N₂, 32 AU, auxiliary gas: N₂, 8 AU, sweep gas: N₂, 4 AU, spray voltage: 3.30 k, capillary temperature: 320°C, auxiliary gas heater temperature: 30°C, scan range: 66.7–1,000 m/z, resolution: 70,000 for full MS and 17,600 for dd-MS2. Molecular annotation was generated by comparing detected and calculated mass ions with a discrepancy of less than 5 ppm using the database from <https://www.chemcalc.org/> and <https://www.chemspider.com/> [10].

Additionally, rigorous filtering and quality control steps were applied to remove artefacts and ensure data reliability. Raw peaks with low signal-to-noise ratio (< 3), poor peak shapes, or Rts outside the gradient were excluded. Peaks detected in blanks or corresponding to solvent or column signals were discarded, and features appearing in blanks at $> 10\%$ of the sample intensity were considered artefacts. Isotopic peaks, adducts (Na⁺, K⁺), and multiply charged ions were consolidated, retaining only monoisotopic protonated species (M+H). Features with mass

deviations >5 ppm relative to ChemCalc and ChemSpider were removed. Only high-confidence, reproducible features were retained, removing sporadic or inconsistent signals. Following these steps, 606 molecular features were detected, of which 461 (76%) were unmatched in ChemSpider, suggesting a high potential for previously undescribed or poorly characterized metabolites.

2.8. Statistical analysis

Statistical analyses were designed to address a priori, biologically meaningful comparisons rather than exhaustive multiple pairwise testing. In the cytotoxicity assays, cell viability of marine fungal extract-treated cells was compared with 1% DMSO as the vehicle control (defined as 0% effect) and with doxorubicin as the positive control within each cell line, providing relevant benchmarks for evaluating cytotoxic activity. For cell cycle and apoptosis analyses, statistical testing was focused on comparisons with the biologically meaningful live cell control (1% DMSO, 0% effect), followed by evaluation of concentration-dependent effects of the SaH 1 extract, and direct comparison with doxorubicin as the positive control (defined as 100% effect).

Cell viability data were analyzed separately for each cell line. Data from T47D, WiDr, and Vero cells met the assumptions of normality and were analyzed using one-way ANOVA, followed by Tukey's HSD *post hoc* test. In contrast, HeLa cell viability data did not meet normality assumptions and were therefore analyzed using non-parametric Kruskal–Wallis tests, followed by Mann–Whitney *post hoc* comparisons. For cell cycle analysis, data for the sub-G1 and G2/M phases met assumptions of normality and homogeneity of variance and were analyzed using one-way ANOVA followed by (least significant difference, *post hoc* statistical analysis) *post hoc* tests, whereas data for the G1 phase, S phase, and polyploidy were analyzed using Kruskal–Wallis tests followed by Mann–Whitney *post hoc* comparisons. Apoptosis parameters (live cells, early apoptosis, late apoptosis, and necrosis) did not satisfy normality assumptions and were therefore analyzed using Kruskal–Wallis tests, followed by Mann–Whitney *post hoc* tests.

In addition to hypothesis testing, effect sizes were calculated to quantify the magnitude of pairwise differences. For pairwise comparisons following parametric analyses, Cohen's *d* was calculated, whereas for non-parametric pairwise comparisons following the Kruskal–Wallis test, effect size *r* (Z/\sqrt{N}) was calculated. Reporting effect sizes was intended to complement *p*-value-based inference and to provide an estimate of biological relevance independent of statistical significance. All analyses were conducted at a 95% confidence level ($p < 0.05$).

The *p*-value was used to assess statistical significance: results with $p < 0.05$ were considered statistically significant (reject H_0), whereas results with $p \geq 0.05$ were considered not statistically significant (fail to reject H_0). Effect sizes were calculated to evaluate the magnitude of differences between treatments. For T47D, WiDr, and Vero cells, Cohen's *d* was used and interpreted as follows: <2 = very small/negligible, ≈ 0.2 = small, ≈ 0.5 = medium, ≈ 0.8 = large, and ≥ 1.2 = very large. For HeLa cells, the *r* statistic was used and interpreted

as: <0.1 = very small/negligible, ≈ 0.1 = small, ≈ 0.3 = medium, and ≥ 0.5 = large effect (Supplementary Data 1). This approach allows both the statistical significance and the practical magnitude of the treatment effects to be evaluated. Because the statistical comparisons were hypothesis-driven and limited to predefined biological references, correction methods designed for extensive multiple testing, such as the Holm–Bonferroni procedure, were not applied.

3. RESULTS AND DISCUSSION

3.1. Extraction of bioactive compounds

Among 17 stably re-culturable marine fungi strains taken from the South Coast of Jember, East Java, Indonesia, only 7 strains generated sufficient extract quantities for further studies. This was mainly due to strain-specific differences in growth and metabolite production. Despite medium optimization, some isolates produced limited biomass or low levels of extractable metabolites under *in vitro* conditions, which are common challenges in culturing marine-derived fungi. The culturable marine fungi that yielded a sufficient amount of ethyl acetate extract are listed in Table 1. These extracts were subsequently tested for their anticancer potential.

3.2. Modulation of cancer cells' proliferation analysis

The ethyl acetate extract of culturable marine fungi isolated from the South Coast of Jember demonstrated differential cytotoxic effects across cancer and normal cell lines. At a concentration of 500 $\mu\text{g/ml}$, the extracts caused variable reductions in cell viability depending on the cell line (Fig. 1). As expected, 1% DMSO, used as the solvent control, did not affect viability in any cell line, while doxorubicin consistently produced the lowest viability, serving as a robust positive control. Among all extracts tested, *A. stromatoides* strain SaH 1 exhibited the strongest and most consistent cytotoxic activity, showing the lowest cell viability across all cancer cell lines.

In T47D breast cancer cells, most marine fungal extracts markedly reduced viability, with SaH 1 showing particularly strong activity approaching that of doxorubicin. Several other extracts exhibited moderate-to-large reductions in viability, indicating broadly similar biological activity.

Table 1. Culturable marine fungi isolated and identified from the South Coast of Jember, Indonesia.

No	Strain	Identity
1	SaH 1	<i>Aspergillus stromatoides</i> EKAP-SaH 1 ^a
2	WoH 6	<i>Penicillium copticola</i> EKAP-WoH 6 ^a
3	WoH 6.2	<i>Penicillium copticola</i> EKAP-WoH 6.2 ^a
4	WoHS 3.1	<i>Curvularia lunata</i> EKAP-WoHS 3.1 ^a
5	WoHS 3.2	<i>Curvularia lunata</i> EKAP-WoHS 3.2 ^a
6	WoP 2	<i>Aspergillus sydowii</i> EKAP-WoP 2 ^b
7	RaP 2	<i>Aspergillus versicolor</i> EKAP-RaP 2 ^a

Note: ^aPuspitasari *et al.* [7]

^bthis study, NCBI Accession Number PV682694.1.

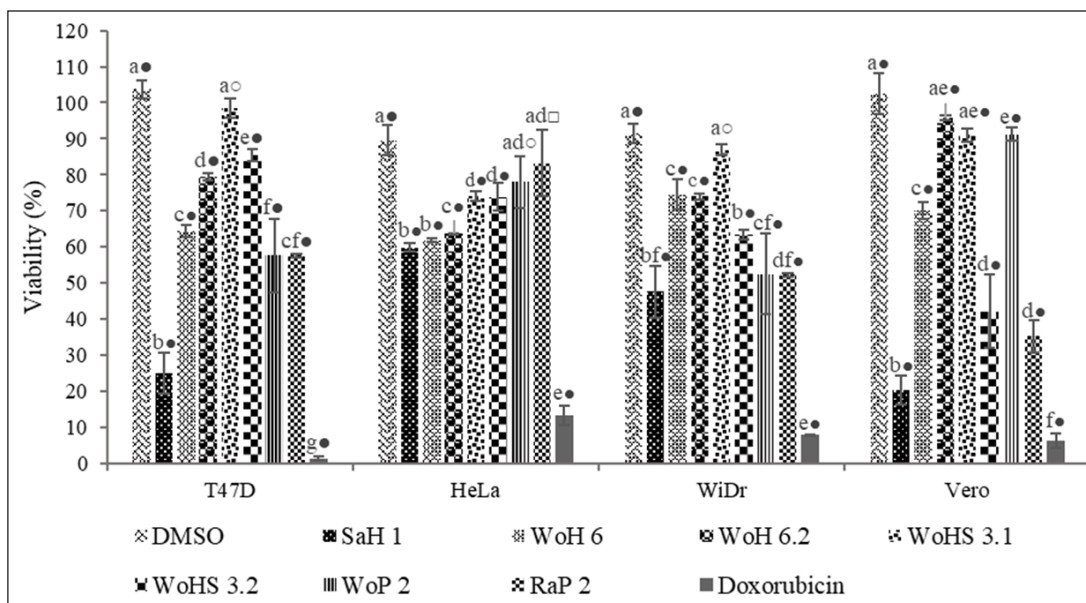


Figure 1. Cell viability of T47D, HeLa, WiDr, and Vero cells treated with 500 µg/ml ethyl acetate extracts of culturable marine fungi. Data are shown as mean ± SE ($n = 3$). Different letters indicate statistically significant differences within each cell line ($p < 0.05$). Effect sizes are indicated separately for each cell line: T47D, WiDr, and Vero – Cohen's d (● very large and large, ○ medium, □ small); HeLa – r (● large, ○ medium, □ small) based on the respective analysis.

In HeLa cervical cancer cells, multiple extracts—including SaH 1, WoH 6, WoH 6.2, WoHS 3.1, and WoHS 3.2—also induced meaningful cytotoxicity relative to DMSO, with large effect sizes indicating strong biological impact. Notably, WoH 6.2 displayed greater cytotoxicity than WoH 6, suggesting that minor differences in metabolite composition may lead to significant activity changes. Conversely, WoP 2 and RaP 2 were less effective, consistent with their smaller effect sizes.

For WiDr colorectal cancer cells, cytotoxic responses were more selective. SaH 1, WoHS 3.2, WoP 2, and RaP 2 significantly reduced viability, whereas WoH 6 and WoH 6.2 were comparatively less active. In Vero normal cells, SaH 1 and several other extracts reduced viability with large effect sizes, whereas most extracts showed limited toxicity. Doxorubicin again induced the strongest cytotoxicity, emphasizing the sensitivity of normal cells to chemotherapeutic stress and highlighting the importance of evaluating therapeutic selectivity.

The IC_{50} of ethyl acetate extract was then calculated to evaluate the cytotoxic activity (Table 2), followed by the (SI, Table 3). DMSO was used as a negative control (0% of effect), while doxorubicin was used as a positive control (100% of effect). DMSO showed no toxicity, with IC_{50} values exceeding 1,000 µg/ml in all cells (Table 2). Doxorubicin demonstrated cell line-dependent cytotoxicity, with IC_{50} values of 0.30 ± 0.05 µg/ml (T47D), 0.02 ± 0.04 µg/ml (HeLa), 0.91 ± 0.31 µg/ml (WiDr), and 15.23 ± 8.16 µg/ml (Vero) (Table 2). IC_{50} values varied across cell lines, consistent with prior studies, categorized as potentially cytotoxic on cancer cell lines and moderate cytotoxic on normal cell lines [23,24].

None of the marine fungal extracts exhibited IC_{50} values below 100 µg/ml (Table 2), indicating limited potential cytotoxicity across all cell lines [17]. Some of the marine fungal extracts exhibited moderate cytotoxicity with $100 \mu\text{g/ml} < IC_{50} <$

1,000 µg/ml on several cancer cell lines [17], i.e., *A. stromatoides* strain SaH 1 and *Penicillium copticola* strain WoH 6 on T47D; *P. copticola* strain WoH 6, *P. copticola* strain WoH 6.2, and *Curvularia lunata* strain WoHS 3.2 on HeLa; *A. stromatoides* strain SaH 1 and *A. versicolor* strain RaP 2 on WiDr; and also, *C. lunata* strain WoHS 3.2 on Vero. The others were revealed to be non-toxic with $IC_{50} > 1,000$ µg/ml [17].

Curvularia lunata strain WoHS 3.1 and *Aspergillus sydowii* strain WoP 2 demonstrated non-toxic activity against all cell lines in this study (Table 2). Previous studies showed that *C. lunata* ethyl acetate crude extract had moderate cytotoxicity against leukemia cell lines ($100 \mu\text{g/ml} < IC_{50} < 1,000 \mu\text{g/ml}$) [25], while isolated curvulomycins from *C. lunata* demonstrated potent cytotoxic activity on prostate cancer cells ($IC_{50} < 20 \mu\text{g/ml}$) [26]. Other studies revealed that *A. sydowii* exhibited strong cytotoxic activity against several cancer cell lines ($IC_{50} < 100 \mu\text{g/ml}$) [27]. These discrepancies may be due to variations in fungal strain origin and culture conditions, which can produce distinct metabolite profiles [28,29]. Of course, they could be useful for bioactivity-guided isolation [30,31], but they may not be active enough at low concentrations.

The lowest IC_{50} value of all marine fungi was obtained from SaH 1 on T47D breast cancer cell lines, indicating its highest cytotoxic activity and aligning with Figure 1. While the IC_{50} values of the crude extract indeed fall within the range of moderate cytotoxicity ($100 \mu\text{g/ml} < IC_{50} < 1,000 \mu\text{g/ml}$), this is expected due to complex metabolite mixtures, limited bioavailability, and reduced cellular uptake. Importantly, such moderate activity does not preclude biological relevance, as bioactivity-guided fractionation of extracts with comparable or even weaker initial IC_{50} values has frequently led to the isolation of highly potent anticancer compounds with IC_{50} values below

Table 2. IC₅₀ values of ethyl acetate extracts of culturable marine fungi isolated from the South Coast of Jember, Indonesia.

No	Strain	IC ₅₀ (µg/ml)*			
		T47D	HeLa	WiDr	Vero
1	SaH 1	298 ± 36 [§]	6,318 ± 2,741 [§]	528 ± 130 [§]	1,509 ± 294 [§]
2	WoH 6	800 ± 16 [§]	610 ± 49 [§]	11,265 ± 6,882 [§]	2,692 ± 1,025 [§]
3	WoH 6.2	1,169 ± 11 [§]	725 ± 56 [§]	6,522 ± 2,142 [§]	11,046,298,592 ± 3,104,282,939 [§]
4	WoHS 3.1	128,089,866 ± 121,463 [§]	13,654 ± 3,402 [§]	1,585 ± 284 [§]	904,041,451 ± 904,038,831 [§]
5	WoHS 3.2	3,791,859,493 ± 3,791,858,427 [§]	771 ± 60 [§]	3,673 ± 2,215 [§]	587 ± 245 [§]
6	WoP 2	11,997 ± 7,787 [§]	1,342 ± 516 [§]	7,779 ± 7,121 [§]	15,514 ± 6,266 [§]
7	RaP 2	22,461 ± 20,707 [§]	11,662,949,637 ± 11,662,948,880 [§]	752 ± 440 [§]	1,450 ± 963 [§]
8	DMSO**	14,613 ± 11,730 [§]	87,068,639,678 ± 40,528,139,628 [§]	1,831 ± 325 [§]	3,287 ± 1,014 [§]
9	doxorubicin**	0.30 ± 0.05 [¶]	0.20 ± 0.04 [¶]	0.91 ± 0.31 [¶]	15.23 ± 8.16 [¶]

*Data was shown as mean ± SE (n = 3).

**DMSO was used as a negative control, and doxorubicin was used as a positive control.

Cytotoxicity classification:

[§]potential cytotoxic: IC₅₀ < 100 µg/ml

[§]moderate cytotoxic: 100 µg/ml < IC₅₀ < 1,000 µg/ml

non-toxic: IC₅₀ > 1,000 µg/ml [24]

Regression method used for IC₅₀ calculation:

[§]probit regression

[¶]curved-fit regression

..... Lowest IC₅₀ values indicate the highest cytotoxic activity among the marine fungal extracts.

Table 3. SI of ethyl acetate extracts of culturable marine fungi isolated from the South Coast of Jember, Indonesia.

No	Strain	SI*		
		T47D	HeLa	WiDr
1	SaH 1	5.1 [§]	0.2 [§]	2.9 [§]
2	WoH 6	3.4 [§]	4.4 [§]	0.2 [§]
3	WoH 6.2	>1,000 [§]	>1,000 [§]	>1,000 [§]
4	WoHS 3.1	7.1 [§]	>1,000 [§]	>1,000 [§]
5	WoHS 3.2	0.0 [§]	0.8 [§]	0.2 [§]
6	WoP 2	1.3 [§]	11.6 [§]	2.0 [§]
7	RaP 2	0.1 [§]	0.0 [§]	1.9 [§]

*SI classification:

[§]SI < 1 → Unselective against cancer cells (affects normal cells) [25].

[§]1 ≤ SI ≤ 3 → Moderate selectivity toward cancer cells [26].

[§]SI > 3 → Selective against cancer cells (unlikely to affect normal cells) [25].

20 µg/ml [30,31]. Thus, despite moderate crude cytotoxicity, SaH 1 shows meaningful anticancer potential.

The ethyl acetate extract of marine fungi showed varying selectivity (Table 3). Some extracts were unselective, some were more selective, but most were selective. Being the most cytotoxic on T47D breast cancer cell lines, *A. stromatoides* strain SaH 1 showed selective activity against breast cancer cells compared to normal cells, with an SI value of 5.1 (Table 3). As noted in Methods, Vero cells serve as a general baseline for cytotoxicity. Although Vero cells provide a baseline for general cytotoxicity, they are not tissue-relevant for breast cancer; therefore, the calculated SIs reflect general cytotoxic selectivity rather than breast cancer-specific selectivity.

Based on the cytotoxicity and selectivity assays, *A. stromatoides* strain SaH 1 on T47D cells showed the highest cytotoxic activity with high selectivity (SI > 3, Table 3). To evaluate whether the cytotoxicity of the *A. stromatoides* strain SaH 1 ethyl acetate extract on the T47D breast cancer cell line was associated with its ability to induce cell cycle arrest or apoptosis, further analyses were conducted on its effects on the cell cycle, apoptosis induction, as well as its metabolite profile. The graphical representation of the cell cycle result was illustrated in Figure 2, while the results of apoptosis induction analysis were presented in Figure 3.

Baseline analysis of untreated T47D control cells showed that the majority of cells were in the G0/G1 phase (44.37%), followed by G2/M (34.03%) and S phase (20.60%), with minimal sub-G1 (0.50%) and polyploid (0.53%) populations. This profile reflects actively proliferating cells with low spontaneous death. Treatment with SaH 1 induced substantial redistribution across cell cycle phases (Fig. 2). The sub-G1 population increased significantly, consistent with DNA fragmentation typically associated with apoptotic cells. Changes in the relative proportions of G0/G1, S, and G2/M phases indicate widespread disruption of cell cycle progression rather than arrest at a single checkpoint.

The increase in polyploid cells at higher SaH 1 concentrations suggests mitotic stress or failed cytokinesis, phenomena often linked to mitotic catastrophe—a form of cell death triggered by severe DNA damage and checkpoint failure. Mitotic catastrophe involves activation of DNA damage response (DDR) pathways (e.g., ATM/p53) leading to apoptosis rather than successful cell division, which has been demonstrated in other cancer models following severe genotoxic insult [32].

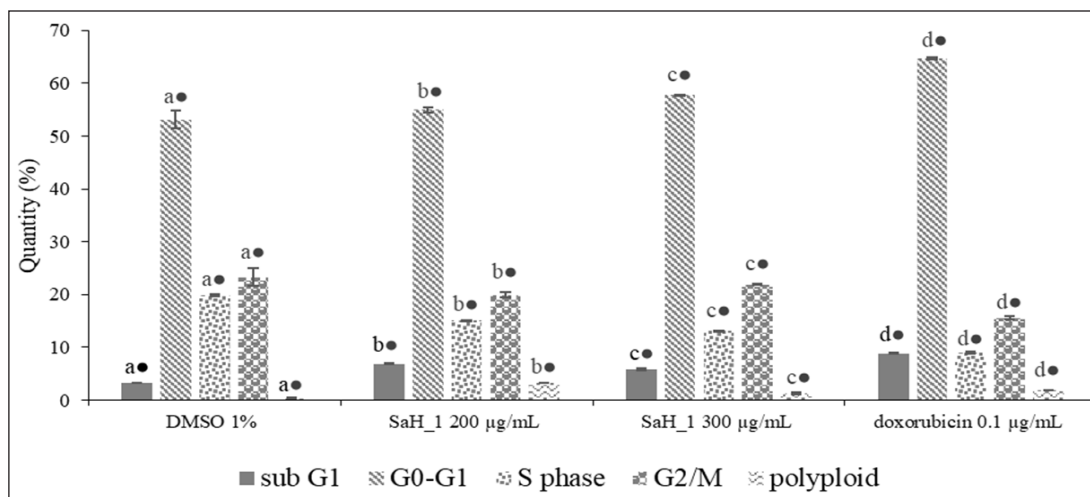


Figure 2. Cell cycle distribution of T47D breast cancer cells treated with SaH 1 ethyl acetate extract. Data are shown as mean \pm SE ($n = 3$). Different letters within the same phase indicate statistically significant differences among treatments ($p < 0.05$), while ● indicates a large effect size ($r \geq 0.50$).

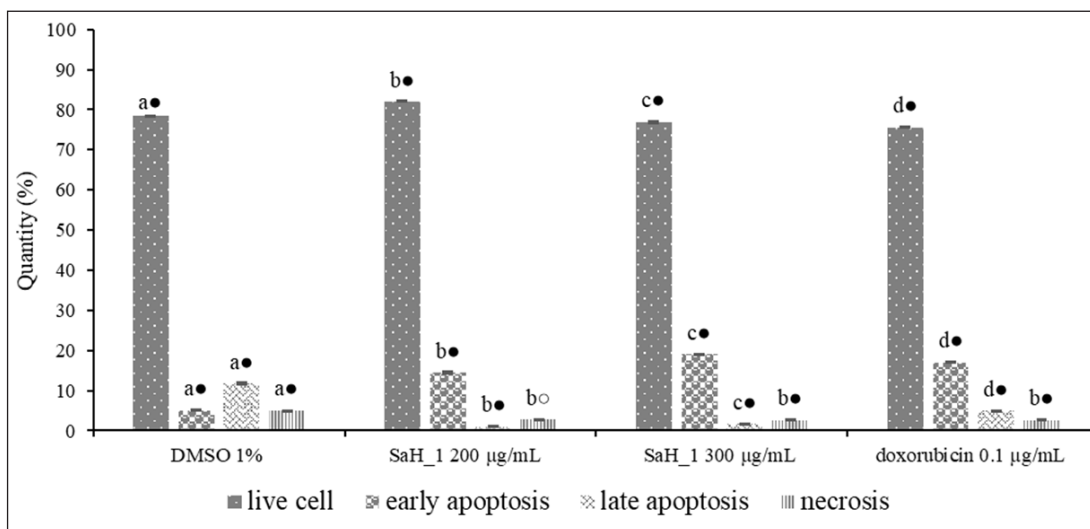


Figure 3. Apoptosis induction in T47D breast cancer cells treated with SaH 1 ethyl acetate extract. Data are shown as mean \pm SE ($n = 3$). Different letters within the same phase indicate statistically significant differences among treatments ($p < 0.05$), ● indicates a large effect size ($r \geq 0.50$), and ○ indicates a medium effect size ($r \approx 0.30$).

Flow cytometric analysis revealed the proportions of apoptotic and necrotic T47D cells following treatment with the fungal extract. While morphological assessment through microscopy was not performed, flow cytometry provided a quantitative, phase-specific evaluation of cell death, allowing precise comparison across treatments. These results align with the observed trends in overall cell viability, supporting the cytotoxic effect of the extract. Baseline apoptosis profiling of untreated T47D control cells revealed live cells (26.5%), early apoptotic (15.3%), late apoptotic (37.9%), and necrotic cells (11.5%) under standard culture conditions. SaH 1 treatment markedly reduced the proportion of live cells while significantly increasing both early and late apoptotic populations (Fig. 3). Although necrotic cells were elevated relative to control, the predominant mode of death was apoptosis, indicating a programmed cell death response rather

than uncontrolled necrosis. This favorable shift—enhanced apoptosis with minimized necrosis—reflects a potentially safer anticancer mechanism, as apoptosis prevents the release of pro-inflammatory mediators and suppresses carcinogenesis [33], whereas necrosis is associated with inflammation that may promote tumor progression and aggressiveness [34,35]. Consequently, limiting necrotic cell death is critical for effective anticancer therapies [36].

The observed increase in sub-G1 and polyploid fractions, along with robust apoptosis induction by SaH 1, suggests that the extract triggers DNA damage-associated checkpoint disruption and engages intrinsic apoptotic pathways, possibly involving p53 activation. These mechanistic profiles align with those reported for genotoxic anticancer agents, supporting the biologically meaningful effects of SaH 1.

Doxorubicin, the positive control, induces cell cycle arrest and apoptosis via DNA intercalation and inhibition of topoisomerase II, leading to double-strand breaks and activation of DDR kinases (ATM/ATR), phosphorylation of checkpoint kinases (CHK1/CHK2), and subsequent p53-mediated cell cycle arrest and apoptosis [37]. Doxorubicin also promotes reactive oxygen species (ROS) production, further contributing to DNA and mitochondrial damage and enhancing apoptotic signaling [38].

In summary, the ethyl acetate extract from marine fungi—particularly SaH 1—demonstrated meaningful anticancer potential through selective cytotoxicity, disturbance of cell cycle progression (Fig. 2), and apoptosis induction (Fig. 3) in T47D cells. These coordinated effects justify future bioactivity-guided isolation of active constituents and molecular characterization.

Consistent with our observations, marine-derived fungal metabolites have demonstrated a potential in modulating the cell cycle and DDR pathways in cancer cells, highlighting their broader therapeutic promise. For example, preussin, isolated from *Aspergillus candidus* KUFA 0062, inhibited the proliferation of breast cancer cells and induced cell cycle arrest at approximately 25 μM . Similarly, asperphenin A from a marine *Aspergillus* sp. triggered G₂/M cell cycle arrest and apoptosis in colon cancer cells via ROS generation. The diketopiperazines waikikiamides A and C also exhibited potent antiproliferative activity, with IC₅₀ values ranging from 0.5 to 1.8 μM [39]. Beyond these specific compounds, marine fungi have been shown to produce over 600 cytotoxic metabolites between 1991 and 2023, particularly from the genera *Aspergillus* and *Penicillium*, illustrating their remarkable chemical diversity and bioactivity potential [40]. Collectively, these findings underscore the value of marine fungi as a rich source of structurally diverse compounds capable of interfering with critical regulatory pathways in cancer cell proliferation and survival.

3.3. Metabolite profile analysis

The metabolite profiling was done using UHPLC–HRMS. The data suggest that the compound exhibits high ionization efficiency, indicating favorable ionization

characteristics under the applied ESI conditions—likely due to its polarity or proton-donating functional groups [41,42].

The *A. stromatoides* strain SaH 1 metabolite profile was depicted in Figure 4. The detailed list of compounds contained in the ethyl acetate of *A. stromatoides* strain SaH 1 ethyl acetate extract was summarized in Supplementary Data 2. UHPLC–HRMS analysis enabled the detection of 606 molecular features, of which 461 (76%) molecular formulas were either not listed in ChemSpider or had no reported compounds associated with those formulas. This high proportion of unmatched formulas indicates that our samples may contain previously undescribed or less-characterized metabolites. However, as these annotations are based solely on accurate mass and database searches, they should be regarded as tentative identifications rather than definitive compound characterizations. Consequently, it is not possible to assign these unannotated features to specific compound classes, such as polyketides or alkaloids. The possibility that some of these metabolites represent novel natural products cannot be excluded, but further structural elucidation—including isolation, tandem mass spectrometry (MS/MS) fragmentation analysis, and nuclear magnetic resonance (NMR) spectroscopy—is essential to confirm their novelty and fully determine their chemical structures.

Amid the various compounds identified in the *A. stromatoides* strain SaH 1 ethyl acetate extract, several have been previously reported in other strains of *Aspergillus* sp., i.e., flavoglaucin [43] and 7 β -hydroxy-DHEA [44]. Notably, flavoglaucin has also been isolated from a terrestrial endolichenic *Aspergillus* sp. obtained from the lichen *Xanthoparmelia conspersa*, confirming that this metabolite is produced by non-marine strains as well [45]. However, there is currently no evidence that terrestrial *Aspergillus* sp. produce 7 β -hydroxy-DHEA, and to our knowledge, no studies have directly compared the anticancer activities of marine- versus terrestrial-derived *Aspergillus* metabolites. These compounds were detected at a Rt of 14.466 minutes, corresponding to the molecular formula C₁₉H₂₈(NO)O₃. Both compounds are known to exhibit anticancer activity [45,46]. The annotation of these two highlighted compounds was based on database comparisons of exact mass and Rt and is therefore considered putative (Level 2 confidence according to the Metabolomics Standards Initiative). No MS/MS spectral matching or *in silico*

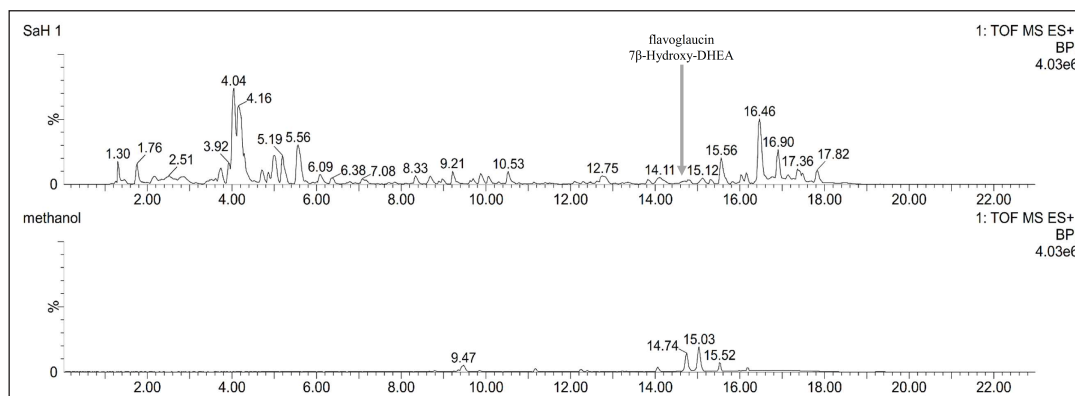


Figure 4. UHPLC–HRMS metabolite profile of SaH 1 ethyl acetate extract (top) and blank methanol solvent (bottom). Peaks corresponding to compounds of interest (flavoglaucin and 7 β -hydroxy-DHEA) are highlighted with arrows (→).

fragmentation analysis was performed in this study, but MS/MS confirmation and/or spiking with authentic standards will be conducted in follow-up work to provide higher annotation confidence. The UHPLC–HRMS analysis employed an untargeted approach to comprehensively profile all metabolites present in SaH 1, reflecting the exploratory nature of this work rather than quantifying specific compounds.

Flavoglaucin is a yellow p-terphenyl benzaldehyde derivative (C₁₉H₂₈O₃) first isolated from *A. glaucus* in 1949 [43]. As a phenolic compound produced by *Aspergillus* sp., flavoglaucin demonstrates anticancer properties, primarily through antiproliferative mechanisms and the induction of apoptosis. *In vitro* studies using the human colorectal cancer cell line HT-29 showed that flavoglaucin significantly inhibited cell proliferation, with IC₅₀ values of 9.17 μM at 24 hours and 34.40 μM at 48 hours. Notably, its potency at 24 hours surpassed that of two commonly used chemotherapeutic agents: 5-fluorouracil (IC₅₀ > 50 μM) and irinotecan (IC₅₀ = 28.00 μM). In addition to growth inhibition, flavoglaucin induced apoptosis, as evidenced by DNA fragmentation in cancer cells. Apoptosis, a form of programmed cell death, plays a crucial role in cancer therapy by selectively eliminating abnormal cells. The biological activity of flavoglaucin is closely associated with its chemical structure, particularly the presence of phenolic groups and a prenyl side chain. The position and saturation level of the prenyl chain at the C-3 position of the flavoglaucin molecule have been reported to influence its cytotoxic effects significantly. These findings suggest that flavoglaucin holds potential as an antiproliferative agent and a chemopreventive compound with possibly fewer side effects than conventional therapies [45].

In silico data demonstrated that flavoglaucin has moderate binding affinity toward cyclin-dependent kinase 2 (CDK-2) and matrix metalloproteinase-13, suggesting a potential but limited contribution to anticancer activity through modulation of these pathways [47]. Furthermore, flavoglaucin exhibited moderate acetylcholinesterase (AChE) inhibition, supported by docking analysis showing hydrogen-bond formation and π–π interactions within the AChE active site [48]. For cancers that exhibit elevated AChE activity—e.g., leukemia, retinoblastoma, brain, non-small cell lung, breast, and ovarian tumors—AChE inhibition may contribute to cytotoxicity by promoting caspase-dependent apoptosis, modulating the phosphatidylinositol 3-kinase/Akt signaling pathway as a downstream component of p53, and influencing specific isoforms, particularly the synaptic variant (AChE-S), which is known to regulate and enhance apoptotic cell death [49]. Taken together, these findings indicate that while flavoglaucin may contribute modestly to growth inhibition through partial interference with cell cycle regulators such as CDK-2, its anticancer potential is more strongly associated with apoptosis induction mediated by AChE inhibition.

7β-hydroxy-DHEA is a hydroxylated metabolite of DHEA, produced via hydroxylation at the C-7 position by enzymatic action in *A. niger* KCH910. This reaction results in two epimers: 7α-hydroxy-DHEA and 7β-hydroxy-DHEA. These metabolites are formed via a minor biosynthetic pathway, likely involving cytochrome P450 enzymes or similar monooxygenases [44].

7β-Hydroxy-DHEA is also an endogenous androgenic metabolite of DHEA, with reported anticancer activity. Its mechanism of action involves inhibition of estrogen-mediated growth signaling in breast cancer cells. While breast cancer cells typically proliferate in response to estrogen via estrogen receptor alpha (ERα) and beta (ERβ), 7β-hydroxy-DHEA does not act through the ERα pathway (as is the case with tamoxifen). Instead, it exerts its effects through ERβ and the membrane-associated estrogen receptor GPR30, which are implicated in estrogen response pathways. By interfering with estrogen signaling, 7β-hydroxy-DHEA disrupts the proliferative stimulus, leading to cell cycle arrest (at G0/G1 phase) and induction of apoptosis in breast cancer cells [46].

Our findings indicate that the anticancer properties of *A. stromatoides* strain SaH 1 ethyl acetate extract might be contributed by flavoglaucin and 7β-hydroxy-DHEA. The lower potency of *A. stromatoides* strain SaH 1 ethyl acetate extract likely reflected both its relatively low abundance of flavoglaucin and the highly complex mixture in which it resides; our UHPLC–HRMS profiling revealed 606 molecular features, 461 (76%) of which are uncharacterized and may impact bioactivity. It is plausible that some of these co-occurring metabolites could antagonize [50,51] flavoglaucin's anticancer effects. While specific antagonistic interactions among marine fungal metabolites have not been well-documented, the substantial metabolic diversity in crude extracts (e.g., 86 compounds identified in a mangrove fungal study, 11 with potent cytotoxicity [52]), and reports of culture-condition-dependent shifts in metabolite activity via one strain many compounds approaches [53], support this hypothesis.

The metabolite profile and anticancer activity observed for the marine-derived *A. stromatoides* strain SaH 1 also highlight several novel aspects of this study. To the best of our knowledge, no UHPLC–HRMS (or comparable high-resolution LC–MS) analysis of ethyl acetate extracts of *A. stromatoides* has been reported previously, and chemical studies on this species remain virtually absent [54]. Unlike the predominantly terrestrial or airborne *A. stromatoides* strains described in earlier studies, this marine-derived strain originates from an ecological niche that can induce distinct and sometimes unique metabolite profiles due to factors such as salinity, pressure, and nutrient-related stressors [55]. Furthermore, the combination of untargeted UHPLC–HRMS metabolomic profiling with anticancer screening provides a broad-spectrum chemical and biological assessment, contrasting with prior work that typically focused on the isolation of only one or two compounds. Although *Aspergillus* is among the most extensively studied fungal genera, chemical diversity is highly species- and habitat-dependent, and many species—including *A. stromatoides*—remain largely unexplored in metabolite databases [56]. Collectively, these points emphasize the novelty of our study, which provides the first UHPLC–HRMS-based metabolomic characterization and anticancer assessment of a marine-derived *A. stromatoides* strain, filling a clear gap in the literature and expanding our understanding of this neglected species.

Although the screening of marine fungi for anticancer activity has been extensively reported, and this study does not

involve the isolation or characterization of a new bioactive compound, it holds significance in a different way. By establishing a molecular landscape of culturable marine fungi from the South Coast of Jember, Indonesia, this work provides a foundation for future chemoprevention research, including targeted compound isolation, purification, and mechanistic studies. However, this study provides novelty by:

- (1) detecting 606 molecular features via UHPLC-HRMS, of which 461 (76%) molecular formulas were not listed in ChemSpider or had no reported compounds, indicating potential for novel metabolites pending further structural elucidation using MS/MS and NMR techniques;
- (2) isolating the fungal strain from the underexplored South Coastal Area of Jember, Indonesia, where ecological variation may contribute to unique bioactivity profiles even within the same species.

Accordingly, future studies will focus on bioactivity-guided fractionation and purification of active constituents, followed by comprehensive structural characterization to confirm compound identities and assess the presence of truly novel metabolites. In parallel, expanded mechanistic investigations and *in vivo* validation will be required to substantiate the anticancer potential observed *in vitro* and evaluate translational relevance. Overall, these findings highlight the chemical diversity of marine fungi from this region and establish a foundation for future compound discovery. Despite the large number of reported anticancer compounds, the demand for new anticancer agents remains high, underscoring the importance of continued screening efforts, particularly from underexplored sources, to identify novel anticancer candidates.

4. CONCLUSION

Based on the results, we can conclude that *A. stromatoides* strain SaH 1, a marine fungal isolate from the South Coast of Jember, exhibited selective cytotoxicity against cancer cells while sparing normal cells. It induced cell cycle arrest at the G0–G1 and G2/M phases and promoted apoptosis without triggering necrosis. Metabolite profiling identified flavoglucin and 7 β -hydroxy-DHEA, known anticancer compounds, and revealed that 76% of metabolites were uncharacterized, suggesting the presence of potentially novel compounds. Future studies will focus on bioactivity-guided fractionation, isolation of active constituents, and structural elucidation using MS/MS and NMR, as well as mechanistic studies, selectivity profiling, and *in vivo* validation to confirm anticancer effects and assess translational potential. Overall, these findings provide preliminary *in vitro* evidence that *A. stromatoides* SaH 1 is a promising source of anticancer metabolites, warranting further investigation.

5. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to

be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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7. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

8. ETHICAL APPROVALS

The study protocol was approved by the Ethical Committee of Medical Research, Faculty of Dentistry, University of Jember (Approval No.: 2524/UN25.8/KEPK/DL/2024; Date: 22 April 2024).

9. DATA AVAILABILITY

All data is available to the authors (<https://drive.google.com/drive/folders/1Fg5zQfK-MRRP5UqMxIYd6eekgix9DIp1?usp=sharing>) and shall be provided upon request.

10. PUBLISHER'S NOTE

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11. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declare that they have not used AI tools for writing and editing the manuscript, and no images were manipulated using AI.

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