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Original Research Article

Metabolomics-Guided Discovery of Anticancer Metabolites from Marine Sponge-Associated *Bacillus safensis*: *In Vitro* and *In Silico* Evaluation

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ABSTRACT

Marine-derived bacteria are valuable sources of novel anticancer agents, particularly those associated with sponges in tropical ecosystems. This study aimed to investigate the cytotoxic and metabolomic profile of *Bacillus safensis* P.039.5, isolated from marine sponge *Petrosia nigricans* collected near Pari Island, Indonesia. Cytotoxic screening against MCF-7 breast cancer cells revealed significant inhibition, with an IC_{50} value of 186.63 µg/mL and a high selectivity index compared to doxorubicin. Metabolite profiling using Liquid Chromatography-High Resolution Mass Spectrometry (LC-HRMS/MS) identified 28 putative compounds, dominated by peptides and alkaloids. Among these, militarinone A, maculosin, and verpacamide A showed the strongest predicted binding affinities in molecular docking studies against five breast cancer-related targets: ER-α, HER2, EGFR, CDK2, and topoisomerase IIα. Militarinone A exhibited the highest binding affinity (-11.11 kcal/mol), exceeding native ligands and reference drugs. To our knowledge, this is the first report of militarinone A detection in *B. safensis* P.039.5, demonstrating the utility of a metabolomics-guided approach to prioritize bioactive compounds from underexplored marine bacteria. These findings highlight the pharmacological potential of *B. safensis* as a sustainable source of multi-target anticancer leads.

Keywords: Marine bacteria, Sponge, *Bacillus safensis*, Cytotoxicity, Molecular docking

Introduction

Breast cancer is the most commonly diagnosed malignancy and remains a leading cause of cancer-related mortality worldwide.[1] In 2022 alone, approximately 2.3 million women were diagnosed with the disease, resulting in an estimated 670,000 deaths globally.[2] About 60% of breast cancer cells exhibit overexpression of estrogenic receptor alpha (ER-α), a critical component in the transcription of nuclear DNA required for mammary gland development and essential for maintaining the breast cancer signalling network.[3–5] The malignant nature of breast tumors allows them to invade surrounding tissues and metastasize to distant organs, making early detection and targeted treatment critical for improving patient outcomes.[6] The development of breast cancer is influenced by multiple risk factors, including genetic and hereditary predispositions.[7] Breast cancer with estrogen receptor-positive (ER+) subtypes making up the vast majority of cases.[8]

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The signaling of estrogen receptors is crucial for the growth and development of ER+ breast cancer, which is the primary driver in about two-thirds of breast cancers, making it an important target for therapy.[9] Existing therapies, such as selective estrogen receptor modulators (SERMs) like tamoxifen and aromatase inhibitors, have greatly enhanced patient outcomes.[10] However, resistance to these therapies can occur due to genetic mutations in the ERα gene, including Y537S and D538G, which result in increased transcriptional activity independent of estrogen.[9] Therefore, alternative approaches are required to discover therapeutic agents that can effectively target ERα and overcome resistance mechanisms. Current treatments for breast cancer primarily include chemotherapy and surgery.[11] However, chemotherapy is often associated with critical challenges, including drug toxicity and the emergence of resistance, limiting its long-term effectiveness.[12,13] For instance, doxorubicin, a widely used anthracycline, is known to cause cardiotoxicity,[14,15] while paclitaxel can induce peripheral neuropathy, significantly affecting patients' quality of life.[16] To address these limitations, intensive research efforts are increasingly focused on marine natural products (MNPs), which hold great promise for discovering new anticancer drugs.[17] At present, over 15 to 20 MNPs-derived drugs have received clinical approval, primarily for cancer treatment largely attributed to funding initiatives by the US National Cancer Institute which began in the 1960s.[18]

Sponges are marine invertebrates that produce MNPs with diverse pharmacological properties.[19] As filter-feeder organisms, sponges absorb microorganisms that can either be digested or retained within their bodies, making most of their biomass composed of symbionts, including marine bacteria.[20] Recent discoveries revealed bacteria as the primary sources of bioactive compounds. Thus, they become the target for the researcher to find a sustainable source for drug development rather than harvesting the sponge directly to minimize the negative

environmental effect while still being able to unlock the discoveries of novel drugs.[21]

Marine sponge-associated bacteria exhibit remarkable potential as producers of bioactive compounds, representing an alternative solution for sustainable natural resources. Bacterial biomass in the high microbial abundance sponge can comprise about 40% to 60% of the sponge's total tissue volume, emphasizing their integral ecological role.[22,23] Previous studies on sponge-associated bacteria for anticancer purposes have highlighted their promising cytotoxic activities. For instance, *Bacillus safensis* HA-MS-105 from the red sea sponge *Amphimedon ochracea* has been reported to show cytotoxic activity against HepG2, HCT, and MCF-7 cell lines.[24] This finding underscores the therapeutic potential of marine sponge-associated bacteria as a source of novel anticancer agents.

Among the diverse taxa isolated from marine sponges, *B. safensis* has emerged as a particularly intriguing candidate due to its ecological adaptability and growing evidence of bioactivity from various marine environments. *B. safensis* is a Gram-positive bacterium found in a wide variety of habitats, including seawater, marine sediments, and in symbiotic relationships with macroorganisms such as sponges.[25,26] Some strains of *B. safensis*, particularly those isolated from marine sources, exhibit a diverse range of bioactivities. For instance, the strain *B. safensis* SDG14, which was isolated from the gut of the marine fish *Sardinella longiceps*, produces a magainin-2-like bacteriocin called BpSl14. This bacteriocin has shown cytotoxic activity against A549 lung cancer cells.[27] Another strain, obtained from the red alga *Halymenia durvillei*, has demonstrated quorum-sensing inhibitor (QSI) and antimicrobial properties.[28] Additionally, a strain isolated from the sponge *Mycale* sp. in Jeju Island, South Korea, produces compounds known as Seongsanamide A-D, which exhibit antiallergic activity.[29] Despite these promising bioactivities, the secondary metabolite repertoire of *B. safensis* remains largely unexplored, particularly in the context of marine anticancer drug discovery.

To address this gap, the present study applies an integrative approach combining in vitro cytotoxic screening, LC-HRMS/MS-based metabolite profiling, and molecular docking analysis to investigate the anticancer potential of a marine sponge-associated *B. safensis* strain. In vitro screening provides the first layer of biological evidence, allowing rapid assessment of the extract's cytotoxic effects and helping to prioritize strains with promising activity against breast cancer cells.[30] LC-HRMS/MS offers a powerful platform for the untargeted detection of structurally diverse secondary metabolites from complex bacterial extracts.[31,32] Molecular docking complements this by providing a structure-based method for predicting ligand-receptor interactions, prioritizing bioactive candidates, and accelerating early-stage drug discovery at relatively low cost.[33] Through this multidisciplinary approach, the study aims to identify and prioritize metabolites with strong predicted binding affinities toward key breast cancer-related proteins, supporting the discovery of novel selective anticancer agents from marine microbial sources.

Materials and Methods

Collection of Marine Invertebrates

Marine invertebrates were collected from four locations close to Pari Island in the Kepulauan Seribu, Jakarta, using SCUBA diving. The sampling locations were situated to the north and south of Pari Island, where the majority of the island's population resides near the local harbor in the southern section. The GPS coordinates of the sampling sites were as follows: Site 1 (Latitude -5.850003, Longitude 106.633065), Site 2 (-5.851216, 106.612787), Site 3 (-5.860029, 106.622764), and Site 4 (-5.870405, 106.612164). Marine samples were collected using the previous method by Wibowo *et al*.[34]

Ethical Approval

All experimental procedures involving human cancer cell lines were conducted following institutional ethical standards. Ethical approval for the in vitro cytotoxicity assays was granted by the Medical and Health Research Ethics Committee (MHREC), Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada, Indonesia (Ref. No. KE/FK/0135/EC/2025). Marine sponge sampling was conducted under relevant permits and in accordance with national environmental regulations.

Isolation of Symbiotic Bacteria

The symbiotic bacteria were isolated using the previously described method by Wibowo *et al*.[34] A total of 1 cm³ from each sample of marine invertebrates was carefully rinsed with sterile seawater to effectively remove any weakly attached bacteria. The samples were homogenized using a sterile mortar and pestle, then subjected to a serial dilution in sterile seawater up to a 10^{-5} concentration, with a dilution factor of 10 at each step. From the diluted samples, 100 µL was streaked onto agar plates with selective isolation media as in the previous study. The plates were incubated at approximately 28°C for 14 days, with colony development monitored daily.

Metabolite Production and Extraction

Marine bacterial isolates were initially grown in 10 mL of Marine Broth (HiMedia) for three days to prepare seed cultures. Subsequently, 1 mL of each seed culture was inoculated into 100 mL of fresh Marine Broth in a 250 mL Erlenmeyer flask and incubated at room temperature under static conditions for 10 days. Following fermentation, the culture broth was extracted with ethyl acetate (pro analysis grade, SmartLab) at a 1:2 (v/v) ratio using a separatory funnel to separate the organic and aqueous phases. The ethyl acetate layer was collected and concentrated using a rotary evaporator, yielding crude extracts that were stored at -20 °C for further analysis.

Anticancer Activity

The cytotoxic activity of marine bacterial extracts was assessed against human breast adenocarcinoma (MCF-7) and normal kidney epithelial (Vero) cell lines using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay.[35] MCF-7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Sigma-Aldrich), 2% penicillin-streptomycin (Elabscience), and 0.5% fungizone (Gibco) under standard conditions (37°C, 5% CO_2). Cells were seeded into 96-well plates at a density of 5,000-10,000 cells per well in 100 µL of medium and incubated for 24 hours to allow cell attachment. Thereafter, 100 µL of bacterial extract at varying concentrations was added to each well and incubated for another 24 hours. Doxorubicin (KalbeMed) at 2 µg/mL served as the positive control, while 1% DMSO (Merck) was used as the solvent control. Post-treatment, MTT solution was added to each well at a final concentration of 0.5 mg/mL and incubated for 4 hours at 37°C. Subsequently, 100 µL of a 10% SDS solution in 0.01 M HCl (Sigma-Aldrich) was added to solubilize the formazan crystals, followed by incubation for 24 hours. Absorbance was measured at 595 nm using a microplate reader (Bio-Rad).[36] Cell viability (%) was calculated using the following formula (Equation 1):

$$\%\ \text{cell viability} = \frac{\text{absorbance of treatment samples}}{\text{absorbance of negative control (DMSO)}} \times 100\% \quad (1)$$

The cell viability percentage was subsequently utilized to calculate growth inhibition percentages and to determine the IC_{50} values.[37]

Calculation of Selectivity Index

The selectivity index (SI) was calculated to assess the differential cytotoxicity of marine bacterial extracts toward cancerous versus normal cells.[38] This index is a critical parameter for determining the therapeutic potential of a compound, as it indicates the degree to which it selectively targets cancer cells while sparing healthy ones. The SI for each extract was determined using the following formula (Equation 2):

$$\text{Selectivity Index (SI)} = \frac{IC_{50}\text{ value in normal cell lines (Vero)}}{IC_{50}\text{ value in cancer cell lines (MCF-7)}} \quad (2)$$

Extracts with an SI value ≥2 demonstrated selective toxicity towards cancer cell lines only. In contrast, extracts with SI value <2 were classified as toxic to both normal and cancer cell lines.[39]

Bacterial Identification

Marine bacterial isolate with the best cytotoxic activity was molecularly identified using 16S rRNA gene sequencing. The DNA of selected isolated was extracted using the Bacteria Genomic DNA Kit (GBB 100, Geneaid). PCR amplification of the 16S rRNA gene will be performed using universal forward and reverse primers, followed by purification and sequencing of the PCR products. The obtained sequences will be compared with sequences of related type strains available in the EzBioCloud server to determine their genetic relationships.[40] Subsequently, the sequences will be deposited in the NCBI database. A phylogenetic tree will then be constructed based on the 16S rRNA gene sequences of the bacteria and their closest strains. Neighbor-joining trees will be generated using MEGA 7 version 7.0.26, and bootstrap values exceeding 50% will be denoted on nodes after 1000 iterations.

Metabolite Profiling Using LC-HRMS/MS

The most cytotoxic extract from marine bacteria was examined using untargeted liquid chromatography-high-resolution mass spectrometry (LC-HRMS/MS) on a Vanquish UHPLC Binary Pump connected to a Quadrupole-Orbitrap Exploris 120 mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). The separation process was performed on a ZORBAX Eclipse Plus C18 RRHD column (2.1 × 100 mm, 1.8 µm) with UV detection set at 254 nm. Heated electrospray ionization (HESI) in positive mode was used with a spray voltage ranging from 3.3 to 3.8 kV, a capillary temperature maintained at 320°C, and nitrogen used as sheath and auxiliary gases (33 and 7 arbitrary units, respectively). The mass scan range was *m/z* 100-1500, with stepped collision energies set at 30, 60, and 80 eV. Gradient elution was performed at a flow rate of 0.3 mL/min using MS-grade water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). Initially, mobile phase B was set at 5%, then gradually increased to 100% over 31 minutes, maintained at 90% for 5 minutes, and finally returned to the initial condition (5% B) by 43 minutes. Data acquisition was conducted in full data-dependent MS2 (ddMS2) mode. The raw data underwent processing using Thermo XCalibur and Compound Discoverer 3.3, with a minimum peak intensity threshold of 10,000. Compound annotation involved spectral alignment, database matching (mzCloud, ChemSpider, PubChem), and the identification of unknowns employing MS/MS fragmentation patterns and predictive modeling via CFM-ID (https://cfmid.wishartlab.com/predict).[41] A mass error threshold of 5 ppm was implemented for filtering compounds.

Molecular Docking Study

Molecular docking was conducted to evaluate the interactions between bioactive compounds and five breast cancer-related protein targets, including estrogen receptor alpha (ER-α) (PDB ID: 1A52), human epidermal growth factor receptor 2 (HER-2) (PDB ID: 3RCD), epidermal growth factor receptor (EGFR) (PDB ID: 3POZ), cyclin-dependent kinase 2 (CDK2) (PDB ID: 2XMY), and topoisomerase IIα (PDB ID: 1ZXM). The crystal structures were retrieved from the Protein Data Bank (https://www.rcsb.org/) and prepared by removing water molecules, ions, and native ligands using UCSF Chimera 1.18.[42] Ligands were selected based on LC-HRMS/MS analysis of bioactive extracts. The docking simulation was carried out using two types of docking methods, AutoDock Vina 1.1.2 and AutoDock Tools 1.5.7. Initial screening was performed using AutoDock Vina 1.1.2 to identify compounds with high binding affinity, followed by a more detailed docking analysis using AutoDock Tools 1.5.7 to refine ligand-protein interactions. The validation of the docking protocol was confirmed by redocking the native ligand into its binding site with an RMSD value less than 2Å, ensuring reliable docking accuracy.[43] The docking grid was set to 40 Å × 40 Å × 40 Å, centered on the protein's active site as defined by the native ligand position.[44] The binding affinities were reported as the lowest binding energy (kcal/mol), in which more negative values indicate stronger interactions.[45] Ligand-protein interactions, including hydrogen bonds and hydrophobic interactions, were visualized using BIOVIA Discovery Studio 24.1.10.[46] Standard inhibitors such as doxorubicin (for topoisomerase IIα), fadraciclib (for CDK2), and lapatinib (for HER2 and EGFR), as well as the respective native ligands were docked in the same method.

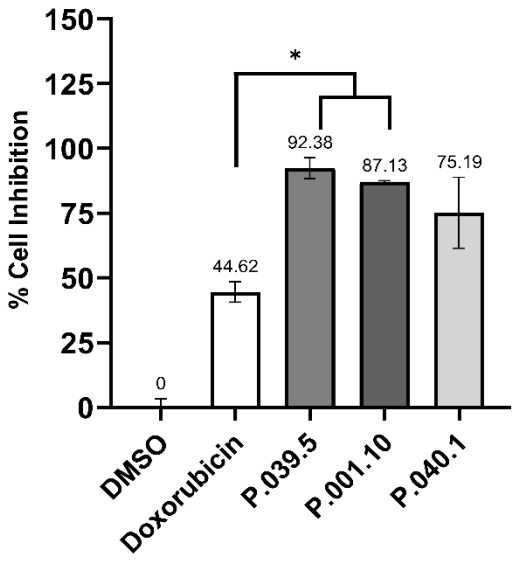
Statistical Analysis

MCF-7 cell growth inhibition was calculated from triplicate assays, and the results were expressed as mean percentage inhibition. Data normality was tested using the Shapiro-Wilk test, while Levene's test assessed homogeneity of variances. Since the data met normality but violated homogeneity ($p < 0.05$), Welch's one-way ANOVA was applied, followed by the Games-Howell post hoc test for group comparisons. A p-value < 0.05 was considered statistically significant.[47] Statistical analyses were performed using IBM SPSS Statistics version 27.0.1, and graphical visualizations were generated in GraphPad Prism version 10.4.1.

Results and Discussion

Screening Anticancer Activity

In this study, we screened 81 marine bacterial extracts (**Table S1**), isolated from marine samples collected near Pari Island, Kepulauan Seribu, Jakarta, as reported by Wibowo *et al*.[34] The screening result displayed three bacterial strains with remarkable inhibition activity, namely P.001.10, P.039.5, and P.040.1, which were isolated from the sponges *Stylissa* sp., *Petrosia nigricans*, and *Dasychalina fragilis*, respectively. Among these, strain P.039.5 exhibited the highest inhibition (92.38%) at 500 µg/mL, significantly exceeding the positive control doxorubicin at 2 µg/mL (44.62% inhibition) (**Figure 1**). ANOVA analysis confirmed that P.039.5 and P.001.10 exhibited significantly higher cytotoxicity than doxorubicin ($p < 0.05$), while 1% DMSO as negative control did not exhibit any significant cytotoxic effect, confirming its role as an appropriate solvent.



	Treatments (Mean ± SD)				
	DMSO	Doxo	P.039.5	P.001.10	P.040.1
% Inhibition	0 ± 3.34	44.62 ± 3.97	92.38 ± 4.07	87.13 ± 0.42	75.19 ± 13.66

Figure 1: Percent growth inhibition of MCF-7 breast cancer cells after treatment with the three most cytotoxic marine bacterial extracts at a concentration of 500 µg/mL. Doxorubicin (2 µg/mL) was used as a positive control, and 1% DMSO served as the solvent control. Data are presented as mean ± SD from triplicate experiments

IC_{50} determination revealed that P.001.10 and P.039.5 showed moderate cytotoxicity, based on National Cancer Institute criteria[48] with IC_{50} values of 164.25 µg/mL and 186.63 µg/mL, respectively. Meanwhile, P.040.1 displayed weak cytotoxicity with an IC_{50} value more than 200 µg/mL (**Table 1**).

Table 1: The IC_{50} and SI value of bacterial extracts and doxorubicin treatment against MCF-7 cell lines

Bacterial Extracts	IC_{50}(µg/mL)	Selectivity Index
P.039.5	186.63	18099.83
P.001.10	164.25	275.43
P.040.1	344.68	201.72
Doxorubicin	2.34	2.35

Although P.001.10 demonstrated a slightly lower IC_{50} than P.039.5, the selectivity index (SI) of P.039.5 (SI = 18099.83) was markedly higher than P.001.10 (SI = 275.43) and doxorubicin (SI = 2.35). A higher SI indicates greater selectivity towards cancer cells, thereby minimizing potential adverse effects on normal cells.[49] These findings emphasize that despite its moderate cytotoxicity compared to doxorubicin (IC_{50} = 2.34 µg/mL), P.039.5 offers greater selectivity, which is crucial for minimizing off-target toxicity. Doxorubicin's potency is attributed to its chemical purity and well-established mechanism of DNA intercalation that induces apoptosis,[50] but its lower SI (2.35) suggests greater toxicity to normal cells.[39]

Strain P.039.5 was isolated from the marine sponge *Petrosia nigricans*, a species previously reported to produce cytotoxic compounds such as petronigrione and methyl tortuoate B that inhibited various cancer cell lines, including HepG2, KB, LU-1, and MCF-7.[51] Similarly, strain P.001.10 was derived from sponge *Stylissa* sp., reported to yield stylissamide A and stylissoside A with strong cytotoxicity against MCF-7 and HepG2 cell lines,[52] while strain P.040.1 from sponge *Dasychalina fragilis* was reported to produce sterol compounds such as haplosamate A,[53] which inhibits membrane type 1 matrix metalloproteinase, a tumor metastasis-related enzyme.[54] These host-specific associations reflect a broader ecological pattern in which marine sponges not only produce bioactive compounds but also harbor symbiotic bacteria that may be the true producers of these metabolites.[20] As nutrient-rich microenvironments, sponges support diverse microbial communities capable of synthesizing unique secondary metabolites with pharmacological potential.[55,56]

Identification of Active Bacterium

The isolate P.039.5, which shows the highest inhibition against MCF-7 cell lines, was identified using the 16S rRNA gene sequence. The obtained partial sequences were assembled to produce 1265 bp of 16S rRNA gene sequence. The 16S rRNA gene sequence of isolate P.039.5 was deposited in GenBank under the accession number PQ804041. BLAST analysis showed 100% sequence similarity with *Bacillus safensis*, followed by *B. australimaris* (99.84%) and *B. pumilus* (99.76%). Phylogenetic analysis placed P.039.5 within the same clade as *B. safensis* and *B. safensis* subsp. *osmophilus* (**Figure 2**), confirming its taxonomic identity as B. safensis.

B. safensis was known as a cosmopolitan species, inhabiting diverse environments, including soils, animals, plants, and aquatic ecosystems.[26] It has shown promising biotechnological applications, such as producing industrial enzyme,[57] producing antibacterial[58] and anticancer compounds,[59] plant-growth-promoting bacteria,[60] and bioremediation agents.[61] The observed moderate cytotoxicity and strong selectivity align with previous studies reporting the anticancer potential of other *B. safensis* strains. For instance, *B. safensis* MB8, isolated from deep-sea sediments in the Bay of Bengal, was shown to produce Bis(2-ethylhexyl) benzene-1,2-dicarboxylate (BEHBD), a compound with cytotoxic activity against MCF-7 cells and an IC_{50} value of 49.8 µg/mL.[59] This supports the potential of *B. safensis* strains as underexplored microbial sources of bioactive compounds with relevance to cancer therapy.

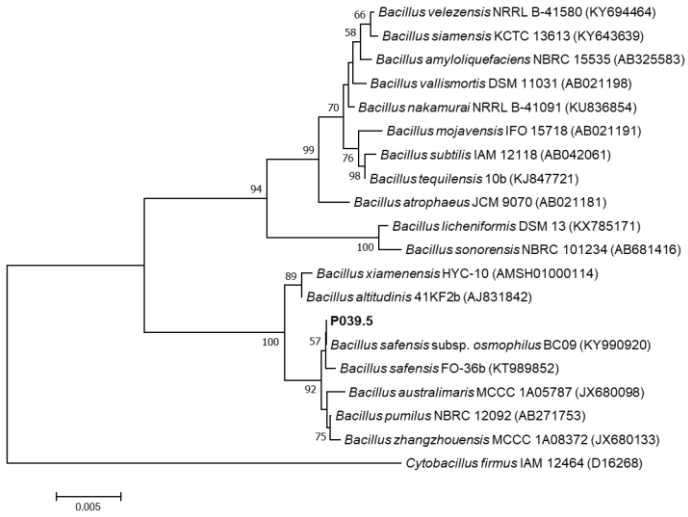


Figure 2: Neighbor-Joining phylogenetic tree based on 16S rRNA gene sequences, showing the phylogenetic position of isolate P.039.5 (shown in bold) and their closely related species. Bootstrap values >50% are shown at nodes. Bar, 0.005 substitutions per nucleotide position. *Cytobacillus firmus* (D16268) was used as an outgroup.

Metabolite Profiling of Crude Extract Using LC-HRMS/MS

Metabolite profiling of *B. safensis* P.039.5 crude extract via LC-HRMS/MS identified 28 putative compounds **(Table 2)**, with six major compounds detected based on peak intensity and relative abundance (**Figure S1**). These include betaine (RT = 0.74 min), trans-4-hydroxy-L-proline (RT = 0.73 min), indospicine (RT = 1.13 min), verpacamide A (RT = 1.36 min), maculosin (6.48), and erucamide (RT = 33.27 min). Among them, betaine was identified as the most abundant compound. Betaine, a trimethylated glycine derivative, was found in almost all living organisms, including microorganisms such as bacteria, archaea, and fungi.[62] This compound is primarily classified as an amino acid derivative due to its structural similarity to glycine and its role in methyl donation and osmoregulation.[62] However, it is also referred to as an alkaloid in certain phytochemical and microbial studies, particularly because it is a naturally occurring nitrogen-containing compound with physiological activity.[63] Despite lacking a heterocyclic ring, it is specifically categorized under non-heterocyclic or ammonium-type alkaloids due to its quaternary ammonium structure.[64] Similarly, trans-4-hydroxy-L-proline and indospicine are categorized as amino acid derivatives known roles in metabolic and toxicological pathways.[65,66] Verpacamide A and maculosin, both classified as diketopiperazines, fall under the broader class of cyclic peptides, which are known for their stability and diverse bioactivities.[67,68] Erucamide, a monounsaturated fatty acid amide with a 22-carbon aliphatic chain, belongs to the lipid or fatty acid derivative category.[69]

The overall metabolite profile revealed that the most abundant compounds belong to a group of peptides and alkaloids. The identified peptides include maculosin, cyclo(leucylprolyl), verpacamide A, glycylprolylhydroxyproline, and prolylglutamic acid. Meanwhile, the identified alkaloids include militarinone A, N-methylproline, 6-hydroxypseudooxynicotine, and betaine. Apart from peptides and alkaloids, several amino acid and fatty acid derivatives, as well as small organic molecules were also detected, further contributing to the complex chemical diversity of *B. safensis* metabolites. This composition aligns with previous reports highlighting sponge-associated marine bacteria as rich sources of structurally diverse and bioactive secondary metabolites, particularly within these two chemical classes.[70,71] Both peptides and alkaloids have demonstrated a wide range of pharmacological activities, including anticancer effects.[20] Bioactive peptides can disrupt cancer cell membranes or trigger apoptosis through pore formation and immune modulation,[72] while alkaloids are known to induce apoptosis, inhibit angiogenesis, and modulate key signaling

Table 2: Identified metabolites of P.039.5 bacterial crude extract using LC-HRMS/MS Q-Orbitrap

No.	RT (minute)	Metabolites	Formula	Molecular Weight	Δ Mass (ppm)	% Area Relative
1.	0.66	N-methylproline	$C_6H_{11}NO_2$	129.07895	-0.20	0.02
2.	0.71	L-(+)-norleucine	$C_6H_{13}NO_2$	131.09471	0.62	0.50
3.	0.73	trans-4-Hydroxy-L-proline	$C_5H_9NO_3$	131.05832	0.59	3.06
4.	0.74	Betaine	$C_5H_{11}NO_2$	117.07897	-0.10	73.01
5.	1.07	1,3-Dimethylbutylamine	$C_6H_{15}N$	101.12051	0.58	1.98
6.	1.07	1-Deoxynojirimycin	$C_6H_{13}NO_4$	163.08458	0.76	1.08
7.	1.07	Glycylprolylhydroxyproline	$C_{12}H_{19}N_3O_5$	285.13277	1.05	1.62
8.	1.07	1-Aminocyclopropanecarboxylic acid	$C_4H_7NO_2$	101.04772	0.42	0.14
9.	1.07	Dihydrothymine	$C_5H_8N_2O_2$	128.05862	0.30	0.07
10.	1.07	Hexylamine	$C_6H_{15}N$	101.12051	0.58	1.98
11.	1.13	Indospicine	$C_7H_{15}N_3O_2$	173.11659	0.97	2.61
12.	1.36	Verpacamide A	$C_{11}H_{19}N_5O_2$	253.15399	0.47	2.79
13.	1.60	Biotin	$C_{10}H_{16}N_2O_3S$	244.08846	1.20	0.82
14.	1.61	4-Methyleneglutamine	$C_6H_{10}N_2O_3$	158.06933	1.21	0.52
15.	1.67	Prolylglutamic acid	$C_{10}H_{16}N_2O_5$	244.10607	0.59	0.02
16.	1.67	4-Hydroxyprolyl-4-hydroxyproline	$C_{10}H_{16}N_2O_5$	244.10607	0.59	0.02
17.	2.08	Pyridoxamine	$C_8H_{12}N_2O_2$	168.09001	0.81	0.54
18.	2.12	L-Phenylalanine	$C_9H_{11}NO_2$	165.07910	0.76	0.90
19.	3.80	6-hydroxypseudooxynicotine	$C_{10}H_{14}N_2O_2$	194.10567	0.73	0.40
20.	5.26	NP-013736	$C_{10}H_{16}N_2O_2$	196.12140	1.14	0.50
21.	6.48	Maculosin	$C_{14}H_{16}N_2O_3$	260.11623	0.53	2.70
22.	6.90	1-(2-Morpholinophenyl)dihydro-1H-pyrrole-2,5-dione	$C_{14}H_{16}N_2O_3$	260.11629	0.76	0.57
23.	7.26	Cyclo(leucylprolyl)	$C_{11}H_{18}N_2O_2$	210.13703	0.94	0.35
24.	15.14	2-Amino-1,3,4-octadecanetriol	$C_{18}H_{39}NO_3$	317.29332	1.03	0.50
25.	18.82	Militarinone A	$C_{26}H_{37}NO_6$	459.26228	0.42	0.62
26.	26.09	cis-Cyclooctene	C_8H_{14}	110.10951	-0.33	0.14
27.	32.89	Adipic acid	$C_6H_{10}O_4$	146.05787	-0.24	0.01
28.	33.27	Erucamide	$C_{22}H_{43}NO$	337.33441	-0.16	2.52

pathways involved in tumor progression and metastasis.[73] In contrast, the crude extract of P.039.5 contains diverse metabolites that may act synergistically or antagonistically, contributing to lower potency but improved selectivity.[74,75] Despite this complexity, such extracts represent promising sources of novel compounds with potentially enhanced safety profiles compared to conventional chemotherapeutics. The mass analysis showed that among the identified putative compounds, two of them were recognized as *Bacillus* sp. derived compounds, namely maculosin and cyclo(leucylprolyl). Maculosin, a diketopiperazine cyclic dipeptide, has been previously reported as a metabolite of *Bacillus pumilus*.[68] Similarly, cyclo(leucylprolyl), another cyclic dipeptide, was identified in *Bacillus amyloliquefaciens*.[76] The identification of these two compounds, which are known metabolites of *Bacillus* species, strengthens the putative annotation of LC-HRMS/MS spectra and further supports the role of *Bacillus safensis* as a producer of bioactive secondary metabolites.

Molecular Docking Study

The identified compounds in Table 2 were docked to evaluate their binding affinity towards ER-α, HER2, EGFR, CDK2, and topoisomerase IIα as the protein target. ER-α is highly expressed in MCF-7 cells and plays a crucial role in hormone-driven tumor growth.[4,9] HER2 and EGFR are members of the receptor tyrosine kinase family, frequently overexpressed in aggressive breast cancer subtypes and associated with enhanced proliferation and poor prognosis.[77,78] CDK2 is a key regulator of the G1-S phase transition and is often dysregulated in breast cancer,[79] while topoisomerase IIα is essential for DNA replication and serves as a target for widely used chemotherapeutic agents.[80] These targets represent interconnected oncogenic pathways, offering a rational framework for evaluating the multi-target therapeutic potential of marine-derived secondary metabolites in anticancer drug discovery.

Initial screening was performed using AutoDock Vina 1.1.2 to predict the binding affinity of each compound, and the complete docking scores are presented in Table S2. Compounds with binding energies close to or even lower than –7.0 kcal/mol were considered to have strong and favorable interactions with the protein targets.[81] Based on this criterion, the top three candidates, namely militarinone A, maculosin, and verpacamide A were selected for further refinement using AutoDock Tools 1.5.7, enabling more detailed evaluation of ligand-protein interactions.

Figure 3 summarizes a comparative analysis of the top three candidate metabolites and standard inhibitors across five breast cancer-related protein targets. Militarinone A exhibited the lowest binding energies across all five targets, particularly topoisomerase IIα (−11.11 kcal/mol), followed by CDK2 (−10.12 kcal/mol) and HER2 (−9.52 kcal/mol). These values exceeded those of the standard inhibitors doxorubicin, fadraciclib, and lapatinib, as well as the respective native ligands, indicating strong and potentially stable interactions. Its ability to interact with active-site residues reflects a binding profile similar to clinically validated inhibitors.[78–80] On the other hand, maculosin showed moderate binding across the five targets, with the best affinity for topoisomerase IIα (−8.12 kcal/mol) and CDK2 (−7.68 kcal/mol), while verpacamide A exhibited slightly lower affinities, though it showed notable interaction with topoisomerase IIα (−8.33 kcal/mol) as well. The lower binding energy indicates a more stable ligand-protein interaction,[82] suggesting that these metabolites could serve as potential candidates for further in vitro and in vivo validation. These strong binding affinities are not solely attributed to hydrogen bonding but also to the combined effects of multiple non-covalent interactions, including van der Waals forces, π-π-alkyl stacking, and hydrophobic packing. Such interactions enhance ligand stability within the binding pocket, improve shape complementarity, and reduce entropy penalties, ultimately resulting in favorable binding energies.[83–85]

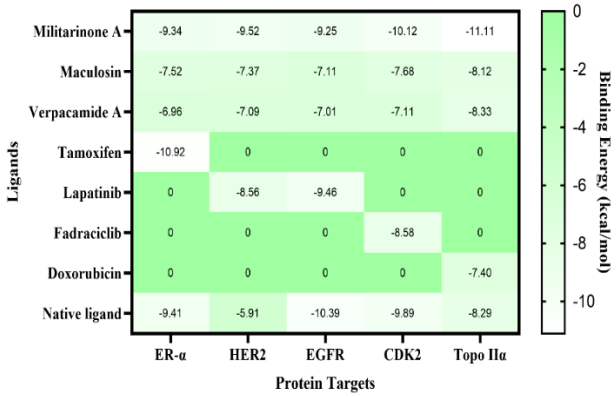


Figure 3: Comparative heatmap of binding affinities (kcal/mol) for the top three candidate metabolites from *B. safensis* P.039.5 and standard inhibitors against five breast cancer-related protein targets. Color intensity reflects the strength of binding affinity, with values below −9 kcal/mol indicating high-affinity interactions. Blank cells (0) represent compounds not docked against the corresponding target.

The docking simulation results of the three most active compounds and standard inhibitors revealed key active sites on each protein. Hydrophilic interaction (**Table 3**) revealed key amino acid residues involved in stabilizing each ligand-protein complex, supported by the visualization as shown in **Figure 4** and **Figures S2-S5**.

In HER-2 complex, militarinone A formed a hydrogen bond with Met801, a key residue also targeted by clinically relevant inhibitors such as lapatinib and native ligand (TAK-285), supporting its potential as a HER2 kinase inhibitor.[78] Meanwhile, in CDK2 complex, militarinone A interacted with Gln131, a hydrogen bond residue commonly involved in stabilizing native ligands such as 2-anilino-4-(thiazol-5-yl)pyrimidine derivatives, which also formed critical hydrogen bonds with Gln131, Asp86, and Lys33, and engaged in hydrophobic interactions with residues like Phe80.[79] These interactions support the potential of militarinone A as a selective ATP-competitive CDK2 inhibitor involved in cell cycle regulation. Additionally, in topoisomerase IIα complex, militarinone A formed hydrogen bonds with Tyr34 and Thr215, which are located in the ATPase domain's active site involved in nucleotide recognition and hydrolysis, alongside

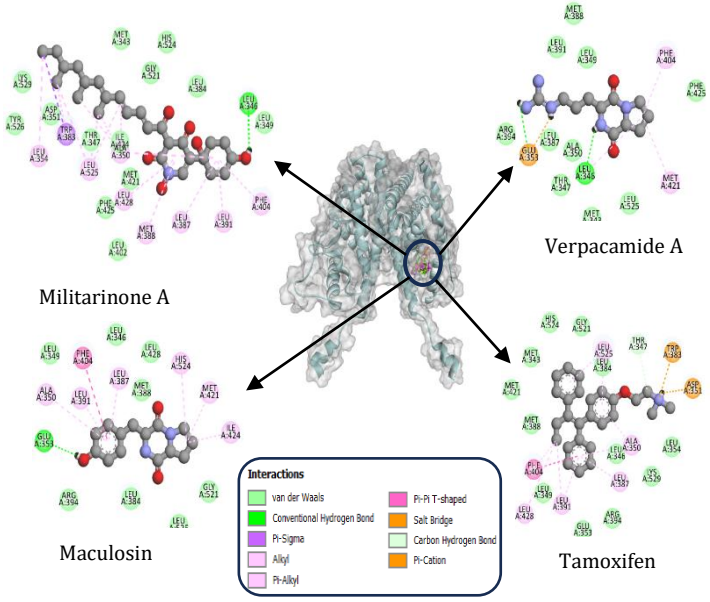


Figure 4: Docking representation of 3D and 2D interactions between amino acids from ERα with active metabolites and standard inhibitor

other residues such as Asn91 and Lys168 that stabilize conformational changes during DNA strand passage.[80] These interactions suggest its potential as a topoisomerase IIα inhibitor capable of disrupting DNA processing and replication in cancer cells.

The putative chemical structure of the three most active metabolites is represented in **Figure 5** as identified by LC-HRMS/MS and spectral database matching. Both verpacamide A and maculosin share a diketopiperazine core structure, a pharmacophore known for its structural rigidity, bioavailability, and receptor-binding versatility.[86,87]

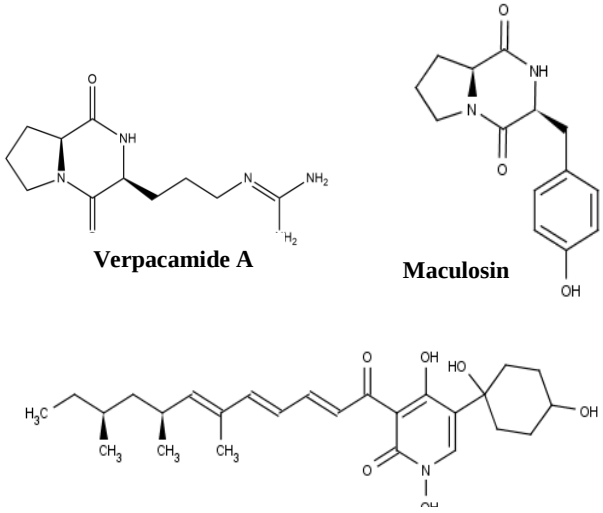


Figure 5: Putative structure of anticancer compounds namely verpacamide A, maculosin, and militarinone A found in *B. safensis* P.039.5 based on the mass spectra. These compounds exhibited potent binding affinities against ER-α, HER2, EGFR, CDK2, and topoisomerase IIα.

These cyclic dipeptides are recognized for their potential to form stable hydrophobic and π-π interactions with active site residues, contributing to their moderate yet consistent binding affinities across multiple targets. In contrast, militarinone A displays a more complex polyketide-derived scaffold, which has been characterized in previous studies as a neurotrophic pyridone alkaloid with conjugated double bonds and

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multiple hydroxyl groups.[88] These features are typical of polyketide biosynthetic pathways and are known to enhance ligand flexibility and the capacity for hydrogen bonding, thereby facilitating interaction with diverse protein binding pockets.[89]

Metabolite Profile of The Best Binding Affinity Compounds

The extracted ion chromatogram of militarinone A revealed a retention time (RT) of 18.816 minutes and a protonated molecular ion at *m/z* 460.26956 [M+H]+ with characteristic MS/MS fragmentation spectrum detected at *m/z* (%): 119.08562 (100), 135.08055 (25.04), 107.08561 (23.55), 91.05428 (8.93), and 69.03358 (8.33) (**Figure 6**). Although militarinone A accounted for only 1.58% of the total relative abundance among the annotated metabolites (**Table 2**), it exhibited the strongest binding affinities across multiple cancer-related protein targets in the docking analysis. This indicates that while not a major metabolite, militarinone A may possess high bioactivity and merits further investigation.

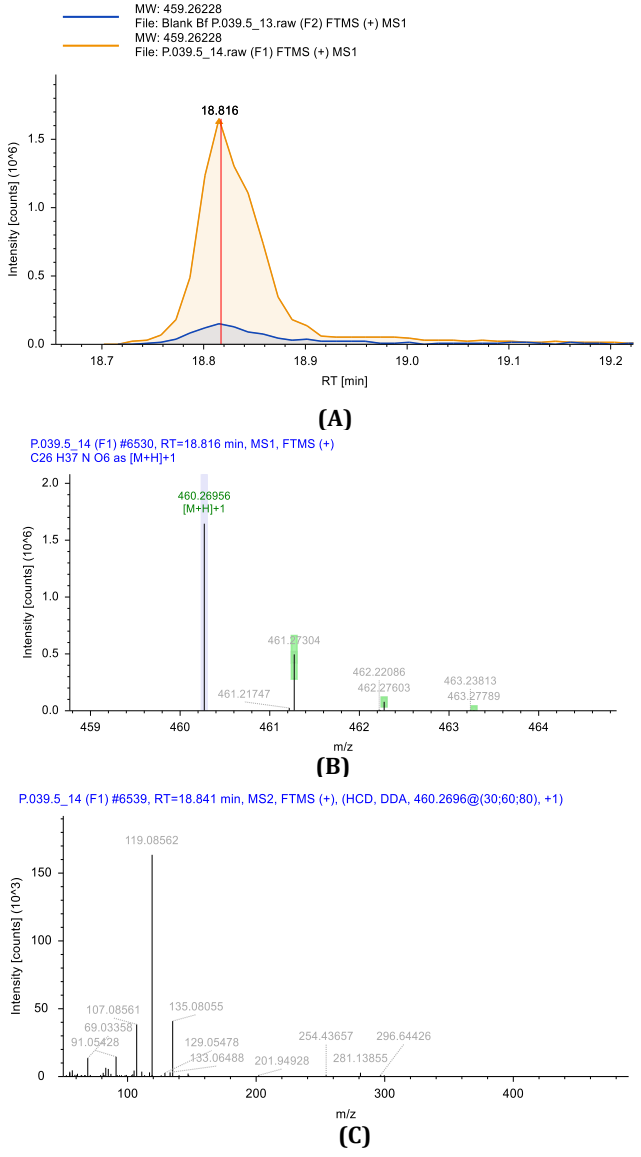

Figure 6: LC-HRMS/MS spectrum of *B. safensis* crude extract corresponding to militarione A. (A - B) The extracted mass spectrum of militarinone A at [M+H]+1 at *m/z* 460.26956 with RT 18.816 min, (C) The MS2 spectrum of militarinone A with its in-source fragments

In addition to militarinone A, two other compounds suspected to play a role in cytotoxic activity against breast cancer cells based on docking simulations are maculosin and verpacamide A. Maculosin was detected at a retention time of 6.477 minutes and a protonated molecular ion at *m/z* (%) 261.1235 [M+H]+. Its MS/MS fragmentation pattern showed key ions at *m/z* (100): 120.08087 (100), 68.04955 (62.14), 86.06008 (45.54), 261.12357 (26.71), 215.11803 (15.83), and 187.12315 (11.18) (**Figure 7**). As one of the major metabolites identified in the extract, maculosin demonstrated strong predicted binding affinities, particularly toward HER2 and topoisomerase IIα, supporting its relevance as a potential bioactive compound in the extract of *B. safensis* P.039.5.

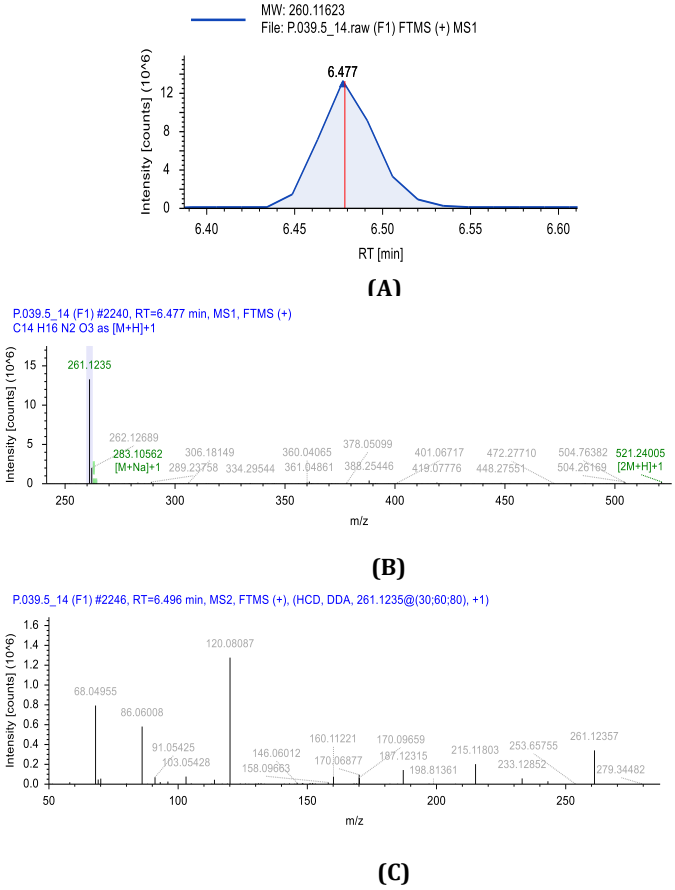

Figure 7: LC-HRMS/MS spectrum of *B. safensis* crude extract corresponding to maculosin. (A - B) The extracted mass spectrum of maculosin at [M+H]+1 at *m/z* 261.1235 with RT 6.477 min, (C) The MS2 spectrum of maculosin with its in-source fragments

Verpacamide A, detected at a retention time of 1.360 minutes with a [M+H]+ ion at *m/z* 254.1612, exhibited characteristic MS/MS fragment ions at *m/z* (%): 195.11302 (100), 254.16142 (65.28), 70.06522 (38.10), 125.07108 (34.16), 98.06013 (27.70), 167.11807 (26.38) (**Figure 8**). As one of the major metabolites identified in the extract, verpacamide A showed notable predicted binding affinities toward EGFR and CDK2. Together with militarinone A and maculosin, this compound exhibited a compelling interaction profile with key breast cancer-related protein targets, reinforcing its potential as a promising candidate for further metabolomics-guided exploration and therapeutic validation.

Among the identified metabolites, militinone A was prioritized based on its strong multi-target binding profile, making it the most promising candidate for further investigation in terms of therapeutic potential and biosynthetic origin. Militarinone A is a pyridone alkaloid originally isolated from the entomopathogenic fungus *Paecilomyces militaris* RCEF 0095,[88] and has been reported to induce apoptosis in N2a neuroblastoma cells via activation of the p53 pathway and AIF translocation.

Table 3: Hydrophilic interaction of the top three highest binding affinity ligand-protein complexes

Ligands	Protein Target				
	ER-α	HER-2	EGFR	CDK2	Topoisomerase IIα
Militarinone A	Leu346	Gln799, Met801, Arg849	Phe723, Gly719	Leu83, Gln131	Tyr34, Thr215
Maculosin	Glu353	Thr798, Asp863, Ser783	Lys745, Met793	Leu83, Lys89	Asn91, Thr147, Ala167, Glu87
Verpacamide A	Leu346, Glu353	Thr798, Asp863, Ser783, Thr862	Lys745, Thr854, Asp855, Phe856	Leu83, Asp86, Gln131, Ile10	Asn91, Gly166, Ser148, Ser149
Tamoxifen	Thr347	ND[a]	ND[a]	ND[a]	ND[a]
Lapatinib	ND[a]	Met801, Asp863	Met793, Thr854, Leu777	ND[a]	ND[a]
Fadraciclib	ND[a]	ND[a]	ND[a]	Leu83, Ile10	ND[a]
Doxorubicin	ND[a]	ND[a]	ND[a]	ND[a]	Ser149, Asp94, Asn150
Native ligand	His524, Glu353, Arg394	Met801, Lys753	Lys745, Met793	Leu83, Asp86, Lys89	Tyr165, Gly164, Asn120, Ala167, Gly166, Gln376, Arg162, Asn163

[a]ND = Not Docked (Standard inhibitors were only docked against their respective protein targets)

Additionally, it promotes neuronal differentiation in PC-12 cells by activating the PI3-K/PKB and MEK/ERK signaling pathways, demonstrating a dual role as both a neurotrophic and anticancer agent.[90] Interestingly, although militarinone A has not been previously reported in bacteria, it was detected in the crude extract of *Bacillus safensis* P.039.5 through LC-HRMS/MS. This unexpected finding raises compelling questions about its biosynthetic origin. While militarinone A is traditionally considered a fungal metabolite, recent studies have shown that marine bacteria, especially from the genus *Bacillus* can harbor cryptic or silent biosynthetic gene clusters (BGCs) such as nonribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) that can support the biosynthesis of complex secondary metabolites.[91]

The presence of militarinone A in *B. safensis* P.039.5 through LC-HRMS/MS can be attributed to two factors: biotransformation processes and horizontal gene transfer (HGT). The biotransformation process suggests that this strain may have the capacity to produce or modify compounds. It is possible that *B. safensis* P.039.5 does not synthesize militinone A de novo, but rather converts exogenous precursors through enzymatic biotransformation. This hypothesis is supported by prior studies showing the ability of *B. safensis* to modify external substrates, such as the conversion of eugenol to vanillin by *B. safensis* SMS1003.[92] Meanwhile, HGT may have enabled the acquisition of metabolite-producing gene clusters from coexisting sponge microbiota. Recent mechanistic and evolutionary studies by Slomka *et al*.[93] demonstrated that bacteria from the genus *Bacillus*, particularly *Bacillus subtilis* can integrate large segments of foreign DNA up to 2% of its genome through natural transformation, especially from phylogenetically related donors. These HGT events often occur in bursts and enable the stable acquisition of complex biosynthetic gene clusters that may offer adaptive advantages under environmental stress. Based on these findings, it is possible that *B. safensis* P.039.5 expresses a transformation-mediated metabolite sourced from an external precursor or acquired the genetic ability to make militinone A by HGT from a co-resident sponge symbiont. This demonstrates the amazing metabolic flexibility and evolutionary adaptability of *Bacillus* species in the marine symbiotic niches, warranting further genomic and transcriptomic investigations to determine the exact biochemical origin of militinone A in this strain.

To strengthen the therapeutic relevance of these findings, future research should focus on bioassay-guided fractionation and purification of the active metabolites from the *B. safensis* P.039.5 crude extract. Structural elucidation using techniques such as NMR and LC-MS/MS will be essential to confirm compound identity and enable structure-activity relationship (SAR) studies.

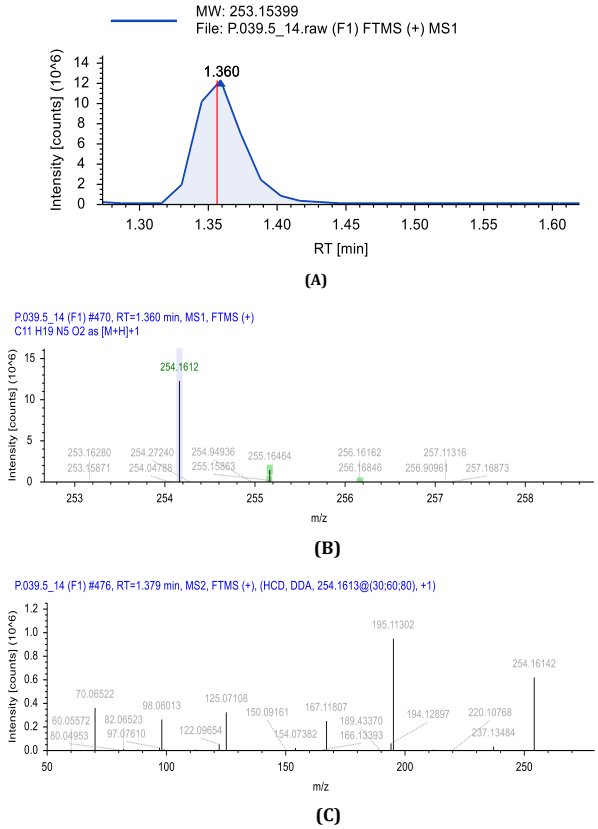

Figure 8: LC-HRMS/MS spectrum of *B. safensis* crude extract corresponding to verpacamide A. (A - B) The extracted mass spectrum of verpacamide A at [M+H]+1 at *m/z* 254.1612 with RT 1.360 min, (C) The MS2 spectrum of verpacamide A with its in-source fragments

Additionally, the anticancer efficacy of the purified compounds should be validated through in vitro mechanistic assays and, ultimately, in vivo models to assess safety and pharmacokinetics. These steps are critical not only for confirming the biological activity of militarinone A and related metabolites but also for advancing sponge-associated bacterial compounds toward preclinical development as selective anticancer agents.

Conclusion

Our study revealed that *B. safensis* P.039.5 associated with the sponge *Petrosia nigricans* exhibited significant anticancer activity against MCF-7 cells. Metabolite profiling identified 28 compounds, predominantly from the peptide and alkaloid groups. Molecular docking study demonstrated that militarinone A exhibited strong binding energy towards all five breast cancer-related protein targets, particularly topoisomerase IIα, CDK2, and HER2 that exceeded native ligands and standard inhibitors. These results highlight the potential of *B. safensis* as a promising source of marine natural products for anticancer drug discovery. However, further research is required to isolate and purify the key bioactive compounds, validate their anticancer properties through in vivo studies, and evaluate their therapeutic potential and safety profiles.

Conflict of Interest
The authors declare no conflict of interest.

Authors' Declaration
The authors hereby declare that the work presented in this article are original and that any liability for claims relating to the content of this article will be borne by them.

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