



Integrative GC–MS, *in silico*, and *in vitro* evaluation of the synergistic anticancer effects of *Holothuria lessoni* extract and low-dose cisplatin on MCF-7 cell line[☆]

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ABSTRACT

Background: Breast cancer is the most prevalent form of cancer affecting women worldwide, and the management is often hindered by chemoresistance, reduced drug responsiveness, and high recurrence rates under standard cisplatin therapy. This study is evaluated the anticancer potential of low-dose cisplatin combined with ethanol extract of *Holothuria lessoni* (HLE), using integrative *in silico* and *in vitro* approaches.

Methods: *Holothuria lessoni* specimens were extracted with ethanol by GC–MS (gas chromatography–mass spectrometry). Putative bioactive compounds were subjected to network pharmacology and molecular docking against AKT1. *In vitro*, cytotoxicity of HLE alone and combined with a fixed sub-IC₅₀ dose of cisplatin (5 μM) was evaluated in MCF-7 breast cancer cells using a resazurin assays. Synergism was assessed by Chou–Talalay analysis.

Results: GC–MS identified 25 bioactive compounds in HLE, predominantly steroids, fatty acid derivatives. Network pharmacology revealed AKT1, MAPK3, and STAT3, as key targets, with Cholest-5-en-3-ol and Ergosta-5,22-dien-3-ol showing high-affinity binding to AKT1. HLE inhibited MCF-7 viability in a dose-dependent manner with an IC₅₀ = 600 μg/mL. Co-treatment with cisplatin 5 μM reduced the IC₅₀ of HLE to 100 μg/mL. Chou–Talalay analysis yielded a combination index (CI) of 0.368 at ED₅₀.

Conclusion: The ethanol extract of *Holothuria lessoni* enhanced cisplatin cytotoxicity in MCF-7 cells through synergistic effects at sub-IC₅₀ concentrations. *In silico* findings indicate that this chemosensitization is primarily mediated by the potential inhibition of AKT1 signaling. These studies support HLE as a promising candidate for combination therapy, though further biochemical validation is required.

1. Introduction

Breast cancer remains the most prevalent malignant tumor affecting women worldwide and continues to exhibit the highest annual incidence, despite advances in early detection and therapeutic strategies [1]. Persistently high morbidity and mortality are primarily driven by

tumor recurrence and resistance to standard chemotherapeutic agents such as cisplatin, which limit overall therapeutic efficacy [2]. At the molecular level, breast cancer is characterized by genetic alterations that disrupt the balance between cell proliferation and survival signaling [3]. In this context, hyperactivation of central node kinases such as AKT1 frequently sustains cell survival, promotes metabolic adaptation,

Abbreviations: ADMET, Absorption, Distribution, Metabolism, Excretion, and Toxicity; AKT1, AKT Serine/Threonine Kinase 1; CASP3, Caspase-3, apoptosis executioner; CASP9, Caspase-9, apoptosis initiator; CI, Combination Index; DMSO, Dimethyl Sulfoxide; ED₅₀, Effective Dose for 50% of population; ER, Estrogen Receptor; GC–MS, Gas Chromatography–Mass Spectrometry; HLE, *Holothuria lessoni* Extract; IC₅₀, Half-maximal Inhibitory Concentration; KEGG, Kyoto Encyclopedia of Genes and Genomes; MAPK3, Mitogen-Activated Protein Kinase 3; MCF-7, Michigan Cancer Foundation-7 human breast cancer cell line; NCBI, National Center for Biotechnology Information; PDB, Protein Data Bank; PPI, Protein–Protein Interaction; QSAR, Quantitative Structure–Activity Relationship; STAT3, Signal Transducer and Activator of Transcription 3; TP53, Tumor Protein p53..

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and directly confers resistance to standard therapeutics. Reduced sensitivity to chemotherapy remains a critical challenge in breast cancer therapy, contributing to diminished treatment outcomes. [4] This inherent drug resistance highlights the need for alternative therapeutic strategies that effectively target chemoresistant breast cancer cells. [5].

Accordingly, current therapeutic approaches increasingly focus on agents that enhance chemosensitivity or target key survival-associated signaling pathways. [6,7]. Among these, AKT1 serves as a pivotal node in the PI3K/AKT/mTOR network, regulating cell cycle progression, metabolic adaptation, and survival signaling that sustain cancer cell viability under chemotherapeutic stress.[8] Cisplatin remains a cornerstone chemotherapeutic agent, exerting cytotoxic effects through DNA cross-link formation that disrupts replication and transcription, ultimately leading to cell cycle arrest and cell death [9]. However, the widely used MCF-7 breast cancer cell line harbors a caspase-3 gene mutation, resulting in impaired caspase-3-dependent apoptotic signaling. Consequently, targeting survival pathways such as AKT signaling has emerged as a promising strategy to disrupt pro-survival mechanisms and restore drug sensitivity in apoptosis-resistant breast cancer models [10–12].

Within this framework, marine-derived natural products have gained attention as potential anticancer agents due to their ability to inhibit proliferation, enhance chemosensitivity and regulate key signaling networks involved in tumor progression [13,14]. However, many natural extracts exhibit limited efficacy as monotherapies and demonstrate improved therapeutic outcomes when combined with conventional chemotherapeutic drugs, enabling enhanced anticancer activity with reduced toxicity [15,16]. Despite evidence supporting the anticancer potential of sea cucumber extracts, their combination with cisplatin remains poorly explored, particularly in apoptosis-deficient models. Therefore, this study evaluated the chemosensitizing potential of combining *Holothuria lessona* extract (HLE) with low-dose cisplatin in MCF-7 breast cancer cells, with a focus on synergistic growth inhibition and potential AKT1 pathway modulation.

2. Method

2.1. Sample collection and extract preparation

The extraction procedure was adapted from the optimized protocol for isolating secondary metabolites from sea cucumber tissues. Specimens of *Holothuria lessona* were collected through daytime diving at Paguyaman Beach, Boalamo, Gorontalo, Indonesia, during October–November 2024. Identification was confirmed based on morphological characteristics and reference to relevant taxonomic keys. *Holothuria lessona* is a species of sea cucumber that is extensively found throughout the Indo-West Pacific region and often inhabits reef flats, coastal lagoons, seagrass beds, and sandflats.

To obtain the sea cucumber extract, the body wall was separated and cut into segments approximately 1 cm in size. The segments were thoroughly dried to reduce residual water content and subsequently subjected to ethanol extraction. The dried material was combined with ethanol at a sample-to-solvent ratio of 1:5 (w/v) to facilitate protein breakdown and ensure efficient extraction of secondary metabolites. The mixture was incubated under constant agitation to maximize extraction efficiency. Subsequently, the ethanolic mixture was concentrated by rotary evaporation at 40 °C (Heidolph Instruments GmbH, Schwalbach, Germany) to remove residual ethanol and obtain a semi-solid crude extract. The concentrate was kept at 4 °C until it was ready for subsequent chemical and biological analyses.

2.2. Seacucumber extract characterization

The analysis was conducted with a GC–MS system (Thermo Fisher Scientific, Chromeleon 7, Version 7.2.10.24543). Chromatographic separation was conducted utilizing an HP-5MS UI capillary column with

30 m length, 0.25 mm internal diameter, as well as 0.25 µm film thickness. The oven temperature was initially set at 50 °C, held for 2 min, then finally increased to 280 °C, with a total run time of 58 min. The injector was operated in split mode with a split flow of 50 mL/min and an inlet temperature of 220 °C. The carrier gas flow rate was maintained at 1.0 mL/min. A 1.1 µL sample was injected using a TriPlus RSH autosampler equipped with a 10 µL syringe and a 57 mm needle. To minimize carryover, both pre- and post-injection wash cycles were performed five times. For mass spectrometric detection, the transfer line and ion source temperatures were maintained at 250 °C and 200 °C, respectively. Electron ionization (EI) was performed in positive ion mode, and full-scan mass spectra were acquired over an *m/z* range of 50–650 with a scan time of 0.2 s. Quantification and identification were conducted using Chromeleon 7 software.

2.3. Cell culture and maintenance

The MCF-7 breast cancer cell line was acquired from the Central Laboratory's Cell BioBank, Universitas Padjadjaran. MCF-7 cells were grown as monolayers in Roswell Park Memorial Institute Medium (Gibco, 11875–093) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich) and 1% penicillin-streptomycin (Sigma-Aldrich, P4333), and maintained in a humidified incubator with 5% CO₂. For subculturing, cells were washed with PBS, detached using Trypsin-EDTA (Gibco, Lot#1297823), and centrifuged at 1000 ×g for 5 min. All procedures were performed under sterile conditions within a biosafety cabinet.

2.4. Cytotoxicity assay

The cytotoxic effects of HLE and cisplatin were evaluated using a resazurin reduction assay. MCF-7 cells were seeded into 96-well plates at 1×10^4 cells/well and allowed to adhere for 24 h before treatment. To determine single-agent cytotoxicity, cells were treated for 24 h with either HLE alone at concentrations ranging from 7.81 to 1000 µg/mL or cisplatin alone at concentrations ranging from 0.78 to 100 µM. Vehicle control (0.1% DMSO) was included in all experiments. To assess potential chemosensitizing effects, combination treatments were performed by exposing cells to HLE at 400, 600, and 800 µg/mL in the presence of a fixed low dose of cisplatin (5 µM), which was selected as a sub-IC₅₀ concentration. Following treatment, resazurin solution was added to each well, and the wells were incubated for 2 h under standard culture conditions. Absorbance was measured at 570 nm with a reference wavelength of 600 nm using a multimode microplate reader (Varioskan LUX, Thermo Fisher Scientific). Cell viability was expressed as a percentage relative to untreated control cells. Dose–response curves were generated and IC₅₀ values were calculated with duplo.

2.5. Chou–talalay drug interaction analysis

Drug interaction analysis was performed using the Chou–Talalay method to quantitatively assess the interaction between HLE and cisplatin. Dose–effect data from single agents treatments and from the non-constant ratio combination of HLE (400, 600, 800 µg/mL) with cisplatin (5 µM) were fitted to the median effect equation:

$$\frac{f_a}{f_u} = \left(\frac{D}{D_m} \right)^m$$

where *f_a* and *f_u* represent the fraction affected and unaffected, respectively, *D* is the dose, *D_m* is the median effect dose (equivalent to IC₅₀), and *m* is the slope of the dose–response curve. [17].

Combination Index (CI) values were calculated at *F_a* = 0.5 across *F_a* = 0.2–0.8 using CompuSyn software (ComboSyn Inc., USA) to evaluate synergy consistency. CI values < 1, = 1, and > 1 indicate synergistic, additive, and antagonistic interactions, respectively. Dose reduction indices (DRI) were also calculated to estimate the extent of dose

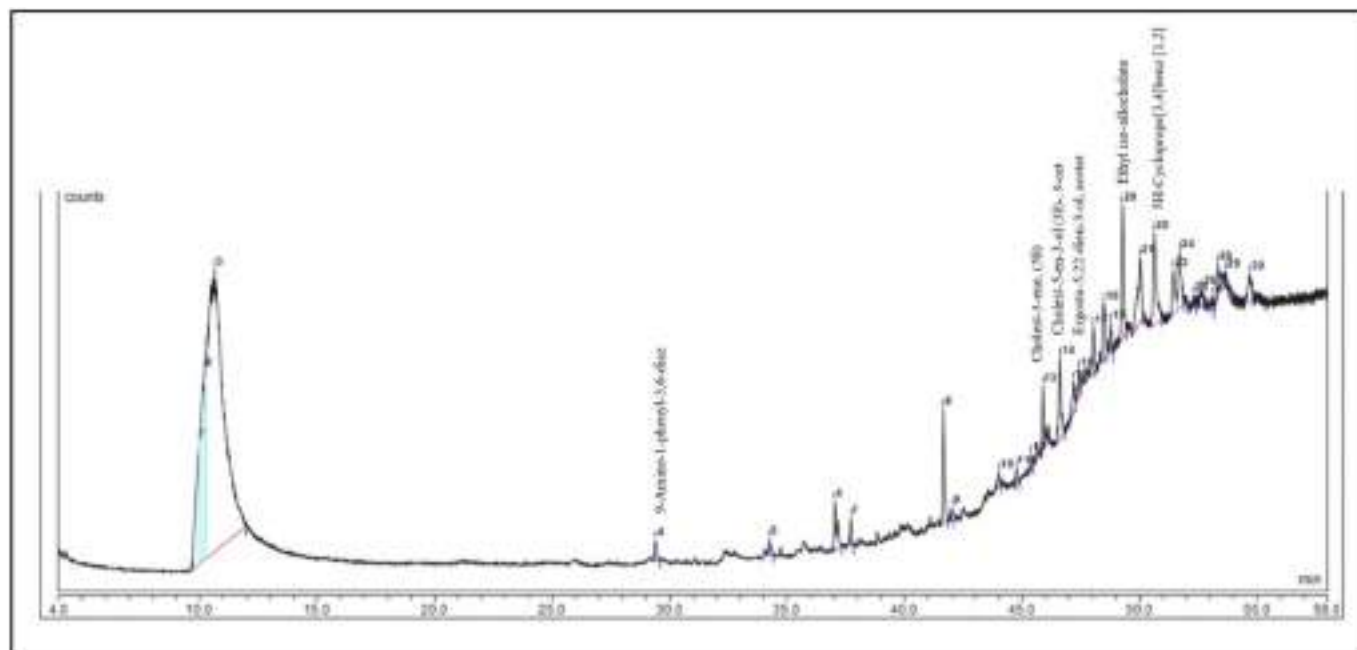


Fig. 1. Representative chromatogram of secondary metabolites detected in *Holothuria lessoni* Extract (HLE). Several major peaks are annotated with identified compounds, including Methyl-8,11,14-heptadecatrienoate, Cholestane-3,5,6-triol, and 3β-Sitosteryl-β-D-glucoside. Peaks corresponding to plasticizers and other contaminants were excluded from further analysis.

reduction achievable for each agent in the combination. 95% confidence intervals for CI values were determined from three independent biological replicates.

2.6. Screening of compounds for drug-likeness

Prediction of drug-likeness and pharmacokinetic properties, including ADMET, for selected compounds was performed using the SWISS-ADME and pkCSM web servers. The SMILES codes for the chosen secondary metabolites were retrieved from PubChem as well as entered into the online tools SWISS-ADME. Parameters such as the Lipinski, HIA, PPB, H-HT, DILI, and FDAMDD were considered.

2.7. Quantitative structure-activity relationship (QSAR) analysis

The analysis of bioactive compounds in *Holothuria lessoni* for anticancer potential was conducted using the WAY2DRUG PASS prediction system. This tool evaluates compounds by comparing the structural features to those of compounds with known properties through Structure-Activity Relationship (SAR) analysis. Predictions of the system become more reliable when there are stronger structural similarities between the analyzed compounds. The method is founded on the principle that compounds with similar structures may show identical properties. The system produces a Pa value (Probability to be Active) that signifies the tendency of a compound to have specific effects, with higher Pa values suggesting greater confidence in the accuracy of the prediction.

2.8. Prediction of protein targets and network analysis

The SEA Search Server and the Comparative Toxicogenomics Database were used to analyze protein targets (Wiegiers et al., 2025). Furthermore, the DisGeNET database and Open Targets were used to find proteins and genes related to breast cancer, specifically ER-positive breast cancer [18]. Overlapping genes between the predicted targets and gene-disease associations (GDAs) were identified [19]. Protein-protein interaction (PPI) networks were then constructed and analyzed using

the the STRING server. Several main features, such as stress, degree, betweenness centrality, and closeness centrality, were assessed for network analysis [20].

2.9. Molecular docking

The 3D structure of the chosen protein target, AKT1 (PDB ID: 4GV1), was downloaded from the RCSB PDB database (<https://www.rcsb.org/>). The control inhibitor Capivasertib (CID: 50985634) [21] was obtained from PubChem (<https://www.pubchem.ncbi.nlm.nih.gov>). Protein preparation involved removing water molecules in BIOVIA Discovery Studio 2025, while the ligand was subjected to energy minimization using PyRx v.1.1. Docking was carried out with AutoDock Vina as implemented in PyRx v.1.1 [22] using targeted docking and an exhaustiveness value of 200. The grid box was positioned at the control ligand's PDB coordinates (center xyz: -21.157, 6.4124, 11.461) with dimensions of 15 × 15 × 15 Å. Docking outputs were reported as binding affinity (interaction energy) between each compound and the protein. Final ligand-protein interactions were visualized in BIOVIA Discovery Studio 25.

2.10. Statistical data analysis

All experiments were performed in three independent biological replicates with triplicate wells. Dose-response curves and IC₅₀ values were determined by nonlinear regression using a four-parameter logistic model in GraphPad Prism v9.0. Synergistic interactions between HLE and cisplatin were evaluated using the Chou-Talalay method, with combination index (CI) and dose reduction index (DRI) values calculated using CompuSyn v1.0.

3. Result

Profiling and Identifications of Secondary Metabolites using GC-MS. GC-MS analysis of the ethanol extract of *Holothuria lessoni* (HLE) revealed 25 distinct compounds with SMILES data available in the PubChem database. Compound identification was based on retention

Table 1
Data mining of secondary metabolites from HLE.

No	Compound	CID
1	7,8-Epoxylostan-11-ol, 3-acetoxy-	541,562
2	Ergosta-5,22-dien-3-ol, aceta	13,889,457
3	Ethyl isoallocholate	6,452,096
4	Glycerin	753
5	Cholest-2-ene	15,557,412
6	Cholest-3-ene, (5 β)-	11,810,787
7	Cholest-5-en-3-ol (3 β)-, 9-oct	304
8	Cholesterol margarate	14,077,841
9	cis-11-Eicosenoic acid, methyl ester	5,463,047
10	cis-13-Eicosenoic acid	5,312,518
11	10-Octadecenoic acid, methyl ester	25,642
12	11-Octadecenoic acid, meth	5,364,432
13	9-Octadecenoic acid, (2-phen	5,366,356
14	16-Octadecenoic acid, methyl	5,364,513
15	Stigmast-5-en-3-ol, oleate	20,831,071
16	6,9,12,15-Docosatetraenoic aci	5,362,672
17	Arachidonic acid	444,899
18	1-Hydroxy-2-(2,3,4,6-tetra-	632,688
19	3',8,8'-Trimethoxy-3-piperidyl	590,815
20	5,8,11,14-Eicosatetraenoic acid, methyl ester, (all-Z)-	6,421,258
21	Cyclopenta[g]-2-benzopyran, 1,3,4,6,7,8-hexahydro-4,6,6,7,8,	91,497
22	Epistephamsine	91,895,297
23	9,10-Secosterolesta-5,7,10(19)-t	5,372,266
24	9-Amino-1-phenyl-3,6-diaz	620,561
25	Methyl 9-eicosenoate	13,908,975

time (RT), mass spectra matching against standard reference libraries, peak intensity, and molecular formula (MF). Relative abundance was determined from peak area percentage. Peaks corresponding to

phthalates and siloxanes were excluded from downstream analysis.

Representative chromatogram is shown in Fig. 1. Several major peaks were annotated, Key bioactive constituents identified included steroids such as cholest-5-en-3 β -ol and cholestane-3,5,6-triol, fatty acid esters such as cis-11-eicosenoic acid methyl ester and Methyl-8,11,14-heptadecatrienoate, and phytosterols such as stigmast-5-en-3-ol oleate and 3 β -Sitosteryl- β -D-glucoside. Eight additional compounds identified as process-related contaminants or plant-derived markers were excluded from downstream analyses. (See Table 1.)

The identified compounds comprised steroids, fatty acid esters, phthalates, glycosides, terpenoids, and heterocyclic compounds. Detailed compound information is provided in Supplementary Table 1.

3.1. Cytotoxicity of HLE and cisplatin on MCF-7 breast cancer cells

The cytotoxic effects of *Holothuria lessoni* extract and cisplatin on MCF-7 human breast cancer cells were evaluated using a resazurin reduction assay after 24 h of treatment. HLE alone exhibited moderate cytotoxic activity, with an IC₅₀ value of 600 μ g/mL. Cisplatin showed stronger potency with an IC₅₀ value of 34.30 μ M. Dose-response curves were generated by nonlinear regression analysis in Fig. 2A and C.

To evaluate potential synergy, cells were treated with HLE (400–800 μ g/mL) combined with a fixed, sub-toxic concentration of cisplatin (5 μ M). The presence of cisplatin shifted the dose response curve of HLE to the left (Fig. 2B), reducing IC₅₀ to 100 μ g/mL, compared to HLE alone.

3.2. Chou–talalay drug interaction analysis

The combination of HLE and cisplatin exhibited strong synergistic

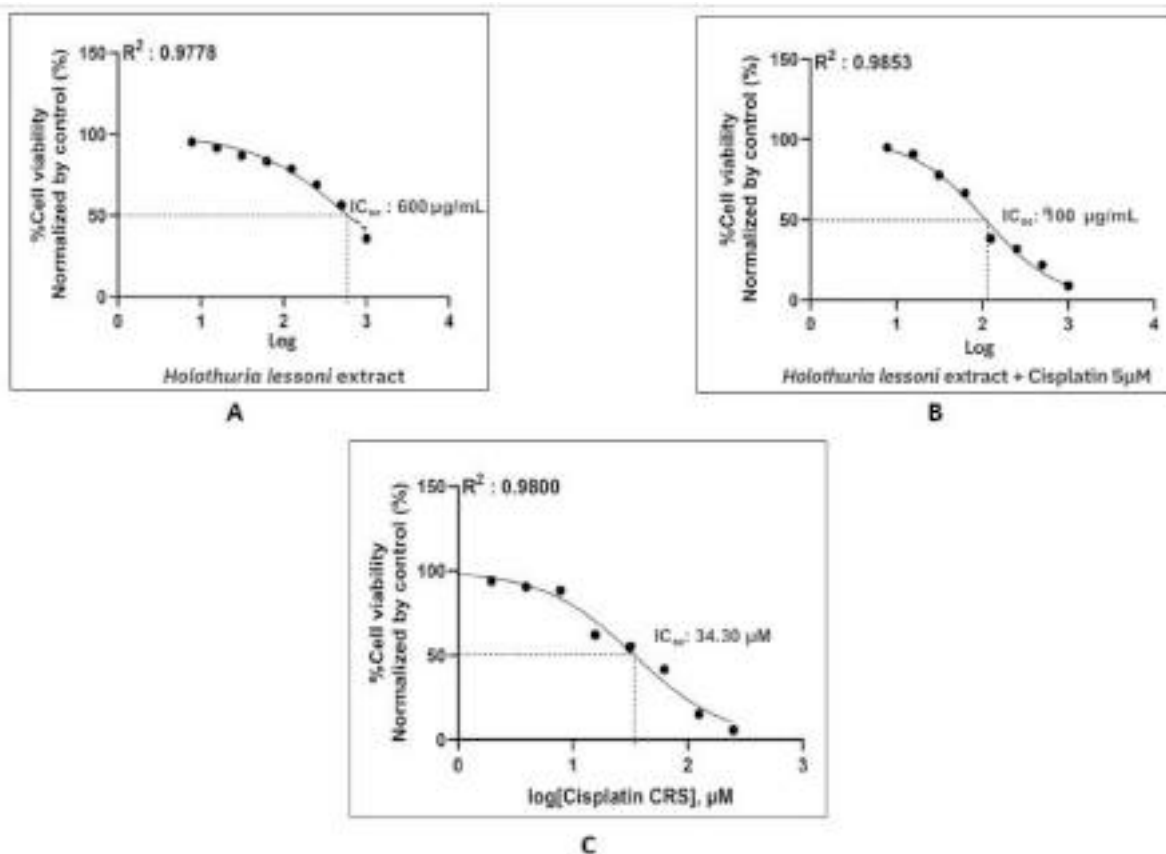


Fig. 2. Dose–response curves of *Holothuria lessoni* extract, cisplatin, and their combination in MCF-7 cells. (A) Cytotoxic effect of *Holothuria lessoni* extract alone (IC₅₀ = 600 μ g/mL). (B) Cytotoxic effect of *Holothuria lessoni* extract in combination with cisplatin 5 μ M (IC₅₀ 100 μ g/mL). HLE concentrations used were 400, 600, 800 μ g/mL (C) Cytotoxic effect of cisplatin alone (IC₅₀ = 34.30 μ M). Cell viability was determined using a resazurin assay after 24 h of treatment and expressed as a percentage of untreated controls.

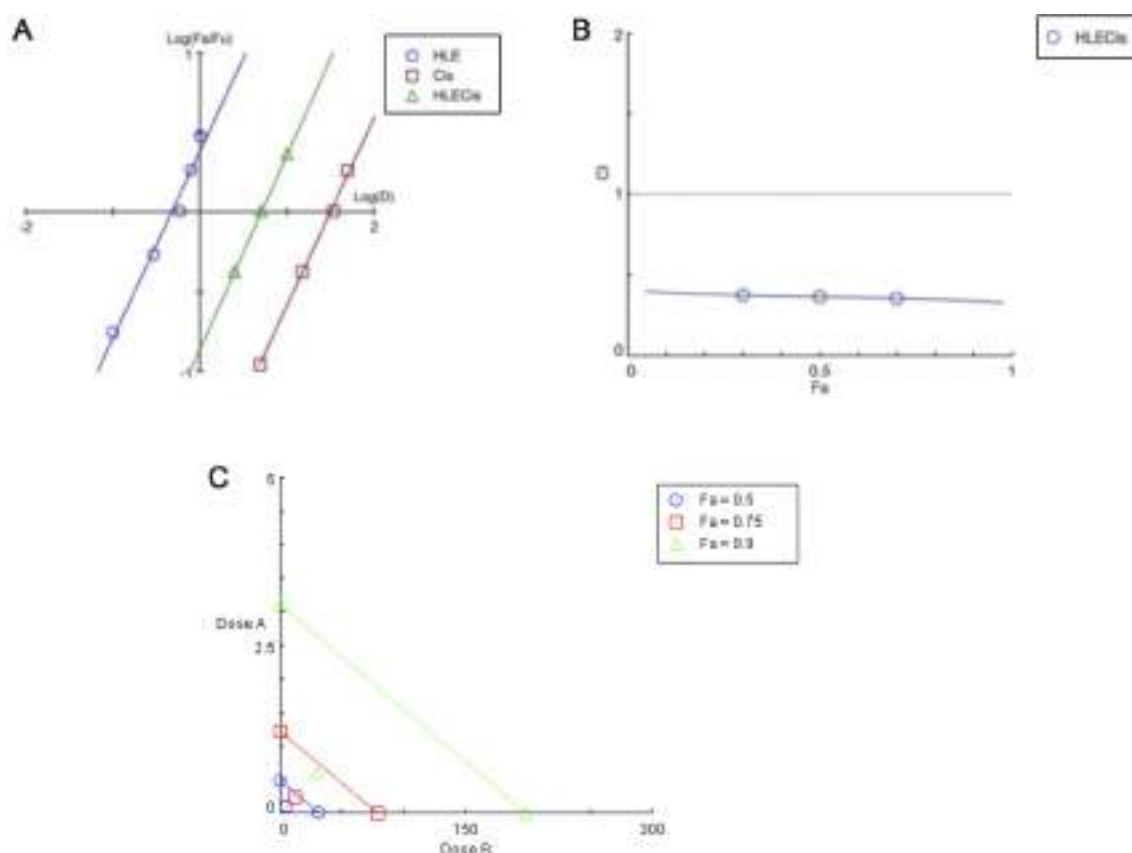


Fig. 3. Chou-Talalay synergistic analysis of HLE-Cisplatin combination in MCF-7 cells. (A) Median-effect plots of HLE, cisplatin, and their combination at 1:50 ratio. The high linear correlation coefficients ($r > 0.98$) validate the median-effect principle. (B) Combination index plot showing CI values < 1 across all Fa levels, with $CI = 0.368$ at $Fa = 0.5$, indicating strong synergism. The dashed line at $CI = 1$ represents additivity. (C) Normalized isobologram at $Fa = 0.5$. The diagonal line represents the line of additivity; the combination data point falls below this line, confirming synergism. All analyses were performed using CompuSyn software.

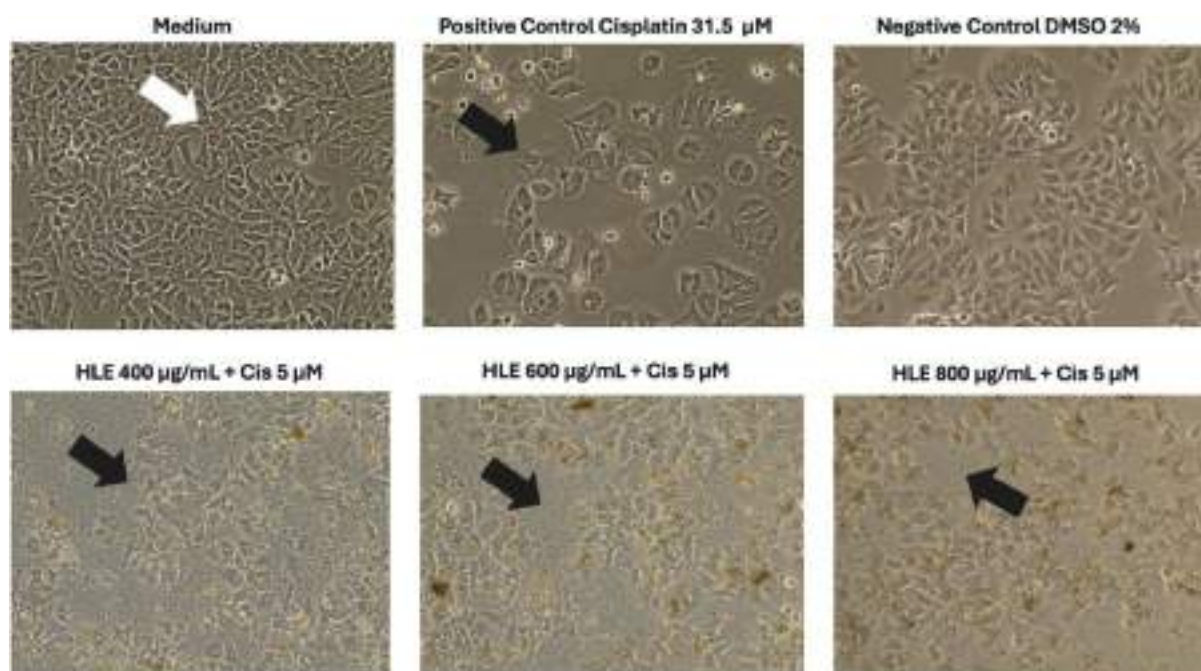


Fig. 4. Representative phase contrast images of MCF-7 cells following treatment with *Holothuria lessoni* extract (HLE) in the presence of cisplatin (5 μM). Images show untreated control (Media + Cell), cisplatin alone (31.5 μM), DMSO vehicle, and HLE-treated wells at the indicated concentrations of 400, 600, and 800 $\mu\text{g/mL}$ in combination with cisplatin 5 μM . White arrows indicate viable cells characterized by intact cell bodies, visible nuclei, and preserved intercellular junctions; black arrows indicate detached cells exhibiting apoptotic-like morphological features, such as cell shrinkage, loss of visible nuclei, and disruption of cell junctions.

Table 2
Screening of compounds for drug-likeness.

No.	Compound	Lipinski	HIA	PPB	H-HT	DILI	FDAMDD
1	7,8-Epoxylostan-11-ol, 3-acetoxy-	Rejected	0.006	99%	0.292	0.047	0.771
2	Ergosta-5,22-dien-3-ol, aceta	Accepted	0.005	87%	0.304	0.858	0.929
3	Ethyl isoallocholate	Accepted	0.003	79%	0.425	0.044	0.887
4	Glycerin	Accepted	0.66	9%	0.051	0.027	0.003
5	Cholest-2-ene	Accepted	0.002	96%	0.065	0.736	0.041
6	Cholest-3-ene, (5 β)-	Accepted	0.002	95%	0.077	0.747	0.036
7	Cholest-5-en-3-ol (3 β)-, 9-oct	Accepted	0.003	98%	0.235	0.19	0.676
8	Cholesterol margarate	Rejected	0.002	97%	0.191	0.878	0.407
9	cis-11-Eicosenoic acid, methyl ester	Accepted	0.021	99%	0.043	0.077	0.031
10	cis-13-Eicosenoic acid	Accepted	0.043	100%	0.045	0.018	0.024
11	10-Octadecenoic acid, methyl ester	Accepted	0.004	97%	0.018	0.056	0.017
12	11-Octadecenoic acid, meth	Accepted	0.008	99%	0.039	0.105	0.04
13	9-Octadecenoic acid, (2-phen	Accepted	0.003	100%	0.051	0.029	0.062
14	16-Octadecenoic acid, methyl	Accepted	0.011	99%	0.041	0.211	0.069
15	Stigmast-5-en-3-ol, oleate	Rejected	0.003	101%	0.215	0.644	0.23
16	6,9,12,15-Docosatetraenoic aci	Accepted	0.128	100%	0.951	0.009	0.974
17	Arachidonic acid	Accepted	0.033	100%	0.251	0.006	0.349
18	1-Hydroxy-2-(2,3,4,6-tetra-	Rejected	0.995	68%	0.027	0.87	0.105
19	3',8'-Trimethoxy-3-piperidyl	Accepted	0.006	92%	0.651	0.959	0.843
20	5,8,11,14-Eicosatetraenoic acid, methyl ester, (all-Z)-	Accepted	0.037	99%	0.32	0.014	0.444
21	Cyclopenta[g]-2-benzopyran, 1,3,4,6,7,8-hexahydro-4,6,6,7,8,	Accepted	0.029	97%	0.073	0.036	0.939
22	Epistephamsine	Accepted	0.04	42%	0.552	0.135	0.877
23	9,10-Secocholesta-5,7,10(19)-t	Accepted	0.005	91%	0.207	0.021	0.92
24	a-D-Galactopyranose, 6-O	Accepted	0.868	12%	0.043	0.041	0.005
25	Methyl 9-eicosenoate	Accepted	0.021	99%	0.043	0.077	0.031

cytotoxicity against MCF-7 cells. The median-effect doses (D_m) for HLE and cisplatin alone were 0.477 mg/mL and 31.55 μ M, with linear correlation coefficients ($r > 0.98$), validating conformity to the mass-action law (Fig. 3A). The combination index (CI) values were below 1 across all effect levels, with a CI of 0.368 at ED_{50} , indicating strong synergistic activity (Fig. 3B). The synergistic interaction was further confirmed by the normalized isobologram at $F_a = 0.5$ (Fig. 3C). Favorable dose reduction was achieved, with DRI values of 4.77–5.16 for HLE and 6.31–6.62 for cisplatin at ED_{50} .

3.3. Morphological alterations following treatment with HLE in breast cancer MCF-7 cell line

Untreated MCF-7 cells exhibited a confluent monolayer with typical epithelial morphology, as shown in Fig. 4. The morphology of cells treated with DMSO was indistinguishable from that of the medium-only control, indicating that DMSO at 2% did not induce cytotoxicity.

In contrast, treatment with *Holothuria lessoni* ethanol extract (HLE) in combination with cisplatin (5 μ M) resulted in concentration-dependent morphological changes. At 400 μ g/mL HLE, cells displayed reduced cell-to-matrix adhesion and initial rounding. At 800 μ g/mL HLE, these alterations were more pronounced, characterized by extensive cell rounding, loss of typical epithelial shape, membrane integrity alterations, and cell detachment, accompanied by a marked decrease in adherent cell number. Cisplatin alone (31.5 μ M), used as a positive control, induced similar morphological features. The specific mode of cell death cannot be determined by morphology alone and requires further biochemical validation.

3.4. Screening of compounds for drug-likeness

The analysis showed that 4 out of 25 bioactive compounds from HLE did not meet drug-likeness criteria as defined by Lipinski's rule of five. Lipinski's rule of five states that an orally active drug should have: [21] molecular weight < 500 Da, [23] $\text{LogP} < 5$, [24] ≤ 5 hydrogen bond donors, and [5] ≤ 10 hydrogen bond acceptors [25]. ADMET analysis suggested that nearly all compounds had good pharmacodynamics and pharmacokinetics (Table 2). Some compounds showed potential toxicity to the liver and tended to be carcinogenic when present as single agents. (See Table 3.)

Table 3

Docking scores of major bioactive compounds identified from HLE and reference compound Capivasertib against the AKT1 protein target.

No.	Ligand	Binding affinity (kcal/mol)
1	Capivasertib	−8.6
2	Cholest-5-en-3-ol (3 β)-, 9-oct	−8
3	Cholest-3-ene, (5 β)-	−7.9
4	Ergosta-5,22-dien-3-ol, aceta	−7.9
5	9-Amino-1-phenyl-3,6-diaz	−7.6
6	Ethyl isoallocholate	−7.5
7	7,8-Epoxylostan-11-ol, 3-acetoxy	−7.5
8	9-Octadecenoic acid, (2-phen	−6.7
9	Epistephamsine	−6.3
10	6,9,12,15-Docosatetraenoic acid	−6.3
11	11-Octadecenoic acid, meth	−5.7

3.5. In Silico study

QSAR-based bioactivity analysis was carried out on compounds that passed the drug-likeness assessment. The analysis showed that several bioactive compounds had high predicted activity, as presented in Fig. 5. A Hierarchical clustering heatmap analysis revealed distinct bioactivity patterns among nine secondary metabolites from HLE. The compounds were clustered based on their predicted activities against five functional groups: Anti-inflammatory, Antineoplastic, Antineoplastic_breast, Proliferative_diseases, and TNF_inhibitor.

Cholest-5-en-3 β -ol and 3 β -Acetoxy-7,8-epoxylostan-11 α -ol exhibited the highest predicted activity scores across multiple groups, particularly for Antineoplastic and Antineoplastic_breast activities. Ethyl 3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-oate showed moderate to high activity for Antiinflammatory and TNF_inhibitor groups. In contrast, 9-Amino-1-phenyl-3,6-diazahomoadamantane and (6Z,9Z,12Z,15Z)-Docosa-6,9,12,15-tetraenoic acid displayed low predicted activities across all groups.

A target proteins were identified as potential key targets from the intersection of *Holothuria lessoni* metabolite targets, GDA targets, and breast cancer-related genes. PPI network analysis using the STRING database revealed that 12 proteins participated in the interaction network, whereas PIK3AP1 showed no interactions and was excluded from further topological analysis. Based on centrality parameters, AKT1,

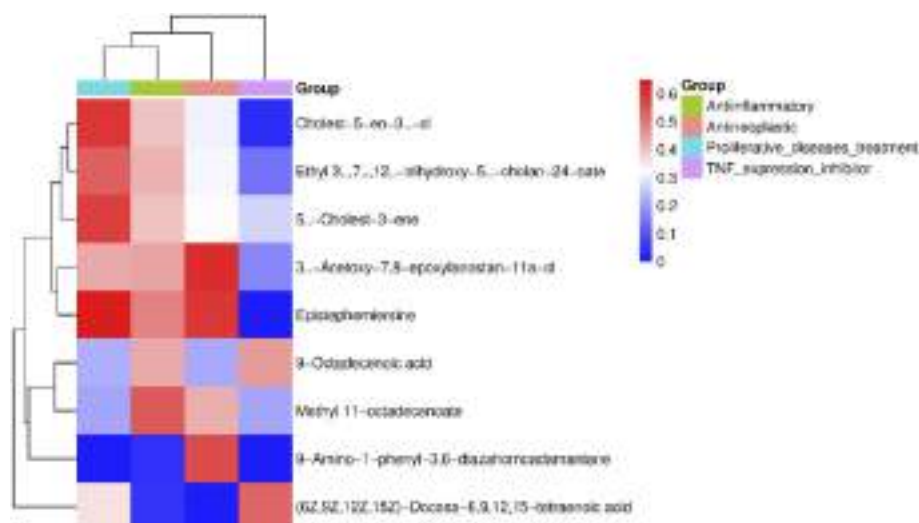


Fig. 5. Heatmap of predicted bioactivities for secondary metabolites from *Holothuria lessoni* extract (HLE). Hierarchical clustering was performed to group metabolites (rows) based on their predicted activity profiles against five pharmacological categories (columns). The color gradient represents the predicted activity score ranging from 0 (low activity, blue) to 1 (high activity, red). Group annotations: Antiinflammatory (orange), Antineoplastic (pink), Antineoplastic_breast (green), Proliferative_diseases_treatment (light blue), TNF_inhibitor (purple). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

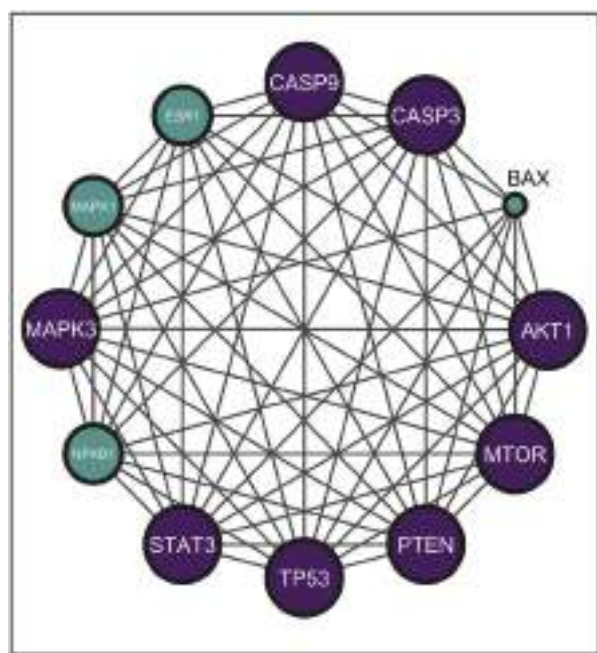


Fig. 6. Protein-protein interaction (PPI) network of *Holothuria lessoni* metabolites in breast cancer. The network was constructed using the STRING database. Node size and color intensity correspond to degree centrality, where larger, purple nodes indicate higher connectivity and topological importance. Edges represent predicted functional associations between proteins. AKT1, MAPK3, STAT3, TP53, CASP3, CASP9, MTOR, and PTEN were identified as hub proteins based on degree, betweenness, and closeness centrality analyses. PIK3AP1 was excluded due to lack of interactions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

MAPK3, STAT3, TP53, and CASP3 were identified as hub proteins, exhibiting the highest degree, betweenness centrality, and closeness centrality values. CASP9, MTOR, and PTEN shared identical topological values with the five hub proteins. The node size and color intensity in the STRING network reflect degree centrality, with larger, darker nodes

representing proteins with higher connectivity. These results are summarized in Fig. 6. (See Figs. 7 and 8.)

Gene ontology (GO) analysis and KEGG enrichment analyses linked the identified target proteins to key biological processes and signaling pathways involved in cell survival, apoptosis, and immune response, which are hallmarks of cancer [26]. Enrichment analysis indicated that *Holothuria lessoni* metabolites potentially modulate these signaling hubs, suggesting their therapeutic relevance for combination with conventional chemotherapies, including cisplatin (Fig. 4A).

Network pharmacology analysis of the 12 interacting proteins identified 8 hub proteins based on degree centrality: AKT1, MAPK3, STAT3, TP53, CASP3, CASP9, MTOR, and PTEN. These proteins represent key nodes in breast cancer progression and serve as potential therapeutic targets. Several of these hub proteins, including AKT1 and MTOR, are well-established regulators of cancer cell survival, proliferation, and apoptosis.[27] Notably, AKT1 was identified as a dominant node, supporting its role as a critical effector in oncogenic signaling and highlighting its potential as a target for novel anticancer agents (Fig. 4B).

3.6. Molecular docking analysis

Docking studies were conducted with AutoDock Vina integrated in PyRx v1.1, using a targeted docking method including exhaustive search parameters and 200 modes. Binding affinity values obtained from the docking simulations showed that several *Holothuria lessoni* -secondary metabolite compounds had substantial interaction with the AKT1 protein, which was considered critical in breast cancer pathogenesis.[3] A total of three secondary metabolites, Cholest-5-en-3-ol, Ergosta-5,22-dien-3-ol and 9-Octadecenoic acid, consistently ranked in the top 10 based on affinities for AKT1, with the best compound showing a binding energy of -8.0 kcal/mol, close to the reference inhibitor Capivasertib (-8.6 kcal/mol). The top nine compounds still had binding affinities ≤ -7.0 kcal/mol, a threshold representing stable and meaningful ligand-protein interactions. Detailed interaction analyses showed that the dominant forces stabilizing the ligand-AKT1 complexes were van der Waals interactions, hydrogen bonds, as well as hydrophobic contacts. These bindings predominantly included key AKT1 active site residues, such as VAL164, MET281, ALA177, and LEU156. The results support a favorable binding environment for the major secondary

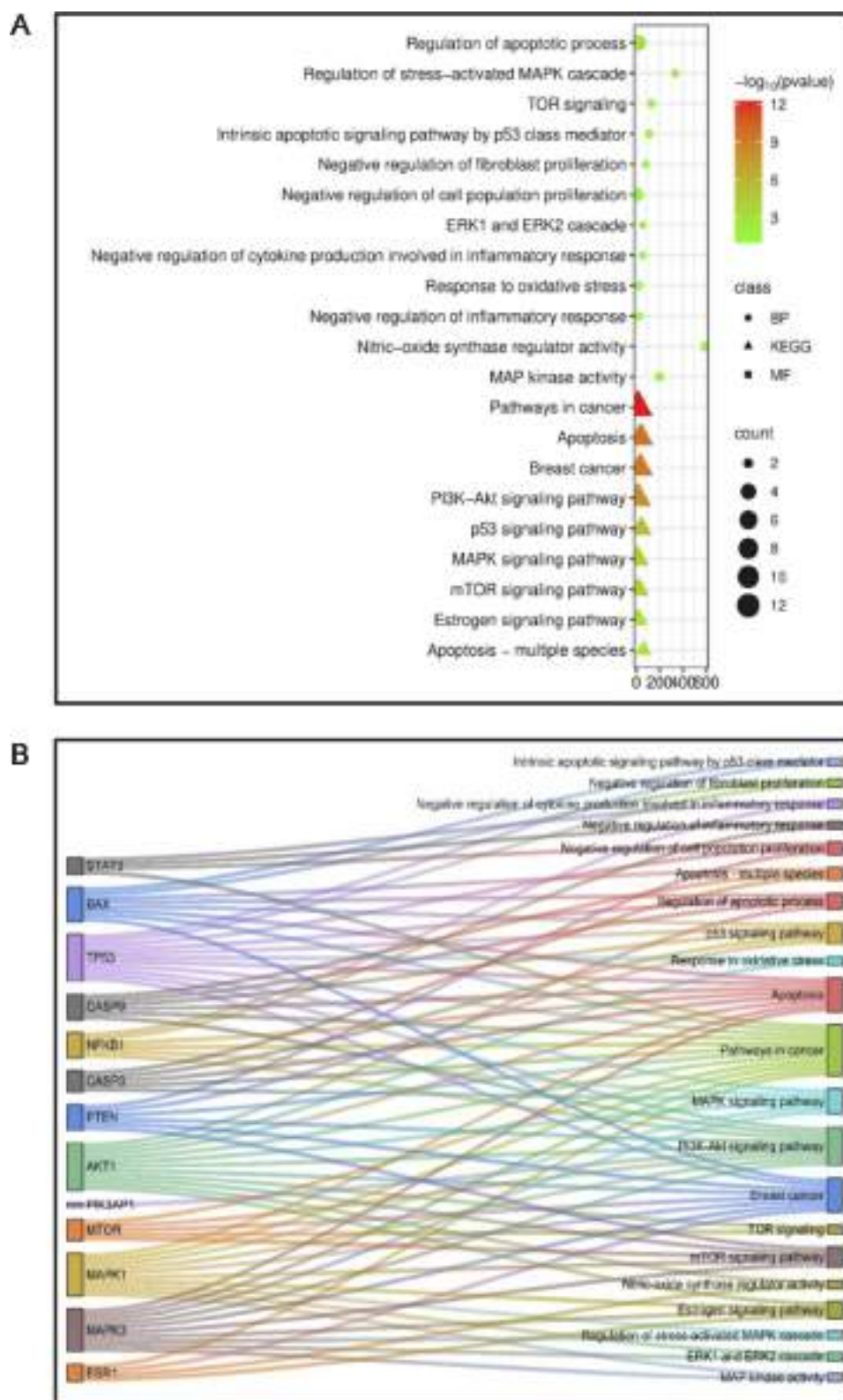


Fig. 7. Functional annotation and pathway analysis of breast cancer-related targets of *Holothuria lessona* metabolites. (A) GO and KEGG pathway enrichment analysis showing the top biological processes, molecular functions, and signaling pathways associated with the 12 target proteins. Dot size represents gene count, and color indicates adjusted *P*-value. (B) Chord diagram illustrating the gene-pathway associations, where the width of each chord corresponds to the number of genes involved in a specific pathway.

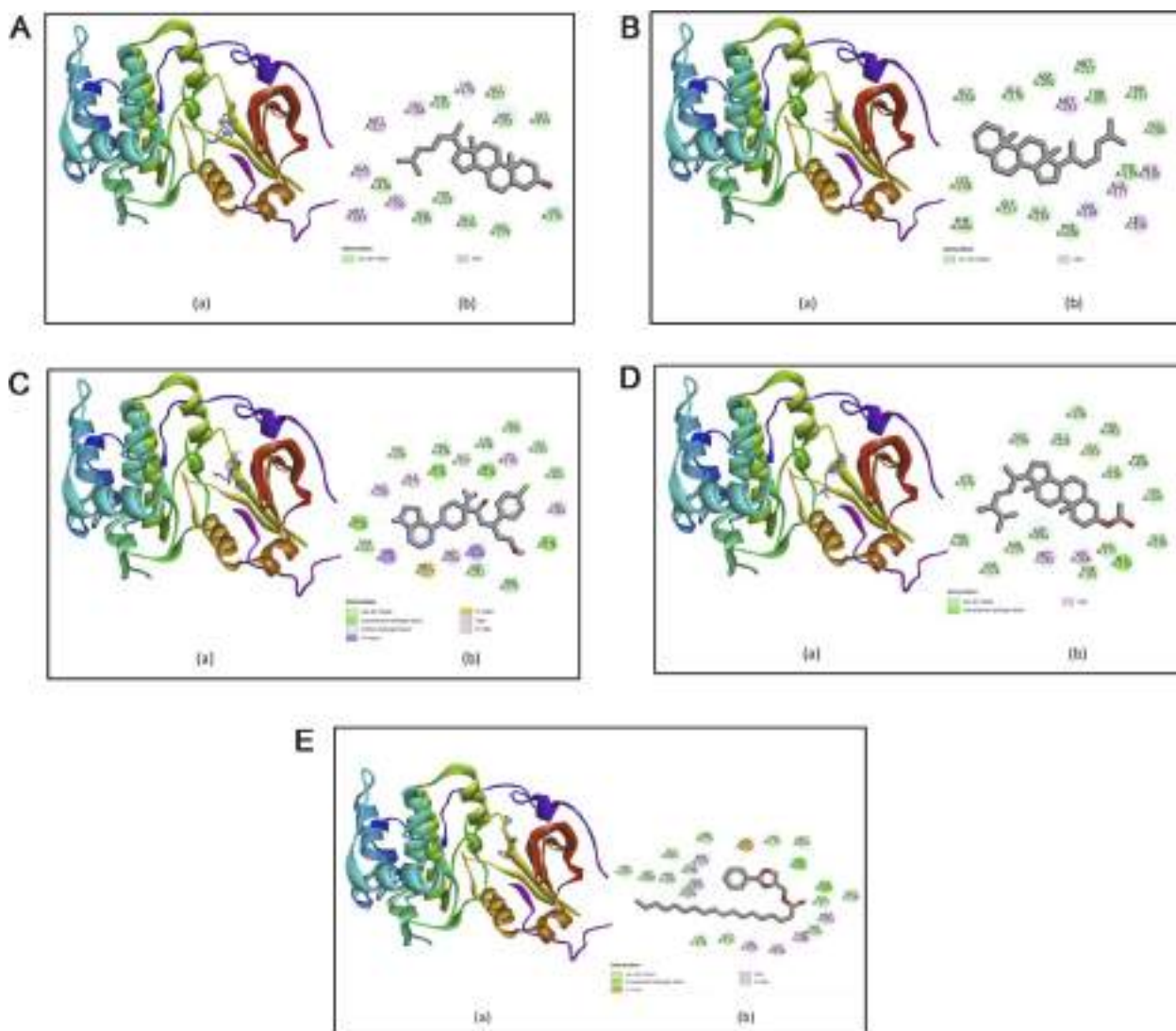


Fig. 8. Molecular docking interactions of selected *Holothuria lessoni*-derived compounds with AKT1 protein. (A) AKT1/Capivasertib complex as positive control. (B) AKT1/Cholest-5-en-3-ol complex. (C) AKT1/Cholest-3-ene complex. (D) AKT1/Ergosta-5,22-dien-3-ol complex. (E) AKT1/9-Octadecenoic acid complex. For each panel, (a) shows the 3D binding pose of the ligand in the active site of AKT1, and (b) shows the 2D interaction diagram depicting hydrogen bonds, hydrophobic interactions, and key amino acid residues. Molecular docking was performed using AutoDock Vina. Binding affinity values are provided in Table 4.

metabolites, signifying the potential as lead candidates for further anticancer development.

The interactions formed between the target protein and each of the control and sample ligands were mainly van der Waals forces, hydrogen bonds, as well as hydrophobic interactions.

4. Discussion

This study evaluated whether a marine-derived secondary metabolite extract can modulate the response of MCF-7 cells to a conventional chemotherapeutic agent. The investigation focused on the potential chemosensitizing effects of an ethanolic extract of *Holothuria lessoni* (HLE) in combination with low-dose cisplatin, rather than on defining a specific cytotoxic mechanism. This approach is appropriate for multi-component natural extracts, whose bioactivities often arise from combined or context-dependent interactions rather than from selective, high-affinity engagement of a single molecular target [28]. Our findings suggest a potential mechanism wherein HLE functions as a chemosensitizer associated with AKT-related signaling. Although direct molecular target engagement remains to be experimentally validated, integration of chemical, computational, and pharmacologic data

provides a coherent and testable framework for future investigations.

GC–MS profiling revealed that HLE is a chemically diverse mixture dominated by steroids, fatty acid esters, and terpenoid compounds [23,29]. This chemical profile differs from classical DNA-reactive anti-cancer agents and aligns more closely with bioactive molecules known to interact with lipid environments or membrane-associated proteins [30]. The predominance of hydrophobic and amphiphilic constituents in HLE suggests these constituents are more likely to influence membrane-associated signaling or enzyme localization than to act as potent, selective inhibitors of constitutively active enzymes.

Computational analyses further contextualized these chemical features within a biological medicine framework and indicated that several HLE constituents are associated with anticancer activity. Network pharmacology linked HLE compounds to proteins implicated in breast cancer-relevant pathways, including AKT1, MAPK3, STAT3, and TP53 [8,31]. These results reflect pathway-level connectivity. Molecular docking analysis indicated that the selected sterol constituents can adopt favorable binding poses within the ATP-binding site of AKT1, with calculated binding energies ranging from -7.2 to -9.1 kcal/mol, comparable to those observed for known AKT1 allosteric inhibitors [32]. Based on network pharmacology and molecular docking analyses, the

lipid-active constituents of HLE are predicted to modulate membrane properties and cellular signaling dynamics, potentially influencing downstream AKT-mediated survival pathways through the PI3K/AKT/mTOR signaling axis [13,33]. This proposed mechanism requires experimental validation at the protein level before definitive conclusions can be drawn.

Consistent with this interpretation, cell-based assays demonstrated that HLE enhanced cisplatin cytotoxicity in MCF-7 cells when cisplatin was applied at sub-IC₅₀ concentrations. The IC₅₀ of HLE alone (600 µg/mL) is indeed relatively high compared with conventional chemotherapeutics, indicating that HLE is not a potent cytotoxic agent. However, the primary finding of this study is its role as a chemosensitizer; co-treatment with sub-toxic cisplatin (5 µM) reduced the IC₅₀ of HLE by 6-fold to 100 µg/mL. This highlights that the therapeutic value of HLE lies in combination therapy to enable cisplatin dose reduction, rather than as a stand-alone treatment [9]. Chou-Talalay analysis indicated synergistic interactions, with a combination index (CI) of 0.368 at ED₅₀ [34]. HLE alone produced dose-dependent growth inhibition, yet its primary role appears to be as a modulator, increasing cellular susceptibility to cisplatin rather than acting as a potent independent cytotoxin (Lin et al., 2022). Minor discrepancies between values derived from Chou-Talalay analysis and those extracted from direct IC₅₀ curve fitting likely reflect differences in how data points are utilized. From a strictly preclinical perspective, combining HLE may lower the effective dose of cisplatin in vitro, which could theoretically contribute to mitigating dose-limiting toxicities such as nephrotoxicity in future translational settings. Similar modulatory effects have been reported for other natural product chemosensitizers, where membrane-associated sterols can lower chemotherapeutic thresholds in vitro [24] [35].

This study was based on chemical profiling, computational analyses, and cell-based viability assays, without direct biochemical validation of pathway modulation. Although in silico analyses point to a potential suppression of the AKT1 pathway, the specific programmed cell death cascades induced by this combination remain unconfirmed. Accordingly, the findings should be regarded as preliminary preclinical in vitro evidence, providing a testable framework for future mechanistic and translational studies.

5. Conclusion

This study indicates that the ethanol extract of *Holothuria lessona* modestly enhances cisplatin efficacy in ER-positive MCF-7 breast cancer cells. Cell-based assays confirmed a synergistic interaction at sub-IC₅₀ cisplatin concentrations, supporting a chemosensitizing role. In silico analyses suggest that this effect may be linked to the potential modulation of the AKT1 signaling pathway. These preliminary findings provide preclinical in vitro evidence, and further mechanistic validation is required before translational application.

CRediT authorship contribution statement

Supandi: Methodology, Investigation, Funding acquisition. **Irena Ujianti:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization. **Tahyatul Bariroh:** Formal analysis, Data curation.

Ethics declaration

The author(s) declare(s) that the study does not involve humans nor animal subjects.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.rechem.2026.103751>.

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