

Anticancer Activity of *Neophrissospongia endoumensis* Fraction: Mechanisms of Apoptosis and Migration Suppression in Breast Cancer Cells

Mostafa Khaledi

Food and Drug Research Center, Food and Drug Administration, Ministry of Health and Medical Education

Ardeshtir Abbasi

Tarbiat Modares University

Ensieh Ghasemi

Islamic Azad University, Tehran

Melika Nazemi

Iranian Fisheries Sciences Research Institute (AREEO)

Bahman Moradipoodeh

Islamic Azad University, Lahijan Branch

Mohammad Javad Hessami

UCSI University

Etim Paul Effiong

effiong@ucsiuniversity.edu.my

UCSI University

Lionel Lian Aun In

UCSI University

Research Article

Keywords: Marine sponge, Breast cancer, Apoptosis, Cytotoxicity, Anticancer

Posted Date: September 21st, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-10969001/v1>

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Additional Declarations: No competing interests reported.

Abstract

Background

Breast cancer remains a leading cause of cancer-related death, and marine natural products are a promising source of anticancer compounds. This study evaluated the cytotoxic effects of methanol, ethyl acetate and hexane extracts of the marine sponge *Neophrissospongia endoumensis* on breast cancer cells and explored their mechanisms of action.

Methods and Results

Cytotoxicity was assessed by MTT assay over 48 h and IC₅₀ values were determined. Apoptosis was evaluated by fluorescence microscopy, and Bax, Bcl-2, VEGF, MMP2 and MMP9 expression by qRT-PCR. Cell migration (wound healing), oxidative stress (ROS) and cell-cycle distribution were also measured. All three extracts showed dose-dependent cytotoxicity; the ethyl acetate extract was the most active crude extract (IC₅₀ = 238 µg/mL) and its hexane fraction was the most potent overall (IC₅₀ = 200 µg/mL). The hexane fraction induced the strongest apoptosis, increased Bax and decreased Bcl-2, and reduced VEGF, MMP2 and MMP9, indicating anti-angiogenic and anti-metastatic activity. It also inhibited migration, elevated ROS and caused G2/M cell-cycle arrest. Together, these effects are consistent with suppression of breast cancer cell proliferation.

Conclusions

N. endoumensis extracts, particularly the hexane fraction, exert anti-tumor effects on breast cancer cells through apoptosis induction, gene-expression modulation, migration inhibition and cell-cycle arrest, supporting marine-derived compounds as candidate anticancer agents.

Introduction

Breast cancer remains a significant global health concern accounting for a large share of cancer deaths worldwide with high incidence rates among women [1]. Despite advances in conventional treatment strategies such as surgery, radiotherapy and cytotoxic chemotherapy, significant challenges persist: tumours acquire drug resistance, healthy tissues are damaged, and numerous side effects burden many patients [1–4]. These limitations have spurred researchers to explore new leads, and the marine habitat has proved a rewarding place to look. Sponges, algae and marine bacteria synthesize vast amounts of secondary metabolites, a great many of which have no structural counterpart on land [5–8]. These natural synthesized molecules demonstrate significant effects against tumour cells by slowing proliferation, inducing apoptosis of cancer cells or simply proving cytotoxic at modest concentrations [9]. The pro-apoptotic effects tend to operate through several multiple mechanisms such as the mitochondrial pathway dysregulation, caspase activation, and death receptor signalling [9]. Because

their scaffolds are so often chemically novel, marine metabolites make appealing starting points for drug discovery [5], and several show antioxidants, anti-inflammatory or neuroprotective activity that could prove useful well beyond oncology [7]. For all that promise, the pharmacology of most of these compounds remains only partly mapped, and much of their therapeutic potential is still untested [6].

Among marine invertebrates, sponges stand out as a rich source of bioactive compounds with significant potential in cancer treatment. Their metabolites tend to exhibit diverse mechanisms of action, targeting multiple hallmarks of cancer such as uncontrolled cell proliferation, apoptosis evasion, angiogenesis, and metastasis [10–12]. Recent research has identified numerous sponge-derived metabolites with confirmed apoptosis-inducing properties, making them promising leads for targeted cancer therapies [11]. These compounds have shown efficacy against various tumour types, including breast cancer, lymphoma, and lung cancer [10, 13]. Mechanistically the effects are usually attributed to a combination of cell-cycle interference, apoptosis induction and anti-inflammatory action [5]. Indeed, marine sponges have been feeding the drug pipeline with bioactive compounds since the 1950s, already delivering approved agents against cancer as well as viral, parasitic and inflammatory diseases [14]. The principal bottleneck is translating these bioactive compounds to clinical use and that gap is where most of the remaining work lies [13, 14].

The present study examines *Neophrissospongia endoumensis*, a sponge that has attracted little attention so far, as a candidate source of anti-breast cancer compounds. Rather than lean on a single readout, we profiled the extracts across a panel of cellular and molecular endpoints: apoptosis, expression of the paired regulators *Bax* and *Bcl-2*, the angiogenic factor *VEGF*, the matrix metalloproteinases *MMP2* and *MMP9*, cell migration, intracellular oxidative stress and cell-cycle distribution. These endpoints together give a fuller account of how the extracts work than any one assay could on its own. Of particular significance is our identification of the hexane fraction as a potent anticancer agent, capable of inducing multiple antitumor effects through distinct molecular pathways. Beyond adding *N. endoumensis* to the list of sponges worth studying, we hope these data offers a practical foundation for isolating the specific molecules responsible and, in time, for evaluating them as breast-cancer therapeutics.

Materials and Methods

1. Preparation of Extracts

Specimens of *N. endoumensis* were rinsed thoroughly after collection to remove debris and surface contaminants, air-dried at room temperature and milled to a fine powder. The material was extracted sequentially with three solvents: methanol, ethyl acetate and hexane. For each solvent, roughly 100 g of powder was left to steep for 72 h at room temperature under constant agitation to improve recovery. Each extract was clarified through Whatman No. 1 filter paper and taken to dryness on a rotary evaporator (Büchi R-210, Flawil, Switzerland) at 40 °C under reduced pressure. The concentrated extracts were kept at –20 °C until analysis [15].

2. Cell Culture

The breast cancer cell line 4T1, obtained from the Pasteur Institute of Iran, was grown in DMEM supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin, and maintained at 37 °C under 5% CO₂ in a humidified incubator. Medium was renewed every two to three days, and once the cultures reached 80–90% confluence they were detached with 0.25% trypsin–EDTA and passaged. Only cells passage 3 were used in the experiments [16].

3. Cytotoxicity Assay

The cytotoxic effects of methanol, ethyl acetate, and hexane extracts were evaluated using the MTT assay. Each of the 96-well plate received 5×10^3 cells, which were left overnight to attach. Following this, cells were treated with varying concentrations of the extracts (step 1: 0 to 2000 µg/mL, step 2: 0 to 5000 µg/mL) for 48 hours. Briefly, four hours after adding 20 µL of MTT (5 mg/mL) per well and incubating at 37°C, the resulting formazan was solubilised in 100 µL DMSO and read at 540 nm on a microplate reader (BioTek Instruments, Winooski, VT, USA). Of the three crude extracts, the ethyl acetate preparation returned the lowest IC₅₀ and was therefore the most cytotoxic.

4. Partitioning of ethyl acetate Extract

Following the initial cytotoxicity assessment, the ethyl acetate extract was carried forward for fractionation. It was redissolved in a small volume of methanol and partitioned between hexane and ethyl acetate by liquid–liquid extraction; the mixture was shaken vigorously and left to resolve into distinct phases. The hexane, ethyl acetate and methanol fractions were collected separately, dried under reduced pressure and stored at –20 °C for the biological assays that followed.

5. Apoptosis Assay

To assess apoptosis, cells were treated with the IC₅₀ concentrations of the ethyl acetate extract and its fractions (methanol, hexane, and ethyl acetate) for 48 hours. Following treatment, cells were stained with acridine orange (AO, 10 µg/mL) and propidium iodide (PI, 10 µg/mL) for 15 minutes at 37°C in darkness [17]. The stained cells were observed under a fluorescence microscope (Olympus IX73, Tokyo, Japan). We examined 5 microscopic fields and calculated their average. Viable cells fluoresced green; early apoptotic cells appeared red, late apoptotic cells intense red, and necrotic cells swollen and uniformly red.

6. Gene Expression Analysis

Transcript levels of the pro-apoptotic gene *Bax*, the anti-apoptotic gene *Bcl-2*, the angiogenic gene *VEGF* and the metastasis-associated genes *MMP2* and *MMP9* were measured by quantitative real-time PCR (qRT-PCR). TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to isolate total RNA following the supplier's protocol. cDNA was synthesized using a reverse transcription kit (Thermo Fisher Scientific, Waltham, MA, USA). Gene-specific primers were designed (Table 1) and used for amplification, and gene expression levels were normalized to the housekeeping gene *GAPDH*. Relative gene expression was calculated using the $2^{-(\Delta\Delta Ct)}$ method.

Table 1. Gene-specific primers for real-time PCR assay.

Gene	Primer sequence
GAPDH	Fwd-5'-GTGAACCACGAGAAATATGACAAC-3'
	Rev-5'-AGTGATGGCATGGACTGTG-3'
Bax	Fwd-5'- TGGAGAAGTGTGAAGGAGGTG -3'
	Rev-5'- CTTCTGCTGTGAGGCGTGAT-3'
Bcl-2	Fwd-5'- GAGTACCTGAACCGGCATCT -3'
	Rev-5'-TTGTTTGGGGCAGGTTTGTC -3'
MMP-2	Fwd-5'-GGACAAGAACCAGATCACATAC-3'
	Rev-5'-CGTCGCTCCATACTTTTAAGG-3'
MMP-9	Fwd-5'-GTGTCTGGAGATTCTGACTTG-3'
	Rev-5'-CCTTGTTACCTCATTTTGG-3'
VEGF	Fwd-5'-GGATCAAACCTCACCAAAGC-3'
	Rev-5'-GCAGGAACATTACACGTCTG-3'

7. Wound Healing Assay

Migration was assessed with a scratch-wound assay. Cells were grown to a confluent monolayer in six-well plates, and a single scratch was drawn across each well with a sterile pipette tip. The wells were then treated with the IC₅₀ concentrations of the extracts and fractions, and the wound was photographed at 0h, 24h and 48h on an inverted microscope (Leica DMI8, Wetzlar, Germany). Cells were treated with mitomycin C at a concentration of 10 µg/mL for 2 hours to inhibit proliferation for feeder cell inactivation. Following treatment, migration inhibition was assessed by examining 5 microscopic fields per sample and calculating the mean value. Gap width was measured and closure quantified in ImageJ (NIH, Bethesda, MD, USA).

8. Measurement of Oxidative Stress

Intracellular reactive oxygen species (ROS) were quantified with the DCFH-DA probe [17]. Following IC₅₀ treatment (48 h), cells were loaded with 10 µM DCFH-DA for 30 min at 37°C and the amount of ROS was measured by flow cytometer (BD FACSCanto II, USA). The data were analyzed using Flowjo version 7.6.1

9. Cell Cycle Analysis

DNA content was profiled by flow cytometry. Treated cells (48 h at the IC₅₀ dose) were harvested and fixed in 70% ethanol overnight at -20 °C, then resuspended in propidium iodide (50 µg/mL) along with RNase A (100 µg/mL) and left in the dark for 30 min. Samples were acquired on a BD FACSCanto II (San

Jose, CA, USA), and the proportion of cells in the G1, S and G2/M phases was determined in Flowjo version 7.6.1

10. Statistical Analysis

All experiments were performed in independent triplicate, and the data were expressed as mean $\pm$ standard error of the mean (SEM). Statistical analysis was conducted using one-way ANOVA followed by Tukey's post-hoc test to determine significant differences between groups. A p-value of <0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism software (GraphPad Software, San Diego, CA, USA).

Results

Cytotoxic Evaluation

Initial cytotoxicity screening was performed using the MTT assay to evaluate the antiproliferative effects of methanol, ethyl acetate, and hexane extracts on breast cancer cells. Cells were exposed to varying concentrations (0-2000 $\mu\text{g/ml}$ and 0-5000 $\mu\text{g/ml}$) of each extract for 48 hours. The results demonstrated a dose-dependent increase in cell death across all extracts tested. In the first phase of screening, the IC_{50} values were determined to be $>2000 \mu\text{g/ml}$, 238 $\mu\text{g/ml}$, and 314 $\mu\text{g/ml}$ for methanol, ethyl acetate, and hexane extracts, respectively (Figure 1). In Figure 1, the ethyl acetate extract exhibited superior cytotoxicity, as evidenced by its lowest IC_{50} value.

Based on the initial screening results, the ethyl acetate extract was selected for further fractionation using liquid-liquid partitioning with methanol, ethyl acetate, and hexane. Secondary screening of these fractions confirmed their cytotoxic effects, with refined IC_{50} values of 2000 $\mu\text{g/mL}$, 320 $\mu\text{g/mL}$, and 200 $\mu\text{g/mL}$ for the methanol, ethyl acetate, and hexane fractions, respectively (Figure 2). These concentrations were established as reference points for subsequent biological evaluations.

The progression from initial extraction to bioactivity-guided fractionation revealed significant cytotoxic potency in the fractions. While the original ethyl acetate extract showed promising activity (IC_{50} : 238 $\mu\text{g/mL}$), subsequent fractionation led to enhanced activity in the hexane fraction (IC_{50} : 200 $\mu\text{g/mL}$), suggesting successful concentration of bioactive compounds through the partitioning process.

Effects of Apoptosis Induced by fractions Obtained by Methanol, Ethyl Acetate, and Hexane on Breast Cancer Cells

In Figure 3A, the morphology and level of apoptosis in cancer cells exposed to fractions obtained by methanol, ethyl acetate, and hexane are shown using a fluorescence microscope. The representation of apoptosis in this figure indicates that cells undergoing early apoptosis exhibit a red coloration due to the uptake of acridine orange, which stains the apoptotic cell membranes red. Late apoptotic cells take up propidium iodide (PI) through their DNA, resulting in a bright red coloration, while necrotic cells appear

enlarged and swollen, taking on a full red color as they undergo collapse. Healthy cells, on the other hand, appear green and do not absorb any dye. As shown in Figure 3B, treatment of cancer cells with fractions obtained by methanol, ethyl acetate, and hexane significantly increased apoptosis compared to the control group; however, the hexane fraction demonstrated greater apoptotic effects than the others.

Effects of Fractions Obtained by Methanol, Ethyl Acetate, and Hexane on Pro-Apoptotic and Anti-Apoptotic Genes in Breast Cancer Cells

As shown in Figure 4, the pro-apoptotic gene *Bax* is illustrated in Figure 4A, while the anti-apoptotic gene *Bcl-2* is shown in Figure 4B. The results from treating cancer cells with fractions obtained by methanol, ethyl acetate, and hexane indicated that all three fractions significantly increased the expression of pro-apoptotic genes (*Bax*) in these cancer cells compared to the untreated group. Among the fractions, the hexane fraction exhibited a notably higher pro-apoptotic effect on gene expression than the others. Additionally, these fractions led to a reduction in the expression of anti-apoptotic genes (*Bcl-2*). This demonstrates that the fractions obtained by methanol, ethyl acetate, and hexane promote apoptosis in breast cancer cells by affecting apoptotic-related genes.

Effects of the Fractions Obtained by Methanol, Ethyl Acetate, and Hexane on Angiogenesis and Metastasis-Related Genes in Breast Cancer Cells

Figure 5 illustrates the effects of the fractions obtained by methanol, ethyl acetate, and hexane on the expression of angiogenesis-related gene (*VEGF*) and metastasis-related genes (*MMP2* and *MMP9*). All three fractions significantly reduced the expression of *VEGF* compared to the control group, indicating their anti-angiogenic effects in this type of cancer (Fig 5A). Additionally, it was observed that all three fractions also significantly decreased the expression of *MMP2* and *MMP9* compared to the control group, suggesting their anti-metastatic effects in this cancer (Fig 5B & C). Similar to the apoptotic gene expression results, the hexane fraction exhibited greater anti-angiogenic and anti-metastatic effects than the other fractions, indicating its stronger anti-cancer properties compared to the others.

Effects of the Fractions Obtained by Methanol, Ethyl Acetate, and Hexane on Migration and Metastatic Potential in Breast Cancer Cells

To evaluate the invasive capacity and migration of cancer cells, we employed a wound healing assay. After treating the cancer cells with all three fractions obtained by methanol, ethyl acetate, and hexane, it was observed that the migration rate in cancer cells significantly decreased compared to the control group, resulting in a delayed closure of the wound gap (Fig 6, Wound healing). Among the fractions, hexane exhibited more pronounced and significant effects. Consequently, it was determined that all three fractions showed considerable anti-tumor effects in breast cancer; however, hexane demonstrated greater potency.

Effects of the Fractions Obtained by Methanol, Ethyl Acetate, and Hexane on Oxidative Stress Levels in Breast Cancer Cells

As shown in Figure 7, cells treated with all three fractions resulted in an increase in oxidative stress and reactive oxygen species (ROS) within the cancer cells, which significantly increased compared to the control group. Furthermore, among the fractions, hexane demonstrated a higher level of ROS in the cancer cells.

Effects of the Fractions Obtained by Methanol, Ethyl Acetate, and Hexane on the Cell Cycle in Breast Cancer Cells

As shown in Figure 8, treatment with all three fractions in cancer cells resulted in cell cycle arrest at various stages. Specifically, all three fractions led to a reduction in the G1 phase and S phase, while increasing the G2/M phase compared to the control group. Among the fractions, the results indicated that the hexane fraction caused a more significant cell cycle arrest in this cancer cell line compared to the others. Consequently, the anti-tumor effects of the hexane fraction were comparatively greater than the other fractions.

Discussion

This study investigated the anticancer effects of *Neophrissospongia endoumensis* extracts on breast cancer cells, revealing that all three solvent fractions (methanol, ethyl acetate, and hexane) induced dose-dependent cytotoxicity, with hexane demonstrating the most potent antiproliferative and pro-apoptotic activity. These results support the initial hypothesis that marine sponge-derived bioactive compounds can exert significant cytotoxic and anti-metastatic effects on breast cancer cells. The observed biological effects include induction of apoptosis, suppression of angiogenesis and metastasis-related gene expression, inhibition of cell migration, increase in oxidative stress, and cell cycle arrest.

The cytotoxic profile, with IC₅₀ values of 238 µg/mL (ethyl acetate extract) and 200 µg/mL (hexane fraction), is consistent with earlier reports of marine sponge extracts exhibiting selective anticancer activity [18, 19]. For instance, *Xestospongia testudinaria* showed an IC₅₀ of 39.8 µg/mL and induced G₀/G₁ arrest and apoptosis in MCF-7 cells, alongside inhibition of migration [20]. Though less potent, the *N. endoumensis* extracts demonstrated comparable functional outcomes across similar assays.

Apoptosis was confirmed through fluorescence microscopy and gene expression analysis. All extracts increased the expression of the pro-apoptotic gene *Bax* and reduced the anti-apoptotic gene *Bcl-2*, with the hexane fraction exerting the strongest effect [21]. These findings are in line with studies on *Dactylospongia elegans*, where the metabolites pelorol and 5-epi-ilimaquinone promoted apoptosis in melanoma cells by upregulating *Bax* and downregulating *Bcl-2* and related anti-apoptotic factors [22]. Additionally, smenospongine, derived from *Spongia pertusa*, was shown to induce intrinsic apoptosis in breast cancer stem-like cells through the p38/AMPKα pathway [23].

Furthermore, angiogenesis and metastasis suppression were observed, with all fractions reducing *VEGF*, *MMP2*, and *MMP9* expression. The hexane fraction showed the most pronounced effect [24]. These results mirror the effects of 4-chloro fascaplysin, a marine sponge alkaloid that inhibited tumor growth

and angiogenesis by disrupting the PI3K/Akt/mTOR pathway in breast cancer cells [25]. Similarly, *Aaptosuberitoides* ethanol extract demonstrated anti-migration and anti-proliferation effects in HER2 + breast cancer models [26].

The wound-healing assay further validated the anti-metastatic capacity of the extracts, highlighting the suppression of cell migration. These effects align with the marine *staurosporine* analogue described by Chen which inhibited both regular and drug-resistant triple-negative breast cancer cell migration and induced apoptosis [27].

Oxidative stress, as evidenced by elevated intracellular ROS levels, likely contributes to the induction of apoptosis and cell cycle arrest. However, caution is warranted, as ROS elevation could reflect generalized cytotoxic stress rather than targeted therapeutic effect. This consideration is consistent with observations from *Monascus* sp. NMK7 metabolites, which induced ROS-mediated apoptosis while affecting mitochondrial membrane integrity and migration inhibition in breast cancer cells [28, 29].

In terms of cell cycle effects, all extracts decreased the proportion of cells in G₁ and S phases and increased accumulation in the G₂/M phase. This suggests interference with cell cycle checkpoints, possibly through DNA damage or mitotic stress. Similar mechanisms were reported with *Xestospongia*-derived jorunnamycin A, which induced apoptosis and inhibited key KRAS-mediated survival pathways including AKT and ERK in pancreatic cancer cells [30], and with emodin derivatives, which induced G₀/G₁ arrest and caspase-mediated apoptosis in MCF-7 cells [31].

Nevertheless, there are several limitations. The relatively high IC₅₀ values (> 200 µg/mL) suggest low potency compared to benchmark compounds. Furthermore, the lack of data on normal cell selectivity prevents conclusions about therapeutic index or safety. The use of crude extracts complicates mechanistic interpretation due to the presence of multiple bioactive and possibly antagonistic components. Additionally, the in vitro monolayer culture model may not fully recapitulate tumor microenvironment complexity, limiting extrapolation to in vivo scenarios.

The results warrant further exploration, particularly via bioassay-guided isolation to identify and structurally characterize the active compounds responsible for the observed effects [32]. Follow-up studies should include caspase inhibition assays, ROS scavenger controls, and evaluations of selectivity using non-cancerous breast epithelial cells. Ultimately, in vivo validation in xenograft or orthotopic models will be essential to assess pharmacokinetics, efficacy, and toxicity under physiological conditions. Comparable bioactivity-guided studies of natural-product extracts against cancer cell lines [33] and selectively cytotoxic, migration-inhibiting anticancer nanoformulations [34] offer useful models for these next steps.

Conclusion

This study provides compelling evidence for the anticancer potential of extracts from the marine sponge *Neophrissospongia endoumensis*, with the hexane fraction demonstrating superior activity against breast

cancer cells through multiple mechanisms. These findings not only align with and expand upon the existing literature on sponge-derived compounds but also highlight the untapped potential of this relatively understudied species in breast cancer therapy. By demonstrating effects on apoptosis, migration, angiogenesis, oxidative stress, and cell cycle progression, this work contributes to the growing body of evidence supporting the role of marine natural products in cancer treatment. However, limitations such as the lack of in vivo data and uncharacterized chemical composition must be addressed in future research to fully realize the therapeutic potential of *N. endoumensis*-derived compounds. In light of recent advancements, such as the discovery of synergistic effects between sponge-derived compounds and existing chemotherapeutics, this study lays a strong foundation for further exploration of *N. endoumensis* as a source of novel anticancer agents. Collectively, these findings support the continued development of marine-derived agents for targeted cancer therapy.

Abbreviations

Bax B-cell lymphoma-2-associated X protein; Bcl-2 B-cell lymphoma 2; VEGF vascular endothelial growth factor; MMP matrix metalloproteinase; MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ROS reactive oxygen species; DCFH-DA 2',7'-dichlorofluorescein diacetate; AO acridine orange; PI propidium iodide; IC50 half-maximal inhibitory concentration; qRT-PCR quantitative real-time polymerase chain reaction; DMEM Dulbecco's modified Eagle medium; FBS fetal bovine serum; SEM standard error of the mean; GAPDH glyceraldehyde-3-phosphate dehydrogenase; DMSO dimethyl sulfoxide; EDTA ethylenediaminetetraacetic acid; ANOVA analysis of variance; HER2 human epidermal growth factor receptor 2; mitomycin C

Declarations

Funding No funding was received for conducting this study.

Competing interests The authors have no competing interests to declare that are relevant to the content of this article.

Ethics approval This study did not involve human participants or animals and used an established breast cancer cell line; therefore, no ethical approval was required. Consent to participate and consent to publish are not applicable.

Data availability The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

Author contributions All authors contributed to the study conception and design. The research question was developed by Melika Nazemi. Study methodology and collection of biological material were carried out by Bahman Moradipoode and Mostafa Khaledi. Data analysis was performed by Melika Nazemi,

Ardeshir Abbasi, Ensieh Ghasemi, Mohammad Javad Hessami and Mostafa Khaledi. Effiong Paul Etim contributed to data interpretation and drafting of the manuscript, and Lionel In Lian Aun provided supervision. All authors read and approved the final manuscript.

Declaration of generative AI use in the writing process During the preparation of this manuscript, the authors used a large language model-based AI assistant to assist with language editing, improving readability, rephrasing of draft text for clarity, and formatting the reference list to the journal's style. After using this tool, the authors carefully reviewed, verified, and edited the content as necessary and take full responsibility for the content of the publication. No generative AI tool was used to produce or interpret the scientific data, figures, or results, and no AI tool is listed as an author or fulfils the criteria for authorship.

References

1. Burguin A, Diorio C, Durocher F (2021) Breast cancer treatments: updates and new challenges. *J Pers Med* 11:808. <https://doi.org/10.3390/jpm11080808>
2. Gyanani V, Haley JC, Goswami R (2021) Challenges of current anticancer treatment approaches with focus on liposomal drug delivery systems. *Pharmaceuticals* 14:835. <https://doi.org/10.3390/ph14090835>
3. Chaudhari R, Patel V, Kumar A (2024) Cutting-edge approaches for targeted drug delivery in breast cancer: beyond conventional therapies. *Nanoscale Adv* 6:2270–2286 <https://doi.org/10.1039/d4na00086b>
4. Zafar A, Khan MJ, Abu J, Naeem A (2024) Revolutionizing cancer care strategies: immunotherapy, gene therapy, and molecular targeted therapy. *Mol Biol Rep* 51:219. <https://doi.org/10.1007/s11033-023-09096-8>
5. Khalifa SAM, Elias N, Farag MA et al (2019) Marine natural products: a source of novel anticancer drugs. *Mar Drugs* 17:491. <https://doi.org/10.3390/md17090491>
6. Yun CW, Kim HJ, Lee SH (2019) Therapeutic application of diverse marine-derived natural products in cancer therapy. *Anticancer Res* 39:5261–5284
7. Karthikeyan A, Joseph A, Nair BG (2022) Promising bioactive compounds from the marine environment and their potential effects on various diseases. *J Genet Eng Biotechnol* 20:14. <https://doi.org/10.1186/s43141-021-00290-4>
8. Tamzi NN, Rahman MM, Das S (2024) Recent advances in marine-derived bioactives towards cancer therapy. *Int J Transl Med* 4:740–781 <https://doi.org/10.3390/ijtm4040051>
9. Ercolano G, De Cicco P, Ianaro A (2019) New drugs from the sea: pro-apoptotic activity of sponges and algae derived compounds. *Mar Drugs* 17:31. <https://doi.org/10.3390/md17010031>
10. Calcabrini C, Catanzaro E, Bishayee A, Turrini E, Fimognari C (2017) Marine sponge natural products with anticancer potential: an updated review. *Mar Drugs* 15:310. <https://doi.org/10.3390/md15100310>

11. Essack M, Bajic VB, Archer JAC (2011) Recently confirmed apoptosis-inducing lead compounds isolated from marine sponge of potential relevance in cancer treatment. *Mar Drugs* 9:1580–1606. <https://doi.org/10.3390/md9091580>
12. Sarkar R, Mukherjee A (2024) Marine metabolites as anticancer agents (chapter 4). In: *Marine bioactive compounds*. IGI Global
13. Ortigosa-Palomo A, Quiñonero F, Ortiz R, Sarabia F, Prados J, Melguizo C (2024) Natural products derived from marine sponges with antitumor potential against lung cancer: a systematic review. *Mar Drugs* 22:101. <https://doi.org/10.3390/md22030101>
14. Perdicaris S, Vlachogianni T, Valavanidis A (2013) Bioactive natural substances from marine sponges: new developments and prospects for future pharmaceuticals. *Nat Prod Chem Res* 1:1–8
15. Khaledi M, Moradipoodeh B, Moradi R, Baghbadorani MA, Mahdavinia M (2022) Antiproliferative and proapoptotic activities of sea cucumber *H. leucospilota* extract on breast carcinoma cell line (SK-BR-3). *Mol Biol Rep*. <https://doi.org/10.1007/s11033-021-06947-0>
16. Capes-Davis A, Freshney RI (2021) *Freshney's culture of animal cells: a manual of basic technique and specialized applications*, 8th edn. Wiley-Blackwell, Hoboken
17. Jayaraman V, Arumugam MK, Balachandran S, Boopathy L et al (2025) Anticancer effects of monacolin X against human liver cancer cell line: exploring the apoptosis using AO/EB and DCFH-DA fluorescent staining. *Luminescence* 40:e70229. <https://doi.org/10.1002/bio.70229>
18. Abuzahrah SS, Bakhsh T, Mekky RH, Elbehairi SEI, Juaid N (2025) Exploring the metabolic profiling and anticancer activities of Red Sea sponges *Echinodictyum asperum* and *Callyspongia siphonella* against human breast cancer cells: a comparative study. *ACS Omega* 10:9402–9425. <https://doi.org/10.1021/acsomega.4c10202>
19. Ibrahim NE, El-Feky AM, Aboelmagd M et al (2026) Cytotoxic activity of marine-derived bioactive compounds from Red Sea sponges supported by LC-MS/MS profiling and molecular docking. *Sci Rep* 16:8949. <https://doi.org/10.1038/s41598-026-39782-z>
20. Alkhilaiwi FA et al (2023) Evaluation of cytotoxicity of the methanolic extract of Red Sea marine sponge *Xestospongia testudinaria* and its related compounds against MCF-7 human breast cancer cells. *Breast Cancer (Dove Med Press)* 15:879–890 <https://doi.org/10.2147/BCTT.S429721>
21. Li P, Wei L, Li M, Liu X, Yang H (2025) ESCO2 inhibition induces cell cycle arrest and apoptosis in breast cancer via the P53-CDK1 axis and the BAX/Bcl2/caspase signaling cascade. *Front Oncol* 15:1585945. <https://doi.org/10.3389/fonc.2025.1585945>
22. Carpi S, Scoditti E, Polini B et al (2022) Pro-apoptotic activity of the marine sponge *Dactylospongia elegans* metabolites pelorol and 5-epi-ilimaquinone on human 501Mel melanoma cells. *Mar Drugs* 20:427. <https://doi.org/10.3390/md20070427>
23. Tang S-A et al (2018) Marine sponge-derived smenospongine preferentially eliminates breast cancer stem-like cells via p38/AMPKα pathways. *Cancer Med* 7:3965–3976. <https://doi.org/10.1002/cam4.1640>

24. Keleş D, Sipahi M, İnanç-Sürer Ş, Djamgoz MBA, Oktay G (2023) Tetracaine downregulates matrix metalloproteinase activity and inhibits invasiveness of strongly metastatic MDA-MB-231 human breast cancer cells. *Chem Biol Interact* 383:110693
25. Sharma S et al (2017) A marine sponge alkaloid derivative 4-chloro fascaplysin inhibits tumor growth and VEGF-mediated angiogenesis by disrupting PI3K/Akt/mTOR signaling cascade. *Chem Biol Interact* 275:47–60. <https://doi.org/10.1016/j.cbi.2017.07.017>
26. Bashari MH, Arsydinilhuda FZ, Ilhamsyah RS, Nugrahani AD, Nurdin RA, Kartika A, Huda F, Abdurahman M, Putri T, Qomarilla N, Atmaja H, Sudji IR, Subhan B, Usman HA, Pamela Y, Ariyanto EF, Meiyanto E (2021) The ethanol extract of marine sponge *Aaptos suberitoides* suppresses cell viability, cell proliferation and cell migration in HER2-positive breast cancer cell line. *Asian Pac J Cancer Prev* 22(S1):25–32. <https://doi.org/10.31557/APJCP.2021.22.S1.25>
27. Chen R-Y, Ding L-J, Liu Y-J et al (2024) Marine staurosporine analogues: activity and target identification in triple-negative breast cancer. *Mar Drugs* 22:459. <https://doi.org/10.3390/md22100459>
28. Nagabhishek SN, Madankumar A (2019) A novel apoptosis-inducing metabolite isolated from marine sponge symbiont *Monascus* sp. NMK7 attenuates cell proliferation, migration and ROS stress-mediated apoptosis in breast cancer cells. *RSC Adv* 9:5878–5890 <https://doi.org/10.1039/c8ra09886g>
29. Ahmad I, Zughaibi TA, Alhashmi MH, Abujamel T et al (2025) Synergistic cytotoxicity of curcumin and plumbagin via reactive oxygen species-mediated apoptosis and cell cycle arrest in cervical cancer cell line. *Results Chem* 18:102720. <https://doi.org/10.1016/j.rechem.2025.102720>
30. Khine HEE, Suriya U, Rungrotmongkol T et al (2025) Jorunnamycin A induces apoptosis in pancreatic ductal adenocarcinoma cells, spheroids, and patient-derived organoids by modulating KRAS-mediated survival pathways. *Sci Rep* 15:11376. <https://doi.org/10.1038/s41598-025-95766-5>
31. Yang K-D, Jin M-J, Quan Z-S, Piao H (2019) Design and synthesis of novel anti-proliferative emodin derivatives and studies on their cell cycle arrest, apoptosis pathway and migration. *Molecules* 24:884. <https://doi.org/10.3390/molecules24050884>
32. Esposito G, Ponticelli M, Milella L, Lela L, Teta R, Pawlik JR, Russo D, Costantino V (2025) Bioassay-guided procedure coupled with HR-ESIMS dereplication for isolation of antiproliferative bromo-tyramine derivative from *Aplysina cauliformis*. *Mar Drugs* 23:187. <https://doi.org/10.3390/md23050187>
33. Ng WL, Tan JK, Gnanaraj C, Shah MD, Nor Rashid N, Abdullah I, Yong YS (2025) Cytotoxicity of *Physalis minima* Linn (Solanaceae) fruit against HCT116 and HT29 colorectal cancer cell lines. *Nat Prod Res* 39:5115–5120. <https://doi.org/10.1080/14786419.2024.2370521>
34. Tan KF, Chia LY, Maki MAA, Cheah S-C, In LLA, Kumar PV (2025) Gold nanocomposites in colorectal cancer therapy: characterization, selective cytotoxicity, and migration inhibition. *Naunyn Schmiedeberg Arch Pharmacol* 398:8975–9003. <https://doi.org/10.1007/s00210-025-03839-z>

Figures

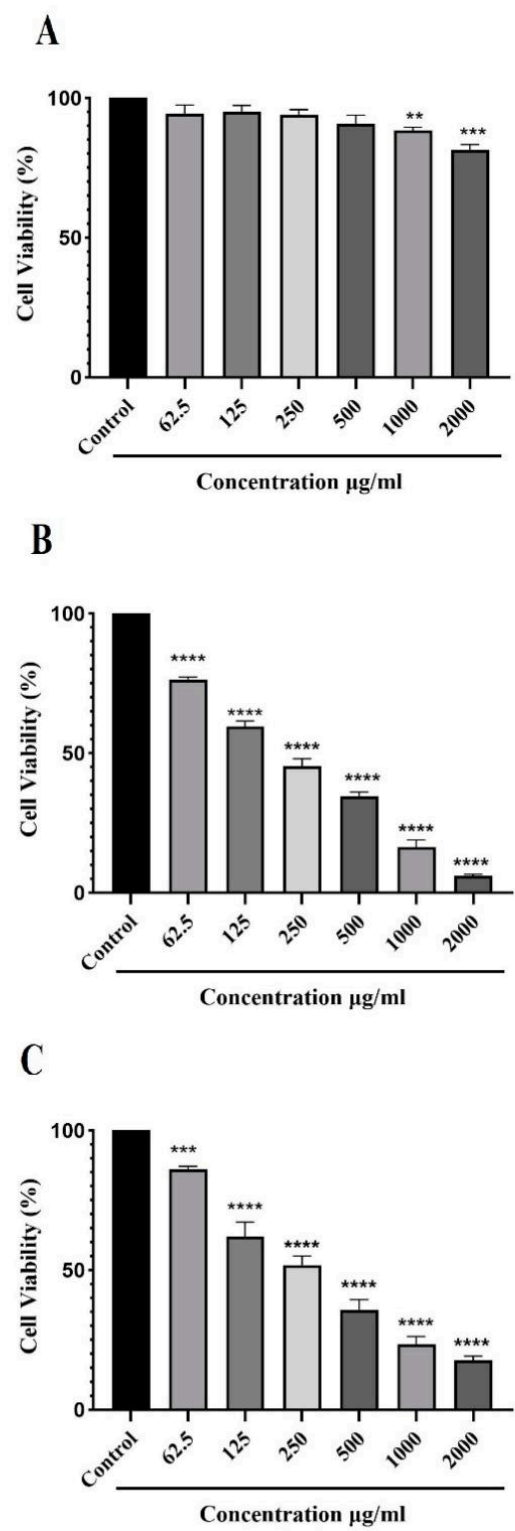


Figure 1

Cytotoxicity of methanol, ethyl acetate and hexane extracts on breast cancer cells. (A) Methanol, (B) ethyl acetate, and (C) hexane extracts were evaluated over a 48-hour period, showing a dose-dependent

increase in cell death. Data are presented as mean $\pm$ SEM for triplicate measurements (significance indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ compared to untreated control).

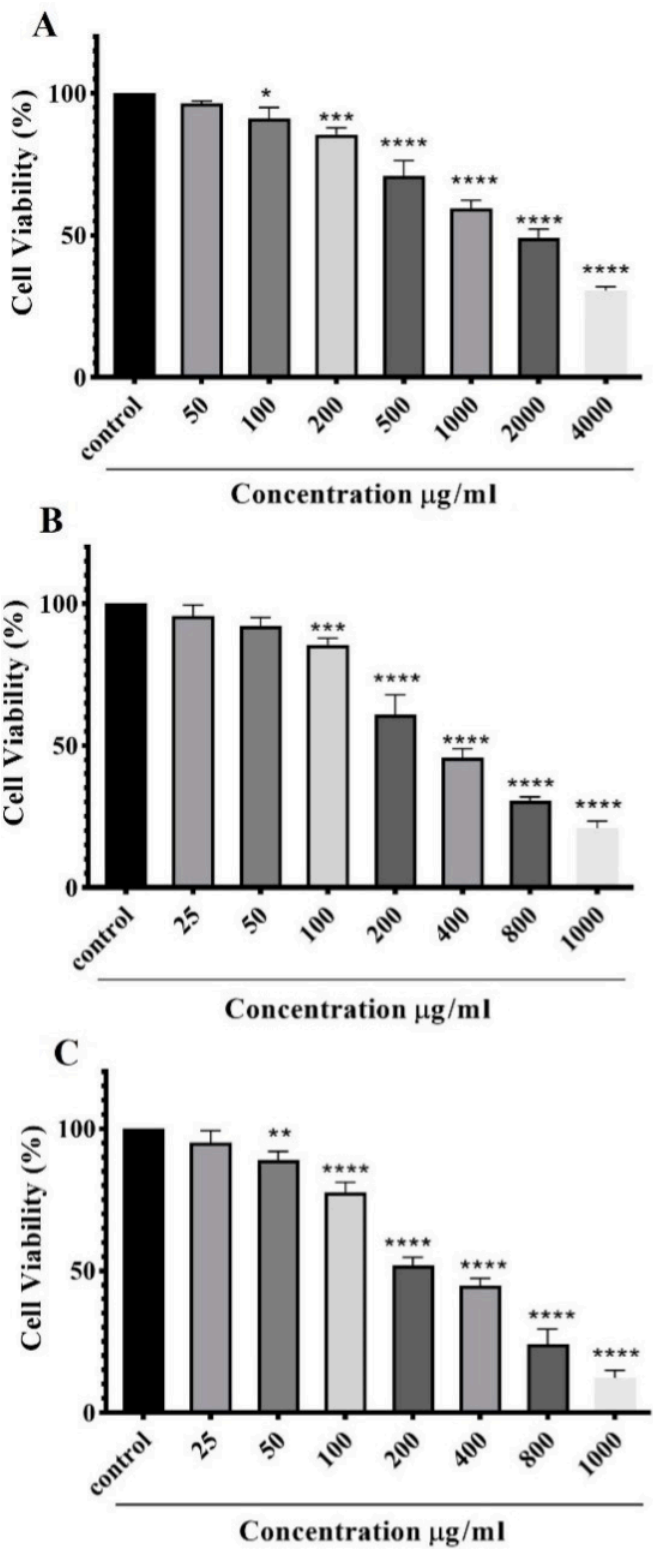


Figure 2

Cytotoxicity of ethyl acetate fractions on breast cancer cells. (A) Methanol, (B) ethyl acetate, and (C) hexane fractions were evaluated over a 48-hour period, showing a dose-dependent increase in cell death.

Data are presented as mean $\pm$ SEM for triplicate measurements (significance indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ compared to untreated control)

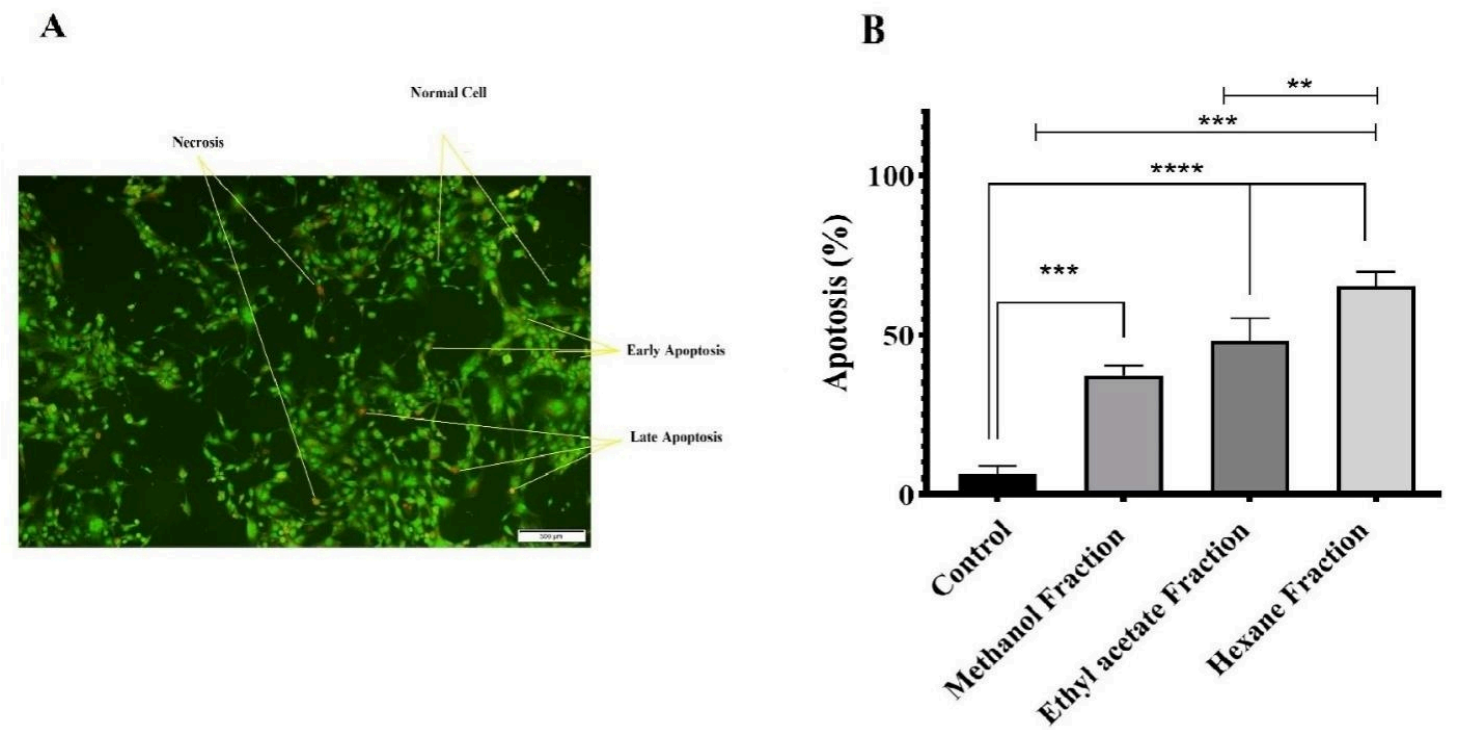


Figure 3

Induction of apoptosis in breast cancer cells treated with *Neophrissospongia endoumensis* fractionated extracts. (A) Fluorescence microscopy images depicting the apoptotic stages of cancer cells treated with methanol, ethyl acetate, and hexane fractions. Early apoptotic cells are marked in red, late apoptotic cells are bright red, and necrotic cells appear enlarged and fully red. (B) Quantification of apoptosis reveals that the hexane fraction induced the strongest apoptotic effect compared to the other fractions. Data are presented as mean $\pm$ SEM for triplicate measurements (significance denoted as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ relative to untreated control)

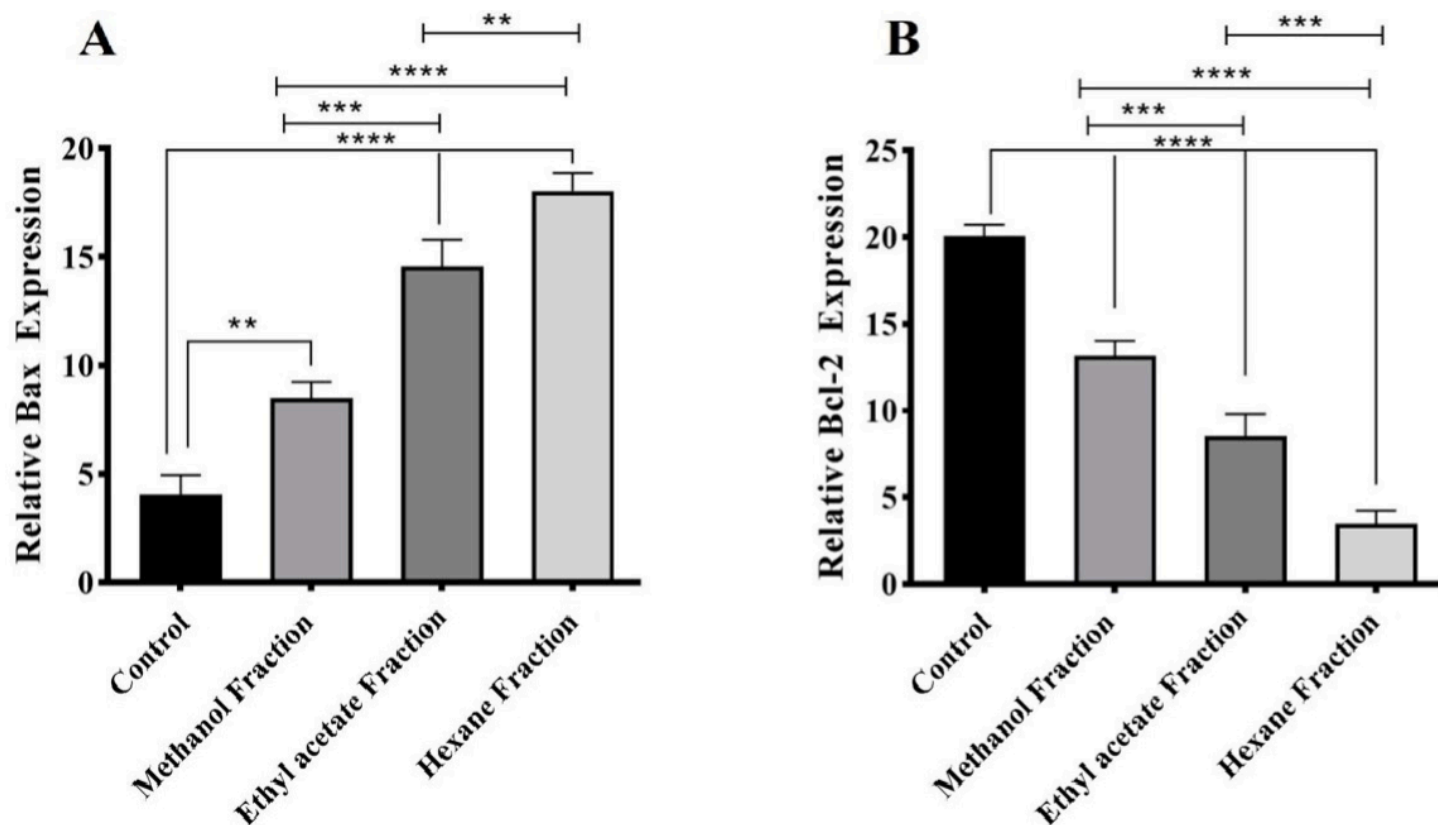


Figure 4

Effects of methanol, ethyl acetate, and hexane fractions on pro-apoptotic and anti-apoptotic gene expression in breast cancer cells. (A) Expression of the pro-apoptotic gene Bax, and (B) expression of the anti-apoptotic gene Bcl-2 in breast cancer cells treated with methanol, ethyl acetate, and hexane fractions. All fractions significantly increased the expression of Bax (pro-apoptotic) compared to the untreated control, with the hexane fraction showing the strongest effect. Additionally, treatment with these fractions led to a decrease in Bcl-2 (anti-apoptotic) expression. Data are presented as mean $\pm$ SEM for triplicate measurements (significance indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ compared to untreated control)

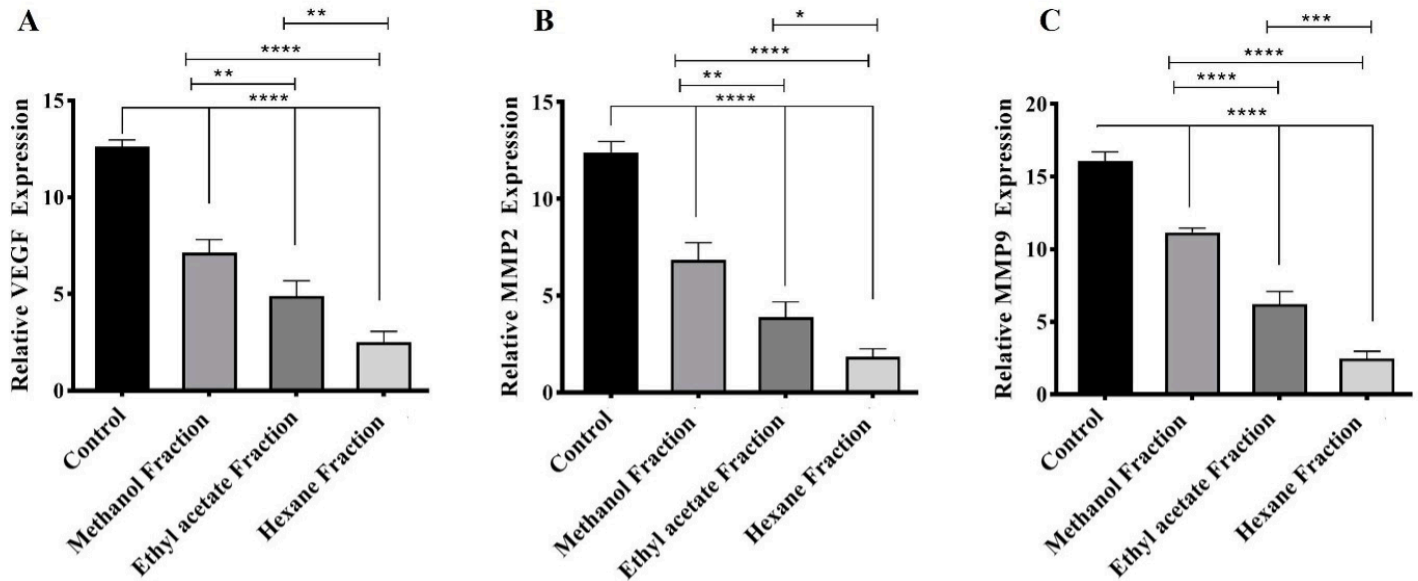


Figure 5

Effects of methanol, ethyl acetate, and hexane fractions on angiogenesis and metastasis-related gene expression in breast cancer cells. (A) Expression of the angiogenesis-related gene VEGF, (B) expression of the metastasis-related gene MMP2, and (C) expression of MMP9 in breast cancer cells treated with methanol, ethyl acetate, and hexane fractions. All three fractions significantly reduced the expression of VEGF, indicating their anti-angiogenic effects, and decreased the expression of MMP2 and MMP9, suggesting their anti-metastatic effects compared to the untreated control. Among the fractions, the hexane fraction demonstrated the strongest anti-angiogenic and anti-metastatic effects. Data are presented as mean $\pm$ SEM for triplicate measurements (significance indicated as *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001 compared to untreated control)

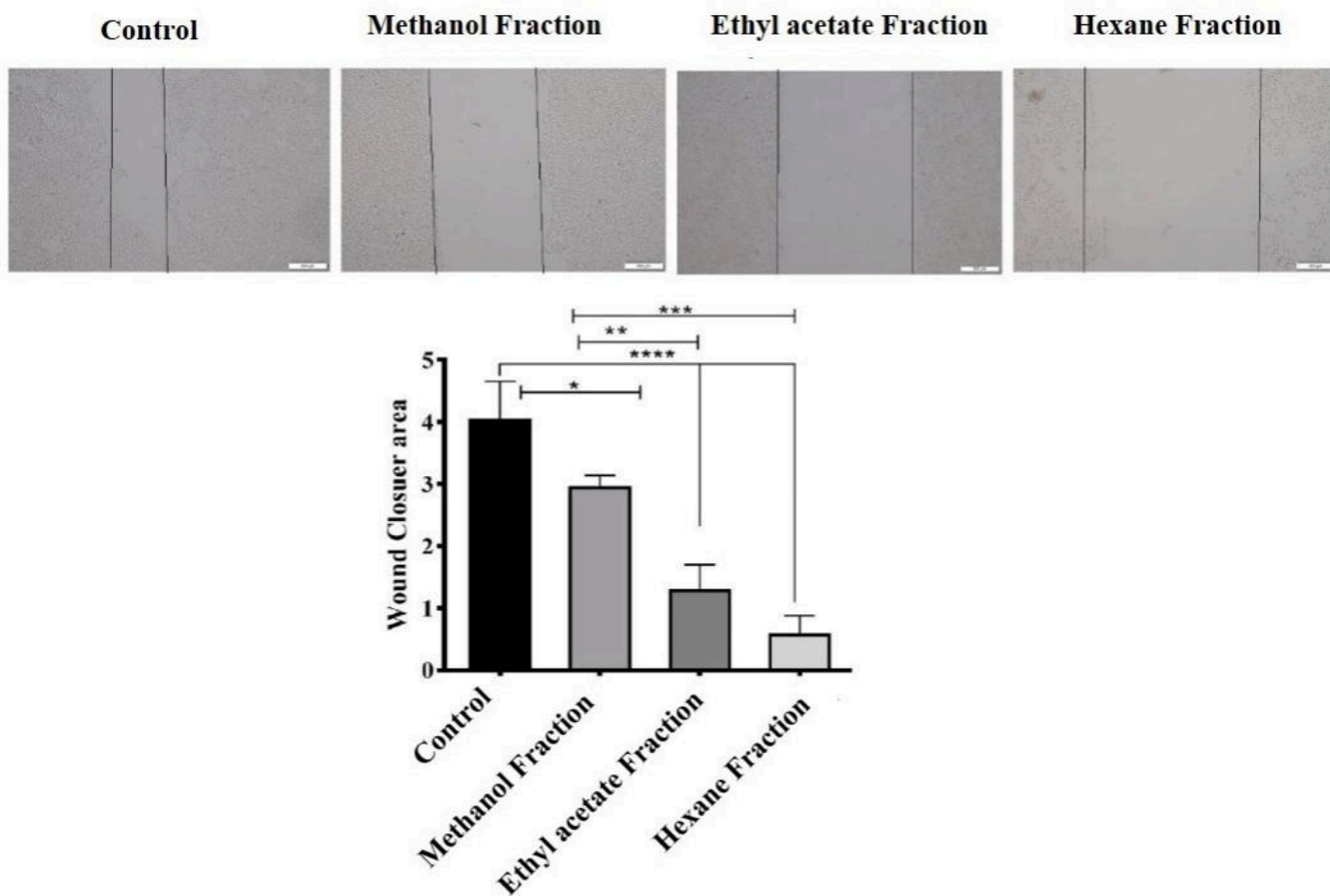


Figure 6

Effects of methanol, ethyl acetate, and hexane fractions on migration and metastatic potential in breast cancer cells. Wound healing assays showed that all three fractions significantly reduced cancer cell migration, delaying wound gap closure compared to the control group. The hexane fraction had the most pronounced effect, demonstrating stronger anti-migratory and anti-metastatic potential. Data are presented as mean $\pm$ SEM for triplicate measurements (significance indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ compared to untreated control)

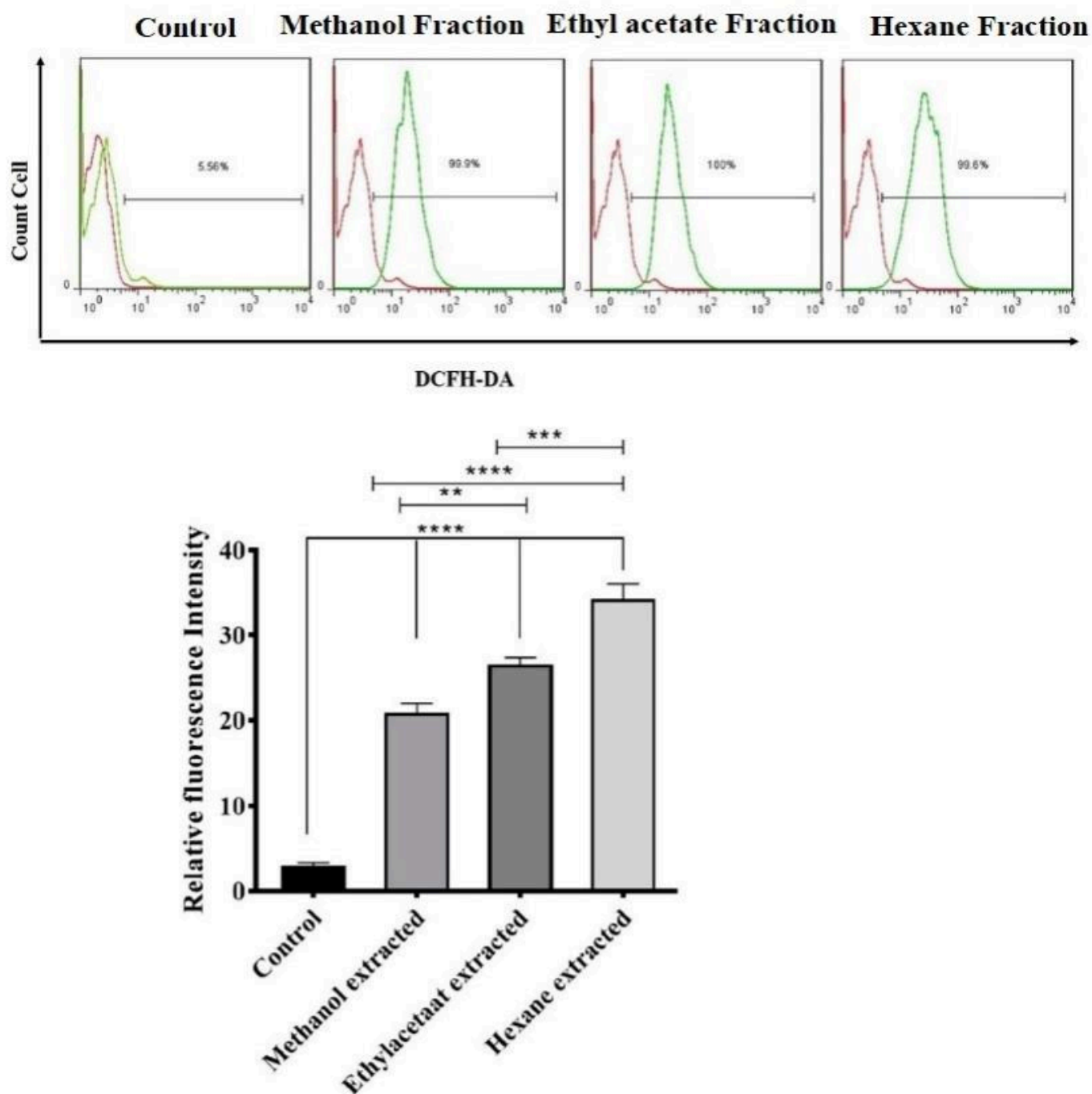


Figure 7

Effects of methanol, ethyl acetate, and hexane fractions on oxidative stress levels in breast cancer cells. Treatment with all three fractions increased oxidative stress and reactive oxygen species (ROS) in cancer cells, with levels significantly higher than the control group. The hexane fraction induced the highest ROS levels among the fractions. Data are presented as mean $\pm$ SEM for triplicate measurements (significance indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ compared to untreated control)

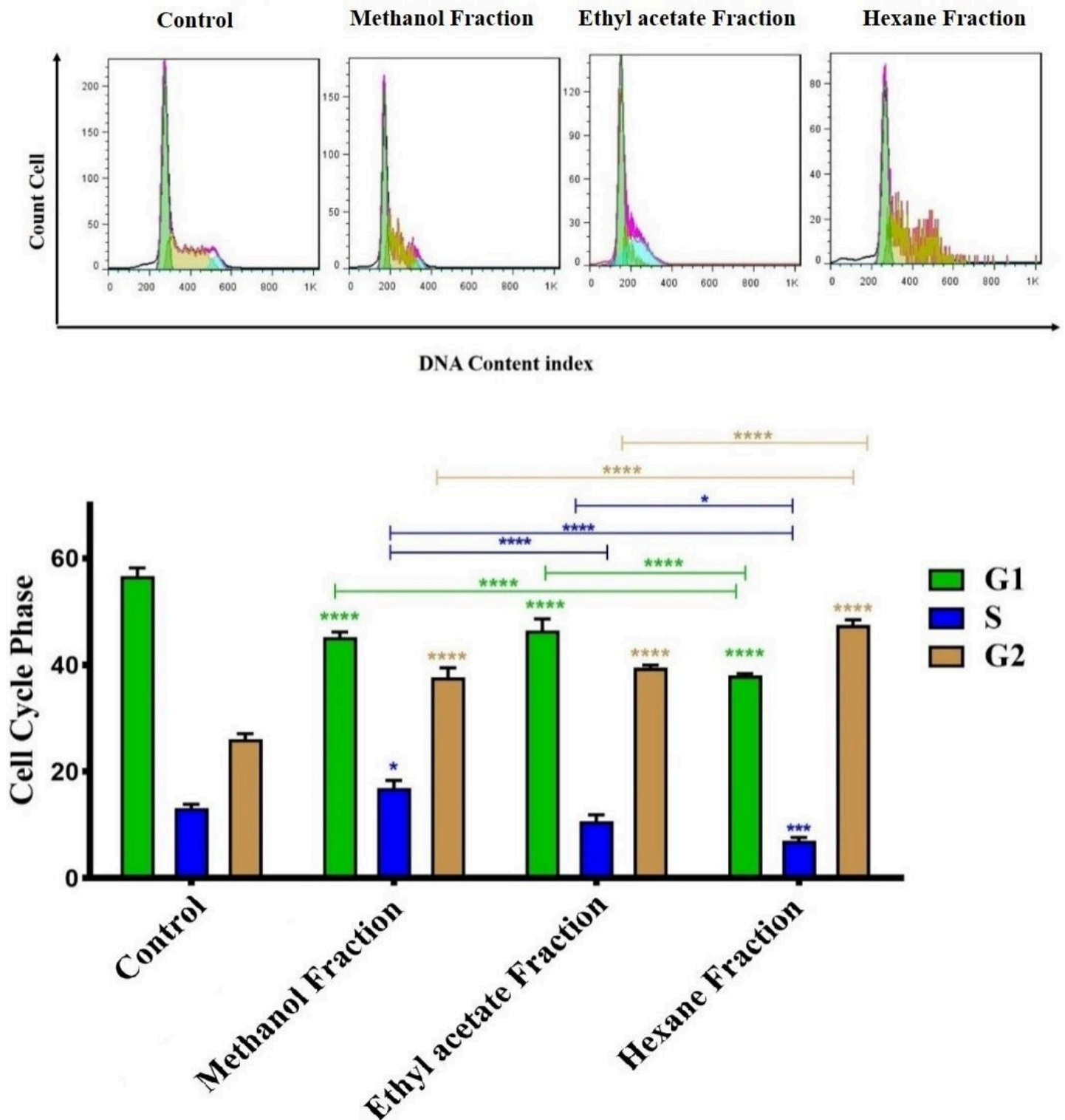


Figure 8

Effects of methanol, ethyl acetate, and hexane fractions on the cell cycle in breast cancer cells. Treatment with all three fractions induced cell cycle arrest, with a reduction in the G1 and S phases and an increase in the G2/M phase compared to the control group. The hexane fraction caused the most significant cell cycle arrest, demonstrating stronger anti-tumor effects than the other fractions. Data are

presented as mean $\pm$ SEM for triplicate measurements (significance indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ compared to untreated control)