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The Impact of *Amphimedon chloros* Ethyl Acetate Extract on MDA-MB-231 Cell Lines' Expression of Bax, Cyclin D, Caspase 3, P21, C-myc, and Bcl2: Mechanism of inhibition of the Growth of Cancer Cells

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ABSTRACT

Numerous marine sponge bioactive compounds exhibit diverse pharmacological properties. This study investigated the ethyl acetate extract of *Amphimedon chloros* for its effects on breast cancer cells (MDA-MB-231), focusing on the expression of key genes involved in apoptosis and cell cycle regulation, including Bax, Cyclin D, caspase 3, p21, C-myc, and Bcl2. The extract's chemical profile was analyzed using Liquid Chromatography-Mass Spectrometry (LC-MS), identifying fourteen compounds such as zamamidine (11.4%), keramamine (10.2%), and methoxyhexadecanoate (9.1%). Cytotoxicity and anticancer activity were assessed on MDA-MB-231 cells and human periodontal ligament fibroblasts (HPLFs). The IC_{50} of the extract against MDA-MB-231 cells was 3.0 µg/mL, indicating significant cytotoxicity, while it did not affect fibroblast viability at concentrations ranging from 50-200 µg/mL. RT-PCR (Real Time-Polymerase Chain Reaction) analysis revealed that the extract increased the expression of Bax, p21, and caspase 3, and decreased the levels of Bcl2 and Cyclin D in MDA-MB-231 cells. These findings suggest that the extract may inhibit cancer cell proliferation by interfering with growth factors signaling pathways, such as PI3K/Akt (Phosphoinositide 3-Kinase/Protein Kinase B) and MAPK (Mitogen-Activated Protein Kinase). The increase in Bax and p21 expression indicates enhanced apoptosis, while the drastic reduction in c-Myc expression points to potential cell cycle arrest. The results support the potential of *A. chloros* as an anticancer agent, with selective cytotoxicity towards cancer cells and significant modulation of apoptotic and cell cycle-related genes.

Keywords: *Amphimedon chloros*, Sponge, Cancer cell lines, Cytotoxicity.

Introduction

In recent times, novel bioactive chemicals from nature have primarily come from a variety of marine species, including sponges, soft corals, and sea slugs.[1,2] Hu and colleagues (2015) examined the overall pattern, the distribution of chemical structures, bioactivity groups, and species distribution of biologically active chemicals found in marine creatures from 1985 to 2012.[3] Researchers found that sponges had the highest ratio of bioactive compounds and that almost 75% of the molecules were obtained from marine invertebrates. Of the 9812 marine natural products that were retrieved from invertebrates between 1990 and 2009, a different study found that sponges had the largest proportion of metabolites (48.8%).[4] Minerals such as silica, calcium carbonate, and a protein known as spongin are the components that make up the skeletal structures of marine sponges.

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These organisms are made up of a layer that resembles gel that is positioned between two layers of rather fragile cells. The size and form of spicules on different sponge kinds are distinguishing characteristics.[5] As filter feeders, sea sponges neutralise potentially hazardous particles by neutralising them with beneficial chemicals. Around 11 sponge genera have been identified due to bioactive chemicals, including Petrosia, Haliclona, and Discodemia. These three genera produce strong anti-inflammatory and anti-cancer compounds.[6] The bioactive materials produced by sponges are chemically varied and include cyclic peptides, alkaloids, terpenes, sterols, and nucleosides.[7–9] In most forms of cancer, increased mechanical targeting activating of the PI3K/Akt/mTOR (Phosphoinositide 3-Kinase/Protein Kinase B/Mammalian Target of Rapamycin) pathway has been observed. These pathways are thought to be involved in the regulations of cellular growth and proliferation.[10] The PI3K/AKT/mTOR signaling pathways control autophagy and apoptosis in malignant cells.[11] The PI3K/AKT/mTOR pathways in malignancies would be the subject of this study's investigation using the sponge extract of *A. chloros* as potential inhibitors. Consequently, to investigate the cytotoxic activity induced by *A. chloros* ethyl acetate extracts, the expression rates of the genes cyclins D1, c-mycs, and p21 as well as the total proteins of PI3K, Akt, and mTOR would be evaluated in breast malignant cells. As a result, it would be feasible to prove the potential cytotoxicity of the *A. chloros* ethyl acetate extracts *in vitro* tests. *A. chloros* may potentially be considered a regulator of different cancer pathways if it has cytotoxic effects on breast cells used as a model. Hence, utilising natural substances to block these pathways, particularly marine sponges extracts like the ethyl acetate extracts of the marine sponge *A. chloros* may be a bold technique with significant therapeutic benefits for those suffering from malignancies.

The objective of this study was to assess the anti-tumour effects of ethyl acetate extract of the sea sponge *A. chloros* against MDA-MB-231, a type of breast cancer cell, using fibroblast cells as a control. The influence on Bax, Cyclin D, caspase 3, p21, C-myc, and Bcl2 expression was also evaluated. RT-PCR is employed to analyze the expression of key genes involved in apoptosis and cell cycle regulation. By assessing the cytotoxic effects of *A. chloros* extract on MDA-MB-231 breast cancer cells and comparing them with human periodontal ligament fibroblasts, this study seeks to validate the extract's potential as a therapeutic agent. Overall, this research aims to elucidate the anticancer effects of *A. chloros* extract, potentially contributing to the development of novel treatments for malignancies by targeting critical cancer pathways.

Materials and Methods

Sponge Collection and Specimen Preparation

Amphimedon chloros was collected in September 2021 from the Red Sea, Gulf of Aqaba, Jordan (29°27′ N, 34°58′ E). The sponge species was identified based on morphological character using Systema Porifera and the World Porifera Database. The *Amphimedon chloros* was ground into powder after cutting it into small parts, weighed, and freeze-dried. An equal proportion of Methanol (99.5%) and dichloromethane (99.5%) (Promega, USA) were used to macerate the freeze-dried sponge (45.0-350.0 g) for 48 hours. Then the solution was filtered and lyophilized. Then the crude extract was suspended in ethyl acetate to provide non-polar fractions. The crude extract was labelled and stored at (-20°C).[12]

Phytochemical screening using LC-MS

Liquid chromatography-mass spectrometry (LC-MS) is an analytical technique that combines mass spectrometry (MS) with liquid chromatography (LC). As the sample passes through chromatographic columns, LC separates the constituent parts. Most of the time, LC cannot positively identify the separated components on its own. Additionally, mass spectrometry is used to detect known and unknown chemicals and characterise their structures by comparison with standard compounds library.[13] The LC-MS analysis was conducted by gradient elution using a mixture of solvents as the mobile phase. Solvent A consisted of 0.1% (v/v) formic acid diluted in water, and solvent B was 0.1% (v/v) formic acid dissolved in acetonitrile. In this procedure, Agilent Zorbax Eclipses XDB-C18 columns (2.1x150 mm x 3.5 m), a temperature of 25℃, injection volumes of 1 L, and ethyl acetate sample concentration of 18 mg/mL were utilised. The masses detectors were equipped with the following components: SIL-30AC autosampler with cooler, LC-30AD pump, a CTO-30 column oven, Electrospray ion-mass spectrometer (ESI-MS) with a Shimadzu LC-MS 8030, skimmer voltage of 65 V, and a fragmentor voltage of 125 V. The extracts from the sponge were injected into the detectors. A drying agent with a purity level of 99.99% and a flow rate of 10 L/min was used under positive ions mode. In this part of the research, nebulizers operating at 45 pounds per square inch (psi) with a capillary temperature of 350°C were also used. The eluents were run through a series of mass/number of ions (m/z) analyses measuring 100 to 1000 times. Results were confirmed with the use of actual, industry-standard chemicals.

Cancer cell lines culture

Human breast cancer (ATCC HTB-26; MDA-MB-231) and periodontal ligament fibroblasts (PDL) cell lines were used in this investigation. The following culture conditions were applied to the cells: 100 µg/mL L-glutamine, 50 µg/mL gentamicin, 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) Buffer, 10% FBS (Fetal Bovine Serum), and 100 µg/mL penicillin and streptomycin.[14]

Cell harvesting and counting

The cells were cultured in incubators (Eppendorf, USA) with 5% CO_2, 37ºC, and humidification. After rinsing with 3-5 mL of phosphates buffer saline (PBS), the cells in 75 cm^2 flasks were subjected to 1-2 mL of trypsin until they separated. To ensure uniform single-cell suspensions, each cell line was given the same quantity of new media and then delicately pipetted for breaking up any clustering. In terms of frequency and cell propagation ratios, each cell line was distinct. Cells were multiplied every two or three days once the correct quantity of cells had been obtained. The cells were counted by bringing the cell suspensions to the margins of the hemacytometer counting chambers after mixing 25 µL of the collected cells with 100 µL of 4% trypan blue dye.[14]

Cytotoxicity assay

The extract from the sponge was tested for cell toxicity. Measurements of cytotoxicity were carried out based on the viability of the cells that were presented in the cultures. At a density of 1104 cells per well, cells were seeded into 96-well plates and cultured in DMEM for 24 hours at 37°C before being exposed to DMEM comprising specific doses of sponge extract for 48 hours.[15,16] Next, the solution was withdrawn, and the MTT test was carried out as described in the following: cells for each well were treated with 20 µL of MTT solutions (5 mg/mL) for 4 hours at 37°C. After that, the MTT solutions were eliminated and 200 µL of dimethyl sulfoxides (DMSO) were introduced to break down the formazan crystals that were intractable. The optical density of the cells was observed at 570 nm and 630 nm. Data were collected from wells in three replicates. The lowest antiproliferative IC_{50} values calculated were utilised to verify selective cytotoxicity using human periodontal ligament fibroblasts as the main cell cultures. The computed IC_{50} antiproliferative activities were presented as the mean values ±SD (n=3) for each experiment, carried out in triplicates.

Gene expression level assay

Cancer cells were grown in a 6-well plate with 1 µg/mL at a density of 1×10^6 cells per well. ILI, TNF, GPx, Catalases, Bax, Cyclins D1, Bcl2, and p21 gene expression fold changes were measured after 24 hours.[14,17] A List of primer sequences used in this test are presented in Table 1.

Ribonucleic Acid (RNA) Extraction and Analysis

The RNeasy Mini kits (QIAGEN, USA) were used for the RNA extraction. After being frozen at -80°C, cell pellets were reconstituted in a 500 µL lysis solution that included 2-mercaptoethanol after being thawed on ice. An equivalent volume (500 µL) of a 70% ethanol solution was added to the lysates and gently vortexed to extract any remaining cell debris. The lysates were then loaded onto RNeasy Mini spin columns and spun for 15 seconds at 10,000 rpm. The whole RNA was caught by the spin columns. The flow-through was disposed of and the collecting tubes were then reattached to the spin columns. After three iterations of the washing process, a fresh collecting tube was employed. The spin column membranes were immediately filled with 50 µL of RNase-free water, and the mixture was centrifuged (Bibby Scientific, UK) for one minute at 10,000 rpm to extract the RNA, and the isolated RNA was collected and kept at -80°C. Spectrophotometric analysis was performed on the isolated total RNA to determine its quantities and purities. A NanoDrop ND-1000 (Thermo Scientific, Wilmington, USA) was utilised to measure optical densities at 260 and 280 nm. The A260/A280 ratios of the majority of the extracted RNA samples ranged from 1.8 to 2.1.

Complementary Deoxyribonucleic Acid (cDNA) synthesis

Utilising a reversed transcriptions technique, cDNA was created (Applied Biosystem, USA). A thermocycler C 1000 (Bio-Rad, USA) was used to incubate 2 µg of total RNA with 1 µL of oligodeoxythimidines primer for 5 minutes at 65 °C. The tubes were then rapidly drained and set on ice. The accompanying reagents were used to create the 20 µL response solutions: a combination of 1.4 mL of 25 mM $MgCl_2$ and 4 mL of 10 mM deoxynucleotides triphosphates (dNTP). Avian myeloblastosis viruses reversed transcriptase (1 µL), recombinants RNasin® ribonucleases inhibitors (1 µL), reversed transcribing 10x buffers (2 µL) (AMV-RT). The following were the thermal circumstances for cDNA synthesis: The microcentrifuge tubes were centrifuged at 37°C for 30 minutes. The solution was agitated for 5 mins at 95°C as part of the denaturation procedure. The microcentrifuge tubes were kept at -80°C for further analysis following a 5-minute incubation period at 4°C. To measure and evaluate the quality of cDNA, a Thermo Scientific NanoDrop ND-1000

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spectrophotometer located in Wilmington, USA was used. The majority of the cDNA samples had ratios (A260/A280) between 1.6 and 1.8, indicating that the relative concentrations at 260 and 280 nm had been established.

Table 1. List of primer sequences used for selected genes

Gene name or symbol	Primer sequence
Cyclin D1	Forward: 5'-ACC TGA GGA GCC CCA ACA A-3'
	Reverse: 5'-TCT GCT CCT GGC AGG CC-3'
BAX	Forward:5'-TGGAGCTGCAGAGGATGATTG -3'
	Reverse: 5'- TGCCACTCGGAAAAAGACCT -3'
P21	Forward: 5'- GACCAGCATGACAGATTTC- 3'
	Reverse: 5'- TGAGACTAAGGCAGAAGATG -3'
BCL-2	Forward: 5'-AAG CCG GCG ACG ACT TCT-3'
	Reverse: 5'-GGT GCC GGT TCA GGT ACT-3'
cmyc	Forward:5'- TGAGGAGACACCGCCCAC-3'
	Reverse: 5'-CAACATCGATTTCTTCCTCATCTTC-3'
caspase-3	Forward: 5′-AGCAAACCTCAGGGAAACATT-3′,
	Reverse : 5′-CTCAGAAGCACACAAACAAAACT-3′

Relative Quantitative RT-PCR Analysis

Incubators were used to cultivate the cells, which were maintained at 37°C with 5% CO_2 and humidity. The cells in 75 cm^2 flasks were allowed to separate for 1-2 minutes after being rinsed with 3-5 mL of phosphate buffer saline (PBS). Each cell line was given an equal amount of fresh medium and then gently pipetted to break up any clustering to guarantee consistent single-cell suspensions. Every cell line is unique with respect to frequency and cell propagation ratio. After getting the right amount of cells, cell proliferation was performed every two or three days. After combining 25 µL of the collected cells with 100 µL of 4% trypan blue dye, the cell suspensions were brought to the edges of the hemacytometer counting chambers to count the cells. The PCR amplifications were performed using the IQ5 multicoloured real-time PCR detection instrument (Bio-Rad, USA). Melting curves ranging from 70 to 95°C indicated the completion of each phase. Using the genes of glyceraldehydes-3-phosphate dehydrogenases (GAPDH) as an internal reference, the expressions of the investigated genes were normalized. The effectiveness of the PCR reactions was evaluated using the linear calibration approach for relative quantifications. The Pffafl technique was utilised to compute the mRNA expression results. All tests were performed in triplicates.

Statistical analysis

The data obtained from three to four separate experiments were expressed as means and standard deviations (SD). To evaluate the differences between the groups, an analysis of variance (ANOVA) was performed using GraphPad Prism 8 (GraphPad Software, Inc., 2018). Dunnett's post hoc test was then used to evaluate differences between the therapy categories and the control group. For every analysis, a p-value of less than 0.05 was deemed statistically significant. Furthermore, a very significant difference was considered to be shown by a p-value of less than 0.001.[18,19]

Results and discussion

The chemical composition of *A. chloros* extract was characterised using LC-MS. As shown in Table 2, fourteen compounds were identified. The results showed that *A. chloros* extract contains zamamidine (11.4%), keramamine (10.2%), methoxyhexadecanoate (9.1%), pyrinodemin (8.9%), kermaphidin (8.7%), nakinadine (8.1%), purine (7.1%), tricosenal (6.4%), and hydroxytricosanoic acid (5.1%). Tricosanoic acid, pentacosenic acid, and hachijodine were identified at lower concentrations. It has been established that *Amphimedon chloros*, classified under the following categories: Kingdom Animalia, Phylum Porifera, Class Demospongiae, Subclass Heteroscleromorpha, Order Haplosclerida, and Family Niphatidae, is a significant source of bioactive metabolites.[20] *Amphimedon* is made up of several species, including viridis, compressa, complanata, and terpenensis, as well as a few uncharacterized species. The *Amphimedon* genus is a rich source of secondary compounds with varied chemical categories such as alkaloids, cerebrososides, ceramides, and terpenes, all of which have valuable biological activities.[20] *Amphimedon* sp., a marine sponge, has been collected in several parts of Japan.[21,22] Through the biological investigation of *Amphimedon* fatty acids and alkaloids, several substances with antitrypanosomal, antibacterial, and anticancer properties have been encountered.[23–25] Previous literature[25,26] includes a variety of chemical and biological research, with a special emphasis on diverse extracts of species within the genus Amphimedon that have not yet been characterised. The results of this research have demonstrated that Amphimedon has an abundant variety of natural products. These natural products include alkaloids, such as manzamine alkaloids, purine-based alkaloids, pyridine-based alkaloids, 3-alkylpyridine glycosides, and macrocyclic lactones/lactams. Furthermore, ceramides and cerebrosides have been determined to be derived from sea sponges that belong to the genus Amphimedon. In addition to that, there have been reports of fatty acids from Amphimedon species.[21,25,27–30]The impact of varying amounts of *Amphimedon chloros* sponge ethyl acetate extract on fibroblasts was examined as a control experiment (Figure 1). None of the concentrations utilised resulted in a definite suppression of the cell viability, except the 50–200 µg/mL concentrations, which exhibited extremely slight effects and were therefore unaffected by this extract. The MDA-MB-231 cell lines were treated with the same quantities of *A. chloros* sponge ethyl acetate extract; however, when concentrations (50-200 µg/mL) were utilised, a promising result was observed in reducing the viability percentage of MDA-MB-231 cells to more than 80% (Figure 2). The results exhibited a dose-dependent decrease in cell viability as signified by cell death. As shown by the MTT assay (Figures 1 and 2), the extract of *A. chloros* showed a high level of cytotoxicity against MDA-MB-231 cancer cells, with an IC_{50} value of 3.0 µg/mL. However, it did not have any cytotoxic impact on the human periodontal ligament fibroblasts (HPLFs) cell lines, which served as the control during the experiment. The *Amphimedon* genus is a rich source of secondary compounds with varied chemical categories such as alkaloids, cerebrososides, ceramides, and terpenes, all of which have valuable biological activities.

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Table 2. LC-MS analysis of *Amphimedon chloros* extract

#	Compound Name	Chemical Structure	M/Z	%
1	Purine	$C_5H_4N_4$	120.1	7.1
2	Methoxyhexadecanoate	$C_{18}H_{36}O_3$	300.5	9.1
3	Hachijodine	$C_{19}H_{34}N_2O$	306.5	3.4
4	Tricosenal	$C_{23}H_{44}O$	336.6	6.4
5	Keramamine	$C_{23}H_{33}N_3$	351.5	10.2
6	Tricosenoic acid	$C_{23}H_{44}O_2$	352.6	4.1
7	Pentacosenal	$C_{25}H_{48}O$	364.6	3.5
8	Hydroxytricosanoic acid	$C_{23}H_{46}O_3$	370.6	5.1
9	Kermaphidine	$C_{26}H_{40}N_2$	380	8.7
10	Pentacosenic acid	$C_{25}H_{48}O_3$	396.6	3.2
11	Ircinol	$C_{26}H_{40}N_2O_2$	412.6	6.4
12	Pyrinodemin	$C_{37}H_{57}N_3O$	559.9	8.9
13	Nakinadine	$C_{27}H_{40}N_2O_2$	424.6	8.1
14	Zamamidine	$C_{49}H_{60}N_6O$	749	11.4

It has been demonstrated that growth factors can encourage the formation of tumours in animals used for experiments. Furthermore, some observational studies have demonstrated that there is a correlation between the effect of various growth factors and the likelihood of an increased death rate due to cancer.[31] The effects of various concentrations of *A. chloros* sponge ethyl acetate extract on the growth of normal cells, fibroblast cell lines, and breast cancer cell lines were evaluated using the MTT assay. Cell viability was unaffected by any of the dosages utilised below 50 µg, while concentrations between 50 and 200 µg/mL had minimal effects with less than 20% inhibition.

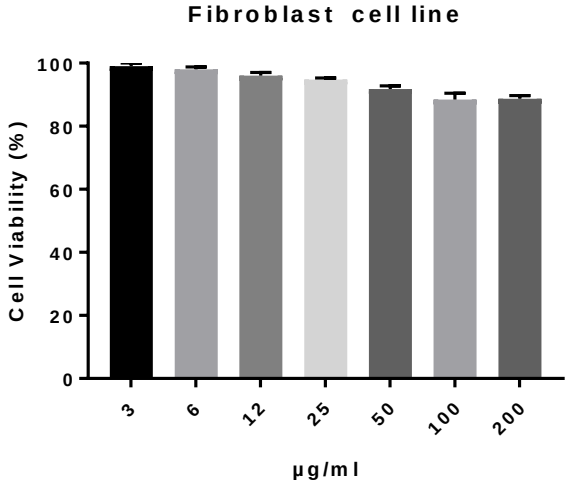

Figure 1: The antiproliferative impact of various concentrations of *Amphimedon chloros* on the Fibroblast cell line investigated using the MTT test. The result is a representation of the proportion of viable cells present under various concentrations of *Amphimedon chloros* extract

A. chloros sponge ethyl acetate extract was applied to the MDA-MB-231 cell lines at the same doses. A noteworthy result was that the viable percentage of the MDA-MB-231 cells dropped to more than 80%, especially at extract concentrations of 50–200 µg/mL extract. A concentration of 3 µg/mL of *A. chloro* extract was employed in gene expression investigations since it was determined to have the least negative impact on cell viability and to minimize any toxicity that cells might experience from exposure to *A. chloro* extract.

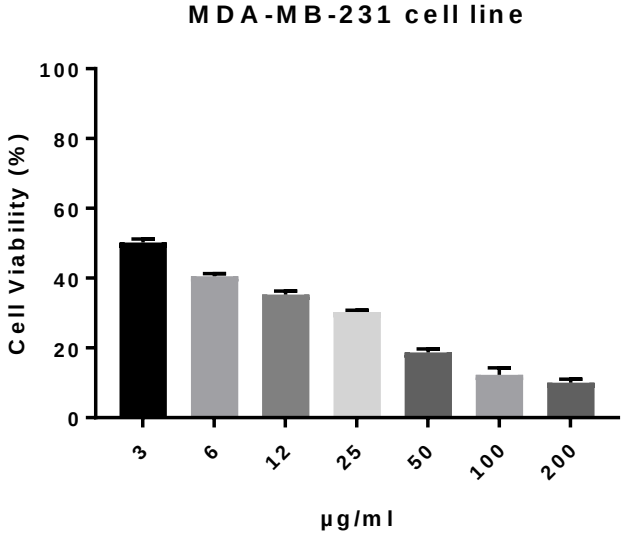

Figure 2: The antiproliferative activity of various concentrations of *Amphimedon chloros* on the MDA-MB-231 cell line evaluated by MTT assay. The result is a representation of the proportion of viable cells present under various concentrations of *Amphimedon chloros* extract

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Similarly, the effect of *Amphimedon chloros* extract on the expression of caspase 3, BCL2, BAX, P21, c-Myc and Cylin D1 in the MDA-MB-231 cell line was evaluated. Interestingly our findings indicated that spongs directly suppressed cell proliferations of breast cancer cell lines (Figure 3). Therefore, further investigation was carried out on the expression of the genes Bax, Cyclin D, caspase 3, P21, C-myc, and Bcl2 in MDA-MB-231 cell lines that had been exposed to sponge extracts to understand the processes underlying the suppressive effect of spongs on cancer cell proliferation. Under the impact of sponge extract, there was an overexpression of the genes Bax, p21, and caspase 3, as well as a downregulation of Bcl2 and Cyclin D. The study revealed that sponges can directly suppress the multiplication of breast cancer cell types. It is possible that the augmentation of apoptosis, which is accomplished by counteracting the anti-apoptotic action of growth factors, could be responsible for this suppressive impact on the further development of breast cancer. Several downstream signaling pathways, including PI3K/Akt and MAPK pathways, are frequently activated by these growth factors.

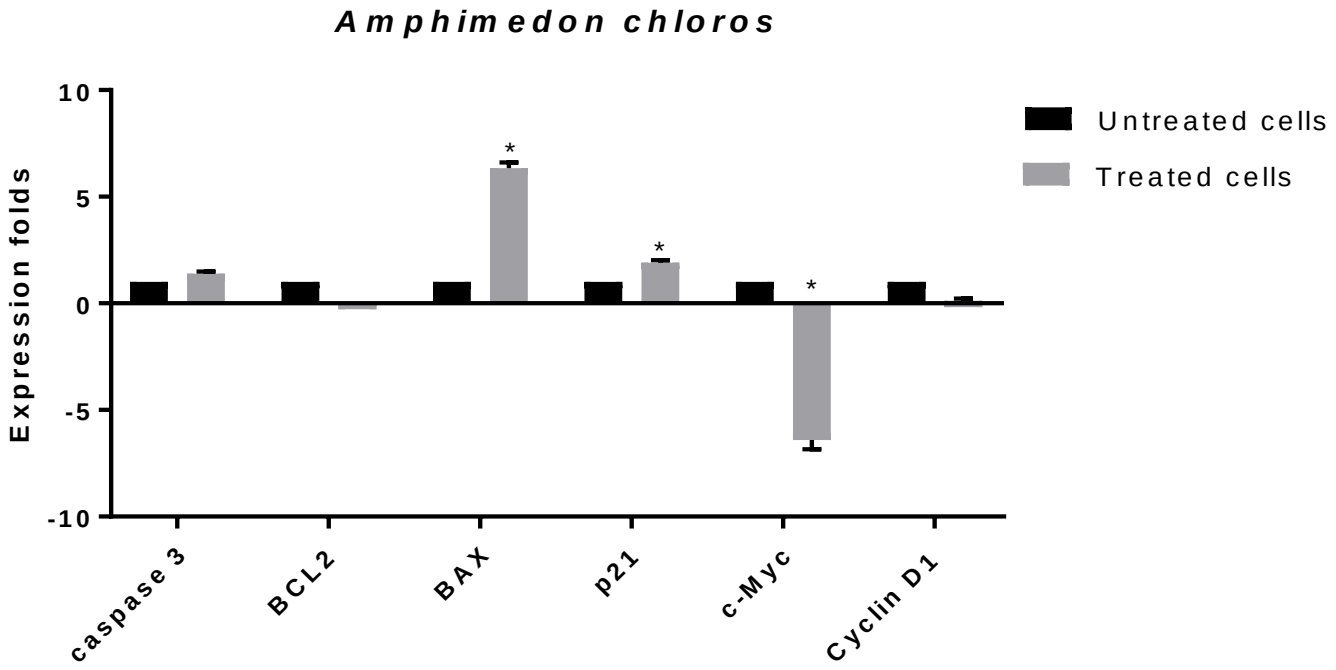

Figure 3: Effect of *Amphimedon chloros* extract on the expression of caspase 3, BCL2, BAX, P21, c-Myc and Cylin D1 in MDA-MB-231 cell line (IC_{50} values). Results were expressed as mean ± SD. * indicates a significant change from control untreated cells for p-values less than 0.05

These pathways are responsible for regulating the cell cycle and inhibiting apoptosis.[32–34] Therefore, further investigation was carried out on the expression of the genes Bax, Cyclin D, caspase 3, P21, C-myc, and Bcl2 in MDA-MB-231 cell lines that had been exposed to sponge extracts to understand the processes underlying the suppressive effect of spongs on cancer cell proliferation. Results showed overexpression of the genes Bax, p21, and caspase 3, as well as a downregulation of Bcl2 and Cyclin D. Based on these findings, it appears that the sponge extract may be able to suppress downstream growth factors signaling pathways, such as the PI3K/Akt and MAPK pathways. Following treatment with the sponge extract, the expression levels of genes such as Bax, p21, and caspase 3 were found to be elevated. On the other hand, the expression levels of Bcl2 and Cyclin D were found to significantly decrease. Taking into consideration these findings, it is evident that the extract has the potential to inhibit the activation of growth factor signaling pathways, specifically the PI3K/Akt and MAPK pathways.[33,35–38] The combined application of PI3K/Akt or MAPK/Erk inhibition has been shown to have a greater influence on tumour growth by forming a negative feedback loop via mTORC1/S6K1 activation and lowering mTORC2 kinase activity toward Akt. It has been observed that IGF-1 promotes beta-catenin migration from the cell membrane to the cytoplasm by inhibiting GSK (Glycogen Synthase Kinase) via both the Akt and MAPK pathways, thereby preventing beta-catenin proteasomal destruction.[39] Thus, inhibiting GSK causes beta-catenin to stabilise and accumulate in the cytoplasm, which is subsequently shuttled into the nucleus and regulates target gene expression.[40] According to Cheng and coauthors,[41] beta-catenin has also been linked to DNA synthesis and cell cycle progression via the G1/S transition by upregulating the expression of p21 and Cyclin D.[41] Moreover, inhibition of GSK induces mitochondrial membrane permeability, which in turn increases the production of proapoptotic genes including caspase 3 and Bax, promoting apoptosis.[42]

Conclusion

According to the findings of this research, the extract of *A. chloros* possesses cancer growth inhibitory potential by inducing apoptosis in tumours and suppressing the overactivation of PI3K/Akt induced by growth factors. Future research would focus on several key areas to build upon these findings. *In vivo* studies are needed to assess the efficacy and safety of *A. chloros* extract in animal models, providing valuable insights into its therapeutic potential. Additionally, a deeper investigation into the molecular mechanisms through which the extract affects the PI3K/Akt pathway and other signaling pathways could uncover further targets and mechanisms of action.

Conflict of Interest

All 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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