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Research Article

Apoptotic Potential of Bioactive Fraction Isolated from *Aliidiomarina taiwanensis* KU31894 against Skin (A375) Cancer Cell lines

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ABSTRACT

In the present study, the marine bacteria *Aliidiomarina taiwanensis* KU31894 was used. The bioactive secondary metabolites extracted from marine bacteria *A. taiwanensis* KU31894 were found to exhibit anticancer activity. The cell-free supernatant of the bacterial culture was extracted with ethyl acetate and showed effective cytotoxicity against skin cancer (A375) cell line, with IC_{50} concentration of 83.01 ± 1.6 µg/mL by MTT assay. Bioassay-guided fractionation of the ethyl acetate was done by silica gel column chromatography using gradients of the solvent system (chloroform: methanol). Fraction 7 exhibited cytotoxicity, with an IC_{50} concentration of 59.13 ± 2.53 µg/mL. The bioactive fraction treated cancer cell lines showed morphological changes such as cell shrinkage, rupture of the nuclear membrane, and chromatin condensation was visualized by AO/EB staining. Flow cytometric analysis of treated cancer cells showed a decreased concentration of DNA in the S phase and results in G0/G1 phase arrest. Apoptotic-related gene expression studies of treated cells illustrated the increased expression of pro-apoptotic gene Bax and tumor suppressor gene p53 and decreased expression of anti-apoptotic genes (Bcl-2 and Bcl-xL). These are the primary molecular mechanism following the implementation of apoptosis. The results concluded that *A. taiwanensis* KU31894 exhibit significant anticancer activity against skin cancer (A375) cells.

Introduction

Skin cancer results from the uncontrolled proliferation of skin cells, and exposure to intense UV rays produces DNA mutations, which is one of the main reasons.[1] The three major types of skin cancer are melanoma (which occurs in pigment-producing cells), squamous cell carcinoma, and basal cell carcinoma. According to numerous studies, a wide diversity of marine microorganisms can produce a diverse range of bioactive secondary metabolites with a range of activities, including antibacterial, anticancer, and anti-inflammatory effects.[2] In recent years, invertebrates, fungi, actinomycetes, marine bacteria, and cyanobacteria have been used to isolate and extract novel bioactive secondary metabolites. The microorganisms allow sustainable production of large quantities of secondary metabolites with reasonable cost by large-scale cultivation and fermentation.[3] Secondary metabolites produced by marine microorganisms have a significant medicinal value.[4] The microbes must create appropriate molecules as a component of their defense system to survive in the hostile marine environment, such as anticancer, antioxidant, and antibacterial substances.[5] A significant discovery for the scientific community could be new physiologically active natural compounds and metabolites from marine microorganisms with high anticancer effects and no harm to normal cells.[6]

The coastal region is the perfect place to gather a variety of valuable species that produce bioactive substances with various complicated and new chemical entities.[7] These structures can be chemically altered and enhanced by understanding the structure-activity connection,

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mechanism of action, drug metabolism, molecular modeling, and combinatorial chemistry studies.[8] These chemicals' ability to induce apoptosis is a crucial target for anticancer therapy.[9] The metabolites derived from marine bacteria are found to exhibit apoptotic potential.[10]

Our previous study established the anti-proliferative and apoptotic potential of *A. taiwanensis* KU31894 against human liver cancer (HepG2) cell lines.[11] The present study investigates the cytotoxic capability of marine microbial extracts against skin cancer cell lines. Hence, our study was the first attempt to explore the bioactive perspective of *A. taiwanensis* KU31894 extracts from the marine environment of Thrissur, Kerala, India, for its anticancer activity against skin cancer.

Materials and Methods

Microorganism

Bacteria, *A. taiwanensis* KU31894, isolated from the marine environment of Thrissur, Kerala, India, available in the culture collection of microbial biotechnology lab, School of Biosciences, Mahatma Gandhi University, Kottayam, Kerala, was used. Phenotypic and molecular identification of the bacteria was made as described earlier.[11] and the 16Sr RNA sequence was deposited in the GenBank with accession number KU31894.

Microbial Production and Extraction of Bioactive Compounds

The microorganism was cultured large-scale in Zobell›s marine broth 2216 (Hi-Media) and incubated at 37°C for 5 days. The secondary metabolites from the culture supernatant were extracted by using an equal amount of ethyl acetate. Then the filtrate and organic solvent were mixed thoroughly by shaking them in a 1000 mL capacity separating funnel and allowed to stand for 1-hour. Two layers were separated; the aqueous layer and the organic layer, which contained the solvent and the bioactive compounds.[12] The extraction was repeated three times, and the organic layer was concentrated by evaporation of the solvent at 40°C by a rotary evaporator (Cyberscan).

Anticancer Study

Cell Lines and Culture Conditions.

The cancer cell lines were initially procured from National Centre for Cell Sciences (NCCS), Pune, India, and maintained Dulbecco's modified Eagles medium (Gibco, Invitrogen). The cell line was cultured in a 25 cm^2 tissue culture flask with Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fasting blood sugar (FBS), L-glutamine, sodium bicarbonate, and the antibiotic solution containing: penicillin (100 U/mL), streptomycin (100 µg/mL), and amphotericin B (2.5 µg/mL). Cultured cell lines were kept at 37°C in a humidified 5% CO_2 incubator (NBS Eppendorf, Germany). The viability of cells was evaluated by direct observation of cells by an Inverted phase contrast microscope. The cells were maintained by routine subculturing in tissue culture flasks. The culture medium was changed routinely, and the cells were split when they reached confluence. Two-day old confluent monolayer of cells was trypsinized, and the cells were suspended in 10% growth medium; 100 µL cell suspension ($5x10^4$ cells/well) was seeded in 96 well tissue culture plate and incubated at 37°C in a humidified 5% CO_2 incubator.

MTT Assay

Cytotoxicity of the extracts was measured by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction assay.[13] Cancer cells were seeded at a density of $5x10^4$ cells per well in 96 well tissue culture plates and incubated for 24 hours. The cells were treated with different concentrations of ethyl acetate extract and fractions (6.25, 12.50, 25.00, 50.00, and 100.00 µg/mL) dissolved in 0.1% dimethyl sulphoxide (DMSO) for 24 hours at 37°C in 5% CO_2 humidified incubator. After 24 hours, 20 µL of 5 mg/mL MTT (pH: 7.4) solution was added to all the wells and incubated for 3 hours in a 5% CO_2 humidified incubator. The medium was aspirated and then added with dimethylsulfoxide (DMSO) to dissolve the purple formazan crystals. The absorbance values were measured using a microplate reader at a wavelength of 540 nm (Elisa Reader- Erba, Germany). The entire plate was observed by an inverted phase contrast microscope (Olympus CKX41 with Optika Pro5 CCD camera), and microscopic observation was recorded as images.

The percentage viability was calculated using the formula:

% of viability = Mean OD Samples x 100/Mean OD of control

Bioassay Guided Fractionation and Purification of the Extract

Bioassay guided fractionation was the repeated chromatography and corresponding bioactivity assays are performed for the identification of bioactive fraction. The silica gel column, which was used as the stationary phase, was suspended in the chloroform, and the fine particles were removed by decantation. The suspension was degassed under a vacuum to clear the air bubbles. The silica gel suspension was carefully poured into the column filled with chloroform without air bubbles and allowed to settle under gravity while maintaining a slow flow rate through the column. The column was stabilized by allowing the solvent chloroform (100%) to pass through the column bed in descending eluent flow. Pre-run was conducted, and the flow rate of the column was adjusted to 0.5 mL/min.

Sample Preparation and Application

The concentrated sample was prepared and loaded onto the silica gel column and eluted with a mixture of chloroform: methanol (v/v) at a flow rate of 0.5 mL/min.

First the column was eluted with 100% chloroform (F1). This is repeated by a 10% reduction in the volume of chloroform and a 10% increase in methanol which finally reaches an elution of 100%. All the collected fractions were screened for bioactivity.

Evaluation of Apoptosis

Acridine Orange/Ethidium Bromide Staining

The bioactive fraction was subjected to the evaluation of apoptosis by acridine orange/ethidium bromide staining. The DNA-binding dyes AO and EB (Sigma, USA) were used for the morphological detection of apoptotic cells.[14] The cells were treated with the IC_{50} concentration for 24 hours, and the cells were washed with cold PBS (pH 7.4) and then stained with a mixture of AO (100 µg/mL) and EB (100 µg/mL) at room temperature for 10 minutes. The stained cells were washed twice with 1X PBS and observed by a fluorescence microscope in the blue filter of a fluorescent microscope (Olympus CKX41 with Optika Pro5 camera).

Cell Cycle Analysis

Analysis of DNA Content and Cell Cycle Distribution using Cell Cycle Kit by Flow Cytometry

Cell cycle analysis and quantification of apoptosis in bioactive fraction treated cells were investigated by flow cytometry using propidium iodide (PI) staining of nuclear DNA. The most accurate method is directly measuring DNA synthesis through a cell cycle kit. The basic principle of the MUSE cell cycle kit is a standard ethanol fixation and detergent permeabilization, sufficient to gain access to the DNA during the active cell cycle. The kit utilizes a premixed reagent that includes the nuclear DNA intercalating stains propidium iodide (PI), which discriminates cells at different cell cycle stages based on the differential DNA content. Cell cycle analysis was determined by the method described by[15] and some modifications. The IC_{50} concentration of bioactive fraction was treated with the A375 cells for 24 hours at 37°C. The samples were then centrifuged at 3000 rpm for 5 minutes. The supernatant was removed without disturbing the pellet. After centrifugation, the cell pellet forms either a visible pellet or a white film on the bottom of the tube. The appropriate volume of PBS was added to each tube (i.e., 1-mL of PBS per 1×10^6 cells), and the contents were mixed by pipetting several times or gently vortexing. The cells were centrifuged at 3000 rpm for 5 minutes. The supernatant was discarded without disturbing the cell pellet, leaving approximately 50 µL of PBS per 1×10^6 cells. Resuspend the pellet in the residual PBS by repeated pipetting several times or gently vortexing. The resuspended cells were added dropwise into the tube containing 1-mL of ice-cold 70% ethanol while vortexing at medium speed. Cap and freeze the tube at -20°C.

DNA Staining for Cell Cycle

After the overnight incubation, the samples were centrifuged at 3000 rpm for 5 minutes at room temperature. The supernatant was removed and 250 µL PBS was added to the pellet and centrifuged. The pellet was taken after discarding the supernatant; 250 µL of cell cycle reagent was added. This was incubated in the dark for 30 minutes (which is light-sensitive). After this, it was analyzed using a flow cytometer. Gating was performed regarding untreated control cells, and samples were analyzed.

Gene Expression Analysis

Isolation of total RNA (Trizol Method)

The IC_{50} concentration of bioactive fraction was treated against A375 cells ($1X10^6$ cells mL^{-1}) for 24 hours at 37°C. A set of untreated control cells was also incubated at 37°C for 24 hours in a CO_2 incubator. After incubation, DMEM media was removed aseptically, and 200 µL of trizol reagent was added to the culture well plate and incubated for 5 minutes. To this, 200 µL of chloroform was added, and shaking was done vigorously for 15 seconds and incubated for 2–3 minutes at room temperature, followed by centrifugation at 14000 rpm for 15 minutes at 4°C. The aqueous layer was collected and 500 µL of 100% isopropanol was added. It was incubated for 10 minutes at room temperature and then centrifuged at 14000 rpm for 15 minutes at 4°C. The supernatant was discarded, and the pellet thus obtained was washed with 200 µL of 75% ethanol. It was then centrifuged at 14000 rpm for 5 minutes at 4°C. The RNA pellet was dried and suspended in TE buffer.[16]

Reverse Transcriptase PCR Analysis

Reverse transcription polymerase chain reaction (RT-PCR) is a modification of polymerase chain reaction commonly used in molecular biology to generate many copies of a DNA sequence. In RT-PCR, however, an mRNA strand is the first reverse transcribed into its DNA complement (cDNA) using reverse transcriptase, and the resulting cDNA is amplified using PCR. The cDNA synthesis was performed using Thermo scientific verso cDNA Synthesis kit (AB-1453/A). About 4 µL of 5X cDNA synthesis buffer, 2 µL of dNTP mix, 1-µL of anchored oligo dT, 1-µL of RT Enhancer, 1-µL of Verso Enzyme Mix, and 5 µL of RNA template (1 ng of total RNA) were added to an RNAse free tube. Then the total reaction volume was made up to 20 µL with the addition of sterile distilled water. The solution was mixed by pipetting gently up and down. The thermal cycler (Eppendorf Master Cycler) was programmed to undergo cDNA synthesis. The following cycling conditions were employed, 30 minutes at 42°C and 2 minutes at 95°C. The cDNA obtained was further amplified using the primers of Bcl2, Bcl-XL, BAX, p53, and β actin. The amplification was done using Thermo scientific amplification kit. The following components were added for each 50 µL reaction: 25 µL of PCR Master Mix (2X), 2 µL of Forward primer

(0.1–1.0 µM), 2 µL of Reverse primer (0.1–1.0 µM), 5 µL of Template DNA (10 pg^{-1} µg). The components were made up to 5 µL with sterile distilled water (nuclease-free). Initial denaturation at 95°C for 3 minutes, followed by denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 1-minute. The process was repeated for 35 cycles and the final extension at 72°C for 5 minutes. After the amplification, the PCR product was separated by agarose gel electrophoresis, and the stained gel was visualized using a gel documentation system (E gel imager, Invitrogen).

Statistical analyses

Statistical analyses were performed by GraphPad Prism 5.03 (GraphPad Software). The comparison of treatment groups and controls was made by means of a one-way analysis of variance (ANOVA) and Tukey›s multiple-comparison posttest. Results were designated significant when the *p-value* (p) < 0.05: * =*p* < 0.05, **= *p* < 0.01, *** = *p* < 0.001, **** = *p* < 0.0001, ns = non-significant. The results are expressed as mean ± SD.

Results

Microorganism

The microorganism identified as *A. taiwanensis* KU31894, available in the culture collection of microbial biotechnology lab, School of Biosciences, Mahatma Gandhi University, Kottayam, Kerala, was used.

Extraction of Bioactive Compounds

The extraction of bioactive metabolites was performed by using ethyl acetate as the organic solvent. The organic phase was dried and used for further assays.

Cytotoxicity Study

The ethyl acetate extract showed significant cytotoxicity against Skin (A375) cancer cells in a dose-dependent manner, and the IC_{50} value was calculated after 24 hours of exposure as 83.01 ± 1.6 µg/mL. L929 (Fibroblast) cells were used as a model for testing cytotoxicity in normal cells and showed less toxicity. The results are shown in Figs 1 and 2.

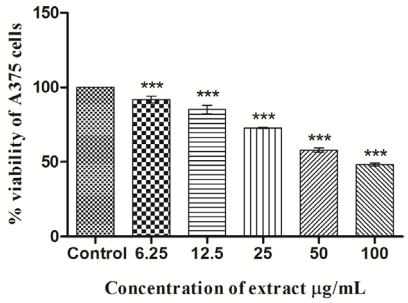

Fig. 1: Cytotoxicity of ethyl acetate extract against A375 cell line. Dose dependent inhibition of A375 cells by ethyl acetate extract. The level of significance was ***= *p* < 0.001.

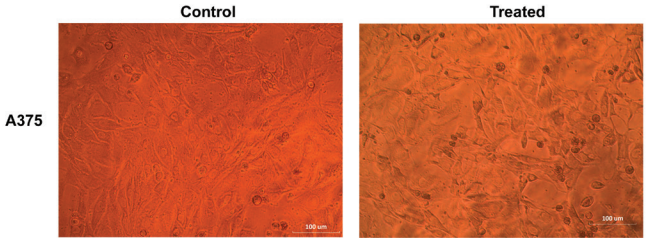

Fig. 2: Morphological observation of A375 cells treated with IC50 concentration of ethyl acetate extract. Treated cells showed rupture of cell membrane, shrinkage of cell, loss of cellular integrity and swollen nuclei, where no visible changes observed in control cells

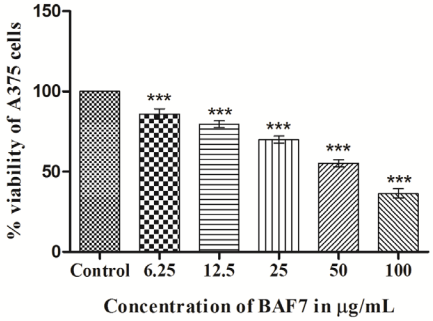

Fig. 3: Cytotoxicity of bioactive fraction against A375 cell line. Dose dependent inhibition of A375 cells by BAF7. The level of significance was ***= P < 0.001.

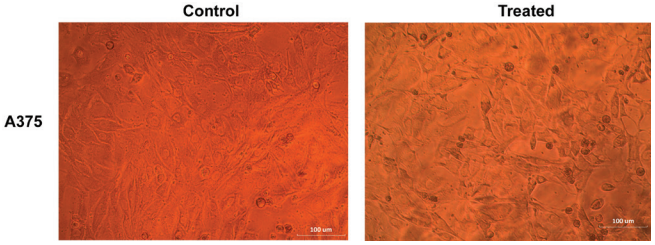

Fig. 4: Morphological observation of A375 cells treated with IC_{50} concentration of bioactive fraction. The treated cells showed shrinkage, loss of cellular integrity and swollen nuclei whereas no visible changes observed in control cells

Bioassay Guided Fractionation

The ethyl acetate extract showed prominent cytotoxicity and was subjected to bioassay-guided fractionation. The fraction 7 (F7) from the collected fractions showed significant cytotoxicity against A375 cells. The IC_{50} values were calculated as 59.13 ± 2.53 µg/mL. The results are shown in Figs 3 and 4.

Apoptotic Assay

Acridine Orange/Ethidium Bromide Staining with Bioactive Fraction Against A375 Cell Lines

A375 cells were exposed to bioactive fraction, and after one day of exposure, the cells were treated with acridine orange/ethidium bromide to determine the apoptosis. Fluorescence microscopy images showed several alterations in the morphology of cells, such as a reduction in their size and volume, contraction of the cell, blebbing of the cell membrane, chromatin condensation,

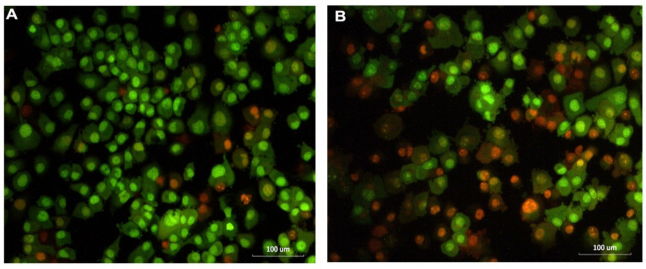

Fig. 5: Micrographs (Magnificationx400) of acridine orange/ ethidium bromide stained A375 cells; A: Untreated cells (control) have normal nucleus of green colour representing live cells B: cells exposed to IC50 concentration of bioactive fraction for 24 hrs showed bright green nucleus; condensed or fragmented chromatin signifying apoptosis.

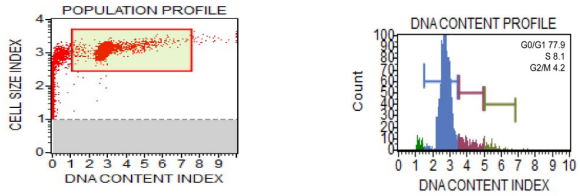

Fig. 6: A) Flow cytometric analysis of untreated A375 cells; B) DNA content of untreated cells

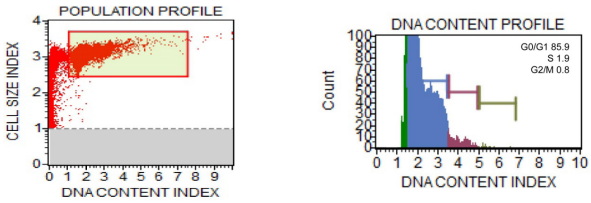

Fig. 7: A) Flow cytometric analysis of treated sample; B) DNA content of treated sample

fragmentation of the nucleus, and development of apoptotic bodies of the cell which were treated (Fig. 5)

Cell Cycle Analysis

A375 cells were treated with IC_{50} concentration of the bioactive fraction 7 and stained with propidium iodide to determine the proportion of cells undergoing cell death or apoptosis by flow cytometry. These studies reveal the distribution of cell populations across various phases of the cell cycle, such as G0/G1, S, and G2/M. The DNA content of the untreated cells is shown in Fig. 6.

The bioactive fraction treated A375 cells showed decreased concentration of DNA in the S phase region, and the results indicated the cell death and accumulation of cells in the G0/G1 phase. Hence G0/G1 phase was arrested as compared with the untreated control cells shown in Fig. 7.

Apoptotic Gene Expression by RT-PCR

Further, to understand the mechanism of apoptotic potential, the expression of significant regulators of apoptosis upon treatment with bioactive fraction was

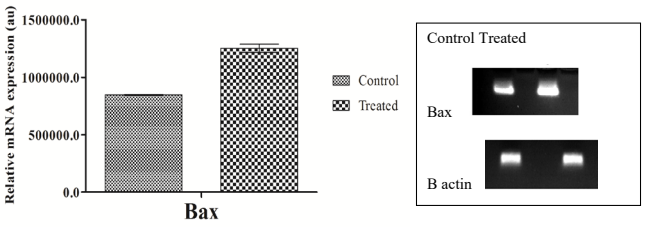

Fig 8: The relative mRNA expression of Bax was analyzed by reverse transcriptase PCR (RT-PCR), β actin was used as internal loading control.

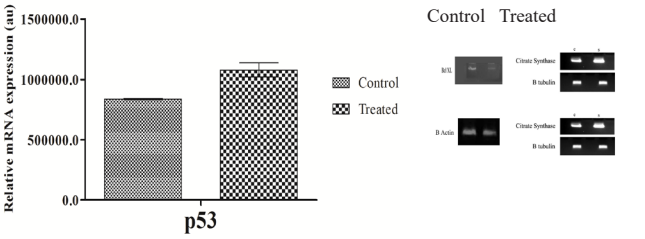

Fig. 9: The relative mRNA expression of p53 was analyzed by reverse transcriptase PCR (RT-PCR), β actin was used as internal loading control

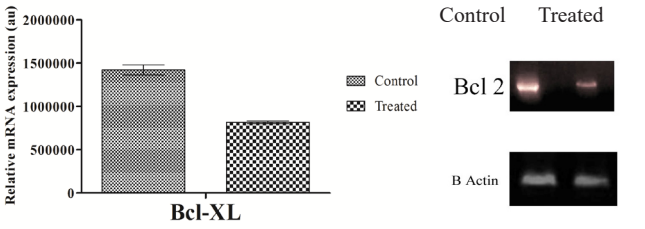

Fig. 10: The relative mRNA expression of Bcl-XL was analyzed by reverse transcriptase PCR (RT-PCR), β actin was used as internal loading control

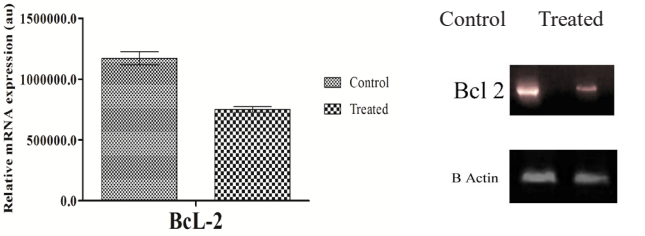

Fig. 11: The relative mRNA expression of Bcl-2 were analyzed by reverse transcriptase PCR (RT-PCR); β actin was used as internal loading control

noted. The treated sample showed increased expression of pro-apoptotic gene Bax and overexpression of tumor suppressor gene p53. From the results, it would be clear that the correlated expression of the Bax and p53 gene significantly activated the apoptotic mechanism. The decreased expression of anti-apoptotic genes such as Bcl-2 and Bcl-XL was also revealed. Suppressing the Bcl-2 and Bcl-xL genes was a significant way of apoptosis. The results are shown in Figs 8 and 11.

Table 1: Cell distribution in control cells

	G0/G1	*S*	*G2/M*	*Debris*
% Gated	77.9	8.1	4.2	63.5
Mean	2870.1	4824.1	4607.4	194.8
% CV	7.1	9.8	8.4	397.8

Table 2: Cell distribution in treated sample

	G0/G1	*S*	*G2/M*	*Debris*
% Gated	85.9	1.9	0.8	83.1
Mean	1841.3	4114.1	4988.8	39.43
% CV	19.2	10.0	9.2	429.4

Discussion

The marine bacteria *A. taiwanensis* KU31894 was used for the determination of the anticancer potential of bioactive secondary metabolites produced. It was a halophilic, aerobic, gram-negative, rod-shaped bacteria belonging to the family Idiomarinaceae, order Alteromonadales, class Gammaproteobacteria, are unusual in that they contain high levels of iso-branched cellular fatty acids.[17] The cell-free supernatant of the bacterial culture was extracted with ethyl acetate as the organic solvent.

Extraction is the process of relocation of compounds from one liquid (water) into another liquid phase (organic solvent). It consists of transferring various compounds contained in a feed solution to another immiscible solvent. The solvent that is enriched in solutes is called extract. The first step in the analysis of natural microbial products is the extraction of chemical components from the culture broth. Solvent extraction (liquid-liquid extraction) is based on the relative solubility of two immiscible solvents and is used to separate compounds.[18] Bioactive secondary metabolites can be easily extracted by using ethyl acetate. Some previous studies state that ethyl acetate extract was found to possess bioactive compounds with pharmacological properties.[19,20]

The ethyl acetate extract of MBTU_MB2 showed remarkable cytotoxicity in a concentration-dependent manner by MTT assay using A375 cells, and the IC_{50} value was calculated as 83.01 ± 1.67 µg/mL. A recent study reported that phenazine 1 carboxylic acid (PCA) produced by marine *Pseudomonas aeuroginosa* GS 33 exhibited anticancer activity against skin cancer melanoma cell line SK-MEL-2.[13]

A gradient of chloroform and methanol was partially used to purify the ethyl acetate extract by silica gel column chromatography. The seventh fraction was found to be more cytotoxic against A375 cell lines with an IC_{50} value of 59.13 ± 2.53 µg/mL. The purified ethyl acetate extract of *Streptomyces coelicoflavus* BC 01 by silica gel column chromatography using chloroform and methanol as the solvent system.[21] An antibacterial compound from marine *Streptomyces sp*. VITAK1 was purified by silica gel column chromatography.[22] Silica gel column chromatography of crude extract of *Pseudoalteromonas flavipulchra* JG1 was conducted by using chloroform: methanol gradient to yield 12 fractions.[23] The anticancer compound was extracted from a new marine bacterium, *Staphylococcus* sp. strain MB30, by silica gel column chromatography using chloroform and methanol as solvent system.[24]

The bioactive fraction was subjected to AO/EB staining to determine the apoptotic model of cell death. The treated group showed morphological characteristics such as cell shrinkage, chromatin condensation, and formation of apoptotic bodies when compared to the untreated control. The same results trend was observed when the 2,4-diacetyl phloroglucinol (DAPG) was isolated from the *P. aeruginosa* strain, and treated against lung cancer cells.[25] Several genes, such as the pro-apoptotic gene (Bax), p53, and anti-apoptotic genes (Bcl-2 and Bcl-xl), are involved in the apoptotic pathways. Overexpression of anti-apoptotic genes has been implicated in different carcinomas. The marine bacterial secondary metabolites can induce the up-regulation of pro-apoptotic genes and down-regulation of anti-apoptotic genes.[24] In our study, the apoptotic-related gene expression patterns of the bioactive fraction-treated cancer cells were noted. The results of apoptotic-related gene expression studies indicated that the treated sample showed increased expression of the pro-apoptotic gene (Bax) and overexpression of p53 related to the induction of apoptosis. The decreased expressions of anti-apoptotic genes (Bcl-2 and Bcl-xL) were also revealed. The results evidenced that the apoptotic induction by the fraction triggered the upregulation of pro-apoptotic proteins and the downregulation of anti-apoptotic proteins. The initiation of apoptosis depends upon the balance between pro-apoptotic and anti-apoptotic proteins.[26] The early reports disclosed that the downregulation of anti-apoptotic Bcl-2 family proteins was observed upon treatment with the novel anticancer metabolite from *Staphylococcus sp.* against lung cancer (A549) cells.[24] The activated Bcl-2 pro-apoptotic proteins (Bax and Bak) increased the outer membrane permeability of mitochondria, facilitating the release of apoptogenic factors into the cytosol. The Bax expression in the cancer cells increased significantly upon the treatment of 2,4-diacteylphloroglucinol (DAPG) isolated from *P. aeruginosa*.[25]

The capability to initiate cell cycle arrest in cancer cells is a vital characteristic of a probable anticancer agent.[27] The molecular mechanism behind the apoptotic potential of a particular bioactive compound was determined by the cell cycle analysis (cell cycle arrest). Our research revealed that the treatment of cancer cells with bioactive fraction arrested major cells at the G1 phase of the cell cycle and reduced DNA content at the S phase. A similar result was observed as the anticancer bioactive compound produced by *Staphylococcus* sp arrests the cell cycle at the G1 phase and induced apoptosis.[24] The bacterial polysaccharide EPS11 induced apoptosis in lung cancer cells (A549) and was confirmed by cell cycle arrest detected in the flow cytometry apparatus.[28]

Conclusion

This study showed that the marine bacteria *A. taiwanensis* KU31894 was an immense producer of therapeutically valuable anticancer compounds. The compounds present in the partially purified bioactive fraction can be used as a potential apoptotic agent against skin cancer.

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