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ANTIPROLIFERATIVE AND APOPTOTIC POTENTIAL OF MARINE BACTERIA *ALIIDIOMARINA TAIWANENSIS* KU31894 ON HUMAN HEPATOCELLULAR CARCINOMA (HEPG2) CELL LINE

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ABSTRACT

Aim: *To determine the antiproliferative and apoptotic effects of marine bacterium, Aliidiomarina taiwanensis KU31894 on human hepatocarcinoma (HepG2) cell lines.* ***Methods:*** *The bacterium was identified by 16S rDNA analysis and the bioactive metabolites were extracted. The cell viability and proliferation were done by trypan blue exclusion and MTT assay against HepG2 cells. The bioassay guided fractionation of the promising extract was performed by silica gel column chromatography. Apoptotic evaluation was done by acridine orange/ethidium bromide staining and confirmed by DNA fragmentation assay. ROS intermediates generation was determined by DCF-DA staining.* ***Results:*** *The bacterium was identified as Aliidiomarina taiwanensis and the sequences were submitted in GenBank under the accession number KU31894. The ethyl acetate extract showed maximum cytotoxicity by trypan blue exclusion method was selected for MTT assay, the IC_{50} concentration was obtained as 75.02 ± 1.7µg/ml. After the fractionation, the fraction F7 showed significant cytotoxicity by MTT assay with the IC_{50} concentration of 48.75 ± 1.5µg/ml. The acridine orange /ethidium bromide staining of the IC_{50} concentration of bio active fraction F7 treated cancer cells exhibited apoptotic characteristics and also showed DNA fragmentation. The high levels of ROS intermediates as evidenced by DCF-DA staining could have played a significant role in the apoptotic induction.* ***Conclusion:*** *Our results demonstrated that the proliferation of cancer cells was significantly reduced with the treatment of bioactive fraction F7 of ethyl acetate extract. Our study concluded that the marine bacteria Aliidiomarina taiwanensis KU31894 has the ability to produce anticancer bioactive metabolites.*

KEY WORDS

Aliidiomarina taiwanensis KU31894; anticancer activity; bioactive compounds; ethyl acetate extract; Marine bacteria

1. INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common malignancy of the liver and is a leading cause of cancer mortality. It is a complex and heterogeneous tumour with reduced prognosis and occurs primarily in patients with chronic liver disease and cirrhosis [1]. The hepatitis B and C virus infection is the common cause of chronic liver disease that eventually leads to liver cancer [2]. Several kinds of treatments might be useful for HCC, such as surgical resection, percutaneous ethanol injection, liver transplantation, transarterial chemoembolization, and radioembolization. Moreover, patients with HCC usually exhibit poor tolerance to systemic chemotherapy because of their unusual liver

function [3]. The progress of drug resistance in hepatocellular carcinoma cells after drug therapy points out the need for the detection of innovative anticancer agents. Therefore, the use of natural products in this respect is expansively under study [4]. Evidently, many scientific societies are now involved in finding out novel anti cancer drugs from natural products for advance chemotherapy and to increased survival rates [5].

In recent years, marine microorganisms have attracted great consideration in the pharmaceutical community as they produce a broad diversity of structurally unique and pharmacologically dynamic metabolites [6]. Extreme habitats provide marine microorganisms with unique physiological and metabolic capabilities, which may not be attributed by their terrestrial counterparts [7]. Different kinds of marine microbial secondary metabolites with remarkable bioactivities such as antimicrobial, antiviral, antitumor or cytotoxic activity were isolated [8]. Bioactive diversities of marine microbial metabolites are probable resource for the discovery of new anticancer drugs and provide enormous applications in pharmaceutical and biomedical research [9]. So far, a significant number of anticancer compounds have been isolated from marine microbes but only a few have been applied in clinical trials and approved for the treatment of different types of cancers.

The present research work communicates, the inhibition of the proliferation of human hepatocellular carcinoma cell line (HepG2) by the bioactive compounds isolated from the marine bacteria *Aliidiomarina taiwanensis* KU31894. The isolate showed promising cytotoxic and apoptotic activity due to the presence of various bioactive compounds.

2. MATERIALS AND METHODS

2.1 Identification of marine bacterium

Marine bacteria designated as MBTU_MB2, isolated from the sea sediments (position Lat 10˚ 21’ 40.64’’N, Lon 76˚ 6’ 18.30’’ E depth 10 m); collected from the sea coast area of Thrissur, Kerala, India was used in the present study. Colony morphology and effect of physiological parameters such as salt concentration, temperature, pH on the growth of the bacteria were recorded [10]. Biochemical characterization of bacteria was determined by using Analytical Profile Index (API)-20 E test strips (bioMerieuix). Molecular identification of the isolate was performed after isolation of the genomic DNA using the method described for gram negative bacteria [11]. The primers used for the amplification of 16s rDNA were 27F (5^1AGAGTTTGATCMTGGCTCAG 3^1) and 1492R (5^1 AAGGAGGTGWTCCARCC 3^1) [12]. PCR was carried out by the thermal cycler under the following conditions- An initialization step at 94ºC for 4 min followed by 30 cycles of 94ºC for 1 min, 55^{0}C for 1 min, 72^{0}C for 2 min followed by final extension at 72ºC for 1 min and holding temperature at 4ºC for 1 min. The amplicon sequences were analyzed using BLAST tool to get the relative identification of each bacterial species [13]. All the sequences were aligned using the multiple sequence alignment program CLUSTAL W [14] and was used for phylogenetic analysis using the MEGA 6 programs for calculating the multiple distance matrixes [15]. The multiple distance matrix obtained was then used to construct phylogenetic trees using neighbor joining method [16]. The sequence was deposited in GenBank.

2.2 Microbial production and extraction of Bioactive Compounds

The isolate was inoculated into 10 ml of Zobell’s marine broth 2216 (HIMedia) and kept in rotary shaker at 120 rpm for 48 hours at 37^{0}C. After incubation the culture was centrifuged at 10000 rpm for 10 minutes at 4^{0}C, the pellet was collected, washed with PBS (phosphate buffer saline), resuspended in 1 ml PBS, and transferred into 500 ml of the same broth. The cell suspension was kept in rotary shaker at 120 rpm for 7 days at 37^{0}C for the production of secondary metabolites. After fermentation the culture broth was centrifuged at 10000 rpm for 10 minutes at 4^{0}C. The culture supernatant obtained was used for extraction of extracellular compounds by liquid-liquid extraction method using equal amount of organic solvents such as Hexane, Chloroform and Ethyl acetate. Filtrate and organic solvent was mixed thoroughly by shaking them in 1000 mL capacity separating funnel and the mixture was then allowed to stand for 30 min. Two layers were formed; the aqueous layer and the organic layer, which contained the solvent and the bioactive compounds. The organic layer was further concentrated by evaporation under vacuum to the least volume [17]. The concentrated extracts were then subjected to cytotoxicity study.

2.3 Anticancer study

2.3.1 Cell Lines and Culture Conditions

HepG2 (Liver cancer / Hepatocellular carcinoma) cell line was initially procured from National Centre for Cell Sciences (NCCS), Pune, India and maintained in Dulbecco's modified Eagles medium (DMEM) (Gibco, Invitrogen). The cell line was cultured in DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate and 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^{0}C in a humidified 5% CO_2 incubator (NBS Eppendorf, Germany).

2.3.2 Cell viability assay

The trypan blue dye exclusion assay (Sigma-Aldrich T8154) is a quantitative method to determine the percentage viability of cells. Briefly, 1×10^4 HepG2 cells were seeded into 96-well plates and treated with different concentration of extracts (6.25, 12.50, 25.00, 50.00, and 100 µg/mL dissolved in 0.1% DMSO) in triplicates and incubated for 3 hours at 37^{0}C. Then, 20 µL of suspension was mixed with equal volume of 0.4% trypan blue and was counted by Neubauer hemocytometer by clear-field microscopy. The stained cells were counted, and percentage of cell death was calculated [18]. Cell counts were expressed as mean ± standard deviation (SD). The most active extract was subjected to MTT assay for long term cytotoxicity evaluation.

2.3.3 Cell proliferation assay

Cytotoxicity of the active extracts was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction assay. Cancer cells (HepG2) were seeded at a density of 5x10^4 cells per well in 96 well tissue culture plate and incubated for 24 hours. The cells were treated with different concentrations of extract (6.25, 12.50, 25.00, 50.00, and 100 µg/mL dissolved in 0.1 % DMSO) in triplicates for 24 hours at 37^{o}C in 5% CO_2 humidifier incubator. After 24 hours, 20 µL of 5mg/ml MTT (pH: 7.4) solution was added to all the wells and incubated for 3 hours. The medium was aspirated and then added with DMSO to dissolve the purple formazan crystals [19]. The absorbance values were measured by using microplate reader at a wavelength of 540 nm (ELISA Reader- ERBA, Germany). Entire plate was observed by an inverted phase contrast tissue culture microscope (Olympus CKX41 with Optika Pro5 CCD camera). The IC_{50} value was calculated by using GraphPad prism software.

2.4 Separation and bioassay guided fractionation

The separation of the most promising extracts was performed by High performance thin layer chromatography (HPTLC) using Solvent systems such as Chloroform: Methanol (7:3; v/v). 15µL of extracts dissolved in methanol and spotted on TLC plate (5.0 x 10.0 cm) [20]. The separated bands were visualized under UV-illuminator and the Retention factor (Rf) value was calculated by using CAMAG TLC Scanner. The extract was then subjected to fractionation on a glass column with silica gel (60– 120 mesh) and the column was packed as per the wet packing system. The extract (500 mg) was adsorbed on silica gel and gently layered on top of the column. Step by step elution was done with chloroform-methanol as solvent system standardized by HPTLC. First the column was eluted with 100% chloroform (Fraction 1). This step was repeated by reducing the chloroform by 10% in each fraction and the methanol was increased by 10% in each fraction until percentage of methanol was 100%. Twelve fractions were collected (each of 30 mL) and selected for determining cytotoxicity by trypan blue exclusion method described earlier. The bioactive fraction obtained was used for further analysis of cytotoxicity by MTT assay as described earlier and the apoptotic potential was also determined by AO/EB staining as described below.

2.5 Acridine Orange/Ethidium Bromide Staining

DNA-binding dyes Acridine Orange (AO) and Ethidium Bromide (EB) (Sigma, USA) were used for the morphological detection of apoptotic cells. After treating HepG2 Cells with the IC_{50} concentration of bioactive fraction for 24 hours, the cells were washed with cold PBS (pH 7.4) and stained with a mixture of AO (100 µg/mL) and EB (100 µg/mL) at room temperature for 10 min [21]. The stained cells were washed twice with PBS and observed using a fluorescence microscope in blue filter of fluorescent microscope (Olympus CKX41 with Optika Pro5 camera).

2.6 DNA Fragmentation Analysis

DNA fragmentation assay was performed to confirm the apoptotic mode of cell death. HepG2 cells were treated with IC_{50} concentration of bioactive fraction for 24 hours. After treatment, cells were trypsinized and collected with PBS. 100 µL of lysis buffer was added to the pellet and incubated for 30 min on ice. After incubation, centrifugation was carried out at 10000 rpm for 30 min at 4^{0}C. The supernatant was collected and

mixed with 25:24:1 mixture of phenol:chloroform:isoamyl alcohol and precipitated with two equivalents of ice cold ethanol and one-tenth equivalent of sodium acetate. This was followed by centrifugation at 12,000g for 20 minutes. The pellet was resuspended in 30 µL of RNase solution (15 µg/mL) and 6 µL of 6x loading dye for 30 min at 37^{0}C which was electrophoresed [22]. The fragmentation pattern was imaged in a Syngene Ingenious gel documentation system (Syngene Bioimaging Pvt. Ltd., Haryana, India).

2.7 Dichlorofluorescein Diacetate (DCF-DA) Staining

HepG2 cells were treated with the IC_{50} concentration of bioactive fraction for 24 hours. The cells were washed with PBS (pH 7.4) and stained with 10 μM of DCF-DA for 30 min at 37^{0}C and wrapped in aluminum foil. The treated cells were then washed twice with cold PBS-EDTA, collected by trypsinization and centrifugation at 1500 rpm for 5 min, and resuspended in PBS-EDTA [23]. The fluorescence intensity was recorded by using a fluorimeter at 470 nm excitation and emission at 635 nm. The Quantification of ROS Generation was also determined using the fluorimeter (Qubit 3.0, Life technologies, USA).

3. RESULTS AND DISCUSSION

3.1 Identification of marine bacterium

The marine bacterium MBTU_MB2, isolated from the sea sediments of Kerala, India was used for the present study was characterized to be halophilic, aerobic, Gram negative and rod-shaped bacteria. After incubation on Zobell's marine agar at 37^{0}C, colonies appeared as light green, circular, convex with entire edges. NaCl was required for growth and the optimum concentration was 3%, and optimum temperature for the growth was observed at 37^{0}C, and the optimum pH was 7. Biochemical characteristics determined using Analytical Profile Index (API)-20 E test strip (bioMerieuix) revealed that the bacterium is in the family *Idiomarinaceae*.

The 16S rDNA of the isolated organism was amplified by using appropriate primers with the help of thermocycler and the amplified product was subjected to agarose gel electrophoresis. The 16S rDNA was sequenced by dideoxy method and BLAST analysis was performed. The sequence obtained was deposited in GenBank with the accession number KU318394. Based on the homology of 16S rDNA sequence, strains with different sequence similarity from relative species were selected and multiple sequence alignment comparison was carried out by BioEdit program. Then, bootstrap values were calculated by neighbor-joining analysis method using MEGA 6 software, and the phylogenetic tree was constructed. The same is shown in Fig. 1. Phylogenetic analysis based on 16S rDNA gene sequences showed that the strain formed a distinct lineage within the class *Gamma proteobacteria* and was most closely related to members of the genus *Idiomarina* in the family *Idiomarinaceae* and 100% similarity towards *Aliidiomarina taiwanensis*.

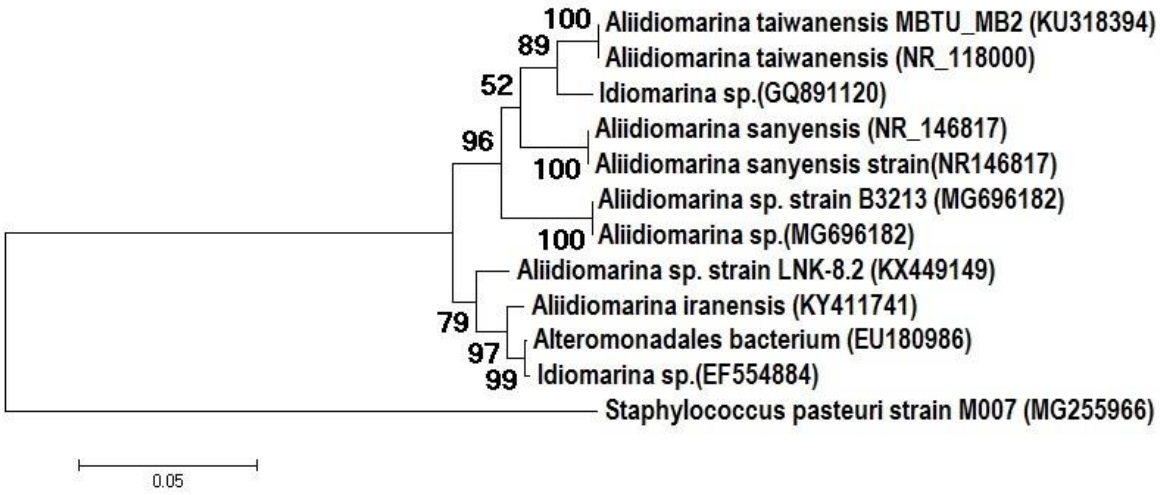

Fig 1: Phylogenetic analysis of marine bacterium MBTU_MB2

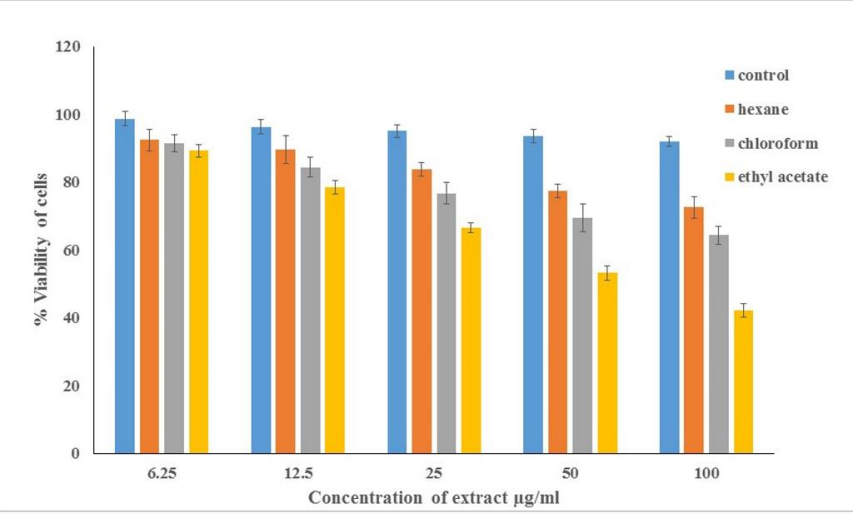

Fig 2: Cytotoxicity of extracts of *Aliidiomarina taiwanensis* KU31894 determined by trypan blue exclusion method. Values are expressed as mean ± SD.

3.2 Microbial production and extraction of bioactive compounds

The cell free supernatant obtained after centrifugation of culture broth of *Aliidiomarina taiwanensis* KU31894 was extracted using organic solvents such as hexane, chloroform and ethyl acetate. Dry weight of the extract calculated per liter of the hexane, chloroform and ethyl acetate extract, respectively, were 0.77g, 0.68g and 0.52g. The dry extracts were evaluated for their cytotoxicity against HepG2 cells.

3.3 Anticancer study

3.3.1 Cell viability assay

The short-term cytotoxicity of hexane, chloroform and ethyl acetate extract of *Aliidiomarina taiwanensis* KU31894 was determined by trypan blue exclusion method. The ethyl acetate extract showed maximum inhibition against HepG2 cells (Fig. 2) and hence this extract was selected for further studies. Whereas the non-viable cells stained blue due to their membrane damages. From the study it was revealed that the ethyl acetate extract showed maximum inhibition against HepG2 cell lines.

3.3.2 Cell proliferation assay

In vitro inhibitory activity of ethyl acetate extract of *Aliidiomarina taiwanensis* KU31894 was determined by MTT reduction assay in Hepatocellular carcinoma (HepG2) cells. Ethyl acetate extract showed promising cytotoxicity against HepG2 cells in a dose dependent manner. The IC_{50} values of ethyl acetate extract were calculated after 24 hours of exposure as 75.02±1.7 µg/mL. The results showed decreased viability and cell growth inhibition in a dose dependent manner.

3.4 Separation and bioassay guided fractionation

The ethyl acetate extract which showed cytotoxicity against HepG2 cells by trypan blue exclusion method and MTT assay was further purified. The solvent system standardization for column chromatography was carried out by High Performance Thin Layer Chromatography (HPTLC). Chloroform: methanol was used as solvent system in the ratio of 7:3 for separation, and Rf values calculated are shown in the Fig. 5. Gradients of chloroform and methanol was used for column chromatography. Twelve fractions were collected, and UV analysis was performed by UV Visible Spectrophotometer (Shimadzu) in the range of 200-800 nm. The cytotoxicity study against HepG2 cells by trypan exclusion method reveals that the fraction 7 showed reduced viability of cancer cells shown in the Fig. 5.

The bioactive fraction F7 evaluated for long term screening of cytotoxicity by MTT assay showed reduced cell proliferation in a dose dependent manner and the IC_{50} values was 48.75±1.5 µg/mL after 24 hours of exposure (Fig. 6). The lowered IC_{50} value of bioactive fraction F7 specified the purity of the fraction. Morphological changes such as membrane damage, altered cell structure and integrity were observed in a concentration dependent manner (Fig. 7). Hence it was taken for further HPTLC analysis and the Rf value of ethyl acetate extract and purified product was calculated as 0.86 (Fig. 4).

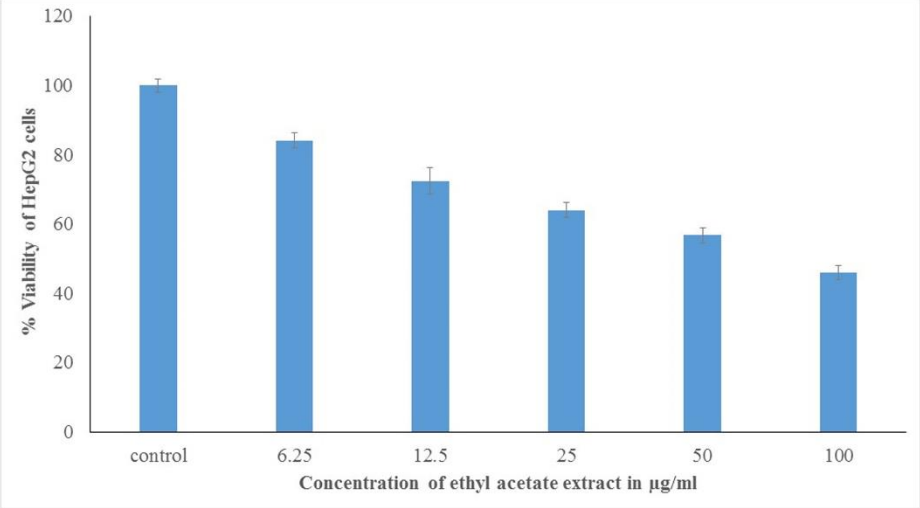

Fig 3: Inhibition of ethyl acetate extract treated HepG2 cancer cells at 24 hours of exposure.

3.5 Acridine orange/ethidium bromide staining

To realize whether the inhibition of cell proliferation by the most potent bioactive fraction F7 was because of the induction of apoptosis, it was subjected to acridine orange/ethidium bromide staining method. HepG2 cells were exposed to bioactive fraction F7 and after one day of exposure the cells were treated with acridine orange/ethidium bromide. Both viable and non-viable cells uptake AO and emits green fluorescence, EB is taken up only by non-viable cells and emits red fluorescence after intercalated into DNA. Fluorescence microscopy images showed the number of alteration in the morphology of cell such as size and volume reduction, cell shrinkage, condensation of chromatin, and breakdown of nucleus and creation of apoptotic bodies of the cell which were treated (Fig. 8). Results of AO/EB clearly indicates that bio active fraction F7 enhance the apoptosis at IC_{50} dose (48.75±1.5 µg/mL).

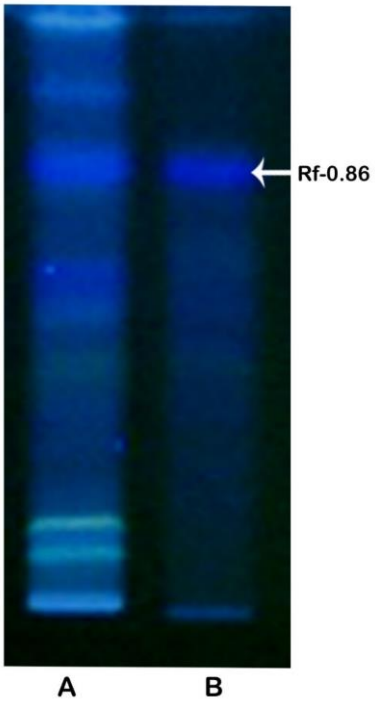

Fig 4: HPTLC chromatogram of ethyl acetate extract and bioactive fraction F7. A. ethyl acetate extract B. bioactive fraction 7. Showing Rf value of the purified metabolite.

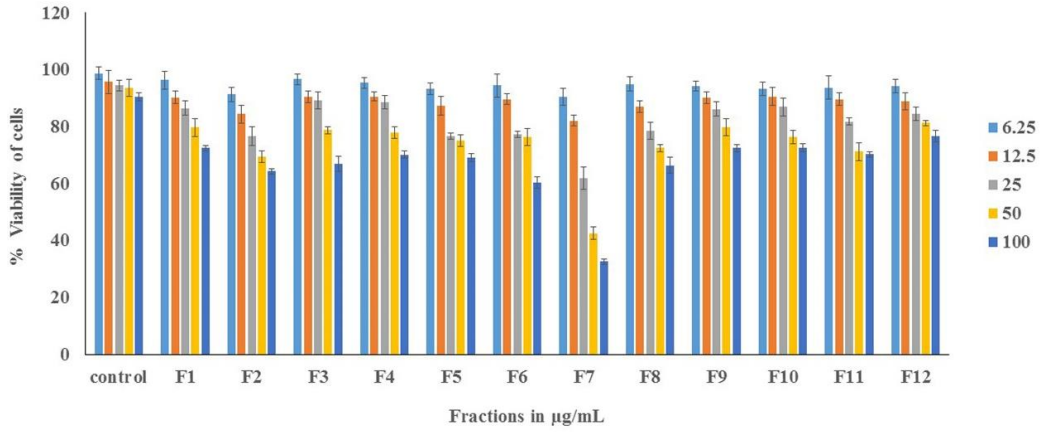

Fig 5: Cytotoxicity of fractions against Hep G2 cells by trypan blue exclusion method.

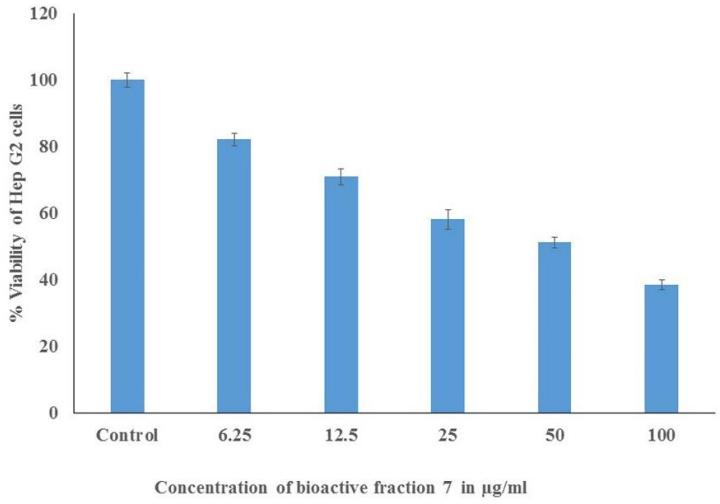

Fig 6: Inhibition of bioactive fraction F7 treated HepG2 cancer cells at 24 hours of exposure

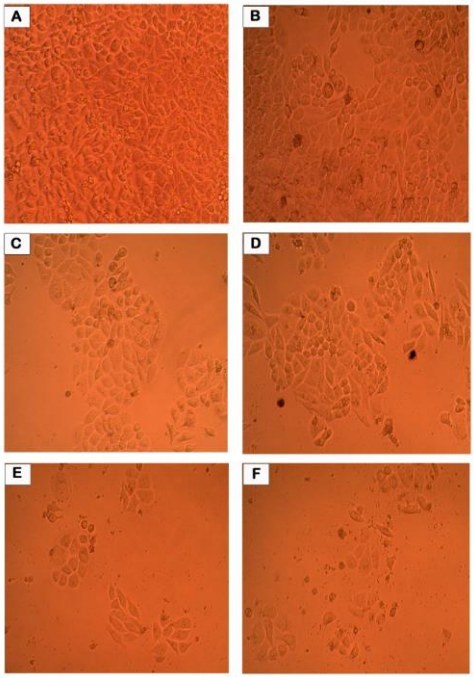

Fig 7: Morphological changes occurred in HepG2 cell lines after treatment with the bioactive fraction F7 for 24 hours.

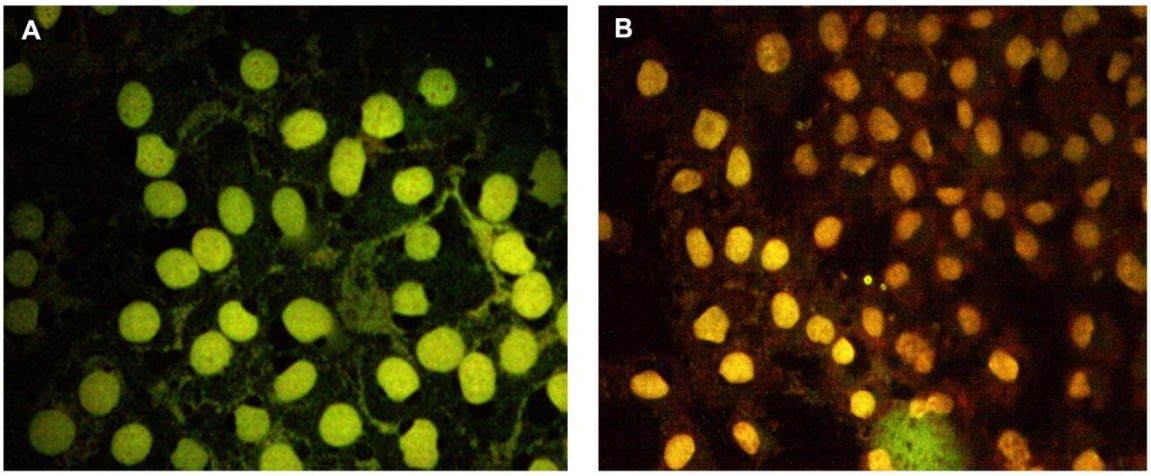

Fig 8: Micrographs (Magnification x 400) of acridine orange/ethidium bromide stained HepG2 cells; A: cells without drug (control) have normal nucleus of green colour representing live cells B: cells exposed to IC_{50} of bioactive fraction F7 for 24 hours showed bright green nucleus; condensed or fragmented chromatin signifying apoptosis.

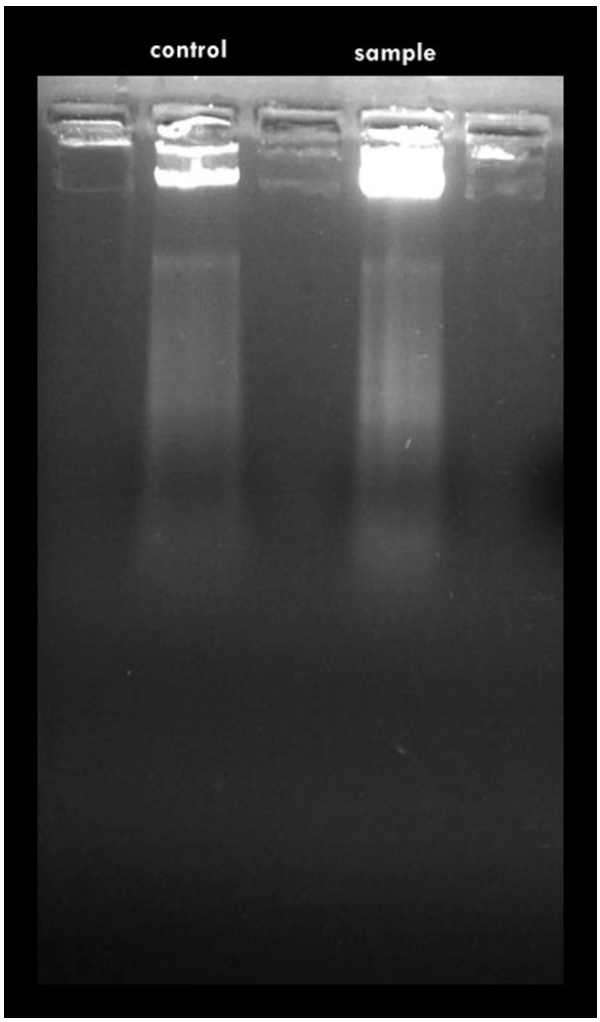

Fig 9: DNA fragmentation analysis by agarose gel electrophoresis: DNA laddering was visualized in HepG2 cell lines after treatment with IC_{50} concentration of the bioactive fraction F7 for 24 hours.

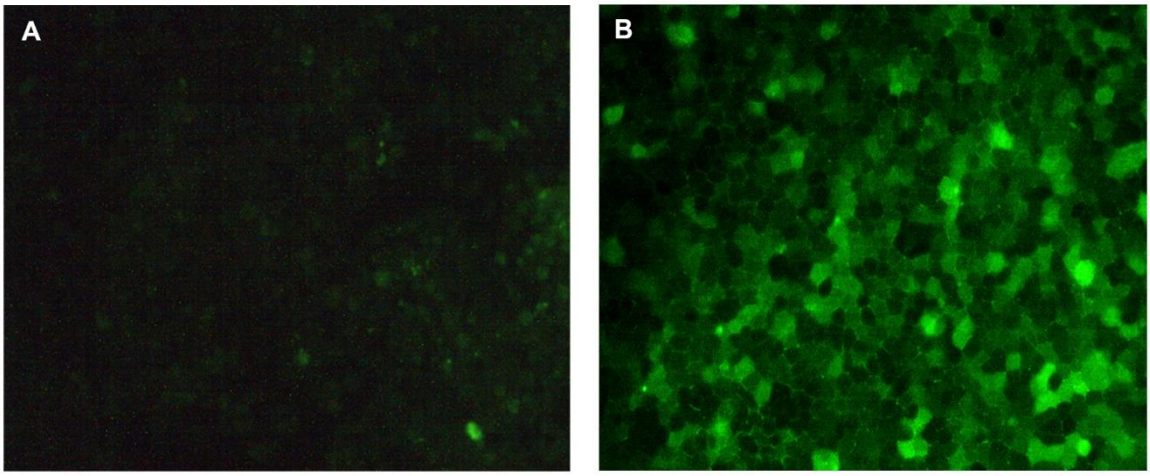

Fig10: DCFDA staining for ROS generation of Hepatocellular carcinoma (HepG2) cells in the presence and absence of IC_{50} value of the bioactive fraction F7 (A) Untreated HepG2 cells. (B) HepG2 cells treated with IC_{50} dose of the bioactive fraction 7 for 24 h.

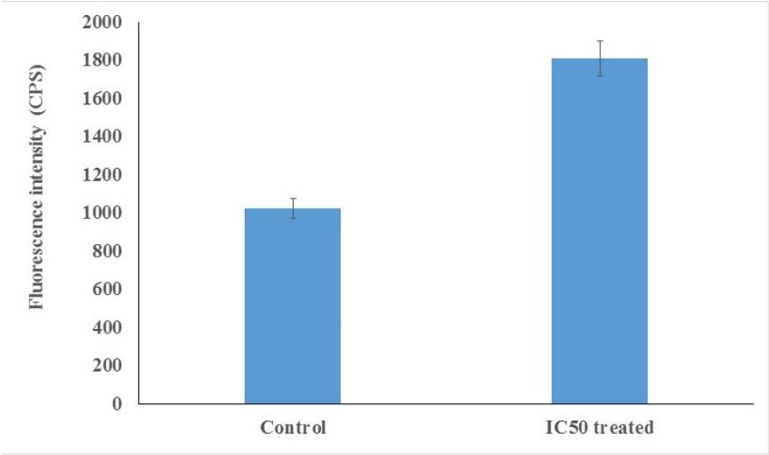

Fig 11: Densitometry graph compare the levels of ROS intermediates in untreated and treated HepG2 cells.

3.6 DNA fragmentation detection assay

HepG2 cells treated with the bioactive fraction F7 showed characteristics of DNA laddering, for which DNA is extracted from the apoptotic cells and separated in an agarose gel as shown in Fig. 9. Treatment with bioactive fraction F7 resulted in degradation of chromosomal DNA into small internucleosomal fragments, and it was the biochemical assurance of cells undergoing apoptosis.

3.7 Dichlorofluorescein diacetate (DCF-DA) staining

Apoptotic induction can also be facilitated through the generation of radioactive oxygen species [24]. It was reported that ROS could play important role in down regulating Bcl-2 as well as prompting the release of cytochrome C and finally leading to the activation of cascade 3 and apoptosis [25]. HepG2 cells were incubated with IC_{50} concentration of bioactive fraction F7 to determine the effect of intracellular ROS generation. The ROS levels were assessed after 24 h of incubation by using 2'7' dichloro dihydrofluorescein diacetate (DCHF-DA) as fluorescent probe. DCHF-DA is cleaved by intracellular esterases into its non-fluorescent form (DCHF). DCHF is then oxidized by intracellular free radicals to produce a fluorescent product (DCF) which emits green fluorescence. In the control (Fig. 10A), no obvious fluorescent image points were observed. By contrast, the treatments of HepG2 cells with IC_{50} dose of the bioactive fraction F7 for 24 hours showed bright fluorescent image points (Fig. 10B). These findings suggest that bioactive fraction F7 can enhance intracellular ROS levels and negative control induces no increase in the ROS level. The DCF fluorescence intensity was measured using a

fluorimeter at 470 nm excitation and emission at 625 nm (Fig 11).

In the present study, increased levels of ROS in the treatment group indicate the apoptotic mode of cell death induced by the bioactive fraction F7. These observations strongly suggest an outline to the possible mechanism of action for the anticancer effect of the isolated bioactive fraction F7 through the contributions in the generation of ROS intermediates and triggering the apoptotic cascade leading to the cell death.

4. CONCLUSION

On the basis of these results, it was confirmed that the proliferation of cancer cells were significantly reduced with the treatment of bioactive fraction F7 of ethyl acetate extract. Based on the results of the present study it was concluded that this *Aliidiomarina taiwanensis* KU31894 bacterium isolates from sea sediment of Kerala, India has the ability to produce anticancer bioactive metabolites that has potential for possible applications in future subject to clinical studies. This is first report about the antiproliferative and apoptotic potential of the marine bacterium, *Aliidiomarina taiwanensis* KU31894. Further it is suggested that there occurs the possibility for the production of drug combinations for challenging different types of cancers mainly liver cancer using these bioactive compounds from marine bacteria.

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