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Caspase mediated cytotoxicity of a yellow pigment produced by *Exiguobacterium alkaliphilum* on human cancer cell lines

[Citotoxicidad mediada por caspasa de un pigmento amarillo producido por *Exiguobacterium alkaliphilum* en líneas celulares de cáncer humano]

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Abstract

Context: Investigation of microbial diversity for the identification of novel bioactive compounds or therapeutics is a more potential and incessant ongoing process for drug development research. Many tumors are generally metastasized at the time of diagnosis and restorative surgery is rarely achieved. As response rates to chemotherapy are low, surgery remains the only effective treatment. Consequently, a substantial need for new therapeutic options is essential. The promotion of apoptosis in cancer cells could potentially lead to the regression and improved prognosis of cancer.

Aims: To assess the *in vitro* anticancer activity of the pigment extracted from *E. alkaliphilum* on cancer cell lines.

Methods: The effect of a pigmented compound produced by *Exiguobacterium alkaliphilum,* isolated from a soil sample was investigated on various human cancer cell lines. Proliferation and viability were analyzed using cell counting and MTT cell proliferation assay. Quantification of lactate dehydrogenase (LDH), a cytosolic enzyme, which is an indicator of cellular toxicity was carried out. Apoptosis was determined by DNA fragmentation, and by colorimetric assays for caspases 3, 7 and 10. The cell cycle analysis was also performed using a flow cytometer, by fixing the cells with propidium iodide. The structural elucidation of the active compound was analyzed by GC-MS.

Results: Pigment extracted from *E. alkaliphilum* showed a time and dose-dependent reduction of proliferation in HepG2, HeLa, MCF7, Jurkat and K562 cell lines with lower IC_{50} concentrations and induction of apoptosis was significantly evident from the caspase assay and DNA fragmentation.

Conclusions: The pigment showed a significant anticancer property on cancer cell lines and this study thereby provides a rationale for pre-clinical and clinical evaluation for the effective treatment of cancer.

Keywords: anticancer; caspase; cell cycle; DNA fragmentation; microbial pigments.

Resumen

Contexto: La investigación de la diversidad microbiana para la identificación de nuevos compuestos bioactivos o terapéuticos es un proceso continuo más potencial e incesante para la investigación del desarrollo de fármacos. En general, muchos tumores tienen metástasis al momento del diagnóstico y rara vez se logra una cirugía restauradora. Como las tasas de respuesta a la quimioterapia son bajas, la cirugía sigue siendo el único tratamiento efectivo. En consecuencia, una necesidad sustancial de nuevas opciones terapéuticas es esencial. La promoción de la apoptosis en las células cancerosas podría conducir a la regresión y al mejor pronóstico del cáncer.

Objetivos: Evaluar la actividad anticancerígena *in vitro* del pigmento extraído de *E. alkaliphilum* en líneas celulares de cáncer.

Métodos: Se investigó el efecto de un compuesto pigmentado producido por *Exiguobacterium alkaliphilum*, aislado de una muestra de suelo en varias líneas celulares de cáncer humano. La proliferación y la viabilidad se analizaron usando el conteo celular y el ensayo de proliferación celular MTT. Se realizó la cuantificación de lactato deshidrogenasa (LDH), una enzima citosólica que es un indicador de toxicidad celular. La apoptosis se determinó mediante fragmentación de ADN y mediante ensayos colorimétricos para las caspasas 3, 7 y 10. El análisis del ciclo celular también se realizó usando un citómetro de flujo, fijando las células con yoduro de propidio. La elucidación estructural del compuesto activo se analizó por GC-MS.

Resultados: El pigmento extraído de *E. alkaliphilum* mostró una reducción de la proliferación dependiente del tiempo y la dosis en las líneas celulares HepG2, HeLa, MCF7, Jurkat y K562 con concentraciones más bajas de IC_{50} y la inducción de apoptosis fue significativamente evidente a partir del ensayo de caspasa y la fragmentación del ADN.

Conclusiones: El pigmento mostró una propiedad anticancerígena significativa en las líneas celulares de cáncer y, por lo tanto, este estudio proporciona una justificación para la evaluación preclínica y clínica para el tratamiento efectivo del cáncer.

Palabras Clave: anticancerígeno; caspasa; ciclo celular; fragmentación de ADN; pigmentos microbianos.

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INTRODUCTION

Battling malignant growth is viewed as a standout amongst the most vital regions of research in medicine and immunology. Natural sources like plants and microorganisms are significant sources of bioactive compounds. Tumor cell resistance to traditional medication treatments is rapidly increasing and numerous medications are not powerful any longer. There is in this manner an incredible interest for creative lead structures from natural sources to create novel medications in the treatment of malignant growth and other intimidating diseases (Newman and Cragg, 2007). Natural products have been known to have the possibility to serve as impressive lead structures and as basis for promising therapeutic agents in the area of cancer treatment (Koehn and Carter, 2005).

Apoptosis plays a vital role in maintaining homeostasis and development of the tissues in organisms (Igney and Krammer, 2002). Inequality between cell proliferation and apoptotic cell death will lead to severe diseases like cancer. Many studies have validated that cancer therapeutics aims to kill the target cells primarily through induction of apoptosis by chemotherapy and γ-irradiation. a replacement direction in cancer treatment has arisen, dedicated to the adjuvant use of natural bioactive compounds like microbial pigments in conventional chemotherapy (Lu et al., 2004).

Microbial secondary metabolites incorporate a wide range of enzymes, antibiotics, pigments etc., which are of extraordinary significance to mankind in many ways like pharmaceutical agents, food industry, bioremediation etc. Bacteria, among all have been looked upon as potential sources of bioactive compounds that hold promising position as targets in screening platforms due to their vast diversity and their ability to produce novel metabolites of pharmaceutical significance.

Bacterial metabolites apart from being used as strong immunoceuticals also serves as a reservoir of effective metabolites, able to penetrating cellular membranes and interfering with unique signal transduction pathways connected to physiological processes such as inflammation, cell differentiation and persistence, carcinogenesis and metastasis (Rocha et al., 2001). In this regard bacterial pigments such as prodigiosins (Nakashima et al., 2005) and violacin (Saraiva et al., 2004) have been reported to possess potential anticancer properties against many cancers.

E. alkaliphilum isolated from soil samples from Wayanad of Kerala, India are Gram stain positive, motile rods to almost coccoid in shape. Colonies of the bacteria on Nutrient agar plates appear round, irregular, shiny and elevated. At 37^{o}C after 24 h of incubation the colonies appear orangish yellow producing pigment (Kulshreshtha et al., 2013).

In this study, *in vitro* anticancer activity of the pigment extracted from *E. alkaliphilum* on cancer cell lines such as human cervical cancer cell line, HeLa; human liver cancer cell line, HepG2; Breast cancer cell line, MCF 7; T lymphocytes cancer cell, Jurkat and human erythromyeloblastoid leukemia cell, K562 was assayed.

MATERIAL AND METHODS

Cell lines and culture

HepG2, HeLa, MCF7, Jurkat and K562 cell lines were procured from National Centre for Cell Sciences, Pune, India. HepG2, HeLa and MCF 7 cells were maintained in MEM medium (HIMEDIA, India), Jurkat and K562 in RPMI medium (HIMEDIA, India) supplemented with 10% Fetal Bovine Serum (HIMEDIA, India). The cells were incubated in a humidified incubator at 37°C with 95% air and 5% CO_2 (Thermo Scientific, USA).

Production of pigment

Exiguobacterium alkaliphilum isolated from the soil source of Wayanad region, Kerala (11.6854° N, 76.1320° E), India and was identified by 16s rRNA partial sequencing. The organism was grown on Nutrient agar plates at 37°C for 48 h to form orangish-yellow colonies. The colonies were then scraped from the plates and dissolved in methanol, centrifuged (Cooling centrifuge, REMI Labor-

atory Instruments, India) at 10000 rpm for 10 min. Supernatant was collected in a pre-weighed vial and kept for vacuum evaporation under reduced pressure in a rotary evaporator (IKA, Germany) at 40°C to obtain the crude pigment.

Molecular characterization and phylogenetic analysis

Exiguobacterium sp. were cultured on nutrient agar for 48 h at 37°C, the cells were harvested and used for genomic DNA isolation. DNA extraction was carried out using genomic DNA isolation kit (Chromus Biotech Pvt. Ltd., Bangalore, India). Sequence specific amplification was performed using universal 16S rDNA primers (Schwieger and Tebbe, 1998). The reaction was set in sterile 0.2 mL PCR vials with the reaction mixture containing the following components: 1.5 µL of genomic DNA, 10 picomoles of 16S rDNA forward primer (5′-AGAGTTTGATCCTGGCTCA – 3′), 10 picomoles of 16S rDNA reverse primer (5′-ACGGCTACCTTGTTACGACT – 3′), 1 µL of 30 mM deoxyribonucleoside 5′ triphosphate (all the dNTPs in equimolar concentration), 2.5 µL of 10X PCR assay buffer and 1 µL of Taq DNA polymerase enzyme (1U) (Chromus Biotech Pvt. Ltd., Bangalore, Karnataka, India). Sterile distilled water was added to make up the volume to 25 µL. The vials were then set in thermal cycler (MJ research, PTC 200) for amplification with the following program: initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation for 1 min at 94°C, annealing for 30 sec at 57°C and extension for 1 min at 74°C. The program was ended with final extension step for 5 min at 74°C. The amplified PCR product was then sequenced for obtaining partial sequence. The sequences were then analysed and Phylogenetic tree was constructed using Phylogeny fr. (https://www.phylogeny.fr) online tool.

Cytotoxicity testing by MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide)] cell viability assay

The cells growing in the exponential phase were harvested after trypsinization for HeLa, MCF 7 and HepG2 cells and direct centrifugation for Jurkat and K562 cells. A volume of 100 µL of cell suspension in culture medium were plated in 96-well microtitre plates and allowed to grow for 24 h prior treatment. Increasing concentrations of the yellow pigment produced by *Exiguobacterium alkaliphilum* (1, 10, 25, 50 and 100 µg/mL) dissolved in 10% DMSO were added in quadruples to different wells of the microtitre plates. Cytotoxicity was analyzed using MTT assay following the standard protocol (Mosmann, 1983). The cells were incubated for 24, 48 and 72 h in the presence of the pigment. The final concentration of DMSO was maintained at 0.4% of the media in each well. Control cells were treated with only DMSO (not exceeding 0.4%) in place of the extracts. After the treatment period, 10 µL of MTT prepared at a concentration of 5 mg/mLin PBS, was added to each well and incubated in the dark for 3 h at 37°C. After 3h, 100 µL of DMSO was added to each well and the absorbance was read at 540 nm using the ELISA plate reader (Lisa Plus Plate Reader, Rapid Diagnostics, India). Decreased cell viability upon the pigment treatment was calculated. IC_{50} value was extrapolated from the graph of concentration vs. viability. The percentage viability was calculated from the following formula [1].

$$\text{Viability (\%)} = \frac{\text{Absorbance of the test sample}}{\text{Absorbance of the control}} \times 100 \qquad [1]$$

The non-toxicity of the pigment on normal cells was evaluated by treating the yellow pigment on healthy lymphocytes. Lymphocytes were isolated based on the method described earlier (Nadumane et al., 2013) and cytotoxicity was tested by MTT assay as mentioned above.

Chromatographic separation and cytotoxicity activity of the bioactive compound

Thin Layer Chromatography (TLC) was carried out using pre-coated TLC plates (Silica gel 60 F 254 Merck) to fractionate the bioactive components from the crude pigment. The silica gel coated sheet was activated at 110˚C for 20 minutes. The pigment dissolved in methanol (20 µL) was spotted on the silica gel coated sheet (Kirchner et al., 1951). Chromatogram was performed with chloroform: acetonitrile (7:3 v/v) solvent system. Different

fractions were separated and collected in different vials by dissolving with 5 mL of methanol. The tubes were gently vortexed and centrifuged at 10,000 rpm for 5 min. The supernatant was transferred into pre-weighed vials and evaporated to dryness in a rotary evaporator (IKA Rotary Evaporator, Germany). Extract concentration was maintained at 1 mg/mL (stock solution) by dissolving in DMSO. The partially purified fractions obtained from preparative TLC were tested for cytotoxicity against the HepG2, HeLa, MCF 7, Jurkat and K562 cells using doxorubicin as the positive control by MTT assay as described earlier (Mosmann, 1983).

Microscopic observation

Cell morphology is one of the indicative factors to assign the health status of the cells. The cells treated with pigment for 48 h were harvested and the number of surviving cells in the population was estimated by the trypan blue dye exclusion method as per standard protocol (Freshney, 2000) using Doxorubicin as the positive control. Briefly, the cells treated with the pigment fraction and doxorubicin were trypsinized, and centrifuged at 1000 rpm for 10 min. The supernatant was discarded, and cells were resuspended in 1 mL of PBS. About 10 µL of 0.4% trypan blue stain was mixed with 10 µL of cells suspended in PBS and the cells were counted on a hemocytometer and observed under 40x light microscope (Labomed, Germany)

DNA fragmentation assay

Cell mediated cytotoxicity and apoptosis is characterized by cleavage of genomic DNA into discrete fragments seen before membrane disintegration. Since DNA fragmentation is the hallmark of apoptosis, assays that can measure the fragmentation are well suited for determining the apoptotic cell death. For analyzing the DNA fragmentation, HeLa, HepG2 and MCF 7 cells grown in 25 cm^2 flasks were treated with the TLC purified pigmented fraction, in order to obtain a final concentration of 10 µg/mL, doxorubicin was used as the reference compound. After 48 h of incubation, the cells were harvested, lysed and DNA was extracted using Mammalian Genomic DNA isolation Kit (HiMedia Ltd). The isolated DNA was electrophoresed on 0.8% agarose gel containing ethidium bromide and visualized with the help of gel documentation system (Herolabs, Germany) (Biase et al., 2002).

Caspase-3, 7, 10 apoptotic assay

The assay is based on spectrophotometric detection of the chromophore *p*-nitroaniline (*p*NA), after its cleavage from the labelled substrate LEHD-*p*NA. The free *p*NA can be quantified using a microtitre plate reader at 405 nm. Comparison of the absorbance of *p*NA from an apoptotic sample with an un-induced control allows determination of the fold increase in the caspase activity. After treating the cells with the bioactive pigmented fraction or doxorubicin for 48 h, the assay was carried out using 2X CasPASE from (G-Biosciences). Negative control cells were treated with DMSO at 0.4%. To each well of the microtitre plate, 50 µL of 2X CasPASE assay buffer and 50 µL of bioactive pigmented fraction treated cell suspension were added. The contents were mixed thoroughly and 5 µL of 1 mM AFC-conjugated substrate was added. Optical density was measured in an ELISA plate reader (405 nm) at zero time point and thereafter every 30 min, until significant differences in the readings were observed. Percentage increase in caspase activity over the length of the reaction time was calculated using the formula [2].

$$\text{Rate of percentage increase} = \frac{OD_{\text{control /sample}} - OD_{blank}}{OD_{\text{blank}}} \times 100 \qquad [2]$$

Lactate dehydrogenase assay

LDH assay is a colorimetric method for assaying cellular cytotoxicity. The cytotoxicity induced by the pigment was analyzed by quantitatively measuring the cytosolic enzyme lactate dehydrogenase (LDH), which is released during cell lysis. Cancer cells treated with the pigment fraction for 48 h were assayed for the LDH activity as per the manufacturer's instructions using Cytoscan LDH Assay Kit (G Biosciences Ltd, USA). The treated cells culture supernatant was collected by tripsinizing, centrifuging at 1000 rpm. A volume of 10 µL of the supernatant was plated in triplicates

to 96 well plate along with the positive and negative controls. The absorbance was measured at 490 nm using a microtitre plate reader and the percentage cytotoxicity was calculated using the formula [3]. The results were compared against doxorubicin as the reference compound.

$$\text{Cytotoxicity (\%)} = \frac{OD_{\text{treated}} - OD_{\text{negative control}}}{OD_{\text{positive control}}} \times 100 \quad [3]$$

Flow cytometry analysis

The effect of pigment fraction on cell cycle was determined by Flow cytometry using PI Staining (Pozarowski et al., 2003) using doxorubicin as a positive control. The pigment/doxorubicin treated cells were harvested by trypsinization followed by centrifugation. Required volume of PBS was added to the cell pellet to obtain a final concentration of 1-2×10^6 cells/mL. The cells were fixed in chilled 70% ethanol at 4°C overnight. Post fixation, the cells were centrifuged at 5000 rpm for 10 min and washed with PBS. The cells were re-suspended in staining solution [50 µg/mL propidium iodide, 30 units/mL RNase, and Triton X-100 (pH 7.8)] and incubated in dark condition at 37°C for 15 min. After incubation, the fluorescence-activated cells were sorted in a FACScan flow cytometer (equipped with a 488 nm argon laser), and the data was analyzed using MACS Quant analyzer.

Chemical characterization by GC-MS

Chemical characterization of pure active compound was performed through GC–MS analysis carried out using Thermo GC - Trace Ultra Ver: 5.0 and Thermo MS DSQ II fitted with a DB 35 - MS Capillary Standard Non - Polar Column, having 0.25 mm ID, 30 m length, and 0.25 µm film thickness. Helium was the carrier gas at a flow rate of 1 mL/min; injector port temperature was 260°C; detector temperature was 260°C; oven temperature was maintained at 70°C for 2 min and then increased to 260°C at the rate of 6°C/min at which temperature the column was maintained for 5 min; splitting ratio was 1:50; ionization voltage was 70 eV. The product obtained was compared to the molecules reported in the literature (Wiley Spectra) and Cancer Resource database (Gohlke et al., 2016).

Statistical analysis

All the experiments were performed in triplicates and the results were calculated as mean ± standard error of mean (SEM) values. Statistical significance was calculated using one-way analysis of variance (ANOVA) to test the null hypothesis. Duncan's multiple range test (DMRT) was done to compare the sample means. The data were considered significantly different from each other when significance level was $p<0.05$ (Gomez and Gomez, 1984).

RESULTS

The bacteria isolated from the soil of Wayanad region, Kerala in India produced a orangish-yellow colored pigment which showed strong anticancer activity against the human cervical cancer cell line, HeLa; human liver cancer cell line, HepG2; T lymphocytes cancer cell, Jurkat; human erythromyeloblastoid leukemia cell, K562 and Breast cancer cell line, MCF 7. Sequence analysis along with fundamental microbiological investigations is the method proven for effective characterization as it gives a trusted identification comparative to the knowledge of the existing organisms. Based on the preliminary morphological and phenotypic observations, the pigment producing organism was identified as a member of *Exiguobacterium* species (Fig. 1).

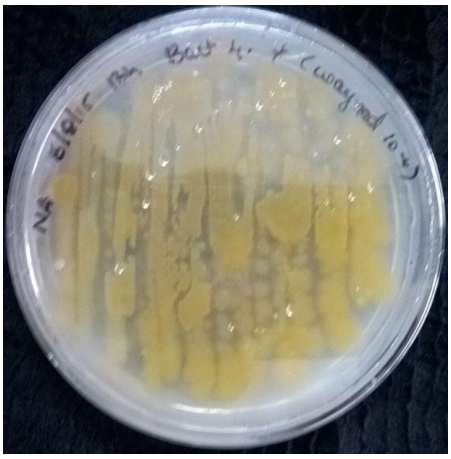

Figure 1. Pure culture of pigment producing *Exiguobacterium alkaliphilum* species.

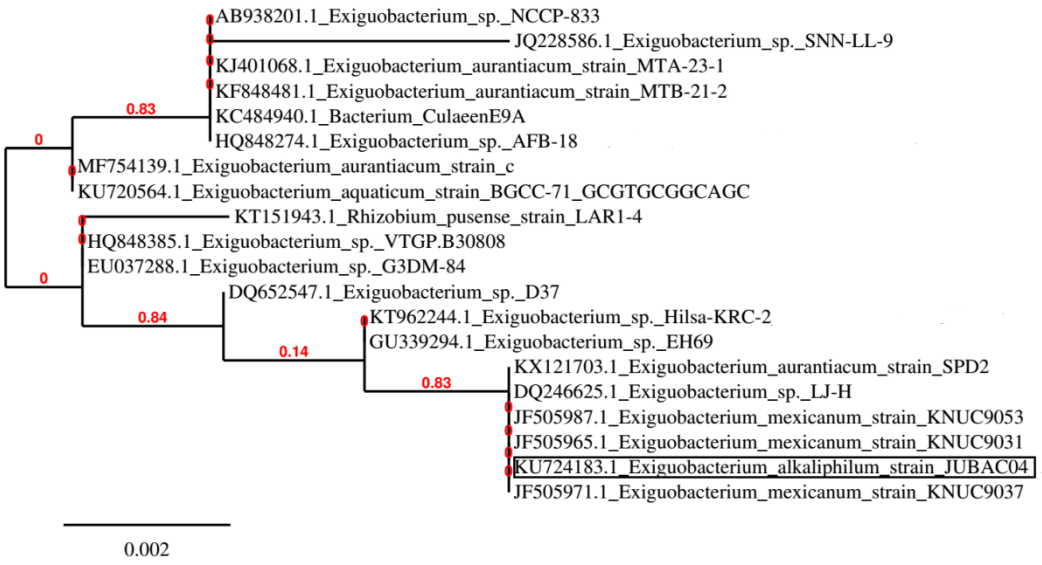

Figure 2. The evolutionary history was inferred using the Neighbour-Joining method using Phylogeny fr. tool.

The gene sequence encoding for the 16s ribosomal RNA was sequenced and analyzed with the sequences of representatives of the *Bacillales* family XII, which confirmed that isolate belonged to the genus *Exiguobacterium* and the sequence similarity was found with *Exiguobacterium alkaliphilum* strain 12/1. The sequence was then submitted to GenBank database with accession number KU724183. The organism was identified as *Exiguobacterium alkaliphilum* JUBAC04. By using neighbor-joining method, the phylogenetic analysis was performed and the strain JUBAC04 showed resemblance to *Exiguobacterium alkaliphilum* with 100% bootstrap level in the phylogenetic tree (Fig. 2).

The pigment producing bacteria was grown on nutrient agar plates and the pigment was extracted using methanol as the solvent. The crude pigment showed an absorbance peak at 210 nm. The partial purification and extraction of the bioactive fraction was carried out using combination of solvents varying in polarity. The separation of fraction conveniently with higher resolution was achieved using acetonitrile and chloroform solvent mixture in the ratio of 3:7 by thin layer chromatography. The pigment produced by *E. alkaliphilum* species showed wakeless anticancer potential on HeLa, HepG2, Jurkat, K562 and MCF 7 cells as indicated by their low IC_{50} concentrations in comparison to doxorubicin being used as a reference compound (Table 1). The pigment also showed no cytotoxicity against healthy lymphocytes owing to its anticancer property.

The pigment extracted from *E. alkaliphilum* when tested for *in vitro* cytotoxicity by using MTT cell viability assay showed significant inhibition of cell growth and proliferation. Treatment of pigment at the same concentration (50 and 100 µg/mL) to normal healthy lymphocytes did not cause any significant change in the cell viability even after 72 h. Treatment of doxorubicin showed highest inhibition on MCF 7 cells after 72h with a percentage viability of 40% (Fig. 3A-C).The inhibition of cancer cell viability using the pigment from *E. alkaliphilum* was comparable to that with doxorubicin.

The induction of apoptosis by the pigment treatment is very well characterized by DNA fragmentation. Appearance of distinct DNA fragments on agarose gel is a clear indication for the induction of apoptosis in pigment treated cells. The DNA isolated from the cells treated with 50 µg/mL of the TLC purified pigment for 48 h showed a smeared pattern when compared to that

Table 1. IC_{50} concentration (µg/mL) of the TLC purified pigment and doxorubicin, on HeLa, HepG2, Jurkat, MCF 7 and K562 cell lines at 24, 48 and 72 h.

Cell Line	24 h		48 h		72 h	
	Pigment	Doxorubicin	Pigment	Doxorubicin	Pigment	Doxorubicin
HeLa	17.31 ± 0.23	3.29 ± 0.10	11.13 ± 0.21	2.77 ± 0.16	4.47 ± 0.15	2.05 ± 0.10
HepG2	9.45 ± 0.11	22.39 ± 0.36	9.31 ± 0.17	11.54 ± 0.23	6.31 ± 0.19	3.2 ± 0.11
MCF 7	9.73 ± 0.12	5.08 ± 0.14	5.70 ± 0.19	1.6 ± 0.09	8.17 ± 0.22	0.81 ± 0.29
Jurkat	44.05 ± 0.73	-	7.37 ± 0.93	-	5.60 ± 0.69	-
K562	34.09 ± 0.56	-	3.85 ± 0.50	-	3.18 ± 0.51	-

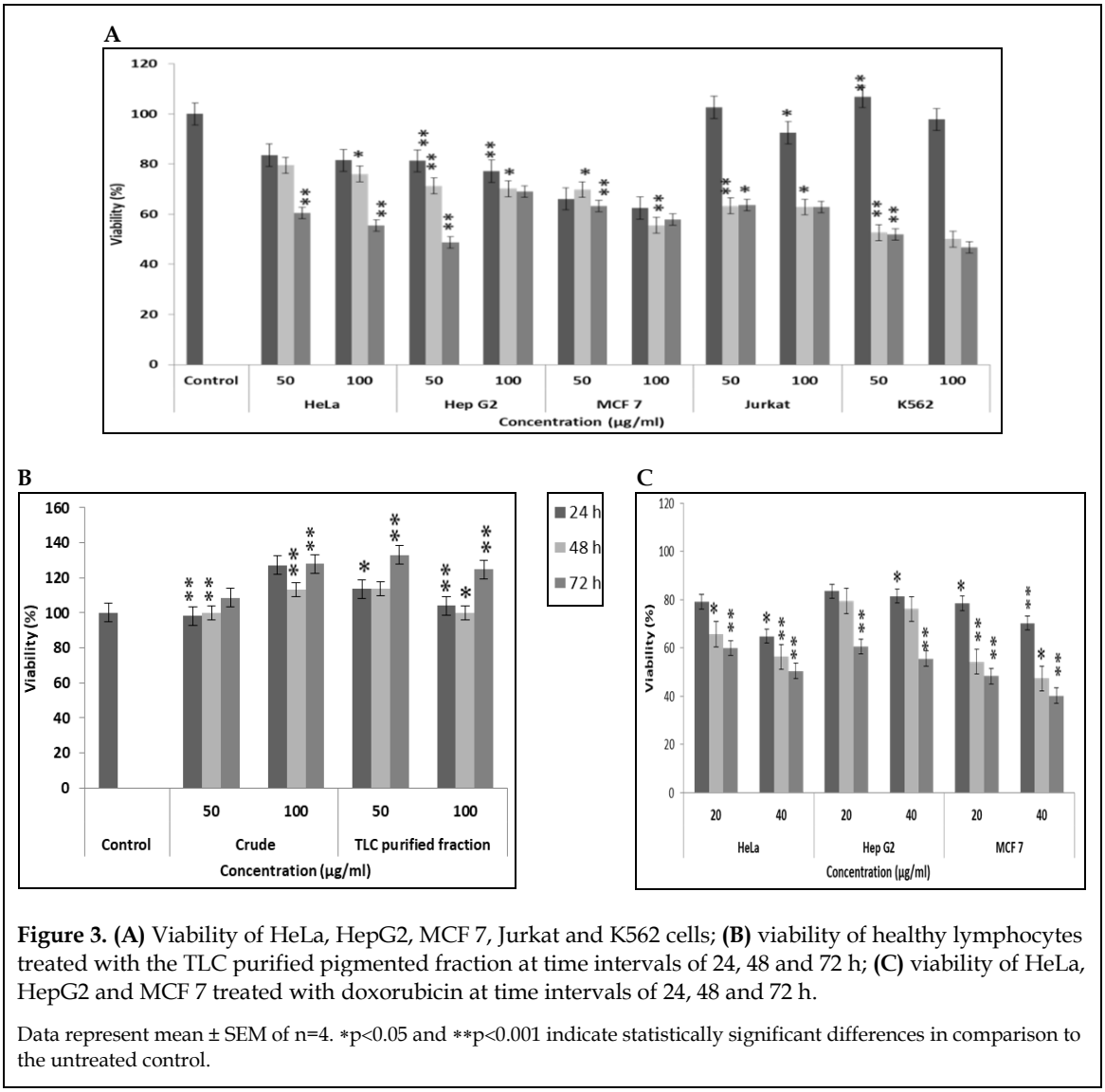

Figure 3. (A) Viability of HeLa, HepG2, MCF 7, Jurkat and K562 cells; **(B)** viability of healthy lymphocytes treated with the TLC purified pigmented fraction at time intervals of 24, 48 and 72 h; **(C)** viability of HeLa, HepG2 and MCF 7 treated with doxorubicin at time intervals of 24, 48 and 72 h.

Data represent mean ± SEM of n=4. *p<0.05 and **p<0.001 indicate statistically significant differences in comparison to the untreated control.

of the DNA isolated from the untreated control cells and doxorubicin treated cells as a positive control (Fig. 4). Along with DNA fragmentation, cells undergoing apoptosis exhibit morphological changes like plasma membrane blebbing, cell body shrinkage and formation of membrane bound apoptotic bodies and increased caspase levels (Cohen et al., 1992). Morphological and microscopic observation of the cells stained with trypan blue stain enables to visualize the morphology and viability of the pigment treated cells as comparable to that of doxorubicin (reference compound) treated cells when compared to the control cells. The treatment caused lethal effect to a greater extent on the viable cancer cells. The count of treated cancer cells was significantly lesser than that of the untreated control cells and comparable to that of the doxorubicin treated cells (Table 2).

The LDH activity obtained from the untreated cells were used as the positive control in assaying the release of lactate dehydrogenase enzyme of the treated cells. The untreated cells served as a source of maximum LDH release and the percentage activity was calculated for the control. The percentage activity compared between the positive, negative control and the treated sample were 71.41, 54.51, 60.29, 73.14 and 62.89% in K562, HeLa, HepG2, MCF 7 and Jurkat cells respectively. The doxorubicin treated cells showed an activity of 46.83, 52.25 and 66.32% in MCF 7, HeLa and HepG2 cells respectively (Fig. 5). It is very clear that the cytotoxicity caused by the pigment on MCF 7, HepG2 or HeLa was higher compared to doxorubicin.

Caspases are a class of aspartate- specific cysteine proteases which are the primary mediators of apoptosis, they also serve as key indicators of apoptosis (Yuan et al., 2003). The activities of caspases in the current study revealed the sharp increase in treated cells as compared to the untreated cells. Treatment with 50 µg/mL concentration of the TLC purified pigment on HeLa and HepG2 cells resulted in 3-fold increase in the final caspase activity as compared to the initial caspase activity whereas with MCF 7 cells the increase was found to be 4 folds. When compared to the increase in caspase activity of doxorubicin treated cells, the increase due to pigment treatment in HeLa, HepG2 or MCF-7 cancer cells were much higher (> double) clearly indicating our isolated pigment as a better inducer of apoptosis than doxorubicin (Fig. 6).

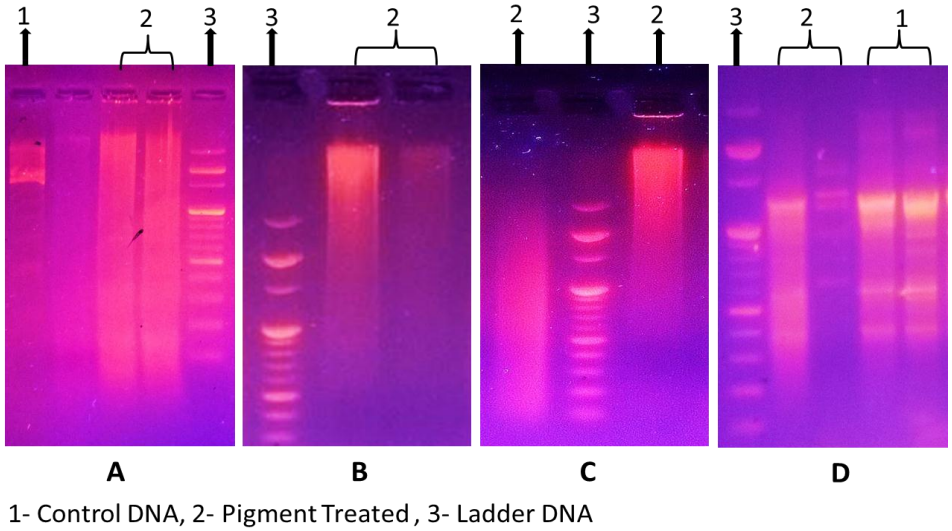

Figure 4. DNA fragmentation was evident by the formation of sheared bands on Hep G2 **(A)**, HeLa **(B)**, MCF 7 **(C)** cells and doxorubicin treated MCF 7 and HepG2 cells **(D)**.

Table 2. Cell concentration (cells/mL) of pigment and doxorubicin treated cancer cells as determined by trypan blue assay.

Cells	Samples	Viable cells	Dead cells
HeLa	Control	11.6 x10⁴	3.5 x10⁴
	Pigment treated	8.5 x10⁴	18.0 x10⁴
	Doxorubicin treated	6.4 x10⁴	21.8 x10⁴
Hep G2	Control	34.3 x10⁴	3.1 x10⁴
	Pigment treated	15.1 x10⁴	46.1 x10⁴
	Doxorubicin treated	13.4 x10⁴	49.2 x10⁴
MCF 7	Control	12.8 x10⁴	2.1 x10⁴
	Pigment treated	4.9 x10⁴	14.8 x10⁴
	Doxorubicin treated	2.8 x10⁴	18.2 x10⁴

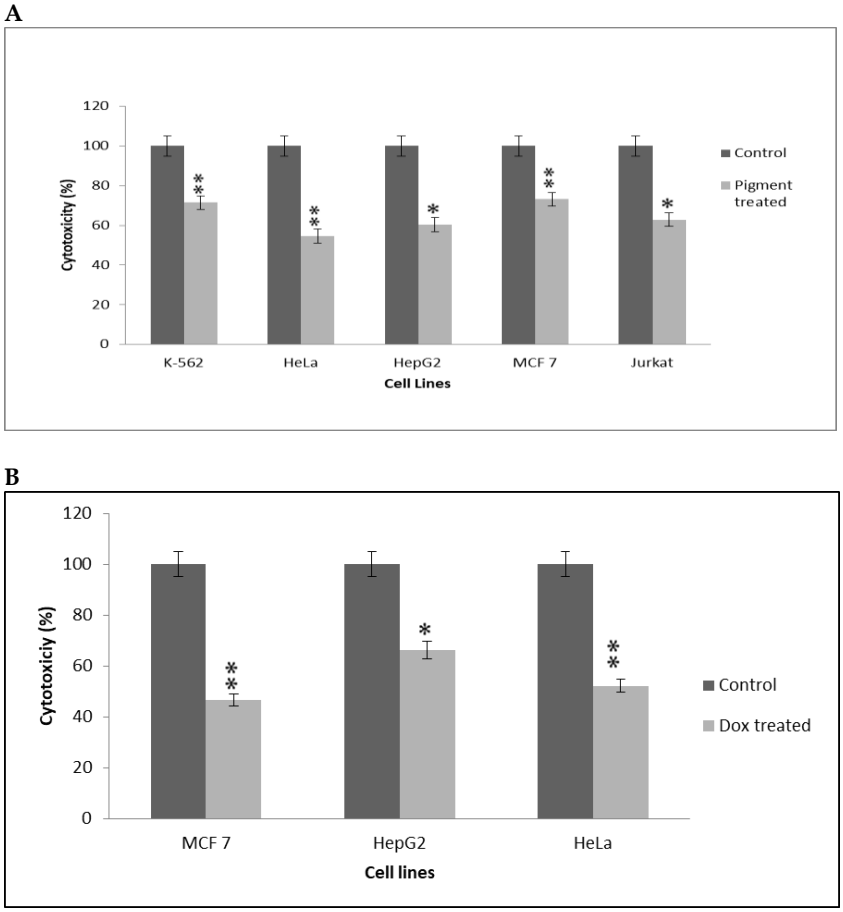

Figure 5. Percentage cytotoxicity of **(A)** TLC purified pigmented fraction calculated from the LDH released on K562, HeLa, HepG2, MCF 7 and Jurkat cell lines and **(B)** doxorubicin treated cells.

Data represent mean ± SEM of n=4. *p<0.05 and **p<0.001 indicate statistically significant differences in comparison to the untreated control.

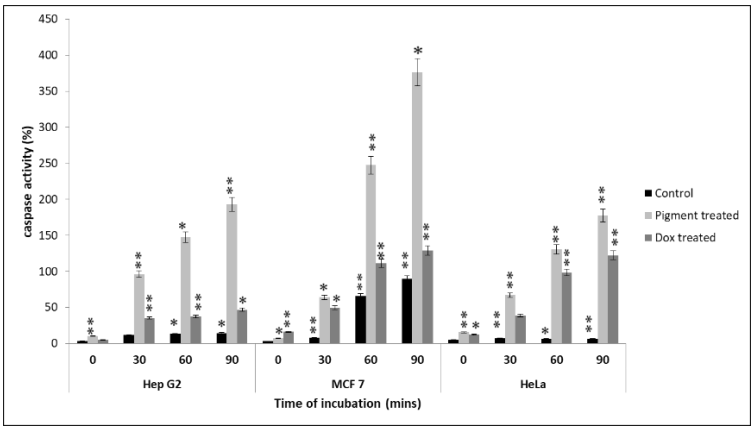

Figure 6. Caspase activity of TLC purified pigment and doxorubicin treated HepG2, MCF 7 and HeLa cells.

Data represent mean ± SEM of n=4. *p<0.05 and **p<0.001 indicate statistically significant differences in comparison to the untreated control.

Table 3. Cell cycle stages of pigment and doxorubicin treated cancer cell lines.

Cells	Control cells			Pigment treated cells			Doxorubicin treated cells		
	% G1 Phase	% G2 Phase	% S Phase	% G1 Phase	% G2 Phase	% S Phase	% G1 Phase	% G2 Phase	% S Phase
HeLa	46.80	9.05	44.15	50.83	17.68	31.49	94.12	5.78	0.10
HepG2	70.10	12.91	17.00	99.32	0.68	0.00	97.54	2.31	0.15
MCF7	62.86	7.95	29.19	99.38	0.09	0.54	99.26	0.74	0.00

Flow cytometric analysis of cell cycle with the recovered cells after treating with 50 µg/mL of pigment revealed that, there was an evident difference in the cell cycle stages between the control and treated cell populations. It was seen that untreated cell population was healthy and multiplying with 44.15, 17.10 and 29.19% of cells in S phase on HeLa, HepG2 and MCF 7 cells respectively (Fig. 7). The treated cancer cell population showed decreased number of cells in S phase. There was an evident G1 phase arrest with 99.32% and 99.38% in HepG2 and MCF7 cells respectively (Table 3). In case of HeLa cells G2 phase arrest was found along with a drastic decline in S phase cells. The results of the cell cycle analysis were comparable against doxorubicin treated cells with an evident G1 phase arrest showing cell population of 94.12, 97.54 and 99.26% in HeLa, Hep G2 and MCF 7 cells respectively. This pattern of cell cycle indicates the inhibition of proliferation and the results show the probable effect of the pigment to cause apoptosis in the cancer cells.

The GC-MS analysis of TLC purified pigment produced by *E. alkaliphilum,* resulted in the identification of predominant compounds. The major compound was lycoxanthin with molecular formula $C_{40}H_{56}O$ and molecular weight 552, which was at 37.68 retention time and peak area of 33.85% (Fig. 8). The compound is first for its identification from a microbial extract however the compound is reported for its anticancer property extracted from plant source. This compound identified is a carotenoid and possess anticancer property.

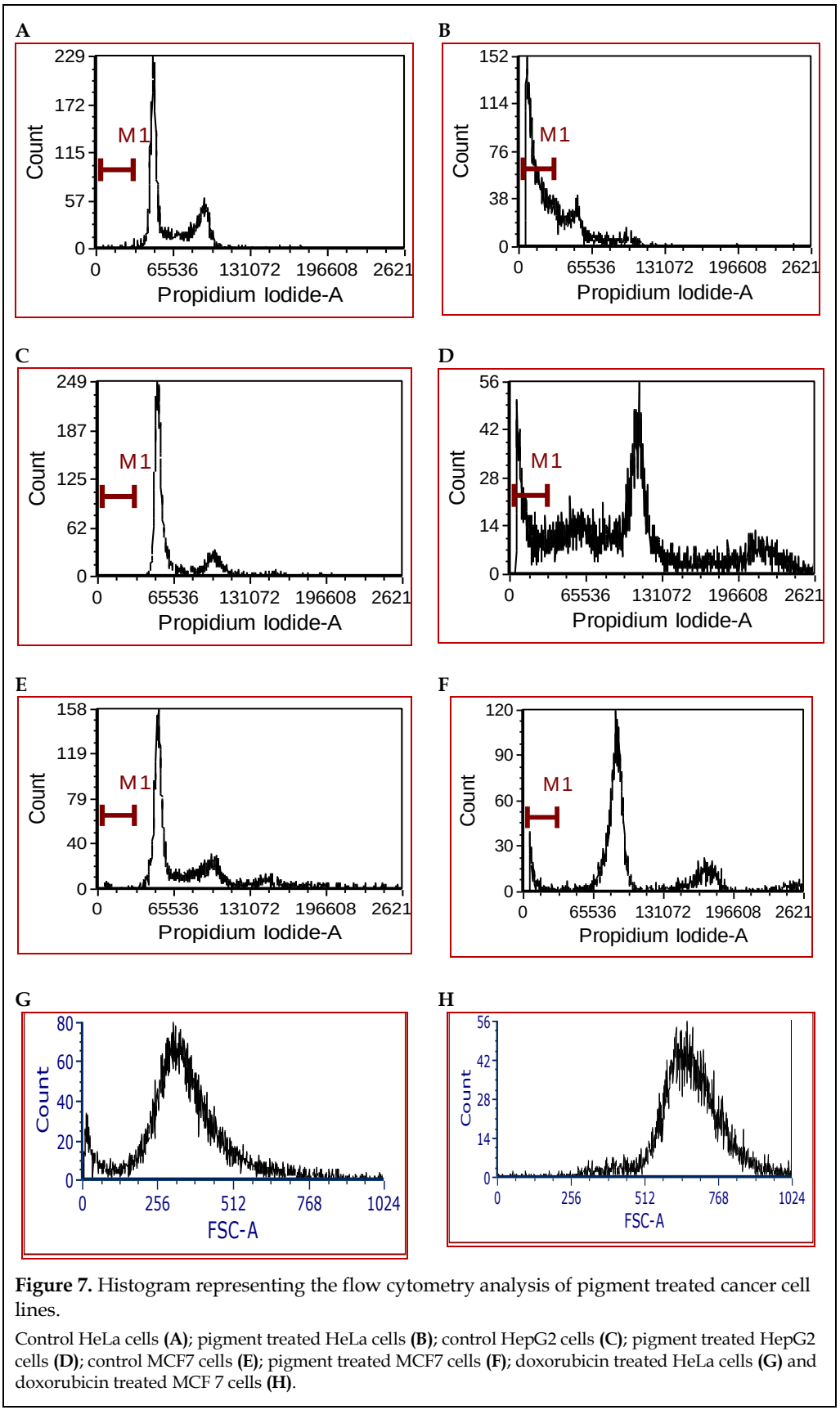

Figure 7. Histogram representing the flow cytometry analysis of pigment treated cancer cell lines.

Control HeLa cells **(A)**; pigment treated HeLa cells **(B)**; control HepG2 cells **(C)**; pigment treated HepG2 cells **(D)**; control MCF7 cells **(E)**; pigment treated MCF7 cells **(F)**; doxorubicin treated HeLa cells **(G)** and doxorubicin treated MCF 7 cells **(H)**.

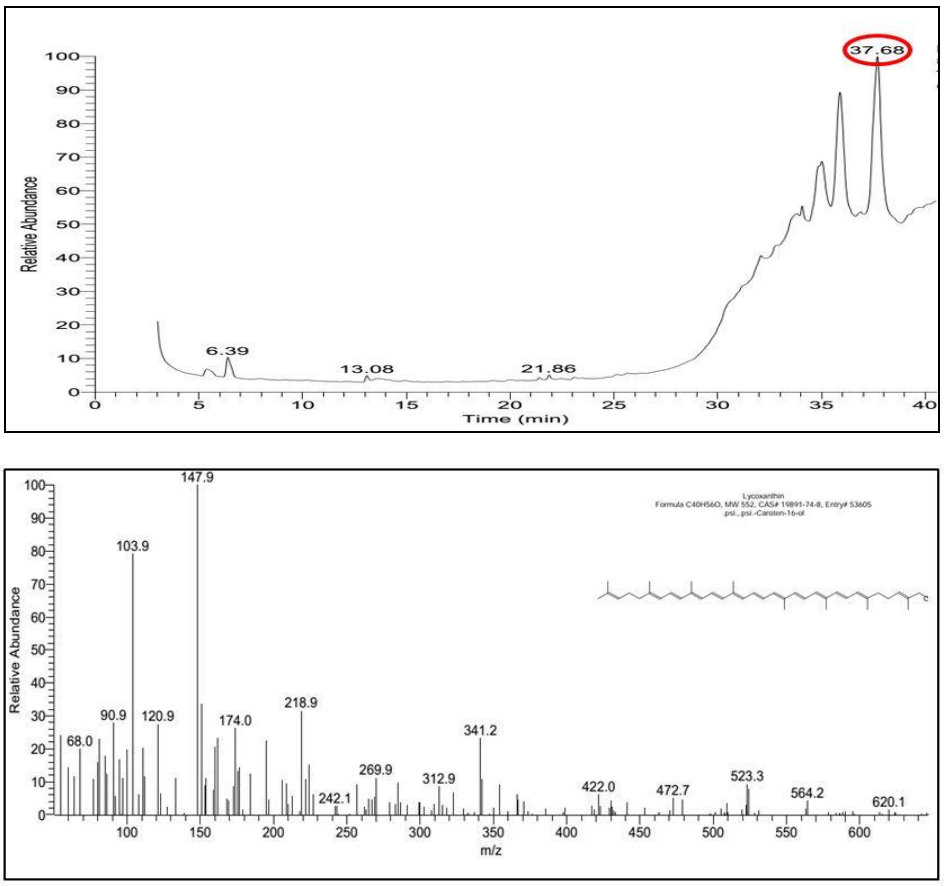

Figure 8. GC-MS chromatogram of the TLC purified pigment produced by *E. alkaliphilum*.

DISCUSSION

Bioactive compounds produced by bacteria possess enormous efficiency as medicinally important products. The study predominantly aimed at analyzing the anticancer potential of the pigment produced by *E. alkaliphilum* to either use as crude bioactive agents for direct use as drugs or to identify the bioactive compounds that can be used as lead structures in the preparation of semisynthetic drugs. In this study we demonstrate the cytotoxic property of TLC purified pigment produced by *E. alkaliphilum* on various human cancer cell lines. The MTT results showed that pigmented bioactive fraction was able to significantly inhibit cell proliferation in a time and dose-dependent manner at concentrations between 10-100 µg/mL.

As the cell's fundamental cell death program, apoptosis plays a vital role in growth control of cells and maintaining tissue homeostasis and consequently, an inactivation or imbalance of essential pathways can result in tumor development and progression (Hengartner, 2000). Moreover, inducing apoptosis in the cells is the primary target of cytotoxic therapies in cancer treatment such as chemotherapy, γ-irradiation or immunotherapy (Apel et al., 2008) and many tumors are subsequently resistant as they develop different escape mechanisms to evade apoptosis (Persidis, 1999). Therefore, the induction of apoptotic response in tumor cells is a significant step in anticancer treatment. In the present study, the pigment treatment caused significant decrease in the percentage of viable cancer cells, which correlated well with the increased caspase activity and their

reduced cell count in comparison to doxorubicin. The pigment treatment also showed non-toxic effect on normal healthy lymphocytes as opposed to its cytotoxic effects to the cancer cells.

The genomic DNA fragmentation is the biochemical hallmark of apoptosis, an irreversible event that constrains the cell to die. The DNA fragments are the result of nuclear endonuclease enzyme cleaving DNA. The pigment treatment induced DNA fragmentation in the cancer cells, proving induction of apoptosis. Caspase activation was also evident in the anticancer effects of the pigment. The increase in the activated effector caspases 3, 7 and 10 confirmed an efficient induction of the apoptotic pathway in the cancer cells.

Cell cycle progression, a series of events in a eukaryotic cell leading to its replication, is terminated at the transition from the G1 to the S phase or from the G2 to the M phase after DNA damage (Hartwell and Weinert, 1989). The analysis of the cell cycle distribution in the current work demonstrated that G1/S-phase was arrested in Hep G2 and MCF7 cancer cells as an effect of the bioactive fraction, whereas G2/M-phase arrest was seen in HeLa cells. This supported the evidence that the suppression of the cell cycle transition is involved in the pigment induced antitumor action observed in the present study on human cancer cells. The GC-MS analysis of TLC purified pigment produced by *E. alkaliphilum* revealed the presence of an important secondary metabolite lycoxanthin, having various pharmacological activities including anticancer activity.

An ideal anticancer drug should specifically target the malignant cells while normal cells in the body should not be damaged. As this is unfortunately rarely achieved, the present data demonstrated that the apoptotic effect of the pigment fraction was only noted in the tumor cells without impairing the normal lymphocyte cells. These outcomes suggested a tumor-specific effect.

CONCLUSIONS

In the current study, the apoptosis mediated cytotoxicity of the pigment produced by *E. alkaliphilum* was assayed on Human cancer cell lines and the bioactive fraction exhibited potential anticancer activity in a dose-dependent manner with lower IC_{50} values and without impairing normal cells. To the best of our knowledge, this is the first report on the anticancer activity of this pigment from *E. alkaliphilum*. Further studies can be carried out on structural elucidation of the active component, optimization of production parameters for scaling up of the pigment yield and also evaluate its anticancer property on animal tumor models.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTION:

Contribution	Bharath PG	Varalakshmi KN
Concepts or ideas	x	x
Design	x	x
Definition of intellectual content		x
Literature search	x	x
Experimental studies	x	
Data acquisition	x	
Data analysis	x	x
Statistical analysis	x	
Manuscript preparation	x	
Manuscript editing	x	x
Manuscript review	x	x

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