Anticancer Effects of Brominated Indole Alkaloid Eudistomin H from Marine Ascidian *Eudistoma viride* Against Cervical Cancer Cells (HeLa)

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Abstract. *Marine invertebrates called ascidians are prolific producers of bioactive substances. The ascidian Eudistoma viride, distributed along the Southeast coast of India, was investigated for its in vitro cytotoxic activity against human cervical carcinoma (HeLa) cells by the MTT assay. The crude methanolic extract of E. viride, with an IC_{50} of 53 µg/ml, was dose-dependently cytotoxic. It was more potent at 100 µg/ml than cyclohexamide (1 µg/ml), reducing cell viability to 9.2%. Among nine fractions separated by chromatography, ECF-8 exhibited prominent cytoxic activity at 10 µg/ml. The HPLC fraction EHF-21 of ECF-8 was remarkably dose- and time-dependently cytotoxic, with 39.8% viable cells at 1 µg/ml compared to 51% in cyclohexamide-treated cells at the same concentration; the IC_{50} was 0.49 µg/ml. Hoechst staining of HeLa cells treated with EHF-21 at 0.5 µg/ml revealed apoptotic events such an cell shrinkage, membrane blebbing, chromatin condensation and formation of apoptotic bodies. Cell size and granularity study showed changes in light scatter, indicating the characteristic feature of cells dying by apoptosis. The cell-cycle analysis of HeLa cells treated with fraction EHF-21 at 1 µg/ml showed the marked arrest of cells in G_0/G_1, S and G_2/M phases and an increase in the sub G_0/G_1 population indicated an increase in the apoptotic cell population. The statistical analysis of the sub-G_1 region showed a dose-dependent induction of apoptosis. DNA fragmentation was also observed in HeLa cells treated with EHF-21. The active EHF-21 fraction, a brominated indole alkaloid Eudistomin H, led to apoptotic death of HeLa cells.*

Treatments for cancer, such as radiation and chemotherapy, have significant side-effects. Hence, less toxic treatments with significantly fewer side-effects have been the main objective of anticancer research. Significantly, the comprehensive understanding of tumor biology and molecular mechanisms have led to new insights into the mechanisms responsible for the disease, modifying the general approach towards cancer treatment.

Apoptosis, a strongly regulated and structurally-distinct form of programmed cell death, has widespread biological significance, hence apoptosis has been recognized as an important target for therapeutic intervention and rational drug discovery (1) and cancer treatment strategies are directed towards reconstituting the tumor cell's ability to undergo apoptosis (2).

Marine natural products have received much attention due to their chemical novelty and great diversity (3). Sponges, bryozoans and tunicates are among the most promising sources of dynamic principles for drug development (4).

Ascidians are prolific producers of bioactive substances and many ascidian compounds have been shown to possess cytotoxic properties. Metabolites such as halocynthiaxanthin and fucoxanthinol from *Halocynthia roretzi* (5), meridianins, brominated 3-(2-aminopyrimidine)-indoles from *Aplidium meridianum* (6), and compounds from *Diplosoma virens* (7)

and *Policlinum indicum* (8), have novel modes of action of inducing apoptosis and cell-cycle arrest. Among the ascidian families of Didemnidae and Polycitoridae, the most prolific producers of biologically-active compounds (9), the genus *Eudistoma*, belonging to the most diverse family Polycitoridae and inhabiting mainly tropical regions (10), has been the source of several cytotoxic alkaloids (11), including eudistomins, eilatin, staurosporine derivatives, methyleudistomins and pibocin (12, 13). With this in mind, we investigated the cytotoxic potential of compounds from the ascidian *Eudistoma viride*, distributed along the Southeast coast of India, and their possible mechanisms of action against HeLa cell lines.

Materials and Methods

Extraction of ascidian. Eudistoma viride Tokioka, 1955 (Chordata, Urochordata, Ascidiacea, Enterogona, Polycitoridae), collected from the Tuticorin coast (Lat 8˚45'N and Long 78˚13'E) of the Southeast coast of India. The ascidian was identified using standard keys (14) and the identity was confirmed by Dr. V.K. Meenakshi of APC Mahalakshmi College, Tuticorin. The ascidians were brought to the laboratory, washed thoroughly with sterile distilled water, cut into small pieces, rinsed with sterile distilled water and air-dried for 24 h at room temperature before extraction with methanol. Methanol was chosen based on preliminary antibacterial activity screening against human bacterial pathogens with hexane, chloroform, diethyl ether, ethyl acetate, acetone and methanol (data not presented). The crude extract, cold steeped overnight at −18˚C and filtered with Whatman No.1 filter paper, was evaporated to dryness in a rotary evaporator (8,15), and used for the preliminary cytotoxic screening against cervical cancer cells (HeLa).

Tumor cell culture. The human cervical carcinoma (HeLa) cell line from the American Type Culture Collection (ATCC, Manassas, VA 20108 USA), obtained from the National Center for Biological Sciences, Bangalore, India, were maintained under a cell density of 1×10^6/ml (to avoid mutations of the cells) in tissue culture flasks with F-12 Dulbecco's modified Eagle's medium (Sigma, Bangalore, India) (17) supplemented with 500 ml fetal bovine serum (Sigma), penicillin (100 U/ml) and streptomycin (100 μg/ml) in a CO_2 incubator. Cells were passaged three times a week and cells cultivated longer than 20 passages (8,18) were not used for the experiments.

In vitro cytotoxicity assay. The MTT assay was followed based on Martinez-Garcia et al (19). HeLa cells (9×10^5 cells/ml in a volume of 100 μl/well) (20) were incubated with methanol crude extract of *E. viride* at concentrations of 25, 50, 75 and 100 μg/ml in a 96-well microplates and incubated in an incubator with 5% CO_2 at 37˚C for 24 h. Then 100 μl of MTT solution [5 mg/ml in phosphate-buffered saline (PBS) (Sigma)] was added to each well and cells were incubated for 2 h at 37˚C under the same conditions. The accumulated formazan crystals were dissolved with the addition of 150 μl DMSO to each well. The culture plate was then gently shaken in the dark at room temperature for 1 h and the absorbance was measured at a wavelength of 570 nm with an ELISA plate reader (BIORAD model 680, Life Science, Guragon, India). Cyclohexamide at 1 μg/ml was used as positive control and a

negative control without ascidian extract was maintained separately. The experiment was carried out in triplicate. Survival of the cells in treated wells was measured as the percentage of absorbance compared to that of the control wells (non-treated cells, taken as 100% survival). The IC_{50} value (effective concentration required to inhibit cell growth by 50%) was calculated from dose–response curves (20).

Chromatographic isolation of cytotoxic compound. Column fractionation: The crude methanolic extract was fractionated using reverse-phase silica gel flash column chromatography (230-400 mesh; LOBA Chemie, Bangalore, India) (16) with a 43×2 cm size column. Based on gradient partitioning (16) and thin layer chromatography (TLC) [pre-coated TLC plates with silica gel, 0.2 mm thickness, 20×20 cm (Merck, Bangalore, India) silica gel] of crude extract, a step-gradient solvent system of increasing polarity from hexane (Hex) to dichloromethane (DCM) to methanol (Meth) (100% Hex, 75% Hex:25% DCM, 50% Hex:50% DCM, 25% Hex:75% DCM, 100% DCM, 75% DCM:25% Meth, 50% DCM:50% Meth, 25% DCM:75% Meth, 100% Meth) was used to fractionate the crude extract. The purity of the fractions was assessed with TLC and the cytotoxicity of the nine eluted column fractions (ECFs) was determined through MTT assay against HeLa cell lines at a concentration of 10 μg/ml, selected at random based on the crude activity profile.

High performance liquid chromatography (HPLC): Fraction ECF-8 (25% DCM:75% Meth), which showed potent cytotoxicity against HeLa cell line, was collected, pooled, concentrated and further purified using semipreparative HPLC. An isocratic solvent system using methanol/water (0.05% trifluoroacetic acid) (10:90) was employed to separate the mixture of compounds (21). The 23 fractions (EHFs) which exhibited prominent peaks during elution in semipreparative HPLC column fractionation were collected and subjected to MTT assay at 2 μg/ml concentration. The fractions thus obtained were spotted onto TLC plates and observed under UV light. Pancal-D was used as a spray reagent (21.6 g ammonium molybdate, 1.6 g ceric sulphate and 50 ml concentrated H_2SO_4 were added under ice-cold conditions and then made up to 500 ml with water). The biologically active TLC fraction EHF-21 was further used for structural elucidation.

Dose- and time-dependent cytotoxicity of fraction EHF-21. The dose-dependent (0.25 to 1 μg/ml) activity of fraction EHF-21 over a 24-h period and subsequently, the time-dependent (at 2, 4, 8, 16 and 24 h) activity of 0.50 μg/ml over a 24-h period were assessed. Controls were maintained separately using cycloheximide (1 μg/ml) as positive control. The MTT assay was carried out to determine the viability of treated and untreated cells.

Hoechst staining. The *E. viride* fraction EHF-21 was added at a concentration of 0.5 μg/ml to a 24-well tissue culture plates with HeLa cells (7×10^5 cells/ml; 1 ml) (18) and observed for different periods of time (4, 8, 16, 24 h). Untreated HeLa cells were maintained as control. The cells were harvested by centrifugation ($110 \times g$; 10 min; 4˚C) at appropriate time periods, 1 ml PBS was used to resuspend the pellets and cells were stained with 10 μl Hoechst solution (dissolved in double distilled water at a stock concentration of 0.1 mg/ml) for 5 min at 37˚C in the dark. The dye was removed by centrifugation ($110 \times g$; 10 min; 4˚C) and by washing with PBS. The condensed chromatin was visualized at 200-

fold magnification with a Nikon EclipseTE 2000-S fluorescence microscope (Nikon Corporation, Kanagawa, Japan) using a filter 02 (λ ex: 365 nm; λ em: 420 nm).

Cell-cycle analysis and quantification of apoptosis. In cell-cycle analysis (22, 23), HeLa cells at 7×10^5 cells/ml, (18) seeded in a 24-well tissue culture plate (1 ml/well), were treated with *E. viride* fraction EHF-21 at 0.25, 0.50, 0.75 and 1 μg/ml for 24 h. Negative and positive (1 μg/ml cycloheximide) controls were maintained separately. After incubation, the cells were harvested by centrifugation (600 ×*g*, 10 min, 4˚C), washed with cold PBS and resuspended in 500 μl hypotonic propidium iodide (PI) (Sigma) staining solution. The tubes were stored at 4˚C in the dark overnight. The cell fluorescence (FL-2) and scatter characteristics (FSC/SSC) were acquired (23) with a FACS calibur instrument (Becton Dickinson, Heidelberg, Germany), equipped with a 488 nm argon-ion laser and analyzed using LYSYS II software (18). The analysis, depicted in a histogram with the number of cells (counts) plotted against the relative fluorescence intensity of PI (FL-2; λ em: 585 nm; red fluorescence), reflected the cell-cycle distribution of the cell population. Untreated control cells were used for the adjustment of instrument settings. Marked regions of sub-G1 cells were evaluated to quantify the percentage of apoptotic cells (18).

Determination of cell size and granularity. HeLa cells (7×10^5 cells/ml) (18), seeded in a 24-well tissue culture plate (1 ml/well), were left 24 h after treating with *E. viride* fraction EHF-21 at a moderately cytoxic concentration of 0.5 μg/ml. Untreated HeLa cells were maintained separately as control. The cells were then harvested by centrifugation (600 ×*g*, 10 min, 4˚C), washed with cold PBS, resuspended in 500 μl of cold PBS and analyzed by flow cytometry. Untreated control cells were used for adjusting cytometer settings (FSC, SSC). The detected signals were illustrated in dot plots (FSC *versus* SSC). Normal and apoptotic cells were differentiated by assessing the distribution of the dots within the plot (24).

DNA fragmentation assay. *E. viride* fraction EHF-21 at concentrations of 0.25, 0.50 and 1 μg/ml were added to 24-well tissue culture plates with HeLa cells and incubated for 24 h to assess the internucleosomal cleavage of DNA (25). Then the cells were washed twice with ice-cold PBS, resuspended in lysis buffer containing 10 mM Tris–HCl (pH 8.0), 20 mM EDTA and 0.5% Triton X-100) (Sigma) and incubated on ice for 30 minutes. Then the suspension was centrifuged at 8400rcf x*g* for 10 min at 4˚C and DNA was extracted with phenol–chloroform, precipitated with 0.1 vol 3 M sodium acetate and 2.5 vol ethanol and stored at −20˚C overnight. DNA was pelleted by centrifugation at 8400rcf for 5 min at 4˚C, rinsed with 70% ethanol and then resuspended in TRIS-EDTA buffer (pH 8.0) (Sigma) containing, 30 μg/ml of RNase (Sigma) and incubated for 6 h at 37˚C. DNA was run on a 2% agarose gel with ethidium bromide staining (26). After electrophoresis, DNA was visualized under UV and documented.

Characterization of the active compound. The mass spectrum for the cytotoxic fraction EHF-21 was obtained in ultraflex MALDI-TOF (Bruker Daltonics, Germany). Infrared spectrum for functional group analysis of Eudistomin H was recorded by using a Vector 22 Brucker FTIR instrument. Solid samples were recorded as KBr pellets and liquid samples were taken as solution spectra in CHCl₃ (27). ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were
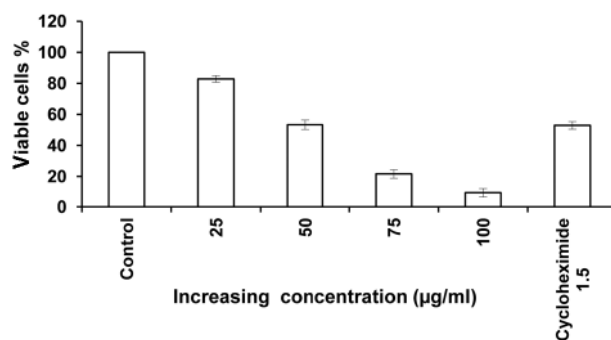

Figure 1. *Dose-dependent cytotoxicity of crude methanolic extract of Eudistoma viride against HeLa cells (mean±SD).*

recorded on a BRUKER DRX 500 solid state NMR spectrometer with proton frequency of 300.013 MHz and probe of 5 mm QNP (^{1}H, ^{13}C, ^{19}F, ^{31}P) instrument using TMS as the internal standard (28). CDCl3 was used as the solvent. By using the generated data, the molecular formula and structural characteristics of the active compound were elucidated.

Statistical analysis. The results were expressed as the mean±SD of three replicates. Student's *t*-test indicated the significance of the experimental results, compared to the control and a value of $p<0.05$ was considered as significant.

Results

The crude methanolic extract of *E. viride* exerted dose-dependent cytotoxicity against HeLa cells (Figure 1) and at 100 μg/ml, it was more potent than cyclohexamide (1 μg/ml), causing low cell viability of 9.2%. The IC_{50} was 53 μg/ml. Among the nine column fractions, the cytotoxic activity was low in fractions ECF-1 (100% Hex) and ECF-2 (75% Hex:25%DCM) with higher hexane content (Figure 2). ECF-4 (25% Hex:75% DCM) also exhibited low activity. Comparatively, fractions ECF-3 (50% Hex:50% DCM) and ECF-5 (100% DCM) had higher cytotoxicity. The fractions with higher methanolic content had higher cytotoxicity, with fraction ECF-8 (25% D CM: 75% Meth) showing prominence. However, the TLC of the active fraction ECF-8 revealed many compounds with different retention time R_f values (results not presented).

In the subsequent semipreparative HPLC of ECF-8, 23 fractions (EHF-1 to EHF-23) with prominent peaks with good retention time were collected and among them, 21 fractions showed more than 50% inhibition of cell proliferation at 2 μg/ml concentration (Figure 3). HPLC fractions EHF-12 to 21 and 22 had prominent cytotoxicity, with fractions EHF-20 and EHF-21 showing potent activity. EHF-21 showed remarkable dose- and time-dependent cytotoxicity against HeLa cells (Figures 4 and 5). At 1 μg/ml concentration, the percentage of viable cyclohexamide-
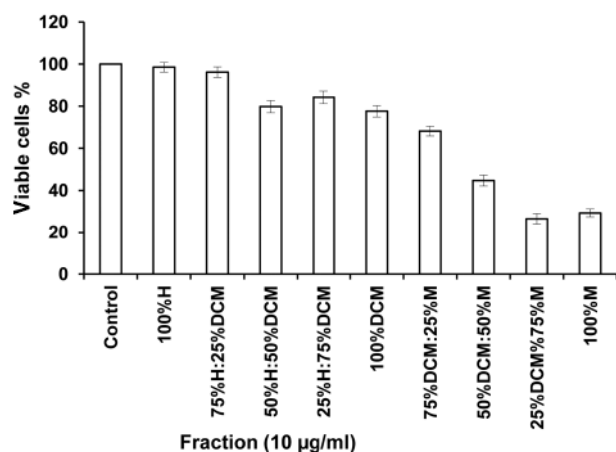
Figure 2. *Cytotoxicity of fractions eluted by column chromatography of methanolic extract of Eudistoma viride against HeLa cells (mean±SD). H: DCM: M.*

treated cells was 51% and that for cells treated with EHF-21 was 39.8%; 0.5 and 0.75 μg/ml EHF-21 led to 48.6% and 44.2% viable cells, respectively. lower than that for cells treated with cyclohexamide at 1 μg/ml. The activity of EHF-21 at 0.5 μg/ml increased with increasing incubation time. The IC$_{50}$ was 0.49 μg/ml.

Hoechst staining of cells treated with fraction EHF-21 and untreated cells displayed differential staining due to the highly condensed chromatin in treated cells and intact normal chromosomes in untreated cells. The apoptotic process in the cells treated with EHF-21 at 0.50 μg/ml showed sequential events from 4 to 24 h period, portraying the apoptotic events in drug-induced apoptosis. This was clearly indicated the characteristic morphological and biochemical features of apoptosis such as cell shrinkage, membrane blebbing, chromatin condensation and formation of apoptotic bodies in HeLa cells. These processes started from 4 h and blebbing appeared from 8 h, leading to formation of apoptotic bodies (Figure 6). The present study showed changes in light scatter *i.e.* a decrease in FSC as a sign of lower cell size and an increase in side scatter as a sign of granularity compared to control cells, indicating the characteristic feature of cells dying of apoptosis (Figure 7). The occurrence of a second population of apoptotic cells in the range of lower FSC and higher SSC compared to control cells was noticed.

In the cell-cycle profiles of HeLa cells, determined by flow cytometry after staining of the DNA with PI, the positive control of cycloheximide-treated HeLa cells showed an increase in the sub G_0/G_1 population, targeting cells in G_0/G_1 and G_2/M phases (Figure 8). Treatment of HeLa cells with EHF-21 at 0.25 μg/ml led to a substantial

elevation in the sub G_0/G_1 population, indicating the induction of apoptosis. The G_0/G_1 population continued to increase with increasing concentration of fraction EHF-21. The arrest was marked in the G_2/M phase on treatment with 0.50 μg/ml EHF-21, which also led to marked arrest of cells in G_0/G_1 and a further increase in the sub G_0/G_1 population. EHF-21 at 0.75 μg/ml led to the marked arrest of cells in S and G_2/M phases and substantial arrest of the G_0/G_1 population. At 1 μg/ml of EHF-21, marked arrest of cells in $G_0/G1$, S and G2/M phases and an elevated sub-G_0/G_1 population indicated an increase in the apoptotic cell population. The statistical analysis of the sub-G_1 region showed dose-dependent apoptosis induction by fraction EHF-21 (Figure 9). At the highest concentration tested (1 μg/ml), the effect of EHF-21 was almost equally potent as the positive control cycloheximide at inducing apoptosis in the HeLa cells. After 24 h, the percentage of apoptotic cells at all concentrations increased, with an increase of 65.21% for 1 μg/ml.

In agarose gel electrophoresis of the chromosomal DNA isolated from HeLa cells treated with fraction EHF-21 (Figure 10), the study clearly established the DNA damage caused by ascidian *E. viride* fraction EHF-21.

The characterization of fraction EHF-21 resulted in the isolation of Eudistomin H as a yellow powder, 6-bromo-1-(3,4-dihydro-2*H*-pyrrol-5-yl)-9*H*-β-carboline, with a molecular formula of $C_{15}H_{12}BrN_3$, established on the basis of interpretation of the ESI-MS, FT-IR spectroscopy and NMR data. The molecular weight was 314 Da. A strong peak at 1017.27 cm^{-1} in the infra-red spectrum clearly illustrated the presence of halogen (bromine). The structure of Eudistomin H is presented in Figure 11.

Discussion

The cytotoxic activity of the extract of *E. viride* was similar to that of *E. vannamei* and *Eudistoma* sp. from Northeast Brazil (29). However, the IC$_{50}$ of 53 μg/ml for the crude methanolic extract was comparatively higher than that reported for *E. vannamei* (<2 μg/ml for CEM, 11.2 μg/ml for HL-60, 23.8 μg/ml for B16 and 14.3 μg/ml for HCT-8 cell lines) (29); Ecteinascidin-743 from Caribbean tunicate Ecteinascidia turbinata (0.5 ng/ml against L1210 leukemia cells) (30, 31) and Lamellarin b from *Didemnum* sp. (4.8 μg/ml against HL-60) (32). However, the activity was much greater than that of pyrrolopyrimidine alkaloid, rigidin E, rigidin, and 1-methylherbipoline, isolated from a Papua New Guinea tunicate *Eudistoma* sp., which led only to minimal growth inhibition at 100 μg/ml against HCT 116 human colon carcinoma cell lines (33). Nevertheless, the IC$_{50}$ of the crude extract was higher than the limit prescribed by the US-NCI by which an extract is recognized as active by an IC$_{50}$ of <20 μg/ml (34).
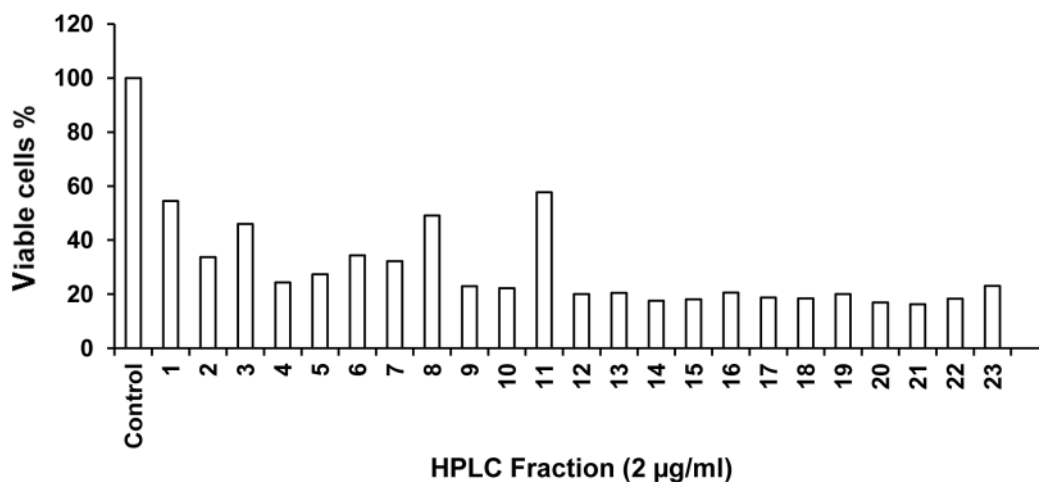
Figure 3. *Cytotoxicity of HPLC fraction of methanolic extract of Eudistoma viride against HeLa cells.*
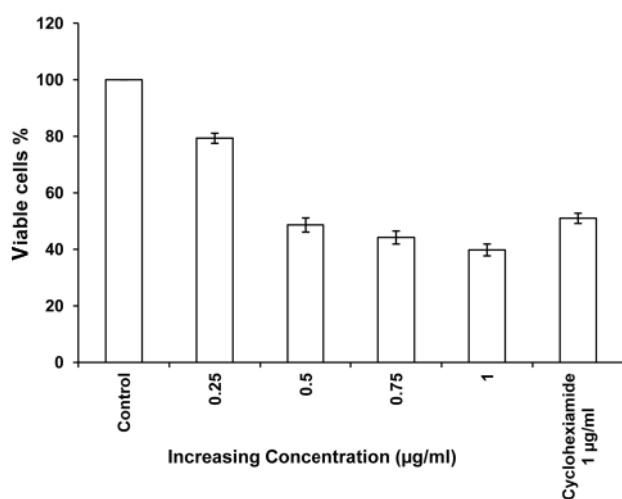

Figure 4. *Concentration-dependent cytotoxicity of fraction EHF-21 against HeLa cells (mean±SD).*
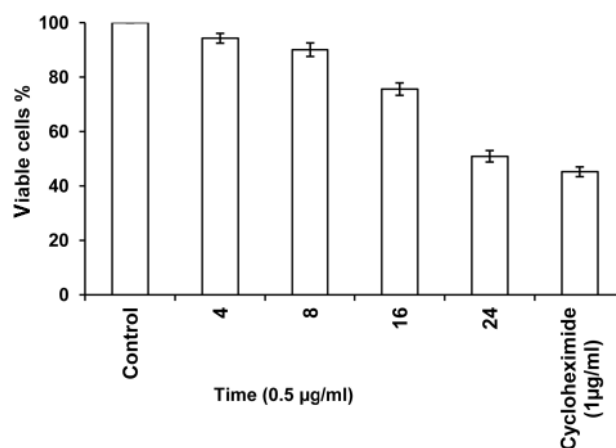

Figure 5. *Time-dependent cytotoxicity of fraction EHF-21 (0.5 µg/ml) against HeLa cells (mean±SD).*

The cytotoxicity of HPLC fractions against HeLa cell lines was comparatively higher than that reported for Hydroperoxysterol 1, from *Eudistoma* sp. against Hep3B (human hepatoma cells) and A549 (human lung adenocarcinoma) and that of hydroperoxysterol 2 towards Hep3B, HepG2 (human hepatocellular carcinoma), MECF7 (human breast carcinoma) and MDA-MB-231 (human breast carcinoma) cells (35). The observation of more than 50% inhibition of cell proliferation by almost 21 HPLC fractions at 2 µg/ml highlights the potential of *E. viride* as a source of effective cytotoxic agents. The IC_{50} value was higher than that of a range of 0.022-4.78 µM for the chlorinated didemnin

classes of compounds from tunicate *Trididemnum* against SK-MEL (melanoma), KB (epidermal carcinoma), BT549 (breast carcinoma) and SK-OV-3 (ovarian carcinoma) cancer cell lines (36) and Tanjungide A (0.19 to 0.33 µM) and Tanjungide B (1.00 to 2.50 µM) from the tunicate *Diazona* cf *formosa* against lung carcinoma (A549), colorectal carcinoma (HT29) and breast adenocarcinoma (MDA-MB-231) cell lines (37). The IC_{50} value of 0.49 µg/ml for the EHF-21 fraction was very well below the 4 µg/ml prescribed by the US-NCI for purified compounds to be considered as active (34).

It has been suggested that susceptibility to apoptosis-inducing effects of chemotherapeutic drugs may depend on
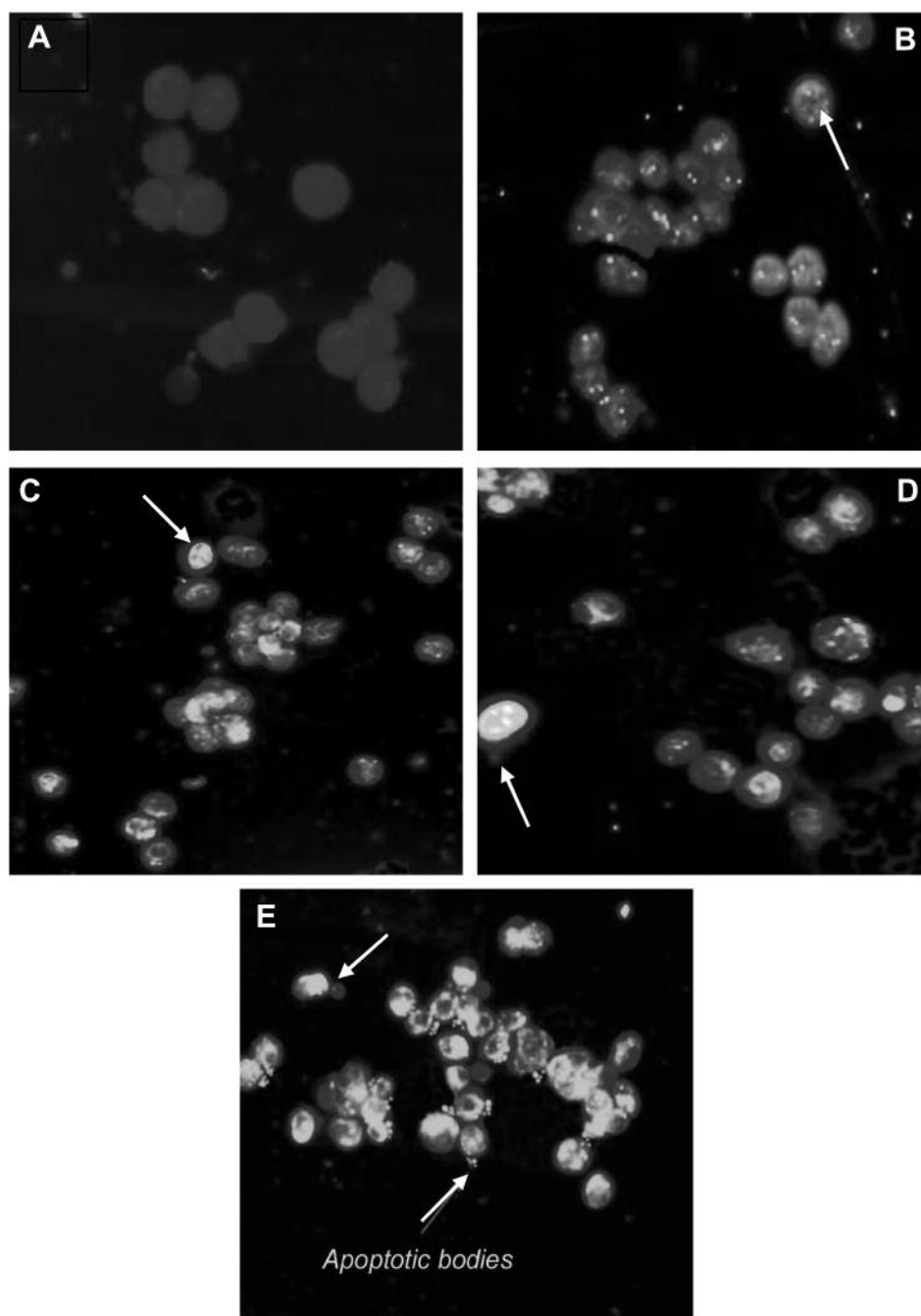
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Figure 6. *Apotopsis induced in HeLa cells by Eudistoma viride fraction EHF-21 at 0.50 μg. A: Control; B: at 4 h, clear chromatin condensation (arrow); C: 8 h, cell shrinkage apparent (arrow); D: 16 h, visible membrane blebbing (arrow); E: 24 h, membrane blebbing and formation of apoptotic bodies (arrows).*

the intrinsic ability of tumor cells to respond by apoptosis (38, 39). In the present study, the EHF-21 fraction was shown to induce apoptosis of HeLa cells in contrast to the induction of both necrosis and apoptosis by substances from *Didemnum psammathodes* (40). Apoptosis induction by

compounds isolated from *E. viride* was found to be due to Ecteinascidin-743 (ET-743) from Ecteinascidia turbinata, which induced a massive apoptosis of Ewing's sarcoma cells (41). Aplidine, from the Mediterranean tunicate Aplidium albicans, at nanomolar concentrations (10-100 nM) induced
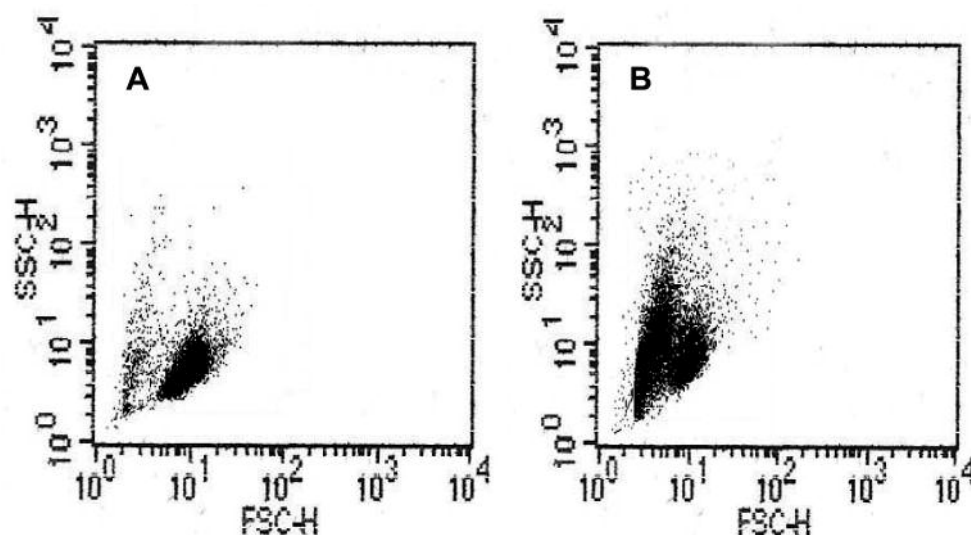
Figure 7. *HeLa cells treated with Eudistoma viride fraction EHF-21 also exhibited change in morphological features in flow cytometric analysis. A: Untreated HeLa cells exhibited uniform cell size and granularity. B: HeLa cells treated with EHF-21 exhibited a second population of cells which denoted the presence of apoptotic cells in the range of lower FSC and higher SSC when compared to untreated cells.*
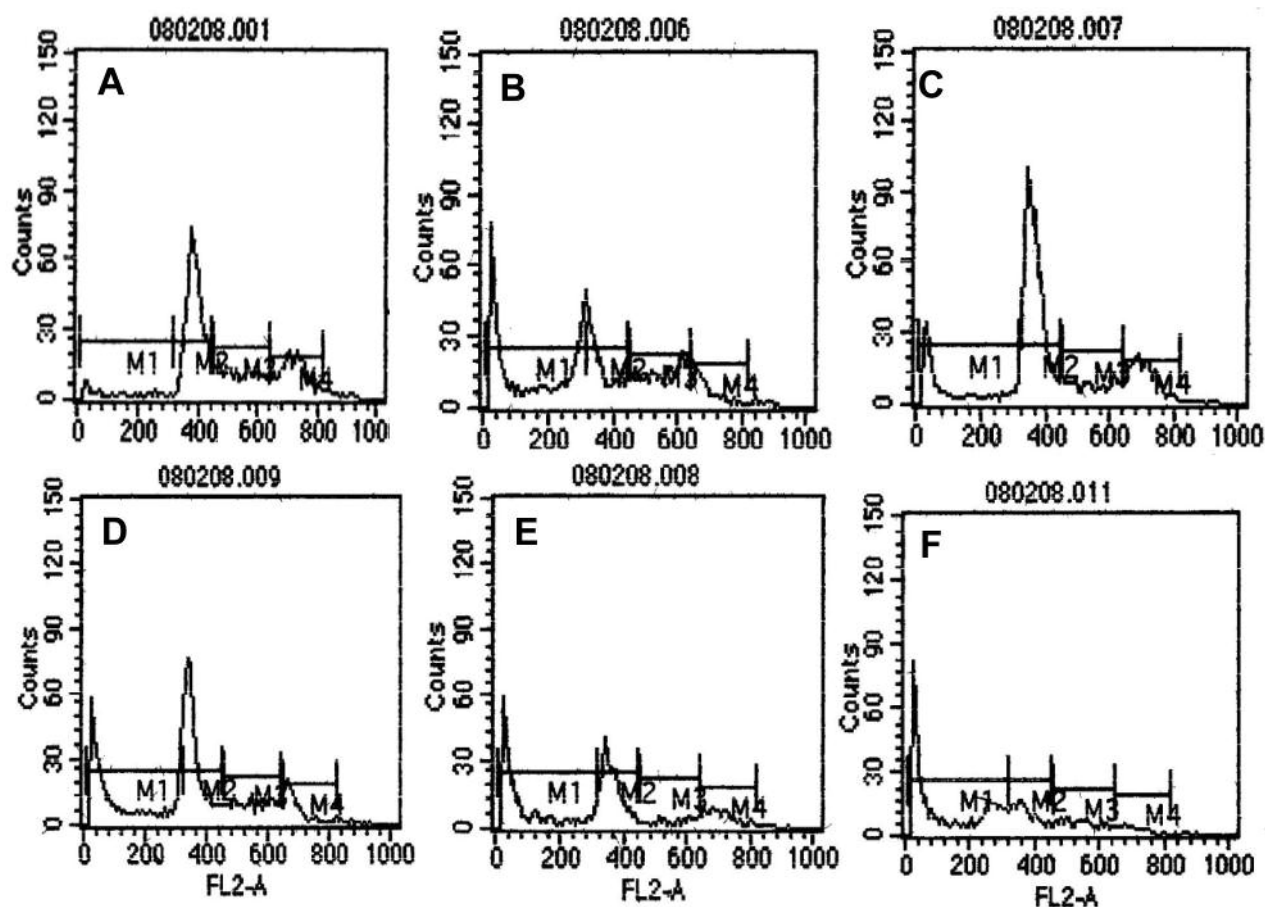

Figure 8. *Cell-cycle analysis indicating the different phases of the cell cycle. A: Untreated control; B: positive control of HeLa cells treated with 1 μg/ml cycloheximide; C-F: HeLa cells treated with fraction EHF-21 at 0.25 μg/ml, 0.50 μg/m, 0.75 μg/ml and 1 μg/ml, respectively. The peak in sub-G_0/G_1 phase of the cell cycle indicates the presence of apoptotic cells. M1: Sub G_0/G_1, M2: G_0/G_1, M3: S, M4: G_2/M.*
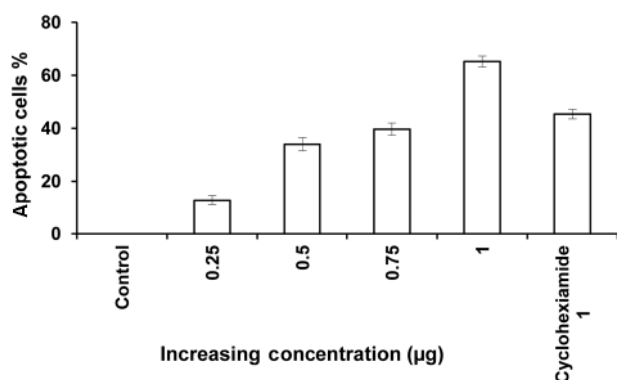
Figure 9. *Dose-dependent (0.25-1 µg) induction of apoptosis of HeLa cells by fraction EHF-21.*
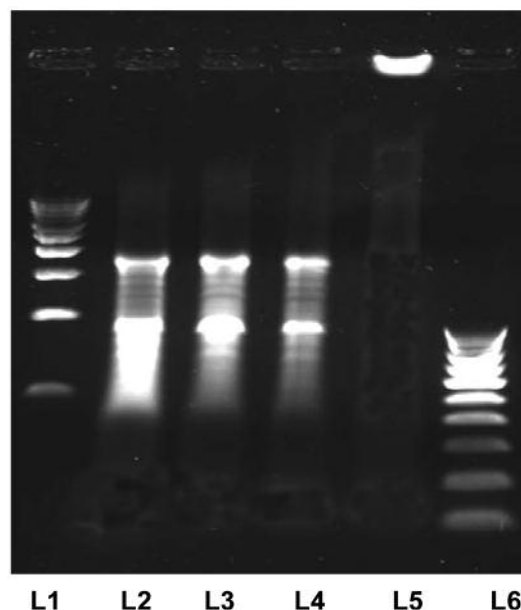

Figure 10. *DNA fragmentation analysis. Lane 1 and 6: 500 bp, 100 bp Molecular weight markers; lanes 2-4: HeLa cells treated with fraction EHF-21 at 0.25, 0.50 and 1 µg/ml; lane 5: untreated HeLa cells. Fragmented DNA of the HeLa cells treated with fraction EHF-21 can be seen in lanes 2-4.*

apoptosis of human leukemic cell lines and primary leukemia cell cultures from patients with leukemia (42) and two compounds from the ascidian *Diplosoma virens* were cytotoxic against HCT116 cells (human colorectal cancer cells) by triggering apoptotic cell death (7).

The appearance of two different cell populations in cells treated with EHF-21 when compared to untreated control cells in flow cytometric analysis indicated that the cells treated with fraction EHF-21 had undergone some cellular changes, either in surface or inner components, coinciding with the observation of production of two different cell populations in ascididemin-treated human leukemia Jurkat T-cells measured by flow cytometry (18). A marked arrest of HeLa cells in G_2/M phase on treatment with 0.50 µg/ml of fraction EHF-21 and significant appearance of cells in the sub-G_0/G_1 phase indicated induction of apoptosis. The arrest of HeLa cells in the G_2/M phase corroborates the observation that some anticancer drugs, when applied *in vivo*, are able to intercalate with DNA in cancer cells and do arrest cancer cells in the cell cycle *via* G_2/M arrest (41, 43, 44). The present study was also in line with the observation that ascidian metabolites didemnin B and aplidine interfered with the synthesis of DNA and proteins and induced cell-cycle arrest (45).

The percentage of apoptotic cells increased gradually with increasing concentration of fraction EHF-21. The interference of fraction EHF-21 with the cell cycle at the G_2/M phase and induction of apoptosis in HeLa cells in a dose-dependent manner correlated with the reported interference of paclitaxel with the cell cycle at the G_2/M phase and induction of apoptosis of HL-60 cells in a dose- and time-dependant manner (46). The marked arrest of HeLa cells in the G_2/M phase by EHF-21 parallels the observation that meridianin B induced proliferation arrest in exponentially growing cells, clearly accompanied by an accumulation in G_0/G_1 phase, whereas meridianin E treatment led to an arrest in the G_2/M phase (6). The mode of

marked arrest of cells in the G_2/M phase concurs with findings with halichondrins, potent tubulin inhibitors, which noncompetitively bind to the Vinca binding site and cause a characteristic G_2-M cell-cycle arrest with concomitant disruption of the mitotic spindle (47, 48). Similarly, Dolastatin 10, isolated from a sea hare, cause cell-cycle arrest in metaphase as it binds to the rhizoxin/maytansine binding site and to the exchangeable guanosine triphosphate site on tubulin (49). The time-dependent increase in activity of EHF-21 fraction in the present study was in line with the cytotoxicity of lissoclinolide, isolated from the marine ascidian *Lissoclinum patella*, which at 2.4 µM resulted in a strong arrest in the G_2/M phase of the cell cycle of HCT 116 human colon tumor cells after 24 h exposure (50).

During apoptosis, a specific nuclease cuts genomic DNA between nucleosomes to generate DNA fragments and the presence of this ladder is a hallmark of apoptotic cell death (51). The ascidian *E. viride* fraction EHF-21 may be a DNA-damaging agent due to its intercalating properties, predominantly targeting cells in the G_2/M and S phases, which correlated with that of Ascididemin, a DNA-damaging agent due to its intercalating properties, which predominantly target cells in the S phase (18). Similarly, the present observation concurs with findings for Aplidine, which interfered with the synthesis of DNA and proteins and induced cell-cycle arrest (44). The inhibition of
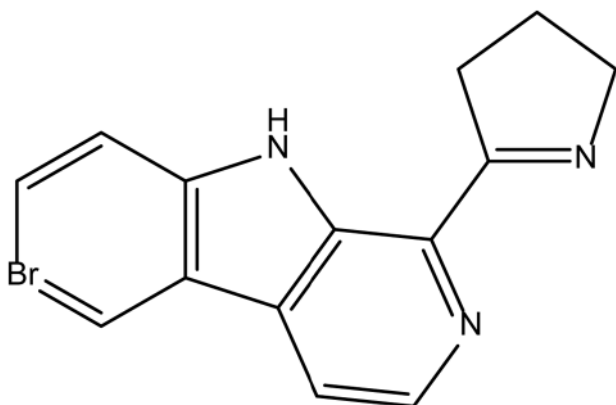
Figure 11. *The structure of Eudistomin H isolated from fraction EHF-21 of methanolic extract of Eudistoma viride.*

topoisomerase II and inducement of topoisomerase II-mediated DNA cleavage as noted in the potent cytotoxicity of Ascididemin, a pyridoacridine alkaloid of *Didemnum* sp. against tumor cells *in vitro* and *in vivo* (52) is also valid for the possible mechanism of activity of *E. viride* fraction. The discovery of a new cell-killing mechanism mediated by DNA nucleotide excision repair (NER) and interaction of ET-743 with the transcription-coupled NER machinery to induce lethal DNA strand breaks (53) corroborate the DNA fragmentation found in HeLa cells treated with *E. viride* fraction EHF-21.

Many novel and bioactive secondary metabolites have been isolated from the ascidian genus *Eudistoma*. Among the metabolites isolated from this genus, the predominant structure class was found to be β-carboline alkaloids (13). Macrolides (54), indoles (12, 55) and aromatic polysulfides (56) have also been reported from the same genus. Among them, the best known metabolites are the eudistomins, which are a series of β-carboline derivatives that display variety of pharmacological activities (57).

The present apotogenic compound, a brominated indole derivative, isolated from the green ascidian *E. viride* is similar to that of β-carboline skeleton. Similarly, the genus *Eudistoma* from Korea yielded seven new beta-carboline-based metabolites with the presence of a benzoyl group attached to the beta-carboline nucleus at C-1, designated as eudistomins Y1-Y7 (58). The isolated compound from *E. viride* was considered potent when compared to that of Pibocin B, the first representative of marine alkaloids with a unique structural feature [(8 beta)-2-bromo-*N-O*-methyl-6,8-dimethylergoline], an *N-O*-methylindole group, isolated from the Far-Eastern ascidian *Eudistoma* sp., which showed moderate cytotoxic activity against mouse Ehrlich carcinoma cells (12). This simple aromatic nitrogenous compound could

be a highly potent metabolite as evidenced from the strong cytotoxic activity. Similar bromine carrying beta-carbolines eudistomin E and eudistalbin A from the marine tunicate E. album were shown to possess cytotoxic activity (ED_{50} less than 5.0 ng and 3.2 μg/ml, respectively) *in vitro* against the growth of KB human buccal carcinoma cells (59). The beta-carboline alkaloid 14-methyleudistomidin C isolated from E. gilboverde exhibited potent cytotoxic activity with IC_{50}s of <1.0 μg/ml against four different human tumor cell lines (60).

Although the mechanism of action of the *E. viride* fraction EHF-21 (Eudistomin H) was not ascertained, the induction of apoptosis and DNA fragmentation indicate the initiation of the pathway through cleavage of caspase-3, as the complex apoptosis mechanism involving extrinsic or intrinsic or perforin/granzyme B pathways ultimately converge on the execution pathway *i.e.* activation of caspase-3 (61) which leads to apoptosis, including chromosomal DNA degradation and formation of apoptotic bodies. But the observation of no cleavage of caspase after treatment with Ritterazine B from ascidian *Ritterella tokioka* and its induction of apoptosis *via* a novel anti-mitotic mechanism (43) poses an interesting question as activation of caspase-independent parallel cell death pathway by granzyme A pathway *via* single-stranded DNA damage, has been reported (62). Hence, the potent apotogenic compound Eudistomin H isolated here, with an IC_{50} of 0.49 μg/ml, could play an important role in overcoming chemoresistance in cancer cells through novel modes of action (63).

Acknowledgements

References

1   Nicholson DW: From bench to clinic with apoptosis-based therapeutic agents. Nature *407*: 810-816, 2000.
2   Ashkenazi A. Pai RC, Fong S, Leung S, Lawrence DA, Marsters SA, Blackie C, Chang L, McMurtrey AE, Hebert A, DeForge L, Koumenis IL, Lewis D, Harris L, Bussiere J, Koeppen H, Shahrokh Z and Schwall RH: Safety and antitumor activity of recombinant soluble Apo2 ligand J Cli Invest *104(2)*: 155-62. 1999.
3   Munro MHG, Blunt JW, Dumdei EJ, Hickford SJH, Lill RE, Li S, Battershill CN and Duckworth AR: The discovery and development of marine compounds with pharmaceutical potential. J Biotechnol *70*: 15-25, 1999.

4 Faulkner DJ: Marine natural products. Nat Prod Rep *18*: 1-49, 2001 .

5 Konish I, Hosokawa M, Sashima T, Kobayashi H and Miyashita K: Halocynthiaxanthin and fucoxanthinol isolated from *Halocynthia roretzi* induce apoptosis in human leukemia, breast and colon cancer cells. Comp Biochem Physiol, C Toxicol, Pharmacol *142*: 53-59, 2006.

6 Gompel M, Leost M, Joffe EBD, Puricelli L, Franco LH, Palermo J, Meijer L Meridianins, a new family of protein kinase inhibitors isolated from the ascidian *Aplidium meridianum*. Bioorg. & Med. Chem Lett *14*: 1703-1707, 2004.

7 Ogi T, Taira J, Margiastuti P and Ueda K: Cytotoxic metabolites from the Okinawan ascidian Diplosoma Virens. Molecules *13*: 595-602, 2008.

8 Rajesh RP, Ramasamy MS and Murugan A: Anticancer activity of the ascidian Polyclinum indicum against cervical cancer cells (HeLa) medicated through apoptosis induction. Medicinal Chemistry *6(6)*: 396-405, 2010.

9 Watters DJ and Van den Brenk AL: Toxins from ascidians. Toxicon *31*: 1349-1372, 1993.

10 Kott P: The Australian Ascidiacea. Part 2. Aplousobranchia (1). Mem Qd Mus *29*: 1-266, 1990.

11 Jimenez PC, V Wilke D, Ferreira EG, Takeara R, de Moraes MO, Silveira ER, da C Lotufo, TM, Lopes NP and Costa Lotufo LV: Structure elucidation and anticancer activity of 7-oxostaurosporine derivatives from the Brazilian endemic tunicate *Eudistoma* vannamei. Mar Drugs *10*: 1092-1102, 2012.

12 Makarieva TN, Dmitrenok AS, Dmitrenok PS, Grebnev BB, Stonik V and Pibocin B: the first N-O-methylindole marine alkaloid, a metabolite from the Far-Eastern ascidian *Eudistoma* species. J Na Prod *64*: 1559-1561, 2001.

13 Menna M, Fattorusso E and Imperatore C: Alkaloids from marine ascidians Molecules *16*: 8694-8732, 2011.

14 Renganathan TK: New record and redescription of a rare colonial ascidian *Eudistoma viride* Tokioka 1955 from the Indian waters. Geobios New Reports *3*: 49-51, 1984.

15 Riguera R: Isolating bioactive compounds from marine organisms. J Mar Biotechnol *5*: 187-193, 1997.

16 Wright AE: Isolation of marine natural products. In methods in biotechnology Natural product isolation. Cannell RPJ, (Ed); Humana press Inc. New Jersy USA Vol *4*: 365-408, 1998.

17 Moore GE and Woods LK: Culture media for human cells: RPMI 1630, RPMI 1634, RPMI 1640 and GEM 1717. TC A Man *3*: 503-508, 1976.

18 Kirschke SO: Investigation of the Apoptosis Signal Transduction Mediated by the Marine Pyridoacridine Alkaloid Ascididemin in Human Leukemic Jurkat T-Cells, PhD.Thesis, Ludwig-Maximilans-Univeritat München Munich, December 2002.

19 Martinez-Garcia M, Diaz-Valdes M, Ramos-Espla A, Salvador N, Lopez P, Larriba E and Anton J: Cytotoxicity of the ascidian Cystodytes dellechiajei against tumor cells and study of the involvement of associated microbiota in the production of cytotoxic compounds. Drugs *5*: 52-70, 2007.

20 Zaeoung S, Plubrukarn A and Keawpradub N: Cytotoxic and free radical scavenging activities of Zingiberaceousrhizomes Songklanakarin. J Sci Technol *27(4)*: 800-812, 2005.

21 Allen JR, Williams JD, Burinsky DJ and Cole SR: Isolation of the components of a complex mixture by means of column switching for their enhanced detection by massspectrometry J Chromatogr A *913*: 209-219, 2001.

22 Vindelov LL, Christensen IJ, Keiding N, Spang Thomsen M and Nissen NI: Long-term storage of samples for flow cytometric DNA analysis. Cytometry *3*: 317-322, 1983.

23 Wesierska-Gadek J, Schloffer D, Gueorguieva M, Uhl M and Skladanowski A: Increased susceptibility of poly (ADP-ribose) polymerase-1 knockout cells to antitumor triazoloacridone C-1305 is associated with permanent G2 cell cycle arrest. Cancer Res *64*: 4487-4497, 2004.

24 Gorman AM, Samali A, McGowan AJ and Cotter TG: Use of flow cytometry techniques in studying mechanisms of apoptosis in leukemic cells. Cytometry *29*: 97-105, 1997.

25 Sri Balasubashini M, Karthigayan S, Somasundaram ST, Balasubramanian T, Rukkumani R and Menon VP: FV peptide induces apoptosis in HEP 2 and HeLa cells: an insight into the mechanism of induction. Journal of Carcinogenesis *5*: 27, 2006.

26 Sambrook J and Russell DW: The Condensed Protocols from Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, USA, 2001.

27 Benkendroff K, Bremner JB and Davis AR: Tyrian purple precursors in the egg masses of the Australian muricid Dicathais orbita , a possible defensive role J Chem Ecol *26*: 1037-1050, 2000.

28 Sera YK, Fujii AK and Shizuri Y: Isolation of haliclonamides. New peptides as antifouling substances from a Marine sponge species Hallclona. Mar Biotechnol *3*: 441-446, 2002.

29 Jimenez PC, Fortier SC, Lotufo TMC, Pessoa C, Moraes MEA, Moraes MO and Costa-Lotufo LV: Biological activity in extracts of ascidians (Tunicata Ascidiacea) from the northeastern Brazilian Ecol coast. J Exp Mar Biol *287(1)*: 93-101, 2003.

30 Rinehart K, Holt T, Fregeau N, Stroh J, Keifer P, Sun F, Li L and Martin D :Ecteinascidins 729, 743, 745, 759A, 759B and 770: potent antitumor agents from the Caribbean tunicate Ecteinascidia turbinata. J Org Chem *55*: 4512-4515, 1990.

31 Wright A, Forleo D, Gunawardana G, Gunasekera S, Koehn F and McConnell O: Antitumor tetrahydroisoquinoline alkaloids from the colonial ascidian *Ecteinascidia turbinate* J Org Chem *55*: 4508-4512, 1990.

32 Ham J and Kang HA: novel cytotoxic alkaloid of lamellarin class from a marine ascidian *Didemnum* sp Bull, Korean Chem Soc *23*: 163-166, 2002.

33 Davis RA, Christensen LV, Richardson AD, Da Rocha RM, Ireland CM and Rigidin E: a new pyrrolopyrimidine alkaloid from a Papua New Guinea tunicate *Eudistoma* species Ma-. Drugs *1*: 27-33, 2003.

34 Suffness M and Pezzuto JM: Assays related to cancer drug discovery. Methods in Plant Biochemistry. Biological Techniques, Academic Press, London Vol *6*: 71-131, 1991.

35 Sung PJ, Lin MR, Chen JJ, Lin SF, Wu YC, Hwang TL and Fang LS: Hydroperoxysterols from the tunicate *Eudistoma* sp Chem Pharm Bull *55*: 666-668, 2007.

36 Ankisetty S, Khan SI, Avula B, Gochfeld D, Khan IA and Slattery M: Chlorinated Didemnins from the Tunicate *Trididemnum solidum*. Mar Drugs *11*: 4478-4486, 2013.

37 Murcia C, Coello L, Fernández R, Martín MJ, Reyes F, Francesch A, Munt S and Cuevas C, 2014: Tanjungides A and B: New antitumoral bromoindole derived compounds from *Diazona* cf *formosa*. Isolation and total synthesis. Mar Drugs *12*: 1116-1130, 2014.

38 Yano H, Mizoguchi A, Fukuda K, Haramaki M, Ogasawara S, Momosaki S and Kojiro M: The herbal medicine sho-saiko-to inhibits proliferation of cancer cell lines by inducing apoptosis and arrest at the G0/G1 phase. Cancer Res *54*: 448-454, 1994.

39 Tseng CJ, Wang YJ, Liang YC, Jeng JH, Lee WS, Lin JK, Chen CH, Liu IC and Ho YS: Microtubule-damaging agents induce apoptosis in HL-60 cells and G_2/M cell arrest in HT 29 cells. Toxicology *175*: 123-142, 2002.

40 Takeara R, Lopes JLC, Lopes NP, Jimenez PC, Costa-Lotufo LV and Lotufo TMC: Constituintes quimicos da ascidia *Didemnum psammathodes* (Sluiter, 1895) coletadana costa cearense (Chemical constituents from the ascidian *Didemnum* psammatodes (Sluiter,1895) collected on the shores of Ceara state. Quit. Nova *30*: 1179-1181, 2007.

41 Scotlandi K, Manara MC, Strammiello R, Landuzzi L, Benini S, Perdichizzi S, Serra M, Astolfi A, Nicoletti G, Lollini PL, Bertoni F, Nanni P and Picci P: c-KIT receptor expression in Ewing's Sarcoma: lack of prognostic value but therapeutic targeting opportunities in appropriate conditions. Journal of Clinical Oncology *21(10)*: 1952-1960, 2003.

42 Gajate C, An F and Mollinedo F: Rapid and selective apoptosis in human leukemic cells induced by aplidine through a FAS/CD95-and mitochondrial-mediated mechanism. Clin Cancer Res *9*: 1535-1545, 2003.

43 Komiya T, Fusetani N, Matsunaga S, Kubo A, Kaye FJ, Kelley MJ, Tamura K, Yoshida M, Fukuoka M and Nakagawa K: Ritterazine B, a new cytotoxic natural compound induces apoptosis in cancer cells. Cancer Chemotherapy& Pharmacol *51*: 202-208, 2003.

44 Erba E, Bassano L, Di Liberti G, Muradore I, Chiorino G, Ubezio P, Vignati S, Codegoni A, Desiderio MA, Faircloth G, Jimeno J and D-Incalci M: Cell-cycle phase perturbations and apoptosis in tumour cells induced by aplidine British J Cancer *86*: 1510-1517, 2002.

45 Sakai R, Kishore V, Kundu B, Faircloth G, Gloer JB, Carney JR, Namikoshi M, Sun F, Hughes RG, Gravalos DG, Quesada TG, Wilson GR and Heid RM: Structure–activity relationships of the didemnins. J Med Chem *39*: 2819-2834, 1996.

46 Ko-Hsiu L, Ko-Huang L, Hsien-Hua L, Keh-Liang L and Jing-Gung C: Induction of caspase-3-dependent apoptosis in human leukemia HL-60 cells by paclitaxel. Clinica chemicia Acta *357(1)*: 65-73, 2005.

47 Bai RL, Paull KD, Herald CL, Malspeis L, Pettit GR and Hamel E: Halichondrin B and homohalichondrin B: marine natural products binding in the Vinca domain of tubulin. Discovery of tubulin-based mechanism of action by analysis of differential cytotoxicity data. J Biol Chem *266*: 15882-15889, 1991.

48 Dabydeen D, Florence G, Paterson I and Hamel EA: Quantitative evaluation of the effects of inhibitors of tubulin assembly on polymerization induced by discodermolide epothilone B, and paclitaxel. Cancer Chemother Pharmacol *53*: 397-403, 2004.

49 Bai R, Pettit GR and Hamel E: Dolastatin 10, a powerful cytostatic peptide derived from a marine animal. Inhibition of tubulin polymerization mediated through the Vinca alkaloid binding domain. Biochem Pharmacol *43*: 2637-2645, 1992.

50 Richardson AD and Ireland CA: A profile of the *in vitro* antitumor activity of lissoclinolide Toxicol & Applied Pharmacol *195*: 55-61, 2004.

51 Wyllie AH: Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature *284*: 555-556, 1980.

52 Matsumoto SS, Biggs J, Copp BR, Holden JA and Barrows LR: Mechanism of ascididemin-induced cytotoxicity. Chem Res Toxicol *16*: 113-122, 2003.

53 Takebayashi Y, Pourquier P, Zimonjic DB, Nakayama K, Emmert S, Ueda T, Urasaki Y, Kanzaki A, Akiyama SI, Popescu N, Kraemer KH and Pommier Y: Antiproliferative activity of ecteinascidin 743 is dependent upon transcription-coupled nucleotide-excision repair. Nat Med *7*: 961-966, 2001.

54 Kobayashi J, Cheng J, Nakamura H, Ohizumi Y, Hirata Y, Sasaki T, Ohta T and Nozoe S: Ascididemin, a novel pentacyclic aromatic alkaloid with potent antileukemic activity from the okinawan tunicate *Didemnum* sp. Tetrahedron Lett *29*: 1177-1180, 1988.

55 Makarieva TN, Ilyin SG and Denisenko VA: Pibocin, the first ergoline marine alkaloid from the far-eastern ascidian *Eudistoma* sp. Tetrahedron Lett *40*: 1591-1594, 1999.

56 Compagnone RS, Faulkner DJ, Carte BK, Chan G, Freyer A and Hemling ME: Pentathiepins and trithianes from two Lissoclinum species and a *Eudistoma* sp inhibitors of protein kinase C. Tetrahedron *50*: 12785-12792, 1994.

57 Cao R, Peng W, Wang Z and Xu A: β-Carboline Alkaloids: biochemical and pharmacological functions. Curr Med Chem *14*: 479-500, 2007.

58 Wang W, Nam SJ, Lee BC and Kang H: Beta-carboline alkaloids from a Korean tunicate *Eudistoma* sp. J Nat Prod *71*: 163-166, 2008.

59 Adesanya SA, Chbani M, Pais M and Debitus C: Brominated β-carbolines from the marine tunicate *Eudistoma* album. J Nat Prod *55*: 525-527, 1992.

60 Rashid MA, Gustafson KR and Boyd MR: New cytotoxic N-methylated β-carboline alkaloids from the marine ascidian *Eudistoma* gilboverde. J Nat Prod *64*: 1454-1456, 2001.

61 Elmore S: Apoptosis: A Review of programmed cell death Toxicol Pathol *35*: 495-516, 2007.

62 Martinvalet D, Zhu P and Lieberman J: Granzyme A: induces caspase-independent mitochondrial damage, a required first step for apoptosis. Immunity *22*: 355-370, 2005.

63 Barthomeuf C, Bourguet-Kondracki ML and Kornprobst JM: Marine metabolites overcoming or circumventing multidrug resistance mediated by ATP-dependent transporters: a new hope for patient with tumors resistant to conventional chemotherapy. Anticancer Agents Med Chem *8(8)*: 886-903, 2008.