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Antiproliferative activity of *Phormidium valderianum* and *Phormidium tenue* (Cyanobacteria) on human cervical cancer cells (HeLa) in vitro

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Abstract

Antiproliferative potential of two cyanobacterial taxa viz *Phormidium valderianum* Gomont and *Phormidium tenue* (Meneghini) Gomont was evaluated on HeLa cells using their crude aqueous extracts. Cytotoxicity was assessed by standard cell viability assays like 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) reduction assay and Trypan blue dye exclusion test. Algal aqueous extract (AAE) treated HeLa cells' morphology was studied by Haemalum and Hoechst 33258 staining. DNA fragmentation assay was also conducted to study the presence of DNA laddering. MTT assay showed gradual decrease in cell viability with increase in treatment amount*; Phormidium valderianum* was found to be more effective than the other. Trypan blue dye exclusion test also validated the same. Distinct changes in both cellular and nuclear morphology were observed. Most of the treated cells lost their characteristic stretched appearance, became round, with condensed nucleus. DNA profile revealed presence of sheared DNA in treated ones but no fragmentation was observed. The results indicated potent antiproliferative activity of the AAEs on HeLa cells which may be good candidates for further investigation to isolate bioactive anticancer compounds.

Introduction

Cancer is one of the most serious threat to human health as it's the second largest death causing disease while cardiac diseases tops the chart (Xu et al. 2009). There was an estimated 12.7 million cancer cases & 7.6 million cancer deaths were reported worldwide (Jemal et al. 2011) and the number is expected to reach 21 million by 2030. Chemotherapy is still the standard treatment method while most of the available chemotherapeutic drugs are coming with serious side effects like hair loss, bleeding, fatigue, infertility, cognitive impairment, sensory abnormalities, lung damage, nervous tissue damage, liver damage, gastrointestinal damages etc. Emerging cancer drug resistance is another serious problem regarding chemotherapy (Gottesmann 2002). That's why finding new antitumor compounds having lesser side effects have been a field of interest for many years. In this context natural products, derived from plants, marine organisms and microorganisms have drawn attentions of many scientists. Some important findings also have gained significance for natural biomolecules in the field of cancer research. According to WHO, 80% of world's population especially in developing countries rely on plant derived (Gurib-Fakim 2006) medicines.

Microalgae have tremendous potential for generating new anticancer drugs, as they are rich source of bioactive compounds which are not utilized yet. They have been used traditionally for medicinal purposes in India, China, Japan, Korea, Ireland and Wales. Marine algae are sources of pharmacologically active metabolites (Cannel 1993; Nekhoroshev 1996; Mayer et al. 1999, 2003; Faulkner 2000 ;) having antimicrobial, antineoplastic, antiviral (Rinehart et al. 1981; Tziveleka et al. 2003), antinflammatory, antitumoral and immunostimulant (Konig et al. 1998) activities.

The cyanobacterial genus *Phormidium* is evenly distributed throughout the Indian coast, having many species mainly marine in nature, rich in different types of pigments specially phycobiliproteins and different types of carotenoids. The crude aqueous extract may contain large amount of water soluble pigment phycocyanins and extracellular polysachharides. In the present study, *Phormidium valderianum* and *Phormidium tenue*, two cyanobacteria were procured from NFMC,Trichy.

HeLa cells are HPV-positive human cervical cancer cells in which there is expression of high risk viral oncoproteins E6 and E7 (DeFilippis et al.2003) expressing low level of P53 and normal level of pRB.

The primary objective of this study is to evaluate the antiproliferative potential of crude aqueous extracts of these two cyanobacteria on HeLa cells by standard anti-proliferative assays. Cell and nuclear morphology were also studied by different staining

procedures. DNA profiling was studied to investigate any change in DNA of the treated cells.

Materials and methods

Cyanobacterial culture

The cyanobacterial strains were maintained in Artificial Sea Nutrients – III (ASN-III) medium in a culture room equipped with 18:6 light/dark cycle for twenty one days at 20ºC. Lighting was provided by cool white 40W fluorescent tube lamps.

After 21 days of inoculation, the biomasses were harvested and with a small portion of the cyanobacteria were sub-cultured. The harvested biomass was then washed with sterile distilled water to eliminate the salts present in culture medium, soaked in blotting paper and then their fresh weight was taken. Aliquots of 2 gm were prepared and stored at -80ºC (Thermo-Scientific) for future use.

Preparation of Cyanobacterial extracts

The aliquots (2 gm) of each cyanobacterium were separately grounded in pre-chilled sterile mortar and crushed with 5 ml of sterile Milli-Q grade water (Purelab Option-Q) using sterile pestle in a cold room (Bluestar) at 4ºC. The whole homogenate of each sample was then transferred to sterile vials and sonicated (Hielscher) for 15 min at 60% amplitude, 1 cycle and subsequently centrifuged (Eppendorf) at 9000 x g for 20 min at 4ºC. The supernatants were taken and filtered through 0.22 µm sterile syringe filters (PALL) to eliminate bacterial contamination. The filtered AAEs were then stored at -20ºC as 0.5 ml aliquots to avoid repeated freeze thawing cycles.

HeLa cell culture

HeLa cells used in this study were a kind gift from Dr. R.N. Boral, Chittaranjan National Cancer Institute, Kolkata, India. They were maintained in monolayer cultures in Minimum Essential Medium Eagle medium (HiMedia) supplemented with 0.035 % (w/v) sodium bicarbonate (HiMedia), 10 % (v/v) fetal bovine serum (HiMedia), 0.03 % (w/v) L-Glutamine (HiMedia), antibiotic antimycotic solution (HiMedia) – 100unit/ml Penicillin, 100µg/ml Streptomycin and 0.25 µg/ml Amphotericin B at 37ºC in a humidified incubator having 5% CO_2.

Cell proliferation assay

Effect of the AAEs on proliferation of HeLa cells were evaluated by standard cytotoxicity measuring assays like MTT assay (Carmicheal et al. 1987) and Trypan Blue dye exclusion test (Freshney R. 1999).

MTT reduction assay

The inhibition of cell proliferation by the crude aqueous extracts was detected by formation of purple formazan crystals through reduction of MTT (Sigma) by mitochondrial dehydrogenase enzymes working only in viable cells. HeLa cells were plated at an initial density of $2x10^4$ cells per well in 96 well flat bottom tissue culture plates (HiMedia) and incubated in 200 µl MEM Eagle medium for 24 hrs at 37ºC with 5% CO_2 with a negative control set i.e. wells with only media but no HeLa cells. After incubation the old medium was discarded and fresh medium was poured in each well having 0-125 µl/ml of each AAE treatment with 25 µl increments. Total 6 treatment sets were performed each in triplicate, incubated again for 24 hours keeping earlier culture conditions. When the treatment period was over 20 µl of 5 mg/ml MTT solution was added to each well and the cells were further incubated for 4 hrs at similar culture conditions. Then the medium was discarded from each well keeping the cells undisturbed followed by addition of 100 µl Dimethyl Sulfoxide (DMSO) to each well including the negative control set and pipetted repeatedly to solubilize the formazan crystals. Absorbance at 490 nm was read by a microplate reader (BioRad iMark) taking the negative control set as blank. Using DMSO as a solubilizing agent for the formazan crystals, the absorbance readings were taken at 490 nm (Pang et al 2010).

The percent inhibition of cell proliferation was calculated as follows:

$$\% \text{ inhibition} = [(1- \text{Absorbance}_{\text{treatment}})/\text{Absorbance}_{\text{control}}] \times 100$$

Trypan Blue dye exclusion assay

The inhibition of cell proliferation by the crude aqueous extract was also checked by using the vital dye Trypan Blue having a negative chromophore interacts only with damaged cell membranes of dead cells. Viable cells exclude this dye. HeLa cells were plated at an initial density of $1x10^5$ cells per well in a 6 well plate (HiMedia) and incubated in 2 ml MEM Eagle medium for 24 hrs at 37ºC with 5% CO_2. After incubation the old medium was discarded and fresh medium was poured in each well having 0 and 250 µl/ml each AAE treatments in duplicate and incubated for 24 hrs keeping earlier culture conditions similar. After incubation the medium of each well containing detached dead cells was collected in separate microcentrifuge tubes along with attached cells that were scraped using sterile cell scraper (Corning). The cell suspensions were centrifuged at 6000 x g, 4ºC to pellet down the cell and rewashed with Phosphate Buffered Saline (PBS) pH 7.4 and centrifuged again, finally dissolved in 0.5 ml PBS in sterile vials. Then 0.1 ml of 0.4 % (w/v) Trypan Blue (Sigma) solution was added to each vial and allowed to stand at

room temperature for 5 min, filled in haemacytometer (Marienfeld-Neubauer) and cell count was performed under 10X magnification of a phase contrast microscope (Olympus).
The percent inhibition of cell proliferation was calculated as follows:

% inhibition = (Number of dead cells/ Number of total cells) x 100

Morphological examination

To study the morphological changes, HeLa cells treated with the AAEs at 250 µl/ml concentration for 24 hrs, were stained with Haemalum and Hoechst 33258. In both methods, HeLa cells were plated at an initial concentration of $5x10^4$ cells per coverslip (sterile, poly-L-Lysine coated) and incubate for 24 hrs at culture conditions previously mentioned before adding treatment.

Haemalum staining

After treatment, cells were first washed with PBS for twice and then fixed in serra solution. They were then stained with 1% acidic Mayer's Haemalum (BDH, Gurr) for 5 min at room temperature and observed under microscope (Olympus).

Hoechst staining

After treatment, cells were first washed with PBS for twice and then fixed in fixing solution (Denatured alcohol: Formaldehyde: Glacial acetic acid = 30:15:1). Then the cells were incubated with Hoechst 33258 (Sigma) (2µg/ml) at dark for 15min at room temperature. After incubation, extra stain was drained out and observed under a fluorescence microscope (Olympus).

DNA fragmentation assay

Cells were seeded at a concentration of 5×10^5 cells per T-25 flask and incubated for 24 hrs. Then they were treated with each AAEs (250µl/ml) and 25µM of R-Roscovitine; 2-(1-Ethyl-2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine ,a potent inhibitor of CDK1 (Federico et al. 2010, Meijer et al. 1997, Zhang et al 2009, Zharskaya et al. 2008);) and a positive inducer of apoptosis (Hahntow et al. 2004; Mgbonyebi et al.1999; Wesierska-Gadek et al. 2009), along with a controlled set for 24 hrs. The treated and control cell samples were collected in micro centrifuge tubes, washed with PBS and resuspended in Tris hydroxylmethyl aminomethane(Tris)-Ethylenediaminetetraacetic acid(EDTA) buffer (TE) pH 8.0 (10mM Tris, 1mM EDTA). Then they were incubated overnight at room temperature with lysis buffer (pH 8.0) containing 20mM EDTA, 50mM Tris, 1% Sodium Dodecyl Sulfate (SDS), 1% Nonidet P40(NP40). Cells were then incubated at 37°C for 1 hour with Ribonuclease A (RNase A). Phenol-chloroform-Isoamyl alcohol (25:24:1) extraction was done to purify DNA from protein contamination and then the DNA was precipitated from aqueous solution with the help of isopropanol and ammonium acetate. The DNA was precipitated by centrifugation at 27000 x g, washed with 70% alcohol and finally dissolved in TE buffer. The DNA samples were then electrophoresed through a 1.5% agarose (Sigma) gel having Ethidium bromide (EtBr) at a final concentration of 0.5 µg/ml, in 1xTris Acetate EDTA buffer (TAE) and subsequently visualized under UV.

Statistical Analysis

Statistical analysis was done by one-way analysis of variance (ANOVA) using the SPSS 11.5 software. Student-Newmans-Keuls (S-N-K) and Duncan's post hoc tests were performed for multiple group comparisons. Significance level was set at $p < 0.05$ in all cases. All data were expressed as mean ± standard deviation (SD), with index letters indicating statistical difference between the means.

Result

Antiproliferative activity of the crude aqueous extract

MTT assay

With the increase in concentration of the extracts, percentage of cytotoxicity was increased. *P. valderianum* showed more cytotoxicity than *P. tenue*. With the highest concentration applied, 11.46 % and 19.33% inhibition to cell proliferation were found using extracts of *P. tenue* and *P. valderianum* respectively (Fig.1).

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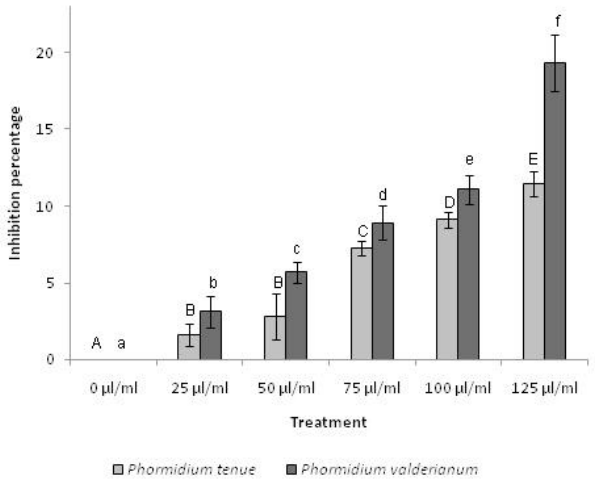

Fig. 1 Antiproliferative effects of the AAEs on HeLa cells showing percent inhibition on cell growth against increasing concentration of the extracts. All data were plotted as mean ± SD of three replicas. Index letters indicates a significant difference ($p<0.05$) in cellular inhibition between different concentrations of an AAE

Trypan Blue dye exclusion test

With 250µl/ml AAEs of *Phormidium tenue* and *Phormidium valderianum*, 27.79% and 41.05% cells were found dead respectively (Fig. 2).

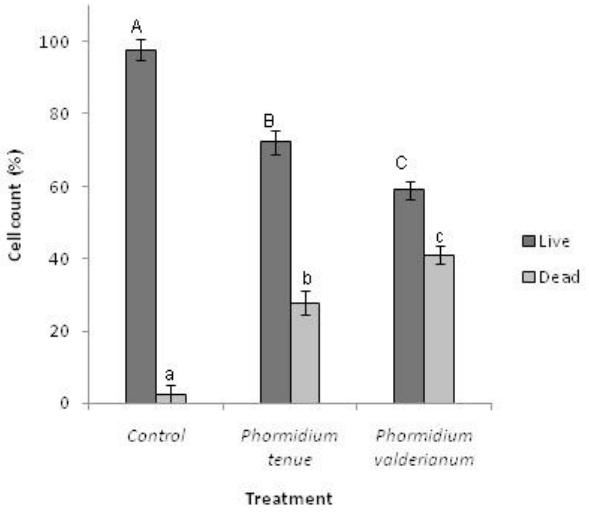

Fig. 2 Antiproliferative activity of the AAEs on HeLa cells showing a comparison between number of live and dead cells in non-treated and treated cells. All data were plotted as mean ± SD of four replicas. Index letters indicates a significant difference ($p<0.05$) in cellular inhibition between different concentrations of an AAE

Morphological Changes in HeLa cells after treatment

Haemalum staining

Cells treated with AAEs showed clear morphological changes. Most of the cells had lost their characteristic elongated appearance; became round with condensed chromosome and lost their cytoplasm. Cytoplasmic bleb like structures were also observed (Fig. 3).

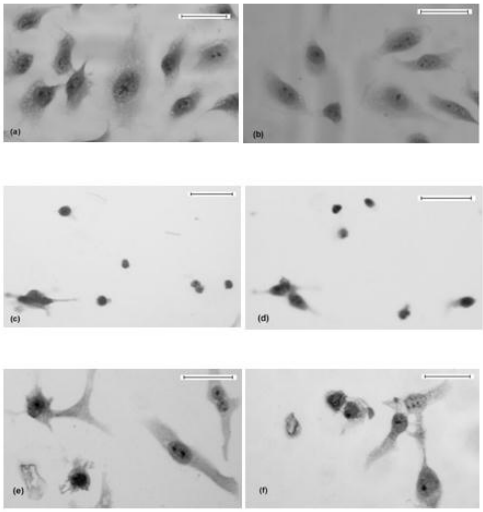

Fig. 3 Morphological changes in HeLa cells stained with Haemalum after treating with AAEs. (a- b) control cells, (c-d) & (e-f) cells treated with P. tenue & P. valderianum respectively. Scale indicates 50 µm

Hoechst staining

Changes in nuclear morphology in treated cells were distinct. Chromosome condensation with micronuclei like structures were observed in treated cells. Cells treated with *P. tenue* nuclei became roundish whereas the controlled ones showed normal elongated shape. More pronounced nuclear condensation was observed in the cells treated with *P. valderianum* (Fig. 4).

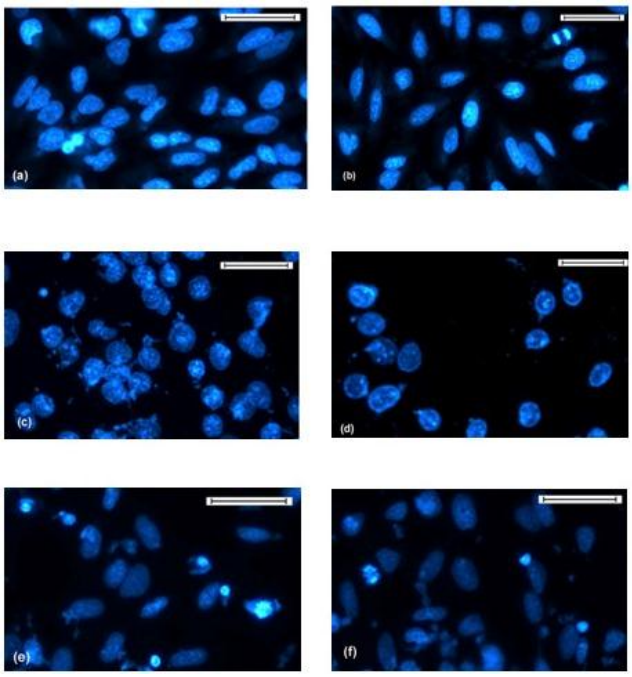

Fig. 4 Morphological changes in HeLa cells stained with Hoechst 33258 after treating with AAEs. (a- b) control cells, (c-d) & (e-f) cells treated with P. tenue & P. valderianum respectively. Scale indicates 50 µm

DNA Profile

Non-treated cells showed clear sharp band of genomic DNA whereas the samples treated with Roscovitine showed clear laddering pattern indicating DNA fragmentation. No fragmentation was visualized in both the AAE treated samples, but signs of DNA shearing were clearly visible indicating DNA damage (Fig. 5).

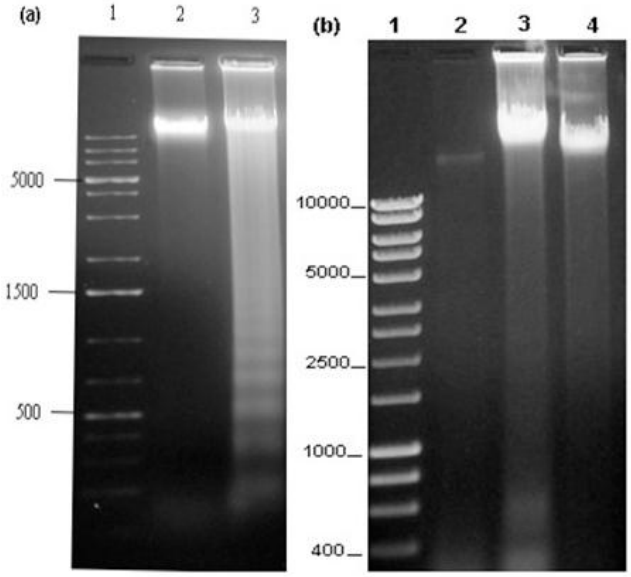

Fig. 5 DNA profile of Hela cells treated with AAEs. (a) Lane 1: 20 kb DNA marker, lane 2: Control DNA, lane 3: DNA from cells treated with 25 µM Roscovitine (b) Lane 1: 10 kb DNA marker, lane 2: Control DNA, lane 2 & 3: DNA from cells treated with 250 µl/ml of AAE of P. valderianum & P. tenue

Discussion

Antiproliferative activity

Both the MTT assay and Trypan Blue dye exclusion test results had showed potent antiproliferative activity of the AAEs. The data observed were found to be statistically significant ($P<0.05$). The inhibition percentage of *P. valderianum* extract was significantly increased in all extract concentrations; whereas that for *P. tenue*, the percentage increased significantly at concentration 75 µl/ml and more. Previously antitumor activity of glycolipids from *P. tenue* was reported (Shirahashi et. al 1993). *P. molle* had been also reported to be cytotoxic to HeLa cells (Dzhambazob et al. 2006). Within cyanobacteria, *Spirulina platensis* & *Aphanizomenon flos-aquae* were also reported to have anticancer bioactive compounds (Li et al. 2006, Bechelii et al. 2011).

Morphological change

AAEs treated HeLa cells showed morphological changes, distinctly different from the normal ones. Both Haemalum and Hoechst stained AAEs treated cells showed highly condensed chromatin, characteristic of apoptotic cells. Similar observation was reported in Yessotoxin treated HeLa cells (Pang et. al 2010) Chromosome condensation was clearly observed in both the treated samples, but more pronounced condensation was seen in *P. valderianum* treated samples. Whereas in *P. tenue* treated samples the overall nuclear shape had been changed to a roundish form.

DNA profiling

Cells treated with a positive apoptosis inducer Roscovitine (Wojciechowski et al. 2003, Wesierska-Gadek et al. 2009, Hahntow et al. 2004, Mgbonyebi et al.1999) showed clear DNA laddering, a characteristic apoptotic marker; but with the microalgal extract we didn't get the fragmented DNA, instead DNA shearing was evident. This observation suggests that the cell death induced by the microalgal extracts is not apoptosis mediated. Their clear cytotoxicity in HeLa cells may be associated with other means of cell death like autophagy, necrosis etc.

Induction of cell death is one of the most prevalent pathways to control overall growth of cancer cells, as most of the cancer cells lack normal progression to cell death. Therefore, drugs are designed to induce cell death either by apoptosis or by other pathways, autophagy, necrosis or necroptosis. For this all over the world work is going on to find new drugs from natural products, as synthetic anticancer drugs cause nonspecific cell death and has several side effects. Plants and marine organisms contribute a lot in new drug discovery to combat cancer.

The present study provides the first report of antiproliferative activity of aqueous extract of these two

species of *Phormidium* on HeLa cells. Results of this study revealed that these algal extracts have potent antiproliferative activity. These results are indeed encouraging for doing further investigation to isolate the active anticancer compounds and find their molecular mechanism to cause cell death in the cancerous cells.

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References

Bechelli, J., Coppage, M., Rosel,l K., Liesveld, J. 2011 Cytotoxicity of Algae Extracts on Normal andMalignant Cells. Leukemia Research and Treatment :1-7

Cannell, J.P.R. 1993 Algae as a source of biologically active products. Pestic. Sci. 39:147–153

Carmicheal, J., DeGraff, W.G., Gazder, A.F. 1987 Evaluation of a Tetrazolium-based Semiautomated Colorimetric Assay: Assessment of Chemosensitivity testing. Cancer Res. 47:936-942

DeFilippis, R.A., Goodwin, E.C., Wu, L., DiMaio, D. 2003 Endogenous human papillomavirus E6 and E7 proteins differentially regulate proliferation, senescence, and apoptosis in HeLa cervical carcinoma cells. J Virol. 77:1551-1563

Dzhambazov, B., Teneva, I., Mladenov, R., Popov, N. 2006 In Vitro Cytotoxicity and amticancer properties of two Phormidium molle strains (Cyanoprokaryota) Trav. Sci. Univ. Plovdiv, Plantarum 39:6:3-16

Faulkner, D.J. 2000 Marine natural products. Nat. Prod. Rep. 17:7–55

Federico, M., Symonds, C.E., Bagella, L., Rizzolio, F., Fanale, D., Russo, A., Giordano, A. 2010 R-Roscovitine (Seliciclib) prevents DNA damage-induced cyclin A1 upregulation and hinders non-homologous end-joining (NHEJ) DNA repair. Mol Cancer 9:208

Freshney, R. 1999 Culture of Animal Cells: A Manual of Basic Technique, Alam R. Liss Inc., New York

Gottesman, M.M. 2002 Mechanisms of Cancer Drug Resistance. Annu. Rev. Med. 53:615-627

Gurib-Fakim, A. 2006 Medicinal plants: traditions of yesterday and drugs of tomorrow. Molecular Aspects of Medicine. 27.1:1-93

Hahntow, I.N., Schneller, F., Oelsner, M., Weick, K., Ringshausen, I., Fend, F., Peschel, C., Decker, T. 2004 Cyclin –dependent kinase inhibitor Roscovitine induces apoptosis in chronic lymphocytic leukemia cells. Leukemia 18:747-755

Jemal,A., Bray, F., Center, M.M., Ferlay, J., Ward, E., Forman, D. 2011 Global Cancer Statistics. Ca Cancer J Clin 60:69-90

Konig, G.M., Wright, A.D. 1995 Marine natural products research. Current directions and future potential. Planta Med; 62:193-211

Li, B., Gao, M., Zhang, X. and Chu, X, 2006 Molecular immune mechanism of C-phycocyanin from Spirulina platensis induces apoptosis in HeLa cells in vitro Biotechnol. Appl. Biochem. 43:155–164

Mayer, A., Lehmann ,V. 1999 Marine pharmacology in 1999: antitumor and cytotoxic compounds. Anticancer Res. 21:2489-2500

Mayer, A.M.S., Gustafson, K.R. 2003 Marine pharmacology in 2000: Antitumor and cytotoxic compounds. Int. J. Cancer 105:291–299

Meijer, L., Borgne, A., Mulner, O., Chong, J.P., Blow, J.J., Inagaki, N., Inagaki, M., Delcros, J.G., Moulinoux, J.P. 1997 Biochemical and cellular effects of roscovitine, a potent and selective inhibitor of the cyclin-dependent kinases cdc2, cdk2 and cdk5. Eur J Biochem. 243:527-536

Mgbonyebi, O.P., Russo, J., Russo, I.H. 1999 Roscovitine induces cell death and morphological changes indicative of apoptosis in MDA-MB-231 breast cancer cells. Cancer Res. 59:1903-2010

Nekhoroshev, M.V .1996 The Black Sea algae are potential source of antitumor drugs. Al'-gologiya 6:86–90

Pang, M.,Gao, Wu. Z., Lv, N., Wang, Z., Tang, X. and Qu, P. 2010 Apoptosis induced by yessotoxins in Hela human cervical cancer cells in vitro. Molecular Medicine Reports 3:629-634

Rinehart, K.L. Jr., Shaw, P.D., Shield, L.S.,Gloer, J.B., Harbour, G.C., Koker, M.E.S., Samain, D., Schwartz, R.E., Tymiak, A.A., Weller, D.L., Carter, G.T. and Munro, M.H.G. 1981 Marine natural products as sources of antiviral, antimicrobial, and antineoplastic agents. Pure and Appl Chem 53:795–871

Shirahashi, H., Murakami, N., Watanabe, M., Nagatsu, A., Sakakibara, J., Tokuda, H., Nishino, H., Iwashima, A. 1993.. Isolation and identification of anti-tumorpromoting principles from the fresh-water cyanobacterium Phormidium tenue. Chem. Pharm. Bull. (Tokyo) 41:1664-1666

Tziveleka, L.A., Vagias, C., Roussis, V. 2003 Natural products with anti-HIV activity from marine organisms. Curr. Top. Med. Chem. 3(13):1512–1535

Wesierska-Gadek ,J., Borza, A., Walzi, E., Kristof, V., Maurer, M., Komina, O., Wandl, S, 2009 Outcome of Treatment of human HeLa cervical cancer cells with roscovitine strongly depends on dosage and cell cycle status prior to the treatment. J Cell Biochem 106:937-955

Xu, H., Yao, L., Sung, H., Wu, L. 2009 Chemical composition and antitumor activity of different polysaccharides from the roots Actinidia eriantha. Carbohydr. Pol. 78:316-322

Zhang, F., Zhang, T., Jiang, T., Zhang, R., Teng, Z.H., Li, C., Gu, Z.P., Mei, Q. 2009 Wortmannin potentiates roscovitine-induced growth inhibition in human solid tumor cells by repressing PI3K/Akt pathway. Cancer Lett. 286:232-239.

Zharskaya, O.O., Barsukova, A.S., Zatsepina, O.V. 2008 Effect of roscovitine, a selective cyclin B-dependent kinase 1 inhibitor, on assembly of the nucleolus in mitosis. Biochemistry (Mosc) 73: 411-419