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IN VITRO CYTOTOXICITY AND ANTICANCER PROPERTIES OF TWO *PHORMIDIUM MOLLE* STRAINS (CYANOPROKARYOTA)

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ABSTRACT

The search for sources of new biologically active compounds is important for the discovery of new drugs relative to the treatment of cancer. Cyanoprokaryota are known to produce anticancer, antiviral and antifungal compounds and antibiotics. Two strains of *Phormidium molle* (Cyanoprokaryota, Oscillatoriales) were investigated for *in vitro* cytotoxic and anticancer properties using 5 cancer (HeLa, Jurkat, U-937, A2058, RD) and 2 normal (3T3, FL) cell lines. Treatment with *Phormidium* extracts or *Phormidium* growth media altered the cytoskeletons and microtubule network of the adherent cells causing dose-dependent destruction of the monolayer and morphological changes. An extract concentration of 10 µg/mL showed significant cytotoxicity on HeLa and A2058 cells 24 h after treatment (41% and 60% respectively) as determined with the MTT assay, while the viability of the other cells was not significantly affected. Both, extracts and growth media, significantly inhibited incorporation of [^{3}H]-thymidine in the adherent cancer cell lines in a dose-dependent manner. No colony formation was observed over a concentration of 6 µg/mL *Phormidium molle* extracts for the cancer adherent cells (HeLa). Chromatographic analyses of the *Phormidium* extracts and growth media showed peaks with retention time between 6 and 15 min, which potentially represent alkaloids. Thus, Cyanoprokaryota could be a novel appropriate choice for development of anticancer therapeuticals.

Key words: *Phormidium molle*, *Cyanoprokaryota*, cytotoxicity, anticancer properties, *in vitro*.

INTRODUCTION

The majority of human cancers are result from exposure to environmental carcinogens as natural and synthetic chemicals, radiation or viruses. Mortality that results from the common forms of cancer disorders is still unacceptably high. This disease is clinically treated by surgery, radiotherapy and chemotherapy. After surgical ablation of progressive cancer, metastasised tumour cells continue to progress. Radioactive rays also affect normal cells to cause serious adverse effects. Most chemotherapeutic agents applied in the treatment of both the primary tumor and metastatic disease, have difficulty to distinguish malignant from normal cells. Thus, there is still need to search for new drugs of better pharmacological indices. Extracts from a broad spectrum of plant species contain substances that posses anticancer activity. Plant products have low toxicity and high medicinal effectiveness. Some examples of plant-derived drugs are vincristin, vinblastin, rubomycin, taxol, camptothecin, etoposide and irinotecan. Most of the identified compounds belong to the classes of alkaloids, polyphenols, triterpens, steroid glycosides or small peptides (Nakamura et al., 1994; Inoue et al., 1995; Mimaki et al., 1996a, 1996b; Kuo & Kuo, 1997; Javed et al., 1998; Rao & Venkatachalam, 2000). In the past few years, combination chemotherapy using anticancer drugs with different modes of action is one of the most common forms of cancer treatment. Natural anticancer substances may target several processes involved in cell proliferation, functions and death.

It has been shown that *Cyanoprokaryota* are a recognized source of potential pharmaceuticals with different activities including cytotoxicity, immunosuppression, antiproliferation, microbial toxicity as well as specific anticancer activities (Jaspars & Lawton, 1998; Mishima et al., 1998; Morliere et al., 1998). Cryptophycin-52, a synthetic analogue of the terrestrial cyanobacterial peptolide cryptophycin-1, recently entered phase II human clinical trials against cancer (Trimurtulu et al., 1994; Barrow et al., 1995; Golakoti et al., 1995; Eggen & Georg, 2002). Dolastatin-10, a modified pentapeptide isolated from the sea hare *Dolabella auricularia* and from marine cyanobacteria (*Lyngbya majuscula*), is another anticancer agent being clinically evaluated (Pettit et al., 1987). Several studies have demonstrated biological activity of *Phormidium*, another genus of *Cyanoprokaryota*. *Phormidium* extracts showed anti-inflammatory (Garbacki et al., 2000), anti-plasmodial (Papendorf et al., 1998) or anticancer properties (Tokuda et al., 1986, 1996; Shirahashi et al., 1993; Williamson et al., 2002). Therefore, *Cyanoprokaryota* are a promising but still unexplored natural source offering a wealth of novel chemicals for lead compounds discovery and new drugs.

The present study was undertaken to evaluate the *in vitro* cytotoxic and anticancer properties of two strains of *Phormidium molle* (*Cyanoprokaryota*).

MATERIAL AND METHODS

Phormidium cultures and extraction

Two strains of *Phormidium molle* (Cyanoprokaryota, Oscillatoriales) were studied: *Phormidium molle* No 8140 and *Phormidium molle* No 5088 (PACC,

Plovdiv Algal Culture Collection). Blue-green algae were grown intensively under sterile conditions using a Z-nutrient medium. Cultures were synchronised by altering light/dark periods of 16/8 hours. The temperature was 33°C and 22°C during the light and dark period, respectively. The intensity of light during the light period was 224 μmol.photon.s^{-1}.m^{-2} (Lux 12000)**.** The culture medium was aerated with 100 litres of air per hour per one litre of medium, adding 1% CO_2 during the light cycle. The period of cultivation was 25 days.

Extracts of the blue-green algae were obtained according to the method of Krishnamurthy et al. (1986) with slight modifications. Briefly, *Phormidium* species were removed from the Z-medium and weighed, then frozen and thawed, and extracted twice (3 h and overnight) with water-methanol-butanol solution (15:4:1, v:v:v, analytical grade) at 22°C while stirring. The extracts were centrifuged at 10,000 rpm for 30 min. The supernatants of the two extracts were pooled and organic solvents removed via speed-vac centrifugation (SAVANT, Instruments Inc. Farmingdate, NY, USA) at 37°C for 2 h. To investigate whether *Phormidium* species release bioactive products into their culture environment, the nutrient solution in which the algae were cultivated during the 25 days was also tested for cytotoxicity and anticancer activity *in vitro.*

The resulting extracts and growth media were freeze-dried and dissolved with mqH_2O to give final concentrations of 2 mg/ml. Samples were sterilized by filtration through a 0.22 μm Millipore filter and stored at -20°C before use.

Cell lines and exposure conditions

Seven different commercially available cell lines were used for the *in vitro* tests: HeLa (human cervical epithelial adenocarcinoma, ATCC CCL-2), Jurkat (human acute T cell leukaemia, ATCC TIB-152), U-937 (human histiocytic lymphoma, ATCC CRL-1593.2), 3T3 (mouse embryonic fibroblasts, ATCC CCL-92), FL (normal amniotic human cells, ATCC CCL-62), A2058 (human metastatic melanoma, ECACC 91100402) and RD (human embryonic rhabdomyosarcoma, ATCC CCL-136).

Cells were cultured in 75 cm^2 flasks in Dulbecco's Modified Eagle's Medium (DMEM, Gibco™, Paisley, Scotland, UK), supplemented with 10% (v/v) heat inactivated fetal calf serum (FCS, PAA Laboratories GmbH, Linz, Austria), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma, Steinheim, Germany), at 37^{o}C with 5% CO_2 in air and high humidity. Cell viability was measured with the trypan blue exclusion test (Berg et al., 1972) prior to seeding.

Prior to exposure, cells were plated in 96-well tissue culture plates at a density of $1.5x10^4$ per 200 μL DMEM medium with 10% FCS. After 24 h (to allow the attachment of the adherent cells) the cultures were exposed to three concentrations of the *Phormidium* extracts or growth media – 2 μg/ml, 6 μg/ml and 10 μg/ml. Control wells were prepared by adding equal amounts of Millipore water to the culture medium. The cells were exposed for 24 h or 48 h prior to analysis.

Cytotoxicity assay in vitro by MTT conversion method

The MTT (3-(4',5'-dimethylthiazol-2'-yl)-2,5-diphenyl-tetrazolium bromide; Sigma, St Louis, MO, USA) assay is based on the capacity of mitochondrial succinyl dehydrogenase to convert the soluble yellow tetrazolium salt into an insoluble purple-blue formazan product inside living cells. Briefly, after the desired time of exposure with *Phormidium* extracts or growth media, 20 µl of MTT solution (5 mg/ml in PBS) were added directly to each well and incubated at 37°C for 3 h. Thereafter, the supernatant was discarded and 0.1 ml of dimethylsulfoxide (DMSO) was added to each well in order to facilitate solubilization of the formazan product. After 15 min at room temperature the plates were shaken, and absorbance was read at 570 nm in a SPECTRAmax®PLUS microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). The mean absorbance taken from cells without any addition was referred to as 100% cell survival (control).

Proliferation assay

Cells were plated and exposed to different concentrations of *Phormidium* extracts or *Phormidium* growth media as described above. During the last 18 h of exposure, cells were pulsed with 1 µCi [^{3}H]-thymidine per well (Amersham Labs, Buckinghamshire, England). After the completion of 24 h or 48 h of exposure, the cultures were harvested in a Filtermate™ cell harvester (Packard Instrument, Meriden, CT, USA). Incorporation of [^{3}H]-thymidine was measured in a Matrix 96 Direct beta counter (Packard). The mean cpm values of triplicates were determined.

Clonogenic assay

Cytotoxicity was assayed by determining the ability of HeLa and 3T3 cells to form colonies after the *Phormidium* extract treatment. The cell culture monolayer was trypsinized and the cell density was adjusted to $5x10^3$ cells/mL. One millilitre of the diluted cell suspension was added to each well of a 24-well microtitre plate and incubated overnight at 37°C in 5% CO_2. The supernatant was discarded and the cells were exposed to different concentrations of the *Phormidium* extracts for 2 h. Cells were washed with fresh medium, followed by addition of 1 mL growth medium. Colonies were allowed to grow for 2 weeks at 37°C in 5% CO_2. The supernatant was then removed, the colonies were stained with 1% crystal violet in 70% ethanol and counted manually. The ability of a cell to form a colony containing more than 50 cells was determined.

Chromatographic analysis

HPLC analysis was performed with an ÄKTA™ explorer 100 Air system (Amersham Pharmacia Biotech AB, Uppsala, Sweden) using an UNICORN V4.00 software. The analytical column was a Discovery® C_{18} (5x4 mm I.D., 5 µm) from Supelco (Bellefonte, PA, USA). The mobile phase consisted of a mixture of solvent A (10 mM ammonium acetate, pH=5.5) and solvent B (10 mM ammonium acetate-acetonitrile, 80:20, v/v) as follows: 0% of B at 0 min, 100 % of B at 35 min to 50 min using a linear gradient. Flow-rate was 0.8 ml/min and UV detection was performed at

238 nm. All runs were carried out at room temperature. The column was reequilibrated with 8 ml of the solvent A between runs. 200 µl of each sample were injected for HPLC analysis.

RESULTS AND DISCUSSION

Effects of the *Phormidium molle* intracellular (extracts) and extracellular (growth media) compounds were evaluated using 5 cancer (HeLa, Jurkat, U-937, A2058, RD) and 2 normal (3T3, FL) cell lines. Jurkat and U-937 cells are growing in suspension in contrast to the rest cell lines, which are adherent. Treatment with *Phormidium* extracts or growth media altered mainly the adherent cells showing dose-dependent destruction of the monolayer and morphological changes (Table 1).

Table 1. *Morphological changes/toxic effects induced by the Phormidium extracts and Phormidium growth media observed microscopically after 24 h treatment*.

Treatment	Concentration (µg/mL)	Morphological changes/toxic effects in*						
		HeLa	Jurkat	U-937	3T3	FL	A2058	RD
Control	-	0	0	0	0	0	0	0
PACC 8140 extract	2	+	0	0	0	+	+++	++
	6	++	0	+	0	++	+++	+++
	10	++++	+	+++	++	++++	++++	++++
PACC 5088 extract	2	+	0	0	0	0	+++	+++
	6	+	0	0	0	+	+++	++++
	10	++++	+	0	+	+	++++	++++
PACC 8140 growth medium	2	+++	0	0	0	0	+++	++
	6	++++	0	0	0	+	++++	++
	10	++++	+	0	0	0	++++	+++
PACC 5088 growth medium	2	++	0	0	0	+	+++	++
	6	++++	0	0	0	+	++++	+++
	10	++++	+	0	0	0	++++	+++

*Average of three independent experiments: 0, normal; +, 20% toxic effect; ++, 40% toxic effect; +++, 60% toxic effect; ++++, 80% toxic effect (toxic effects were judged to have occurred when there was cell shrinkage, cell wall breakage and/or leakage of cell components).

Comparing the effects of both extracts and growth media on normal and cancer adherent cell lines, the normal cells were less affected, which indicated that the *Phormidium* bioactive compounds have selective mode of action. Control cells as well as most treated normal adherent cells had fibroblast-like shape, with an elongated morphology. In contrast, treated cancer cells tended to round up, and many blebs were detected on cell membranes. There were no structural alterations in the cell cultures growing in suspension (Jurkat and U-937). Thus, *Phormidium molle* contains bioactive compounds that affect the cytoskeletons and microtubule network of adherent cancer cells leading to inhibition of the cell division.

Comparative cytotoxicity of the extracts and growth media prepared from the two *Phormidium* strains to normal and cancer cells *in vitro* was investigated using the MTT assay as a measure of cellular viability after exposure. Figure 1 shows the mean value of cell viability 24 h after treatment calculated as a percentage of controls.

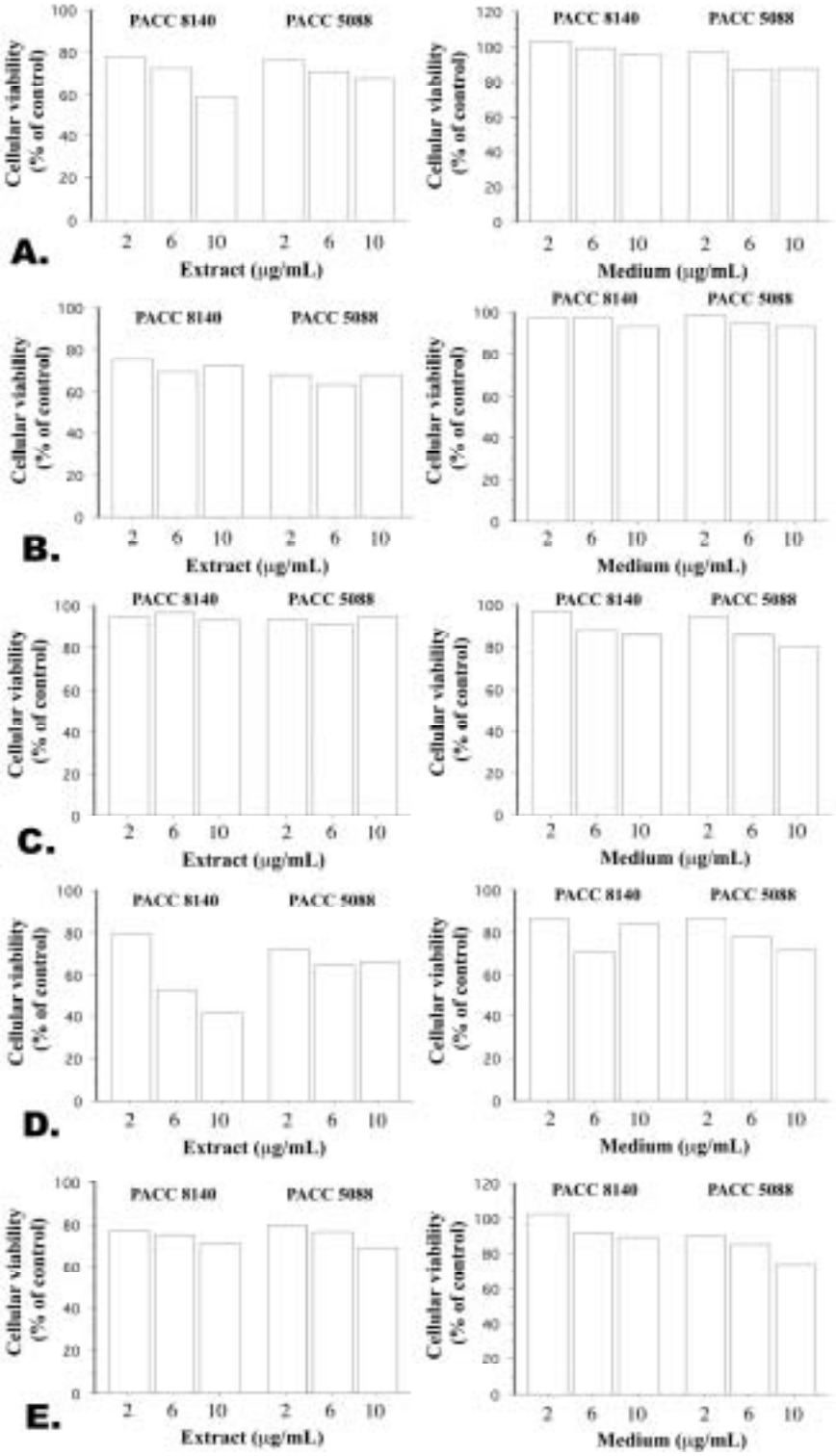

Figure 1. *Viability of mammalian cell lines treated with Phormidium molle extracts or growth media for 24 h as determined with the MTT assay and expressed as mean value in percentage of control (based on at least 4 replicates). (**A**.) HeLa cells; (**B**.) Jurkat cells; (**C**.) RD cells; (**D**.) A2058 cells; (**E**.) U-937 cells.*

Similar results were obtained after exposure to *Phormidium* samples for 48 h (data not shown). Treatment with an extract concentration of 10 µg/mL of *Phormidium molle* PACC 8140 for 24 h showed significant cytotoxicity on HeLa

(Fig. 1A) and A2058 (Fig. 1D) cells (41% and 60% respectively), while the viability of the other cells was not significantly affected compared to the control (less than 30%). Cytotoxicity of the extract PACC 5088 and both growth media was not so efficient as that observed in the case of PACC 8140 extract showing moderate effects against all the cell lines used (Fig. 1). This suggests that the ingredients are not present in sufficient concentration to exacerbate or potentate toxicity. When the cells were treated with 15 µg/mL *Phormidium* extracts for 24 h, rapid decrease of the cell viability was observed (Fig. 2A). *Phormidium* growth media from both strains showed strongest effect on RD cells, where the cytotoxicity was more than 40% (Fig. 2B).

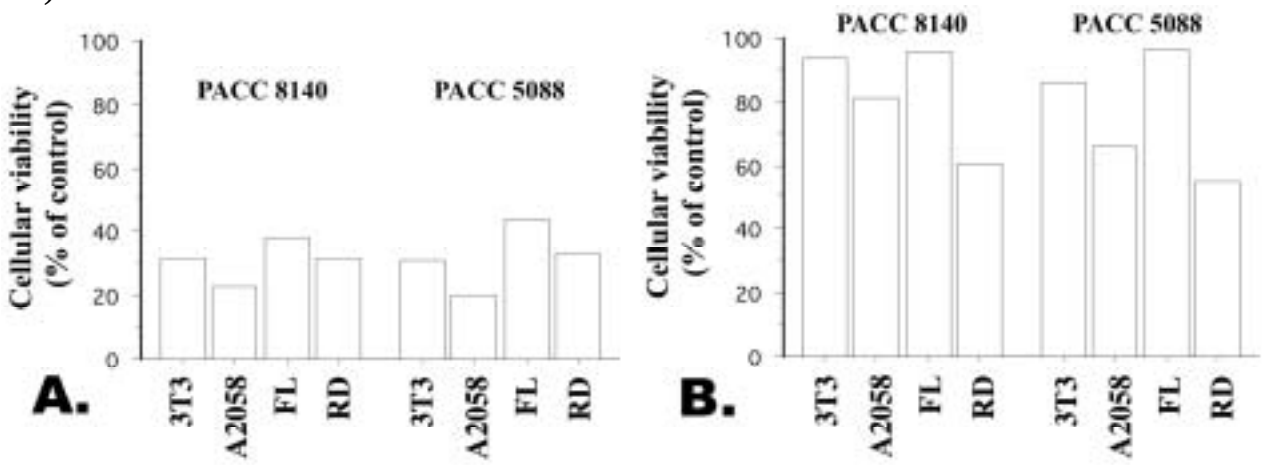

Figure 2. *Viability of different cell lines treated with 15 µg/mL Phormidium molle extracts (**A**.) or growth media (**B**.) for 24 h as determined with the MTT assay and expressed as mean value in percentage of control.*

In addition to the MTT conversion method, we decided to test proliferation capacity of the cells after treatment. Both, extracts and growth media, significantly inhibited incorporation of [^{3}H]-thymidine in the adherent cancer cell lines in a dose-dependent manner (Fig. 3a, 3b). Proliferation inhibition might reasonably be related to the cell adhesion because non-adherent cells were less inhibited than the adherent cells. However, *Phormidium molle* strains probably contain bioactive compounds similar to the cryptophycins that inhibit cancer cell proliferation suppressing microtubule dynamics (Panda et al., 1998; Mayer & Gustafson, 2003). Recently, Teruya et al., (2005) have isolated novel 2-alkylpyridine alkaloids (Phormidinines A and B) from *Phormidium sp*. with potential biological activities.

In the clonogenic assay, no colony formation for cancer adherent cells (HeLa) was obtained over a concentration of 6 µg/mL *Phormidium molle* extracts (Table 2), whereas clonogenic efficacy of 3T3 cells was not significantly affected. These results confirmed the cytotoxicity and anticancer activity of tested extracts.

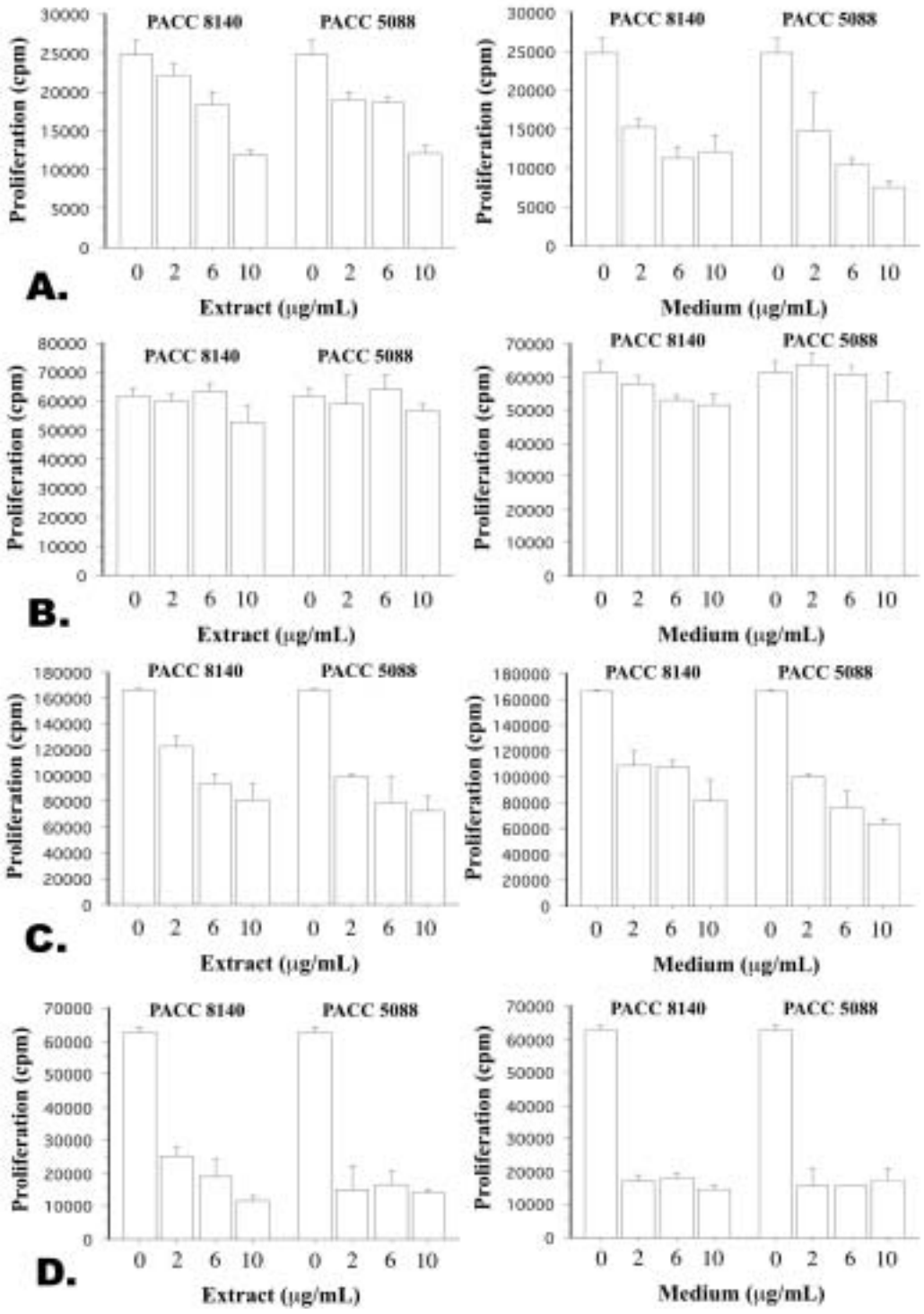

Figure 3a. *[^{3}H]-thymidine incorporation into cells after 24 h treatment with Phormidium molle extracts or growth media. One experiment is shown for which each bar represents the average of three culture wells, with vertical lines indicating the standard deviation. (**A**.) HeLa cells; (**B**.) Jurkat cells; (**C**.) RD cells; (**D**.) A2058 cells.*

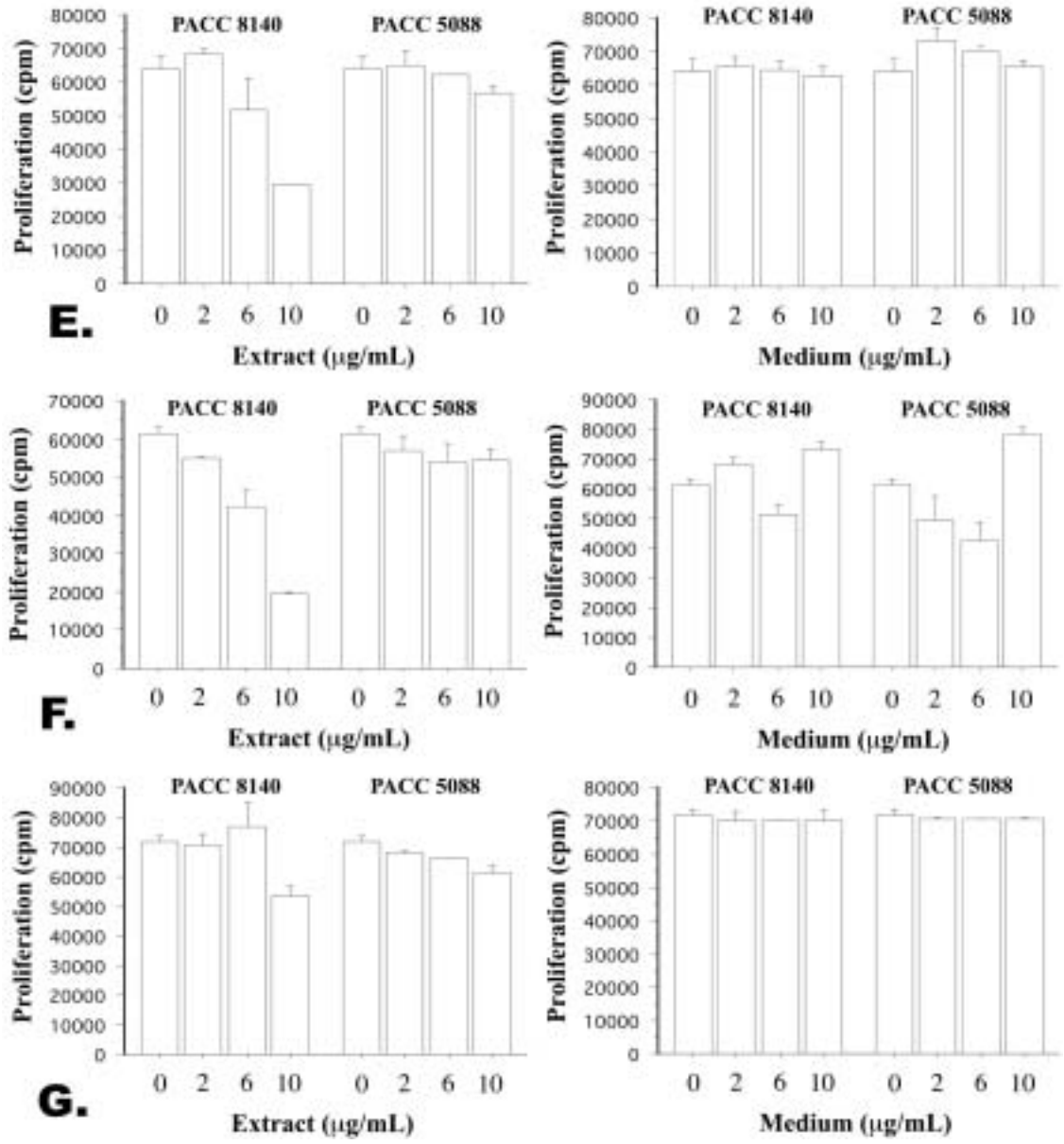

Figure 3b. *[^{3}H]-thymidine incorporation into cells after 24 h treatment with Phormidium molle extracts or growth media. One experiment is shown for which each bar represents the average of three culture wells, with vertical lines indicating the standard deviation. (**E**.) U-937 cells; (**F**.) FL cells; (**G**.) 3T3 cells.*

Chromatographic analyses of the *Phormidium* extracts and growth media showed peaks with retention time between 6 and 15 min (Fig. 4), which potentially represent alkaloids. These results are in agreement with other reports on the anticancer activity of several alkaloids and their mechanisms of action.

The data of the present study demonstrated significant antiproliferative effects of intracellular and extracellular compounds prepared from *Phormidium molle* strains on several adherent cancer cell lines, which shows that *Cyanoprokaryota* may be useful source for development of new anticancer agents.

Table 2. *Cytotoxic effects of Phormidium extracts on HeLa and 3T3 cells by using clonogenic assay. Values are mean of three independent experiment ± SE.*

Treatment	**Concentration (µg/mL)**	**HeLa (No. of colonies)**	**3T3 (No. of colonies)**
Control	-	56±6.30	68±3.22
PACC 8140 extract	2	8±0.12	58±2.32
	6	2±0.18	46±3.24
	10	0±0.00	44±2.66
PACC 5088 extract	2	12±1.14	55±1.55
	6	3±1.01	47±2.17
	10	0±0.00	42±3.34

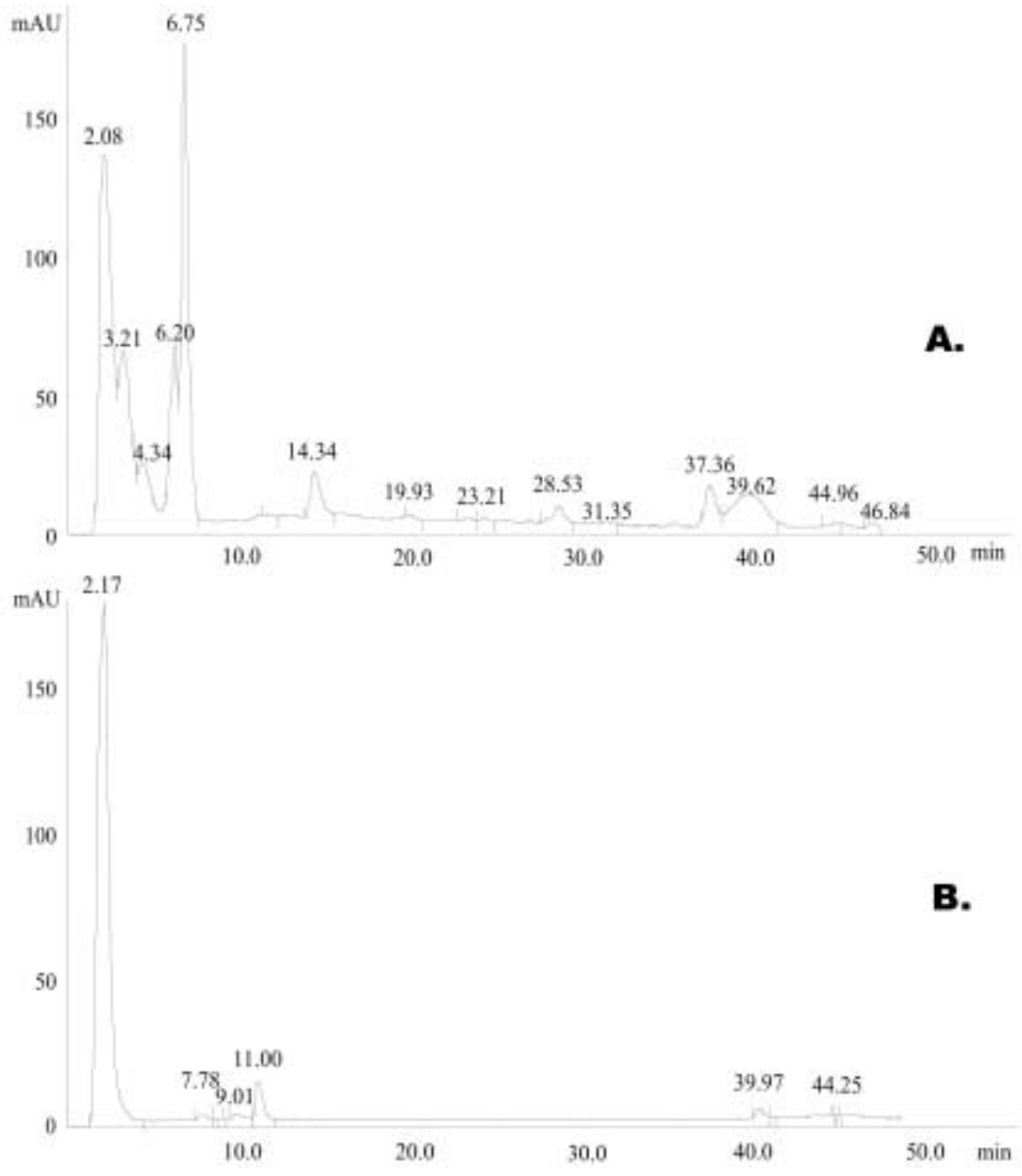

Figure 4. *HPLC profile of Phormidium molle extract (**A**) and growth medium (**B**).*

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IN VITRO ЦИТОТОКСИЧНОСТ И АНТИТУМОРНА АКТИВНОСТ НА ДВА ЩАМА *PHORMIDIUM MOLLE* (CYANOPROKARYOTA)

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(Резюме)

Cyanoprokaryota са известни като продуценти на антивирусни, антитуморни и противогъбични вещества, както и на различни антибиотици. В настоящата работа са изследвани два щама *Phormidium molle* (Cyanoprokaryota, Oscillatoriales) за *in vitro* цитотоксичност и антитуморна активност чрез използването на 5 туморни (HeLa, Jurkat, U-937, A2058, RD) и 2 нормални (3T3, FL) клетъчни линии. Третирането с екстракти от *Phormidium* или хранителни среди, в които са култивирани щамовете *Phormidium*, оказва влияние върху цитоскелета на адхезивните клетки, предизвиквайки доза-зависимо увреждане на клетъчния монослой, както и морфологични изменения. 24 часа след третиране с 10 μg/mL екстракт бе установена значителна цитотоксичност върху клетъчни линии HeLa и A2058 (41% и 60% респективно), докато преживяемостта на останалите клетъчни линии не бе повлияна. Както екстрактите, така и хранителните среди значително инхибират инкорпорирането на радиоактивен тимидин в адхезивните туморни клетъчни линии. Клоногенният тест при туморните адхезивни клетки HeLa показа, че при третиране с концентрации на екстракта над 6 μg/mL липсва формиране на колонии. Хроматографският анализ на тестваните екстракти и хранителни среди от *Phormidium molle* показа пикове между 6 и 15 мин, които представляват алкалоидни компоненти. Получените резултати определят Cyanoprokaryota като важен източник на компоненти с антитуморна активност.