ISSN : 0974 - 7508 Volume 7 Issue 3

Trade Science Inc.

Natural Products

An Indian Journal

Full Paper

NPAIJ, 7(3), 2011 [137-146]

Callus culture of oleander retains pharmacological activities of the plant

N.Hovhannisyan*[1], M.Mkrumyan[1], A.Yesayan[1], R.Aroutiounian[1], R.A.Newman[2], R.Grigoryan[3], N.Sarkisyan[3], G.Gasparyan[3]

[1]Yerevan State University, 1, A. Manoogian str., Yerevan 0025, (ARMENIA)

[2]Department of Experimental Therapeutics and Pharmaceutical Development Center, University of Texas, M. D. Anderson Cancer Center, Houston, TX 77054, (USA)

[3]Institute of Molecular Biology of the Armenian National Academy of Sciences, 7, Hasratyan str., Yerevan 0014, (ARMENIA)

E-mail: bionellibiotech@yahoo.com

Received: 18th June, 2011 ; Accepted: 18th July, 2011

ABSTRACT

There is growing interest in the proposed antineoplastic activity of *Nerium oleander* components. Earlier we have developed an oleander callus culture. The objectives of this work were to reveal whether the cultured tissue is able to generate secondary metabolites representative of the parent plant under *in vitro* conditions and whether these products retain biological activities of the source. The callus tissue produces oleandrin, oleandrigenin and odoroside, the main oleander secondary metabolites. The levels of cytotoxicity of callus and plant leaf extracts for human cell lines *in vitro* are comparable. Callus extract expresses species-specific cytotoxicity characteristic of the parent plant. The both extracts tested are toxic for human (normal embryo fibroblasts, Jurkat, K-562, L-41, KB cell lines) and monkey (COS-7 cell line) cells, while rat PC-12 cell line has low sensitivity to the extract of leaves and is fully resistant to callus extract. The extracts were shown to induce apoptosis. The anti-genotoxic and anti-mutagenic activity of polysaccharide fractions of oleander leaves and callus culture was revealed and can be considered to be novel findings. Thus, oleander callus culture retains the main activities of the plant. This culture can be used as a biotechnological source of biologically/pharmacologically active compounds of oleander. © 2011 Trade Science Inc. - INDIA

KEYWORDS

Callus culture;
Cytotoxicity;
Genotoxicity;
Oleander;
Testing *in vitro*.

INTRODUCTION

Nerium oleander Linn, an ornamental plant belonging to the Apocynaceae family, is distributed in the Mediterranean area, sub-tropical Asia and America. The plant is known to be toxic[20] and reputed to be a therapeutic agent in folk medicine.

Currently there is growing interest in the proposed antineoplastic activity of oleander extracts and components. A hot water oleander extract called Anvirzel™ was patented in the United States[36] as an antitumor agent. This multi-component mixture contains polysaccharides[56], proteins, sugars[38] and a number of other bioactive compounds (cardiac glycosides such as oleandrin, odoroside, the aglycone oleandrigenin[1-3,34,53,57], steroids[15], triterpenes[9], *etc.*). Oleandrin and

Full Paper

oleandrigenin were shown to be cytotoxic *in vitro*[38] in a species-specific manner[31,40].

The effects of Anvirzel™ *in vitro* and mechanisms of its action were studied in detail[10,21,33,34,38,45]. Anvirzel™ and its principal cardiac glycoside component oleandrin were shown to be potent inducers of apoptosis[25,32,38,46,47]. To date, Anvirzel™ has progressed through a Phase I trial in the United States[26]. A supercritical CO_2 extract of *Nerium oleander* termed PBI-05204 is completing a Phase I clinical trial in advanced cancer patients at the University of Texas, M. D. Anderson Cancer Center (Houston, TX USA). Plant cell, tissue, and organ cultures are known to be capable of producing and accumulating many of chemical compounds replicating that found in the parent plant in the nature[41]. Therefore, these cultures provide an alternative method of production of phytochemicals of therapeutic importance[4,17,29,48]; including potential anticancer agents[54]. The advantages of *in vitro* techniques are evident and include independence from environment pollutants and interfering compounds that occur in the field-grown plant, control of plant vegetation cycle and plant diseases as well as an opportunity to obtain pure and standardized products under controlled conditions. This biotechnological approach is particularly applicable to *Nerium oleander* as the plant was found to accumulate heavy metals from soil and atmosphere[28, 42].

There are few reports on oleander tissue culturing[16,44]. We have also propagated callus cultures initiated from oleander leaves. The growth conditions and characteristics of these cultures were determined (optimum growth media, cell growth rate, *etc*.) and have previously been described[13,14]. The objectives of the present work were to reveal whether the cultured plant tissue is able to generate secondary metabolites representative of the parent plant under rigorously controlled *in vitro* conditions and whether these products retain the source biological activities.

MATERIAL AND METHODS

Callus culure initiation and propagation

To induce sterile plantlet culture axillary buds were aseptically cultivated on modified Murashige-Skoog medium containing 2 mg/l indolyl-acetic acid and 0.2 mg/l kinetin. Plantlet cultures were then expanded by clonal micropropagation. Mericlones were transferred to the hormone-free Murashige-Skoog medium. Oleander mericlones were cultivated in a climatic chamber with illumination (1000 lux) and a controlled temperature of 26 ± 2°C

Leaves of aseptically growing plantlets were used to induce callus tissue growth on modified Murashige-Skoog medium containing 1.0 mg/l 2,4-dichlorophenoxyacetic acid and kinetin. Cultures were kept in darkness at 26 ± 2^{0}C. To sub-culture, callus cultures were periodically (every 20 days) cut into pieces and transferred to fresh medium. To prepare hot water extracts, leaves of 4-week-old plantlets or 20-days-old callus cultures were air-dried at 70^{0}C and boiled in 0.9 % NaCl solution or Hanks' balanced solution (Sigma-Aldrich, St. Louis, MO) (0.5: 10, w/v) for 3 h. The extracts were filtered and stored at -10^{0}C for further use[36].

Animal cell culture

Normal human embryo fibroblasts (22nd passage, subconfluent culture, 2 days after cell seeding) were used in these studies. Additional cell lines included PC-12 (rat pheochromocytoma), COS-7 (fibroblast-like African green monkey kidney cells transformed by SV40), Jurkat (human T-lymphoblastic leukemia), K-562, KCL-22 (both from human chronic myeloid leukemia), and KB (oral epidermoid carcinoma) kindly provided by Prof. J. Masters (Institute of Urology and Nephrology, University College London, UK) and Dr. T. Liehr (Institute of Human Genetics and Anthropology, Germany). Cells were routinely maintained in the growth media RPMI-1640 (cell lines PC-12, Jurkat, K-562 and KCL-22) or DMEM (normal human embryo fibroblasts, cell lines COS-7 and KB) supplemented with 10% fetal bovine serum (media and serum from Sigma-Aldrich) and 50 μg/ml gentamycin at 37^{0}C.

Determination of main secondary metabolite oleandrin content in plant leaves and callus tissues

Concentrations of secondary metabolites were determined in leaves of 4-week-old plantlets and 20-day-old callus cultures.

Extraction procedure

(1) A small amount of leaf and callus samples was

weighed to the nearest 0.1 mg and placed in a 15ml glass screw cap round bottom centrifuge tube. (2) 5 ml of absolute ethanol was added to each tube. The mixture was vortexed for 10 min. (3) Contents within the tubes were sonicated for 1 h in an ultra-sonic bath, (4) then mixed for 15 min and centrifuged at 2.500 rpm in a Brinkman centrifuge for 5 min. (5) Supernatant was removed and transferred to a 13x100 mm glass tube and 5 ml of 50: 50 methanol: methylene chloride (v/v) was added to tubes with extracted solid material. (6) Steps 3, 4, 5 were repeated. (7) Extracts were combined and evaporated to dryness with nitrogen gas at room temperature.

Sample preparation

Dried samples were reconstituted with 1 ml of HPLC grade methanol. Aliquots (100 µl) of each sample were diluted 1:10 using acetonitrile and 10 mm/ml ammonium acetate solution, pH 3.0 (final ratio of organic to buffer 60: 40). Subsequent dilutions were made using 40: 60 acetonitrile: 10 mM ammonium acetate solutions, pH 3.0. Diluted samples were transferred into standard polypropylene sample vials for analysis by LC/MS/MS.

Oleandrin standard preparation

Oleandrin (Sigma-Aldrich) stored at -20 C in 1 mg/ml solution in methanol was used to prepare the calibration standards for quantification of oleandrin in extracted samples. Stock material (10 µl of 1 mg/ml) was serially diluted using 40: 60 acetonitrile: 10 mM ammonium acetate, pH 3.0. Calibration curves were constructed from concentrations of 5, 10, 25, 50, 100, 250 and 500 ng/ml oleandrin. Standards were transferred into standard polypropylene samples vials and analyzed by LC/MS/MS.

LC/MS/MS instrumentation and method setup

The presence and quantitative determination of oleandrin was carried out by LC/MS/MS analysis of the peaks with co-elution of authentic standard compounds. LC/MS/MS was performed using a Waters QuattroPremier tandem mass spectrometer with an electrospray ion source and Waters Acuity UPLC system. The separation of oleandrin was achieved using a Waters UPLC BEH C18 (1.7 um, 2.1x50 mm) analytical column maintained at 50^0C. The solvent system for column elution consisted of solvent A: 10 mM ammonium acetate with 0.2% (v/v) formic acid, pH 3.0, and solvent B: acetonitrile (HPLC grade). Mobile phase was delivered at 35 µl/min starting at 75% A and 25% B, followed by 5% A and 95% B at 4 min which was continued until 8.5 min, then programmed back to starting conditions until 20.5 min.

Oleandrin in oleander plant and callus tissue extracts and as reference standards were detected using electrospray ionization in positive ion mode with ion transition. The mass spectrometer was operated in the multiple reaction monitoring mode (MRM) which was optimized for the precursor-product ion transition of *m/z* 577.9 > 373.8 for oleandrin.

Isolation of glycoside fraction and determination of total glycoside content

Aliquots (2 g) of dried and powdered leaves or callus tissue were extracted by boiling in 35 ml ethanol: water (2: 3) mixture for 20 min. After cooling and filtration, the filtrate volume was brought up to 50 ml with the same mixture and filtered. The completeness of glycoside extraction was tested by Legal reaction[52]. The glycoside fraction was stored in the dark at 4^0C.

To evaluate the glycoside content in the fraction isolated, the method generally applicable for glycosides[30,52]; was applied. The total glycosides were calculated as oleandrin. Briefly, 2 ml of the filtrate was applied to a column filled with Al_2O_3 and eluted 4 times with 0.5 ml ethanol and finally with 16 ml ethanol. The column effluent was collected into a volumetric flask and added with ethanol to 25 ml. An aliquot (4 ml) of this glycoside containing fraction was mixed with 4 ml of the Kedde reagent and the absorbance of the mixture was measured in 2 min with a spectrophotometer SF-56 (LOMO, Russia) against the blank (4 ml of the Kedde reagent) at 540 nm. Previously the calibration curve was developed. To this end, 10 mg of oleandrin was dissolved in 100 ml ethanol. The oleandrin solutions (1, 2, 3, or 4 ml) were mixed with 4 ml of the Kedde reagent and the absorbance of samples was measured. The curve was applied to calculate total glycoside content (in percent) in a sample as:

$$a \times 1562.5 / e \times (100 - t)$$

where 'a' is glycoside content, mg; 'e' is the weight of a sample, mg; 't' is the percent loss of liquid from the sample.

Full Paper

Isolation of polysaccharide fraction

Plant leaves and callus tissue were dried at 70^{0}C. Samples (500 mg) of dry leaves and callus were treated with methanol: chlorophorm mixture (1: 1) in a Soxhlet apparatus to remove low-molecular components. The dry residue was extracted four times with water at 50^{0}C. Collected water extracts were concentrated in a rotary evaporator and centrifuged at 8,000 rpm. Polysaccharides were precipitated with ethanol (1: 2, v/v) and centrifuged at 8,000 rpm. The sediment was dissolved in 1 ml water and stored at + 4^{0}C.

Analysis of in vitro cytotoxicity and species-specificity of water extracts, glycoside and polysaccharide fractions of plant leaves and callus cultures

Extracts, glycoside or polysaccharide fractions (100 μl/ml) were added to human and animal cell cultures 24-48 h after cell seeding. After incubation of cultures for 24 h the cell viability was determined by the vital dye (Trypan blue, Sigma-Aldrich) exclusion test[6]. This simple technique identifies dead cells with compromised cell membrane integrity. It is known to overestimate the number of living cells; nevertheless, it is appropriate for comparative experiments. Monolayer cells were detached with trypsin-EDTA solution (Sigma-Aldrich) and suspended. The number of living cells was counted and expressed as a percent of the control (untreated culture).

To develop the concentration-effect curve (concentration of callus extract as oleandrin content against living cell number, % of the control) the K-562 cell line was incubated with various concentrations of callus extract (from 10 to 100 μl/ml that corresponds to oleandrin content from 6 to 60 ng/ml according to glycoside fraction analysis) for 24 h and the cell viability was evaluated.

The species-specificity of extracts' cytotoxic effect was determined by comparing the responses of rodent (PC-12), primate (COS-7) and human (Jurkat, K-562, and KB) cell lines.

Analysis of the cell death mode (necrosis or apoptosis) induced by water extracts of plant leaves and callus cultures

Water extracts of plant leaves or callus tissue (100 μg/ml) were added to the K-562 cell line 24 h after cell seeding. Non-treated cell cultures were used as a control.

i) Morphological analysis. Timed (8, 12, 16, 20, and 24 h) triplicate samples after addition of extracts to cell cultures were transferred to slides, fixed with ethanol: acetic acid (3: 1), air-dried and stained with Giemsa (Sigma-Aldrich). The number of cells appearing as apoptotic bodies was counted and expressed as a percentage of 1,000 cells examined.

ii) Gel-electrophoretic analysis of DNA fragmentation pattern. Cells were treated with extracts for 4, 8, and 12 h. DNA was extracted and purified by[43]. DNA electrophoresis was performed in 1.6% agarose gel for 1 h at 75 V. DNA was stained with ethidium bromide (Sigma-Aldrich) and visualized by UV-illumination.

Genotoxicity and anti-genotoxic effect of water extracts and polysaccharide fractions of plant leaves and callus cultures

To determine the genotoxicity of extracts and polysaccharide fractions the cytokinesis block variant of the *in vitro* micronucleus (MN) induction test[7,37]; was applied. MN test is known to detect agents that modify chromosome structure and segregation in such a way as to lead to induction of MN in interphase cells. Treatment of cultures with the inhibitor of actin polymerization cytochalasin B results in the "trapping" of cells at the binucleate stage where they can be easily identified.

Various concentrations of materials tested combined with cytochalasin B (Sigma-Aldrich) dissolved in ethanol (not more than 10 μl ethanol per 1 ml of the medium so as not to affect the cell viability and growth) to the final concentration 3 μg/ml were added to cultures of KCL-22 cell line 44 h after seeding. Cell cultures incubated with cytochalasin B only were used as negative controls; positive controls consisted of cultures incubated with cytochalasin B and 1 μl/ml bleomycin (Bleomycinum, Tomen Corporation), an antibiotic and chemotherapeutic drug that is known to induce DNA strand breaks and chromosome aberrations[5,39,49]. Earlier the dose-effect curve for bleomycin effect on KCL-22 cell line was developed and the above mentioned concentration was selected as effective one. After 28 h incubation the cells were fixed with ethanol: acetic acid (3: 1), spread on slides, air dried, and stained with Gi-

emsa (Sigma-Aldrich). The number of binucleate cells with MN was counted in 1,000 binucleate cells in the same cultures. Only micronuclei not exceeding 1/3 of the main nucleus diameter, not overlapping with the main nucleus, and with distinct borders were included in the scoring[8,12].

Bleomycin (1 μg/ml) was added to cultures of KCL-22 cell line 44 h after seeding in order to analyze anti-genotoxic effect of extracts and polysaccharide fractions. Cytochalasin B and various concentrations of plant materials tested (0.1 or 1 μl/ml of water extracts and 1-1,000 μl/ml of polysaccharide fractions) were added to the cultures simultaneously, 2 hours before and 2 hours after addition of bleomycin. Cells were incubated for 28 h and the MN frequency was determined as mentioned above. The negative and positive control cultures were treated with distilled water or 1 μg/ml bleomycin, respectively.

Anti-mutagenic effects of water extracts and polysaccharide fractions of plant leaves and callus cultures

To study mutagenic and anti-mutagenic effects of extracts and polysaccharide fractions, the Ames bacterial reverse mutation test[23,27,35] without metabolic activation was applied. The strain ÒÀ 1535 of *Salmonella typhimurium*[24]; recommended by[35] was used to detect point mutations, which involves substitution of DNA base pairs resulting in the shift from hisitidine auxotrophy to prototrophy.

Minimum agar containing the Vogel-Bonner minimum medium E (the medium components from Sigma-Aldrich) and glucose and overlay agar containing biotin were used. Aliquots (0.1-1 ml) of the test solutions, 0-0.9 ml of sterile buffer solution (total volume 1 ml) and 0.1 ml of fresh bacterial culture containing approximately 10^8 viable cells were mixed with 2.0 ml of overlay agar and poured over the surface of a minimum agar plate. The plates were incubated at 37^0C for 48 h and the number of colonies per plate was counted. Mutations were induced by 0.5 μg/ml bleomycin added to the overlay agar. Earlier the dose-effect curve for bleomycin effect on the bacterial strain used was developed and the concentration mentioned was selected as an effective one. Antimutagenic activity of extracts and polysaccharide fractions (concentrations 0.1; 1; 10; 100 and 1,000 μl per plate) was estimated by measuring the decrease in frequency of mutation events (expressed as revertant colony number) induced by bleomycin. The negative and positive control cultures were combined with distilled water and 0.5 μg/ml bleomycin, respectively. Triplicate cultures were used at each experimental point/dose level. The results were statistically analyzed using the Student's one-tail t-test.

RESULTS

Secondary metabolite oleandrin content in callus culture

According to results of LC/MS/MS chromatographic analysis, the callus tissue contained the main oleander secondary metabolites oleandrin (Figure 1), oleandrigenin and odoroside (not shown). The oleandrin content in 20-days callus culture was notably less (about 13%) than in plant leaves.

Cytotoxicity and species—specificity of water extracts, glycoside and polysaccharide fractions of plant leaves and callus cultures

The cytotoxicity of callus extract was shown to be concentration-dependent (Figure 2). The IC_{50} value (concentration inducing 50% inhibition of cell viability) for K-562 cell line was about 7.5 μl/ml (corresponding to oleandrin content 6 ng).

It was revealed that the cytotoxicity of both plant leaves and callus tissues belonged mainly to the glycoside fraction. The polysaccharide fraction possessed low cytotoxicity (Figure 3). In spite of low oleandrin content in callus tissue in comparison with leaves (see Figure 1), the cytotoxicity of callus extract and glycoside fraction did not reduced.

The toxicity of oleander was demonstrated earlier to be species-specific as human cells *in vitro* are sensitive to plant extracts while rodent cells are not[31,34,40];. It would be of interest to reveal whether this uncommon feature remains in plant tissues *in vitro*. If yes, it will testify to the identity of activities of the plant and its callus culture.

The sensitivity of human (normal embryo fibroblasts, cell lines Jurkat, K-562, L-41 and KB), monkey (cell line COS-7) and rat (cell line PC-12) cells to extracts of plant leaves and callus tissue were com-

Full Paper

pared (TABLE 1).

Both extracts tested (100 µl/ml, oleandrin content 75 ng) were toxic for human and monkey cells; the most sensitive among them was human leukemia K-562 cell line. Rat PC-12 cell line was slightly sensitive to the extract of plant leaves and expressed full resistance to callus extract. The cytotoxicity of callus extracts in all

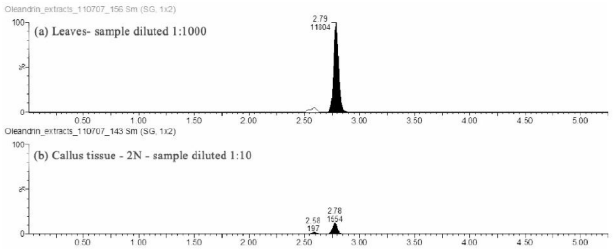

Figure 1 : LC/MS/MS chromatograms of leaf (a) and callus (b) samples

cases was comparable to the leaf extracts. So, cultured oleander tissues retained the species-specific toxicity characteristic of the parent plant.

The mode of cell death (necrosis or apoptosis) induced by water extracts of plant leaves and callus cultures

In the K-562 cell line treated with extracts of plant leaves and callus tissue, the number of apoptotic cells increased sharply to 12 h when more than 90% of cells contained apoptotic bodies (TABLE 2). The dynamics of this index was similar in both cases (treatment with plant and callus extracts). Later (24 h) the cells disappeared, perhaps, because of development of secondary necrotic processes[50] and disintegration of dead cells.

Electrophoresis of DNA isolated from cells exposed to extracts of plant leaves and callus tissue for 8 and 12

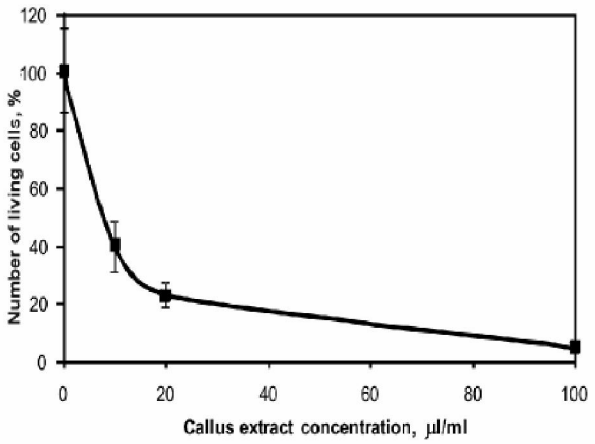

Figure 2 : K-562 cell line viability as a function of the concentration of callus extract

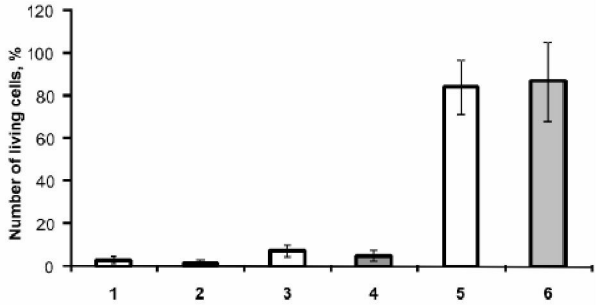

Figure 3 : The relative cytotoxicity of water extracts (columns 1 and 2), glycoside (columns 3 and 4) and polysaccharide (columns 5 and 6) fractions of plant leaves (white columns) and callus tissue (dark columns) for KCL-22 cell line

h demonstrated DNA fragmentation (Figure 4). DNA fragments were distributed as discrete bands (so called 'laddering') that suggested the development of apoptotic processes in affected cells[22].

Both extracts tested were shown to induce apoptosis. Pro-apoptotic activity of the water plant

TABLE 1 : Species-specificity of cytotoxic effect of water extracts of plant leaves and callus tissue[a]

Cell lines	Control, number of living cells x 10^6	Extract of plant leaves		Extract of callus tissue	
		Number of living cells x 10^6	% of control	Number of living cells x 10^6	% of control
Normal human embryo fibroblasts	0.03 ± 0.005	0.004 ± 0,002	12 ± 0.8	0.002 ± 0.001	6.4 ± 0.3
Jurkat	1.26 ± 0,024	0.3 ± 0,012	23.8 ± 0.5	0.42 ± 0,038	32.8 ± 1.6
K-562	0.89 ± 0,018	0.01 ± 0,001	1 ± 0.3	0.03 ± 0,012	3.8 ± 0.1
L-41	0.05 ± 0,005	0.01 ± 0,003	20 ± 0.6	0.014 ± 0,006	28 ± 0.8
KB	0.16 ± 0,065	0.02 ± 0.002	12 ± 0.5	0.02 ± 0,005	15 ± 1.1
COS -7	0.07 ± 0,025	0.03 ± 0,015	43 ± 0.5	0.02 ± 0,003	28.5 ± 0.1
PC-12	0.23 ± 0,028	0.14 ± 0,017	64.5 ± 0.7	0.23 ± 0,098	100 ± 1.3

[a] Here and below the results are presented as Mean ± SE

extract was revealed earlier[25,46,55]. Our results indicate that this activity was retained in and may be extracted from plant tissues *in vitro*.

Genotoxicity and anti-genotoxic effect of water extracts and polysaccharide fractions of plant leaves and callus cultures

Water extracts and polysaccharide fractions of plant leaves and callus cultures were shown not to be genotoxic for KCL-22 cell line as they did not induce MN formation (data not shown). Water extracts did not inhibit bleomycin-induced MN generation (TABLE 3). Addition of polysaccharide fractions before or after treatment of cells with bleomycin also did not change the MN frequency (data not shown).

On the contrary, polysaccharide fractions added to the cell cultures together with bleomycin reduced the number of cells bearing MN from more than two to about five times. This effect seemed to be concentration-dependent in the range studied (1-1,000 μl/ml). So, polysaccharide fractions partly protected the cells from genotoxic effect of bleomycin decreasing the rate of appearance of daughter cells bearing as heavy DNA/chromosome damage as MNs.

TABLE 2 : Apoptosis-inducing activity of water extracts of plant leaves and callus tissue

Hours	Number of cells with apoptotic bodies, %	
	Extract of plant leaves	Extract of callus tissue
0	2.3 ± 0.02	2.3 ± 0.03
12	94.9 ± 1.6	92.1 ± 1.7
16	99.5 ± 1.4	97.7 ± 1.9
20	99 ± 0.9	95.3 ± 1.2

Anti-mutagenic effects of water extracts and polysaccharide fractions of plant leaves and callus cultures

In preliminary experiments it was found that extracts and polysaccharide fractions of plant leaves and callus tissue are non-toxic for *S. typhimurium* as their addition to the culture medium did not change the number of bacterial colonies grown (data not shown). It suggested the adequacy of the applied test system for evaluation of genotoxic effects. The materials tested were also demonstrated not to induce any significant increase in revertant colony number (data not shown). In other words, both extracts and polysaccharide fractions did not express any detectable genotoxic activity for *S. typhimurium*.

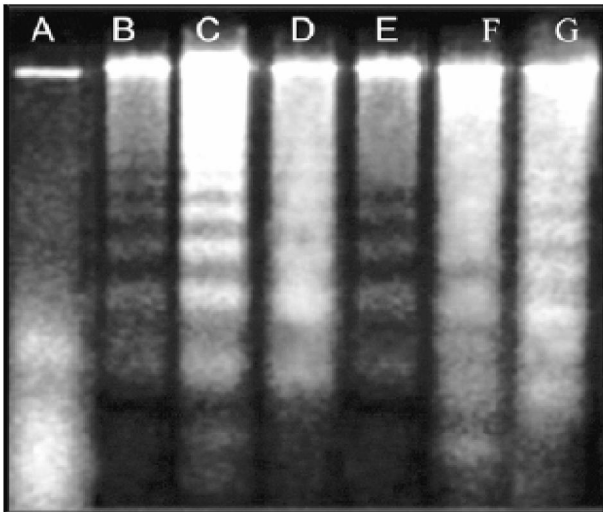

Figure 4 : Pattern of DNA fragmentation in K-562 cells. Control, untreated cells (A) and cells treated with extracts of plant leaves (B, C, D) and callus tissue (E, F, G). Duration of exposure to extracts: 4 h (B, E), 8 h (C, F), and 12 h (D, G).

Bleomycin was shown to induce reverse mutations in bacterial cultures elevating the number of revertant colonies about seventy times in comparison to untreated cultures (TABLE 4). Addition of extracts or polysaccharide fractions before or after the mutagen did not change the revertant colony number (data not shown).

TABLE 3 : Anti-genotoxic activity of water extracts and polysaccharide fractions of plant leaves and callus tissue in KCL-22 cell line

Treatment	Concentration, µl/ml	MN number in 1,000 binucleate cells	MN number, % of positive control
Negative control (intact cell culture)		5 ± 0,5	
Positive control (bleomycin, 1µg/ml)		314 ± 2,38	
Bleomycin + water extract of plant leaves	1	296 ± 1,44	94.3 ± 0.3
	0.1	312 ± 1,61	99.3 ± 0.45
Bleomycin + water extract of callus tissue	1	301 ± 0,29	95.9 ± 0.079
	0.1	310 ± 1,21	98.7 ± 0.35
Bleomycin + polysaccharide fraction of plant leaves	1	132 ± 0,86*	42 ± 0.57
	10	112 ± 0,95*	35.7 ± 0.78
	100	76 ± 1,08*	24.2 ± 1.35
	1,000	68 ± 0,75*	21.7 ± 0.95
Bleomycin + polysaccharide fraction of callus tissue	1	124 ± 0,49*	39.5 ± 0.34
	10	115 ± 0.49*	36.6 ± 0.38
	100	85 ± 1,03*	27.1 ± 1.12
	1,000	71 ± 0,63*	22.6 ± 0.75

*** p > 0.05 (comparison with positive control)**

Full Paper

The same extracts and fractions added together with bleomycin essentially reduced the frequency of mutation events. Differences in activity of materials obtained from plant and callus culture were minute. Isolated polysaccharides were more effective than whole extracts (the decrease in the number of revertant colonies changed from 21.8 to 52.5% and from 42.5 to 76.6% as a function of the positive control, accordingly). The effect of polysaccharides was concentration-dependent in the range studied (0.1-1,000 µl per plate). The anti-genotoxic (TABLE 3) and anti-mutagenic (TABLE 4) activities of polysaccharide fraction of oleander leaves and callus culture are considered to be novel findings.

DISCUSSION

In this work the activities of oleander callus culture were tested and compared with those of oleander leaves. Water extract of callus culture was shown to be multi-component mixture containing oleander secondary metabolites (oleandrin, oleandrigenin and odoroside) and polysaccharides. The extract was cytotoxic for mammalian cells *in vitro* in a concentration-dependent and species-specific manner and was a potent inducer of apoptotic cell death. The cytotoxicity of extract was demonstrated to belong solely to the glycoside fraction whereas the polysaccharide fraction was practically non-toxic. The features listed were earlier described to be specific for water extracts of oleander leaves[32,38]. It is reasonable to assert that oleander cells propagated as callus culture continue to synthesize specific secondary metabolites and retain their distinctive activities.

Oleander leaf and callus extracts were for the first time tested for genotoxic and mutagenic activity using two *in vitro* cellular models (KCL-22 cell line, the MN induction test; *S. typhimurium* culture, the Ames test, respectively). Both extracts were found to be non-genotoxic and to express no mutagenic activity. The combination of high cytotoxicity with low genotoxicity/mutagenicity is believed to be advisable for chemotherapeutic agents not to incite secondary mutations and DNA/chromosome damage in the process of therapy[11,51].

Anti-genotoxic and anti-mutagenic activities of extracts were also analyzed in the same cell cultures treated with the mutagenic chemotherapeutic agent bleomycin. The plant and callus extracts added before or after bleomycin were shown not to be able to protect the cells from induced chromosome/DNA damage. In other words, they had no protective or therapeutic effect. The same extracts applied simultaneously with bleomycin did not diminish the number of MN in KCL-22 cell line but reduced (1.5-2 times) the incidence of revertant colonies in *S. typhimurium* culture. Thus, they seemed to interfere with the mutagen and partially prevent the emergence of point mutations. The polysaccharide fractions isolated from plant leaves and callus tissue were observed not to stop the development of bleomycin-induced genetic damages when added before or after the mutagen. Added together with bleomycin, polysaccharides substantially (approximately 2-5 times) decreased both the MN (in KCL-22 cells)

TABLE 4 : Anti-mutagenic activity of water extracts and polysaccharide fractions of plant leaves and callus tissue in *S. typhimurium* culture

Treatment	Concentration, µl per plate	Number of revertant colonies/plate	Number of revertant colonies, % of positive control
Negative control (intact cell culture)		16.2 ± 3.3	
Positive control (bleomycin, 0.5 µg per plate)		1135.4 ± 92.2	
Bleomycin + extract of plant leaves	0.1	733.9 ± 27.5*	64.6 ±3.1
	1	652.0 ± 26.7*	57.4 ± 3.0
	10	567.4 ± 25.6*	50 ± 2.9
	100	482.7 ± 26.4*	42.5 ± 2.3
	1,000	869.0 ± 21.7*	76.6 ± 1.9
Bleomycin + extract of callus tissue	0.1	756.4 ± 31.2*	66.6 ± 2.8
	1	647.3 ± 28.5*	57 ± 2.5
	10	782.6 ±23.9*	68.9 ± 2
	100	795.3 ± 24.6*	70 ± 2.2
	1,000	566.0 ± 29.4*	49.9 ± 2.6
Bleomycin + polysaccharide fraction of plant leaves	0.1	583.9 ± 19.4*	51.4 ± 1.7
	1	468.6 ± 23.5*	41.3 ± 2.0
	10	389.4 ± 27.3*	34.3 ± 2.4
	100	351.3 ± 12.8*	30.9 ± 1.2
	1,000	247.0 ± 14.6*	21.8 ± 1.3
Bleomycin + polysaccharide fraction of callus tissue	0.1	595.3 ± 23*	52.3 ± 2.0
	1	489.0 ± 25*	43.1 ± 2.2
	10	427.4 ± 23.3*	37.6 ± 2.0
	100	358.6 ± 13.9*	31.6 ± 1.2
	1,000	253.4 ± 14.8*	22.3 ± 1.3

***p > 0.05 (comparison with positive control)**

and revertant colony (in *S. typhimurium* culture) numbers in a concentration-dependent manner. Earlier it has been shown that polysaccharides from few other medicinal plants (*Aloe barbadensis* Miller, *Lentinus edodes, Ganoderma lucidum, Coriolus vercicolor*[18] and *Curcuma zedoaria*[19] also express the anti-genotoxic activity. In our experiments the differences in the effect of polysaccharides separated from plant leaves and callus tissue were not essential.

Thus, oleander polysaccharides themselves were revealed to possess the anti-genotoxic activity which did not manifest in the extracts. Plant medicines are usually used as whole extracts. Their effect often decreases after fractionation or even after purification and, so, cannot be evaluated as a mere sum of constituents' action. The possibility of interactions between components and their pharmacological effects should not be ignored as well. The observed 'masking' of anti-mutagenic activity of polysaccharides in whole extract may be a result of neutralizing influence of other components. The oleander polysaccharides by themselves may be considered as a substance exerting anti-genotoxic and anti-mutagenic effects of pharmacological value.

Thus, in the present work it was demonstrated that oleander tissue in callus culture retains the main activities of the plant. It can be concluded that this culture can be used as a biotechnological source of biologically/pharmacologically active compounds of oleander to provide standardized and pure medicines.

ACKNOWLEDGEMENTS

The authors would like to acknowledge Prof. John Masters (Institute of Urology and Nephrology, University College London, UK) and Dr. Thomas Liehr ((Institute of Human Genetics and Anthropology, Germany) for generous providing cell lines used in the present work. The authors are grateful to researchers of the Experimental Therapeutics of the Pharmaceutical Development Center (the University of Texas, M. D. Anderson Cancer Center) for help.

REFERENCES

[1] F.Abe, T.Yamaguchi; Chem.Pharm.Bull, **26**, 3023-3027 **(1978)**.
[2] F.Abe, T.Yamaguchi; Chem.Pharm.Bull, **27,** 1604-1610 **(1979)**.
[3] S.Begum, B.S.Siddiqui, R.Sultana, A.Zia, A.Suria; Phytochem., **50**, 435-438 **(1999)**.
[4] F.Dicosmo, M.Misawa; Biotechnol.Adv., **13**, 425-453 **(1995)**.
[5] R.T.Dor; Sem.Oncol., **2**, 3-8 **(1992)**.
[6] A.Doyle; 'Cell and Tissue Culture: Laboratory Procedures in Biotechnology', John Wiley & Sons, Chichester, **(1998)**.
[7] M.Fenech; Mutat.Res., **455**, 81-95 **(2000)**.
[8] M.Fenech, A.A.Morley; Mutat.Res., **147**, 29-36 **(1985)**.
[9] L.Fu, S.Zhang, N.Li, J.Wang, M.Zhao, J.Sakai, T.Hasegawa, T.Mitsui, T.Kataoka, S.Oka, M.Kiuchi, K.Hirose, M.Ando; J.Nat.Prod., **68,** 198-206 **(2005)**.
[10] S.A.Jortani, R.A.Helm, R; Clin.Chem., **42**, 1654-1658 **(1996)**.
[11] G.Gasparyan, G.Hovhannisyan, R.Ghazaryan, L.Sahakyan, A.Tovmasyan, R.Grigoryan, N.Sarkissyan, K.Margaryan, S.Haroutiunian, R.Arutyunyan;. Int.J.Toxicol, **26**, 497-502 **(2007)**.
[12] J.A.Heddle; Mutat.Res., **18**, 187-190 **(1973)**.
[13] N.A.Hovhannisyan; Vestnik Biotechnologii (Moscow), **3**, 10-17 **(2007)**.
[14] N.Hovhannisyan, M.Mkrtumyan, G.Gasparyan; Biotechnologiya (Moscow), **5**, 66-71 **(2007)**.
[15] A.M.Huq, A.Jabbar, M.A.Rashid, C.M.Hasan, C.Ito, H.Furukawa; J.Nat.Prod., **62**, 1065-1067 **(1999)**.
[16] A.K.Ibrahim, S.Khalifa, I.Khafagi, D.Youssef, I.Khan, M.Mesban; J.Enzyme and Microbial Technol., **41**, 331-336 **(2007)**.
[17] S.Karuppusamy; J.Med.Plant Res., **3**, 1222-1239 **(2009)**.
[18] H.S.Kim, S.Kasew, B.M.Lee; Carcinogenesis **20**, 1637-1640 **(1999)**.
[19] K.I.Kim, J.W.Kim, B.S.Hong, D.H.Shin, H.Y.Cho, H.K.Kim, H.C.Yang; Mol.Cells, **10**, 392-398 **(2000)**.
[20] S.D.Langford, P.J.Boor; J Toxicol **109**, 1-13 **(1996)**.
[21] S.K.Manna, N.K.Sah, R.A.Newman, A.Cisneros, B.B.Aggarwal; Cancer Res., **60**, 3838-3847 **(2000)**.
[22] B.Mannheim; 'Apoptosis and Cell Proliferation', 2nd Edition, Biochemica, Germany, **(1998)**
[23] D.Maron, B.Ames; Mutat Res., **113**, 173-215 **(1983)**.
[24] J.McCann, N.Spingarn, J.Kobori, B.Ames; Proc. Nat Acad Sci., **72**, 979-983 **(1975)**.

Full Paper

[25] D.J.McConkey, Y.Lin, L.K.Nutt, H.Z.Ozel, R.A.Newman; Cancer Res., **60**, 3807-3812 **(2000)**.

[26] T.Mekhail, H.Kaur, R.Ganapathi, G.T.Budd, P.Elson, R.M.Bukowski; Invest New Drugs, **24**, 423-427 **(2006)**.

[27] H.A.Milman, E.K.Weisburger, (Eds); 'Handbook of Carcinogen Testing', 2nd Edition, Noyes Data Corp Park, Ridge, **(1994)**.

[28] M.D.Mingorance, S.O.Rossini; J of Environmental Monitoring and Assessment, **119(1-3)**, 57-68 **(2006)**.

[29] V.Mulabagal, H.S.Tsay; Int.J.Appl.Sci.Eng., **2**, 29-48 **(2004)**.

[30] L.Nerium Oleander; Web. http://www.inchem.org/documents/pims/ plant/pim366.htm, **(1997)**.

[31] R.Newman, A.Cisneros, E.Felix, M.Vijjeswarapu, Y.Lin, P.Yang, P.Azadi; J.Herbal Pharmacol, **1**, 1-12 **(2001)**.

[32] R.A.Newman, Y.Kondo, T.Yokoyama, S.Dixon, C.Cartwright, D.Chan, M.Johansen, P.Yang; Integrative Cancer Therapies, **6**, 354-364 **(2007)**.

[33] R.A.Newman, P.Yang, W.N.Hittelman, T.Lu, D.Ho, D.Ni, D.Chan, M.Vijjeswarapu, S.Dixon, E.Felix, C.Addinton; J.Exp.Ther Oncol, **5**, 167-181 **(2006)**.

[34] R.A.Newman, P.Yang, A.D.Pawlus, K.J.Block; Mol Interventions, **8**, 36-49 **(2008)**.

[35] OECD Guideline 471; Bacterial Reverse Mutation Test, Web. www.oecd.org/ dataoecd/18/31/ 1948418.pdf **(1997)**.

[36] Ozel HZ; Inventor Extracts of Nerium Species, Methods of Preparation and Use Therefore. US Patent 5,135,745, Aug 4 **(1992)**.

[37] J.M.Parry; A proposal for a new OECD guideline for the *in vitro* Micronucleus Test Web. http://www.swan.ac.uk/cget/ejgt/articleMNUKEMS Protocol.htm, **(1998)**.

[38] S.Pathak, A.S.Multani, S.Narayan, V.Kumar, R.A.Newman; Anticancer Drugs, **11**, 455-463 **(2000)**.

[39] L.F.Povrik, M.J.Austin; Mutat Res., **257**, 127-143 **(1991)**.

[40] P.B.Raghavendra, Y.Sreenivasan, S.K.Manna; Mol.Immunol., **44**, 2292-2302 **(2007)**.

[41] R.S.Rao, G.A.Ravishankar; Biotechnol Adv., **20**, 101-153 **(2002)**.

[42] S.O.Rossini, M.D.Mingorance; J.Chemosphere, **65**,177-182 **(2006)**.

[43] J.Sambrook, D.W.Russell; Molecular Cloning, 'A Laboratory Manual', 3rd Edition, Cold Spring Harbor, Laboratory Press Cold Spring Harbor, New York, **(2001)**.

[44] I.Santos, I.Guimaraes, R.Salema; J.Plant Cell, Tissue and Organ Culture, **37**, 83-86 **(1994)**.

[45] J.A.Smith, T.Madden, M.Vijjeswarapu, R.A.Newman; Biochem.Pharmacol., **62**, 469-472 **(2001)**.

[46] Y.Sreenivasan, A.Sarkar, S.K.Manna; J Biochem Pharmacol, **66**, 2223-2239 **(2003)**.

[47] Y.Sreenivasan, P.B.Raghavendra, S.K.Manna; J.Clin Immunol **26**, 308-322 **(2006)**.

[48] S.Srivastava, Plant Tissue Culture; An Overview Web. http://www.pharmainfo.net/reviews/plant-tissue-culture-overview, **(2007)**.

[49] C.H.Takimoto, E.Calvo; Principles of Oncologic Pharmacotherapy. In: R.Pazdur, L.D.Wagman, K.A.Camphausen, W.J.Hoskins (Eds); Cancer Management; A Multidisciplinary Approach, 11th Edition, CMP Medica, London, **(2008)**.

[50] I.Vermes, C.Haanen, D.J.Richel , M.R.Schaafsma, E.Kaisbeek-Batengurg, C.P.Reutelingsperger; Acta Haematologica, **98**, 8-13 **(1997)**.

[51] R.Vogel, G.Laschinski, H.Spielman, W.Ehrlich, B.Drevenstedt, J.Klosa, H.Kroger; Mutat Res., **264**, 225-230 **(1991)**.

[52] H.Wagner, S.Bladt; Plant Drug Analysis, 2nd Edition, Springer, Berlin, **(1995)**.

[53] X.Wang, J.B.Plomley, R.A.Newman, A.Cisneros; Anal.Chem., **72**, 3547-3552 **(2000)**.

[54] M.Wink, A.W.Alfermann, R.Franke, B.Wetterauer, M.Distl, J.Windhovel, O.Krohn, E.Fuss, H.Garden, A.Mohagheghzaden, E.Wildi, P.Ripplinger; Plant Genetic Resources, **3**, 90-100 **(2005)**.

[55] K.Winnicka, K.Bielawski, A.Bielawska; Acta.Pol. Pharm., **63**, 103-115 **(2006)**.

[56] M.Sh.Yui, S.W.Lai, K.F.Lin, J.N.Fang, W.H.Yuen, R.C.Chang; Int.J.Mol.Med., **14**, 917-924 **(2004)**.

[57] M.Zhao, L.Bai, L.Wang, A.Toki, T.Hasegawa, M.Kikuchi, M.Abe, J.Sakai, R.Hasegawa, Y.Bai, T.Mitsui, H.Ogura, T.Kataoka, S.Oka, H.Tsushima, M.Kiuchi, K.Hirose, A.Tomida, T.Tsuruo, M.Ando; J.Nat.Prod., **70**, 1098-1103 **(2007)**.