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ANTIMUTAGENIC ACTIVITY OF POLYSACCHARIDE FRACTION OF *NERIUM OLEANDER* (L.) CALLUS CULTURE

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The antimutagenic effect of *Nerium oleander* intact plant and callus culture hot water extracts and polysaccharide fractions on the mutagenicity of sodium azide and bleomycin was investigated *in vitro* using *Salmonella typhimurium* TA 1535 as an indicator bacterium. It was shown that *N. oleander* callus tissue retains the antimutagenic effect investigated and specific for intact plant. So, *N. oleander* callus culture may be used as an alternative source of biological active substances for the mutagenesis and carcinogenesis prevention.

Ուսումնասիրված է դափնավարդի ՝ *Nerium oleander*-ի ինտակտ բույսի և կալուսային հյուսվածքի ջրային լուծամզվածքների և պոլիսախարիդային ֆրակցիայի հակամուտագենային ակտիվությունը նատրիումի ազիդով և բլեոմիցինով ինդուկցված մուտացիաների դեմ պայմաններում *Salmonella typhimurium* -ի TA 1535 շտամի բջիջների վրա:

Nerium oleander-ի կալուսային հյուսվածքը պահպանում է ինտակտ բույսին բնորոշ հակամուտագենային ակտիվությունը: Հետևաբար *Nerium oleander*-ի կալուսային հյուսվածքը կարող է օգտագործվել որպես հակամուտագենային և հակաուռուցքային ակտիվությամբ օժտված կենսաբանորեն ակտիվ միացությունների այլընտրանքային աղբյուր:

Изучена антимутагенная активность водных экстрактов и полисахаридной фракции интактного растения и каллусной культуры *Nerium oleander* на мутагенный эффект азида натрия и блеомицина *in vitro* на клетках *Salmonella typhimurium* шт. ТА 1535.

Показано, что каллусная культура *N. oleander* сохраняет антимутагенную активность, свойственную интактному растению. Следовательно, каллусная культура *N. oleander* может использоваться как альтернативный источник биологически активных веществ с антимутагенной и антираковой активностью.

Antimutagen – Salmonella - Nerium oleander – bleomycin – sodium azide - polysaccharides

There has been increasing interest in anticarcinogens of plant origin in recent years. It is now becoming clear that higher plants contain a variety of preformed secondary metabolites that represent a structurally diverse array of mutagenic, antimutagenic, and desmutagenic substances.

Study of such substances can lead to much interesting details about the processes of mutagenicity and antimutagenicity. In addition, detection and identification of agents known as antimutagens, which inhibit mutagenesis, are very important because DNA damage is crucial in variety of diseases and degenerative processes. Especially, antimutagen agents present in human dietary products and in preparations for cancer therapy are of great importance because they can act as preventive substances.

The plant *Nerium oleander* (L) is known in the folk medicine. Hot water extracts of *N. oleander* patented as Anvirzel were shown to be cytotoxic for human cancer cells [10]. The extract has been found to comprise several polysaccharides with very potent immune stimulating properties [10]. The cytotoxic and apoptosis – inducing properties of *N. oleander* plant and callus tissue culture hot water extracts for human transformed cell lines *in vitro* have been specified by us early [5-7]. In the present study the antimutagenic efficacy of *N. oleander* intact plant and callus tissue hot water extracts and polysaccharide fraction were tested. The Ames mutagenicity test has been used as a simple primary screen for antimutagenesis [4]. Two known mutagens, bleomycin and sodium azide were used to chemically induce reversion mutation as a positive control.

Materials and Methods. *Nerium oleander callus culture hot water extracts and polysaccharide fractions:* Leafs of intact plant and callus tissue obtained by us early was air-dried at 70^0 and extracted (0.5: 10, w/v) by boiling in 0.9 % NaCl solution or Hanks' balanced solution for 3 hours [7, 10]. The extracts were filtered and used in different concentrations.

For the isolation of polysaccharide fractions dried intact plant leafs and callus tissues of N. oleander were crushed and extracted with ethanol 96% in Soxlet apparatus for removal of low-molecular compounds. Than air-dried reminder was cooked (0.5: 10, w/v) in distilled water for 3 hours. The solution was mixed with ethanol (96%) in a 1:1 ratio and allowed to set for 12 hours at room temperature. The formed gel suspension was centrifuged (5000 rpm) for 10 min. The reminder was taken up in distilled and again mixed with ethanol (96%) in a 1:1 ratio. After allowing the solution to set for at least 12 hours, the gel suspension was centrifuged (5000 rpm) for 10 min. The reminder was taken up in distilled water and dried by evaporation. The dried polysaccharide extract was obtained. Previously the experiments were resolved in 0.9% NaCl solution in different concentrations [1, 10].

Bacterial strains for Salmonella antimutagenicity assays: *Salmonella typhimurium* strain TA1535 original stock cultures were tested for genetic markers, including sensitivity or resistance to UV radiation (uvrB) and sensitivity to crystal violet (deep rough character, rfa). Strains were routinely characterized for spontaneous reversion characteristics and reversion rates in response to sodium azide or bleomycin TA1535.

Bacterial antimutagenicity assay: For testing of bacteritoxicity of hot water extracts and polysaccharide fraction of intact plant and callus tissue of N. oleander the test extracts and fractions at concentrations from 0.1 μl/per plate to 1000 μl/per plate and 100 μl ($2x10^9$ cell/ml) of the bacterial overnight culture were added into the 2 ml of molten bacterial complete medium, and the mixture was plated on a bacterial complete medium; after incubation at 37°C for 24h, the number of bacterial colonies was scored. Average numbers of colonies and standard errors were calculated.

For testing of mutagenicity of hot water extracts and polysaccharide fraction of intact plant and callus tissue of *N. oleander,* 100 μl bacterial overnight culture, 500 μl of phosphate buffer, and the test extracts and fractions (0.1 μl/per plate to 1000 μl/per plate) were all added directly to 2ml of the histidine/biotin supplemented soft agar for plating. For testing of antimutagenic activity of extracts and fractions of *N.oleander,* 100 μl bacterial overnight culture, a known mutagen (optimal concentration), 500 μl of phosphate buffer, and the test extracts and fractions (0.1 μl/per plate to 1000 μl/per plate) were all added directly to 2ml of the histidine/biotin supplemented soft agar for plating. As a negative control 100 μl of distilled water were used, as a positive control the known mutagens bleomycin (0.5 μg/per plate) and sodium azide (1.5 μg/per plate) were used, which dissolved in 0.9% aqueous NaCl before testing. Initially, the concentrations of these mutagens were tested and optimal concentrations were calculated. When the agar solidified, the plates were inverted and incubated at 37 °C in the dark for 72 h. Numbers of revertant colonies were scored manually. Average numbers of revertant colonies and standard errors were calculated. All experiments were done in duplicate or triplicate. We have accepted an antimutagenicity result as positive if there is a dose–response effect seen, and if the data at a given dose of an antimutagen is significantly lower than seen in experiments without an antimutagen [8].

Results and Discussion. Table 1 illustrates, that hot water extracts and polysaccharide fraction of intact plant and callus tissue of *N. oleander,* aren't toxic for bacterial strains which allows using this test - system for studying of antimutagenic activity of metabolites from intact plant and callus culture of *N.oleander*.

Table 1. Number of colonies *S. typhimurium* TA1535 per plate treated with *N. oleander* intact plant and callus culture hot water extracts and polysaccharide fractions

Tested paterial	Amount μl/per plate					Control
	1000	100	10	1	0.1	
Plant water extract	738.0 ± 16.8	600.0 ± 14.9	802.0 ± 11,.1	804.0 ± 18.8	980.0 ± 14.9	800.0± 12.6
Callus water extract	741.0± 16.8	593.0 ± 14.5	905.0 ± 22.3	907.0 ± 15.6	882.0 ± 24.6	
Plant polysaccharide fraction	845.0± 15.5	912.0 ± 24.1	981.2 ± 17.6	798.0 ± 15.7	811.0 ± 16.7	
Callus polysaccharide fraction	831.0± 12.1	935.0 ± 21,3	887.4 ± 16.5	915.0 ± 25.3	873.0 ± 17.1	

Possible mutagenic activity of *N. oleander* callus culture and intact plant was investigated. Results obtained was shown (Tab.2.) that extracts and fractions of intact plant and callus culture of *N. oleander* in dose range from 0.1 μl/per plate to 1000 μl/ per plate in experiments without metabolic activation were not induce increasing in number revertants of *S.typhimurium* TA1535 in comparison to spontaneous (negative controls) and inducible (positive controls) levels of reversions. Results obtained suggest that studied plants extracts and fractions are not characterized with mutagenic properties.

Table 2. Number of revertant colonies *S. typhimurium* TA1535 per plate treated with f *N.oleander* intact plant and callus culture hot water extracts and polysaccharide fraction

Amounts of extracts μl/per plate	Number of revertant colonies per plate for each variant of experiments			
	Negative control (Spontanous levels)		Negative control (Spontanous levels)	
	14.8±4.0		66.2±3.3	
	Positive control (Sodium azide)		Positive control (Bleomycin)	
	985.4±18.4		1162.4±22.2	
	Plant water extract	Callus water extract	Plant water extract	Callus water extract
1000	16.3±6.8	10.3±2.5	66.3±15.6	93.3±8.6
100	15.3±5.9	11.7±5.1	64.0±16.7	86.3±11.9
10	15.7±5.1	16.7±6.7	62.0±1.7	55.3±3.1
1	19.7±3.8	11.7±2.5	71.7±17.8	61.0±4.4
0.1	16.7±11.,2	18.3±7.4	62.7±9.3	72.3±4.0
	Plant poly-saccharide fraction	Callus poly-saccharide fraction	Plant polysac-charide fraction	Callus poly-saccharide fraction
1000	17.1±5.9	14.1±3.5	59.3±13.4	73.3±5.6
100	14.2±4.9	13.7±5.1	47.0±13.2	79.2±10.6
10	15.5±5.2	16.4±7.7	61.2±2.5	58.2±3.1
1	16.2±3.9	17.1±3.5	63.7±13.5	59.0±3.3
0.1	13.1± 8.2	19.1±6.4	55.2±6.3	65.3±3.0

Table 3 illustrates, that hot water extracts and polysaccharide fraction of *N. oleander* intact plant and callus culture are possesses antimutagenic activity in comparison with positive controls.

Hot water extracts are less active then polysaccharide fractions which suggests that antimutagenic activity caused by components of polysaccharide fraction. Dose-response curves were also shown.

It is known that, bleomycin binds to guanosine–cytosine-rich portions of DNA. In the presence of iron (Fe(II)) and molecular oxygen, this complex can lead to highly reactive free radicals and Fe(III) [3, 11]. The free radical product of bleomycin is able to induce double strand breaks in DNA through oxidation of the deoxyribose sugar backbone, thereby generating chromosomal aberrations [3,11]. Sodium azide is able to generate hydroxyl radicals both extracellularly and intracellularly. The radicals are capable of inducing chromosomal aberrations through oxidative base damage as well as through direct strand breakage in the DNA [2]. As *N. oleander* intact plant and callus tissue polysaccharide fraction components (pectines, D- galacturonic acid etc) are known antioxidants, the possible explanation of their inhibitory effect on the mutagenicity of bleomycin and sodium azide is that they reduce the concentration of mutagenic oxidation products by free radical scavenging [12].

Table 3. Number of revertant colonies of TA1535 per plate in case of studying of antimutagenic activity of *N.oleander* intact plant and callus culture hot water extracts and polysaccharide fractions

Controls and amounts of extracts μ/per plate	Number of revertant colonies per plate for each variant of experiments			
	Negative control (Spontanous levels)		Negative control (Spontanous levels)	
	14.8±4.0		16.2±3.3	
	Positive control (Sodium azide)		Positive control (Bleomycin)	
	1150±82.4		1135.4±92.2	
	Sodium azide + plant water extract	Sodium azide+ callus water extract	Bleomycin + plant water extract	Bleomycin + callus water extract
1000	876.1±15.3	966.7±26.7	869.0±21.7	566.0±29.4
100	546.3±23.3	876.3±22.5	482.7±26.4	795.3±24.6
10	671.1±24.1	851.7±25.1	567.4±25.6	782.6 ±23.9
1	945.6±23.5	735.3±27.4	652.0±26.7	647.3±28.5
0.1	993.6±26.2	854.7±22.5	733.9±27.5	756.4±31.2
	Sodium azide+ plant polysac-charide fraction	Sodium azide+ plant polysac-charide fraction	Bleomycin + plant polysac-charide fraction	Bleomycin + callus polysac-charide fraction
1000	233.1±16.9	244.4±13.6	247.0 ± 14.6	253.4±14.8
100	345.5±15.2	356.3±15.5	351.3±12.8	358.6±13.9
10	443.5±24.3	486.1±17.6	389.4±27.3	427.4±23.3
1	496.6±15.9	527.5±22.6	468.6±23.5	489.0±25.0
0.1	576.6± 23.2	586.6±26.6	583.9 ±19.4	595.3±23.0

By using the Ames test in *Salmonella typhimurium* TA 1535 cells the ability of *N. oleander* callus culture to prevent mutagenicity caused by sodium azide and bleomycin was revealed. *N. oleander* callus tissue retains the antimutagenic effect investigated and specific for *N. oleander* intact plant. These results allow to assume that the protection against sodium azide and bleomycin mediated mutagenicity may be one of the mechanisms by which *N. oleander*

intact plant and callus tissue extracts exert their anticarcinogenic activities. So, *N. oleander* callus culture may be used as an alternative source of biological active substances for the mutagenesis and carcinogenesis prevention. Polysaccharides can be mixed with various pharaceutically acceptable carriers to form injectables, capsules, tablets and various other administrative forms [10].

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