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EXPERIMENTAL ARTICLES

Induction of Oxidative Stress and SOS Response in *Escherichia coli* by Vegetable Extracts: the Role of Hydroperoxides and the Synergistic Effect of Simultaneous Treatment with Cisplatinum

I. V. Manukhov[a], V. Yu. Kotova[a], D. G. Mal'dov[b], A. V. Il'ichev[b], A. P. Bel'kov[b], and G. B. Zavil'gel'skii[a,1]

[a] *State Research Institute for Genetics and Selection of Industrial Microorganisms State Unitary Enterprise, 1st Dorozhnyi pr., 1, Moscow, 117545 Russia*

[b] *Sky-Ltd Medical Center, ul. Kosmonavtov, 18/2/ Moscow, Russia*

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Abstract—Plasmids containing a transcription fusion of *Escherichia coli* PatG, SoxS and recA promoters to the *Photorhabdus luminescens lux* operon (*lux*CDABE) were constructed. The bioluminescence method of assessing oxidative stress and SOS response in *E. coli* cells was applied to test the genotoxicity of cisplatinum and vegetable extracts. Strains MG1655 (pKatG-lux) and MG1655 (pSoxS-lux) were used in the oxidative stress procedure. Strain MG1655(pRecA-lux) was used to test the genotoxicity of the chemicals. All vegetable extracts induced oxidative stress and SOS response. A marked synergistic response was observed when MG1655 (pRecA-lux) cells were exposed to both cisplatinum and vegetable extracts; the level of luminescence measured in the presence of both inducers was much higher than the sum of the levels of luminescence observed with vegetable extracts or cisplatinum alone. The hydroperoxide content in vegetable extracts and in X63-Ag8.6.5.3 myeloma cells was determined. Vegetable extracts were shown to inhibit the HeLa cell growth.

Key words: lux biosensor, *Escherichia coli*, reporter gene, oxidative stress, SOS response, vegetable extract, cisplatinum.

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Extracts from medicinal plants are widely used in folk medicine. Tinctures of aconite, St. John's wort, and rosewort, for example, are used as antitumor agents. In oncology, platinum complexes are used as antitumor preparations; they form cross-links between the DNA purine bases [1, 2]. Platinum compounds, as well as DNA-tropic compounds, induce SOS response in bacterial cells [3, 4].

The goal of the present work was to study the effect on bacterial cells of alcohol vegetable extracts in complex with DNA-tropic compounds. Specific lux biosensors (*Escherichia coli* cells bearing hybrid plasmids with reporter *lux* genes under control of induced promoters) were constructed as the major object of the study. Such biosensors are presently widely used for the study of the mechanisms of toxic effects of various compounds, as well as for environmental monitoring [5–8]. High sensitivity and specificity are the main advantages of lux biosensors. The sensors used in the present work were (i) *E. coli* biosensors bearing pPkatG'::lux and pPsoxS'::lux plasmids used for detection of the oxidative stress induced by hydroperoxide compounds or superoxide ion radical and (ii) *E. coli* biosensor bearing pPrecA'::lux plasmid used to assess the SOS response.

Other issues investigated in this study included the effect of vegetable extracts on HeLa cells and determination of alkyl hydroperoxide content both in the extracts and in tumors and other organs of mice with transplanted X63-Ag8.6.5.3 myeloma in the case of oral administration of the extracts.

MATERIALS AND METHODS

Construction of plasmids and bacterial strains. The DNA fragments containing promoters P_{katG}, P_{soxS}, and P_{recA}, and the corresponding regulatory segments were obtained by PCR amplification of nucleotide sequences from the genome of *E. coli* MG1665 F$^-$*ilvG rfb-50 rph-1* [9] (Table 1). *E. coli* strain TG-1 was used at the first stage of transformation with the hybrid plasmids.

The constructs containing these DNA fragments transcriptionally fused with *Photorhabdus luminescens*

[1] Corresponding author; e-mail: zavilgel@genetika.ru

Table 1. PCR primers

5'–3' sequence	Gene prior to the sequence
GATCTACATCTCTTTAACCAACAA GTGCTCCCCTCTACAGTGTTACCG	*kat*G
CATGCGGGGTAATTTCTTTTCCA GACATAAATCTGCCTCTTTTCA	*sox*S
cacgtggaattcACCGTGATGCGGTGCGTCGTC cacgtgggatccCATGCCGGGTAATACCGGATA	*rec*A

*lux*CDABE reporter genes were created using the pDW201 promoterless multicopy vector with ori_{colE} and the *bla* marker gene (ampicillin resistance) [10].

The PCR fragments were incorporated into the T vector constructed by treating the pUC18 plasmid with *Sma*I restriction endonuclease with subsequent bonding of 3'-ddT to both ends by deoxynucleotidyl transferase.

Transformation of *E. coli* TG-1 cells was carried out by the calcium method according to [11]. The orientation of incorporated fragments was determined by cutting the hybrid plasmids at *Bam*HI–*Eco*RI sites. The DNA fragments containing promoter regions were then excised from the T vector and incorporated into the pDW201 vector. The following hybrid plasmids were thus constructed: pKatG-lux, pSoxS-lux, and pRecA-lux, with *P. luminescens lux*CDABE genes located under control of the corresponding induced promoters. The hybrid plasmids were introduced into *E. coli* K12 MG1665 cells by transformation.

Cultivation. Bacteria were grown to the early exponential phase in Luria–Bertani broth (LB) with ampicillin (100 μg/ml) at 30°C with aeration.

Assessment of the oxidative stress and SOS response. An overnight culture of the lux biosensor was diluted with fresh LB medium to 10^7 cells/ml and grown for 2–3 h at 30°C. Then the samples (200 μl) were transferred to special cuvettes; 4 μl of distilled water was added to the control cuvette, and 4 μl of a vegetable extract or chemical compounds at different concentrations, to the experimental cuvette. The samples were then placed into a luminometer and the levels of bioluminescence were determined at specified time intervals. The samples were incubated at room temperature. The level of bioluminescence was determined according to [12].

Enzymes and chemical compounds. All the chemical reagents were of analytic grade. Hydrogen peroxide was obtained from Ferraine. Mitomycin C, cisplatinum (*cis*-diamminedichloroplatinum, *cis*-$[Pt(NH_3)_2Cl_2]$, paraquat, aconitine, and catalase were obtained from the Sigma Chemical Co. All the test solutions were prepared immediately before the experiment.

Preparation of vegetable extracts. The following medicinal plants were used to prepare the extracts: aconite (*Aconitum karakolicum*), touch-and-heal (*Hypericum perforatum* L.), rosewort (*Rhodiola rosea* L.), live-forever (*Sedum purpureum* L.), Circassian walnut (*Juglans regia*), and licorice (*Glucyrhizae glabra* L.)

Aconite extract (5%) was prepared by covering dry pips with 70% ethanol twice, both times with seven-day incubation. The amount of plant material was 10% (wt/vol). Extracts from live-forever, green skin of Circassian walnut, and rosewort were prepared according to the same procedure, but with single extraction. Touch-and-heal was first covered with boiling water and incubated for 24 h; the water extract was then collected, the wet material was extracted with 70% ethanol, and both extracts were combined. Water extract of rosewort was obtained by extraction with hot water alone.

Determination of alkyl hydroperoxide s by the thiobarbituric method. The products reacting with thiobarbituric acid were revealed in vegetable extracts, as well as in homogenates of liver, spleen, brain and myeloma X63-Ag8.6.5.3. Vegetable extracts (0.2 ml) or tissue homogenates (containing 0.5 mg of protein as determined by the Lowry method) were mixed with 1 ml of the solution containing equal volumes of 0.67% thiobarbituric acid and 30% trichloroacetic acid. After heating for 20 min at 100°C, the samples were centrifuged for 15 min at 5000 *g*. The supernatant absorption at 535 nm was then determined. All the vegetable extracts reacted with thiobarbituric acid. Absorption spectra of the pigments present in the extracts change after heating; aliquots of the extracts heated under the same conditions without thiobarbituric acid were therefore used as controls.

Cell cultures and cell treatment with plant extracts. Human uterine neck adenocarcinoma cells (HeLa) were grown in DMEM medium with 100%

serum at 37°C with 5% CO_2 in the gas phase. For experiments, the trays with 0.5 ml of the incubation medium were inoculated with ~2×10^4 cells and incubated for 24 h. Vegetable extracts were then introduced. After 48-h incubation with the extracts, methyl tetrazolium blue (MTT) was added to the medium. Formazan formation in the mitochondria was determined by measuring OD_{570} of cell lysates. For control, 70% ethanol or distilled water was used for ethanol and water extracts, respectively.

The effect of vegetable extracts on development of transplantable tumors in mice was determined in four groups of male mice of the BALB/c line (1.5 month old, grown in the retainer of the Central Research Institute of Tuberculosis, Russian Academy of Medical Sciences). The culture of X63-Ag8.6.5.3 myeloma cells was injected subcutaneously (4×10^6 cells per mouse). The first group was given orally two portions of aconite extract (5 μl of 5% extract with 180 μg/ml aconitine in 0.2 ml of water) on the 12th and 14th day of the experiment. The second group was given orally four portions of the extract on the 8th, 10th, 12th, and 14th day. The third group received orally six portions of the extract on the 4th, 6th, 8th, 10th, 12, and 14th day. The control group was given four portions of 70% ethanol (5 μl) in 0.2 ml of water. The condition of the animals was monitored daily.

RESULTS AND DISCUSSION

Oxidative Stress

Biosensor *E. coli* MG1665 (pKatG'::lux), a sensitive and specific sensor for hydroperoxide compounds [6], was used to assess the oxidative stress caused by vegetable extracts. Six ethanol extracts from different plant species were used, along with one water extract. Hydrogen peroxide was used as an inductor of oxidative stress (positive control). The capacity of cisplatinum and aconitine to induce oxidative stress was also studied.

All the vegetable extracts used in the present work induced oxidative stress in bacteria. Threshold values of the concentrations causing reliable two- to threefold induction of the PkatG promoter are listed in Table 2. Figure 1 shows the dependence between time and intensity of the bioluminescence of *E. coli* MG1665 (pKatG'::lux) cell suspension for the extracts of aconite and rosewort, two of the most active inducers of oxidative stress.

Preliminary catalase treatment practically eliminated the effect of hydrogen peroxide and significantly decreased the inductive effect of vegetable extracts (Fig. 1). Ethanol extracts from touch-and-heal, live-forever, Circassian walnut, and licorice also induced oxidative stress, albeit to a lower degree (Table 2). The

Table 2. Threshold concentrations of vegetable extracts required for two- to threefold induction of MG1655 (pPkatG'::lux) biosensor

Vegetable extract	Extract concentration in the sample*, %	Degree of induction
Aconitum karakolicum	0.4 ± 0.3	2.2
Sedum purpureum	1.5 ± 0.4	2.6
Rhodiola rosea, ethanol extract	0.4 ± 0.3	2.1
Rhodiola rosea, water extract	2.0 ± 0.6	3.0
Glucyrhizae glabra	0.6 ± 0.3	2.5
Juglans regia	1.5 ± 0.5	2.8
Hypericum perforatum	0.5 ± 0.3	2.0
H_2O_2	5 μM	2.1

* Average of three independent experiments.

Table 3. Alkyl hydroperoxide content of vegetable extracts

Plant species	Alkyl hydroperoxide content (μmol) in the sample (250 μl)
Aconitum karakolicum	51.2
Rhodiola rosea	22.3
Hypericum perforatum	66.0
Glucyrhizae glabra	34.6
Sedum purpureum	3.7
Juglans regia	1.0

DNA-tropic agents, cisplatinum and mitomycin C, did not induce oxidative stress (data not shown).

It should be noticed that both hydrogen peroxide and vegetable extracts also induce the bioluminescence of the lux biosensor with PsoxS promoter. The degree of induction is, however, significantly less than in the case of paraquat, a source of superoxide ion radicals (data not shown).

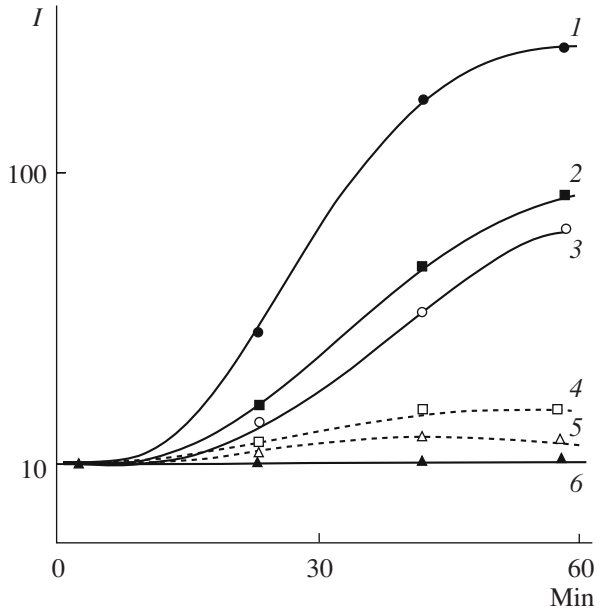

Fig.1. Induction of oxidative stress in *E. coli* MG1655 (PkatG::lux) biosensor cells treated with vegetable extracts. H_2O_2 4.0×1^{-4} M (*1*); *Aconitum karakolicum* (*2*); *Rhodiola rosea* (*3*); *Aconitum karakolicum* and *Rhodiola rosea* extracts after catalase treatment (*4* and *5*); control (10 μl distilled water) (*6*). The X axis shows incubation time at room temperature; the Y axis, bioluminescence intensity (I, μV).

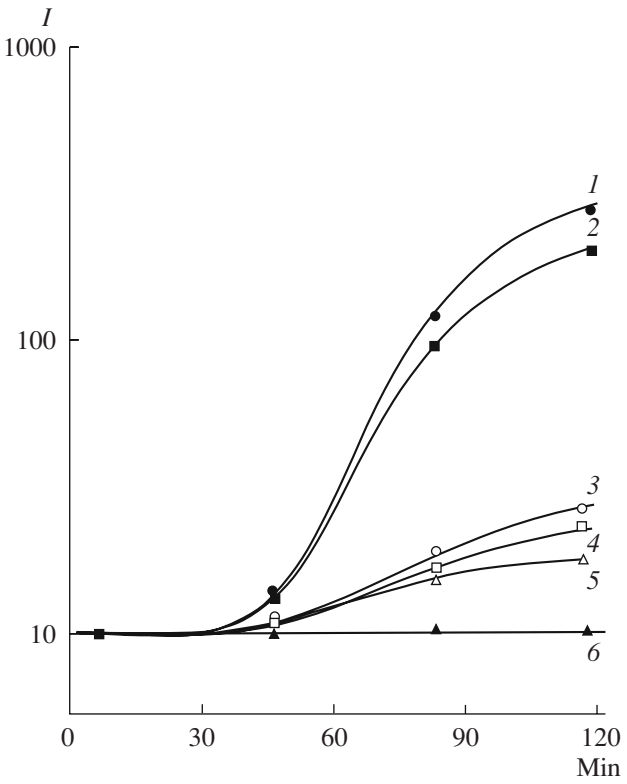

Fig.2. Induction of SOS response in *E. coli* MG1655 (pPrecA'::lux) biosensor cells treated by DNA-tropic agents and vegetable extracts. Mitomycin C (10^{-7} M) (*1*); cisplatinum (10^{-5} M) (*2*); *Aconitum karakolicum* extract (*3*); *Hypericum perforatum* extract (*4*); *Rhodiola rosea* (*5*); control (distilled water) (*6*). The X axis shows incubation time at room temperature; the Y axis, bioluminescence intensity (I, μV).

Alkyl Hydroperoxide Content in Vegetable Extracts

The data on alkyl hydroperoxide content in vegetable extracts are listed in Table 3. Calibration with malonic aldehyde was used as a standard for calculations.

The amount of compound reacting with thiobarbituric acid in the extracts from different plant species varies significantly; it is higher in aconite, touch-and-heal, and rosewort. Thiobarbituric acid reacts with hydrophobic and amphiphilic alkyl hydroperoxide s but does not react with hydrogen peroxide.

Table 4. Formation of alkyl hydroperoxides in mouse liver caused by application of aconite extracts

Number of aconite extract applications	Alkyl hydroperoxide concentration, μM	Standard deviation
0	0.02	±0.002
2	0.044	±0.007
4	0.046	±0.006

Notes: 0 designates control (mice not receiving aconite extract); 2 and 4 designate twofold and fourfold application of the extract, respectively.

In mice with transplanted X63-Ag8.6.5.3 myeloma treated with aconite extract, alkyl hydroperoxides were revealed in the tumor and organs (liver, spleen, and brain); their content increased somewhat with an increased amount of the preparation (Table 4). Alkyl hydroperoxides were not revealed in the organs and tumor of the control group receiving no aconite extract.

SOS Response

To detect bacterial SOS response, MG1655 (pPrecA'::lux) biosensor was used. Cisplatinum and mitomycin C were used as DNA-damaging agents. Capacity of a commercial aconitine preparation to cause SOS response was also studied. Cisplatinum forms monoadducts with the bases of one DNA strand [2], while mitomycin C forms both single-strand monoadducts and cross-links between DNA strands

Table 5. SOS response induction by combined application of cisplatinum and vegetable extracts to MG1655 (pPrecA'::lux) biosensor cells

Extract	–Cisplatinum	+Cisplatinum (7.5 μg/ml)	Expected additive effect
Control	0 (1.4)	6.5	6.5
Hypericum perforatum	0.3 (1.7)	8.0	6.8
Aconitum karakolicum	0.5 (1.9)	11.0	7.0
Rhodiola rosea, ethanol extract	0.3 (1.7)	8.5	6.8
Rhodiola rosea, water extract	0.2 (1.6)	8.2	6.7
Glucyrhizae glabra	0.2 (1.6)	8.5	6.7
Juglans regia	0.3 (1.7)	7.2	6.8
Sedum purpureum	0.5 (1.9)	9.0	7.0
H_2O_2	1.2 (2.6)	12.5	7.7

Note: In columns 2 to 4, the values of bioluminescence (*I*) in mV are given. In column 2, the values in parentheses represent the *I* values; preceding numbers indicate *I* sample–*I* control. The values are averages of at least three independent experiments. Standard deviation of the measurements was ~15%. Column 4 shows the values of SOS response induction calculated with the assumption of additive action of vegetable extracts and DNA-tropic compounds. Bioluminescence values are given in mV (background, 5–10 μV).

[4]. These defects block DNA transcription and replication in the cell.

The minimal (threshold) concentrations resulting in an SOS response level twice as high as in the control (untreated cells) were 1, 200, and 0.003 μg/ml for cisplatinum, aconitine, and mitomycin C, respectively.

Table 6. Inhibition of HeLa cell growth by vegetable extracts

Vegetable extract	Extract concentration in the sample, %*	Degree of inhibition, %
Aconitum karakolicum	2.8 ± 0.3	90
Sedum purpureum	2.0 ± 0.4	30
Rhodiola rosea, ethanol	2.0 ± 0.6	78
Glucyrhizae glabra	0.4 ± 0.2	70
Hypericum perforatum	1.2 ± 0.3	90

* Average values of three independent experiments.

Figure 2 demonstrates the kinetic curves of SOS response of MG1655 (pPrecA'::lux) biosensor treated with DNA-tropic agents, cisplatinum and mitomycin C, as well as with hydrogen peroxide and some ethanol extracts from plants. Although vegetable extracts induce a SOS response of the lux biosensor, it is weaker than in the case of cisplatinum or mitomycin C. The effect of ethanol extracts was noticeable at 2–4% concentration (vol/vol) in the broth with the biosensor cells. The data on SOS response induction by vegetable extracts are presented in Table 5.

Synergistic Effect of Combined Treatment of Lux Biosensor Cells with Vegetable Extracts and DNA-Tropic Agents

The possibility of synergistic induction of oxidative stress and SOS response in combined treatment of lux biosensors with a DNA-tropic agent and a vegetable extract was studied. The preparations of aconitine, cisplatinum, and mitomycin C were tested in combination with each of the six extracts; H_2O_2 was used as a control.

Oxidative stress (induction of PKatG promoter) caused by vegetable extracts was not affected by the

Table 7. Inhibition of HeLa cell growth by aconite extract with cisplatinum

Aconite extract concentration, %	Cisplatinum concentration, μg/ml	Degree of inhibition, %	Expected additive effect
0	0	0	–
0	30.0	30	–
0.32	0	7	–
0.32	30.0	60	35
0.64	0	25	–
0.64	30.0	80	46

Note: Average values of three independent experiments are presented. Standard deviation did not exceed 10--15% of the measured value.

DNA-tropic agents, cisplatinum and mitomycin C (data not shown).

In the case of MG1655 (pPrecA'::lux) biosensor the picture was different. In the combination of cisplatinum and vegetable extracts, a pronounced synergistic effect was observed (Table 5). Addition to MG1655 (pPrecA'::lux) cells of an extract in the concentration inducing a weak (1.5-fold) SOS response together with cisplatinum resulted in increased SOS response induction which significantly exceeded their additive effect. Thus, in the case of combined application of ethanol vegetable extracts (oxidative damage to nucleotides) and cisplatinum (formation of monoadducts), a synergistic effect of DNA-tropic action occurs.

Synergistic SOS response was not observed in the case of combined treatment of MG1655 (pPrecA'::lux) biosensor cells with vegetable extracts and mitomycin C or aconitine (data not shown).

Inhibitory Effect of Vegetable Extracts on Growth of HeLa Cells

Analysis of the effect of vegetable extracts of HeLa growth revealed that all the extracts with significant hydroperoxide content efficiently suppressed cell growth (Table 6). Table 7 lists the result of combined treatment with aconite extract and cisplatinum. A pronounced synergistic effect can be seen: the degree of cell growth inhibition during combined treatment with aconite extract and cisplatinum was approximately twice as high as the additive effect of these agents.

Since cisplatinum is highly toxic not only to bacterial cells but to many types of human tumor cells [1], the synergism of SOS response in combined application of vegetable extracts and cisplatinum is of special interest.

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