Extracts of medical plants suppress the SOS response and reduce mutagenesis in *E. coli*

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Abstract. One of the promising directions in the fight against the emergence and spread of farm animal microbiota resistance factors is the development and search for feed additives that can inhibit the SOS-response. SOS-response is one of the main mechanisms of the occurrence of mutations in bacteria. Plants used in traditional medicine can be a promising source of safe substances that reduce the SOS-response of bacteria. A screening of plants potentially containing substances with anti-SOS activity was performed. During the initial screening, the *E. coli* MG 1655 pRecA-lux biosensor strain with ciprofloxacin as RecA inducer was used. Seven plants were identified whose extracts reduced the expression of the RecA operon. In further experiments on bacteria exposed to antibiotics, we identified four plants whose exstracts significantly reduced the mutagenesis rate of clinical *E. coli* strains: Austrian broom (*Cytisus austriacus*), greater celandine (*Chelidonium majus*), walnut (*Juglans regia*) and smooth sumac (*Rhus glabra*).

1 Introduction

The use of antibiotic drugs in animal husbandry is a serious problem leading to an increase of bacteria antibiotic resistance. There are two ways in which the use of antibiotics in animals contributes to increase of resistance in humans' pathogens. Firstly, the uncontrolled use of antibiotics leads to their accumulation in meat and thereby entering the human body. Persistent presence of sublethal low levels of antibiotics contribute to the development of antibiotic resistance in human microbiota. Secondly, the microbiota of farm animals acquires resistance itself, so resistance genes can be transmitted to the environment, and thus, to humans. To reduce the second pathway of the spread of antibiotic resistance, feed additives should be able to reduce the rate of resistant forms appearance in animal husbandry [1, 2].

One of the promising directions in the fight against the emergence and spread of resistance factors is the development and search for inhibitors of the SOS response, i.e. one of the main mechanisms of the occurrence of mutations in bacteria, which gives them an evolutionary advantage and material for the selection of resistant strains [3]

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In many studies, RecA protein is considered as the main target for inhibition, since it is necessary for DNA repair and other processes, such as the induction of toxin biosynthesis, the synthesis of virulence factors [4], and the induction of hypermutagenesis [5], as well as horizontal gene transfer and biofilm formation [6]. It also participates in recombination processes that enhance the integration of exogenous DNA into the bacterial genome [5].

Alam and others [5] characterized some RecA inhibitors that block antibiotic-induced activation of the SOS response. In one study [7], an effective type 2 inhibitor screening system (preventing ATP hydrolysis by RecA) based on fluorescence analysis was proposed. After the *in vitro* screening of more than 35,000 molecules, authors reported that suramin and suramin-like substances have such activity.

However, a significant obstacle to the use of suramin may be due to its negative charge: suramin may not be able to cross the cell walls of certain bacteria, such as Mycobacteria. It is reported that other compounds that were previously identified as RecA inhibitors are either not cell-permeable or have been found to have a pleiotropic effect on living bacteria.

In a similar screening process [8], computer simulation was used to identify and develop substances with the necessary activity. Using genetic algorithms, the authors presented a method for de novo designing potential inhibitors.

However, the issue of the bioavailability of new inhibitors and safety for eukaryotic cells remains a problem point in these studies. Therefore, the search for substances with the ability to inhibit the SOS response in natural sources, in particular among plant metabolites, looks like a promising direction. A number of studies show that in the plant world such substances are quite widespread [9-12].

The aim of our study was to study the SOS-inhibitory activity of 10 plant extracts, as well as to test a two-component screening system for potential SOS-inhibitors, based on a lux-biosensory test as the first stage. Since it uses the *in vivo* model, it is possible to immediately evaluate the bioavailability and safety of the studied extracts.

Plants selected for study are: Chinese cabbage (*Brassica rapa subsp. Pekinensis*), that is known to produce an active metabolite, synapic acid; greater celandine (*Chelidonium majus*), a source of alkaloids, phenolic compounds and biologically active proteins [13-15], for which an antimutagenic effect was shown [16]; smooth sumac (*Rhus glabra*), a producer of biologically active organic acids and polyphenols [17-19]; walnut (*Juglans regia*), as a source of quinones, phenols and tannins [18, 20, 21]; black mulberry (*Morus nigra*) as a source of anthocyanins, rutin, and other biologically active substances [22]; sweet corn (*Zea mays*) as a source of organic acids, tocopherols and phenolic compounds [23]; European smoketree (*Cotinus coggygria*) - as a producer of phenolic compounds, tannins and flavonoids [24], common basil (*Ocimum basilicum*) - as a source of various essential oils [25]; and two species of *Cytisus* – *C. austriacus* and *C. ruthenicus* - as sources of isoflavonoid compounds and alkaloids [26]. All of these plants grow or are cultivated in the south of the European part of the Russian Federation and easily could be used as raw materials for the production of drugs. These species considered to be sources of biologically active metabolites and are used as medical plants in traditional medicine.

2 Materials and methods

2.1 Preparation of plant extracts

For various raw materials, based on their properties, optimal extraction methods were selected.

1) Ethanol extract (*C. majus*, *Z. mays*, *O. basilicum*, *C. saustriacus*, *C. ruthenicus*): an aqueous solutions of 40%, 50%, and 70% ethanol were used as an extractant.

a) Ethanol extract from dry material: 25 ml of an aqueous ethanol solution was added to 2g of dry grass, chopped with scissors; the mixture was processed on a SonicsVibracells ultrasound machine in a pulsed mode (10 sec. exposure to ultrasound, 10 sec. break) for 40 minutes at 100 W. The resulting extract was filtered through a paper filter.

b) Ethanol extract from fresh raw materials (unripe walnut fruit (*Juglans regia*): 20 ml of 70% ethanol was added to 12 g of ground raw material; treated with ultrasound as described above; filtered through a paper filter.

2) Aqueous extract (*R. glabra*, *C. coggygria*, *J. regia* green pericarp, *C. majus*): 40 ml of distilled water was added to 3 g of homogenized grass, kept in a water bath for 20 min, cooled for 45 min, filtered through paper filter.

3) Raw juice (*B. rapa*, *M.nigra*): raw materials were homogenized; the pulp mass was squeezed through a double layer of gauze and filtered through a paper filter.

2.2 Spectrophotometric study of extracts

UV-Vis spectrometry via Beckman Coulter DU 800 spectrophotometer was performed to evaluate the output of active compounds in preparations.

2.3 Bioluminescent test

We used a slightly modified protocol of the bioluminescent test, described in detail in [27], using *E. coli* strain MG 1655 pRecA-lux, which emits light in response to recA- induction promoter.

A solution of ciprofloxacin (pharmaceutical grade, KRKA, Slovenia) in deionized water at a concentration of 10-4 mg / ml was used as an inducer. For luminescence measurements, an LM-01T microplate luminometer (Immunotech) was used.

The induction factor SOS response (I_s) was calculated by the formula:

$$I_s = Le / Lk - 1 \quad (1)$$

where:

Lk is the luminescence intensity of the control sample (in arbitrary units);

Le – luminescence intensity of the experimental sample (in arbitrary units).

A sign of the statistical significance of the SOS induction effect was considered the statistically significant excess of Le over Lk, estimated by the t-criterion.

The level of SOS-inhibitory activity ($A, \%$) was calculated by the formula:

$$A = (1 - I_a / I_p) \cdot 100\% \quad (2)$$

where: I_a is the induction factor of the SOS response in the presence of an inhibitor.

I_p is the induction factor of the SOS response without an inhibitor.

All experiments were performed in three independent replicates.

2.4 Mutagenesis Assay

Clinical isolate of *Escherichia coli*, strain IP-1, was used as test bacteria. The isolate was kindly provided by Pokudina I. O., Laboratory of Biomedicine, Southern Federal University and was isolated from a patient with dysbiosis.

A Rif^S→Rif^R model was used to estimate the effect of plant extracts on the level of induced mutagenesis.

An overnight test culture was grown in LB liquid medium at a temperature of 37 ° C for 18-20 hours. The following samples were used: (1) control (2) plant extract (in a range of

10-fold dilution, see Table 1); (3) Ciprofloxacin at a concentration of 0.1 µg / ml; (4) Ciprofloxacin at a concentration of 0.1 µg / ml + test extract. The concentration of 0.1 µg/ml of ciprofloxacin was previously shown to induce SOS response in *E.coli* [28].
100 µl of the test culture was plated on agarized LB plates without rifampicin and with 50 µg/ml rifampicin in four replicates. Colony counts were performed after 48 hours.
Survival was calculated by the formula:

$$Survival, \% = N_{sample}/N_{control} \cdot 100\%$$

(3)

where Nsample is the number of colonies after incubation with an inductor, Ncontrol is the number of colonies after incubation without an inductor.
The mutant frequency was calculated by the formula:

$$Frequency = Na/Nw$$

(4)

where Na is the number of colonies in the medium with antibiotic, Nw is the number of colonies in the medium without antibiotic.
The reliability of the mutagenic effect was evaluated by the statistical significance of the differences (t-test p <0.05) by the number of colonies between experiment and control.

3 Results

3.1 Screening of plant extracts containing substances that suppress ciprofloxacin-induced SOS response in E. coli strains

Table 1 presents data on the SOS-inhibitory properties of the studied extracts.

Table 1. SOS-inhibitory activity of plant extracts, %.

Source	Volume fraction of extract in solution,%						
	0.0000 1	0.0001	0.001	0.01	0.1	1	10
<i>C.coggygria</i> , aqueous extract of leaves	0	0	0	0	5.02	43.53	45.07
<i>J.regia</i> , green pericarp extracted with 70% ethanol	Bacteri cidal	Bacteri cidal	Bacteri cidal	Bacteri cidal	Bacteri cidal	Bacteri cidal	Bacteri cidal
<i>J.regia</i> , aqueous extract of green pericarp	74.72	72.02	74.7	78.3	76.9	Bacteri cidal	Bacteri cidal
<i>C.austriacus</i> 50% ethanol extract	62.36	84.9	70.9	67.4	55.48	51.9	67.3
<i>C.ruthenicus</i> 50% ethanol extract	Bacteri cidal	Bacteri cidal	Bacteri cidal	Bacteri cidal	Bacteri cidal	Bacteri cidal	Bacteri cidal
<i>C.majus</i> aqueous extract	0	0	0	0	19.09	15.16	19.71
<i>C.majus</i> 40% ethanol extract	46.2	43.3	32.5	50.5	57.63	48.98	86.25
<i>M.nigra</i> , raw juice	Bacteri cidal	Bacteri cidal	Bacteri cidal	Bacteri cidal	Bacteri cidal	Bacteri cidal	Bacteri cidal
<i>Z.mays</i> 40% ethanol extract of leaves	59.39	47.42	44.29	35.47	44.47	Bacteri cidal	Bacteri cidal
<i>B.rapa</i> subsp. <i>pekinensis</i> raw juice	72.40	75.02	72.56	75.60	81.44	Bacteri cidal	Bacteri cidal
<i>R.glabra</i> aqueous extract	78.29*	81.53*	82.8	81.9*	83.67	90.16	100.0 0

<i>R. glabra</i> 50% ethanol extract	Bacteri cidal	Bacteri cidal	Bacteri cidal	Bacteri cidal	Bacteri cidal	Bacteri cidal	Bacter icidal
<i>O. basilicum</i> aqueous extract	0	26.83	30.37	31.75	16.58	30.00	Bacter icidal

* - although the induction of the SOS response was reduced, in these several cases, the extract itself caused the SOS response, so its effect cannot be considered as SOS-inhibitory.

As a result of the first stage of screening, it was found that seven extracts show SOS-inhibitory activity. *B. rapa* subsp. *pekinensis* raw juice (average value of inhibitory activity 75.40%) and aqueous extract of *J. regia* green pericarp (average value 75.33%) have the highest levels of protective activity, and aqueous extract of *O. basilicum* is the weakest inhibitor (average value 27.1%). Some of the extracts were bactericidal (toxic to bacterial cells) in highest concentrations, and four of the thirteen extracts were bactericidal even in a 0.00001-fold dilution.

Based on these data, we selected sources and concentrations suitable for studying the effect of extracts on mutagenesis.

3.2 Assessing the ability of plant extracts to inhibit the development of antibiotic resistance in clinical isolates of bacteria

The parameters of the spontaneous and induced mutation rates of antibiotic resistance under the action of extracts of medicinal plants on clinical isolates of Escherichia coli are presented in table 2.

Table 2. RifS→RifR mutation frequencies in *E.coli* IP-1.

	Rif- medium		Rif+ medium	
	CFU/ml	Survival rate, %	CFU/ml	RifR mutation frequencies
<i>B. rapa</i> subsp. <i>pekinensis</i> raw juice, 10% of extract in solution				
Control	32.3±3.4·10 ⁶	100.00	4.3±1.5	1.33·10 ⁻⁵
Plant extract	11.2±1.1·10 ⁶	34.67*	1.2±0.5	1.07·10 ⁻⁵
Ciprofloxacin added	7.9±2.1·10 ⁶	24.46*	2.0±0.8	2.53·10 ⁻⁵ *
Plant extract and ciprofloxacin added	1.8±1.2·10 ⁶	5.57*	0.6±0.3	3.33·10 ⁻⁵ *
<i>B. rapa</i> subsp. <i>pekinensis</i> , 0.1% of extract in solution				
Control	34.4±4.4·10 ⁶	100.00	3.9±1.0	1.13·10 ⁻⁵
Plant extract	35.8±3.7·10 ⁶	104.07	4.2±1.2	1.17·10 ⁻⁵
Ciprofloxacin added	8.9±3.1·10 ⁶	25.87*	2.4±0.8	2.70·10 ⁻⁵ *
Plant extract and ciprofloxacin added	8.6±3.0·10 ⁶	25.00*	2.1±0.7	2.44·10 ⁻⁵ *
<i>C.austriacus</i> , 10% of extract in solution				
Control	38.0±3.5·10 ⁶	100.00	7.1±2.4	1.87·10 ⁻⁵
Plant extract	9.3±2.2·10 ⁶	24.47*	1.9±2.3	2.04·10 ⁻⁵
Ciprofloxacin added	7.0±2.3·10 ⁶	18.42*	3.4±1.2	4.86·10 ⁻⁵ *
Plant extract and ciprofloxacin added	1.6±0.9·10 ⁶	4.21*	0.4±0.1	2.50·10 ⁻⁵ *
<i>C.austriacus</i> , 0,1% of extract in solution				
Control	34.3±2.6·10 ⁶	100.00	6.1±1.9	1.78·10 ⁻⁵
Plant extract	32.0±2.7·10 ⁶	93.29	5.4±1.4	1.69·10 ⁻⁵
Ciprofloxacin added	6.6±1.2·10 ⁶	19.24*	3.9±1.1	5.91·10 ⁻⁵ *
Plant extract and ciprofloxacin added	5.4±1.2·10 ⁶	15.74*	1.2±0.9	2.22·10 ⁻⁵ *
<i>Z.mays</i> , 0,1% of extract in solution				
Control	36.2±3.4·10 ⁶	100.00	5.4±1.0	1.49·10 ⁻⁵

Plant extract	29.2±3.2·10 ⁶	80.66*	5.8±1.7	1.99·10 ⁻⁵
Ciprofloxacin added	9.1±1.3·10 ⁶	25.14*	2.6±0.9	2.86·10 ⁻⁵ *
Plant extract and ciprofloxacin added	6.8±0.9·10 ⁶	18.78*	2.2±1.2	3.24·10 ⁻⁵ *
<i>C. majus</i> 40% ethanol extract, 0,1% of extract in solution				
Control	38.0±3.1·10 ⁶	100.00	5.6±2.1	1.47·10 ⁻⁵
Plant extract	40.9±3.2·10 ⁶	107.63	5.2±0.9	1.27·10 ⁻⁵
Ciprofloxacin added	6.5±1.2·10 ⁶	17.11*	3.1±0.8	4.77·10 ⁻⁵ *
Plant extract and ciprofloxacin added	6.8±1.3·10 ⁶	17.89*	1.4±0.6	2.06·10 ⁻⁵ *
<i>J.regia</i> , aqueous extract of green pericarp, 0,1% of extract in solution				
Control	31.2±2.4·10 ⁶	100.00	3.8±1.4	1.22·10 ⁻⁵
Plant extract	28.4±2.6·10 ⁶	91.03	3.6±2.0	1.27·10 ⁻⁵
Ciprofloxacin added	6.0±1.1·10 ⁶	19.23*	3.7±1.2	6.17·10 ⁻⁵ *
Plant extract and ciprofloxacin added	5.5±0.8·10 ⁶	17.63*	2.5±0.5	4.55·10 ⁻⁵ *
<i>R.glabra</i> aqueous extract, 0,1% of extract in solution				
Control	37.6±3.8·10 ⁶	100.00	4.7±1.3	1.25·10 ⁻⁵
Plant extract	31.5±3.4·10 ⁶	83.78	4.0±1.1	1.27·10 ⁻⁵
Ciprofloxacin added	7.8±1.1·10 ⁶	20.74*	3.9±0.9	5.00·10 ⁻⁵ *
Plant extract and ciprofloxacin added	6.5±0.9·10 ⁶	17.29*	1.4±0.4	2.15·10 ⁻⁵ *
<i>O. basilicum</i> , 0,1% of extract in solution				
Control	35.4±3.8·10 ⁶	100.00	6.5±2.0	1.84·10 ⁻⁵
Plant extract	36.1±2.0·10 ⁶	101.98	6.8±0.6	1.88·10 ⁻⁵
Ciprofloxacin added	8.4±0.7·10 ⁶	23.73*	4.4±1.1	5.24·10 ⁻⁵ *
Plant extract and ciprofloxacin added	8.5±1.5·10 ⁶	24.01*	4.9±0.9	5.76·10 ⁻⁵ *

* Statistically significant differences from control, p <0.05.

The frequency of spontaneous mutagenesis was 1,22-1,87·10⁻⁵.

Ciprofloxacin increased the number of rifampicin-resistant mutants, and the frequency of induced mutagenesis in a series of experiments increased 2.5-4 times above spontaneous mutagenesis. This is consistent with the data obtained earlier [28].

Extracts of *B. rapa* and *C.austriacus* at a concentration of 10% led to a significant decrease in bacterial survival (35% and 25% of cells survived, respectively). This, together with the previously mentioned luminescence drop in a biosensor test, indicates the bactericidal properties of plant extracts at a given concentration. On the other hand, this led to a significant drop in the survival rate in the experiment, which makes the experimental results difficult to interpret.

Therefore, in further studies, we used an extract concentration of 0.1% for all plants to unify the results. At this concentration, the survival of the studied bacteria did not differ significantly from the control.

According to the results, extracts of *C. majus*, *C. austriacus*, *J.regia*, *R.glabra* were able to reduce the amount of RifR mutations in *E.coli* IP-1 isolate.

3.3 Spectrophotometric study

The results of a spectrophotometric study of the extracts showed rather similar patterns. A typical spectrum is shown in Fig. 1-2.

Fig. 1. Light absorption by an extract of *C. majus* in the wavelength range of 200-800.

Fig. 2. Light absorption by an extract of *C. majus* in the wavelength range of 560-800 (different scale).

The figures show the absorption curves of the extract of *C. majus* in the wavelength range 200–800 nm. In the ultraviolet region, several absorption bands and peaks of 220, 270, 330 nm are observed. The curve also shows several peaks of maximum absorption in the visible light range (610, 670 nm).

In the absorption spectrum of the *C.austriacus*, there are three maxima similar to the previous ones (maxima at 210, 270, 340 nm) and a maximum in the visible region at 665 nm.

R.glabra extract have a maximum at 265 nm in the ultraviolet region and an absorption band at 200-220 nm. In the visible light spectrum, maxima of 610 and 670 nm are observed.

The absorption spectrum of *J.regia* extract in the visible spectrum is difficult to analyze due to the presence of dark pigments. In the ultraviolet spectrum, an absorption band at 203 nm and a number of small peaks are observed.

4 Discussion

Evidence that plants are capable of secreting metabolites with antimutagenic and SOS inhibitory activity has been appearing for some time [9-12]. This may be due to the long co-evolution of bacteria and plants that produce phytoncides as agents of antagonistic interactions with bacteria. Bacteria adapted to the action of phytoncides, but did it slower if exposed to SOS inhibitors.

As inhibitors of the SOS response, substances such as saponins, polyphenols, terpenoids, isoflavonoids, indoles, etc. can be considered [29, 30].

In our study, plants with a wide range of metabolites-potential inhibitors of the SOS response, were used. Seven of ten plant extracts demonstrated the ability to reduce SOS response induced by ciprofloxacin. The remaining four extracts were toxic to bacterial cells, therefore, they can be used more likely as antibacterial agents.

After the primary screening on biosensors, we tested their effect directly on the model of ciprofloxacin-induced resistance to rifampicin. Such processes of cross-induced resistance, as it were shown before [31], might be the part of a global problem of antibiotic resistance. The proposed test system allows one to immediately determine the potential of the studied inhibitor in preventing the adaptation of bacteria to antimicrobial agents.

The UV-Vis spectra of the studied extracts in most cases contained peaks in the ultraviolet region up to 220 nm, corresponding, according to the literature, to phenolic compounds; a peak in the region of 265-270 nm, corresponding to the absorption bands of catechins and tannins of the catechin group [32, 33], as well as flavonoids [33]; and a peak at 330-350 nm corresponding to flavones/flavonoles [34, 35].

There were also mild peaks on 650-670 nm that may indicate the presence of aromatic ketones and amines (Berger, Sicker et al., 2009). All of these compounds may exhibit antimutagenic and SOS inhibitory properties.

It was shown that of *C. majus*, *C. austriacus*, *J.regia*, *R.glabra* extracts significantly reduced the frequency of *E. coli* induced mutagenesis, although none of these extracts showed a 100% reduction. The extracts of *B. rapa* subsp. *pekinensis*, *Z. mays* and *O. basilicum* did not show significant changes in the frequencies of induced mutagenesis, although they showed a decrease in the SOS response in bioluminescent biosensor tests. As far as the process of appearance and fixation of mutation in bacterial genome is a complex multistage system [37], it is influenced by many factors, and the level of mutagenesis is connected with, but is not equal to the intensity of the SOS response. That is why the two-component screening system is necessary to effectively assess the potential of the studied preparations.

5 Conclusions

A screening of plants potentially containing substances with SOS-inhibitory activity was performed. During the initial screening using the biosensor strain *E. coli* MG 1655 pRecA-lux, seven plant extracts with the ability to reduce the expression of the RecA gene were selected. On the induced mutagenesis model, extracts of *C. majus*, *C. austriacus*, *J.regia*, *R.glabra* significantly reduced the mutagenesis frequency in *E. coli*. These plants can be considered as sources of metabolites with SOS-inhibitive ability: phenolic compounds, catechins and tannins, flavonoids, flavonoles, aromatic ketones and amines. The two-

component test system allows to evaluate the bioavailability and safety or potential toxicity of extracts. These plants could be used in the development of preparations for adjuvant therapy in the treatment of antibiotics, which will slow down the development of resistance to them.

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