Environmental and biological assessment of plant extracts in Rosaceae family as promising feed components

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Abstract. Today, the whole world is facing the problem of antibiotic resistance, including in agricultural production, affecting its efficiency. This fact determined the search for new means of combating bacterial pathogens by affecting the bacterial population through the regulation of density-dependent chemical communication. Medicinal plants can be used as such “regulators”, as feed components. This paper examines toxicological and other properties of Prunus padus fruit and Sanguisorba officinalis herb extracts as likely components of animal feed. The testing of P. padus fruit extract on Escherichia coli K12 MG1655 (pXen7) strain showed the inhibitory action on the microorganism cells of all concentrations of the studied substance. The EC$_{20}$ was 0.625 mg/ml. the S. officinalis herb extract showed a pronounced antibacterial effect on E. coli K12 MG1655 (pXen7). P. padus fruits had the maximum toxicological effect on Stylonychia mytilus population at the concentration of 10.0 mg/ml throughout the incubation period, the effect of S. officinalis herb extract was evident at 2.5; 5.0 and 10.0 mg/ml. Both substances in subinhibitory concentrations suppressed the quorum-dependent biosynthesis of violacein pigment (for P. padus fruits the EC$_{100}$=8.05 mg/ml, for S. officinalis grass EC$_{100}$=0.22 mg/ml).

1. Introduction
The multiple drug resistance of bacterial pathogens is a major public health problem that the whole world is facing these days. This is largely due to the use of antibiotics in livestock and the efficiency of agricultural production in general. In this regard, a large number of studies are devoted to the search for alternative methods of infectious diseases prevention with conventional antibacterial agents aimed at inhibiting the pathogen growth [1]. This alternative includes the phenomenon known as the “quorum sensing”. Quorum sensing is a density-dependent chemical communication in bacteria whereby molecular genetic mechanisms in a bacterial cell are regulated [2]. This approach can be quite effective because many pathogens use quorum-sensing to realize their pathogenic potential [3]. Modern science has a large amount of data on the ability of medicinal plants to inhibit quorum-dependent processes in bacteria [4]. Plant metabolites can be a valuable tool for the treatment and prevention of animal infections [5, 6]. The ability of the digestive system of ruminants to control bacteria is known by inactivation of N-acylhomoserin lactones regulating the “quorum sensing” and activation of the enzyme system under the action of plant extracts [7, 8]. However, the presence or absence of toxic properties in these plants that appear when used in the creation and use of feed additives remains to be seen. In this regard, the purpose of this study was to analyze impact of the
habitat on activity of Prunus padus fruit and Sanguisorba officinalis herb extracts.

2. Materials and methods

2.1. Medicinal plants

The objects of the study were dry extracts of medicinal plants of Rosaceae family – Prunus padus fruits and Sanguisorba officinalis herbs. The methodology of obtaining these extracts includes the following: 1) mixing 6 g of dry matter with 50 ml of hot sterile distilled water; 2) boiling in water bath for 15 minutes; 3) cooling at room temperature for 45 minutes; 4) centrifugation at 1000 rpm for 10 min to remove solid particles; 5) filtering through a polyether sulfone syringe filter (Members Solutions LLC, USA) with a pore size of 0.4 μm; 6) drying the obtained extracts at 60 °C. For biological analyses, the samples thus obtained were dissolved in sterile distilled water.

2.2. Bacterial strains and protista

The recombinant luminescing strain Escherichia coli K12 MG1655 (pXen7) received at the transformation of cells of a host strain by hybrid plasmid pUC18 with the built-in EcoRI DNA fragment with the size of about 7 thousand n.p. containing structural genes of bioluminescence of soil microorganism Photorhabdus luminescens ZM1 was used to study the toxic effect of dry extracts [9].

In order to determine the toxicological effect of dry extracts of medicinal plants in addition to the bacterial strain E. coli K12 MG1655 (pXen7) the study was carried out on a hydrobiont model – representative of protozoa ciliates Stylonychia mytilus. This protist was exposed to the studied substances at the concentrations from 0.019 to 10.0 mg/ml (tenfold dilution from 10.0 mg/ml) for 1, 6, 12 and 24 hours.

To assess antibacterial and quorum-regulating activities of dry extracts a wild strain Chromobacterium violaceum ATCC 31532 with the two-component system of CviI/CviR autoinduction with a growth on nutrient mediums realizing the “quorum sensing” effect followed by the formation and accumulation of a blue-violet violacein pigment in bacterial cells with an absorption maximum at 590 nanometers was used as a tester strain [10].

2.3. Study of toxicological activity

E. coli K12 MG1655 (pXen7) was cultured for 24 hours on LB agar (Sigma, USA) at 37 °C, 100 μg/ml ampicillin serving a selective factor was added to the medium. The cells were then suspended in 0.9 % NaCl solution to an optical density of 0.5 RU at 450 nm, at the same time the measurements were carried out in plastic transparent wells on AIFR-01 UNIPLAN (ZAO PICON, Russia). Thereafter, 500 μl of final bacterial suspension was added to 1000 μl of LB broth without further rooting.

Tenfold dilutions of dry plant extracts at a final concentration of 10.0 mg/ml to 0.0097 mg/ml were prepared to study the effects of the analyzed substances in white opaque plate wells. Thereafter, diluted extract solutions in a volume of 50 μl and 50 μl of water were added to the test wells, and 50 μl of the prepared strain suspension and 100 μl of LB broth were added to all samples.

The plate was then placed in the measuring unit of the Infinite 200 Pro luminometer (Tecan, Austria). The measurements were done in kinetic mode for 120 minutes at 37 °C. The results were first processed using Magellan™ Luminometer software, and further processing of data was performed using Excel 2010 (Microsoft Inc.).

The diagrams of strain luminescence kinetics were built on the basis of the obtained values, at the same time the time of the experiment was fixed along X axis in minutes, while the intensity of luminescence was marked along Y axis (RU). The primary luminescence values were normalized against the control. Normalization allowed avoiding the nature of curve in the control sample and directly assessing the effect of the substance sampled. The calculation was carried out according to the following formula:

\[ I_{\text{norm}} = \frac{I_{\text{exp}}^{60} \times I_{\text{kontrol}}^{0}}{I_{\text{exp}}^{0} \times I_{\text{kontrol}}^{60}}, \]
where $I_{60}^{\text{exp}}$ – luminescence index in the test sample at 60 minutes of the experiment; $I_{0}^{\text{exp}}$ – luminescence index in the test sample at 0 min of the experiment; $I_{60}^{\text{control}}$ – luminescence index in the control sample at 60 minutes of the experiment; $I_{0}^{\text{control}}$ – luminescence index in the control sample at 60 minutes of the experiment.

The second test object for determining the toxicological activity of the test extracts was the culture of *S. mytilus* freshwater infusory cells (wild strain) in the exponential growth phase. The studied test functions included: survival rate, number (biomass). The initial culture of *S. mytilus* cells was cultured on Lozin-Lozinsky medium with the addition of yeast (Saccharomyces cerevisiae) of the nutrient medium: NaCl-0.1 %; KCl-0.01 %; CaCl$_2$-0.01 %; MgCl$_2$-0.01 %; NaHCO$_3$-0.02 %.

The sensitivity of the culture of *S. mytilus* cells to the toxicant action was determined by the time of their death recorded by stopping the movement of the protozoa, which was accompanied by the damage of integrity and lysosomes of the cell. The number of cells in 5 ml of medium containing intact infusion culture (without substances) was monitored in all experiments. The total number of cells in 5 ml of infusoria medium was calculated using a light microscope (MT 5300L). The cells taken in the stationary growth phase were incubated at 20±2 °C in the medium with addition of substances for 24 hours.

2.4. Study of antibacterial and anti-quorum activity

In the experimental series aimed at characterizing the antibacterial and anti-quorum activity of each substance used, the double dilutions of dry extracts (in the concentration range of 10.0 mg/ml to 0.0097 mg/ml) in distilled water were prepared in the cells of a 96-well plate from transparent plastic, which were mixed with the equal volumes of LB broth (Sigma, USA). Then, 20 μl of a daily culture *C. violaceum* ATCC 31532 was added to each well, and further sprouted at 27 °C for 24 hours. Each study included additional LB broth samples containing no test substances and used as positive (test strain growth) and negative (sterile) controls. The results of the experiment were assessed using the multifunctional microplate reader Infinite 200 Pro (Tecan, Austria), sequentially recording the optical density of the bacterial biomass at 450±5 nm, and the quantitative presence of violacein pigment after its ethanol extraction at 600±5 nm. The negative control absorption values were subtracted. The antibacterial effect was expressed by MIC$_{100}$ and MIC$_{50}$ – minimal inhibiting concentrations causing 100 and 50 % inhibition of test strain growth relative to the positive control. In turn, the rate of suppression of the “quorum sensing” system was expressed by EC$_{100}$ and EC$_{50}$ values corresponding to similar rates of inhibition of violacein pigment biosynthesis.

2.5. Statistical processing of the study results.

All experiments were performed in at least five repetitions. The results were processed by variation statistics in Excel for Windows 10.

3. Results and discussion

3.1. Analysis of effects of *P. padus* fruit and *S. officinalis* herb extracts on recombinant luminescent strain *E. coli* K12 MG1655 (pXen7)

The testing of *P. padus* fruit extract on recombinant luminescent strain *E. coli* K12 MG1655 (pXen7) showed that almost all concentrations of the test substance had an inhibitory effect on the cells of the microorganism. Thus, at a maximum concentration of 10.0 mg/ml, 40% suppression of the luminescence level was observed, with an increase in the dilution degree, the bioluminescence level increased, which indicated a decrease in toxic effect. At the concentration from 5.0 mg/ml to 1.25 mg/ml, 20 % of test strain cells were killed, and a further decrease in the concentration showed no more than 10 % inhibition of the biosensor.

The analysis of the time factor on the substance-cell system revealed that the action of a substance occurred during the first 15 minutes (a given time sufficient to activate lux-operon) and continued throughout the accounting period (180 minutes).

The calculation of the EC$_{50}$ index did not reveal the concentration causing 50 % cell death, but it can be assumed that an increase in the concentration above 10.0 mg/ml would reveal this parameter. The EC$_{20}$ value was 0.625 mg/ml.
The analysis of bioluminescent testing of *S. officinalis* herb extract showed a pronounced antibacterial effect on *E. coli* K12 MG1655 (pXen7). Thus, at high concentrations of 10.0 mg/ml to 1.25 mg/ml, a suppression of the biosensor glow from 80 to 60 % was observed. A further decrease in the concentration of the analyzed substance was characterized by a gradual reduction in luminescence. Thus, the apparent dose-dependent effect of *S. officinalis* extract can be noted. With respect to the time dependence of the effect, it should be noted that inhibition develops during the first 60 minutes and continues until the end of the experiment.

The calculation of the EC_{50} parameter showed 50 % cell death at the concentration of 0.625 mg/ml. With increased dilution, the number of surviving cells increased, and at the minimum concentration of 0.0097 mg/ml, the percentage of cells reached the original level.

3.2. Assessment of biological effects of *P. padus* fruit and *S. officinalis* herb extracts on *S. mytilus* cell population

The study of the effect of the dry extract of *P. padus* fruit on *S. mytilus* population revealed that the maximum toxicological effect was observed at the concentration of 10.0 mg/ml throughout the incubation period. At the concentration of 5.0 mg/ml of extract, 50 % survival of the test object was established. The high survival rate of *S. mytilus* cells was observed for a time period from 1 to 24 hours in the concentration range from 0.019 to 2.5 mg/ml (Figure 1a).

The assessment of the concentration ranges of the *S. officinalis* herb extract, in which the effect of inhibition of the test object growth was observed, showed that the effect of the analyzed extract was exhibited at 2.5; 5.0 and 10.0 mg/ml at all stages of *S. mytilus* incubation, which corresponds to 20–39 % reduction in the biomass of the protist cell population. The exception was the first hour of incubation at 2.5 mg/ml extract, where 50 % survival is noted. The maximum activity level of *S. mytilus* cells was recorded at the concentrations from 0.019 to 0.625 mg/ml over the entire time range from 1 to 24 hours of cultivation (Figure 1b).

**Figure 1.** Effects of different concentrations of plant extracts on the biomass of *S. mytilus* cell population: a – *P. padus* fruits; b – *S. officinalis* herb

3.3. Analysis of the effect of dry extracts of *P. padus* fruit and *S. officinalis* herb on growth and quorum-dependent biosynthesis of violacein in wild strain *C. violaceum* ATCC 31532

The cultivation of *C. violaceum* ATCC 31532 in a liquid nutrient medium in the presence of a wide range of concentrations of test extracts followed by the assessment of the optical density (OD_{450}) of the grown population and pigment formation (OD_{610}) allowed assessing their effect on growth and quorum-dependent biosynthesis of violacein pigment (Table 1).

The results showed low growth-inhibitory activity of the test extracts, as evidenced by the values of MIC_{100}=10.0 mg/ml for *P. padus* fruit and unattainable for *S. officinalis* herb. At the same time, it was found that both test compounds in subinhibitor concentrations suppressed quorum-dependent biosynthesis of violacein pigment, but the effect was different. Thus, for *P. padus* fruits, the EC_{100} value was 8.05 mg/ml, and for *S. officinalis*, the EC_{100} reached 0.22 mg/ml. The MIC_{50}/EC_{50} ratio most fully characterizing the concentration range of plant extracts causing inhibition of quorum-dependent biosynthesis of violacein pigment in the absence of growth-inhibiting effect made 1.3 for *P. padus* fruit and 2 for *S. officinalis* herb.
Table 1. Concentrations of *P. padus* fruit and *S. officinalis* herb extracts (mg/ml) causing growth suppression and quorum-dependent violacein pigment biosynthesis in *C. violaceum* ATCC 31532

| Dry extracts of medicinal plants | Antibacterial activity, mg/ml | Quorum-dependent activity, mg/ml |
|----------------------------------|-------------------------------|----------------------------------|
|                                  | MIC<sub>100</sub> | MIC<sub>50</sub> | EC<sub>100</sub> | EC<sub>50</sub> |
| *P. padus* (fruits)             | 10.0             | 8.05         | 10.0          | 7.5          |
| *S. officinalis* (herb)         | –                | 0.22         | 0.08          | 0.04         |

The treatment with popular agents created on the basis of medicinal plants as an alternative to traditionally used pharmaceutical preparations has been known since time immemorial [11]. Today, the interest in these drugs is primarily related to the growing threat of antibiotic resistance and the search for an alternative to antibiotics. However, despite the popularity and widespread use of medicinal plants, the safety of these treatments has recently come into question due to available data on complications such as hepatotoxicity [12] and nephrotoxicity [13, 14] caused by the administration of plant agents, as well as fatal consequences [15, 16].

The studies demonstrated the presence of toxicological, antibacterial and anti-quorum properties in the studied extracts of medicinal plants with only a small difference in the effective concentrations depending on the choice of the test object. Thus, in the study of *P. padus* fruit and *S. officinalis* herb extracts on the recombinant luminescent strain *E. coli* K12 MG1655 (pXen7) and the prostatic *S. mytilus*, the highest level of toxicity was at 10.0 mg/ml for *P. padus* and from 10.0 to 1.25 mg/ml for *S. officinalis*, with its subsequent reduction as the concentration decreased.

In turn, the testing of drug extracts on violacein-producing *C. violaceum* ATCC 31532 strain made it possible to conclude that the antibacterial activity of *P. padus* fruits had a similar effect with the results obtained at other test objects, and in contrast, had a difference in activity for *S. officinalis* herb, which MIC<sub>100</sub> was not achieved in this concentration range.

Against this background, both extracts demonstrated anti-quorum activity against *C. violaceum* ATCC 31532, the highest for *S. officinalis* herb. The obtained result is well consistent with the data described for another herbaceous plant in Acanthaceae family with respect to *Pseudomonas aeruginosa* [17]. A similar effect is also known for *Acacia hockii* of Fabaceae family [18].

4. Conclusion
The obtained results confirm and supplement already available data on antibacterial, antioxidant activities of *P. padus* [19, 20] and *S. officinalis* [21, 22], and significantly expand the perception of their use as “sensing quorum” inhibitors [23]. However, it should be noted that the data identified cannot yet serve as a recommendation for the use of *P. padus* fruit and *S. officinalis* herb extracts as likely components of animal feed and require further study.

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Conflict of Interest: The authors declare that they have no conflict of interest.

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