Toxicological, antibacterial and anti-quorum activity of extracts of medicinal plants Betula spp., Hypericum spp. and Angelica spp.

K S Inchagova, D B Kosyan, E A Rusakova and G K Duskaev

Federal Research Centre of Biological Systems and Agrotechnologies RAS, 29, 9 Yanvarya St., Orenburg 460000, Russia

1 E-mail: gduskaev@mail.ru

Abstract. Quorum sensing plays an important role in the infectious process development in plants, animals and humans. Inhibiting this phenomenon may be a new way to treat bacterial infections in therapeutic practice. Similar studies are being conducted all over the world and several approaches to inhibiting quorum-dependent processes are currently being presented. Folk remedies, which include, in particular, medicinal plants, can become an alternative tool for fighting bacterial pathogens to traditionally used antibacterial substances that have lost their former effectiveness, due to the widespread spread of antibiotic resistance. In this work, the toxicological, antibacterial and anti-quorum properties of the extracts of Betula spp. leaf, Hypericum spp. herb and Angelica spp. root were studied. Testing the extracts of Betula spp. leaf, Hypericum spp. herb and Angelica spp. root showed a minor toxicological effect on the recombinant luminescent strain Escherichia coli, which is manifested at high concentrations of the substance being tested. Similar results were obtained on another test object Stylonychia mytilus, for which the maximum toxicological effect was observed in the concentration range of 5.0-10.0 mg / ml for Betula spp. leaf and Hypericum spp. herb, and 10.0 mg / ml for Angelica spp. root. Against this background, quorum-inhibiting activity of the studied plant extracts was registered, increasing in the series Angelica spp. → Hypericum spp. → Betula spp.

1. Introduction
Currently, many studies of microbiologists are devoted to a new treatment strategy for the bacterial infections, based on the regulation of bacterial gene expression, which occurs at a sufficiently high population density. This phenomenon is known as “quorum sensing” [1]. In most plant, animal, and human pathogens, quorum sensing determines their ability to induce an infectious process [2]. To date, a large number of substances with quorum-inhibiting activity are known, which also include plants and their metabolites. Thus, the work of B. K. Tiwary shows the ability of leaf extracts of Astilbe rivularis, Fragaria nubicola and Osbeckia nepalensis to inhibit the pigment synthesis of violacein in Chromobacterium violaceum and pyocyanin in Pseudomonas aeruginosa [3]. There is also data of phenolic compounds of plant origin as regulators of homoserinlactone mediated intercellular communication in C. violaceum and P. aeruginosa [4]. Against this background, for some medicinal plants the possibility of their use is described not only as inhibitors of “quorum sensing”, but also as biologically active additives that can have a preventive effect in the fight against infectious diseases, in feeding farm animals [5, 6]. However, many of them have a toxic effect [7, 8]. It makes the use of plants in medicine and veterinary medicine difficult and requires more careful study.
In this regard, the purpose of this study was to study the toxicological, antibacterial and anti-quorum activity of *Betula spp.* leaf extracts, *Hypericum spp.* herb. and *Angelica spp.* roots.

2. Materials and methods

2.1. Medicinal plants

The objects of research were dry extracts of medicinal plants: *Betula spp.* leaf, *Hypericum spp.* herb and *Angelica spp.* root, belonging to the families Betulaceae, Hypericaceae and Apiaceae, respectively. The method of obtaining these extracts included: 1) mixing 6 g of dry mass with 50 ml of hot sterile distilled water; 2) boiling in a water steam for 15 minutes; 3) cooling at room temperature for 45 minutes; 4) centrifugation at 1000 rpm for 10 minutes to remove solid particles; 5) filtration through a polyethersulfone syringe filter (Membrane Solutions LLC, USA) with a pore size of 0.4 μm; 6) drying the obtained extracts at +60°C. For biological analyses, the samples obtained in this way were dissolved in sterile distilled water.

2.2. Bacterial strains and protists

*Escherichia coli K12 MG1655 (pXen7) (EC)* test strain capable of luminescence was used to assess the potential toxicity of the studied dry extracts [9]. In addition, a representative of the ciliary protozoa *Stylonychia mytilus (SM)* was used to study the negative effect of the studied compounds. For this, the body was exposed to a concentration range from 0.019 to 10.0 mg/ml (tenfold dilution from 10.0 mg/ml) for 1, 6, 12, and 24 hours, and then the general condition was evaluated.

A wild strain of *Chromobacterium violaceum ATCC 31532 (CV)* with a two-component CviI/CviR auto-induction system was used as a test strain for assessing the antibacterial and quorum-regulating activities of dry plant extracts. When growing on nutrient media, it implements the "quorum sensing" effect, accompanied by the formation and accumulation of the blue-purple pigment violacein in bacterial cells with a maximum absorption at 590 nm [10].

2.3. Toxicological activity research

The strain EC was grown for 24 hours on LB agar (Sigma, USA) at 37 °C in medium supplemented with 100 μg/ml ampicillin as a selective factor. Then, the cells were suspended in a 0.9% NaCl solution until an optical density of 0.5 rel. units. The measurements were carried out at 450 nm in transparent plastic wells on AIFR-01 "UNIPLAN" (CJSC PIKON, Russia). Next, 500 μl of the bacterial suspension was added to 1 ml of LB broth without further growth.

To evaluate the action of the test substances in wells of the plate, ten-fold dilutions of dry plant extracts were made at a final concentration of 10.0 mg/ml to 0.0097 mg/ml. Then, dilutions of extracts in a volume of 50 μl were added to the experimental wells, and 50 μl of water to the control wells; then, a suspension of strains in a volume of 50 μl and 100 μl of LB broth was added to all samples. Then, measurements were made using an Infinite 200 Pro luminometer (Tecan, Austria) for 120 minutes at 37 °C.

The obtained results were initially processed using the software of the Magellan™ tablet luminometer, and further processing of the obtained data was carried out using the computer program “Excel 2010” (Microsoft Inc.).

Based on the obtained values, graphs of the kinetics of the luminescence of the strains were plotted, while the time of the experiment in minutes was plotted along the X axis, and the luminescence intensity (rel. units) was noted along the Y axis. (rel. units). The initial value of luminescence was normalized to control. Normalization allowed us to “get rid” of the curve character in the control sample and evaluate the effect of the analyzed substance directly. The calculation was performed using the formula:
\[ I_{\text{norm}} = \frac{I_{\text{exp}}^{60} \times I_{\text{control}}^{0}}{I_{\text{exp}}^{0} \times I_{\text{control}}^{60}} \]

where \( I_{\text{exp}}^{60} \) is the luminescence index in the experimental sample at 60 minutes of the experiment; 
\( I_{\text{exp}}^{0} \) is the luminescence index in the experimental sample at 0 minute of the experiment; 
\( I_{\text{control}}^{60} \) is the luminescence index in the control sample at 60 minutes of the experiment; 
\( I_{\text{control}}^{0} \) is the luminescence index in the control sample at 60 minutes of the experiment.

A freshwater infusorian cell culture \( SM \) (wild strain) was used in the exponential growth phase. For this, the initial culture of \( SM \) cells was cultured on Losin-Lozinsky medium supplemented with yeast (Saccharomyces cerevisiae).

The parameters determined included: survival, number (biomass).

The sensitivity of the culture of \( SM \) cells with respect to the toxicant was evaluated by the time of their death, recorded by the cessation of protozoa movement, then morphological changes in the cell structure were noted. The number of cells in 5 ml of medium containing an intact culture of ciliates (without adding substances) served as a control in all experiments. Counting the total number of cells in 5 ml of medium containing ciliates was carried out using a light microscope (MT 5300L). Cells taken in the stationary phase of growth were incubated at a temperature of 20 ± 2 °C in a medium with the addition of substances for 24 hours.

2.4. Research of antibacterial and anti-quorum activity

During an experimental series aimed at characterizing the antibacterial and anti-quorum activity of each of the substances used, double dilutions of dry extracts (in the concentration range from 10.0 mg/ml to 0.0097 mg/ml) were prepared in cells of a 96-well transparent plastic tablet in distilled water, which were mixed with equal volumes of LB-broth (Sigma, USA). Then 20 µl of daily culture of \( CV \) was added to each well, and additional growth was made at 27°C during the day. Each study included additional samples of LB-broth that did not contain the studied substances and were used as positive (growth of the test strain) and negative (sterile) controls. The results of the experiment were evaluated using a multifunctional microplate reader “Infinite 200 Pro” (“Tecan”, Austria), sequentially registering the optical density of bacterial biomass at 450±5 nm, and the quantitative presence of the pigment violacein after its ethanol extraction at 600±5 nm. The absorption values of the negative control were subtracted. The antibacterial effect was expressed by the values of MIC_{100} and MIC_{50} which are the minimal inhibitory concentrations that cause 100% and 50% suppression of the growth of the test strain relative to positive control. In turn, the intensity of suppression of the quorum sensing system was expressed in the values EC_{100} and EC_{50}, corresponding to similar intensities of inhibition of the biosynthesis of the pigment violacein.

2.5. Statistical processing of research results

All experiments were performed in at least five repetitions. The results obtained were processed using the methods of variation statistics Statistica 10.0 RU.

3. Results and discussion

Analysis of bioluminescent testing data for extracts of \( Betula \) spp. leaf, \( Hypericum \) spp. herb and \( Angelica \) spp. root showed a minor toxicological effect against the recombinant luminescent strain of \( EC \). Thus, testing of \( Betula \) spp. leaf extracts demonstrated a 30% reduction in bioluminescence at a maximum concentration of 10.0 mg/ml of the analyzed substance. At dilutions from 5.0 mg / ml to 0.31 mg / ml, a decrease in the level of toxic action was observed from 25% to 10%. Further reduction of the concentration did not have a negative impact, since the bioluminescence value slightly exceeded the control sample.

The results of bioluminescent analysis of the extract of \( Hypericum \) spp. herb also showed insignificant toxic activity of the test extract, the maximum concentration of which (10.0 mg/ml) caused
37% death of the test organism. With increasing dilution of the test substance from 5.0 mg/ml to 0.15 mg/ml, the percentage of surviving cells increased from 75% to 95%. Further dilution to 0.0097 mg/ml did not cause suppression of the luminescence, so the bioluminescence level corresponded to the control level.

The assessment of the time factor showed that the toxic effect developed within 60 minutes. At high concentrations, the negative effect was maintained throughout the time period; at lower concentrations, after 1 hour, the luminescence level was restored to its original level.

As for the extract of Angelica spp. roots, there was a small antibacterial effect on the cells of the test strain. Thus, the assessment of the concentration dependence showed that at the maximum concentration of the extract of 10.0 mg/ml, 50% death of the microorganism occurred. With an increase in the number of dilutions from 5.0 mg/ml to 0.078 mg/ml, the number of surviving cells increased and reached almost 100%. Further dilution of the test substance to 0.0097 mg/ml demonstrated no toxic effect on the test strain.

Analysis of the time factor effect during contact of the substance-cell system showed that the toxicological effect was achieved within 15 minutes required for activation of the lux-operon, and was maintained throughout the accounting period (180 minutes).

Testing of the studied plant extracts on SM showed results similar to those obtained for EC. Thus, exposure to 5.0 and 10.0 mg/ml of the test extract of Betula spp. leaf resulted in a decrease in the activity and survival of the test object during the entire exposure period. 50% survival of SM was observed at concentrations of 2.5 mg/ml during 6, 12 and 24 hours of exposure. This trend in survival rate was maintained at a concentration of 1.25 mg/ml during 1 hour of exposure. Analysis of the effect of this extract in the concentration range from 0.313 to 0.625 and from 0.019 to 0.156 mg/ml demonstrated a high survival rate of the population of SM cells (40-69% and 70-100%).

Researching the extract of Hypericum spp. herb, it was found that the maximum effect on the population of SM cells (0-39% survival rate) was exerted by this extract in concentrations from 5.0 to 10.0 mg/ml. At a concentration of 2.5 mg/ml, there was a 50% survival rate of the test object. The survival rate remained unchanged during the period from 1 to 24 hours of incubation at concentrations from 0.019 to 1.25 mg/ml. Assessment of the impact of Angelica spp. roots made it clear that at a concentration of 10.0 mg/ml, this extract had a pronounced toxic effect, causing a decrease in the activity of SM cells throughout the entire period of exposure. The survival rate of the test object was 0-39%. The concentration of the test substance equal to 5.0 mg/ml contributed to 50% survival of the population of SM cells. At concentrations of 2.5 and 1.25 mg/ml, 40-69% survival of the test object was observed during the entire exposure period. Further decrease in the concentration of Angelica spp. root extract contributed to 70-100% survival rate of SM cells (table 1).

**Table 1.** Influence of the extracts of Betula spp. leaf, Hypericum spp. herb, and Angelica spp. roots on SM the cell population.

| Concentration, mg/ml | Exposure time, hour |
|----------------------|---------------------|
| 10.0 | 5.0 | 2.5 | 1.25 | 0.625 | 0.313 | 0.156 | 0.078 | 0.039 | 0.019 |
| **Tox** | **Tox** | **LC<sub>50</sub>** | **LC<sub>50</sub>** | **LOEC** | **LOEC** | **NOEC** | **NOEC** | **NOEC** | **NOEC** |
| **Tox** | **Tox** | **Tox** | **Tox** | **LC<sub>50</sub>** | **LC<sub>50</sub>** | **LC<sub>50</sub>** | **LC<sub>50</sub>** | **LC<sub>50</sub>** | **LC<sub>50</sub>** |
| **Tox** | **Tox** | **Tox** | **Tox** | **LOEC** | **LOEC** | **NOEC** | **NOEC** | **NOEC** | **NOEC** |
| **Tox** | **Tox** | **Tox** | **Tox** | **NOEC** | **NOEC** | **NOEC** | **NOEC** | **NOEC** | **NOEC** |
| **Tox** | **Tox** | **Tox** | **Tox** | **NOEC** | **NOEC** | **NOEC** | **NOEC** | **NOEC** | **NOEC** |
| **Tox** | **Tox** | **Tox** | **Tox** | **NOEC** | **NOEC** | **NOEC** | **NOEC** | **NOEC** | **NOEC** |

Note. Tox = 0-39% the survival rate of the test object; LC<sub>50</sub> = 50% the survival rate of the test object; LOEC = 40-69% the survival rate of the test object; NOEC = 70-100% the survival rate of the test object [11].
The obtained results, conducted in a liquid nutrient medium in the presence of a wide range of concentrations of the studied plant extracts, indicated a low growth-inhibiting activity of all tested compounds, whose MIC\textsubscript{100} was at the level of 10.0 mg/ml (the exception was Angelica spp. root extract, its MIC\textsubscript{100} was not reached). Against this background, it was noted a fairly high quorum-inhibiting activity of the extracts of Betula spp. leaf and Hypericum spp. herb along with low activity of the extract of Angelica spp. root. So, for the extracts of Betula spp. leaf the EC\textsubscript{100} value was 0.63 mg / ml, for Hypericum spp. herb EC\textsubscript{100} reached 2.5 mg / ml, and for the extract of Angelica spp. root EC\textsubscript{100} value was 10.0 mg / ml (table 2). The MIC\textsubscript{50} / EC\textsubscript{100} ratio, which most fully characterizes the range of concentrations of plant extracts that cause suppression of quorum-dependent biosynthesis of the pigment violacein in the absence of growth-inhibiting effect, increased in the series Angelica spp. (1.25) → Hypericum spp. (4.85) → Betula spp. (28.41).

**Table 2.** The concentration of the extracts of Betula spp. leaf, Hypericum spp. herb and Angelica spp. root (mg / ml), which cause inhibition of growth and quorum-dependent biosynthesis of the pigment violacein in CV.

| Dry extracts of medicinal plants | Characteristics of antibacterial activity, mg / ml | Characteristics of anti-quorum activity, mg / ml |
|--------------------------------|-----------------------------------------------|-----------------------------------------------|
|                                | MIC\textsubscript{100} | MIC\textsubscript{50} | EC\textsubscript{100} | EC\textsubscript{50} |
| Betula spp. (leaf)             | 10.0             | 6.25             | 0.63             | 0.22             |
| Hypericum spp. (herb)          | 10.0             | 4.17             | 2.5              | 0.86             |
| Angelica spp. (root)           | -                | 9.38             | 10.0             | 7.5              |

Testing the extracts of Betula spp. leaf, Hypericum spp. herb and Angelica spp. root allowed to reveal the presence of toxicological, antibacterial and quorum-inhibiting properties in the studied medicinal plants. In the course of studies on the recombinant luminescent strain of EC and the protozoan SM, a range of concentrations was recorded at which the maximum manifestation of the toxic effect of the tested extracts occurs, equal to 5.0-10.0 mg/ml for Betula spp. leaf, Hypericum spp. herb, and 10.0 mg/ml for Angelica spp. roots. With a subsequent decrease in the concentration of the tested substances, there was a decrease in the detected effect, approaching the control values.

The antibacterial activity of the studied extracts obtained on the violacein-producing strain of CV was characterized by high values of MIC\textsubscript{100} (10 mg / ml) and confirmed the results obtained on other test objects. The exception was the root extract of Angelica spp., whose MIC\textsubscript{100} was not achieved in this study.

At the same time, the ability of the studied plant extracts to inhibit quorum-dependent synthesis of the pigment violacein in CV was registered, increasing in the series Angelica spp. → Hypericum spp. → Betula spp. The result is in good correlation with the previously described quorum-inhibiting effect of Quercus cortex on CV [12] and the detected anti-quorum activity of Mangifera indica extract [13].

4. **Conclusion**

Thus, in the ongoing discussion about the ability of extracts of Betula spp. leaf [14, 15], Hypericum spp. herb [16, 17] and Angelica spp. root [18, 19] to have a therapeutic effect in the treatment of infectious conditions in humans and animals, the results obtained significantly complement and expand the idea of them as a new class of drugs that can not only have a direct antibacterial effect on the pathogen population, but also affect the profile of gene expression, thereby blocking a cascade of adverse reactions.

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