Quorum Sensing Suppression of Chromobacterium Violaceum when exposed to combinations of dry plant extracts

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Abstract. The study of density dependent chemical communication in bacteria called the quorum sensing is a promising area in science since most microorganisms that can cause infectious diseases use QS to realize their pathogenic potential. Currently, there is an extensive search for drugs that can inhibit QS dependent processes. Medicinal plants and their components are of particular interest. The main methodological approach aimed at studying the QS inhibitory activity of the studied compounds in this work was the use of a strain of Chromobacterium violaceum ATCC 31532, which is able to respond to potential QS inhibitors by synthesis of violacein pigment. The object of the study is dry extracts of oak bark (Quercus cortex), birch leaves (Folia betulae) and St. John's wort herb (Hyperici herba). The QS inhibitory activity of dry extracts of oak bark, birch leaves and St. John's wort herb is shown. For the first time, it is justified to use compositions based on oak bark and birch leaves, as well as oak bark and St. John's wort to suppress the quorum sensing in pathogenic and conditionally pathogenic bacterial microflora.

1. Introduction
The development of new methods of combating diseases caused by antibiotic-resistant pathogens is one of the main problems that scientists are currently facing [1]. As an alternative to natural and chemically synthesized direct-acting bactericides, an approach is currently being considered that consists not in destroying, but in changing the physiology of microorganism in the direction of suppressing (decreasing) its ability to parasitize and damage biological substrates. In particular, the foregoing relates to the search and creation of inhibitors of quorum sensing (QS) in bacteria, assessed as an attractive alternative to traditional means of fighting infections especially caused by multiresistant strains of microorganisms [2]. So Shukla and Bhathena in their work report that extracts rich in hydrolyzable tannins Phyllanthus emblica, Terminalia bellirica, Terminalia chebula, Punica granatum, S. cumini and Mangifera indica (flower) exhibit broad-spectrum anti-QS activity that affects the activity acyl homoseryl lactones (AHL) in a wide range of subinhibitory concentrations [3]. Another study showed that dichloromethane extract from the roots of Cordia gilletii bark suppresses the production of pyocyanin, a QS-dependent virulence factor P. aeruginosa, and specifically inhibits the expression of several QS-regulated genes [4]. There is also evidence of the QS-inhibiting activity of 4-hydroxy-3-methoxybenzaldehyde (vanilla) from Vanilla planifolia [5], anacardic acids extracted from Pimpinella anisum (anise) [6], Anacardium occidentale (cashew), eugenol from Syzygium...
aromaticum (cloves), bay leaves and other plants [7, 8]. The foregoing indicates the promise of studying medicinal plants as inhibitors of quorum sensing.

The aim of our study is to develop a composition of extracts of medicinal plants used in traditional medicine and exhibiting an additive effect in regulating the quorum sensing of pathogenic and putrefactive bacteria.

2. Materials and Methods

2.1. Bacterial strains

The wild-type strain Chromobacterium violaceum ATCC 31532 with the two-component QS system, where the synthase generated under the control of the cviI gene ensures the formation of an auto-inducer N-hexanoyl-L-homoserine lactone (C6-AHL), and the receptor encoded by the cviR gene is used as the main object of study in its perception, followed by QS-dependent transcription of a number of target genes [9]. In particular, the vioABEDC operon, responsible for the formation of blue-violet pigment violacein with a maximum absorption at 570-600 nm, the amount of which in the bacterial culture allows the direct assessment of activity of the QS system, is under such control.

2.2. Plant components

When influencing the density-dependent communication system C. violaceum ATCC 31532, water extracts were used or similarly obtained and subsequently dried extracts from oak bark (Quercus cortex), birch leaves (Folia betulae) and St. John’s wort (Hyperici herba).

The extracts used were prepared on the basis of dry plant material, carrying out aqueous extracts from it, after which, in case it is needed, additional drying of the obtained extracts was carried out.

When preparing aqueous extracts from the coarse parts of plants (in particular, oak bark), 6 grams of crushed plant material pre-sterilized in an autoclave at 1 atmosphere for 30 minutes was poured with sterile distilled water in a volume of 50 ml. The next step was boiling in a water bath for 30 minutes, then cooling for 10 minutes, after which the resulting broth was filtered through a sterile cotton-gauze filter in a glass dish.

When preparing water extracts from the soft parts of plants (birch leaves and St. John’s wort herb), a similar sequence of actions was used, except that boiling in a water bath was carried out for 15 minutes and infusion took place for 45 minutes.

If necessary, the obtained extracts were evaporated in a water bath until a thicker consistency was obtained, after which they were completely dried to obtain a powder.

The composition was obtained by mixing aqueous extracts in a ratio of 50.0 wt.% when mixing two extracts.

When conducting an experimental series of studies in a liquid nutrient medium, two-fold dilutions of dry extracts of oak bark, birch leaves and St. John’s wort were prepared. Each study included additional samples of LB broth that did not contain these components and were used as positive (growth of the test strain) and negative (sterile) controls. The experimental results were evaluated using the Infinite 200 Pro multifunctional microplate reader (Tecan, Austria), after which the data were evaluated and archived using the standard software - magellanTM Software V7.2. The optical density of bacterial biomass was measured at 450±5 nm, and the quantitative presence of violacein pigment at 600±5 nm, after its preliminary ethanol extraction. Negative control absorbance values were subtracted. The antibacterial effect was expressed by the values of MIK100 and MIK50 - the minimum inhibitory concentrations, causing 100% and 50% inhibition of growth of the test strain relative to the positive control. In turn, the intensity of suppressing the system of quorum sensing was expressed by EC100 and EC50 values corresponding to similar intensities of inhibition of violacein pigment biosynthesis.

To study the combined effect of extracts of oak bark and birch leaves, oak bark and St. John’s wort herb, birch leaves and St. John’s wort herb, a series of two dilutions of substance “a” and substance “b” were introduced in directions perpendicular to each other in the cells of a 96-well plate, so that each of them formed an individual ratio “oak bark: birch leaves”, “oak bark: St. John’s wort herb” or “St. John’s wort: birch leaves”. Control samples were dilution series containing only substance “a” or...
only substance “b”. Then, the cells were filled with a liquid nutrient medium containing C. violaceum ATCC 31532 and incubated at 27°C for 24-48 hours for the growth of the bacterial culture to develop with the development of the quorum sensing effect estimated by the formation of the blue-violet pigment violacein extracted from the bacterial biomass.

The combined effect of the studied extracts was evaluated in accordance with the method proposed by R.J. Tallarida [10]. The principle of this method is to construct an isobologram by which it is possible to determine the nature of interaction of two substances by comparing the experimental points whose coordinates are the concentration of the substance and the value of EC50. The combination of substances causes an additive effect if the point is located on the isobole, a superadditive effect if it is under the isobole and an infra-additive effect if the point is above the isobole.

3. Results and discussions

In a series of experiments, it was found that aqueous extracts from medicinal plants develop direct antibacterial activity against the indicator strain C. violaceum ATCC 31532, characterized by the values MIC\textsubscript{100} = 5, 10, and 10 mg/ml dry matter for oak bark, birch leaves, and St. John’s wort herb, respectively. At the same time, it was found that all the studied extracts suppressed the QS-dependent biosynthesis of the violacein pigment, although the severity of the effect was different. Thus, the ratio of the concentrations responsible for the complete suppression of growth (MIC\textsubscript{100}) and pigmentation (EC\textsubscript{100}) was 16 for oak bark, 4 for birch leaves, and 2 for St. John’s wort. The MIC\textsubscript{50}/EC\textsubscript{50} ratio, which most fully characterizes the range of concentrations of dry extracts that suppress the QS-dependent biosynthesis of the violacein pigment in the absence of growth-inhibiting effect, was at the level of 22.2 for oak bark, 13 for birch leaves and 12 for St. John’s wort. The results of the analysis of such activity are illustrated in Fig. 1 and in a generalized form they are given in Table 1.

| Active compositions | Characteristics of antibacterial activity, mg/ml | Characteristics of anti-QS activity, mg/ml |
|---------------------|---------------------------------|---------------------------------|
|                     | MIC\textsubscript{100} | MIC\textsubscript{50} | EC\textsubscript{100} | EC\textsubscript{50} |
| Oak bark            | 5.00              | 1.11              | 0.31              | 0.05              |
| Birch leaves        | 10.00             | 7.02              | 2.50              | 0.53              |
| St. John’s wort herb| 10.00             | 7.44              | 5.00              | 0.61              |

The results of studies conducted in a cross-concentration matrix of the studied extracts concentrations in growing cultures of C. violaceum ATCC 31532 (Fig. 2) revealed different bioactivity in the test for suppressing QS-dependent violacein pigment biosynthesis. So, the combination of the oak bark with the birch leaves and the ak bark with St. John’s wort showed a super-additive effect, which manifests itself in the location of most of the calculated indices of combined exposure under a straight line connecting the EC\textsubscript{50} values (Fig. 3a, b). An isobolographic analysis of the composition “birch leaves: St. John’s wort” showed an insignificant additive character of the action of such compositions, and therefore, this composition is less attractive for its use in the fight against bacteria that use QS to realize their pathogenic potential (Fig. 3c).
Figure 1. The impact of oak bark, birch leaves and St. John’s wort herb on the growth and QS-dependent biosynthesis of violacein pigment in C. violaceum ATCC 31532 culture. Designations: along the abscissa — concentration of active compounds (mg/ml); along the ordinate axis on the left, the solid line in the graphs (1) is the optical density of biomass (OP$_{450}$); on the ordinate axis on the right, the dotted line on the graphs (2) is the optical density of pigment (OP$_{600}$).

Figure 2. The regulatory effect of St. John’s wort herb in combination with the oak bark on the synthesis of violacein pigment by C. violaceum ATCC 31532 culture when testing on a liquid nutrient medium; K1 is an action of St. John’s wort herb without St. John’s bark, K2 is an action of the oak bark without St. John’s wort, K3 is a pure culture.

Thus, the results of the study show the promising nature of using dry extracts of medicinal plants and their combinations as inhibitors of collective behavior in bacteria. In the literature there are many examples of the use of drugs for the treatment of bacterial infections. In particular, a plant composition for the prevention or treatment of metabolic disorders of carbohydrates and/or fats is known [11], an antibacterial and quorum-sensitive molecular composition obtained from bark extract (oak bark) [12] is confirmed to have an antibacterial and quorum-sensitive regulatory activity some traditional East European medicinal plants [13], to increase the immunity of birds [14], etc. Moreover, the main...
argument for the formation of such compositions is the presence in each of their plant extracts of the corresponding biological activity, as well as the additive effect upon their sharing.

The revealed QS-inhibitory activity of the dry extracts presented in this work is in good agreement and complements the data on phytoextract compositions containing at least two dried aqueous or alcoholic extracts from oak bark (*Quercus cortex*), birch buds (*Betulae gemmae*), and eucalyptus leaves (*Eucalyptus folia*) with a certain ratio of components [15].

![Figure 3. Isobolographic analysis of the combined effect of combinations of the oak bark with the birch leaves (a) and St. John’s wort herb (b), as well as the birch leaves with St. John’s wort herb (c) on the QS-dependent biosynthesis of violacein pigment in C. violaceum ATCC 31532 culture. Isobols are presented as direct lines connecting the concentrations of each of the compounds that cause the same biological effect (50% inhibition of violacein biosynthesis; EC$_{50}$).](image)

### 4. Conclusion

A practically oriented aspect of the application of the obtained additive compositions based on the extracts of oak bark, birch leaves and St. John’s wort is the suppression of the quorum sensing in pathogenic and conditionally pathogenic bacterial microflora involved in the development of infectious diseases of plants and animals. To this end, this composition can be introduced into the body to provide a systemic effect and also be applied topically to affect certain areas (for example, as part of dressings for treating wounds, when treating an operative field, etc.). The composition of the extracts can be used in the form of aqueous solutions, solids (powders, feed additive), as well as applied to various carriers.

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