

Assessment (in vitro) toxicity of small molecules of plant origin

Ludmila Vlasenko*, and Kseniya Atlanderova

Federal Research Centre of Biological Systems and Agrotechnologies of the Russian Academy of Sciences, Orenburg, Russia

Abstract. Small molecules of plant origin can have different effects on bacterial cells. At present, it is of great interest to determine the toxic effects of such compounds in order to assess the potential of their use in veterinary medicine and medicine. The aim of this work was to evaluate the toxicity of various chemically synthesized small molecules of plant origin using a bacterial luminescent biosensor based on *Escherichia coli* and a cell culture of the freshwater ciliate *Stylonychia mytilus*. Cinnamic aldehyde had the greatest toxic effect on the *E. coli* MG1655 pXen7 *lux*-biosensor, which was expressed in a significant decrease in the luminescence level of the strain compared to the control. Quercetin in the concentration range used did not affect the luminescence intensity of the *lux*-biosensor. Coumarin and vanillin were characterized by a similar manifestation of the toxic effect. Similar results were also confirmed using *S. mytilus* as a test object. The results obtained expand the understanding of the possible toxic effect of phytochemicals, which can be used in the development of feed additives in animal husbandry (as analogues of feed antibiotics).

1 Introduction

Phytochemicals have become the subject of in-depth study in recent years. The special properties of these compounds determine the possibility of their use in various fields of human activity, including the pharmaceutical, cosmetic, agricultural and food industries [1–3]. However, this makes it necessary to conduct appropriate tests and determine their danger [4], which can also be useful in justifying the use of small molecules of plant origin as feed additives. Currently, various test systems are used to assess the toxicity of substances: microorganisms, aquatic organisms, and insects [5–7]. At the same time, bacterial luminescent biosensors (*lux*-biosensors) have significant potential in this context, using which, by changing the level of their luminescence, one can quantify the toxic effect of various compounds. A convenient tool is also unicellular ciliates, which react to exposure with a whole range of changes. The possibility of using these objects in assessing the toxicity of phytochemicals was described earlier [4, 8], but the data are relatively scarce.

Therefore, the goal of this work was to determine the toxicity of various chemically synthesized small molecules of plant origin using the *E. coli* MG1655 pXen7 *lux*-biosensor and the *Stylonychia mytilus* test system as test objects.

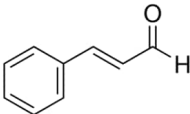
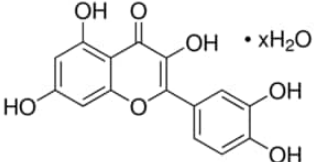
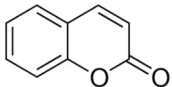
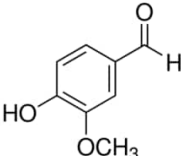
* Corresponding author: lv.efremova@yandex.ru

2 Materials and methods

2.1 Small molecules of plant origin

Small molecules of plant origin - cinnamic aldehyde, quercetin, coumarin and vanillin - were represented by their chemically synthesized counterparts manufactured by Sigma-Aldrich (Table 1). To create suspensions of the studied substances, weighed portions of 0.2 M were placed in glass containers using 45% ethanol as a solvent. Further work was carried out using a concentration of substances of 0.0125 M, containing a safe amount of ethanol for the *lux*-biosensor (which does not have its own effect on the level of bioluminescence).

Table 1. General characteristics of small molecules of plant origin.

Test substances	Chemical formula	Structural formula	Molar mass
Trans-cinnamaldehyde (cinnamaldehyde), 99 %, C80687	C ₉ H ₈ O		132.16 g/mol
Quercetin hydrate (quercetin), ≥95 %, 337951	C ₁₅ H ₁₀ O ₇ ·xH ₂ O		302.24 g/mol
Coumarin, ≥99 %, C4261	C ₉ H ₆ O ₂		146.14 g/mol
Vanillin, 99 %, V1104	C ₈ H ₈ O ₃		152.15 g/mol

2.2 *Lux*-biosensor

A laboratory luminescent strain *E. coli* K12 MG1655 (pXen7) is used in the work, which was obtained by transforming cells of the host strain with a hybrid plasmid pUC18 with an embedded *Eco*RI DNA fragment containing the *lux*-CDABE genes of the soil microorganism *Photobacterium luminescens* ZM1 [9]. Previously, this strain was used in the study of the toxicity of nanocarbon compounds [10].

2.3 Bioluminescent test

Lux-biosensor *E. coli* K12 MG1655 pXen7 was cultivated on LB-agar (AppliChem, Germany) with ampicillin at 37°C for 18-24 hours; then transferred to LB-broth and grown to an early exponential growth phase (optical density 0.4 units at 450 nm). Aliquots of bacterial suspensions, 100 µl each, were added to the wells of a FluoroNunc plate (Thermo Fisher Scientific, USA) containing 100 µl of pre-diluted test substances or water (control).

The plates were placed in the measuring block of an Infinite 200 microplate reader (Tecan Austria GmbH, Austria), where for 180 minutes (with an interval of 5 minutes) at 37°C, the bioluminescence intensity was dynamically recorded, estimating it in relative light units; RLU). The obtained data were initially processed using the software of the Magellan instrument.

Toxicity index (TI) was calculated to the equation:

$$TI = (RLU_{K_0} \times RLU_{O_n}) / (RLU_{K_n} \times RLU_{O_0}), \quad (1)$$

where RLU_{K_0} and RLU_{O_0} – luminescence values of control and experimental samples at the 0-th minute of measurement, RLU_{K_n} and RLU_{O_n} – glow values at the n-th minute of measurement. Based on this, the EC50 parameter was determined by a graphical method in the “substance concentration–TI” coordinate system, corresponding to the concentration of the test substance that causes 50% decrease in the bioluminescence level of the *lux*-biosensor compared to the control.

2.4 Protozoa test

The ciliates *Stylonychia mytilus* were used as a test object, which were grown on Lozin-Lozinsky medium at 23°C with the addition of dried yeast as feed. The cessation of the movement of protozoa, the violation of their integrity, which ultimately led to cell lysis, indicated the toxic effect of the substances under study. When performing studies, 20 µl of the medium containing a daily culture of ciliates was mixed with 20 µl of the test substances or Lozin-Lozinsky medium (control) and incubated at a temperature of 23 °C for 180 minutes. An intermediate count was made after 60, 120 and 180 minutes of contact using a light microscope (MT 5300L).

2.5 Methods of statistical processing of research results

Statistical processing of the study results was carried out using Microsoft Excel (Microsoft Corporation, USA).

3 Results

Testing of small molecules of plant origin against the *E. coli* K12 MG1655 pXen7 *lux*-biosensor made it possible to identify and quantify the toxicity of the studied compounds. Of all the substances, only quercetin did not affect the luminescence level of the *lux*-biosensor in the entire range of concentrations and exposure time used. Cinnamaldehyde had the greatest toxic effect, which resulted in a significant decrease in the luminescence level of the strain compared to the control even at the 60th minute of the study (EC50<0.78 mM). A less pronounced effect was detected for coumarin (EC50=2.70±0.21 mM) and vanillin (EC50=2.40±0.19 mM).

Table 2. The values of the toxicological parameter EC50 (mM) determined for the studied small molecules of plant origin in assessing their effect on the *lux*-biosensor *E. coli* K12 MG1655 pXen7

Test materials	60 minutes	120 minutes	180 minutes
Cinnamaldehyde	<0.78	<0.78	<0.78
Quercetin	–	–	–
Coumarin	3.10±0.28	2.80±0.25	2.70±0.21
Vanillin	2.90±0.54	2.50±0.17	2.40±0.19

Notation: «–» no toxic effect.

The use of *Stylonychia mytilus* also made it possible to detect various degrees of toxicity of the studied substances. Thus, when the test organism was exposed to cinnamic aldehyde at concentrations of 0.78–3.10 mM, its toxic effect was observed from the first minutes of contact, which was accompanied by a change in the shape of ciliates to a round one and a cessation of movement, and by 180 minutes led to cell lysis (0-39% survival). A less pronounced toxic effect was recorded when using quercetin: only at a concentration of 3.10 mM and 180 minutes of contact, LC50 was observed - a concentration that ensures 50% survival of the object. For coumarin and vanillin, a similar toxic effect was recorded. At the same time, the test substances at a concentration of 0.78 mM did not significantly affect the survival of *Stylonychia mytilus* throughout the entire duration of the experiment, with an increase in concentration to 1.56 mM, the survival of the test organism was 40-69%; a subsequent increase in concentration led to a decrease in survival, which was manifested in the cessation of the movement of ciliates.

4 Discussion

According to the results of the study of the toxicity of various chemically synthesized small molecules in relation to the *E. coli* K12 MG1655 pXen7 *lux*-biosensor and *Stylonychia mytilus* cells, consistent data were obtained. It was found that cinnamic aldehyde has the greatest toxic effect. Similar results have been previously described for *E. coli* [11] and *Staphylococcus aureus* [12]. According to the literature, the antibacterial effect is explained by the presence of an aldehyde group in the compound, which contributes to the development of oxidative stress in target cells [13]. The use of cinnamic aldehyde in agriculture has been described using the example of its effective destruction of *E. coli* O157:H7 in drinking water for cattle, but concerns have been raised about the taste of water containing this substance consumed by animals [14]. According to the results of this study, quercetin turned out to be a less toxic compound. However, the available literature describes its antibacterial action against pathogenic bacteria such as *Salmonella enterica*, *E. coli*, *Pseudomonas aeruginosa*, *S. aureus* [15], while the mechanism of toxicity is described as a change in the permeability of bacterial cells, disruption nucleic acid synthesis and reduced enzyme activity [16, 17]. It has been established that the introduction of quercetin into the diet affects the microbiome of the caecum of broiler chickens, in particular, the number of *P. aeruginosa*, *S. enterica*, *S. aureus* and *E. coli* decreases, against the background of a significant increase in bacteria of the genus *Lactobacillus* and *Bifidobacterium* [16]. Along with this, there is evidence of a positive effect of quercetin on the physiological processes of plants, such as seed germination, proper growth and development [18].

According to the results of this work, coumarin and vanillin were characterized by average values (among the studied substances) of toxicity. According to the literature, simple coumarins (in particular, coumarin and umbelliferone) have a weak antibacterial effect [19]. However, there are data on the toxic effect of umbelliferone against enteropathogenic bacteria, which makes it a promising and, most importantly, safe compound for the treatment of various gastrointestinal pathologies [19] and for other practical purposes [2]. Recent studies have shown that the possible mechanism of action of this compound is the formation of reactive oxygen species [20]. The same mechanism of antibacterial action was described for vanillin [21], which also did not show a significant toxic effect on the test organisms used. However, there are data on the inhibition of biofilm formation by vanillin in *P. aeruginosa* and the associated suppression of Quorum sensing, which contributed to the growth of *Caenorhabditis elegans* [22].

5 Conclusion

Currently, the study of the influence of phytochemicals is a very promising area of research. The obtained data on the toxicity of small molecules of plant origin can be further used for the targeted development of new and safe components for their inclusion in the diet of animals (including cattle) as an alternative to feed antibiotics.

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