International Journal of GEOMATE, March, 2019 Vol.16, Issue 55, pp. 73 - 79
ISSN: 2186-2982 (P), 2186-2990 (O), Japan, DOI: https://doi.org/10.21660/2019.55.4708
Special Issue on Science, Engineering & Environment

ASSESSMENT OF N-(3-OXOHEXANOYL)-L-HOMOSERINE LACTONE AND VEGETABLE MOLECULES *IN VITRO*

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ABSTRACT: The ban on the use of antibiotics in the feed of farm animals has promoted an active search for new substances with similar properties. Particular attention of researchers has been devoted to the search for these substances among medicinal plants. The effect of substances of vegetable origin, such as coumarin and coniferyl alcohol which both form part of oak bark, on the activity of acyl homoserine lactone (AHL) molecules has been investigated. Reporter luminescent test systems were used to quantify the stress effects using bioluminescent analysis. It was found that the absorption spectrum of coumarin is characterised by the presence of four peaks at 205, 215, 275, 310 nm. The interaction with coniferyl alcohol was characterised by a shift in the absorption maxima (250 and 265 nm). The introduction of a molecule of AHL into the system led to the disappearance of the peak at 250 nm and a significant decrease in the absorption at 265 nm. The formation of a complex of ruminal fluid and coumarin led to a decrease in the expression efficiency of the sensory promoter luxI to a level of 476626 RLU using the same concentration of GSL (homoserine lactone). The combination of ruminal fluid and coniferyl alcohol led to a decrease in the quantum yield of the *E. coli* strain to the level of 345896 RLU following the use of GSL at a concentration of 10^{-4} M. The use of natural plant components (synthetic analogues) enhances the level of inhibition of quorum sensing (QS) system activity by the inactivation of regulatory molecules.

Keywords: Coumarin, Coniferyl Alcohol, Quorum Sensing

1. INTRODUCTION

The ban on the use of antibiotics in the feed of farm animals has promoted an active search for new substances with similar properties. Particular attention has been given to the search for these substances among medicinal plants, which were previously used in the treatment of animal diseases. Therefore, substances from natural sources targeted at certain types of molecules have been extracted and found to be useful for preventing insulin resistance [1]. Methylated quercetin derivatives, which are usually found in fruits and vegetables, and which have antioxidant and anti-inflammatory properties (block the enzymes responsible for inflammation) [2] were identified. Other investigators [3] observed the inhibition of secretory phospholipase A 2 by bioactive molecules from the extract of *Boerhaavia diffusa L* both *in vitro* and *in vivo*. The possible application of polyphenolic extracts for plant protection is considered in view of the absence of any toxicity of these compounds. The inhibition of *Pseudomonas savastanoi* by polyphenolic extracts isolated from plant residues was registered [4]. Studies on the extraction, identification and evaluation of cytotoxicity of the new terpene saponin from *Salicornia bigelovii Torr*, a potential chemotherapy drug for cancer treatment [5], were conducted. It was noted that natural indoles inhibit the activation of T cells of the staphylococcal enterotoxin [6].

When assessing molecules of plant origin, it is necessary to take into account the species of plants. This establishes a 100-fold difference in toxicity between saponin-rich extracts of different plant species [7] and individual vegetative parts [8].

The safety of plant matter is also assessed using laboratory animals. Thus, it was established that extracts from tea flowers do not contain toxic substances, based on the assessment of mutagenicity, and acute and subchronic toxicity in rats [9]. A significant number of studies are being conducted to assess the toxicity of plant extracts and their anti-inflammatory properties, for example in *Parinari kerstingii Engl* [10], their toxicological effects [11], and the anti-tumour and cytotoxic activity of essential oils (*Haplophyllum tuberculatum A. Juss*) [12], as well as acute and subchronic studies of the toxicity of the aqueous extract of *Desmodium adscendens* (Sw) DC [13].

The subchronic toxicity, immunoregulation and anti-tumour effect of Nordamnacantal and anthraquinone, extracted from the stems of *Morinda citrifolia L.* [14], and the antifungal and cytotoxic activity of selected medicinal plants from Malaysia were detected [15].

Substances with anti-quorum activity have been isolated from plant extracts, with Zingerone (ginger substance) not only having a marked effect on the production of quorum sensing signalling molecules by *Pseudomonas aeruginosa* clinical isolates, but also showing a significant effect on the activation of reporter QS strains [16].

In our studies, we used substances with confirmed antibacterial and QS activity that had been previously isolated from the extract of *Quercus cortex* [17].

The aim of the work was to estimate the toxicity of chemically synthesised small molecules isolated from the extract of *Quercus cortex* using strains of *Escherichia coli* JLD271 pAL103 bacteria as test objects, including against the background of cattle ruminal fluid.

2. MATERIALS AND METHODS

The bacterial strains of *E. coli* K12, transformed by plasmids with cross-linking receptor genes (*rhlR*, *lasR* from *Pseudomonas aueruginosa* and *luxR Vibrio fisheri*) and luminescence genes (*luxCDABE*) of *Photorhabdus luminescens,* as well as additional molecular factors such as aqueous oak extract containing coniferyl alcohol and coumarin (Fig. 1), which are part of the oak bark, were used; the ability to inhibit the production of violacecine from *Chromobacterium violaceum* was subsequently detected.

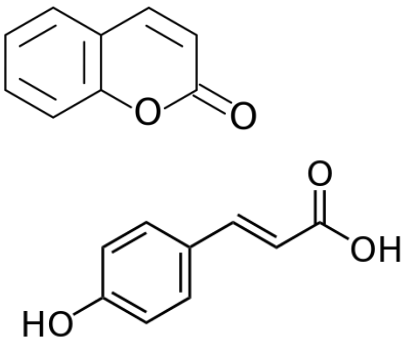

Figure 1 – Structural formulas of coumarin (left) and coniferyl alcohol (right).

Cultivation was performed on LB agar (Sigma, USA), with the addition of 10 μg/ml doxycycline for 18 hours at 37°C. Then, cells were transferred to LB broth (Sigma, USA) and incubated for 90 minutes at 37°C to reach the early exponential phase.

The reporter luminescent test systems (E. coli K-12 pAL103) were used as the material for the study, due to the possibility of the quantitative evaluation of stress effects using widely applied bioluminescent analysis.

As objects of research we used:

1. Autoinducers - N-(3-oxo)-hexanoyl-L-homoserine lactone (oxo-C6-GSL) in the concentration range from 10^{-8} to 10^{-4} M.
2. Bovine ruminal fluid, subjected to centrifugation at 5000 rpm for 10 minutes.

Equipment: luminometer LM-01T (Immunotech, Czech Republic), with the level of luminescence expressed in RLU (relative luminescent units); spectrophotometer Fluorat-02M "Panorama" (Lumex, Russia); multicentrifuge CM-6M.

Statistical processing was performed using the program "Statistica 10 RU", calculating the average value (M), standard deviation (σ), and standard deviation error (m). The significance level was considered reliable at $p < 0.05$.

3. RESULTS

At the first stage, the absorption spectra of N-(3-oxo)-hexanoyl-L-homoserine lactone and plant molecule complexes were evaluated. The change in the absorption spectra of these compounds was studied in the range from 200 to 400 nm, with the most typical and widespread AHL of gram-negative bacteria: N-(3-oxo)-hexanoyl-L-homoserine lactone. The absorption spectrum of coumarin was found to be characterised by the presence of four peaks corresponding to 205 nm (I = 0.255), 215 nm (I = 0.269), 275 nm (I = 0.211), and 310 nm (I = 0.112) (Table 1, Fig. 2).

The results of interactions with coniferyl alcohol turned out to be somewhat different and were characterised by a shift in the absorption maxima (Table 2).

It was found that the original substance has two significant approximate absorption maxima at 250 nm and 265 nm, with an intensity of 1.389 and 1.293 relative units, respectively. In addition, there are insignificant peaks at 205 nm, 220 nm and 230 nm (Fig. 3).

However, the introduction of AHL molecules into the system led to the disappearance of the peak at 250 nm and a significant decrease in the absorption at 265 nm. In this case, the peculiarity of this interaction was the formation of a pronounced maximum at 220 nm with an absorbance value of 0.853 relative units, which exceeds the initial level by 66%. As a result, the curve acquired qualitatively different characteristics compared to the original version.

In the second stage, studies were performed on ruminal tissue with natural and chemically synthesised inhibitors added, including the aqueous extract of oak and solutions of coumarin and coniferyl alcohol. In this case, N-(3-oxo)-hexanoyl-L-homoserine lactone (oxo-C6-GSL) was used as an autoinducer in the concentration range from 10^{-8} to 10^{-4} M.

Table 1. The absorption intensity of coumarin and its complex with N-(3-oxo)-hexanoyl-L-homoserine lactone (oxo-C6-HSL).

Name	Maxima of the absorption curve			
	205 nm	215 nm	275 nm	310 nm
Coumarin	0.255	0.269	0.211	0.112
Coumarin + oxo-C6-HSL	0.106	0.134	0.100	0.069
Character of change	-58%	-50%	-53%	-38%

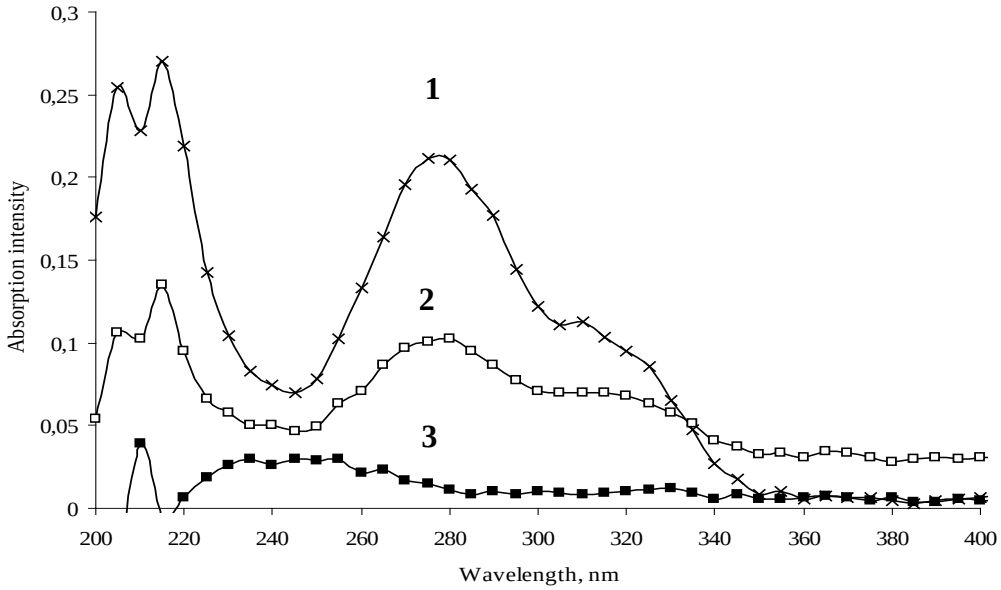

Figure 2 – Absorption spectrum of coumarin (1), N-(3-oxo)-hexanoyl-L-homoserine lactone (3) and their complex in the aqueous medium (2).

Table 2. Intensity of absorption of coniferyl alcohol and its complex with N- (3-oxo)-hexanoyl-L-homoserine lactone (oxo-C6-GSL).

Name	Maxima of the absorption curve				
	205 nm	220 nm	230 nm	250 nm	265 nm
Coniferyl alcohol	0.442	0.513	0.584	1.389	1.293
Coniferyl alcohol + oxo-C6-HSL	0.430	0.853	0.719	0.568	0.862
Decrease percentage	-3%	+66%	+23%	-59%	-33%

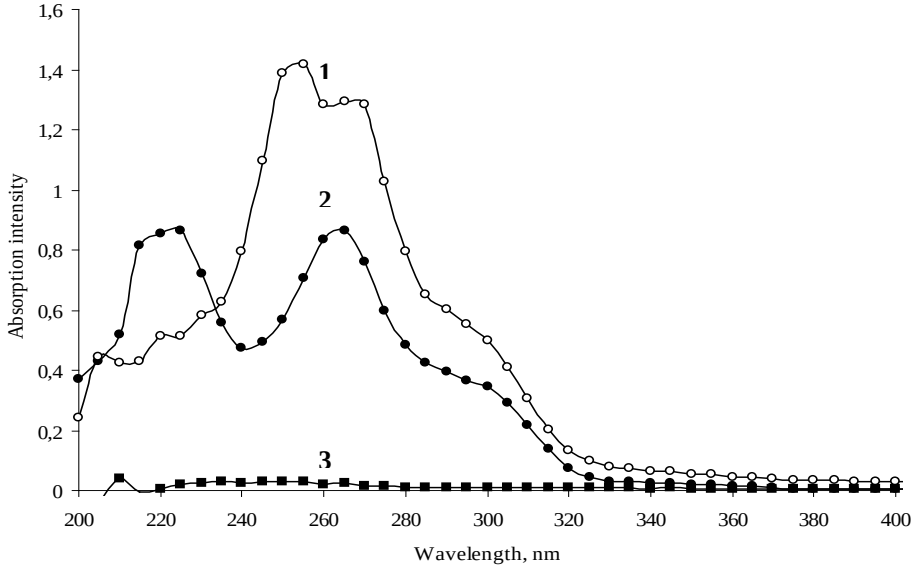

Figure 3 – Absorption of coniferyl alcohol (1), N-(3-oxo)-hexanoyl-L-homoserine lactone (3) and their complex in aqueous medium (2).

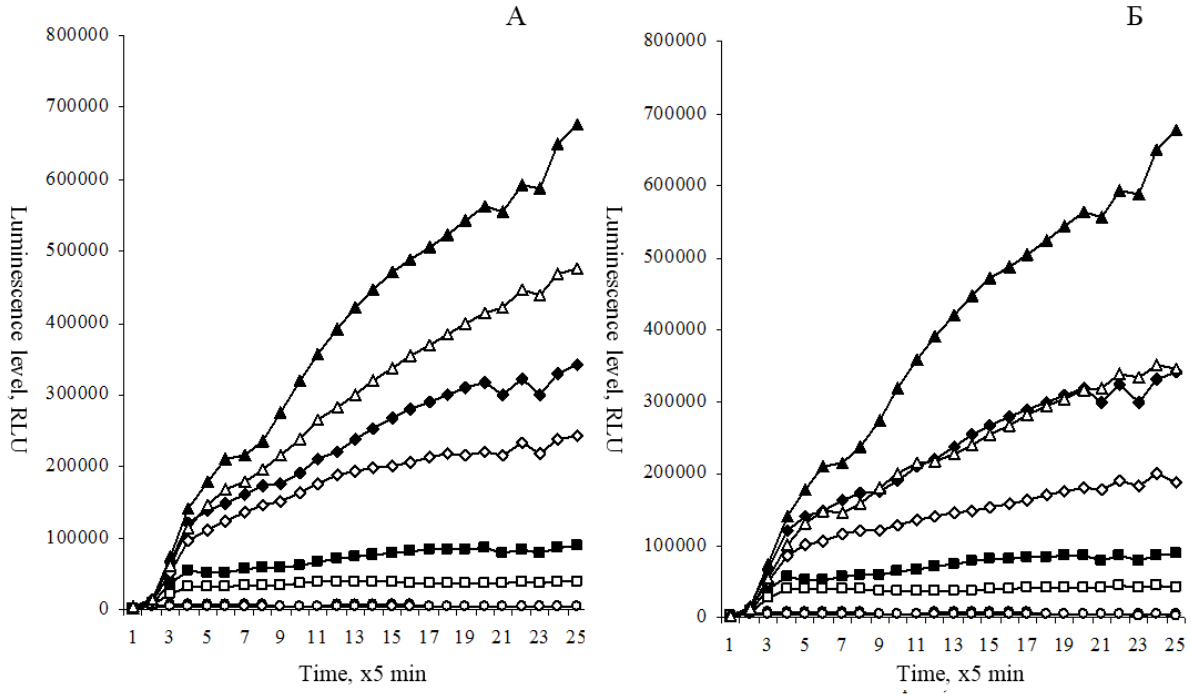

Figure 4 – Enhancement of inhibition activity of N-(3-oxo)-hexanoyl-L-homoserine lactone molecules by ruminal fluid (black figures) in combination with coumarin (A) and coniferyl alcohol (B) (white figures).

In this context, a sample of ruminal tissue diluted to 12.5% served as a control group, and the level of luminescence of *E. coli* JLD271 pAL103 was 676031 ± 10521 RLU at an autoinducer concentration of 10^{-4} M (Fig. 4).

The formation of a complex of ruminal fluid and coumarin led to a decrease in expression of the *luxI* sensory promoter to a level of 476626 ± 13791 RLU using the same concentration of oxo-C6-GSL, indicating an additional 29% of signalling molecules. Likewise, lower concentrations reduced the luminescence level to 241603 ± 7791 RLU at 10^{-5} M and to 39960 ± 2791 RLU at 10^{-6} M, which is an

additional inhibition of 29% and 55% of the molecules, respectively (Fig. 4A).

The creation of a similar system representing the combination of ruminal tissue and coniferyl alcohol also led to a decrease in the quantum yield of the E. coli strain JLD271 pAL103 to a level of 345896 ± 11669 RLU using oxo-C6-GSL at a concentration of 10^{-4} M, indicating the decrease of an additional 48% of signalling molecules. Likewise, lower concentrations reduced the luminescence level to 187329 ± 5238 RLU at 10^{-5} M and to 42525 ± 1988 RLU at 10^{-6} M, which is an additional inhibition of 45% and 52% of the molecules, respectively (Fig. 4B).

On the other hand, the formation of a mixture of ruminal tissue and oak bark extract, representing a whole set of different molecules, demonstrated an even more pronounced change in anti-quorum activity (Figure 5).

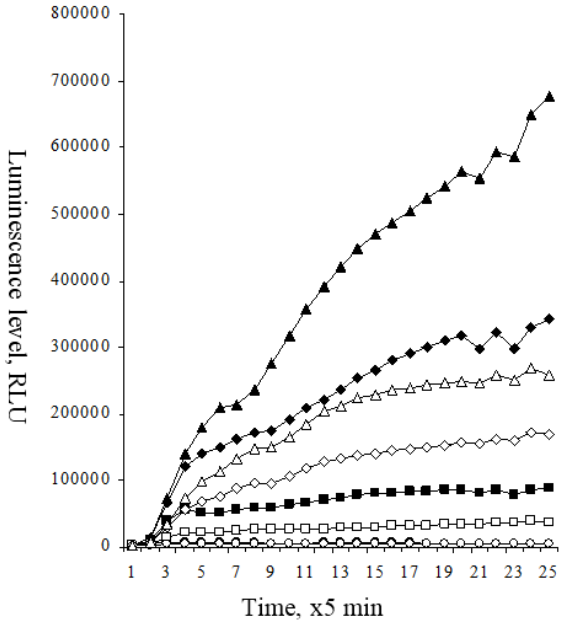

Figure 5 – Increased activity inhibition of N-(3-oxo)-hexanoyl-L-homoserine lactone with ruminal fluid (black figures) in combination with an extract of the oak bark (white figures).

Therefore, the effect of inhibition is increased by 61% in the maximum concentration of oxo-C6-HSL, corresponding to a level of 259479 ± 854 RLU, which is 57% of that at 10^{-6} M.

4. DISCUSSION

Because the feed substrate of cattle is formed from plant material, its composition or individual components can influence the signal molecules or receptor proteins of representatives of the microbial community. Therefore, to study the effect of plant extracts directly on ruminal tissue, it may be relevant to undertake investigations into the intercellular communication between bacteria in the rumen. At the same time, scientists have revealed a new class of substances in plant extracts that can effectively prevent the development of infectious and inflammatory processes in animal bodies due to suppression of the system of intercellular communication in bacteria.

In the first stage, the absorption spectra of N-(3-oxo)-hexanoyl-L-homoserine lactone and plant molecule complexes were evaluated. The formation of mixtures of coumarin and AHL did not lead to qualitative changes in the absorption spectrum, but resulted in a decrease in amplitude instead. This is due to the formation of aggregates that increase the area to more than that of 340 nm and reduce the concentration of coumarin in the system by about half of the initial amount.

The results of the interaction with coniferyl alcohol turned out to be somewhat different, apparently due to covalent interactions, which leads to the formation of new types of compounds with different characteristics; in the context of our work, it potentially leads to the inactivation of mechanisms of intercellular communication using acyl derivatives of homoserine lactone.

In the second stage, studies on ruminal tissue were performed with natural and chemically synthesised inhibitors added, including the aqueous extract of oak and solutions of coumarin and coniferyl alcohol. The formation of a complex of ruminal fluid with coumarin and coniferyl alcohol resulted in a decrease in the expression of the sensory promoter and a decrease in the quantum yield of *E. coli* strain JLD271 pAL103. This can probably be explained by the dose-dependent inhibition of luminescence, as shown by the results of previous studies [18] on the assessment of toxicity degree in cattle ruminal fluid, using indicator luminescent strains designed on the basis of *E. coli.*

Inhibition can be associated with an ambiguous action of the molecules. Therefore, an earlier toxicity assessment (*in vitro*) of *Quercus cortex* inhibitory molecules showed that coumarin did not affect the luminescence kinetics of the *E. coli* strain MG1655 pXen7 [19], which is different from the results of the *E. coli* strain JLD271 pAL103. At the same time, it exerted a toxic effect on *Stylonychia mytilus*, 24 hours before the triplicate dilution (0.1-0.025).

The EC50 values for coniferyl alcohol with respect to *E. coli* strain MG1655 pXen7 were 0.54 mg/ml [19]; a similar inhibition of luminescence was confirmed in this study.

In view of the limited information on the effect of coumarin on microorganisms living in the gastrointestinal tract of animals, an analysis of its possible complex effect on the body was carried out. For example, coumarins in *Hydrangea paniculata*

caused the inhibition of caspase splitting, the infiltration of neutrophils and macrophages in the tissues of kidneys and the production of cytokines and chemokines, and could be metabolised into two biologically active compounds, umbelliferone and esculetin [20,21]. Thus, the probability of the interaction of coumarin with biologically active substances present in the composition of ruminal tissue is high, which can lead to the formation of new compounds.

It should also be taken into account that coumarin can exert a selective effect on certain microorganisms. Thus, coumarin derivatives were assessed as potential inhibitors of the production of the virulent factor of the *Pseudomonas aeruginosa* pyocyanin; the results show that coumarin derivatives suppress the growth of *P. aeruginosa* [22,23].

The presence of a quorum inhibitory effect can be considered here as a possible mechanism of action, as confirmed earlier [17]; this is from the coumarin extracted from oak bark.

This was also confirmed in recent studies comparing seven structurally related coumarins and inhibition of the quorum sensitivity of *Pseudomonas aeruginosa* and *Chromobacterium violaceum* [23,24].

As for coniferyl alcohol, its effect on gastrointestinal tract microorganisms is unknown, while at the same time there is evidence that it reduces the growth of Nicotiana cells at high concentrations [25]; it is also metabolised by BY-2 cells into several compounds as proposed earlier.

5. CONCLUSION

The data obtained made it possible to state that the use of natural plant components and their synthetic analogues increases the inhibition level of Quorum Sensing activity by inactivating regulatory molecules. In the future, this will make it possible to determine the nature of diet and the type of feed substrates used, as well as additives which act as additional factors in regulation of the activity of biochemical communication channels in the ruminal microbial community of ruminant animals.

6. ACKNOWLEDGEMENTS

The study was performed according to the project No.0761-2018-0003

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