ISSN:0975-1459
Journal of Pharmaceutical Sciences and Research
www.jpsr.pharmainfo.in

Assessment of (*in vitro)* Toxicity of Quorum-Sensing Inhibitor Molecules of *Quercus cortex*

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Abstract

Molecules of plant origin are characterized by various forms of action on the bacterial cells. This is caused by the constant interaction and adaptation of organisms to each other. There are studies of toxicity assessment of plant extracts and their main components (phenolic compounds, catechins, triketones) for defining the potential of biological properties, promising molecules for veterinary medicine and medicine.

The aim of this work was to assess the toxicity of various chemically synthesized small molecules isolated from the extract of *Quercus cortex* by using test strains of the bacteria *B. subtilis*, *S. typhimurium*, *E. coli* and the test system *Stylonychia mytilus*. Phytochemical analysis of the plant extract was performed with a gas chromatograph with mass-selective detector GQCMS 2010 Plus (Shimadzu). The studies used the substances previously identified by the team members with confirmed antibacterial and Quorum sensing activity from the extract of *Quercus Cortex*. Three laboratory strains of luminescent bacteria (*E. coli* K12 MG1655 (pXen7), *Salmonella typhimurium* LT2, and *B. subtilis* 168) were studied in the paper. We used a culture of cells of the freshwater infusoria *Stylonychia mytilus* (wild strain) in the phase of exponential growth as a test object.

Scopoletin had no effect on three strains used in the study (no luminescence changes of more than 20%). In the presence of propylresorcinol, the luminescence of *B. subtilis* EG168-1 was completely inhibited in the range of 0.345-1.38 mg/mL, at this time EC50 was 0.028 mg/mL, EC50 in antiarol was 0.48 mg/mL, and EC50 in coniferylic alcohol was 0.54 mg/mL. Antiarol had an effect on *B. subtilis* EG168-1 with EC50 = 0.32 mg/mL. Vanillin and coumarin did not affect the kinetics of luminescence of *E.coli* MG1655 pXen7 and *S.typhimurium* LT2 pACXen, while the value of EC50 was not found for *B.subtilis* EG168-1. Vanillin, scopoletin and antiarol did not have a toxic effect on the cell culture of *Stylonychia mytilus.* The toxic effect of coumarin was observed 24 hours before the triplicate dilution (0.1-0.025). Propylresorcinol caused toxic action during all periods of the study, while coniferyl alcohol caused toxic action in 3 hours, with the survival rate of cell cultures of 40-69% and dilution of up to five times.

Thus, plant metabolites can be a valuable tool for the treatment and prevention of infections and can help to develop new and safe components for inclusion in antimicrobials.

Keywords: cytotoxicity, molecules, *Quercus cortex, B. subtilis, S. typhimurium, E. coli, Stylonychia mytilus.*

INTRODUCTION

Molecules of plant origin are characterized by various forms of action on bacterial cells. This is caused by the constant coexistence and adaptation of organisms to each other. These are phytoncides that have particular interest as these substances are capable of suppressing growth or destroying bacterial cells. For example, *Artemisia parviflora* extract had a pronounced bactericidal effect against *Pseudomonas aeruginosa*, *Escherichia coli* and *Shigella flexneri* [1], determined by the presence of terpenic compounds. Antibacterial activity against *Staphylococcus aureus, Bacillus subtilis, Pasturella multocida* and *Escherichia coli* had been detected by the method of disk diffusion of some fractions of *Colebrookia oppositifolia* [2], *Vitex agnus castus* and *Myrsine africana* [3].

Some Gram-positive and Gram-negative microorganisms are the model ones, and are used in assessing the toxicity of plant compounds [4,5]. There are studies to assess the toxicity of plant extracts (*Acacia nilotica, Anogeissus leiocarpus*) and their main components (phenolic compounds, catechins, triketones) in order to study the potential of anthelmintic insecticidal properties, promising molecules for food industry [6-9].

In the world practice, various test systems such as microorganisms, cellular and subcellular elements, various hydrobionts, insects are proposed for the evaluation of toxicity of substances [10,11], since these are unified fundamental mechanisms being in the basis of the complex of response reactions of the organism having a protective nature and providing adaptation to changing conditions in response to the impact of various adverse factors, including toxic agents [12, 13]. Ecotoxicological tests are accompanied by chemical analysis in order to determine differences between nominal and actual concentrations of the test compounds. They are widely used, including for the evaluation of substances used in livestock production [14,15]. In this respect, unicellular infusorians have a large contact surface with respect to their dimensions. They immediately come into the contact with toxicant, reacting to chemical action with a whole complex of changes. The aim of the work was to assess the toxicity of various chemically synthesized small molecules isolated from the extract of *Quercus cortex* by using strains of bacteria *B. subtilis* EG168-1, *S. typhimurium* LT2 pACXen, *E. coli* MG1655 pXen7 as test objects and test system *Stylonychia mytilus*.

MATERIALS AND METHODS

Phytochemical analysis of the plant extract was performed with a gas chromatograph with a mass-selective detector GQCMS 2010 Plus (Shimadzu, Japan) on HP-5MS column. GCMS Solutions software, GCMS PostRun Analysis software were used

to interpret the results of the study. A set of CAS, NIST08, Mainlib, Wiley9 and DD2012 Lib libraries was used to identify the compounds. Quantitative presence of the individual identified components was estimated by the relative value (%), which related the peak area to the total area of the extract.

In order to carry out the study, we used dry extract that was dissolved in methanol before carrying out XMC with subsequent administration to the analytical cell of the chromatograph by using a Hamilton 1700 microsyringe. 35 substances were isolated in the extract of the oak bark (Table 1).

Table 1. Chemical compounds identified in XMS analysis of the oak bark extract in methanol

No.	Name of the identified substance (according to IUPAC)
1	Propantriol-1,2,3 *
2	Decane*
3	2-furancarboxylic acid*
4	1,3,5-triazine-2,4,6-triamine*
5	Pentadecane*
6	2,3-dihydroxypropanal*
7	Butanedioic acid*
8	2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one*
9	2-amino-9- [3,4-dihydroxy-5-(hydroxymethyl) oxolan-2-yl] -3H-purin-6-one*
10	Cyclopentane-1,2-diol**
11	1,2: 5,6-diandihydrogalacitol**
12	5-hydroxymethylfurfural*
13	acetylcysteine,-2-acetamido-3-mercaptopropanoic acid*
14	1-methylundecyl ester of 2-propenoic acid**
15	2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one**
16	1- (2-hydroxyethyl)-4-methylpiperazine**
17	6- (4-hydroxy-6-methoxy-2-methyl-tetrahydro-pyran-3-yloxy)-2-methyl-dihydro-pyran-3-one**
18	1,2,3-trihydroxybenzene* (pyrogallol)
19	2-methyl-5-nitro-pyrimidine-4,6-diol**
20	4-hydroxy-3-methoxybenzaldehyde* (vanillin)
21	2-Amino-9- [3,4-dihydroxy-5- (hydroxy-methyl) oxolan-2-yl] -3H-purin-6-one*
22	1,6-anhydro-β-D-glucopyranose*
23	1- (β-D-arabinofuranosyl) -4-O-trifluoromethyl uracil**
24	4-hydroxy-3-methoxybenzoic acid**
25	1,6-anhydro-β-D-glucofuranose*
26	4-propyl-1,3-benzenediol * (propylresorcinol)
27	1,2,3,4,5-cyclohexanepentol*
28	4- (hydroxymethyl) -2,6-dimethoxyphenol*
29	4- (3-hydroxy-1-propenyl) -2-methoxyphenol* (coniferyl alcohol)
30	9 - [(2R, 3R, 4S, 5R) -3,4-dihydroxy-5- (hydroxymethyl) oxolan-2-yl] -3H-purine-2,6-dione*
31	7-hydroxy-6-methoxy-2H-1-benzopyran-2-one* (coumarin)
32	methyl-α-D-glucopyranoside *
33	2H-1-benzopyranon-2* (scopoletin)
34	2-ethoxy-6- (methoxymethyl) phenol**
35	3,4,5-trimethoxyphenol** (antiarol)

Note: * identified components with the probability of more than 90%
** identified components with the probability of less than 90%

At the same time, in our studies we used the substances previously identified by the team members with confirmed antibacterial and QS activity from the extract of Quercus Cortex [16] (Table 2).

Table 2. Molecules of chemically synthesized substances

No.	Substances*	Concentration in 1 mL of extract of *Quercus cortex*, mg
1	2-n-Propylresorcinol, 98% AVH27024 (propylresorcinol)	1.38
2	Vanillin, 99% of AC14082-1000	0.53
3	7-Hydroxycoumarin, 99% AC12111-0250 (coumarin)	0.48
4	3,4,5-Trimethoxyphenol, 98.5% AC18914-0050 (antiarol)	1.79
5	Scopoletin, 95% AC30290-0010	0.30
6	Coniferyl alcohol, 98% AL22373-5	4.45

Note: * Manufacturer: Acros

Bacterial strains

Three laboratory strains of luminescent bacteria were used in the work, including those previously tested in assessing the toxicity of various compounds.

Laboratory luminescent biosensor of *E. coli* K12 MG1655 (pXen7) was obtained by transforming the host strain cells with a hybrid plasmid pUC18 with a built-in EcoRI DNA fragment of about 7,000 bp in size containing the structural bioluminescence genes of the soil microorganism *Photorhabdus luminescens* ZM1 [17]. Previously, this strain was used to study the toxicity of carbon nanotubes [18, 19].

The second used strain was based on *Salmonella typhimurium* LT2. At this time, the cells of this host strain were transformed with the plasmid pACxen containing luminescent genes' cassette of *Photorhabdus luminescens* ZM1 and providing a constitutive level of luminescence.

A third biosensor constructed by using *B. subtilis* 168 host strain was constructed with the help of *luxCDABE* genes of *P. luminescens* and integrated ribosome-binding sites providing efficient translation in the cells of Gram-positive bacteria, as well as kanamycin-resistance gene. Clone with the highest luminescence intensity was selected and designated as *B. subtilis* EG168-1.

Bioluminescent test

Luminescent bacterial biosensors were cultivated on LB agar with the addition of a selectable marker at 37 °C for 18-24 hours. Thereafter, the cells were suspended in LB broth and adjusted to the density of 0.5 relative units at 450 nm by using a StatFax 303+ photometer (Awareness Technology, USA). Aliquots of bacterial suspensions of 90 μL were added to Microlite 2+ wells (Thermo, USA) containing pre-injected test substances in the serial dilutions. The plates were placed in the measuring unit of luminometer LM-01T (Immunotech, Czech Republic) and incubated without stirring at 37 °C for 60 min, simultaneously measuring the bioluminescence intensity estimated by relative light units (RLU) at 5 min intervals.

Test on protozoan

As a test object, we used a culture of cells of the freshwater infusorian *Stylonychia mytilus* (wild strain) in phase of exponential growth. The number of test functions included: survival, number (biomass). An initial culture of *Stylonychia mytilus* cells was cultivated on Lozin-Lozinsky medium (1 g per 1 liter of distilled water) with the addition of yeast (*Saccharomyces cerevisiae*) nutrient medium: NaCl - 0.1%; KCl - 0.01%; $CaCl_2$ - 0.01%; $MgCl_2$ - 0.01%; $NaHCO_3$ - 0.02.

Sensitivity of the culture of *Stylonychia mytilus* cells to the action of the toxicant was determined by the time of their death. The latter was diagnosed by the cessation of protozoa movement, which was accompanied by violation of the integrity and lysosomes of the cell. The number of cells in 5 mL of medium

containing an intact culture of infusorians (without the addition of substances) served as a control in all the experiments. Total number of cells in 5 mL of medium containing infusoria was counted by using a light microscope (MT 5300L). Cells taken in a stationary growth phase were incubated at the temperature of 20±2 °C in a medium supplemented with substances for 24 hours. Interim counting was carried out after 10, 180, 360 and 1440 minutes.

Statistics

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

RESULTS

The toxicity assessment of the chemical compounds studied using constitutively luminescent bacteria showed the differences in the activity of the compounds themselves, as well as the specificity of their action. Among all the molecules used, only scopoetin had no effect on three strains used in the study, which was expressed in the absence of luminescence changes of more than 20%. However, luminescence was inhibited to the greatest extent in the presence of propylresorcinol. Despite the typical dose-dependent characteristic of the suppression of the luminescence of bacterial strains, sensitivity level was not the same for *B. subtilis* EG168-1 as compared to *E. coli* MG1655 pXen7 and *S. typhimurium* LT2 pACXen. For this gram-positive strain, the luminescence was completely inhibited in the range from 0.345 to 1.38 mg/mL propylresorcinol. At this time, concentration of the test substance that resulted in 50% inhibition of luminescence relative to the control groups (EC50) was equal to 0.028 mg/mL. For gram-negative biosensors, this compound was not so toxic, and complete suppression was recorded for the maximum used concentration. Twice reduction in this value provided suppression of *E.coli* MG1655 pXen7 by 46% and suppression of *S.typhimurium* LT2 pACXen by 21% from the initial level. Thus, EC50 was 0.71 and 1.06 mg/mL, respectively.

A high degree of specificity for Gram-positive bacteria was exhibited by coniferyl alcohol, with respect to *B.* subtilis EG168-1, its effect was characterized by EC50 equal to 0.54 mg/mL. The effect on *E. coli* MG1655 pXen7 and *S. typhimurium* LT2 pACXen did not change the nature of the kinetics of their luminescence and no toxic effects were observed.

Finally, antiarol showed a different effect on the bacterial cultures used. With respect to *E. coli* MG1655 pXen 7, this substance was found to be low-toxic with EC50 equal to 1.62 mg/mL, and concentrations less than 0.45 mg/mL no longer had an effect on the bacterial luminescence. Biosensor based on *S. typhimurium* LT 2 was more sensitive and the EC50 parameter for it was equal to 0.95 mg/mL. The highest toxicity of 3,4,5-trimethylhydroxyphenol has been shown in *B.subtilis* EG168-1. EC50 for the latter substance was 0.32 mg/mL, and the effect was leveled when the concentration of substance had been reduced down to 0.15 mg/mL or less (Table 3).

Table 3. EC50 values of the test compounds for three types of bacterial luminescent biosensors, mg/mL

Biosensor	Active substance					
	vanillin	propylresorcinol	coniferyl alcohol	coumarin	antiarol	scopoletin
E. coli MG1655 pXen7	-	0.71	-	-	1.62	-
S.typhimurium LT2 pACXen	-	1.06	-	-	0.95	-
B. subtilis EG168-1	-	0.028	0.54	-	0.32	-

Table 4. Biological effect of *Quercus cortex* compounds on the cell culture *Stylonychia mytilus*

Name	Concentration (M)			
	Tox	LC50	LOEC	NOEC
10 min				
Vanillin	-	-	-	0.1-0.0002
Propylresorcinol	0.1-0.025	0.0125	0.00625	0.003-0.0002
Coniferyl alcohol	-	-	-	0.1-0.0002
Coumarin	-	-	-	0.1-0.0002
Scopoletin	-	-	-	0.1-0.0002
Antiarol	-	-	-	0.1-0.0002
180 min				
Vanillin	-	-	-	0.1-0.0002
Propylresorcinol	0.1-0.025	0.00625	0.0125	0.003-0.0002
Coniferyl alcohol	0.1-0.05	0.00125	0.025	0.003-0.0002
Coumarin	-	-	-	0.1-0.0002
Scopoletin	-	-	-	0.1-0.0002
Antiarol	-	-	-	0.1-0.0002
360 min				
Vanillin	-	-	-	0.1-0.0002
Propylresorcinol	0.1-0.025	0,00625	0,0125	0.003-0,0002
Coniferyl alcohol	3.2-0.003	0,00625	0,0125	0.003-0,0002
Coumarin	-	-	-	0.1-0.0002
Scopoletin	-	-	-	0.1-0.0002
Antiarol	-	-	-	0.1-0.0002
1440 min				
Vanillin	-	-	-	0.1-0.0002
Propylresorcinol	0.1-0.025	0,0125	0,00625	0.003-0,0002
Coniferyl alcohol	3.2-0.003	0,00625	0,0125	0.003-0,0002
Coumarin	0.1	0.025	0.05	0.0125-0.0002
Scopoletin	-	-	-	0.1-0.0002
Antiarol	-	-	-	0.1-0.0002

Note: Tox was the concentration causing 0-39% survival of the object; LC50 was the concentration causing 50% survival of the object; LOEC was the concentration causing 40-69% survival of the object; NOEC was the concentration causing 70-100% survival of the object [20].

The remaining two types of compounds did not have significant effect on bacterial biosensors. Due to the last fact, they can be attributed to the group of non-toxic substances. Thus, vanillin and coumarin did not affect the luminescence kinetics of *E. coli* MG1655 pXen7 and *S. typhimurium* LT2 pACXen in the whole concentration range, while for *B.* subtilis EG168-1 only the maximum concentrations ensured the registration of suppression of luminescence by 42% and 34%, respectively. At this time EC50 parameter was not achieved.

The final ranking of compounds according to the averaged EC50 for three strains made it possible to build a line of substances according to an increase in their toxicity: scopoletin - coumarin - vanillin - coniferyl alcohol - antiarol - propylresorcinol.

The use of different type of test system also revealed different effects of chemically synthesized small molecules of Quercus Cortex (Table 4).

During all periods of the study, such substances as vanillin, scopoletin and antiarol did not have a toxic effect on the cell culture of *Stylonychia mytilus.* The toxic effect of coumarin was observed only 24 hours before the triplicate dilution (0.1-0.025). Propylresorcinol caused toxic effects during all periods of the study. On the dilution of up to five times, the survival rate of cell cultures was 40-69%. LC50 for this compound, as a function of time, was 0.00625-0.0125 mM. Manifestation of the toxic effect of coniferyl alcohol was detected only in three hours. At the same time (as in the case with propylresorcin) the survival rate of cell cultures was 40-69% with dilution of up to five times. For a given compound, LC50 as a function of time was equal to 0.00125-0.00625 mM.

DISCUSSION

According to the results of the studies, when assessing the toxicity of different doses of chemically synthesized small molecules isolated from the extract of *Quercus cortex,* identical data were obtained using both strains of bacteria *B.* subtilis EG168-1, *S. typhimurium* LT2 pACXen, *E. coli* MG1655 pXen7, and *Stylonychia mytilus*.

The mechanism of action of compounds on the cell can be explained from the chemical point of view. Chemical process is associated with the cellular transport of compounds, which ultimately leads to oxidative stress and damage to cells as a result of oxidative reactions. Thus, it was found that ATP-binding transporters were involved in the transport of lignin precursors (coniferyl alcohol) through plasma and vacuolar membranes [21,22].

Coniferyl alcohol is a common monolignol. The toxicity of monolignol has been previously known, which is confirmed by the results of our studies, and at high concentrations this substance is toxic even for plant cells [23].

From the literature sources it is known that coumarin derivatives, small molecules isolated from *Juglans mandshurica* plant and obtained chemically, have less pronounced cytotoxic effect on the liver cells [24], as well as in tests performed on *Danio rerio* [25]. According to the results of the studies, coumarin was also less toxic, since these effects were observed only in 24 hours and at high concentrations. Recent studies have shown that the possible mechanism of action of this compound is the generation of reactive oxygen species that play a key role in cellular apoptosis induced by dihydroxycoumarin [26,27]. Perhaps in our study concentration of coumarin was not that significant, which led to an increase in the time of the manifestation of its toxic effects.

In vivo studies of acute toxicity for vanillin and its derivatives, significant safety reserves were detected. They were marked by the lack of systemic and behavioral toxicity up to 300 mg/kg for the first 30 minutes, 24 hours, or 14 days after administration [28]. This was consistent with the results of the conducted studies, we did not find vanillin toxicity for *Stylonychia mytilus,* while EC50 was not achieved for *E. coli* MG1655 pXen7, *S. typhimurium* LT2 pACXen and *B.* subtilis EG168-1 in the entire concentration range. In our case, this was probably caused by lower concentration. At the same time, there is a need for the further study of this compound, in view of the mechanism of its antifungal action, based on mitochondrial dysfunction and the onset of oxidative stress [29].

At the same time, we know some influence of two phenolic compounds of vanillin (4-hydroxy-3-methoxybenzaldehyde) on the development of drug resistance in *Salmonella typhimurium* [30]. In our case, this requires additional study.

The studies are known showing that special compounds isolated from the oak bark (*Melia volkensii*) - toosendanin and kulactone - showed antimicrobial activity with the minimal inhibitory concentration against *Escherichia coli, Staphylococcus aureus,* while at the same time nothing was said about *scopoletin* isolated from the same bark. In our study, we have found a similar effect of *scopoletin* in all the tests used [31]. Scopoletin refers to coumarins being a group of important natural compounds that provide antimicrobial activity of plants and act as the protective mechanism against abiotic stresses [32].

One of the mechanisms of the toxic effect of scopoletin is significant accumulation of intracellular reactive oxygen species [33].

Recent studies have shown that coumarins have toxic effect on the larvae of *Drosophila melanogaster,* while some species have antibacterial and cytotoxic effects [34, 35]. This fact was consistent with the data obtained by us, where the toxicity of this substance manifested itself in 24 hours at the maximum concentration. Previously, researchers had already noted that chemically synthesized compounds, including coumarins, possessed the same biological properties as those isolated from plants [36, 37], which confirmed the prospects of their use.

The experiments conducted show that the high sensitivity of unicellular microorganisms to the toxic effect of *Quercus cortex* compounds allows the use of infusoria in biological tests to assess the toxicity of biologically active substances.

CONCLUSION

Plant metabolites can be a valuable tool for the treatment and prevention of infections and can contribute to the development of new and safe components for their inclusion in antimicrobials. Due to the latest advances in technologies effective for the cultivation and collection of plant metabolites, the study of quorum-sensing inhibitor molecules can be a very promising direction of the study.

ACKNOWLEDGEMENTS

The study was carried out with the financial support of the Russian Science Foundation (Grant No. 16-16-10048).

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